Annealing Prion Protein Amyloid Fibrils at High Temperature Results in Extension of a Proteinase K-resistant Core

Olga V. Bocharova, Natalia Makarava, Leonid Breydo, Maighdlin Anderson, Vadim V. Salnikov, and Ilia V. Baskakov

From the Medical Biotechnology Center, University of Maryland Biotechnology Institute and the Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland 21201

Self-assembly of polypeptides into amyloid structures has been implicated in diverse biological functions, including colonization and biofilm formation of *Escherichia coli* (8), melanosome biogenesis (10), and maintenance of long-term memory (11). Furthermore, amyloid fibrils are utilized as natural biomaterials, e.g. oocyte eggshells in insects and fish (9). The self-propagating conversion of several prion proteins into amyloid forms underlies a non-mendelian type of inheritance in yeast and fungi (12). In additions, the unique physical properties of amyloid structures generated from synthetic peptides have been exploited for creating novel biomaterials and nanodevices (13).

Conversion from a native fold into an amyloid state is a complex process that involves several consecutive steps (14). Because small oligomeric aggregates have been shown to be inherently toxic and have been implicated in a number of conformational diseases (15–17), the initial steps of polymerization, such as early oligomerization and nucleation, have been a recent subject of intense studies (18–21). When the conversion from a native to amyloid state is completed, mature amyloid structures are believed to represent the most conformationally rigid and thermodynamically stable folds (22).

Using amyloid fibrils generated from full-length mammalian recombinant prion protein (rPrP), we show that substantial conformational rearrangement may take place within an apparently mature amyloid structure. This conformational rearrangement can be induced by brief exposure to 80 °C (or a longer incubation at 37 °C) and consists of a substantial extension of the proteolytically resistant core and an increase in the amount of β-sheet-rich structure. We postulate that the amyloid structure can exist in two states that are separated by a high energy barrier. The two amyloid states differ in the conformation of the prion protein. The energy barrier may be a feature of polypeptides in general (7).

Naturally produced amyloid structures are now found in a variety of organisms, including prokaryotes, insects, fish, and mammals (8–10).

More than 15 severe maladies, including prion, Alzheimer, and Parkinson diseases, are related to the formation of specific β-sheet-rich protein aggregates known as amyloid fibrils (1, 2). Recent studies demonstrated that a broad range of proteins unrelated to any known conformational disease are capable of forming β-sheet-rich amyloid forms both in vitro and in vivo (3–6). This finding has led to the proposition that the ability to fold into amyloid structures is not a unique property of certain proteins associated with degenerative maladies; rather, it may well be a feature of polypeptides in general (7).

Amyloids are highly ordered, rigid β-sheet-rich structures that appear to have minimal dynamic flexibility in individual polypeptide chains. Here, we demonstrate that substantial conformational rearrangements occur within mature amyloid fibrils produced from full-length mammalian prion protein. The rearrangement results in a substantial extension of a proteinase K-resistant core and is accompanied by an increase in the β-sheet-rich conformation. The conformational rearrangement was induced in the presence of low concentrations of Triton X-100 either by brief exposure to 80 °C or, with less efficacy, by prolonged incubation at 37 °C at pH 7.5 and is referred to here as “annealing.” Upon annealing, amyloid fibrils acquired a proteinase K-resistant core identical to that found in bovine spongiform encephalopathy-specific scrapie-associated prion protein. Annealing was also observed when amyloid fibrils were exposed to high temperatures in the absence of detergent but in the presence of brain homogenate. These findings suggest that the amyloid fibrils exist in two conformationally distinct states that are separated by a high energy barrier and that yet unknown cellular cofactors may facilitate transition of the fibrils into thermodynamically more stable state. Our studies provide new insight into the complex behavior of prion polymerization and highlight the annealing process, a previously unknown step in the evolution of amyloid structures.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

1 Both authors contributed equally to this work.

2 Present address: Kazan Inst. of Biochemistry and Biophysics, Russian Academy of Sciences, Kazan 420111, Russia.

3 Present address: Kazan Inst. of Biochemistry and Biophysics, Russian Academy of Sciences, Kazan 420111, Russia.

4 To whom correspondence should be addressed: Medical Biotechnology Center, University of Maryland Biotechnology Inst., 725 W. Lombard St., Baltimore, MD 21201. Tel.: 410-706-4562; Fax: 410-706-8184; E-mail: Baskakov@umbi.umd.edu.

