The yeast prion Ure2p retains its native α -helical conformation upon assembly into protein fibrils *in vitro*

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The yeast inheritable phenotype [URE3] is thought to result from conformational changes in the normally soluble and highly helical protein Ure2p. *In vitro*, the protein spontaneously forms long, straight, insoluble protein fibrils at neutral pH. Here we show that fibrils of intact Ure2p assembled *in vitro* do not possess the cross β -structure of amyloid, but instead are formed by the polymerization of native-like helical subunits that retain the ability to bind substrate analogues. We further show that dissociation of the normally dimeric protein to its constituent monomers is a prerequisite for assembly into fibrils. By analysing the nature of early assembly intermediates, as well as fully assembled Ure2p fibrils using atomic force microscopy, and combining the results with experiments that probe the fidelity of the native fold in protein fibrils, we present a model for fibril formation, based on assembly of native-like monomers, driven by interactions between the N-terminal glutamine and asparagine-rich region and the C-terminal functional domain. The results provide a rationale for the effect of mutagenesis on prion formation and new insights into the mechanism by which this, and possibly other inheritable factors, can be propagated.

Keywords: amyloid fibrils/atomic force microscopy/conformational transition/prion/Ure2p

Introduction

How proteinaceous factors cause inheritable disorders in yeasts as well as in higher eukaryotes is a fascinating biological question and, for man, a challenging problem of grave medical importance (Prusiner, 1997). The realization that a number of proteins with prion-like characteristics are found in yeasts has opened the door to detailed structural and biological studies of the conformational changes that accompany fibril formation, and the mechanism of protein-based inheritance (Cox, 1994; Prusiner, 1997). The [URE3] phenotype is one of a number of inheritable prion factors in the yeast *Saccharomyces cerevisiae*. It causes disorder in a signal transduction cascade that regulates nitrogen catabolism (Magasanik, 1992). The phenotype was detected 25 years ago by the non-Mendelian behaviour of a heritable

ability of yeast cells to take up ureidosuccinate, which is structurally analogous to the poor nitrogen source allantoate, on media with a good nitrogen source (Lacroute, 1971). The [URE3] phenotype is believed to result from a conformational change of the protein Ure2 that renders it inactive and insoluble (Wickner, 1994; Masison and Wickner, 1995). Importantly, Ure2p^[URE3] has gained the capacity to convert the active form of the protein into its inactive form (Taylor *et al.*, 1999). Thus, the Ure2p^[URE3] state is a protein-based genetic element, independent of any nucleic acid change, i.e. a prion. It is lost by growth of yeast cells in low concentrations of guanidinium chloride (GdnHCl) and the frequency with which it appears increases by overproducing Ure2p (M.Aigle, cited in Cox *et al.*, 1988; Wickner, 1994).

Insights into the molecular mechanism underlying the unusual protein mode of inheritance of [URE3] came from biochemical and cell biological characterization of Ure2p. The protein is formed from two domains: an N-terminal amino acid sequence that is rich in asparagine and glutamine residues, and a C-terminal part that shares a low degree of sequence identity with glutathione S-transferases (GSTs) (Coschigano and Magasanik, 1991). Deletion of the N-terminal 65 amino acids of the protein has no effect on the function of the protein, while overexpression of the N-terminal region induces *de novo* appearance of the [URE3] phenotype (Masison and Wickner, 1995). This demonstrates that whilst the N-terminal region is not required for the nitrogen regulation function of the protein (Coschigano and Magasanik, 1991), it is an essential part of the mechanism of prion propagation of the intact protein. By contrast, overexpression of the C-terminal region of the protein (residues 65–354) that comprises the functional domain (Masison and Wickner, 1995) in isolation, does not induce the [URE3] phenotype (Masison *et al.*, 1997). The physical boundary between the two domains was redefined more recently using purified soluble native Ure2p (Thual *et al.*, 1999). Thus, the N-terminal region extends from residues 1 to 93, while the compactly folded C-terminal region extends from residues 94 to 354 (Thual *et al.*, 1999, 2001). Chemical denaturation studies performed on full-length Ure2p (Ure2p 1–354) and its C-terminal region (Ure2p 95–354) revealed that the N-terminal domain does not affect the overall stability of the Ure2p molecule (Perrett *et al.*, 1999; Thual *et al.*, 2001), demonstrating that the two domains are structurally independent. Ure2p in solution forms a native, soluble dimer by interactions between the C-terminal domain (Perrett *et al.*, 1999; Bousset *et al.*, 2001a; Thual *et al.*, 2001; Umland *et al.*, 2001). This domain is highly (60%) helical (Figure 1) and has a fold similar to that of the β class of GSTs (Board *et al.*, 2000). It binds glutathione (GSH) and related compounds with high affinity in a cleft running along the

Fig. 1. X-ray structure of the C-terminal domain (residues 95–354) of Ure2p. The site of Cys221 engineered into the protein to stabilize the dimer is shown in yellow. The two subunits in the dimer are coloured blue and green. The structure also shows the position of GSH (in red), which binds between two subdomains within each subunit. The figure was drawn with Molscript (Kraulis, 1991) and Raster3d using the crystal coordinates deposited in the Protein Data Bank, accession No. 1jzr (Bousset *et al.*, 2001b).

domain interface that resembles the active site of all GSTs (Bousset *et al.*, 2001b).

