Mouse Prion Protein Polymorphism Phe-108/Val-189 Affects the Kinetics of Fibril Formation and the Response to Seeding

EVIDENCE FOR A TWO-STEP NUCLEATION POLYMERIZATION MECHANISM

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Background: Alleles Prnpα and Prnpβ of mouse prion protein (PrP) influence the incubation period of prion disease.

Results: PrPα and PrPβ, products of these alleles, aggregate differently in vitro.

Conclusion: The polymorphism at 108/189 influences the oligomeric stages of PrP polymerization.

Significance: Elucidating the mechanism of PrP aggregation is relevant to understanding prion disease susceptibility, prion strains, and species barriers.

Prion diseases are fatal neurodegenerative disorders associated with the polymerization of the cellular form of prion protein (PrPC) into an amyloidogenic β-sheet infectious form (PrPSc). The sequence of host PrP is the major determinant of host prion disease susceptibility. In mice, the presence of allele a (Prnpα, encoding the polymorphism Leu-108/Thr-189) or b (Prnpβ, Phe-108/Val-189) is associated with short or long incubation times, respectively, following infection with PrPSc. The molecular bases linking PrP sequence, infection susceptibility, and convertibility of PrPC into PrPSc remain unclear. Here we show that recombinant PrPα and PrPβ aggregate and respond to seeding differently in vitro. Our kinetic studies reveal differences during the nucleation phase of the aggregation process, where PrPβ exhibits a longer lag phase that cannot be completely eliminated by seeding the reaction with preformed fibrils. Additionally, PrPβ is more prone to propagate features of the seeds, as demonstrated by conformational stability and electron microscopy studies of the formed fibrils. We propose a model of polymerization to explain how the polymorphisms at positions 108 and 189 produce the phenotypes seen in vivo. This model also provides insight into phenomena such as species barrier and prion strain generation, two phenomena also influenced by the primary structure of PrP.

Prion diseases are protein folding disorders that include Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy in cattle, chronic wasting disease in cervids, and scrapie in sheep. These diseases have several similarities to other protein folding neurodegenerative disorders, such as Alzheimer disease, but the hallmark of prion diseases is the infectious nature of the protein responsible for the neurodegeneration (1). The protein in question is the prion protein (PrP),2 and its infectious form is generated when the monomeric, predominantly α-helical, and soluble form (PrPC) is structurally converted into an amyloid structure, oligomeric in nature, high in β sheet, and partially protease-resistant (PrPSc). Many different forms of PrPSc can be generated, and this structural promiscuity is thought to be the molecular basis for prion “strains,” defined by distinct incubation time, phenotype, and/or pathology (2–4). Although certain structures of PrPSc may be better able to convert PrPC from select species, once the process has begun, it is relentless. More PrPC is converted into PrPSc, PrPSc accumulates, and neuronal death follows.

The primary structure of the host PrPSc is a major determinant of the host prion disease susceptibility. For example, subtle differences in PrPSc sequence are sufficient to render some mammals, such as rabbits and horses, immune to prion disease (5–7). Sometimes this species barrier can be crossed, although generally it is an inefficient process, with newly infected species having prolonged incubation periods (7–9). Interestingly, with sequential passages through the new host species, incubation periods become shorter and stabilize at a new and constant incubation period for that host (9). During these passages, it is believed that a process of conformational adaptation or a selection of one subtype of PrPSc conformation is taking place (10). This adaptation is influenced primarily by the sequence of host PrP (7).

The PrP sequence can also vary within a species; these polymorphisms too can influence disease susceptibility or even phenotype. In humans, methionine homozygosity at codon 129 of the prion protein gene (Prnp) increases susceptibility to prion

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2 The abbreviations used are: PrP, prion protein; PrPSc, cellular form of prion protein; PrPC, disease-associated form of prion protein; ThT, thioflavin T; p-FTAA, 4′-3″-bis(carboxymethyl)(2,2′;5′,2″-terpyridine)-5″-dicarboxylic acid; TEM, transmission electron microscopy; GdnHCl, guanidinium hydrochloride; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; a.u., arbitrary units; S, slow; F, fast.
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...disease. Codon 129 also determines the phenotype of a genetic form of Creutzfeldt-Jakob disease caused by the D178N mutation (11). The effect of Prnp polymorphism on disease susceptibility can be found in other species too, including codon 132 in cervids (12) and codons 136/154/171 in sheep (13–17).

