



The fibrils of Ure2p homologs from *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* have similar cross- β structure in both dried and hydrated forms

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ABSTRACT

The ability to convert into amyloid fibrils is a common feature of prion proteins. However, not all amyloid-forming proteins act as prions. Here, we compared two homologs of the yeast prion protein Ure2 from *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*, ScUre2p and SpUre2p, which have different prion propensities *in vivo*. We also addressed the controversial issue of whether hydrated fibrils of Ure2 show a fundamentally different X-ray diffraction pattern than dried samples. Using Fourier transform infrared spectrometry (FTIR) and wide angle X-ray scattering of dried and concentrated hydrated fibrils, we compared the fibril structure of ScUre2p and SpUre2p. The results show that fibrils of ScUre2p and SpUre2p have a similar cross- β core under dried and hydrated conditions, with the same inter-strand and inter-sheet spacings. Given the different prion propensity of the two Ure2p homologs, this suggests that the detailed organization of the cross- β core may play an important role in the efficiency of prion propagation.

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1. Introduction

Certain proteins can readily convert from their soluble functional conformation to highly ordered amyloid structures. Formation of amyloid structure is associated with a number of neurodegenerative disorders, such as Alzheimer's, Parkinson's and the prion diseases, and is also associated with biological functions, such as curli formation in bacteria (Barnhart and Chapman, 2006; Chiti and Dobson, 2006; Tuite and Serio, 2010). Prions are proteins that can transmit their abnormal pathologic conformation to normal molecules of the same protein, without the assistance of nucleic acid (Prusiner, 1998; Uptain and Lindquist, 2002; Wickner et al., 2004). The discovery of fungal prions has provided a powerful experimental model for probing and understanding the complex biological, biochemical and biophysical aspects of the prion phenomenon (Perrett and Jones, 2008; Wickner et al., 2007).

The non-Mendelian element [URE3] in yeast has been shown to be the prion form of the nitrogen metabolism regulator Ure2p

(Wickner, 1994). In the presence of a good source of nitrogen, normal soluble Ure2p interacts with the transcription factor Gln3 and prevents it from entering into the nucleus. As a consequence, the genes needed to utilize poor nitrogen sources are repressed (Blinder et al., 1996). When Ure2p switches to the prion state, the regulatory function of Ure2p is lost and this loss-of-function phenotype allows the detection of its prion state [URE3]. Ure2p is composed of two structural domains (Perrett et al., 1999; Thual et al., 1999). The unstructured N-terminal region of Ure2p, also referred to as the prion domain (PrD), is required for its prion properties *in vivo* (Masison and Wickner, 1995) and for amyloid formation *in vitro* (Jiang et al., 2004; Taylor et al., 1999). In the soluble state, the PrD is sensitive to proteases; however, in the amyloid fibril state, the PrD becomes highly resistant to proteases (Baxa et al., 2003; Thual et al., 1999), indicative of a dramatic conformational change in the PrD. The C-terminal region of Ure2p is the globular functional domain, showing structural similarity to glutathione transferases (GSTs) (Bousset et al., 2001; Umland et al., 2001) and this region is necessary and sufficient for its regulatory function in yeast (Coschigano and Magasanik, 1991). It was found that Ure2p has glutathione peroxidase and glutaredoxin activities *in vitro* and the formation of amyloid fibrils does not disrupt these enzymatic activities (Bai et al., 2004; Zhang and Perrett, 2009).

ScUre2p and SpUre2p are homologous Ure2 proteins that exist in the closely related hemiascomycetous yeasts, *Saccharomyces*

Abbreviations: FTIR, Fourier transform infrared spectroscopy; PrD, prion domain; WAXS, wide angle X-ray scattering.

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cerevisiae and *Saccharomyces paradoxus*, respectively (Baudin-Baillieu et al., 2003). They have identical C-terminal functional domains and differ by only a few residues in their PrD (Fig. 1). *In vivo*, SpUre2p shows a lower propensity to behave as a prion (Talarek et al., 2005) and its prion propagation can be greatly affected by its cellular concentration (Crapeau et al., 2009). Further, *in vitro* and *in vivo* studies suggested that the fibrils of ScUre2p and SpUre2p have different mechanical resistance (Immel et al., 2007). By using a combination of kinetic theory with solution and biosensor assays, the rate constants involved in the fibril formation of ScUre2p and SpUre2p have been compared directly (Wang et al., 2011). It was found that a faster elongation rate but slower breakage rate accompanies the reduced prion propensity of SpUre2; and the breakage rate was found to be influenced by the structure of the fibril seeds, which can be transmitted through self-propagation.