Native soluble Ure2p assembles *in vitro* into fibrils that exhibit the characteristics of amyloid in that they are resistant to proteolysis (Taylor *et al.*, 1999; Thual *et al.*, 1999), are 20 nm wide and >1 μ m long (measured by negative stain electron microscopy), bind thioflavin-T (Bousset *et al.*, 2001b) and Congo Red, and show yellow–green birefringence in polarized light (Taylor *et al.*, 1999; Thual *et al.*, 1999, 2001). Assembly of the fibrils follows a nucleation-growth mechanism that can be seeded with pre-formed fibrils formed from soluble full-length Ure2p (Thual *et al.*, 1999). Finally, an increase in the β -sheet content of the protein has been reported upon its assembly into mixed filaments made of a synthetic peptide corresponding to Ure2p 1–65 and full-length Ure2p, although the resulting fibrils were reported to retain significant α -helical structure (Taylor *et al.*, 1999).

Despite the wealth of information now known about the structure and function of the soluble C-terminal domain of Ure2p, relatively little is known about the molecular events that lead to the assembly of full-length Ure2p into fibrils. Here we provide a model for the assembly mechanism of fibrils of Ure2p based on a combination of biochemical and biophysical assays. The data indicate the remarkable and important result that hydrated fibrils formed from Ure2p at neutral pH are not built on the cross β framework of amyloid (Sunde *et al.*, 1997), but are made instead by the assembly of native-like, functional units, in a manner more reminiscent of the assembly of other biological polymers (Holmes *et al.*, 1990; Nogales

Fig. 2. Ure2p loses its ability to assemble into fibrils when cross-linked in a dimeric form. (A) Analysis on a non-reducing 10% SDS–polyacrylamide gel of Ure2pC221 in the presence of the reducing agent DTT (lane 1) and following its cross-linking by H_2O_2 (lane 2). Cross-linked Ure2pC221 following its reduction in the presence of β -mercaptoethanol (lane 3). The molecular weight markers are shown on the left. The apparent molecular mass of cross-linked Ure2pC221 (116 kDa) is higher than the expected molecular mass of Ure2p dimer (80.4 kDa). This must reflect an abnormal shape of the cross-linked dimer. The polypeptide with apparent molecular mass 70 kDa is a contaminant. (B and C) Negative-stained electron micrographs of oligomers made from the reduced (B) and cross-linked (C) forms of Ure2pC221. Bar, 0.2 μ m.

et al., 1999). The results have importance in that they demonstrate that extreme caution is needed when categorizing protein fibrils as amyloid, and revolutionize current models of the evolution and propagation of the [URE3] phenotype in yeast.

Results

The monomeric form of Ure2p is an early precursor of amyloid fibrils

Ure2p exists as an equilibrium mixture of monomers and dimers at 0.4 mg/ml, pH 7.5 and 15°C (Thual *et al.*, 1999). To establish the identity of the species that assembles into fibrils, we generated a Ure2p variant in which Ser221 was mutated to Cys. In the crystal structure of Ure2p 95–354 (Bousset *et al.*, 2001a), Ser221 from one polypeptide chain in the Ure2p dimer faces the same residue in the partner polypeptide chain (Figure 1). The calculated distance between the two C_α of the newly introduced Cys residues in the Ure2pC221 variant is 4.1 Å, compatible with the establishment of a disulfide bond between these residues under oxidative conditions. Figure 2A shows the behaviour of the Ure2pC221 variant under reducing and oxidizing conditions. The molecular mass of Ure2pC221 in the presence of dithiothreitol (DTT) is identical to that of authentic Ure2p (40.2 kDa). By contrast, under oxidizing conditions, Ure2pC221 migrates on the gel as an entirely dimeric species, indicating quantitative oxidation of the two newly introduced Cys residues into a disulfide bond (Figure 2A, lane 2). In accord with this conclusion, such a species is not generated upon incuba-

tion of wild-type Ure2p under the same oxidizing conditions (data not shown) and treatment of the Ure2pC221 disulfide-bridged dimer with β -mercaptoethanol yields a polypeptide that has a molecular mass of 40.2 kDa. Thus, Ure2pC221 cross-linking is reversible by a reducing agent and involves formation of a disulfide bond, presumably between the newly introduced unique Cys residues. The apparent molecular mass of cross-linked Ure2pC221 (116 kDa) is higher than the expected molecular mass of Ure2p dimer (80.4 kDa). This must reflect an abnormal mobility of the cross-linked dimer during electrophoresis, as Ure2p is devoid of additional Cys residues. This conclusion is confirmed by measurement of the molecular mass of cross-linked Ure2pC221 (80.7 kDa) by mass spectrometry.