Given the clear influence of PrPC sequence on prion disease, the next question is by what mechanism is this influence conferred? The PrPC sequence may dictate which PrPSc conformations are permissive or preferred, or it may influence how readily the conversion process can occur under the guidance of a given PrPSc structure. Although the mechanism of prion conversion into amyloids remains obscure, the canonical model proposed for amyloid formation is nucleated polymerization (18) consisting of two phases: (i) a nucleation phase where monomers undergo conformational change and self-associate to form oligomeric nuclei; and (ii) an elongation phase, in which nuclei rapidly grow by the further addition of monomers, forming larger fibrils until saturation (19). PrPC sequence may regulate either or both of these phases.

In mice, the presence of Prnp allele a (PrnpA, Leu-108/Thr-189) or allele b (PrnpB, Phe-108/Val-189) dramatically influences the incubation period of prion infection (20, 21). Typically, PrnpA mice exhibit shorter incubation times (~100–200 days) when compared with PrnpB mice (~255–300 days) (22–24), although the opposite trend was seen in one study (21). The reason for this discrepancy is not clear, but different prion strains were used; in the latter study, the strain used for inoculation was first passaged in PrnpB mice, whereas PrnpA mice were used as the source of strains for the other studies (25, 26).

As the differences in the primary structure of PrPA and PrPB are the main distinguishing features in these studies, it follows that this polymorphism is the major factor determining the incubation period in vivo. Both residues have been implicated in prion disease, either by playing a role in the initial stages of PrPC conversion (27–29) or by influencing the susceptibility to prion infection (13–16).

We hypothesized that different polymerization kinetics could explain the different incubation periods of these two alleles and that we would be able to detect these kinetic differences in vitro by putting recombinant mouse PrPA or PrPB into fibril-forming assays. Having found this to be the case, we then proceeded to use this in vitro model of conversion to explore the potential mechanisms of fibril formation process in each allele type.

In this work, we compare for the first time the kinetics of amyloid fibril formation of recombinant mouse PrPA and PrPB. From these results, together with the conformational and structural analysis of the formed fibrils, we propose different mechanisms of polymerization for these two isoforms of mouse PrP.

EXPERIMENTAL PROCEDURES

Expression and Purification of Mouse PrP (89–230)—The codon-optimized synthetic genes corresponding to the C-terminal domain of mouse prion protein (89–230) for allele A and B were expressed in Escherichia coli BL21 (DE3) as described earlier (30). The E. coli BL21 (DE3) cells were grown in 25 ml of LB plus 100 μg/ml ampicillin overnight. 10 ml was used to inoculate 500 ml of LB plus 100 μg/ml ampicillin and set to shake at 225 rpm at 37 °C until it reached an A600 of about 1.0. Cells were pelleted down and resuspended in fresh Terrific Broth plus 100 μg/ml ampicillin medium and set to shake at 225 rpm at 37 °C for 1 h before induction with 1 mM isopropyl-β-d-galactopyranoside. Induced cells were grown for a further 12–18 h to reach an A600 of about 2.0. These cells were harvested by centrifugation at 4,500 × g for 25 min at 4 °C. The cell pellet was resuspended in 100 ml of lysis buffer (8x 10 mm Tris, 100 mm Na2PO4, pH 8.0) using vortex and incubated at room temperature for 1 h before sonication using a microtip at 30% amplitude for 10 cycles of 30 s. The cell debris was removed by centrifugation at 15,000 × g for 1 h. The supernatant was incubated with 30 ml of nickel-nitrilotriacetic acid resin with continuous stirring for 30–45 min and transferred to a column. On-column refolding was done by slow gradual change from 8 to 0M urea (10 mM Tris, 100 mM Na2PO4, pH 6.3) (31, 32). Contaminants were removed using 5 column volumes of 10 mM Tris, 100 mm Na2PO4, 50 mM imidazole, pH 6.3. The His-tagged prion proteins were eluted using 10 mM Tris, 100 mm Na2PO4, 500 mM imidazole, pH 6.5. The sample was dialyzed against 10 mM Tris, 2 mM CaCl2, pH 6.3, and the His tag was removed with thrombin by incubation for 24 h at 4 °C. The samples were dialyzed against 10 mM ammonium carbonate, lyophilized, and kept at −80 °C until use.

