Heterogeneous Seeding of a Prion Structure by a Generic Amyloid Form of the Fungal Prion-forming Domain HET-s(218–289)*

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The fungal prion-forming domain HET-s(218–289) forms infectious amyloid fibrils at physiological pH that were shown by solid-state NMR to be assemblies of a two-rung β-solenoid structure. Under acidic conditions, HET-s(218–289) has been shown to form amyloid fibrils that have very low infectivity in vivo, but structural information about these fibrils has been very limited. We show by x-ray fiber diffraction that the HET-s(218–289) fibrils formed under acidic conditions have a stacked β-sheet architecture commonly found in short amyloidogenic peptides and denatured protein aggregates. At physiological pH, stacked β-sheet fibrils nucleate the formation of the infectious β-solenoid prions in a process of heterogeneous seeding, but do so with kinetic profiles distinct from those of spontaneous or homogeneous (seeded with infectious β-solennoid fibrils) fibrilization. Several serial passages of stacked β-sheet-seeded solutions lead to fibrillization kinetics similar to homogeneously seeded solutions. Our results directly show that structural mutation can occur between substantially different amyloid architectures, lending credence to the suggestion that the processes of strain adaptation and crossing species barriers are facilitated by structural mutation.

Prions are proteinaceous infectious particles; proteins that take on a specific, self-propagating, aberrant fold (1). The term prion was originally coined to describe the protein-only infectious agent found in scrapie, a neurodegenerative disease of sheep. Prions have since been implicated in related diseases in bovines, cervids, and humans, diseases collectively known as the transmissible spongiform encephalopathies. The infectious agents are made up of aggregates of the aberrantly folded mammalian prion protein, PrP.2 In the transmissible spongiform encephalopathies, the prions form unbranched fibrillar assemblies known as amyloids (2), which are typically poorly ordered. Amyloids have a common cross-β architecture consisting of β-strands that extend roughly perpendicular to the fiber axis, forming sheets that run parallel to the fiber axis (3, 4). Accumulated amyloids are associated with a number of diseases in addition to transmissible spongiform encephalopathies, including Alzheimer disease, Parkinson disease, and type II diabetes (5). Although these amyloid diseases do not appear to be communicable between individuals, there is increasing evidence that the associated aberrantly folded proteins share self-propagating infectious properties similar to those of the transmissible spongiform encephalopathy-associated prions (6, 7).

In addition to disease-related proteins and peptides, functional prions and amyloids have been identified in a number of different organisms. Examples include curli fibers in Escherichia coli and other Enterobacteriaceae (8), Pmel17 associated with melanosome maturation in humans (9), and a number of peptide hormones stored in secretory granules (10), although these amyloids do not appear to have the in vivo self-propagating activity of prions. Functional amyloids in yeast and fungi, however, have been shown to be prions by their propagation of phenotype through inheritance, by contact with prion-infected individuals, and by inoculation of prion fibrils prepared in vitro (11). Among these functional prions are the glutamine/asparagine-rich prions of Saccharomyces cerevisiae, including Sup35p and Ure3p, and HET-s from the fungus Podospora anserina. The prion-forming domain of HET-s, residues 218–289, is distinct from the Saccharomyces prion-forming domains because it has no particular amino acid composition bias, so insights from HET-s into interactions between sequence, structure, and infectivity are more likely to be applicable to pathogenic prions and amyloids.

HET-s(218–289) can be fibrillized into prions or noninfectious amyloids by controlling the pH of the fibrillization buffer.