The propagation of prion disease between different species is highly sequence specific (Chien et al., 2003). Normally, prion proteins can adopt more than one prion conformation, which is thought to be the basis of different prion strains (King and Diaz-Avalos, 2004; Tanaka et al., 2004). The ability of prions to form distinct strains is responsible for the initiation and elimination of species barriers (Tanaka et al., 2005), which is an extremely important and challenging research area to explore. To date, X-ray diffraction has played an important role in the elucidation of the structure of amyloid fibrils and the combination of X-ray diffraction with other structural techniques has enabled modeling of the structure of amyloid systems (Morris and Serpell, 2010). In this paper, we probed the internal structure of the intact fibrils and proteolytically resistant fibril cores formed by ScUre2p and SpUre2p, using Fourier transform infrared spectroscopy and wide angle X-ray scattering. Our results show that the N-terminal region of Ure2p undergoes a dramatic conformational change to form the cross- β core in the fibrillar form of Ure2p and the fibrils of ScUre2p and SpUre2p possess similar cross- β cores, with the same inter-strand and inter-sheet spacings. These results suggest that the different breakage rates observed for ScUre2p and SpUre2p *in vitro* (Wang et al., 2011) relate to details within the cross- β structure, such as the arrangement of β -strands, or the association of cross- β structures which, in turn, may determine the prion propensity *in vivo*.

2. Methods

2.1. Protein purification and fibril formation

ScUre2 and SpUre2 proteins were expressed in *Escherichia coli* with an N-terminal His tag and purified under native conditions

as described previously (Immel et al., 2007; Perrett et al., 1999). The fibril formation process was monitored by ThT assays as described previously (Zhu et al., 2003). Mature fibrils of full-length Ure2p, denoted as ScUre2-F or SpUre2-F, were formed by incubating 40 μ M ScUre2p or SpUre2p, respectively, at 4 °C without shaking in 50 mM Tris-HCl, pH 8.4, 200 mM NaCl for at least 7 days, until no further changes in ThT fluorescence could be detected indicating fibril formation had reached a plateau. Fibril cores of Ure2p, denoted as ScUre2-FC and SpUre2-FC, were produced by incubating ScUre2-F and SpUre2-F, respectively, with 0.1 mg/ml proteinase K (Sigma-Aldrich) at 37 °C for 16 h.

2.2. FTIR (Fourier transform infrared spectroscopy)

Samples of Ure2p fibrils and fibril cores were centrifuged at 18,000g (Microfuge 22R centrifuge, Beckman) at 4 °C for 30 min to separate them from any soluble protein. The fibril pellets were then resuspended in water and dried on the diamond crystal of an FTIR spectrometer (Universal ATR-FTIR, Perkin Elmer) forming a hydrated film that was monitored in attenuated transmission mode. A total of 128 interferograms were collected per spectrum from 4000 to 650 cm^{-1} with a resolution of 1 cm^{-1} . Data in the amide I region were smoothed using a five point moving average and normalized to aid comparison between samples. Differences in absorbance prior to normalization were minor with no systematic trends between samples.

2.3. X-ray diffraction

Samples of Ure2p fibrils and fibril cores were concentrated by centrifugation at 18,000g and resuspended in water or buffer (50 mM Tris-HCl, pH 8.4, 200 mM NaCl). Dried stalk samples were prepared by air-drying an aliquot (10–20 μ l) of concentrated fibrils (in water) between two wax-filled capillary ends as described previously (Squires et al., 2006; Sunde et al., 1997). Aligned hydrated samples were prepared in thin-walled soda lime glass capillaries (10 mm wall thickness, 0.3 mm diameter, Hampton Research) by gently spinning the fibrils (in buffer) from the top of a funnel-shaped tube to its sealed bottom as described previously (Squires et al., 2006).