The oligomeric state of the Ure2pC221 variant was also examined by analytical ultracentrifugation in sedimentation velocity experiments, and compared with that of unmodified Ure2p under reducing and oxidizing conditions. Under reducing conditions, Ure2pC221 exists as a mixture of 70% dimer ($S_{20,w} = 4.6S$) and 12% monomer ($S_{20,w} = 3.0S$), as does authentic Ure2p (data not shown). A species with a sedimentation coefficient of 3.0S, compatible with the sedimentation coefficient of the monomeric form of the protein, coexists with the dimeric form under these experimental conditions (Thual *et al.*, 1999). By contrast, under oxidizing conditions, the monomeric form of Ure2pC221 is no longer observed, the only species detected in solution being the dimeric form of the protein. No significant changes in species distribution were observed upon incubation of authentic Ure2p under oxidizing conditions.

The ability of the reduced and oxidized forms of Ure2pC221 to assemble into higher molecular weight species was next examined by negative stain electron microscopy. Under reducing conditions, Ure2pC221 assembles into fibrils that are indistinguishable in size and morphology from those assembled from wild-type Ure2p (Figure 2B). By contrast, oxidized Ure2pC221 assembles into amorphous aggregates under these conditions (Figure 2C). Wild-type Ure2p forms fibrils under both oxidizing and reducing conditions (data not shown). We conclude from these experiments that Ure2p loses its ability to assemble into amyloid fibrils when the monomer $\leftrightarrow$ dimer equilibrium is displaced toward the dimeric form. Thus, the monomeric form of the protein appears crucial for its assembly into fibrils *in vitro*.

Previous results have shown that the N-terminal domain of Ure2p is required for its assembly into amyloid fibrils (Thual *et al.*, 2001). The assembly reaction may thus involve conformational changes limited to the N-terminal region of the protein or, alternatively, conformational changes may extend throughout the protein, including the C-terminal domain in the assembly-competent full-length monomer. To distinguish between these possibilities, the conformational properties of native soluble Ure2p, Ure2p 95–354 and fibrils of Ure2p were examined by FTIR spectroscopy (Figure 3). The spectrum of the Ure2p 95–354 is centred on a large peak at 1645 cm^{-1} , typical of a protein with high helical content (Figure 3B). In accord with this, Fourier deconvolution of the spectrum (Table I) indicates a helical content of 63%, a β -sheet content of 22%, and 15% other, which is broadly consistent with the

Fig. 3. Soluble and fibrillar Ure2p have similar secondary structural contents. FTIR spectra of soluble full-length Ure2p (A), Ure2p 95–354 (B) and fibrils assembled from full-length Ure2p (C). Curve fit spectra are presented in each case.

Table I. Secondary structure content of different forms of Ure2p estimated from FTIR spectroscopy measurements

	α -helix (%)	β -sheet (%)	Other (%)
Ure2p ^{95–354} soluble	63	22	15
Ure2p ^{1–354} soluble	60	17	23
Ure2p ^{1–354} filaments	74	20	6

X-ray crystal structure of the protein (9% β -sheet, 55–60% helix, ~30% coil) (Bousset *et al.*, 2001a). The FTIR spectrum of native, soluble, full-length Ure2p is also consistent with a highly helical structure (Figure 3A). Fourier deconvolution of this spectrum indicates that the full-length protein contains 60% α -helix, 17% β -sheet and 23% other (Figure 3A; Table I). Comparison of these data with those of Ure2p 95–354 (Figure 3B; Table I) suggests, therefore, that the N-terminal domain might not be completely unstructured in the soluble full-length protein. Most remarkably, however, the spectrum of hydrated fibrils of Ure2p is very similar to that of its soluble counterpart, suggesting that the fibrillar state is also predominantly helical (Figure 3C). Indeed, Fourier deconvolution of the spectrum of this protein indicates that the fibrils contain only 20% β -sheet structure, whilst the helical content of the fibrils is increased slightly relative to that of the soluble form (Figure 3C; Table I). Most importantly, bands typically expected for the cross β -structure of amyloid (1623–1618 cm^{-1}) (Zurdo *et al.*, 2001) account for less than ~2% of the total. We conclude, therefore, that these Ure2p fibrils do not comprise a cross β -core that is typical of amyloids. In accord with this, a 4.7 Å band is not observed in X-ray fibre diffraction images of these fibrils (L.Bousset, F.Briki, J.Doucet and R.Melki, submitted).