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2 The abbreviations used are: PrP, prion protein; recPrP, recombinant PrP; PrPSc, PrP scrapie; ssNMR, solid state NMR.
At physiological pH, HET-s(218–289) forms infectious fibrils that exhibit a high degree of order and homogeneity. These features make infectious HET-s(218–289) amenable to solid state NMR (ssNMR) analysis, which resulted in an atomic resolution structure: a repeating two-rung β-solenoid (13). At low pH, HET-s(218–289) forms noninfectious amyloids that have heterogeneous morphologies and properties (12, 14). ssNMR studies of low pH fibrils indicated that they have structures significantly different from those of the physiological pH fibrils (15). Low resolution models of one- and three-protofilar fibrils have been obtained by cryo-electron microscopy, although these studies have suggested structures similar to the β-solenoid (16). In previous studies, we and others showed that particular low pH conditions can cause HET-s(218–289) to degrade, with the resulting peptides forming stacked β-sheet structures similar to those commonly found in short amyloidogenic peptides (17, 18).

In this study, we show by x-ray fiber diffraction that HET-s(218–289) polymorphism consists of two different architectures: two-rung β-solenoids and single-layered stacked β-sheets. Under the conditions of this study, stacked β-sheet HET-s(218–289) fibrils are preferentially formed at low pH, although formation of β-solenoids is possible with the addition of seed fibrils. Seeding with stacked β-sheets in otherwise β-solenoid-forming conditions did not result in the formation of stacked β-sheet fibrils, but did alter fibrillization kinetics. Differences in seeding behavior and infectivity between low and high pH fibrils has been previously shown (12), but structural information on those fibrils was limited to morphological features shown by low resolution negative-stain electron microscopy (EM). Our use of fiber diffraction allows for direct probing of internal architecture and subunit structure (19), which is not necessarily related to ultrastructural morphology (20). We propose that the distinct fibrillization kinetics from stacked β-sheet-seeded solutions are a result of heterogeneous seeding: the fibrillization of amyloid nucleated by a different amyloid structure. Similar effects have been seen with inoculation of recombinantly formed PrP amyloids into animals (21), leading to suggestions that heterogeneous seeding is the mechanism underlying prion strain adaptation and infection across species (22, 23). Taken together, our fiber diffraction and kinetics results demonstrate that interactions between polymorphic amyloids can occur despite distinct differences in structural architecture.

**Experimental Procedures**

**Preparation of Recombinant HET-s(218–289)**—HET-s(218–289) was expressed into inclusion bodies as a C-terminally His6-tagged construct in BL-21 E. coli cells and purified under denaturing conditions as described previously (17). Purified protein was moved into non-denaturing fibrillization buffers using freshly prepared Sephadex G-25 Superfine (GE Healthcare). To prevent cross-seeding under non-denaturing conditions, Sephadex media were used only once.

P H 2.0 fibrillization buffers were citric acid or phosphoric acid at final concentrations of 50 mM, adjusted with HCl or NaOH. For pH 7.5 fibrils, HET-s(218–289) was purified into 150 mM acetic acid, pH 2.5, and titrated to pH 7.5 by the addition of 3 M Tris. For seeding experiments, pH 2.0 samples were prepared as above, and pH 4.0 samples were prepared by purification into 50 mM sodium acetate, pH 4.0.

The 13C, 15N isotopically labeled HET-s(218–289) was expressed as described previously (24) and purified under denaturing conditions from inclusion bodies (17). Purified protein was concentrated and washed several times with 150 mM acetic acid, pH 2.5, using Centricon centrifugal devices with a 3-kDa cut-off (Millipore). For infectious fibril formation, the pH was adjusted to 7 with 3 M Tris-HCl, pH 7.