X-ray diffraction images of dried fibril stalks and freshly prepared hydrated fibril samples were collected on the Macromolecular Crystallography beamline (MX1) of the Australian Synchrotron, Victoria, Australia (wavelength 0.95363 Å) using a Blu-Ice control system (McPhillips et al., 2002). The sample-to-detector distance was 300 mm, with exposure times of 10–50 s. Diffraction patterns

ScUre2p	MMNNGNQVSNLSNA LRQVIGNRNSNTTT DQSNINFEFSTGVNN NNNNSSSSNNNN.....
SpUre2p	MMNNGNQVSNLSNA LRQVIGNRNSNTTT DQSNINFEFSAGVNN NNNNSSSSNNNNNNN
ScUre2p	..YQNNNSGRNGSQN NDNENNIKNTLEQHR QQQQAFSDMSHVEYS RITKFFEQPLEGYT
SpUre2p	NNAQNNNSGRNGSQS NDNGNNIKDTLEQHR QQQQAFSDMSHVEYS RITKFFEQPLEGYT
ScUre2p	LFSHRSAPNGFKVAI VLSELGFHYNTIFLD FNLGEHRAPEFVSVN PNARVPALIDHGMDN
SpUre2p	LFSHRSAPNGFKVAI VLSELGFHYNTIFLD FNLGEHRAPEFVSVN PNARVPALIDHGMDN
ScUre2p	LSIWESGAILLHLVN KYKGTGNPLLWSSD LADQSQINAWLFFQT SGHAPMIGQALHFRT
SpUre2p	LSIWESGAILLHLVN KYKGTGNPLLWSSD LADQSQINAWLFFQT SGHAPMIGQALHFRT
ScUre2p	FHSQKIASAVERYTD EVRRVYGVVEMALAE RREALVMELDTENAA AYSAGTTPMSQSRFF
SpUre2p	FHSQKIASAVERYTD EVRRVYGVVEMALAE RREALVMELDTENAA AYSAGTTPMSQSRFF
ScUre2p	DYPVWLVDKLTADI LAFVPWNNVDRIGI NIKIEFPEVYKWKTH MMRRPAVIKALRGE
SpUre2p	DYPVWLVDKLTADI LAFVPWNNVDRIGI NIKIEFPEVYKWKTH MMRRPAVIKALRGE

Fig. 1. Sequence alignment of Ure2p from *S. cerevisiae* and *S. paradoxus*. The differences in amino acid sequence between the two homologs lie within the N/Q-rich N-terminal prion domain (boxed area), while the C-domains are identical.

were converted to tiff files using the program fit-2d (Hammersley/ESRF) and radially integrated to generate one dimensional scattering patterns using Matlab code, as described previously (Gras and Squires, 2011; Squires et al., 2006). The X-ray scattering patterns for dried fibrils were radially integrated in either the equatorial and meridional direction using a 30° sector in the azimuthal direction on either side of the reflection. The position of maximal intensity, d , was used to determine the position of reflections and the difference in the position of d either side of the pattern was used to estimate the error in the position of the reflection. The X-ray scattering patterns for hydrated fibrils were integrated in the radial direction for all azimuthal angles. Patterns were then scaled relative to the background pattern obtained from a buffer filled capillary. The scaling factor was the ratio of the sample to buffer at the point $1/d = 0.15 \text{ \AA}^{-1}$, as described previously (Squires et al., 2006). The background pattern was then subtracted from the scaled hydrated pattern and the position of reflections, d , defined using the position of maximum intensity. One dimensional patterns for dried and hydrated fibrils were normalized using the intensity of the inter-strand peak.

3. Results

3.1. The fibrils of ScUre2p and SpUre2p have a β -sheet core

The secondary structure content of ScUre2-F and SpUre2-F fibrils were examined by FTIR spectroscopy (Fig. 2). A broad peak was observed at $1646\text{--}1650 \text{ cm}^{-1}$, typical of proteins with a high helical content (Barth and Zscherp, 2002). This structure can be attributed to the fibrils as no native protein is expected to be present under these conditions (Bai et al., 2004; Zhang and Perrett, 2009). The proteolytically resistant fibril cores of ScUre2-F and SpUre2-F were then produced by proteinase K digestion. As shown in Fig. 2, following proteinase K digestion, the peak shifted to $1627\text{--}1628 \text{ cm}^{-1}$, indicating that the proteolytically resistant fibril cores of ScUre2p and ScUre2p have a high β -sheet content. This result implies that the high helical content of ScUre2-F and SpUre2-F fibrils is mainly produced by the C-terminal globular domain of Ure2p ($\sim 30 \text{ kD}$), whose signal is superimposed onto that of the fibril core of Ure2p ($\sim 10 \text{ kD}$) before digestion with proteinase K. The degradation of the C-terminal domain of Ure2p fibrils enabled the structure of the fibril core to be exposed facilitating the study of the region critical for fibril formation and stability.

