Strain-specific morphologies of yeast prion amyloid fibrils

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Mass per length (mpl) measurements on single amyloid fibrils that specifically propagate the [VH], [VK], and [VL] strains of the yeast prion [PSI] reveal unanticipated differences in their structures. Many fibrils have ≈ 1.0 prion molecule per 4.7-Å cross- β repeat period, which is consistent with a self-replicating model built by parallel β -sheet hydrogen-bonding of like prion peptide segments, but other fibrils are definitely heavier. The predominantly straight fibrils of the dominant [VH] strain have a bimodal mpl distribution, corresponding to components with ≈ 1.0 and 1.2 prions per repeat. Fibrils of the weaker [VK] strain, which are almost all wavy, have a monodisperse mpl distribution with a mean of 1.15 prions per repeat. The recessive [VL] strain sample has ≈ 1.05 prions per repeat in single fibrils and includes $\approx 10\%$ double fibrils, which are rare in the duplicate [VH] and [VK] samples. All of these samples were assembled from purified recombinant Sup35 prion protein by seeded growth on nuclei extracted from yeast bearing the three [PSI] strains. Infectious and noninfectious spontaneously assembled fibrils of the recombinant prion protein also display different heterogeneous morphologies. The strain-specific morphological differences we have observed directly confirm the structural prediction of the protein-only prion theory but do not have an obvious molecular explanation.

mass per length | scanning transmission electron microscopy | cross- β ply | [PSI] propagation | nucleated assembly

Demonstration of protein-only transmission of distinct yeast [PSI] prion strains (1, 2) indicates that the differences between strains must somehow be embodied in distinguishable, self-propagating structural features of the infectious amyloid fibrils formed by seeded growth *in vitro* with recombinant Sup35 prion protein. Induction of prion disease in transgenic mice by injection of amyloid aggregates of recombinant mouse prion protein (3) supports the hypothesis (4) that strains of the mammalian transmissible spongiform encephalopathies are caused by self-propagating misfolded forms of the prion protein. Considering all of the evidence associating both mammalian and yeast prions with infectious amyloid fibers, and the many atomic models proposed for amyloid structures (cf. refs. 5 and 6) since the cross- β polypeptide folding pattern (7, 8) was recognized as characteristic of pathological amyloid fibers (9, 10), it is surprising that molecular explanations of how amyloid fibers are actually constructed and why they are so stable remain elusive.

Seeded amyloid fibril growth from soluble protein interacting with nucleating amyloid fragments (which is the presumed mechanism of prion propagation *in vivo*) was first demonstrated *in vitro* with fibrous insulin (11) before its cross- β conformation was identified (12). Now such self-nucleated growth is considered a defining characteristic of amyloid fiber assembly (cf. refs. 13–15). Many normal proteins form amyloid aggregates that are involved in a variety of diseases (cf. ref. 16), but so far, only the mammalian prion protein (4) and a few yeast (17) and fungus (18) proteins have been identified as forming infectious prions. Does the difference between an infectious and noninfectious amyloid depend on how the cross- β fibrils are formed, or how the cell machinery acts on the aggregates? Cellular processes are

critical in the propagation of the yeast [PSI] prion (19), and particular mutations in the Sup35 prion-forming domain can inactivate one [PSI] strain but not another (20). Thus, both details of the peptide structure and the cellular environment are important for propagation of strains of the [PSI] prion amyloid. Our focus in this study is on the morphology of strain-specific amyloid fibrils assembled *in vitro* from recombinant Sup35 prion protein by initial seeding with infectious nuclei extracted from yeast cells bearing each of three [PSI] prion strains (1). These fibrils transform yeast cells, producing the same phenotype of the colonies used to purify the infectious nuclei.

The yeast prion state [PSI] results from self-propagating aggregation of the translation termination factor Sup35p, thereby removing it from action; this leads to an enhanced read-through of nonsense mutations (i.e., nonsense suppression) (21). The prion strains [VH], [VK], and [VL] are distinguished by how their suppression activity is affected when they are propagated in cells bearing various point mutations or deletions in the 61-residue N-terminal prion-determining sequence of the Sup35 protein (20). [PSI] strains can also be distinguished in terms of the dominance of one strain over another (20), the thermal stability of their infectious amyloid aggregates in the presence of SDS detergent (2), or by the strength of their suppression activity (22). According to these criteria, [VH] is usually a strong strain, whereas [VK] and [VL] are weak (20), and the order of dominance ([VH] > [VK] > [VL]) (1) and thermal instability ([VH] > [VK] $\sim$ [VL]), which we have determined, are comparable. Correlation of thermal instability with dominance and suppressor strength implies that greater structural lability of infectious amyloid fibrils allows their more efficient fragmentation by cellular machinery to generate the nuclei that specifically sop up Sup35 molecules, thereby selectively propagating the more frangible fibrils.

