Fungal prions are infectious filamentous polymers of proteins that are soluble in uninfected cells. In its prion form, the HET-s protein of Podospora anserina participates in a fungal self/non-self recognition phenomenon called heterokaryon incompatibility. Like other prion proteins, HET-s has a so-called "prion domain" (its C-terminal region, HET-s-(218–289)) that is responsible for induction and propagation of the prion in vitro and for fibril formation in vitro. Prion fibrils are thought to have amyloid backbones of polymerized prion domains. A relatively detailed model has been proposed for prion domain fibrils of HET-s based on a variety of experimental constraints (Ritter, C., Maddelein, M. L., Siemer, A. B., Luhrs, T., Ernst, M., Meier, B. H., Saupe, S. J., and Riek, R. (2005) Nature 435, 844–848). To test specific predictions of this model, which envisages axial stacking of β-solenoids with two coils per subunit, we examined fibrils by electron microscopy. Electron diffraction gave a prominent meridional reflection at (0.47 nm)−1, indicative of cross-β structure, as predicted. STEM (scanning transmission electron microscopy) mass-per-unit-length measurements yielded 1.02 ± 0.16 subunits per 0.94 nm, in agreement with the model prediction (1 subunit per 0.94 nm). This is half the packing density of ~1 subunit per 0.47 nm previously obtained for fibrils of the yeast prion proteins, Ure2p and Sup35p, whence it follows that the respective amyloid architectures are basically different.

When mycelia of filamentous fungi fuse, various het loci screen this process for genetic similarity (i.e. self-recognition), a process termed heterokaryon incompatibility (1). If, in an initial fusion reaction, the mycelia are determined to have different genotypes, programmed cell death is triggered, aborting the fusion; on the other hand, if the mycelia are determined to have the same genotypes, fusion proceeds. The het-s locus, one of several het loci in Podospora anserina can have two possible alleles, het-s or het-S, coding for the two proteins HET-s and HET-S, respectively. Both HET-s and HET-S are 289-residue proteins, differing in just 13 amino acids (2), but only HET-s is known to form prions (1, 3), and only het-s strains harboring the prion form are active in heterokaryon incompatibility toward het-S strains. Recombinant HET-s protein forms fibrils in vitro that are amyloid by the characteristics of Congo red binding, resistance to proteinase K, and high β-sheet content (4, 5). This material shows infectivity when introduced into naïve, i.e. uninfected mycelia (5). HET-s has two domains: residues 1 to approximately 220, which form a well folded and presumably globular domain; and the C-terminal prion domain (6, 7). In the soluble state of HET-s, its prion domain is unfolded. This property is shared by the yeast prion proteins Ure2p (8) and Sup35p (9, 10), whose prion domains are larger, enriched in Asn and Gln residues, and located at their N termini. In the absence of the remainder of HET-s, its prion domain has the propensity to fibrillize and to infect (5, 6, 11). These are also properties that HET-s holds in common with Ure2p and Sup35p (12–14). When expressed in P. anserina, the HET-s prion domain alone can propagate the prion, but is not functional in heterokaryon incompatibility. To restore this function, at least parts of its N-terminal domain are needed (15).

Recently, a β-solenoid model has been proposed for HET-s-(218–289) fibrils, based on mutational analysis, hydrogen/deuterium exchange, and solid-state NMR spectroscopy (11, 16). Generically, solenoid proteins are axially coiled structures with each coil containing a set of secondary structure elements (17, 18). In β-solenoids, these elements are β-strands and turns; special cases are the β-roll, with two strands and turns per coil, and the β-helix with three strands and turns per coil (17, 18, 19). Several β-solenoid models have been proposed for different amyloids (20, 21). Such models may be classified in terms of the number of protofibrils per fibril; the number of coils per subunit in a protofibril; and the particular β-solenoid fold (22). The model proposed for HET-s fibrils envisages a single protofibril and a β-roll fold with two coils per subunit (Fig. 1A; 16). The observation that the HET-s prion domain sequence has two similar subdomains was accommodated by postulating that...
HET-s Prion Fibril Structure

each subdomain forms a coil of the solenoid, and the experimental determination that each subdomain has two β-strands led to the proposed β-roll fold (Fig. 1a).

This model makes predictions that are testable, i.e. the presence of cross-β structure in the fibril; and that its axial packing density should correspond to one subunit per 0.94 nm. Cross-β structure may be detected by electron diffraction (23, 24). Axial packing density may be determined from mass-per-unit-length measurements made by dark-field scanning transmission electron microscopy (STEM)3 (25, 26). In this study, we have applied these techniques to fibrils of the prion domain HET-s-(218–289), and of HET-s-(157–289), a construct containing an additional segment that, when appended to the prion domain, restores competence in heterokaryon incompatibility (15). Our observations lend strong support to the model.