Ure2p fibrils retain a native C-terminal domain

The FTIR results suggest the intriguing possibility that fibrils of Ure2p could be comprised of native-like subunits. To test this possibility, the ability of the fibrils to bind the substrate mimic acetyl-2-dimethylaminonaphthalene-glutathione (ADAN-GSH; Bousset *et al.*, 2001b) was assessed. Using this substrate analogue, we have recently shown that native Ure2p binds GSH with high affinity (22 μ M) (Bousset *et al.*, 2001b). The GSH binding site is located in the catalytic domain of the protein, which is made of two subdomains connected by a short linker region (Bousset *et al.*, 2001a), each of which is involved in the substrate binding site (Figure 1). ADAN-GSH is thus an excellent tool for probing whether the fibrils contain native-like subunits.

Figure 4A shows the titration of Ure2p fibrils with ADAN-GSH. The data show clearly that fibrillar Ure2p binds ADAN-GSH with a 1:1 stoichiometry and an apparent equilibrium dissociation constant of 25 μ M. Moreover, the substrate binds competitively with unmodified GSH, confirming the fidelity of the substrate binding site in the protein fibrils (Figure 4B). Fibrils of Ure2p were also treated with 2 M GdnHCl. Under these conditions, soluble full-length Ure2p is partially unfolded and loses its ability to bind substrate (Thual *et al.*, 2001). Interestingly, fibrils of Ure2p are also partially unfolded in 2 M GdnHCl, as judged by their intrinsic tryptophan fluorescence (data not shown). Under these conditions, the fibrils remain assembled, as judged by atomic force microscopy (AFM) (Figure 6), electron microscopy (EM) and thioflavin-T binding, yet also lose the ability to bind ADAN-GSH (Figure 4). The fibrillar form of Ure2p that persists following this treatment has lost its GSH-binding capacity, which indicates that partial unfolding of the protein results in the loss of the ligand binding site. Taken together, these data demonstrate that fibrils of Ure2p contain native-like catalytic domains. In accord with these results, whilst fibrils of Ure2p are more resistant to protease digestion than their soluble counterpart, the digestion patterns obtained upon treatment of the fibrils with proteinase K are identical to those obtained by digestion of the soluble forms of the protein with the same protease (Thual *et al.*, 1999).

AFM imaging reveals that Ure2p fibrils are flexible, stable and periodic

To examine the morphology of mature fibrils of Ure2p and the nature of early assembly states, fibrils were grown in solution at pH 7 and samples were taken at early and late times in the growth and examined by tapping-mode AFM of hydrated samples in air. At the earliest time points of fibril growth (in the lag phase, open arrow, Figure 5A), a heterogeneous distribution of globular and rod-shaped particles is observed (Figure 5B). The smallest globular particles are 12–14 nm in diameter (measured at half-maximal height), and the rod-shaped particles appear as multiples of this elementary unit. The repeat period along these rods and their width at half-height is 12–14 nm. AFM can accurately measure distances on repetitive structures without compromise from the finite size of the tip, thus implying that these units are globular with a radius of 6–7 nm. At late stages in fibril growth (closed arrow, Figure 5A), these modular rod-like species have converted

Fig. 4. Fibrillar Ure2p binds GSH in a manner similar to native soluble Ure2p. (A) Saturation of Ure2p fibrils by ADAN-GSH (solid symbols). The solid curve depicts the calculated isotherm for binding of ADAN-GSH to a single category of sites (10 μ M) in Ure2p fibrils and an equilibrium dissociation constant of 25 μ M. Inset: double-reciprocal plot of the data. Open symbols and dashed line: titration of fibrils with ADAN-GSH in the presence of 2 M GdnHCl. Under these conditions, the fibrils remain assembled, yet lose the ability to bind the ligand. (B) Competition between GSH and ADAN-GSH for binding to fibrils of Ure2p. ADAN-GSH (20 μ M) bound to fibrils of Ure2p (3.8 μ M) was chased by increasing concentrations of unlabelled GSH in 50 mM Tris pH 7.5 and 100 mM KCl.

into long (>0.5 μ m), semi-flexible fibrils (Figure 5C), which are typically ~35 nm in width (Figure 5D). All fibrils in this case show a rather regular repeating period along their length that ranges from 23 to 33 nm. Examples of a profile along a fibril and a cross-section through two fibrils are shown in Figure 5E and F, respectively.

The mature fibrils formed from full-length Ure2p were treated with 4 M GdnHCl or proteinase K, under which conditions the fibrils remain assembled, and distributions of their height, width and repeat period were measured using AFM (Figure 6). The fibrils formed under native conditions have an average height of 12.7 nm, an average width of 44 nm and show a broad distribution of repeat distances, averaging at 50 nm. Despite the loss of their

Fig. 5. AFM image analysis of the formation of Ure2p fibrils. (A) Time course of Ure2p (60 μ M) assembly into fibrils in 50 mM Tris pH 7.5 and 100 mM KCl monitored by thioflavin-T binding. (B) Tapping-mode AFM amplitude image (error signal) of Ure2p oligomers at an early stage of assembly, shown by the open arrow in (A). (C) Tapping-mode AFM amplitude image (error signal) of Ure2p fibrils at a late stage of assembly, shown by the closed arrow in (A). (D) Tapping-mode AFM height image of the image shown in (C). (E) Fibril height profile along the axis of the fibril in the white frame in (D) showing its periodicity. The repeating unit of the fibril is 30 nm. (F) Cross-section profile for the fibrils presented in (D) performed in the region indicated by the dotted line. The height of the two fibrils is 11 nm and their diameter at half-height is 38 nm. The images were acquired in air. Scale bar, 200 nm.