The molecular architecture of the amyloid fibrils formed in our studies (1) by the recombinant Sup35 prion domain fused with GFP is similar to that of *in vitro* self-assembled Ure2p constructs that have been analyzed in a detailed electron microscopy study by Baxa *et al.* (23). They established that the N-terminal 70-residue prion-defining domain of this yeast prion protein (17) forms the amyloid core and the globular C-terminal portion could be replaced in fusion constructs by four other globular proteins, including GFP, with no evident effect on the amyloid core structure. Mass per length (mpl) measurements by scanning transmission electron microscopy (STEM) (23) indicated that, within the experimental uncertainty, there might be just one prion molecule for each 4.7-Å cross- β repeat period of the amyloid core, independently of the size of the attached C-terminal domain. These results suggested a simple model for the amyloid core in which segments of a sinuously folded N-terminal domain should form a single layer of regularly

Abbreviations: STEM, scanning transmission electron microscopy; TEM, transmission electron microscope; mpl, mass per length; TMV, tobacco mosaic virus; X- β ply, cross- β ply.

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repeating cross- β -sheets (24). Our expectation was that STEM mpl measurements on the three strains of amyloid fibrils formed by the Sup35 construct (1) all would have one prion molecule per cross- β repeat distance, thereby implying that the differences between prion strains should be determined by how the N-terminal domain was folded to form the cross- β segments. When our preliminary measurements indicated a more complicated story, great care was taken to ensure the reliability of the STEM mpl measurements by adequate sampling and careful calibration. Complementary information about fibril morphology was obtained from micrographs of negatively stained and frozen hydrated specimens. Our goal was to determine whether the morphologies of the amyloid fibrils propagating the [VH], [VK], and [VL] strains are distinguishably different.

Materials and Methods

Protein Sample Preparation. Solutions of pure, recombinant His₅-Sup35(1–61)-GFP-Strep(II) (molecular mass 37,229 Da) were assembled into fibrils corresponding to the three different strains, [VH], [VK], and [VL], of the yeast prion [PSI], using seeds purified from yeast cells, as described (1). The concentrations of the recombinant protein solutions used for assembly were typically ≈ 10 – $40 \mu\text{M}$. After incubation, the fibrils from all of the samples were sonicated for 10 s with 6 W of power output. The fibril samples were centrifuged at $80,000 \times g$ on top of 1 ml of a 30% (wt/vol) sucrose cushion for 2 h and resuspended in 100 μl of buffer E (100 mM Tris-HCl/1 mM EDTA/2.5 mM dethiobiotin, pH 8.0). To assess reproducibility of the measurements, two separate batches of infectious [VH] and the [VK] strain fibrils were prepared by using seeds extracted from independently grown yeast colonies. Spontaneously assembled fibrils were prepared by extended incubation in buffer E at 4°C (SH sample) and by incubation in buffer B (20 mM Tris-HCl/100 mM NaCl/500 mM imidazole, pH 7.6) at 22°C (SK sample), followed by centrifugation and resuspension in buffer E. Yeast cells transformed with SH and SK showed the [VH] and [VK] phenotypes, respectively (1).

Electron Microscopy. Dark-field images of unstained, freeze-dried specimens were recorded for mpl measurements (25) at the STEM facility at the Brookhaven National Laboratory, using a scanning pixel size of $(20 \text{ \AA})^2$. Tobacco mosaic virus (TMV) particles, which have a mpl of $13.1 \text{ kDa/\AA}$, were added to all of the STEM samples to have an internal calibration standard. The calculated electron dose in the STEM images was $\approx 10 \text{ e}^-/\text{\AA}^2$ for a $20\text{-\AA}$ pixel size. Images of negatively stained filaments with 2% methylamine vanadate (Nanoprobes, Upton, NY) were collected by STEM using 5- to $20\text{-\AA}$ pixel sizes.