PrP Fibril Formation—Lyophilized samples of recombinant PrPA and PrPB were dissolved in 6 M guanidinium hydrochloride (GdnHCl) at a protein concentration of 5 mg/ml. Stock solutions were diluted in 50 mM sodium phosphate buffer, pH 7.0, to reach a final concentration of 2 M GdnHCl. The final protein concentration was 0.5 mg/ml unless otherwise indicated. Fibril formation reactions were carried out in 96-well plates (white plate, clear bottom, Costar 3610) covered with thermal adhesive sealing film (08-408-240; Fisher Scientific) at reaction volumes of 200 μl/well. The samples were incubated at 37 °C with continuous shaking at 500 rpm in the presence of 10 μM ThT. Fluorescence measurements were taken at 445/482 nm excitation/emission and 475 nm cutoff on a M5 SpectraMax fluorescence plate reader (Molecular Devices). At least three replicates were measured. For seeding experiments, the reaction was carried out in the presence of sonicated preformed fibrils (1 min in a cup sonicator). Estimation of lag phase was done as reported previously (33). In brief, the data were fitted to the limiting forms of the hyperbolic cosine solution of the equation developed by Bishop and Ferrone (34). The data corresponding to the nucleation stage were fitted to the quadratic equation ½B2At2, whereas those corresponding to the elongation stage were fitted to the exponential function ½AeBt, where t is time and A and B are fitting coefficients. The lag phase was defined as the time point where the fitting curves for quadratic and exponential equation intersect. If the time value for the intersection point is equal to or less than zero, there is an absence of lag phase.

Prefibrillar Oligomer Formation—To monitor the formation of ThT-negative oligomers, aggregation reactions were carried out in 1.5-ml test tubes (protein LoBind, Eppendorf) at reaction volumes of 1.2 ml. The samples were incubated at 37 °C with continuous shaking at 500 rpm, and 50 μl of sample was withdrawn at different times and incubated for 15 min at room
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Results

The presence of chaotropic agent GdnHCl at this concentration has been demonstrated to improve aggregation efficiency (36). Sigmoidal kinetic profiles typical for amyloid fibril formation were obtained for both PrP<sup>a</sup> and PrP<sup>b</sup> when the process was followed by measuring changes in ThT fluorescence intensity (Fig. 1A). A clear difference in lag phase was observed, with PrP<sup>a</sup> taking much longer to develop ThT signal than PrP<sup>b</sup>. Even with variability between preparations, PrP<sup>b</sup> always had longer lag phase (supplemental Fig. S1). Quantitative analysis of the lag phases was performed as described previously (34) (see "Experimental Procedures" and supplemental Fig. S2). Lag phases were 36 (± 4.3) h for PrP<sup>a</sup> and 140 (± 23.0) h for PrP<sup>b</sup> (Fig. 1C). Of note, these lag phase differences were not specific to the denaturing GdnHCl condition as PrP<sup>a</sup> also aggregated more quickly than PrP<sup>b</sup> in acetate buffer, pH 4.0, under the same temperature and shaking conditions (supplemental Fig. S3).

PrP<sup>a</sup> and PrP<sup>b</sup> Lag Phases Differ in Response to Seeding—In nucleated polymerization, the rate-limiting step is nucleus formation. Therefore, the addition of preformed nuclei should eliminate the lag phase and push the process into the elongation phase. To test whether lag phase could be eliminated in our reactions, we added preformed fibrils of homologous PrP (PrP<sup>a</sup> seeds to PrP<sup>a</sup> monomers; PrP<sup>b</sup> seeds to PrP<sup>b</sup> monomers). When the reaction was seeded with 0.5% (v/v) of homologous fibrils (Fig. 1B), the variability between replicates was lower than in unseeded reaction. As expected, the lag phase was eliminated for PrP<sup>a</sup>; however, although the lag phase for PrP<sup>b</sup> was substantially reduced from 140 to 20 h, it was not eliminated (Fig. 1C). Using separate preparations and different concentrations of seed, these differences were still observed. As little as 0.01% (v/v) of seed was sufficient to eliminate the lag phase for PrP<sup>a</sup> (Fig. 1D). PrP<sup>b</sup>, increasing seed from 0.01 to 0.1% (v/v) did shorten the lag phase from 23.0 (± 0.8) h to 17.3 (± 1.1) h, but further increase of seed concentration to 0.5 and 2% (v/v) had no effect.