Fig. 6. Tapping-mode AFM image analysis using height data (z-piezo movement) of native, GdnHCl- and proteinase K-treated Ure2p fibrils. (A, E and I) AFM height images of native, GdnHCl- (4 M) and proteinase K- (10 μ g/ml) treated Ure2p fibrils (60 μ M), respectively. The dimensions of the fibrils were analysed as described in Materials and methods. The panels show the height, width and periodicity distributions, respectively, for native fibrils (B, C and D), fibrils treated with 4 M GdnHCl (F, G and H) and fibrils treated with proteinase K (J, K and L). The number of individual objects measured is 3745 in (B), 3435 in (C), 158 in (D), 444 in (F), 407 in (G), 29 in (H), 356 in (J), 95 in (K) and 23 in (L).

ability to bind substrate analogues, fibrils incubated in the presence of 4 M GdnHCl and then deposited onto mica for AFM imaging have a slightly smaller height than their untreated counterparts (~10 nm), their average width is 60 nm and the average repeat distance is broadly distributed at ~54 nm. When the fibrils are treated in solution with proteinase K for 50 min, which is sufficient for complete digestion of the assembled form of Ure2p (Thual *et al.*, 1999), and then deposited onto mica and imaged by AFM, the fibrils are smaller in height (7.0 nm), the width is 57 nm and the average repeat is 60 nm. We conclude from these data that treatment of the assembled form of Ure2p by denaturing agents and proteases is not sufficient to disrupt their fibrillar structure. Moreover, the reduction in height and corresponding increase in width upon treatment are consistent with the rigidity of the fibril scaffold being compromised, allowing greater compression of the fibrils by the AFM tip. The differences between untreated, GdnHCl- and proteinase K-treated fibrils revealed by AFM imaging are not resolved when these fibrils are examined in the electron microscope.

Discussion

The data described above provide new and novel insights into the mechanism of assembly of Ure2p into protein fibrils. Most importantly, we show that despite having many properties akin to amyloid, including a long, straight morphology, the ability to bind thioflavin-T and Congo Red, and to show yellow–green birefringence upon Congo Red binding, the latter thought until recently to be highly specific for the cross β -structure of amyloid (Khurana *et al.*, 2001), the fibrils formed by incubation of full-length Ure2p under physiologically relevant conditions are highly helical in nature. Indeed, further analysis has shown that the precursor of fibril assembly is an obligate monomer that retains the native properties of its C-terminal domain in the fibrillar state. The data therefore indicate that extreme caution should be taken when ascribing long, straight protein fibrils as the cross β -structure of amyloid. Moreover, the data suggest that the protein fibrils from Ure2p that may confer the [URE3] phenotype comprise an array of native-like monomers that retain their ability to bind substrate analogues.

Although the exact function of Ure2p is unknown, the protein is believed to be inactive in its assembled form. Inactivation of Ure2p could be caused either by the loss of the tertiary structure of its functional domain prior to its assembly into fibrils, to the steric inhibition of active sites by the packing of Ure2p into the fibrillar state, or by the inability of the fibrillar form of the protein to interact with partner proteins in a manner similar to its soluble form. Our finding that the secondary structure of fibrils of Ure2p is virtually identical to that of the soluble dimeric form, together with the observation that Ure2p fibrils can bind GSH analogues, suggests that only minor conformational changes occur upon fibril assembly, at least under the conditions studied here. These findings contrast markedly with the increase in β -sheet content observed in fibrils formed from approximately stoichiometric amounts of Ure2p and a synthetic peptide reproducing Ure2p 1–65 (Taylor *et al.*, 1999), as well as fibrils assembled from a fusion protein between GST and Ure2p 1–69

(Schlumpberger *et al.*, 2000). The simplest explanation for this discrepancy is that the conformational properties of Ure2p 1–65 differ significantly from those of the full-length prion domain that extends from residues 1 to 94 and, in the full-length protein, is bound covalently to the rest of the protein.