5 The abbreviations used are: rPrP, recombinant prion protein; PK, proteinase K; GdnHCl, guanidine hydrochloride; AFM, atomic force microscopy; PrPSc, scrapie-associated prion protein; α-rPrP, α-helical recombinant prion protein.
**Annealing Prion Protein Fibrils**

Annealing Procedure—Preformed rPrP fibrils (in 10 mM sodium acetate (pH 6.0)) were supplemented with Tris-HCl (pH 7.5) to a final concentration of 100 mM, and then Triton X-100 (Fisher) was added to a final concentration of 0.01–1% as specified. Samples were heated in a water bath at 80 °C for 5–60 min and spun down for 2 s on microcentrifuge to restore the initial volume. In an alternative format, the amyloid fibrils were incubated for 24 h at 37°C in 100 mM Tris-HCl (pH 7.5). Samples were treated with proteinase K (PK) at 37°C for 1 h in 100 mM Tris-HCl (pH 7.5) at a 1:50 PK/rPrP ratio. Digestion was stopped by quenching with 2 mM phenylmethylsulfonyl fluoride, followed by addition of 4X sample buffer and 9 mM urea to a final urea concentration of 2.25 M. Samples were heated at 95 °C for 15 min and analyzed by SDS-PAGE on precast 12% NuPAGE gels (Invitrogen), followed by silver staining.

Total RNA and mRNA were purified from healthy mouse brains using the RNeasy lipid tissue kit and the Oligotex direct mRNA kit (Qiagen Inc.) according to the manufacturer’s protocols. The concentration and purity of RNA samples were determined by measurement of absorbance at 260 and 280 nm (A_{260}/A_{280} > 2.0). The mixture of tRNAs was from bovine liver (Calbiochem), and heparan sulfate (fast-moving fraction) was from porcine intestinal mucosa (Sigma catalog no. H 9902). 5% normal brain homogenates were prepared in phosphate-buffered saline (pH 7.2) in the absence of detergents using a Potter-Elvehjem polytetrafluoroethylene/glass tissue grinder. Syrian hamster brains and antibody 3F4 were kindly provided by Dr. Rohwer.

Partial Unfolding of Am*-rPrP (Amyloid Fibrils with an Extended PK-resistant Core)—Am*-rPrP fibrils were incubated for 1 h at 37°C with 2, 3, or 4 M guanidine hydrochloride (GdnHCl) in 100 mM Tris-HCl (pH 7.5). One-half of each sample was diluted 4-fold into 100 mM Tris-HCl (pH 7.5), incubated for 6 h at 37°C to allow renaturation, and diluted 4-fold further into 100 mM Tris-HCl (pH 7.5) prior to PK digestion. To assay PK resistance of partially denatured fibrils, the second half of each sample was diluted 8-fold into 100 mM Tris-HCl (pH 7.5) containing 2, 3, or 4 M GdnHCl, respectively, to maintain the initial concentrations of GdnHCl. The control sample was not subjected to denaturation and was diluted and treated with PK as described for the other samples. To compensate for a loss of PK activity in the assays carried out under partially denaturing conditions, PK concentrations in the samples containing GdnHCl was adjusted accordingly (PK/rPrP ratios of 1:29 and 1:34 for the assays carried out in the absence and presence of GdnHCl, respectively). The PK concentration in these samples was optimized in preliminary experiments. PK digestion (1 h at 37°C in 100 mM Tris-HCl (pH 7.5)) was stopped by quenching with 2 mM phenylmethylsulfonyl fluoride, followed by addition of 4X sample buffer and 9 mM urea to a final urea concentration of 2.25 M. Samples were heated at 95 °C for 15 min and analyzed by SDS-PAGE on precast 12% NuPAGE gels, followed by Western blotting with Fab fragments P and R1.