FIGURE 1. Time courses of PrP<sup>a</sup> and PrP<sup>b</sup> fibril formation monitored by ThT fluorescence. PrP (22 μM) was incubated in 50 mM phosphate buffer, pH 7.0, 2 μM GdnHCl at 37 °C and 500 rpm in the absence or in the presence of seeds. A and B, kinetics of unseeded (A) and seeded (B) polymerization reactions of PrP<sup>a</sup> (empty circles) and PrP<sup>b</sup> (filled circles). C, lag phases for the kinetics of PrP<sup>a</sup> (empty bars) and PrP<sup>b</sup> (filled bars) plotted in A and B. D, lag phase of PrP<sup>a</sup> (left) and PrP<sup>b</sup> (right) aggregation process in the presence of 0.01, 0.1, 0.5, or 2% preformed homologous fibrils. The fibril formation process was followed by ThT fluorescence, and the lag phase was estimated as the intersection of quadratic and exponential fitting curves as described under “Experimental Procedures.” Three replicates were plotted for each reaction. Error bars indicate ± S.E.
Cross-seeding Experiments Reveal Similar Efficiency of Seeding by PrP<sup>a</sup> and PrP<sup>b</sup> Fibrils—To test whether PrP<sup>b</sup> fibrils are simply less efficient at seeding than PrP<sup>a</sup> fibrils, we added pre-formed fibrils of heterologous PrP to our reactions (PrP<sup>b</sup> seeds were added to PrP<sup>a</sup> monomers; PrP<sup>a</sup> seeds were added to PrP<sup>b</sup> monomers) (Fig. 2). The lag phase was eliminated when PrP<sup>a</sup> was seeded with PrP<sup>b</sup> fibrils, indicating that PrP<sup>b</sup> fibrils can indeed seed efficiently. Surprisingly, an identical residual lag phase was observed for PrP<sup>b</sup> regardless of whether reactions were seeded with heterologous PrP<sup>a</sup> or homologous PrP<sup>b</sup> fibrils.

Indications for a Receptive Substrate in the PrP<sup>b</sup> Aggregation Mechanism—The residual lag phase in seeded PrP<sup>b</sup> reactions indicated that the starting PrP<sup>b</sup> monomers were not the immediate substrate being incorporated into fibrils during the elongation phase. Rather, a secondary conformation must have formed first, and it was only this receptive substrate that could be polymerized by the seeds. The generation of this receptive substrate could be (i) a “seed-independent” process, where a set amount of time is required for the starting monomers to form this substrate; or (ii) a “seed-influenced” process, where the fibrils actually facilitate receptive substrate formation. To distinguish between these possibilities, we used a delayed seeding reaction in which PrP<sup>b</sup> was put under aggregation conditions and preformed PrP<sup>b</sup> fibrils were added at different times during the lag phase. We predicted that in a seed-independent process, a set amount of time would be required to form the receptive substrate followed by the same onset of exponential growth phase regardless of when the seeds were added. For a seed-influenced process, we predicted that the onset of exponential growth would correlate with the timing of seed addition. As shown in Fig. 3A, the latter phenomenon was demonstrated. Later seed addition produced longer lag phases (seed time 0, lag phase 9.4 ± 0.3 h; seed time 5 h, lag phase 12.2 ± 0.5 h; seed time 10 h, lag phase 16.2 ± 0.5 h). Also of note was that the later the seed addition, the less time required between seed addition and onset of exponential phase (time 0, lag phase 9.4 h; time 5 h, lag phase 7.2 h; time 10 h, lag phase 6.2 h).