Frozen hydrated samples for transmission electron microscopy (TEM) were prepared by adding 1 μl of a 1:10 dilution of the fiber preparation to a glow-discharged quantifoil grid (Quantifoil, Jena, Germany) with $1\text{-}\mu\text{m}$ holes. The grids were plunged into ethane slush after blotting the excess solution and transferred to the electron microscope by using a Gatan 926 cryoholder (Gatan, Pleasanton, CA). The samples were observed by using a CM30-FEG microscope (FEI, Hillsboro, OR) operating with an accelerating voltage of 300 kV. Images were recorded at a magnification of $\times 45,000$ on Kodak SO163 film plates using various defocus values (approximately -1.0 to $-3.5 \mu\text{m}$) for phase contrast. Film plates were scanned with an Optoscan scanner (Intergraph, Madison, AL) using a $7\text{-}\mu\text{m}$ step size, corresponding to $2.5 \text{ \AA}$ per pixel. Images of frozen hydrated fibrils were computationally straightened by using the SUPRIM package (26) and boxed, and their power spectra were calculated.

Negatively stained samples were prepared by adding 1 μl of a 1:50 dilution of the fibril preparations to a glow-discharged carbon-coated grid. After 1 min, the excess solution was blotted, washed with a drop of water, and stained with 1% (wt/vol)

uranyl acetate. Negatively stained samples were observed on a CM120 electron microscope operating with an accelerating voltage of 100 kV.

Data Processing. Measurements from the STEM dark-field images of unstained samples were made with the program PCMASS26 (27). Unencumbered segments of fibril and TMV images were selected in boxes $500 \text{ \AA}$ long by $280 \text{ \AA}$ wide. The program subtracts the background from the images automatically and provides a measurement of electron scattering proportional to the mass inside a box. Reliable absolute scaling of the electron scattering mass requires an internal calibration standard in the images. Only those images in which the mpl of TMV was $13.1 \pm 1.4 \text{ kDa/\AA}$, as indicated by the Brookhaven National Laboratory Facility instrument calibration before rescaling, were used for data collection. For each STEM image of the freeze-dried, unstained specimens, we collected as many measurements as possible from the TMV particles present, calculated the average mpl of the TMV particles, and used the scaling factor necessary to bring the TMV mpl average to $13.1 \text{ kDa/\AA}$ throughout the micrograph. This calibration procedure was tested by using half of the TMV particles present in each micrograph to calculate the scaling factor, and the other half as test measurements, recovering the known mpl of TMV in the control. A further test was done by using images of earthworm hemoglobin hexamers with a mass of 3.75 MDa (28), which yielded self-consistent scaling when calibrated by using the TMV included in the micrographs. (See Fig. 2, which is published as supporting information on the PNAS web site, for details of the mpl calibration.)

The morphologies of the prion fibrils were assessed visually, and the mpl measurements were classified according to the morphologies. The data were analyzed by using the program SIGMASTAT in the following way: For each morphology class in each sample, we first ran a Kolmogorov-Smirnov test to verify the normality of the distribution and computed the mean value, variance, and standard error. Comparisons between populations were made by running t tests in the case of normal distributions with similar variances, otherwise comparisons were made by running Mann-Whitney rank sum tests. All of the comparisons were made by using a 99% confidence interval. Histograms were produced by using a binning window of $0.25 \text{ kDa/\AA}$.

Results

Fibril Morphology. The amyloid fibrils that propagate the three [PSI] prion strains, [VH], [VK], and [VL], have similar overall morphology as visualized in electron micrographs of freeze-dried, negatively stained, and frozen hydrated specimens (Fig. 1 *A–D*). Single fibril diameters measured from negatively stained specimens were ≈ 170 – $180 \text{ \AA}$, whereas, in micrographs of freeze-dried and frozen hydrated specimens, the diameters were $\approx 160 \text{ \AA}$. In negatively stained STEM and frozen hydrated TEM images (Fig. 1 *C* and *D*), the globular GFP domains, of diameter $\approx 40 \text{ \AA}$, could be resolved, clustered around the central $\approx 45\text{-\AA}$ diameter amyloid core formed by the Sup35(1–61) prion domain to which they are covalently connected. These features appear indistinguishable from those of the fibrils formed by spontaneous self-assembly of the Ure2p(1–65)-GFP construct (23), which has a similar size prion domain and the same globular C-terminal domain as our Sup35 construct. Fourier transforms of our micrographs showed no indication of any regular order in the packing of the GFP domains tethered to the amyloid fibril core, which has a very regular $4.7\text{-\AA}$ cross- β periodicity evident from diffraction data (1, 29). Clear structural differences among the three strains of infectious amyloid fibrils were evident from comparison of the mpl measurements on the four distinguishable fibril shapes summarized in Fig. 1 and Table 1, as described below, but the image and diffraction data provide, as yet, no insight into the atomic details of the molecular packing differences.