EXPERIMENTAL PROCEDURES

Plasmids—Construction of Escherichia coli expression plasmids for C-terminally histidine-tagged versions of both polypeptides has been described (6, 15). In brief, the reading frame of HET-s-(218–289) and HET-s-(157–289) was amplified by PCR and cloned into pET-21a using NdeI and HindIII restriction sites. The resulting plasmids code for Met-HET-s-(218–289)-His6 and Met-HET-s-(157–289)-His6, respectively. It has been determined that the C-terminal histidine tag does not affect the biological activity of HET-s in P. anserina (4).

Preparation of HET-s-(218–289) and HET-s-(157–289) and Fibril Formation—The histidine-tagged polypeptides were expressed in E. coli BL21 (DE3) pLysS and isolated, as has been described for full-length HET-s (4). Both polypeptides were pure as judged by SDS gels stained with Coomassie Blue. Mass spectrometry was used to verify their identities, but showed that the N-terminal methionine was cleaved off from HET-s-(218–289), resulting in molecular masses of 8519.4 Da (HET-s-(218–289)) and 15189.8 Da (HET-s-(157–289)). For fibril assembly, denaturant-containing buffer was exchanged to buffer without denaturants at the desired pH value. For fibril formation at neutral pH, a buffer of 20 mM phosphate, 150 mM NaCl, pH 7.4 was used, whereas fibril formation at lower pH values was performed either in 50 mM phosphate at pH 2.0 or in 20 mM citrate at pH 2.0, pH 3.3, or pH 3.9, respectively. Cross-linked fibrils of HET-s-(218–289)-His6 and Met-HET-s-(157–289)-His6 were present, they were fitted either by including additional independent Gaussian components or by fitting Gaussians to the residual distribution left after subtracting the first fitted Gaussian. The number of free parameters was thereby assigned corresponding width and peaks (mass-per-unit-length values). The number of free parameters was thereby reduced to 8 from the 18 that would be needed if all the Gaussians were to be fitted independently.

Conventional Transmission Electron Microscopy and Electron Diffraction—For negative staining, samples were sonicated in a bath sonicator (Crest Tru-Sweep) for 10–20 min, adsorbed to freshly glow-discharged carbon-coated grids, rinsed with water, and stained with 1% uranyl acetate. Sonication was found to be necessary for reproducible dispersal of singlet fibrils. Micrographs were recorded on a Philips CM120 microscope. For electron diffraction, specimens were changed to water by ultracentrifugation (20,000 g × 30 min), immediately embedded in sucrose, and diffraction patterns recorded from areas of ~3 μm diameter at low electron doses of 1–4 e/Å2 on a Philips CM120 microscope. Spacing were calibrated with graphitized carbon and evaporated thallous chloride.

Scanning Transmission Electron Microscopy—Freshly prepared fibrils were lightly sonicated (5 s, 60 watts) and checked for optimal length and dispersal by negative staining as described above. Samples deemed to be adequate were frozen rapidly in liquid nitrogen and stored until further processing. Freeze-dried specimens were prepared according to the standard method of the Brookhaven STEM facility (www.biology.bnl.gov/ stem/stem.html). Briefly, titanium grids were coated with a thick holey (5–10 μm) carbon film that supported a thin (2–3 nm) continuous carbon film. The thin carbon film was prepared by ultra-high vacuum evaporation onto a freshly cleaved crystal of rock salt and was kept under water and not exposed to air at any point to avoid unspecific adsorption of material. 2 μl of tobacco mosaic virus solution (100 μg/ml) was injected into the drop on the grid and incubated for 1 min. After washing with water four times, 2 μl of the specimen was injected into the drop and incubated for 1 min. Ten more washes in water were performed, and after the final wash, the grid was pinched between the two pieces of filter paper (leaving a retained layer of solution of less than 1 μm) and immediately plunged into liquid nitrogen slush. The grid was transferred to an ion-pumped freeze dryer, freeze-dried overnight by gradual warming to ~80 °C and transferred under vacuum to the STEM. Grids were scanned at −150 °C on a custom-built STEM at 40 kV with a probe focused to 0.25 nm with a dwell time of 30 μs/pixel.