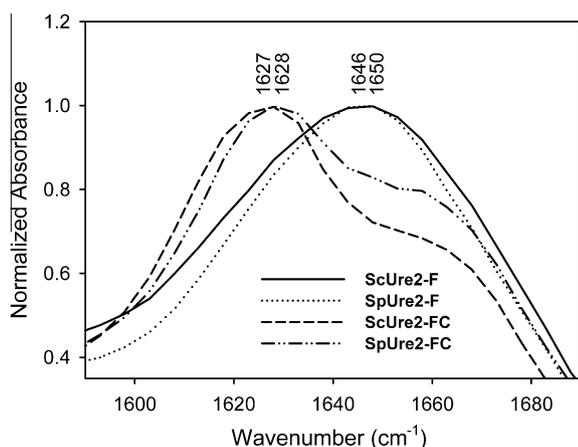


Fig. 2. FTIR spectra of fibrils and fibril cores of ScUre2p and SpUre2p. FTIR spectra of fibrils assembled from full-length ScUre2p before (ScUre2-F, solid line) and after (ScUre2-FC, dashed line) digestion with proteinase K and fibrils of full-length SpUre2p before (SpUre2-F, dotted line) or after (SpUre2-FC, dash-dot-dot line) digestion with proteinase K.

3.2. Fibrils of ScUre2p and SpUre2p have a similar cross- β structure

The structure of ScUre2p and SpUre2p fibrils and proteolytically resistant fibril cores were probed further by wide angle X-ray scattering. The patterns from dried ScUre2-F and SpUre2-F fibril samples are both anisotropic and resemble the classical pattern of other amyloid fibrils, with a strong sharp peak at 4.8 ± 0.1 or $4.9 \pm 0.1 \text{ \AA}$ respectively and a relatively broad peak around 10.3 ± 0.1 or $10.5 \pm 0.1 \text{ \AA}$ respectively (Fig. 3 and Table 1). Most importantly, the meridional or equatorial orientation of the reflections at 4.8 \AA corresponding to the inter-strand spacing, or $\sim 10.0 \text{ \AA}$ corresponding to the inter-sheet spacing, indicate a cross- β structure within ScUre2-F and SpUre2-F fibrils, with similar inter-strand and inter-sheet spacings.

Further, the proteolytically resistant fibril cores of ScUre2p and SpUre2p produced by proteinase K digestion show clear reflections with greater alignment compared to the reflection patterns of ScUre2p-F and SpUre2-F (Fig. 3 and Table 1). These patterns contained a meridional reflection at 4.9 ± 0.1 or $4.8 \pm 0.1 \text{ \AA}$ respectively, and a well defined broad equatorial reflection at 9.8 ± 0.3 or $10.4 \pm 0.6 \text{ \AA}$ respectively. Consistent with the FTIR results, this alignment indicates that the degradation of the C-terminal domain is helpful in observing the structural details of the fibril cores. An additional broad reflection at $\sim 3.9 \text{ \AA}$ is present at the meridian in patterns from both ScUre2-FC and SpUre2-FC. This reflection has been observed at the meridian or off-meridian position for a number of other aligned fibrils, including Ure2p10-39 (Baxa et al., 2005). A small shoulder is observed in a similar position in the patterns for ScUre2-F and SpUre2-F fibrils but the enhanced intensity of this reflection for digested fibrils is further evidence of more ordered samples with greater alignment following treatment with proteinase K.