A number of models have been suggested to explain the assembly of proteins into fibrillar arrays (Horwich and Weissman, 1997). For the human prion protein PrP, for example, formation of its fibrils involves large-scale conformational changes from the all α -helical conformation of its soluble C-terminal domain to a predominantly β -sheet structure in the corresponding fibrillar state (Prusiner, 1997). For other proteins, however, fibril formation may involve the assembly of native-like subunits, for example, in the assembly of microtubules and actin filaments, or more subtle conformational rearrangements, such as domain swapping (Carrell and Gooptu, 1998; Huntington *et al.*, 1999; Liu *et al.*, 2001). The extent of the conformational change that accompanies Ure2p dissociation into its component monomers is unclear. Dissociation could involve major conformational changes that lead to the loss of the tertiary and/or secondary structures of Ure2p, or partial unfolding of a limited region of the protein that yields new surfaces competent for assembly. In each case, however, assembly into filaments results in the formation of native-like secondary structure (as judged by FTIR) that is both stable and able to bind substrate analogues. Whatever the conformational changes at this stage of assembly, it is clear that simplistic models for assembly in which the N-terminal domains of Ure2p stack to form a cross β -core from which the C-terminal domains protrude, as schematized in Speransky *et al.* (2001), are not consistent with the data presented here. Such a model would require an increase in the β -sheet content of the fibrils by >20% (assuming that the N-terminal 65 amino acids form β -sheet structure upon assembly), formation of an FTIR band typical of the cross β -structure of amyloid, and dramatic changes in width of the fibrils upon treatment with protease or GdnHCl, none of which is observed here experimentally.

A model for fibril assembly from Ure2p that accounts for all the data presented here and elsewhere (Taylor *et al.*, 1999; Thual *et al.*, 1999, 2001) is shown in Figure 7. Our model is based on the assumption that limited structural perturbation occurs during assembly (see above). The crystal structure of the C-terminal domain of the Ure2p dimer shows that the N-terminal region of one polypeptide chain faces the flexible cap region from the partner polypeptide chain (Figures 1 and 7), suggesting that these two regions might interact transiently (Bousset *et al.*, 2001a). Such an interaction may limit the conformational dynamics of the N-terminal region of Ure2p, preventing intermolecular interactions with adjacent Ure2p dimers. We demonstrate here that the acquisition of assembly-competent properties requires the dissociation of Ure2p dimer. Upon dissociation, the flexibility of the N-terminal part of Ure2p is expected to increase, and the relatively hydrophobic surface area of ~4000 Å² that is involved in the dimer interface would become exposed to the solvent. Based on these observations, a number of models for fibrillar assembly can be envisaged, two of which are depicted in Figure 7. In these models, the highly flexible

Fig. 7. Molecular models for the assembly of Ure2p into fibrils. The proposed models are based on the observations presented here that: (i) dissociation of the Ure2p dimer occurs prior to assembly into fibrils; (ii) the fibrils formed under the conditions used lack a cross β -core; and (iii) the observation that Ure2p retains a native C-terminal domain in the fibrils. The dissociation of native Ure2p dimer exposes a hydrophobic surface area to the solvent (red). This area can establish a large number of hydrogen bonds with the flexible N-terminal domain (blue) from either the same Ure2p molecule or another Ure2p polypeptide chain, leading to intra- (case A) or inter- (case B) molecular interactions, respectively. In case A, the N-terminal domain binds to the same monomer, forming a surface that favours its interaction with the flexible cap region (green) from another Ure2p molecule, leading to the association of two Ure2p monomers through non-native interactions. In case B, the hydrophobic surface area exposed by dissociation of a given Ure2p monomer interacts with the N-terminal domain from another Ure2p polypeptide chain, forming a non-native dimer. In both cases, assembly can proceed indefinitely by addition of Ure2p monomers to both ends of the non-native dimer, given that each additional monomer exposes a binding site similar to that masked by its association. The oligomers generated are helical since two neighbouring monomers have the same relative orientation. In both cases, the GSH binding site (pink) is maintained in a native conformation in the fibril and is available for binding its ligand.

and poorly structured N-terminal region binds either to another Ure2p molecule (Figure 7, scheme B) or to the same polypeptide chain (Figure 7, scheme A), rationalizing the view that the N-terminal domain is required for oligomerization (Masison *et al.*, 1997; Taylor *et al.*, 1999; Thual *et al.*, 2001). In the first case, strong intermolecular linkages that can propagate in an unlimited manner are established, whilst in the latter case, the N-terminal region of Ure2p establishes intramolecular interactions. Such an interaction may generate a polar surface area with which the polar cap region from another Ure2p molecule, in a conformation similar to that in the crystal structure or following limited conformational rearrangement of the poorly structured flanking loops, could bind. This would lead to strong intermolecular linkages by the establishment of a large number of hydrogen bonds and subsequent highly ordered protein assembly. In either scenario, however, the GSH binding site on Ure2p would remain unaffected by polymerization, accounting for the ability of the protein to bind this substrate in the fibrillar state. Such an aggregation process could either lead to the formation of the nucleation units observed at early times using the AFM, which would then condense into fibrils, or propagate indefinitely by addition of monomeric Ure2p molecules to both ends of the fibrils. Such an assembly process is reminiscent of that described previously for the Sup35p

NM fragment (Serio *et al.*, 2000; Xu *et al.*, 2001). Based on the size of the Ure2p monomer (180 nm^3) and that of the smallest globular particles detected in the early stages of assembly using AFM (diameter 12–14 nm), the number of Ure2p monomers needed to form a nucleation unit (1435 nm^3) is 4–6.