**X-ray Fiber Diffraction**—Fiber diffraction specimens were prepared by hanging 12 µl of ~20 mg/ml fibril solutions between sanded, silanized glass capillaries and allowing them to dry at nominal 100% relative humidity. High humidity of specimens was maintained at all times (25). Fibril solutions were prepared by three cycles of ultracentrifugation and resuspension in final buffers. pH 2.0 specimens were resuspended in 5 mM concentrations of their buffers. All other specimens were resuspended in 5 mM sodium acetate, pH 4.0. Fiber diffraction data were collected at the Biological Small-Angle X-ray Scattering beamline 4-2 at the Stanford Synchrotron Radiation Lightsource or the BioCAT beamline at the Advanced Photon Source. Processing of fiber diffraction patterns was performed with WCEN (26). For plots, diffraction patterns were first mapped to reciprocal space (27) and circular symmetrically background-subtracted. Meridians and equators were integrated as 30° sector plots. Scale factors for each pair of plots were determined by least squares fitting of the equators from 0.03 to 0.25 Å⁻¹. Data lower than ~33 Å⁻¹ (d < 0.03 Å⁻¹) resolution were not plotted because of beam divergence and x-ray scatter around the beamstop. Patterns were compared by calculating correlation coefficients; these coefficients have been shown to be a reliable measure of structural similarity (28). For β-solenoid patterns from different samples, made in the same way but at different times and measured at different synchrotrons, correlation coefficients were between 0.98 and 1.00.

**Solid State NMR—**13C-13C solid state NMR correlation spectrum (proton-driven spin diffusion, with a 50-ms mixing time) of uniformly 13C, 15N-labeled HET-s(218–289) was recorded at 700-MHz 1H frequency. The contours were cut at the 5 σ noise level. The black crosshairs were placed at the previously reported chemical shift assignments for the amino acids indicated (29). The overlap between the previously published chemical shifts and those observed in our experiments (within 0.2 ppm) is indicative of the same structure for the core of the HET-s fibrils studied here and those with their structure previously solved by ssNMR.

**Molecular Modeling**—The stacked β-sheet model was constructed by manipulating the β-solenoid ssNMR structure, Protein Data Bank (PDB) ID 2KJ3 (13), in Swiss-Pdb Viewer (30). Calculated diffraction patterns were produced using DISORDER, a program that calculates Fourier-Bessel transforms (31) and simulates disorientation (32). DISORDER and its associated documentation are available for download from the FiberNet website. B-factors of 4000 Å² were used for disordered regions (residues 217–225, 247–259, 282–295) to simulate significant disorder. Remaining structured residues used B-factors of 10 Å² to simulate high order. Residue numbers include the starting Met and C-terminal His₆ tag in the construct. Plots for
calculated diffraction were made as described above except that reciprocal space mapping and background subtraction were not required.

**Fibrillization Kinetics Assays**—For fibrillization kinetics, assays were carried out using a modified version of an assay described elsewhere (33), performed in triplicate. HET-s(218–289) monomers were purified into 500 mM acetic acid and fractionated into 60-nmol aliquots as determined by absorbance at 280 nm (A_{280}). Aliquots were immediately lyophilized, and the resultant powder was dissolved in 200 μl of hexafluoropropanol, lyophilized again, and stored at −20 °C. For assays, powder was resuspended in 1.5 ml of 175 mM acetic acid. Assays were started by the 1:1 addition of 1 mM Tris-HCl, pH 8.0, and mixing by inversion, providing a final protein concentration of 20 μM. For seeded assays, 6 nmol (for a final concentration of 2 μM, a 1:10 molar ratio) of seed fibrils were moved into the Tris-Cl buffer by ultracentrifugation and resuspension and probe-sonicated for 30 s with ~90 watts of power immediately prior to use. Stacked β-sheet fibrils were first washed with 50 mM citric acid, pH 2.0, purified three times by centrifugation, and resuspended to remove any monomers or small aggregates without inadvertently forming β-solenoids. Serially seeded assays were performed by treating fibrils from previous passages as described above. Seeds from triplicates were serially passaged individually. To prevent cross-contamination, assays were performed with disposable cuvettes.

Unseeded and stacked β-sheet-seeded first passage assays were monitored using optical density at 400 nm (A_{400}) measured every 10 min for 240 min. Assays seeded with β-solenoid or serially passaged stacked β-sheet-seeded solutions were measured at 1-min intervals for 20 min followed by measurements every 10 min up to 120 min. Assays to assess early stacked β-sheet seeding kinetics were measured at 1-min intervals for 20 min followed by 10-min intervals until 240 min was reached. Prior to each measurement, cuvettes were mixed by inverting several times. For display, data were fit to a Boltzmann function in Equation 1.

\[ y = \frac{A_1 - A_2}{1 + e^{x - x_0/dx}} + A_2 \]  

\( A_1 \) is the low limit, \( A_2 \) is the high limit, \( x_0 \) is the half-amplitude point, and \( dx \) is the width. Error bars represent the S.D. between triplicates.