3.3. Hydrated and dehydrated fibrils of ScUre2p and SpUre2p have a similar cross- β structure

Several studies have shown that the dehydration process during sample preparation for X-ray diffraction or repeated drying on a surface may greatly affect the structure of amyloid fibrils for some proteins, such as Sup35p, IAPP and A β (1–42) (Kishimoto et al., 2004; Maurstad et al., 2009). To explore the effect of the dehydration process on the structure of Ure2 fibrils, hydrated forms of both fibrils and proteolytically resistant fibril cores of ScUre2p and SpUre2p were subjected to X-ray diffraction. The diffraction patterns of the hydrated fibrillar samples and positions of the reflections are shown in Fig. 4 and Table 1. Isotropic diffraction patterns were acquired from both fibrils and proteolytically resistant fibril cores of ScUre2p and SpUre2p. Well defined reflections at approximately 4.8 \AA were obtained from all hydrated samples and weak reflections at approximately 10 \AA were observed from hydrated ScUre2-F and SpUre2-F, consistent and within error of the positions determined from dried samples. These results indicate that the stacks of β -sheets in dried Ure2p fibrils are not an artifact produced by the dehydration process. Nor are the fibrils of Ure2p a β -helix in the hydrated state as suggested for the fibrils of Sup35p (Kishimoto et al., 2004) and a poly-L-glutamine peptide (Perutz et al., 2002). The absence of 10 \AA reflections from hydrated ScUre2-FC and SpUre2-FC is likely due to the lower intensity from ScUre2-FC and SpUre2-FC samples, so that the peak is too weak to be observed above the background noise in these patterns. An additional reflection is also observed at $\sim 4.0 \text{ \AA}$ for ScUre2p fibrils that have been digested with proteinase K. The position of this peak is similar to that observed at $\sim 3.9 \text{ \AA}$ for the dried ScUre2-FC and SpUre2-FC samples.