Fig. 1. Data for the three strains {[VH] (a), [VK] (b), and [VL] (c)} of yeast [PSI] prion amyloid fibrils. (A–D) Electron micrographs of single fibrils. Freeze-dried, STEM (A); negatively stained, TEM (B); negatively stained, STEM (C), and frozen hydrated, TEM (D). (E) Histograms of mpl measurements from freeze-dried STEM image data as in A and F. (F) Gallery of the different types of fibrils for the three strains, showing the histograms for the corresponding fibril populations, which are color-coded: cyan, lanky; magenta, hunky; red, wavy; and green, jaggy. Gaussian fits to fibril type distributions are superimposed on the total population histograms in E with the corresponding color codes. One prion molecule per 4.7-Å repeat corresponds to 7.93 kDa/Å, as marked. (G) Gallery of TMV, which is used as internal calibration standard for the STEM measurements. (Scale bars, 500 Å.) See Figs. 3 and 4, which are published as supporting information on the PNAS web site, for double fibril data and high-magnification STEM images, respectively.

Strain mpl Histograms. Independently of the characterization of fibril shapes, the histograms of the mpl distributions for the [VH], [VK], and [VL] amyloid fibrils (Fig. 1E) exhibit distin-

guishing properties of the three prion strains. Nucleation of the amyloid assembly with infectious extracts from yeast bearing the [VH] strain produced almost exclusively single fibrils (only $\approx 1\%$

Table 1. Summary of STEM measurements

Fibril type	Strain type							
	[VH]1	[VH]2	[VK]1	[VK]2	[VL]	SH	SK	S0
Lanky								
mpl(kDa/Å) ± SEM	8.07 ± 0.08	8.08 ± 0.09	8.75 ± 0.14	8.52 ± 0.2	8.28 ± 0.06	8.04 ± 0.08	8.5 ± 0.07	8.1 ± 0.04
SD, %	6	9	9	5	11	9	8	7
Prions/repeat ± SEM	1.02 ± 0.01	1.02 ± 0.01	1.10 ± 0.02	1.07 ± 0.03	1.04 ± 0.01	1.01 ± 0.01	1.07 ± 0.01	1.02 ± 0.01
Measurements/total	116/494	232/616	131/1,145	26/538	860/1,466	227/427	129/582	176/616
Fibrils/total	42/116 (36%)	63/145 (43%)	18/264 (7%)	6/133 (5%)	146/232 (63%)	67/152 (44%)	37/114 (32%)	54/333 (16%)
Hunky								
mpl(kDa/Å) ± SEM	9.50 ± 0.1	9.30 ± 0.12	9.54 ± 0.09	9.37 ± 0.06	9.44 ± 0.1	9.48 ± 0.06	9.30 ± 0.09	N/A
SD, %	9	11	9	N/A	7	7	10	N/A
Prions/repeat ± SEM	1.20 ± 0.01	1.17 ± 0.02	1.20 ± 0.01	1.18 ± 0.07	1.19 ± 0.01	1.19 ± 0.01	1.18 ± 0.01	N/A
Measurements/total	275/494	271/616	121/1,145	8/538	212/1,466	66/427	52/582	N/A
Fibrils/total	43/116 (37%)	54/145 (37%)	20/264 (7.5%)	2/133 (1.5%)	27/232 (12%)	18/152 (12%)	9/114 (6%)	N/A
Wavy								
mpl(kDa/Å) ± SEM	8.02 ± 0.2	7.25 ± 0.13	9.10 ± 0.05	9.10 ± 0.09	8.38 ± 0.09	8.54 ± 0.08	8.20 ± 0.03	N/A
SD, %	5	5	7	7	10	10	4	N/A
Prions/repeat ± SEM	1.01 ± 0.03	0.91 ± 0.02	1.15 ± 0.01	1.15 ± 0.01	1.05 ± 0.01	1.07 ± 0.01	1.04 ± 0.01	N/A
Measurements/total	38/494	44/616	882/1,145	490/548	328/1,466	51/427	306/582	N/A
Fibrils/total	17/116 (15%)	20/145 (14%)	225/264 (85%)	121/133 (91%)	34/232 (15%)	22/152 (14%)	59/114 (52%)	5/333 (1.5%)
Jaggy								
mpl(kDa/Å) ± SEM	7.90 ± 0.15	7.90 ± 0.13	7.9 ± 0.4	7.7 ± 0.4	8.04 ± 0.14	7.54 ± 0.06	7.90 ± 0.07	7.43 ± 0.03
SD, %	5	5	N/A	N/A	8	8	5	9
Prions/repeat ± SEM	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.05	0.97 ± 0.05	1.01 ± 0.2	0.95 ± 0.01	1.00 ± 0.01	0.94
Measurements/total	65/494	69/616	11/1,145	14/538	66/1,466	83/427	95/582	414/616
Fibrils/total	11/116 (9.5%)	8/145 (5.5%)	1/264 (0.4%)	2/133 (1.5%)	5/232 (2%)	45/152 (29%)	9/114 (6%)	274/333 (82%)