PrP<sup>a</sup> Seeding Induces an Initial Linear Increase in ThT Fluorescence—Looking at the ThT fluorescence levels immediately after seed addition in the delayed seeding experiment, we observed a small but steady linear increase over time prior to the exponential growth phase (Fig. 3B). The immediate jump in ThT can be attributed to the addition of seed as the seed mixture contained some ThT. However, the subsequent rate of increase of ThT was higher when seed was added later. The increase (in ThT a.u./hour) was 6.8 ± 0.6, 14.4 ± 1.7, and 20.7 ± 2.7, for seeding times of 0, 5, and 10 h, respectively.

ThT-negative Oligomers Are Formed Prior to the Exponential Growth of PrP<sup>b</sup> Fibrils—To determine whether oligomeric species that do not bind ThT (ThT-negative oligomers) might be contributing to the early fibrillation process, we examined seeded and unseeded reactions with the anionic oligothiophene derivative p-FTAA, which has been reported to bind prefibrillar oligomers of recombinant PrP (37). In the absence of seeds, an increase in p-FTAA signal was observed ~20 h before the increase in ThT signal, demonstrating the early formation of ThT-negative oligomeric species (Fig. 4). When the reaction was carried out in the presence of seeds, p-FTAA-positive oligomers appeared immediately, whereas ThT-positive fibril formation began 6 h later (Fig. 4).

PrP<sup>a</sup> Aggregation Is Not Predictably Influenced by Protein Concentration—In unseeded reactions undergoing nucleation polymerization, a higher monomer concentration should produce a shorter lag phase, a higher slope at elongation phase, and a higher plateau (19). Therefore, we followed the kinetics of unseeded aggregation as a function of concentration of PrP
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**FIGURE 3. Kinetics of PrPb aggregation seeded at different times.** A, monomeric PrPb (22 μM) was aggregated in the absence (filled circles) or in the presence (empty squares, triangles, and circles) of 0.5% (v/v) homologous seeds. The seeds were added at the beginning of the reaction (squares), after 5 h (triangles), or after 10 h (empty circles). The kinetics were normalized, and the percentage of ThT fluorescence was graphed. B, the same data were plotted as absolute fluorescence using a different y scale to focus on the processes happening before the exponential phase. The slopes were calculated by fitting the data with linear regression. The arrows in A and the dotted lines in B indicate the times at which the seeds were added. Three replicates were averaged for each reaction. Error bars indicate ± S.E.

Monomeric PrPa kinetics were proportional to monomer concentration (Fig. 5). PrPa kinetics were proportional to monomer concentration (Fig. 5, A and C). For 11, 16, and 27 μM PrP, respectively, the lag phases were 36.7 (± 4.3), 26.3 (± 1.2), and 18.0 (± 1.7) h, the slopes for elongation phase were 6.1 (± 0.8), 88.8 (± 7.9), and 191.0 (± 45.2) ThT a.u./h, and the plateaus were 1180 (± 32), 2790 (± 99), and 4800 (± 111) ThT a.u. Importantly, these kinetic parameters changed linearly with monomer concentration, whereas an exponential correlation is expected from a pure nucleated polymerization model. For PrPb, no clear correlation was observed between these parameters and protein concentration for PrPb (Fig. 5, B and C). In fact, the highest concentration (27 μM) PrPb reactions consistently had the longest lag phases, opposite to what the nucleated polymerization model predicts. There was also significantly more kinetic variability among PrPb replicates as shown in the non-averaged replicate curves in Fig. 5B.

**FIGURE 4. PrPb prefibrillar (ThT-negative) oligomer formation monitored by p-FTAA.** Monomeric PrPb (20 μM) was put into aggregation assays in 1.5-ml test tubes. 50 μl was withdrawn at different times and incubated with p-FTAA (squares) or ThT (circles) to monitor the formation of prefibrillar oligomers and fibril formation, respectively. The assay was carried out in the absence (filled symbols) or in the presence (empty symbols) of 1% (v/v) of seeds.

Monomeric PrPb and PrPb Share Denaturation/Renaturation Characteristics—A correlation between the thermal lability of mouse PrP and conversion efficiency has been reported (38), and distinct unfolding properties of PrPa and PrPb monomers might explain their different kinetic profiles. We therefore compared the denaturation profiles of PrPb and PrPb by following the 222 nm intensity as a function of temperature or Gdn-HCl concentration. Loss of the 222 nm minimum correlates with loss of α-helix structure. Thermal denaturation and renaturation of PrP produced identical curves for both PrP isoforms (supplemental Fig. S4A, inset); Gdn-HCl denaturation curves for PrPb and PrPb were also identical (supplemental Fig. S4D).