A Ure2p mutant that greatly induces prion formation has been described recently (Fernandez-Bellot *et al.*, 2000). This mutant encodes a protein in which 10 amino acids are substituted. Interestingly, none of these mutations taken alone strongly induces prion formation. By contrast, the [URE3] phenotype is induced significantly by a URE2 allele harbouring the eight substitutions located in the C-terminal region of the protein. The substitutions F218L, H237R, T249S, V271E, M272L and Y282C are of particular interest here. The first three substitutions lie in the inter-dimer interface, while the last three are located in the cap. In each case, the substitutions either reduce hydrophobicity or increase charge, suggesting that they would favour dimer dissociation and disrupt interactions between the cap and the rest of the polypeptide chain. Moreover, when two point mutations located in the N-terminal part of Ure2p are combined with several mutations in its C-terminal part, prion formation is induced. These findings support the view that Ure2p assembly into fibrils is mediated by the interaction of the

inter-dimer interface and the cap with the N-terminal part of the protein. The persistence of Ure2p fibrils in the presence of high denaturant (GdnHCl) concentrations or after proteolytic treatment indicates that the intermolecular interactions within Ure2p fibrils are strong enough to maintain the fibrillar architecture despite these treatments. These observations, together with the findings that Ure2p assembly into fibrils is favoured by increasing the temperature, i.e. the reaction is endothermic (Thual *et al.*, 1999), and that disassembly does not occur upon dilution of the fibrils (L.Bousset and R.Melki, unpublished observations), suggest that a large number of hydrogen bonds and/or hydrophobic interactions stabilize the fibrillar scaffold.

How does full-length Ure2p assemble into fibrils? Our data favour a model in which assembly proceeds by the interaction of non-overlapping areas on the surface of Ure2p. Following the association of two Ure2p monomers, a third molecule of the same kind binds with one of the two molecules to make a trimer; thus, a linear polymerization of the protein takes place. Along the polymer, two neighbouring monomers have the same relative position and orientation; the polymer is therefore helical. This reaction has a character similar to the crystallization of a solute molecule in solution. It involves an energetically unfavourable nucleation step, followed by a more favourable elongation reaction that proceeds exponentially in a closed system until equilibrium is reached between the monomeric and polymeric species. At that stage, polymers of various lengths coexist with dispersed unit molecules whose concentration is the critical concentration (C_c). Because Ure2p assembly is essentially irreversible (L.Bousset and R.Melki, unpublished observations), the kinetics of polymerization is accounted for by monomer association to polymer ends and can be adequately described by a simplified form of Oosawa's equation for reversible assembly (Oosawa and Asakura, 1975):

$$-dc/dt = k[F]c \quad (1)$$

and

$$K_{eq} = 1/C_c \quad (2)$$

where c is the monomer concentration, $[F]$ is the concentration of elongating ends, K_{eq} is the polymerization equilibrium constant and k is the rate constant for monomer association to filament ends. Ure2p molecules are organized in unit cells in the fibrils, as are most proteins in crystals. Based on our AFM data, if we assume that the smallest globular particle is a unit cell, the number of monomers in each cell is 4–6. Alternatively, if the repeats in the fibrils are unit cells, the number of monomers in the unit cell would be much greater, of the order of 100 or more. Further thermodynamic and kinetic characterization of Ure2p assembly is needed to test the models for assembly proposed here and to derive a better understanding at the molecular level of the mechanism of assembly of this prion protein both *in vitro* and *in vivo*.

Materials and methods

Ure2p purification

Recombinant full-length Ure2p (Ure2p 1–354) and the functional part of the protein (Ure2p 95–354) were overexpressed as soluble proteins in

Escherichia coli, purified as previously described (Thual *et al.*, 1999, 2001) and stored at -80°C in buffer A (20 mM Tris-HCl pH 7.5, 250 mM KCl, 1 mM DTT, 1 mM EGTA) at a concentration of 10 mg/ml.

Construction of Ure2pC221 variant expression vector in *E.coli*

The Ure2pC221 variant expression vector was obtained by site-directed mutagenesis by replacing the TCA codon encoding a serine residue at position 221 with a TGC codon encoding a cysteine. This mutation was achieved in pET-URE2 expression (Thual *et al.*, 2001) vector using the QuikChange site-directed mutagenesis kit (Stratagene) and the primers 5'-GGTTGTTCTTCCAAACGTGCGGGCATGCGCAATG-3' and 5'-CATTGCGCATGCCCGACGTTTGAAGAACAACC-3'.