Measurements of mpl of fibrils seeded from three [PSI] strains. The molecular mass of our molecule is 37,229 kDa. The rows labeled "measurements/total" correspond to the number of measurements made from unencumbered, 500-Å-long segments, while "fibrils/total" corresponds to fibrils that were visually classified. [VH] 1 and 2 and [VK] 1 and 2 refer to two independently nucleated samples of each strain type; SH and SK refer to spontaneously self-assembled fibrils, incubated under different conditions which respectively propagated [VH] and [VK] strain phenotypes. S0 corresponds to a noninfectious sample. The SDs are reported as percentages of the mean ($100\sigma/\langle\text{mpl}\rangle$), while the SEM is $\sigma/\sqrt{n}$, where n is the number of measurements. N/A, not applicable. See Fig. 5, which is published as supporting information on the PNAS web site, for plots of the fibril proportions for the different strain types.

of the fibrils were double) with a bimodal mpl distribution; this distribution can be fit by two Gaussians of comparable magnitude whose mean mpl values correspond to 0.99 and 1.17 prion molecules per 4.7-Å cross-β repeat distance. In contrast, seeding the recombinant protein solution with [VK] strain nuclei produced a monodisperse normal distribution of single fibrils (3 doubles per 397 recorded) whose mean mpl value corresponds to 1.15 prions per repeat. Seeding with [VL] strain nuclei produced a larger proportion of double fibrils (20 doubles per 232 recorded) compared with the [VH] and [VK] strains; and the Gaussians that fit the single and double fibril measurements have mean values of 1.06 and 1.94 prions per repeat, respectively. The standard deviations in the mpl measurements of the prion fibrils were 4–11% of their mean values (see Table 1). For comparison, the standard deviation of the calibrating TMV distribution, which provides a measure of intrinsic experimental variation, was 3% of its mean.

Fibril Shapes. The fibril shapes were initially classified as straight or wavy, but the bimodal mpl histogram of the straight [VH] fibrils (Fig. 1E) showed the presence of two normally distributed populations, whose members can be visually distinguished in the electron micrographs; the two straight fibril types have been designated lanky and hunky. Furthermore, a minor population of fibrils designated jaggy, which are distinguishable from the wavy type, were observed in [VH] strain specimens with low frequency and rarely in [VK] and [VL] specimens. The wavy fibrils have periodicities ranging from 1,000 to 2,500 Å and amplitudes ≈150 Å, comparable to the fibril diameter; the jaggy fibril periodicity is only ≈500 Å, appearing as small amplitude (150 Å) sharp bends or smoother ripples (Fig. 1F). Double fibrils, observed

most frequently in the [VL] specimen (see Fig. 3), consist of a coiled pair of single strands with variable periodicity ≈2,000 Å.