**Seed Type Influences the Conformational Stability of PrPb Fibrils More than PrPb Fibrils**—We used a conformational stability assay to study differences between PrPb and PrPb fibrils generated under unseeded and seeded conditions. In this assay, increasing concentrations of Gdn-HCl are used to gradually denature the fibrils, rendering them more susceptible to proteolysis with proteinase K. Structural differences in fibrils can affect the denaturation process and generate distinct proteolysis bands.

All proteinase K-treated PrP fibrils yielded prominent bands of ~13 and 18 kDa (Fig. 6, A–C). In unseeded reactions, the 18-kDa band was more intense and more sensitive to protease action, disappearing in the range of 5.4–5.8 M Gdn-HCl. The 13-kDa band was still visible at 6 M Gdn-HCl (Fig. 6A). Similar results were obtained for PrP fibrils generated in the presence of either homologous or heterologous seeds (Fig. 6, B and C). The pattern was not exactly replicated; the relative band intensities were reversed in the homologous seeded reaction, the 13-kDa band being more intense than the 18-kDa band, and in
the heterologous seeded reaction, the intensities of the two bands were comparable. Also, two other faint bands were only seen in the unseeded reactions.

In contrast, differences in PrPb fibril stability were more apparent and highly dependent on the seeding conditions. Unseeded PrPb fibrils had a pattern similar to unseeded PrPa fibrils, with a resistant 13-kDa band and a more intense 18-kDa band that disappeared after 5.4–5.8 M GdnHCl treatment (Fig. 6D). However, under homologous seeding conditions (Fig. 6E), the 13-kDa band remained resistant and the 18-kDa band was not present at all, indicating a dramatically different structural state in these PrPb fibrils. Under heterologous seeding conditions (Fig. 6F), the 18-kDa band was present, was more intense, and disappeared with 5.8 M GdnHCl, as in the unseeded reactions. The usually resistant 13-kDa band was more sensitive, disappearing by 5.4 M GdnHCl.

Seed Type Influences the Kinetics of Fibril Formation for PrPb but Not for PrPa—Based on our conformational stability assay results, we speculated that if different seeds could strongly influence the stability of PrPb fibrils generated, different seeds might also affect PrPb aggregation kinetics more strongly than PrPa kinetics. Because of the variability of unseeded PrPb fibril reactions, we were able to generate two PrPb seeds with distinct kinetic profiles. The “slow” (S) fibrils had an elongation phase slope of 4.1 ThT a.u./h, whereas the “fast” (F) fibrils had one of 13.6 ThT a.u./h (Fig. 7, dashed and dotted curves). The lag phases for S and F fibril formation were 15 and 19 h, respectively. S and F seeds were used to seed PrPa and PrPb reactions.

As expected, PrPa lag phase was eliminated regardless of the type of seed used (Fig. 7A), whereas for PrPb, residual lag phases between 7 and 9 h occurred with seeding (Fig. 7B). For PrPa, the kinetics of reactions seeded with S or F were almost identical, with slopes of 12.5 and 13.5 ThT a.u./h, respectively (Fig. 7A). These slopes were also similar to those obtained for the elongation phase of unseeded reactions of PrPa (Fig. 7A, open triangles). In contrast, two distinct kinetic profiles were obtained for PrPb seeded with S or F fibrils, the slopes being 4.1 and 8.7 ThT a.u./h (Fig. 7B). The slope of the S-seeded reaction is identical to that of the original S fibril formation, whereas that for the F-seeded reaction is intermediate between the original S and F fibril formation kinetics.