Analysis of STEM Images—Digital dark-field micrographs of freeze-dried specimens were recorded with 512 × 512 pixels at raster steps of 1.0 or 2.0 nm per pixel. The images were analyzed using the PCMass (available from the Brookhaven STEM resource; 28) and PIC (29) programs. Boxes of 25 nm length and appropriate width (16 nm for singlet HET-s fibrils and 22 nm for tobacco mosaic virus particles and thicker HET-s fibrils) were used for the measurements. The resulting data were normalized to the known mass-per-unit-length of tobacco mosaic virus (131.4 kDa/nm).

Histograms were calculated with 1-kDa/nm bins. A Gaussian distribution was then fitted to the main peak using the Marquardt-Levenberg algorithm, as implemented in SigmaPlot (Systat Software). If smaller peaks corresponding to doublet and triplet fibrils were present, they were fitted either by including additional independent Gaussian components or by fitting Gaussians to the residual distribution left after subtracting the first fitted Gaussian. The results are given in Table 1. The more complex distribution found for fibrils at pH 2.0 was fitted, based on the assumption that the largest peak represented triplet fibrils and that the other masses observed were different multiples (doublets to septuplets) of the same basic fibril. In this case, all Gaussian components were assigned corresponding width and peaks (mass-per-unit-length values). The number of free parameters was thereby reduced to 8 from the 18 that would be needed if all the Gaussians were to be fitted independently.

Measurement of Variation in Fibril Width Along Its Length—Fibrils for measurements were selected from unstained STEM images. We used PCMass (28) and Bsoft (30) to measure their widths at 5-nm steps along the fibril. At each point, a transverse

3 The abbreviation used is: STEM, scanning transmission electron microscopy.
HET-s Prion Fibril Structure

RESULTS AND DISCUSSION

In negative stain, HET-s-(218–289) and HET-s-(157–289) fibrils appear smooth-surfaced and are 4.4 ± 0.3 nm and 5.9 ± 0.4 nm, respectively, in diameter (Fig. 1, c and d). The model (16) predicts a fibril cross-section with a long dimension given by strands β2 and β4, which have 11 residues, corresponding to a strand length of 3.85 nm and a short dimension given by stacking two β-sheets (~2 nm). Allowing some uncertainty in edge detection in the micrographs and the possibility of additional contributions to fibril width from flexible inter-strand loops and ends (31), these numbers are compatible with the measured width of HET-s-(218–289) fibrils. The greater width of HET-s-(157–289) fibrils presumably reflects the presence of the N-terminal appendages. Measurement of diameters from unstained STEM images yielded 4.9 ± 0.4 nm and 5.8 ± 0.4 nm for HET-s-(218–289) and HET-s-(157–289), respectively, in good agreement with the data from negative stain. No systematic variations in width along a given fibril were observed for either construct.

Fibrils of both constructs have a marked tendency to bundle into large cable-like structures with diameters of ~20 to >200 nm (supplemental Fig. 1; 6, 15). The average center-to-center spacing of fibrils in a bundle may be measured in terms of spacings of reflections in diffraction patterns calculated from these images. In this way, we measured a spacing of 4.9 ± 0.3 nm for bundles of HET-s-(218–289) fibrils (supplemental Fig. 1a). Again, this number is in good agreement with diameters estimated from negatively stained as well as unstained STEM images of singlet fibrils.

Electron diffraction shows a strong meridional reflection at a spacing of (0.47 nm)−1 with both specimens, establishing the presence of cross-β structure (Fig. 1, e and f; supplemental Fig. 1). Images of the specimen areas that yielded these electron diffraction patterns (e.g. Fig. 1f) showed that they contained reflection at (0.47 nm)−1 (arrow). Similar data (not shown) were obtained for HET-s-(157–289) fibrils. g and h, dark-field micrographs and distributions of STEM mass-per-unit-length measurements of unstained, freeze-dried, specimens of (g) HET-s-(218–289) and (h) HET-s-(157–289) fibrils made at neutral pH. The thicker straight rods (white) are calibration particles of tobacco mosaic virus. Bar = 100 nm. Gaussian distributions were fitted to the histograms (solid curves); black arrows indicate the positions of each Gaussian peak.