Electron Microscopy and Fourier Transform Infrared Spectroscopy—Electron microscopy and Fourier transform infrared spectroscopy were performed as described previously (24).

Atomic Force Microscopy (AFM)—Fibrils were imaged with a PicoSPM LE atomic force microscope (Molecular Imaging Corp., Phoenix, AZ) operating in MAC (alternating magnetic field) mode using a MAC II silicon cantilever (tip radius of <7 nm and spring constant of 2.8 newtons/m) and a liquid cell (Molecular Imaging Corp.). Each sample was diluted to 0.25 mg/ml in 10 mM Tris-HCl (pH 7.5), deposited on a Fisherbrand glass coverslip (22-mm circumference), left to adhere for 30–40 min, and then washed three times with filtered ultrapure H₂O. The slide was placed in the fluid cell and immersed in 200 μl of 10 mM

**FIGURE 1. Heat treatment extends PK-resistant cores in amyloid fibrils.** a, mouse rPrP (23–230) was folded into Am-PrP, the β-sheet-rich form (β-rPrP), or α-rPrP and subjected to the annealing procedure for 15 min at 80 °C in 1% Triton X-100 and 100 mM Tris-HCl (pH 7.5), followed by PK digestion (150 PK/rPrP ratio). In contrast to a previous study in which PK digestion was performed at pH 5.5 (56), in this study at pH 7.5, β-sheet-rich rPrP did not show any notable PK-resistant core (lanes 6 and 7). b, shown is the time and temperature dependence of the annealing reaction. Am-PrP fibrils were incubated for 5–60 min at 20–90 °C as indicated in 1% Triton X-100 and 100 mM Tris-HCl (pH 7.5), followed by PK digestion (150 PK/rPrP ratio). In contrast to a previous study in which PK digestion was performed at pH 5.5 (56), in this study at pH 7.5, β-sheet-rich rPrP did not show any notable PK-resistant core (lanes 6 and 7). c, the time dependence of the annealing reaction carried out at 37 °C. Am-PrP fibrils were incubated for 0–24 h in 1% Triton X-100 and 100 mM Tris-HCl (pH 7.5), followed by PK digestion. d, shown is a diagram illustrating the major steps of in vitro polymerization of rPrP. The first step, referred to as “conversion,” is the formation of Am-PrP fibrils in which region ~23–140 adopts a PK-sensitive conformation (25), whereas the second step, referred to as annealing, leads to conformational rearrangements in which region ~90–140 adopts a PK-resistant conformation.

Tris-HCl (pH 7.5). All imaging was performed at a scan rate of 0.5 lines/s at a drive frequency of 25–30 kHz.