Fibril-Type Histograms. Histograms of the mpl measurements for the different fibril types (Fig. 1F) exhibit detailed differences among the three strains of prion amyloids. For the [VH] fibrils, the mpl distribution of the visually selected hunky fibrils corresponds closely to the heavy component identified by fitting two Gaussians to the histogram of all of the measurements from the [VH] samples (Fig. 1E). Although there is considerable overlap of the lanky and hunky distributions caused by the variation in the electron scattering measurements along the length of the fibrils, the two straight fibril types can be generally distinguished by their overall appearance and their mpl averaged over several 500-Å segments. The larger standard deviation of the hunky compared with the lanky [VH] fibrils implies greater intrinsic variation in the mpl of the hunky fibrils. The standard deviation of the lanky [VH] fibrils is similar to that of the wavy [VK] distribution, but the breadth of the lanky [VL] distribution is significantly broader. It is evident that the amyloid fibrils from the three prion strains differ in the proportion of the four distinguishable fibril types and, generally, in their mpl distributions and mean values for each type. The apparent uniformity in the mean mpl and breadth of the distributions for the hunky fibrils in specimens of the three strains may be caused by the visual criteria used to select this type. Although lanky and hunky appear as distinct populations for [VH] fibrils, they may represent just the lighter and heavier range of a more continuous distribution for the [VK] and [VL] straight fibrils.

Duplicate Samples. Samples of [VH] and [VK] fibrils made in duplicate from seeds extracted from independent colonies

showed a high degree of reproducibility in their populations, both in the proportion of the different fibril types and the mpl measurements (Table 1, columns [VH]1, [VH]2, [VK]1, and [VK]2). There was no significant difference among the mpl measurements for the fibril types observed in the duplicate [VH] and [VK] specimens, except for the wavy type in the pair of [VH] samples, where there was a 10% difference, which is about twice what might be statistically expected for this infrequently observed type. Comparison of the frequency of the four designated fibril types can be made either in terms of the ratios of the number identified visually or by the number of measurements of each type relative to the total. The two ratios are not the same because the number of measurements on any fibril depends on its unencumbered length within the field of view. The average number of mpl measurements per fibril ranged from about three to six for the different fibril types. There do not appear to be significant differences in the frequency of occurrence of the four types of fibrils in the duplicate specimens listed in Table 1.

Comparison of Strain Fibril Types. Wavy fibrils, which are most readily distinguished from the other fibril types, showed the largest differences in mpl and proportion among the three prion amyloid strains: in [VH] samples, they constituted $\approx 15\%$ of the fibrils and their mpl corresponds to ≈ 0.96 prions per repeat; in [VK] samples, nearly 90% of the fibrils were the homogeneous wavy type with 1.15 prions per repeat; and the wavy [VL] fibrils, which constituted $\approx 15\%$ of the sample, have 1.05 prions per repeat (Table 1). There were no noticeable differences in the range of wavelengths among wavy filaments corresponding to the three strains, and Fourier transforms of individual images of ice embedded wavy filaments did not show significant regularity in their periodicities. Lanky [VL] fibrils with 1.04 prions per repeat, which were the predominant type ($\approx 60\%$) in this sample, are $\approx 2\%$ heavier than their [VH] counterpart ($\approx 40\%$ of the sample) and have, as already noted, a broader distribution; lanky [VK] fibrils constituted only $\approx 5\%$ of these samples and, with 1.07 prions per repeat, are heavier than those of the other two strains (Table 1). The frequency of jaggy filaments in [VK] and [VL] samples was very low (1–2%) and, as with the [VH] sample, where they were more frequent (5–10%), all jaggy mpl measurements correspond to ≈ 1.0 prion per repeat (Table 1). The apparent uniformity of the [VK] and [VL] hunky fibrils may be caused by the selection criterion, as noted in the description of their histograms. If no distinction was made between the lanky and hunky types for these strains, the differences between the mpl of their straight fibrils would be accentuated, corresponding to 1.15 and 1.07 prions per repeat for the [VK] and [VL] strains, respectively.