**RESULTS**

**HET-s(218–289) Polymorphism Consists of Two Architectures**—Recombinantly expressed HET-s(218–289) was fibrillized at pH 7.5 as described previously (34) or at pH 2.0 in either 50 mM citric acid buffer or 50 mM phosphoric acid buffer. X-ray fiber diffraction patterns were obtained from each of these fibril types (Fig. 1). Diffraction patterns were of two distinct types. The meridional diffraction from pH 7.5 HET-s(218–289) (Fig. 1A) includes the definitive cross-β reflection at ~4.7 Å and a second reflection at 9.4 Å, demonstrating that the repeating unit of these fibrils is two β-strands thick. Meridional diffraction from pH 2.0 fibrils (Fig. 1, B and C) contains only the cross-β reflection, indicating that the repeating unit of these fibrils is only one β-strand thick. The equatorial diffraction from pH 7.5 fibrils consists of a series of strong intensity maxima, indicating a roughly cylindrical cross-sectional structure (35, 36). Equatorial diffraction from both types of pH 2.0 sample is dominated by an intensity maximum at ~10 Å, which is generally interpreted as representing the spacing between β-sheets stacked perpendicular to the fiber axis (17, 37). The pH 7.5 diffraction patterns are in good qualitative agreement with the two-rung β-solenoid ssNMR structure, and we have used ssNMR to verify that our pH 7.5 HET-s(218–289) sample indeed has the same chemical shift assignments as those published (13) (Fig. 2).

For quantitative comparisons between diffraction patterns, meridional and equatorial intensities were obtained by integrating along 30° arcs. Meridional diffraction data (Fig. 1D) from the pH 2.0 samples clearly show only the ~4.7 Å (\( d^* = 0.21 \) Å⁻¹) cross-β reflection, whereas the pH 7.5 pattern also includes diffraction maxima from a meridional ~9.4 Å (\( d^* = 0.11 \) Å⁻¹) reflection and an off-meridional intensity from the same layer line, indicating the presence of a two-rung helical structure. Equatorial data represent the cylindrically averaged Fourier transforms of the fibril electron density; the data are continuous along the equator, showing that the samples are non-crystalline. Equatorial data from the different pH 2.0 samples (Fig. 1E) show roughly the same intensity distributions, although the precise locations and widths of maxima vary, indicating slight differences in the structures. Quantitative equatorial comparisons were made by calculating correlation coefficients between each pair of plots. Correlation between the pH 2.0 equators (correlation coefficient = 0.91) is high (28), and together with the meridional data, indicates that these specimens share the same overall architecture, although the disorientation and low resolution of the data preclude comparisons of atomic structures. Correlation between pH 7.5 and pH 2.0 citric acid or phosphoric acid equatorial plots is low (0.68 and 0.48, respectively), and taken together with the presence or absence of 9.4 Å meridional reflec-
tions, clearly demonstrates that the fibrils formed at different pH values have distinct architectures.