TEM of PrP Fibrils—Fibril production was confirmed using TEM, and differences between PrPa and PrPb samples were also noted. All PrPa samples contained straight fibrils of similar length (≈600–800 nm) and width (≈10 and 20 nm in narrow and wide regions) regardless of whether the fibrils were produced under seeded or unseeded conditions and regardless of whether S or F seeds were used (Fig. 8, A–C, and Table 1). However, although unseeded PrPb fibrils (Fig. 8D) had grossly similar structures to PrPa fibrils (Table 1), different features occurred with seeding. PrPb fibrils formed in the presence of F seeds (Fig. 8E and Table 1) were shorter (≈100 nm) and uniform in width (≈15 nm); those seeded with S seeds (Fig. 8F and Table 1) were of intermediate length (≈300 nm) and sometimes curved, in contrast to the straight fibrils of PrPa.
DISCUSSION

In mice, the polymorphisms at positions 108 and 189 are major determinants of prion disease incubation time (20, 21), but the molecular basis for this phenomenon is unclear. We hypothesized that the effect of host PrP sequence on incubation time was related to the mechanism of prion conversion, involving the oligomerization of PrP^C into PrP^SC. In this study, we provide evidence that PrP aggregation does not follow the canonical nucleation polymerization and that in vivo findings can be explained by different polymerization kinetics and seeding responses of the two mouse PrP isoforms.

PrP^A and PrP^B differ only at residues 108 and 189, and we did not detect differences in α-helical content or thermal/chemical stability between the two monomers. Instead, the primary differences were found between their kinetic and seeding behaviors.

Based on our kinetic data, neither PrP isoform follows true nucleation polymerization. This model predicts that fibril mass is proportional to \( f^2 \) during nucleation, meaning that there is never a flat lag phase (39, 40). Both PrP^A and PrP^B had prolonged flat lag phases in unseeded conditions. This better fits a double-pathway model of aggregation kinetics by proposing the existence of “conformationally active monomers,” which randomly form different polymerization pathways to receptive substrates. This variability suggests that a number of pathways to receptive substrate formation are possible and that several different receptive substrates can be produced, each of which then produces a different kinetic profile and fibril structure. This model could explain the variability seen in our data, but not why we cannot eliminate the lag phase with seeding nor why we do not see a correlation between PrP^B kinetics and monomer concentration. Rather than monomers, our data suggest that conformationally active oligomers may be the necessary precursors to the formation of receptive substrates.

If the production of conformationally active oligomers is the rate-limiting step, dependent on structural change from the monomer state, it follows that changes in monomer concentration will not facilitate the process of fibril growth, as found in our study. Also, the addition of seed to starting monomers does not immediately lead to exponential growth in PrP^B because starting monomers must first form conformationally active oligomers and then receptive substrates; only the receptive substrates are incorporated into fibril growth. The presence of these early oligomers (ThT-negative and ThT-low oligomers) was confirmed by p-FTAA fluorescence. A similar mechanism, “nucleated conformational conversion,” has been described for the yeast prion element [PSI+], where nuclei are formed by conformational rearrangement of less structured oligomeric intermediates that are in equilibrium with monomers (43).

Of note, although seeding does not eliminate PrP^B lag phase, seed addition does greatly accelerate the time to exponential growth phase, meaning that the seed must be affecting the process in some manner. Seed addition produces an immediate and rapid increase in p-FTAA fluorescence and a slower linear increase in ThT fluorescence (prior to the exponential growth phase), with later addition yielding both a higher rate of ThT increase and a shorter time to onset of exponential growth phase. Given this, we propose the following mechanism (Fig. 9). Starting monomers gradually aggregate into larger and larger oligomers (which are ThT-negative but p-FTAA-positive). When these ThT-negative oligomers bind seeds, they become conformationally active and acquire some low ThT fluorescence (ThT-low, p-FTAA-positive), but do not yet become fully receptive substrates (and therefore do not undergo exponential growth). As larger ThT-negative oligomers are expected to accumulate over time, seeds that are added to the process later will bind larger ThT-negative oligomers that in turn become conformationally active, thus producing higher rates of ThT increase. The larger oligomers may also be closer to adopting a
receptive substrate conformation, which would explain why there is an apparent acceleration to exponential phase after delayed seed addition.

It should be noted that PrP<sup>a</sup> could fundamentally share the mechanism that we propose for PrP<sup>b</sup> and still display the kinetic and seeding tendencies we observed. If PrP<sup>a</sup> converts to conformationally active oligomers and then to receptive substrates more efficiently, and is not rate-limiting, the conformationally active oligomers would not be detectable in our seeded kinetic studies. The efficient formation of receptive substrates might correlate with less off-pathway aggregation, explaining the shorter lag phases and reduced variability seen in unseeded PrP<sup>a</sup> reactions. It would also explain why PrP<sup>a</sup> is not overly influenced by seed type; there is less time for seed and oligomer to interact.