Spontaneously Assembled Fibrils. Two different infectious aggregates, prepared by extended incubation of the Sup35(1–61)-GFP construct without seeds, one at 4°C in 0.1 M buffer, and the other at 22°C in 0.6 M buffer, displayed, respectively, the [VH] and [VK] phenotypes when used to transform [psi-] cells (1). STEM measurements on their different fibril types are listed in Table 1, columns SH and SK (see also Fig. 6, which is published as supporting information on the PNAS web site). The mpl of the lanky and hunky SH fibrils were statistically indistinguishable from the seeded [VH] samples, but the proportions of lanky were enhanced and hunky were diminished in SH compared to [VH]; the proportion of wavy fibrils was unchanged but their mpl in the SH sample was $\approx 10\%$ greater than in [VH]; the most distinctive change in the SH sample was the greater proportion of jaggy fibrils ($\approx 30\%$) with $\approx 5\%$ decrease in mpl compared with the seeded [VH] samples. In the SK sample, which has low infectivity (1), just over half of the fibrils were the wavy type compared with $\approx 90\%$ in the [VK] samples and the number of prions per repeat was 1.04, with an increased standard deviation compared with 1.15 in the seeded samples. The lanky and jaggy fibrils became more common in the SK sample in comparison with the seeded

[VK] samples, whereas the proportion of hunky fibrils remained relatively low.

One spontaneously assembled amyloid sample, incubated in buffer B (0.6 M), failed to show any infectivity. STEM measurements on fibrils from this sample, designated S0, are listed in Table 1. In this preparation, the jaggy fibrils constituted 82% of the total, with 16% lanky and 2% wavy. Using fragments of this preparation to seed assembly of the recombinant Sup35 construct propagated fibrils that were predominantly the jaggy type.

Discussion

The infectious strain-specific amyloid fibrils formed by seeded growth with the recombinant Sup35 prion protein are clearly structurally different. However, our observations on the fibril morphologies engender more questions than answers regarding the molecular basis of the strain-specific structural differences. Self-perpetuation of an amyloid fibril with one prion molecule per $4.7\text{-}\text{\AA}$ cross- β repeat period could be explained by in-register parallel β -sheet hydrogen-bonding of like peptide segments, but what sort of specific recognition mechanism would generate one and a fraction prion molecule per repeat? Why do the fibrils specifically seeded by [VH] strain nuclei have a bimodal distribution, $\approx$ half with 1.0 prion per repeat in lanky fibrils and $\approx$ half with 1.2 prions per repeat in hunky fibrils, whereas the [VK] seeded fibrils are almost all wavy with ≈ 1.15 prions per repeat? Are both the lanky and hunky [VH] fibrils infectious? Why are $\approx 10\%$ of the [VL] seeded fibrils double, with slightly less than twice the mpl of the single form, whereas doubles are very rare in the [VH] and [VK] fibril populations? What is the significance of the jaggy fibrils that predominate in a noninfectious preparation and are more evident in spontaneously aggregated than in seeded infectious populations? Are the heterogeneities of the samples listed in Table 1 the result of unrecognized artifacts that confuse some underlying regularity in the strain-specific amyloid fibril structures?

At present, attempting to answer any of these questions requires indulgence in fanciful speculation, as is still customary in the amyloid structure field because so few hard facts are available. We fancy models for self-propagating prion amyloid cores in which peptide segments directed across the fibril axes are periodically hydrogen-bonded in cross- β stacks similar to the regularly parallel packing of the Sup35(7–13) peptide GNNQQNY in orthorhombic and monoclinic nanocrystals (30, 31). Determination of the structure of a monoclinic crystal form of this peptide (32), in which cross- β stacks are tightly paired, has provided an atomic resolution view of what an amyloid spine may look like. To distinguish such a periodic peptide stack from other β -sheet structures, we call it a “cross- β ply,” abbreviated as X- β ply. The axial, H-bonded repeat period of the crystalline Sup35(7–13) X- β ply is $4.87 \pm 0.02\text{ }\text{\AA}$, and neighboring plies are axially half-staggered. Tight pairing interactions on one side of a ply may be conserved in the orthorhombic and monoclinic lattices but must be symmetrically distinct on the other side. Half-stagger of the peptide β -strands extinguishes meridional diffraction on the $4.87\text{-}\text{\AA}$ layer plane and gives rise to an intense off-meridional $4.7\text{-}\text{\AA}$ spacing reflection from all of the crystals. If pairs of half-staggered X- β plies occur in our prion amyloid fibrils, the true axial period may be $\approx 4.9\text{ }\text{\AA}$, which would increase our calculated number of molecules per X- β repeat in Table 1 by 4%, accentuating the differences with our favored single-layer repeating model. Tilting of X- β ply axes by 30° or more to the fibril axis in some improbably tight helix is an unlikely explanation of how 1.15–1.25 prion molecules could fit in a $4.7\text{-}4.9\text{-}\text{\AA}$ length; tacking additional prion domains on the average every four to seven layers of a periodic X- β core is a messy (but possible) way of enhancing the mpl to fit our data for the heavier fibrils.