FIGURE 1. Two classes of models for amyloid fibrils. a, the stacked β-solenoid model of Ritter et al. (16), with two coils of a β-roll per subunit, in near-lateral view. b, stacked β-serpentine (34), with a 4-stranded serpentine, in near-axial view. c and d, electron micrographs of fibrils of (c) HET-s-(218–289) and (d) HET-s-(157–289) prepared at neutral pH and negatively stained with uranyl acetate. Bar = 100 nm. e, electron diffractogram from a field (f) of partially oriented unstained HET-s-(218–289) fibrils shows a strong meridional
HET-s Prion Fibril Structure

TABLE 1
Mass-per-unit-length measurements of unstained HET-s fibrils by STEM

| Construct and conditions | Mass per unit length | Relative amounts of each observed species | Subunits/0.94 nm (for multiple fibrils, measured density was divided by number of singlet fibrils) |
|-------------------------|---------------------|----------------------------------------|-------------------------------------------------------------------------------------------------|
| HET-s-(218–289), pH 7.4 | 9.20 ± 1.44* (singlet) | 100.0 | 1.02 ± 0.16 |
| HET-s-(157–289), pH 7.4 | 13.80 ± 2.02 (singlet) | 88.8 | 0.85 ± 0.13 |
| | 30.47 ± 3.73 (doublet) | 10.0 | 0.95 ± 0.16 |
| | 39.79 ± 1.02 (triplet) | 1.2 | 0.82 ± 0.03 |
| HET-s-(218–289), pH 3.9 | 9.00 ± 1.53 (singlet) | 86.3 | 0.99 ± 0.17 |
| | 17.50 ± 2.50 (doublet) | 12.7 | 0.97 ± 0.20 |
| | 24.70 ± 1.93 (triplet) | 1.0 | 0.91 ± 0.12 |
| HET-s-(218–289), pH 3.3 | 9.52 ± 1.18 (singlet) | 92.7 | 1.05 ± 0.13 |
| | 18.79 ± 2.57 (doublet) | 5.5 | 1.04 ± 0.20 |
| | 24.85 ± 1.75 (triplet) | 1.8 | 0.91 ± 0.11 |
| HET-s-(218–289), pH 2.0 | 8.60 ± 1.97 (singlet) | 0.0 | |
| | 17.19 ± 2.79 (doublet) | 0.8 | |
| | 25.79 ± 3.42 (triplet) | 75.8 | |
| | 34.39 ± 3.95 (quadruplet) | 0.1 | |
| | 42.98 ± 4.41 (quintuplet) | 2.6 | |
| | 51.58 ± 4.83 (sextuplet) | 18.4 | |
| | 60.18 ± 5.22 (septuplet) | 2.3 | |

*Standard deviations calculated from the widths of the Gaussian fits.

A Position and standard deviation of other peaks were not fitted independently (cf. Experimental Procedures).

b Bundles of HET-s fibrils with diameters of 50–100 nm (supplemental Fig. 1a). Electron diffraction from single bundles confirmed the meridional position of the (0.47 nm)−1 reflection (supplemental Fig. 1, b and c).

In STEM analysis of HET-s-(218–289) fibrils prepared at pH 7.4 (Fig. 1g), the distribution of mass-per-unit-length measurements has a single peak at 9.20 ± 1.44 kDa/nm (S.D.). With a subunit mass of 8.52 kDa, this corresponds to 1.02 ± 0.16 subunits per 0.94 nm (cf. Table 1 for a summary of all STEM measurements). The data for HET-s-(157–289) fibrils prepared at pH 7.4 (Fig. 1h) have a major peak at 13.8 ± 2.02 kDa/nm, corresponding to 0.85 ± 0.13 subunits per 0.94 nm (the subunit mass is 15.19 kDa), with a few higher values corresponding to bundles of two or three fibrils (Table 1).

Our primary interest was in fibrils formed at near-neutral pH, the same condition as was used in the studies that led to the current model. However, in view of the tendency of the fibrils to aggregate (which was eventually resolved, see above), we also explored other ionic conditions, including preparations made at acidic pH (Fig. 2). Fibrils formed at pH 3.9 and 3.3 were found to have less tendency to aggregate, and these preparations were also subjected to STEM mass analysis, yielding major peaks at 9.00 ± 1.53 kDa/nm (0.99 ± 0.17 subunits per 0.94 nm) and 9.52 ± 1.18 kDa/nm (1.05 ± 0.13 subunits per 0.94 nm), respectively, in addition to small amounts of doublet and triplet fibrils (Fig. 2, a and b; Table 1). Thus, to within experimental error, these fibrils have the same axial packing density as fibrils assembled at neutral pH.

We also made some observations on fibrils prepared at pH 2.0. Negative staining showed that they vary in width, but the narrowest have diameters of ~8–9 nm and thus are thicker than singlet fibrils formed at higher pHs. Under favorable staining conditions, these minimum-size pH 2 fibrils appear to consist of two or three narrower fibrils with diameters of 4–5 nm (e.g. Fig. 2d; blown-up micrograph at right). These fibrils (the majority; T in Fig. 2c) vary little in width along their lengths. However, other fibrils are wider and exhibit periodic variations in projected width (H in Fig. 2c; arrow in Fig. 2f). STEM mass-per-unit-length data for HET-s-(218–289) fibrils made at pH 2.0 had a major peak at 25.8 ± 3.4 kDa/nm (Fig. 2f). The simplest interpretation of this peak is that it represents triplets of fibrils that, individually, have the same axial density as fibrils prepared at pH 7.4, 3.9, or 3.3.