**RESULTS**

Previously, we reported that amyloid fibrils produced from full-length rPrP in vitro have abnormally short PK-resistant cores (residues 138–230, 152–230, and 162–230; 12-, 10-, and 8-kDa bands, respectively) (Fig. 1a, lanes 4 and 5) compared with that of classical scrapie-associated prion protein (PrPSc) residues ~90–230 (25). Despite substantially shorter PK-resistant cores, the fibrils of rPrP retain the physical properties typical of amyloid structures and display remarkable resistance to thermal denaturation (23). Specifically, the β-sheet component of amyloid structures (the band at 1618–1622 cm⁻¹) remains stable even upon heating to 80 °C, whereas the loop, β-turns, and α-helical components (the band at 1661 cm⁻¹) undergo reversible melting at 80 °C (23). As judged by electron microscopy, the amyloid form maintained fibrillar morphology even after heating to 80 °C (see Fig. 3).
Because heating induced only local unfolding in the amyloid fibrils while preserving their structural integrity, we were interested in determining the extent to which the rigid fibrillar structure could change in response to high temperatures. In an attempt to detect any conformational changes, we employed an array of biophysical techniques, including a PK digestion assay. After screening a variety of experimental conditions, we found that the PK-resistant core elongated substantially upon brief exposure of the fibrils to 80 °C in the presence of 1% Triton X-100 (Fig. 1a, compare lanes 2 and 3). Specifically, we observed a gradual decrease in the amounts of 10- and 12-kDa PK-resistant bands accompanied by a parallel increase in the amount of a new band at 16 kDa (Fig. 1b). When 1% Triton X-100 was used in the absence of heat treatment, the 8-kDa band was no longer produced in the PK digestion assay, suggesting that Triton X-100 on its own stabilized the amyloid form to a certain extent (Fig. 1a, compare lanes 2 and 4). However, no 16-kDa band was detected in the absence of heat treatment. The new PK-resistant fragment of 16 kDa could be stained with Fab fragments P and R1, specific to residues 96–105 and 225–230, respectively, but not with polyclonal antibody specific to residues 79–97 (data not shown). To ascertain the new location of the PK cleavage site, the 16-kDa fragment was subjected to N-terminal sequencing, which revealed that the PK-resistant fragment of 16 kDa (Fig. 1b). When 1% Triton X-100 was used in the absence of heat treatment, the 8-kDa band was no longer produced in the PK digestion assay, suggesting that Triton X-100 on its own stabilized the amyloid form to a certain extent (Fig. 1a, compare lanes 2 and 4). However, no 16-kDa band was detected in the absence of heat treatment. The new PK-resistant fragment of 16 kDa could be stained with Fab fragments P and R1, specific to residues 96–105 and 225–230, respectively, but not with polyclonal antibody specific to residues 79–97 (data not shown). To ascertain the new location of the PK cleavage site, the 16-kDa fragment was subjected to N-terminal sequencing, which revealed that the PK-resistant core started at Gln97. By analogy to the annealing of fourier transform infrared spectroscopy revealed that the annealing of fibrils displayed partial disassembly of laterally associated filaments and the appearance of segments with irregular morphologies (Fig. 3b).

Next, we were interested to test whether annealing could be achieved at physiologically more relevant temperatures. We found that extension of the PK-resistant core occurred even at 37 °C (in 1% Triton X-100), but with a lower efficacy and only after a prolonged incubation time (>24 h) (Fig. 1c). To optimize experimental conditions, different concentrations of Triton X-100 were tested at 80 °C. At a fixed concentration of rPrP, the yield of annealing increased with a decrease in detergent concentration to as low as 0.01%, at which the molar ratio of Triton X-100 to rPrP was 25:1 (supplemental Fig. 1). The optimal concentration of detergent was found to be 0.05%, which was used in subsequent experiments.

Several biophysical mechanisms, including high order assembly of fibrils into more complex supramolecular structures, may account for the annealing effect. To test whether heating can induce the assembly of fibrils into higher order structures, we employed AFM performed in a liquid cell. As judged by AFM, brief heating to 80 °C caused substantial fragmentation of fibrils into shorter pieces, which was also evident from the electron microscopy imaging, and spatial regrouping of fibrils into small clusters (Fig. 4, a and b). However, separate fibrils were clearly visible (Fig. 4b, inset). Detailed analyses of single fibrils by AFM revealed a slight increase in fibril width and height (Fig. 4c). A rearrangement of the fibrillar substructure and/or binding of Triton X-100 molecules may account for these minor changes in fibrillar shape. However, no signs indicating additional assembly of fibrils were found. Taken together, our data illustrate that brief exposure of amyloid fibrils to high temperatures induces conformational rearrangements within the fibrillar substructure rather than higher order assembly into more complex amyloid structures.