Naked amyloid fibril cores have a high affinity for sticking together. This is very clear in micrographs of the Ure2p core fibrils generated either by proteolytic removal of the globular C-terminal domains or by assembly of just the N-terminal (1–89) prion domain (23). Predominance of single fibrils formed by most Ure2p constructs and our Sup35p construct can be accounted for by the shielding of the amyloid core by the globular C-terminal domains. Baxa *et al.* (23) obtained evidence that the globular domains in their Ure2p constructs are connected to the amyloid core by a flexible linker consisting of a C-terminal portion of the prion domain; furthermore, they found a higher proportion of double fibrils formed by the Ure2p(1–65)-GFP compared with the Ure2p(1–80)-GFP construct, which suggests that shortening the tether may have exposed part of the sticky core, allowing more fibrils to pair.

Our observation of significantly more double fibrils in the [VL] sample compared with the [VH] and [VK] strain samples [which all were assembled from the same Sup35(1–61) construct] may be correlated with greater thermal stability (and recessive prion strength) of the fibrils from the [VL] strain relative to their peers. If the amyloid core is formed by parallel stacking of peptide segments in X- β plies and if more of the C-terminal portion of the Sup35(1–61) sequence in the [VL] strain is assembled in a stable ply than in [VH] and [VK] fibrils, then the GFP domains on shortened tethers may not completely shield distal portions of the core, thus allowing more [VL] fibril pairing. Because [VL] double fibrils have less than twice the mpl of singles, pairing of lean fibrils may be favored; alternatively, doubles may be formed by nucleation of a second fibril on a regularly periodic amyloid core and molecular crowding may impede full-scale completion of a dual. This latter possibility suggests a mechanism for formation of the hunky [VH] and the [VK] fibrils by incipient addition of prion domains at occasional gaps in the globular shield of a periodic core, but it does not explain coexistence of the lanky and hunky [VH] fibrils, nor why the [VK] fibrils are wavy and relatively homogeneous.

Much as we fancy a simple model of single layers of peptide segments periodically stacked in X- β plies to propagate yeast prion amyloid cores, similar to the serpentine model of Kajava *et al.* (24) but with fewer ordered segments whose sequences should define each strain, it is evident that the structure of all amyloid fibers show a high degree of variability and lateral disorder, coupled with the very regular X- β periodicity. The x-ray fiber patterns of oriented self-assembled Sup35 constructs show no indication of any lateral

order in wet specimens, and only limited coherence in dried fibers (29) corresponding to, at most, irregular pairing of X- β plies, as indicated by modeling of similar equatorial fiber diffraction data from another dried amyloid (33). The diversity in lateral packing of Sup35(7–13) X- β ply pairs observed in various highly ordered nanocrystalline forms (31) may exemplify variations that could occur in amyloid fibrils. Fluctuation theory predicts that the percent rms thermal variation in molecular volume should be inversely proportional to the square root of the volume (34). Such fluctuations in ply separation, if randomly distributed along the length of wet amyloid fibrils, may be large enough to obscure the local lateral order in the average Fourier transform without affecting the long-range axial X- β order.

Diversity in the polarity and kinetics of Sup35 protein self-assembly, as observed by microscopically following single amyloid fibril growth from seeds (35), is likely to be involved in generating the confusing variety of fibril morphologies we have identified. The self-propagating polymorphism reported in Alzheimer's β -amyloid fibrils (36), which mimics seeded [PSI] prion propagation (1, 2), involves differences in amino acid residue interactions detected by solid-state NMR and morphological heterogeneities discerned from mpl measurements, analogous to those of our Sup35 fibrils. Establishing that the amyloid fibrils propagating the [VH], [VK], and [VL] prion strains have distinguishably different morphologies provides direct confirmation of the basic structural prediction of the protein-only prion hypothesis (4), but the differences we have observed cannot be accounted for by any of the many atomic models proposed for amyloid structures. We have not attempted to answer all of the questions we have posed regarding the diversity of strain-specific morphologies of the Sup35 amyloid fibrils because we admit to being confused about how to find plausible molecular explanations for all of these puzzles, which present fundamental challenges requiring more focused experiments to begin to understand the intrinsic variations of pathological amyloid structures.

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