A singlet HET-s-(218–289) fibril at pH 2.0 would have 0.95 ± 0.22 subunits per 0.94 nm. The denser fibrils appear to be mostly bundles of six singlet fibrils (or two triplets; Fig. 2c, histogram). The interpretation that the pH 2 fibrils are mainly triplets and pairs of triplets is consistent with the observation that the putatively triplet fibrils do not vary much in projected width; this is a property of three-stranded ropes, where the individual strands have approximately circular cross-sections (supplemental Fig. 2b). However, the widths of the peaks in the STEM mass data for pH 2 fibrils are greater than those obtained at other pHs, and other interpretations of these data are possible.

The pH at which HET-s prion domain fibrils are grown appears to influence the interaction between fibrils but not to change the overall architecture of singlet fibrils. The observation that fibrils are more dispersed between pH 3.3 and 3.9 than at neutral pH suggests that interactions between charged side chains are involved in their aggregation. There are 8 negatively charged residues and one histidine residue (plus 6 histidines in the his-tag) in the HET-s-(218–289) sequence that could have a pK_a value in this range, and all except one are predicted to be on the surface of the β-roll or in flexible loops (16, 31).

In summary, mass-per-unit-length data impose a stringent constraint on viable fibril models, when the subunit mass is known. The STEM data obtained for fibrils, assembled from both constructs near neutral pH, and also for HET-s-(218–289) fibrils at acidic pHs, are consistent with the value (1.0 subunits per 0.94 nm) predicted in the stacked β-solenoid model of Ritter et al. (16). The fact that appending an additional N-terminal
HET-s Prion Fibril Structure

50 residues to the 72-residue prion domain did not alter the axial packing density in the fibril is consistent with the proposition that HET-s prion domains form an amyloid backbone that is decorated peripherally with other domains, if present. A similar backbone/periphery arrangement has been found in yeast prion filaments of Ure2p and Sup35p (10, 32–34).

However, the backbone architecture of HET-s fibrils appears to differ from those of Ure2p and Sup35p in at least one basic respect, axial packing density. Prior STEM analyses have determined one subunit per 0.47 nm for Ure2p fibrils (21) and similar values for the several classes of fibril distinguished for Sup35p (22). Although the present data provide strong support for the β-solenoid model for HET-s (16), it is also clear that this model does not apply to the yeast prion fibrils.

An alternative model, called the superpleated β-structure (35) or alternatively, the stacked β-serpentine (Fig. 1b), represents a class of cross-β fibril structures that satisfies the mass-per-unit-length data on Ure2p fibrils and most of the Sup35p fibril subtypes. Corresponding β-strands, in successive subunits, stack axially to generate parallel β-sheets. Topologically, this model differs from that of Ritter et al. (16) in terms of its serpentine fold and in having one subunit (not half a subunit) per 0.47 nm layer.

There are other reasons to suspect that HET-s fibrils differ structurally from those of the yeast prions. The amino acid compositions of the latter prion domains are rich in uncharged polar residues (Asn, Gln) and have a relative dearth of hydrophobic and charged residues. In these respects, they differ from the HET-s prion domain, which is rich in charged residues, consistent with a higher ratio of surface-exposed residues to buried residues, as in the β-solenoid model. In contrast, this ratio should be lower for β-serpentines with three or more strands per subunit (35). Moreover, there is no reason to suppose that the prion domains of Ure2p or Sup35p subdivide into two (or more) similar subdomains, which is a key consideration in the proposal of two coils per prion domain in the HET-s model.

Finally, we note that the Ure2p and Sup35p prion domains are redundant, as indicated by their prionogenic properties being unaffected by deletions (36, 37) or sequence randomization (37, 38). In contrast, the only portion of HET-s that tolerates deletions is the central loop (16; Fig. 1a). Furthermore, the yeast prions exhibit strain variants (12–14, 39, 40) that have been correlated with amyloid polymorphisms (12, 22, 27, 34, 41). The β-serpentine model readily explains such polymorphisms; for instance, with the serpentine deriving from different segments of the prion domain. It remains to be seen whether HET-s fibrils also exhibit similar polymorphisms and strain variations. In this respect, the two-coil β-solenoid appears to be less amenable to polymorphism.

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