Diffraction patterns from stacked β-sheet amyloids have historically been interpreted with the aid of data from mechanical manipulation of specimens, as in the case of super- contracted epidermin (38), or with the help of constraints provided by crystallinity and simple repeating amino acid sequences, as in the case of the lacewing fly egg stalk (39). These approaches are not available for HET-s, but we can identify architectural features by comparing experimental data with data calculated from models of stacked β-sheets: an approach we have used qualitatively in previous work (35). To provide a quantitative comparison with the analysis described here, we constructed a stacked β-sheet model of HET-s(218–289), retaining the β- and random-coil regions from the β-solenoid, but rearranging them to form a stacked β-sheet structure (Fig. 3A). Apart from avoiding close atomic contacts, no other constraints were used. The model is not meant to represent the actual structure of pH 2.0 fibrils, but is intended to demonstrate the general appearance of diffraction from stacked β-sheets. A diffraction pattern of the model was calculated with large temperature factors (B-factors) in the random-coil regions, making the β-strands the primary contributors to the pattern. The calculated diffraction pattern (Fig. 3B) shows the ~4.7 Å meridional reflection and the ~10 Å equatorial intensity maximum expected from a stacked β-sheet.
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Fibrils formed at pH 2.0 seeded with pH 7.5 solutions of HET-s(218–289) in pH 4 sodium acetate, in which HET-s(218–289) was seeded with preformed solutions yielded different structure. Although this confirms that stacked β-sheets do not act as efficient structural templates at higher pH, it does not address the issue of nucleation effects, that is, whether or not heterogeneous seeding occurs.

Unseeded and Seeded Solutions Fibillize with Distinct Kinetics—Fibillization of HET-s(218–289) was initiated by adding Tris-HCl to monomer solutions in acetic acid, bringing the solution to pH 7.5. Aggregation was monitored by apparent fibrillization kinetics of seeded and unseeded assays. A and B, raw (A) and normalized (B) assays of unseeded, β-solenoid-seeded, and stacked β-sheet-seeded fibrils. OD, optical density. C and D, raw (C) and normalized (D) stacked β-sheet-seeded assay early kinetics with extra readings. Solid line and (○): unseeded fibillization. Dashed line and (▲): fibillization with stacked β-sheet seeds. Dotted line and (■): fibillization with β-solenoid seeds. Dash-dotted line and (●): fibillization with β-solenoid seeds and early kinetics readings. Error bars represent standard deviation of triplicates.

Unseeded and Seeded Solutions Fibillize with Distinct Kinetics—Fibillization of HET-s(218–289) was initiated by adding Tris-HCl to monomer solutions in acetic acid, bringing the solution to pH 7.5. Aggregation was monitored by apparent A400 (Fig. 5), a measure of light scattering. In seeded experiments, β-solenoid or stacked β-sheet seeds were mixed into the Tris-HCl solution; stacked β-sheet seed fibrils were from citric acid buffer because HET-s(218–289) in this buffer displayed no propensity to form β-solenoids at pH 2. X-ray fiber diffraction from the resultant fibrils was used to verify β-solenoid structure qualitatively (Fig. 6).

A400 and normalized values of A400 are shown in Fig. 5 as a function of elapsed time for unseeded solutions and solutions seeded with stacked β-sheet fibrils or β-solenoid fibrils. The unseeded kinetics appeared to follow a roughly sigmoidal curve with a brief lag phase. Solutions seeded with β-solenoid fibrils exhibited no apparent lag phase and much faster fibillization than unseeded solutions. Solutions seeded with stacked β-sheet fibrils exhibited a rapid initial rise in A400 (Fig. 5A), but proceeded to completion (Fig. 5B) more slowly than either the unseeded or the β-solenoid-seeded solution. The rapid initial rise is seen more clearly in Fig. 5, C and D, for which the stacked β-sheet-seeded experiments were repeated with increased sampling in the first 20 min. Light scattering and rate to com-
pletion are slightly greater in the experiments with increased sampling, probably because of increased fibril fragmentation during mixing prior to each reading, leading to an increased number of nuclei (40).

Although the final $A_{400}$ values for each seeding condition were significantly different (Fig. 5A), supernatants from ultracentrifugation of fibril solutions after 4 h showed no detectable protein by $A_{280}$, indicating complete fibrillization. These observations indicate that the differences in final $A_{400}$ values were due to differences in aggregate size. The rapid early increase in $A_{400}$ with no lag phase for both the $\beta$-solenoid-seeded and the stacked $\beta$-sheet-seeded solutions indicates that seeding is occurring in the initial aggregation process.