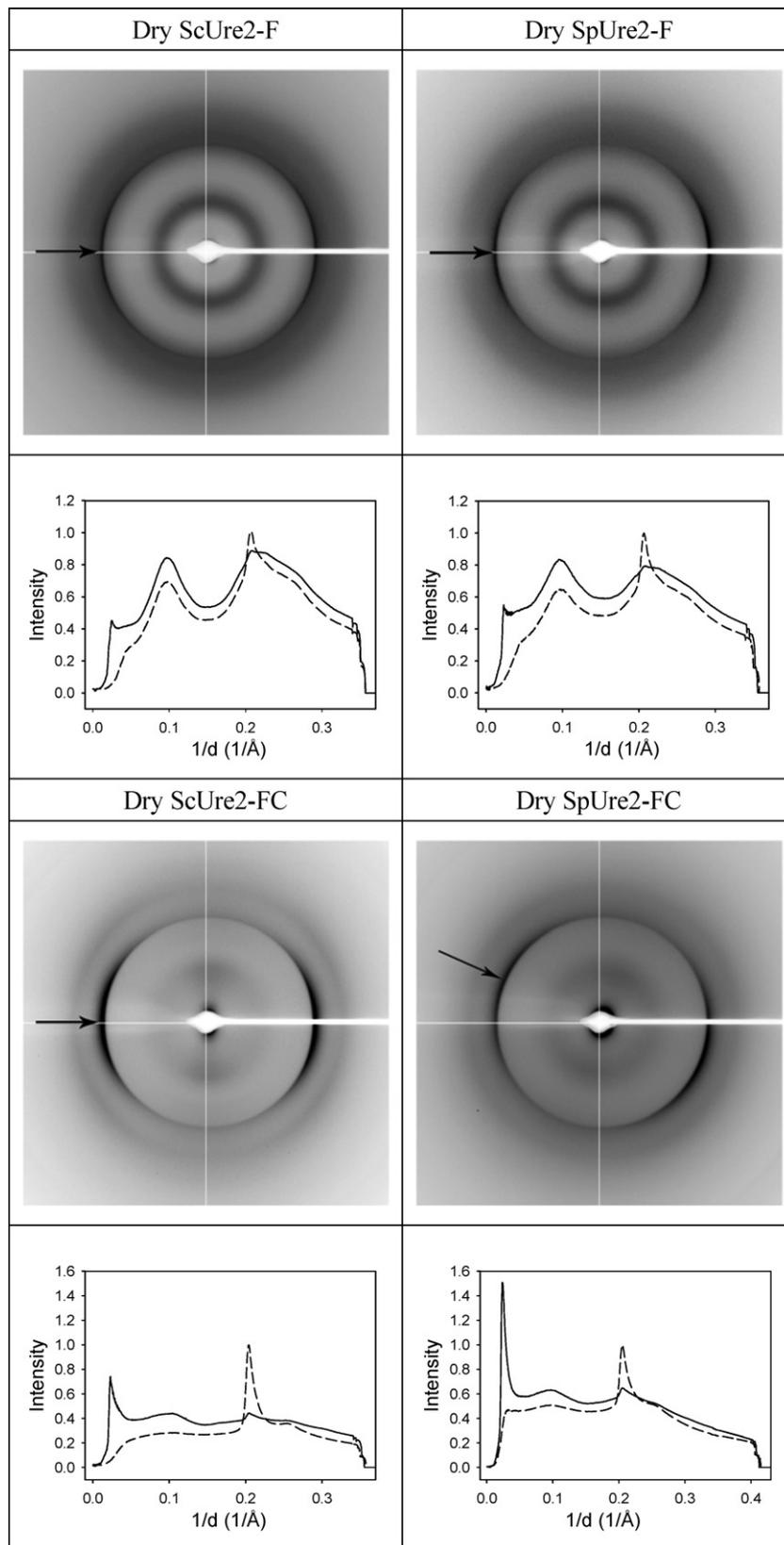


Fig. 3. X-ray diffraction patterns for dehydrated fibrils and fibril cores of ScUre2p and SpUre2p. Two and one dimensional WAXS patterns from dried Ure2 fibrils and proteolytically resistant fibril cores. Full-length ScUre2p or SpUre2p were incubated to form mature fibrils (ScUre2-F and SpUre2-F) and then digested with proteinase K (ScUre2-FC and SpUre2-FC) as described in Section 2. The meridional directions are shown by the arrows. One dimensional patterns are in the equatorial (solid line) and meridional (dashed line) directions. The intensities of the 1D diffraction were normalized relative to the maximum intensity observed for the inter-strand peak at around 4.8 Å. The positions of reflections are summarized in Table 1.

Table 1
Position of WAXS reflections from Ure2p fibril samples.

	ScUre2-F		SpUre2-F		ScUre2-FC		SpUre2-FC	
	D	H	D	H	D	H	D	H
Dry/hydrated								
Reflection 1	10.3 ± 0.1	10.7 ^b	10.5 ± 0.1	10.7 ^b	9.8 ± 0.3	–	10.4 ± 0.6	–
Reflection 2	4.8 ± 0.1	4.8 ^b	4.9 ± 0.1	4.9–4.8 ^{a,b}	4.9 ± 0.1	4.9 ^b	4.8 ± 0.1	4.8 ^b
Reflection 3	~4.0 ^c	–	~4.0 ^c	–	4.0 ± 0.1	~4.0 ^b	3.9 ± 0.1	–
Alignment	+	–	+	–	++	–	++	–

Units are in Å.

^a A broad reflection was detected.

^b Patterns were isotropic and radial integration was performed for all azimuthal angles. Errors in the position of reflections determined for isotropic samples are expected to be similar to those estimated using two 30° sectors for aligned samples.

^c These reflections correspond to a small shoulder.

4. Discussion

Amyloid fibrils are characterized by a typically protease-resistant cross- β architecture, where the β -strands of the β -sheet run perpendicular to the long axis of the filaments (Sunde et al., 1997). Evidence of this characteristic cross- β diffraction pattern by X-ray diffraction has been used as criteria for fibril formation (Nilsson, 2004). Currently, there are three models with respect to chain conformation and chain packing: the classic cross- β models, β -helix-like structures and native-like structures; a comparison of calculated and experimental X-ray diffraction patterns suggests that the cross- β structure is most likely to be the core structure of amyloid fibrils (Jahn et al., 2010). Ure2p is one of the best characterized yeast prion proteins and the amyloid fibril structure of Ure2p has been studied by using many biochemical and biophysical techniques (Lian et al., 2006). Two models for Ure2 fibrils have been proposed as a result of these recent studies; the “amyloid backbone” model states that the PrD of Ure2p polymerizes into an amyloid core fibril that is surrounded by C-terminal domains in their native conformation (Baxa et al., 2003, 2005; Kajava et al., 2004); and the “native-like fibril” model claims that the extent of conformational change in the PrD of Ure2p is limited and that Ure2p fibrils assembled under physiological conditions are devoid of a cross- β core (Bousset et al., 2003). In this study, well defined cross- β diffraction patterns were obtained from fibrils of ScUre2p (as demonstrated before Baxa et al., 2005) and SpUre2p, in both dehydrated and hydrated forms (Figs. 3 and 4 and Table 1). In addition, the diffraction patterns of proteolytically resistant Ure2p fibril cores were consistent with those of intact fibrils, strongly supporting the amyloid backbone model.