Assembly of Ure2p into fibrils

The assembly of full-length Ure2p was achieved by incubation of the protein (75 μM) at 4°C , without shaking, for a week in buffer A. The assembly reaction was monitored using thioflavin-T binding (McParland *et al.*, 2000) using a Quantamaster QM 2000-4 spectrofluorometer (Photon Technology International, NJ). Ure2p fibrils were also examined following negative staining with 1% uranyl acetate on carbon-coated grids (200 mesh) in a Philips EM 410 electron microscope.

AFM

Aliquots of full-length Ure2p (75 μM) at various stages of the assembly reaction were removed from the growth solution, deposited onto freshly cleaved mica and left for 1 min at room temperature. The specimens were then rinsed with deionized water and dried with a 1 bar pressure nitrogen stream at a distance of a few centimetres. Samples produced in this way are coated with a thin water film, which can leave biomolecules in a hydrated state (J.Heddle, T.Maxwell and N.H.Thomson, unpublished data).

Tapping-mode AFM in air was performed using a Nanoscope IIIa Multimode system (Digital Instrument/Veeco, Santa Barbara, CA) equipped with an E-scanner. Olympus rectangular cantilevers (Digital Instrument/Veeco), 160 μm long, with a resonant frequency in the range of 232–311 kHz and nominal spring constants in the range of 12–103 N/m (typical 42 N/m) were excited slightly below the resonant frequency. Samples were imaged at scan rates below 2 Hz, and 512×512 pixels were collected per image. Image data were collected for both the z-piezo signal (height data) and the error signal (residual change in cantilever amplitude).

The Nanoscope image files were examined using WSxMv2.0 (Nanotec Electronica S.L., www.nanotec.es) and flattened before subsequent processing. Files were then transformed to ASCII xyz files for further analysis. An in-house C program was then used to process flattened images. The program finds the highest positions on each scan line (maximum) and the two adjacent lowest positions (minimum) for each maximum (not necessarily on the same scan line). All maxima are assumed to correspond to the height of the fibrils, and the distance between the nearest minima at half-maximal height is assumed to correspond to the width of the fibrils. This program yields averaged values of fibril height and width that are in very good agreement with random manual measurements using WSxMv2.0. To determine the unit repetition along the fibrils, two-dimensional profiles along the fibril axis were taken manually using the WSxMv2.0 software.

Binding of ADAN-GSH to Ure2p fibrils

Binding of ADAN-GSH to Ure2p fibrils in buffer A was monitored as described previously (Bousset *et al.*, 2001b). Competition experiments were performed by two means. ADAN-GSH was added to increasing amounts of GSH and the mixture used in the binding assay. Alternatively, increasing amounts of unlabelled GSH were added to Ure2p fibrils saturated by ADAN-GSH and the decrease in fluorescence monitored.

FTIR spectroscopy

Ure2p 1–354 and Ure2p 95–354 were eluted in D_2O using an UNO Q6-R column (Bio-Rad). Ten milligrams of each purified polypeptide were loaded onto the ion exchange column, washed with four column volumes of 20 mM Tris-HCl pH 7.1, 100 mM KCl, 1 mM DTT and 1 mM EGTA in D_2O , and eluted using the same buffer containing 250 mM KCl.

Ure2p does not assemble into fibrils in D_2O . We therefore assembled Ure2p into fibrils in H_2O as described above and then transferred the fibrils into D_2O . This was achieved by ultracentrifuging the fibrils at 100 000 g for 20 min and resuspending the pellets containing the fibrils in 20 mM Tris pH 7.1, 100 mM KCl, 1 mM DTT and 1 mM EGTA in D_2O . This operation was repeated three times to ensure the sample was in D_2O .

The spectra of the soluble forms of Ure2p 1–354, Ure2p 95–354 and Ure2p fibrils in D₂O were recorded on a Nicolet 560 FTIR spectrometer using attenuated total reflectance mode. The background consisted of buffer A in D₂O. One thousand and twenty-four interferograms were collected with a resolution of 2 cm⁻¹. Second derivative spectra were calculated from smoothed primary spectra. Spectral analyses were performed using OMNIC Series Software version 5.0 (Nicolet).

Additional methods

Protein concentrations were determined by the Bradford method (Bradford, 1976). Alternatively, the concentrations of full-length Ure2p as well its C-terminal domain were determined spectrophotometrically (HP 8453 diode array spectrophotometer; Hewlett-Packard) using an extinction coefficient of 0.67 mg/cm² at 280 nm and molecular weights of 40 200 and 29 900 Da for full-length and Ure2p 95–354, respectively. Standard SDS–PAGE was performed in 10% gels following the method described by Laemmli (Laemmli, 1970).

Cross-linking of the Ure2pC221 variant was achieved by incubation in 30 mM H₂O₂ at 4°C for 120 min.

Proteolytic digestions were performed on Ure2p fibrils (3 mg/ml) in 50 mM Tris pH 7.5, 100 mM KCl, 1 mM EGTA and 1 mM DTT at 37°C using proteinase K (10 µg/ml).

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