Serial Seeding from Stacked Sheet Fibrils Increases Fibrillation Rates—Fibrils formed by seeding with stacked $\beta$-sheet fibrils (first passage) were used as seeds in subsequent fibrillation kinetics assays, to four passages (Fig. 7). Consecutive passages showed progressively faster kinetics, but reached a maximum by the third passage, for which the kinetics were within experimental error of the kinetics shown by $\beta$-solenoid-seeded solutions (Fig. 5).

DISCUSSION

Under the conditions studied, fibrillized HET-s(218–289) folded into two distinct architectures: a two-rung $\beta$-solenoid usually formed near neutral pH, and a single-rung stacked $\beta$-sheet structure formed only at low pH. Variations of these architectures have been reported (16, 17); our results do not contradict those studies because different fibrillation conditions can lead to significant structural diversity in amyloids (41). We have shown that, depending on the fibrillation buffer, $\beta$-solenoids can act as structural templates at pH 2. In contrast, stacked $\beta$-sheet fibrils were unable to propagate their own structures under $\beta$-solenoid-forming conditions, but they did alter fibrillation kinetics, removing the lag phase associated with unseeded solutions and apparently bypassing or accelerating an initial nucleation phase to function as heterogeneous seeds.

Although aggregation was rapid in the initial stages of heterogeneously seeded growth, the solutions did not reach the high $A_{400}$ values observed for homogeneous $\beta$-solenoid seeding (Fig. 5). The initial rapid kinetics are probably related to the exten-
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The concomitance between structural complexity and self-propagation is likely to be common to prions in general. Gln/Asn-rich yeast prions may be an exception; scrambling of the prion domains in Ure2p and Sup35p often yields functional prions, indicating that the prion-forming activity is largely independent of the precise sequence (43–45). The robust infectivity and phenotypic propagation of these prions may simply be due to the Gln/Asn-rich sequence. Amide ladders formed from Gln/Asn side-chain interactions between subunits appear to play a key role in prion assembly and infectivity, and the particular spacing of amide interactions within the sequence appears to define the prion phenotype (45, 46). Fiber diffraction from the prion-forming region of Sup35p has suggested that this structure collapses into a stacked β-sheet on dehydration, indicating that the hydrated form does not possess a well packed core (47). These considerations, however, do not apply to HET-s, PrP, or many other self-propagating amyloidogenic molecules because there are major differences in function and composition between the Gln/Asn-rich yeast prions and other prions including HET-s. Yeast prions sequester protein, leading to a loss of function, whereas HET-s causes a gain of function, that is, heterokaryon incompatibility (48, 49). This gain of function is similar to what is seen with PrPSc, where the diseased state is not caused by the loss of cellular PrP (PrPC) function, but by the gain of toxicity (50). In terms of composition, infectious holding of HET-s(218–289) seems to be driven by the formation of salt bridges and a packed hydrophobic core (13) rather than a large number of amide interactions. Interactions between formal charges also appear to play a role in the formation of recPrP as subtle changes in pH affect nucleation and the formation of oligomeric structures that are off-pathway from amyloid formation (51, 52).

The ability of the low pH amyloid form of HET-s(218–289) to nucleate the wild-type infectious structure in vitro shows that amyloids with decidedly different architectures can interact with one another and that heterogeneous seeding does not require in vivo cofactors, but can be achieved in minimal systems. Although the form studied here is to some extent contrived, being apparently a low energy state that compensates for repulsive charge interactions that are unlikely to occur in vivo, the heterogeneous seeding indicates that there is a preferred prion structure that can be reached regardless of the structure of the nucleating agent. In the case of recPrP, this adaptation requires several passages through animals (53), but HET-s(218–289) arrives at the infectious fold immediately, although reproduction of fibrillization kinetics requires several passages. Heterogeneous seeding can thus provide a relatively simple mechanism for strain adaptation and interspecies prion transmission.

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