Antibody-binding studies have indicated that the prion domain of Ure2p is buried within Ure2 fibrils with its C-terminal exposed (Speransky et al., 2001) and several studies show that the C-terminal region of Ure2p maintains its functional fold in the fibrillar form (Bai et al., 2004; Baxa et al., 2002; Bousset et al., 2002; Zhang and Perrett, 2009; Zhang et al., 2008). It was observed that there are differences between the isolated PrD and the authentic protein (Loquet et al., 2009) and that the diameter of Ure2 fibrils can be reduced from 25 to 4 nm by proteinase K digestion, revealing cores primarily composed of residues 1–70 of Ure2p (Baxa et al., 2003). In this study, instead of observing the fibrils produced by the isolated PrD of Ure2p (such as Ure2p1-89/93), we directly detected the fibril cores produced by proteinase K digestion using FTIR and X-ray diffraction. As shown in a previous study (Bousset et al., 2002), the FTIR spectrum of Ure2 fibrils contained a high α -helical content. However, fibril cores produced by proteinase K digestion showed a high β -sheet content. Consistent with the “amyloid backbone” model, β -sheet structure was observed after the C-terminal regions of Ure2p were degraded. These results provide further evidence that the N-terminal domain of Ure2p undergoes significant conformational change, forming the cross- β core of the fibril.

The basis of prion strains is another critical question in prion biology. Intriguingly, multiple prion strains can arise from the same prion protein, distinguished by distinct incubation periods and patterns of neuropathology in mammals (Collinge and Clarke, 2007), or different mitotic stability and phenotype intensity in yeast (Brachmann et al., 2005; DePace and Weissman, 2002). Moreover, different molecular structures between multiple prion strains were also detected by various methods, such as FTIR (Jones and Surewicz, 2005), solid state Nuclear Magnetic Resonance (ssNMR) (Petkova et al., 2005), fluorescence (Krishnan and Lindquist, 2005) or mass spectrometry (Myers et al., 2006). Our previous studies have shown that the breakage propensity of seed fibrils can be transmitted to daughter fibrils during the propagation of ScUre2p and SpUre2p (Wang et al., 2011). This implies that there may be some structural differences in the fibril that lead to the observed differences in strength. Differences between recombinant (rec) PrP amyloid and highly infectious brain-derived prions were also found by X-ray diffraction (Wille et al., 2009). Given the low infectivity of rec PrP amyloid, this may imply that there are structural differences between prion and non-prion amyloid fibrils. However, the values for inter-strand and inter-sheet spacing we acquired from ScUre2-F(C) and SpUre2-F(C) are indistinguishable. This suggests that the fibrils of ScUre2p and SpUre2p have similar cross- β structure in both air-dried and hydrated conditions. Their different prion properties may arise from their detailed cross- β structure, such as the hydrogen-bond network of β -strands, β -sheet arrangement, and/or association of fibrils into protofilaments. Comparison of the X-ray diffraction patterns of homologous Ure2 fibrils not only provides more information on the structural model of Ure2p fibrils but also reveals that those amyloid fibrils with different propagation properties can possess quite similar cross- β core structures, and so the detailed organization of the cross- β core may play an important role in amyloid propagation. To elucidate this detailed information for the cross- β core, further techniques such as ssNMR, small angle X-ray scattering and cryo reconstruction could be helpful.