To test whether the annealing is reversible and whether the Am*-rPrP fibrils are able to transform back into the initial Am-rPrP conformation after partial denaturation, we conducted a partial unfolding experiment. In this experiment, the Am*-rPrP fibrils were subjected to denaturation with 2, 3, or 4 M GdnHCl, followed by PK analysis of the partially denatured and renatured samples. In Am*-rPrP fibrils exposed to 2, 3, or 4 M GdnHCl, the 16-kDa band disappeared upon treatment with PK, whereas the original PK-resistant core (12-kDa band)
remained intact (Fig. 5). When diluted out of GdnHCl, the fibrils were able to refold back to the Am*-rPrP state as judged by the appearance of the 16-kDa band. The renaturation into the Am*-rPrP form was efficient only if the denaturation took place at 2 or 3 (but not 4) M GdnHCl. Therefore, 4 M GdnHCl seems to be critical for reversibility of refolding to the Am*-rPrP state. Notably, the Am-rPrP fibrils did not adopt the Am*-rPrP conformation upon dilution under partially denaturing conditions (supplemental Fig. 2) unless they were treated using the annealing procedure. This experiment illustrates that, under partially denaturing conditions, the central region (residues ~97–140) "opens up" and becomes accessible to PK; however, it still "remembers" the Am*-rPrP conformation and refolds back upon dilution out of GdnHCl. Most important, these findings illustrate that the PK-sensitive conformation that the central region adopts under partially denaturing conditions in the Am*-rPrP state is not equivalent to the PK-sensitive conformation of the same region in the Am-rPrP fibrils.

Taken together, these results indicate that region 97–140 is partially exposed to the solvent in the Am-rPrP state and that this region plays a key role in the transformation of fibrils from the Am-rPrP to Am*-rPrP state. Thereafter, we were interested in whether the interaction of the Am-rPrP fibrils with other macromolecules interferes with the transition from the Am-rPrP to Am*-rPrP state. Several classes of polyanions, including heparan sulfate and RNA, have been shown to bind selectively to the binding sites within the N-terminal region of PrP (residues 23–140) (26, 27). We tested the effect of both heparan sulfate and RNA on the efficacy of annealing. As judged by a decrease in the intensity of the 16-kDa band, the presence of either heparan sulfate (heparan sulfate/rPrP molar ratios of 0.26 and 2.6) or mouse brain total RNA (RNA/rPrP ratios of 2 × 10^{-3} to 2 × 10^{-5}) significantly attenuated the annealing process (Fig. 6, a and b). Inhibition of the annealing process was accompanied by aggregation of Am-rPrP fibrils into clumps (data not shown). mRNA from mouse brain or a mixture of tRNAs from bovine liver also inhibited formation of the Am*-rPrP state (data not shown).
and cooled down; however, the 16-kDa band appeared when the brain homogenate was heated again in a mixture with Am-rPrP fibrils (data not shown). Remarkably, the annealing was also observed in the absence of Triton X-100 or any other detergent when Am-rPrP fibrils were exposed to 80 °C in a mixture with brain homogenate (Fig. 7b). However, in the absence of Triton X-100, the efficacy of annealing decreased. The amount of the 16-kDa band was reduced further when Am-rPrP fibrils and brain homogenate where heated separately (Fig. 7b, third and fourth lanes). Notably, the 16-kDa band appeared upon long-term incubation of Am-rPrP fibrils in a mixture with brain homogenate at 37 °C in the absence of Triton X-100 (Fig. 7c). The 10- and 12-kDa bands were still visible; however, their intensities were reduced upon incubation at 37 °C (supplemental Fig. 3). Taken together, these data indicate that brain homogenate may contain a cofactor that stimulates transition from the Am-rPrP to Am*-rPrP state, that the effect of the cofactor is specific with respect to the amyloid fibrils (but not α-rPrP), and that the cofactor should be present at the moment of heating.