We are proposing that the seed induces structural change without immediately triggering polymerization. Such a process has been described in “surface-catalyzed nucleation,” where monomers nonspecifically bind the lateral aspects of fibrils with subsequent conformational rearrangement to form on pathway oligomers, and ultimately nuclei, which can bind the ends of fibrils and proceed with exponential growth (44, 45). Such lateral association is a recognized process in recombinant PrP fibril formation (46).

Somewhat unexpectedly, our conformational stability assay and TEM experiments revealed that PrP<sup>b</sup> may be structurally influenced by seeds to a greater extent than PrP<sup>a</sup>; the morphology and stability of PrP<sup>b</sup> fibrils did not vary as much as PrP<sup>b</sup> fibrils. Also, PrP<sup>b</sup> more closely mimicked the kinetics of the seeds used.

The purpose of this study was to gain insight into how polymorphisms of PrP translate into phenotypic differences in prion disease by studying kinetic profiles of fibril formation in vitro. The long lag phases for PrP<sup>b</sup> correlate with long incubation times in Prnp<sup>b</sup> mice (21–25). In addition, if conformationally active oligomers are part of the PrP aggregation mechanisms, they may present a new therapeutic target, one that lies structurally in between monomeric and fibrillar states. Finally, our data may also inform the study where a shorter incubation time was seen in Prnp<sup>a</sup> mice. Here, strain properties may be key, and we have found that PrP<sup>a</sup> appears to be more strongly influenced by the type of seed used. Importantly, this seed influence could have risk implications as hosts presumed to be less susceptible based on PrP sequence may simply need to be exposed to the appropriate strain. A real life example of this can be found in the rise of chronic wasting disease. At present, chronic wasting disease does not appear to cross the species barriers into humans, but there is growing evidence for distinct strains of chronic wasting disease, and the influence of human PrP polymorphism on their transmission characteristics is unknown.

### TABLE 1

Quantitative analysis of PrP<sup>a</sup> and PrP<sup>b</sup> fibrils generated under unseeded and seeded conditions

| PrP<sup>a</sup> fibrils | PrP<sup>b</sup> fibrils |
|-------------------------|-------------------------|
| **No. of fibrils analyzed** | **Unseeded** | **Fast seeds** | **Slow seeds** | **Unseeded** | **Fast seeds** | **Slow seeds** |
|                         | 21            | 27            | 23        | 15        | 18        | 20       |
| **Fibril width narrow region** | 10.7 ± 0.8 | 9.6 ± 0.6   | 8.8 ± 0.6 | 10.2 ± 0.6 | 14.5 ± 0.7 | 8.4 ± 0.4 |
| **Fibril width wide region** | 23.3 ± 0.8 | 20.7 ± 1.1  | 19.5 ± 1.1 | 19.2 ± 1.1 | 14.5 ± 0.7 | 20.0 ± 1.1 |
| **Fibril length**        | 821 ± 126    | 681 ± 82     | 602 ± 67 | 616 ± 86  | 102 ± 13   | 332 ± 58  |

![FIGURE 9. Proposed mechanism of PrP<sup>b</sup> fibril formation in unseeded and delayed seeding conditions.](image-url)

A, the unseeded reaction is shown, where monomers (dark triangles) first form ThT-negative (p-FTAA-positive) oligomers of increasing size and ultimately form conformationally active oligomers (light triangles, ThT-low, p-FTAA-positive) followed many hours later by receptive substrates (white squares, ThT-high), which go on to form fibrils. B, a typical seeded reaction is shown, with seed added at time 0. When monomers bind seed, they are immediately converted into a conformationally active state (ThT-low, p-FTAA-positive). As more monomers bind and are converted, a sufficient number or size is reached such that receptive substrates form. C and D, with delayed seeding, larger ThT-negative (p-FTAA-positive) oligomers are already present when seed is added, so immediate conversion of the larger oligomers occurs (becoming ThT-low, p-FTAA-positive), giving a greater linear increase in ThT fluorescence signal over time and allowing receptive substrates to form in a shorter time period after seed addition.
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