Acknowledgments

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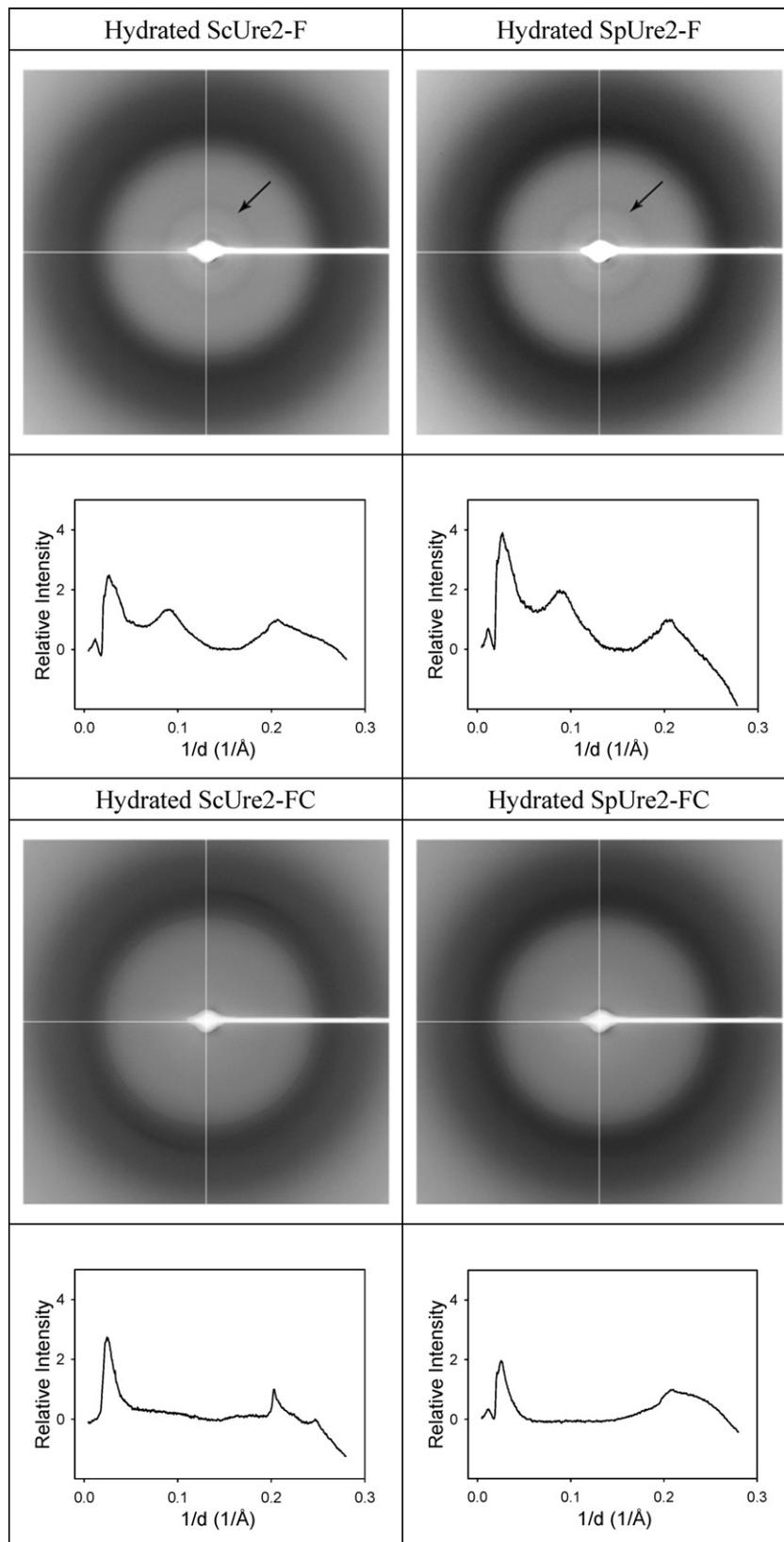


Fig. 4. X-ray diffraction patterns for hydrated fibrils and fibril cores of ScUre2p and SpUre2p. Two dimensional WAXS patterns and background-subtracted one dimensional diffraction patterns from hydrated Ure2 fibrils and proteolytically resistant fibril cores. Full-length ScUre2p or SpUre2p were incubated to form mature fibrils (ScUre2-F and SpUre2-F) and then digested with proteinase K (ScUre2-FC and SpUre2-FC) as described in Section 2. The intensities of the one dimensional diffraction patterns were normalized to the inter-strand peak at around 4.8 Å. The positions of reflections are summarized in Table 1. Reflections at 4.8 Å can be observed in all hydrated samples. The reflections at approximately 10 Å appear in the diffraction pattern of ScUre2-F and SpUre2-F indicated by the arrows.

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