DISCUSSION

We have reported that substantial conformational rearrangements occur within the apparently mature amyloid structure of rPrP amyloid fibrils even after the conversion from the native α-helical to amyloid state is completed. Such rearrangements are facilitated by brief exposure to high temperatures and result in the extension of the PK-resistant core within the amyloid state. Amyloid structures are known to resist heating up to 100 °C (28). Exposure to high temperatures is also known to facilitate the complex processes that involve complementary inter- or intramolecular interactions, such as annealing of nucleic acids and protein domain swapping (29). To our knowledge, the ability of polypeptides to undergo substantial conformational rearrangements within mature amyloid structures at high temperatures has never been demonstrated before. In previous studies, large conformational changes were observed in fibrils produced from the yeast prion protein Ure2p (30). Ure2p fibrils are different from rPrP fibrils in one key aspect: in the fibrillar form, Ure2p retains its native α-helical structures that are devoid of a cross-β-core (30). Heat treatment results in a substantial conformational change consisting of formation of a cross-β-core and a decrease in α-helicity (30). In this respect, the heat-induced transition of Ure2p fibrils is similar to the transitions from a native α-helical to β-sheet-rich state described previously for other proteins, including rPrP (31, 32). On the other hand, our finding emphasizes that substantial conformational rearrangements may occur even after the conversion of α-rPrP into mature amyloid structures is completed.

The annealing of amyloid fibrils consisted of a transition of the PK-resistant core, an increase in the amount of β-sheet-rich structure, and some minor changes in fibrillar shape. We have not found any evidence of higher order assembly of fibrils into more complex supramolecular structures. Therefore, transition from the Am-rPrP to Am*-rPrP state appears to involve only conformational rearrangements within the fibrillar substructure rather than higher order assembly.

What molecular mechanisms account for the annealing of amyloid fibrils? Because the transition between two amyloid states is facilitated at high temperatures, the Am-rPrP state appears to be separated from the Am*-rPrP state by a high energy barrier. Using an Arrhenius plot of the data from Fig. 1 (b and c), we estimated that the activation energy for annealing of Am-rPrP fibrils was at least 30 kcal/mol. A short exposure to high temperatures (or a longer incubation at 37 °C) helps to overcome this barrier and to reach a lower energy Am*-rPrP state. One possible explanation is that the transition that separates the Am-rPrP and Am*-rPrP states may involve an alignment of β-strands (33). Temperature-induced alignment was reported previously for amyloid fibrils produced from short peptides, including Aβ peptide encompassing residues 16–22 (34) and PrP-derived peptides spanning residues 109–122 (33). This alignment is accompanied by an increase in the amount of β-structures and is found only in fibrils in which the β-strands are able to align at residue 117 upon a cycle of heating and cooling (33). Melting of β-structures was observed for fibrils produced from derivatives of the same peptides that were unable to undergo this alignment. The alignment process is irreversible: once aligned, the β-sheet arrangement does not alter, indicating that the aligned conformation is thermodynamically more stable than the unaligned one (33). Temperature-induced alignment of β-strands may account in part for the transition to the Am*-rPrP state observed in this study.

An alternative mechanism responsible for the annealing effect may involve domain swapping (35). Domain swapping normally results in the formation of an intersubunit interface that is identical to the intrasubunit interface in the original state and an additional interface that is unique to the domain-swapped state. Our data are consistent with a model in which exposure of Am-rPrP to 80 °C in the presence of Triton X-100 induces local unfolding within the central region (residues 109–122) while preserving the structural integrity of fibrils (Fig. 8). We know from our previous studies that the C-terminal region is essential for maintaining fibrillar structure (25). Upon subsequent cooling, the central region refolds; however, it adopts a swapped conformation, in which it is largely protected from proteolytic digestion due to a newly formed interface (Fig. 8). Therefore, the swapping requires local unfolding within the central region. It is uncertain, however, whether annealing also involves rearrangement within the rigid cross-β-fibrillar core or occurs outside of this core. Domain swapping has been previously pro-
posed to be involved in protein misfolding, aggregation, and conversion to amyloid fibrils (35–38). However, the model presented here suggests that domain swapping may actually occur at a late stage of polymerization and involves an already mature amyloid structure, rather than accounting for the amyloid polymerization itself. We acknowledge that the domain swapping model is not monolithic, and alternative interpretations of the data are possible.

To what extent does the Am*-rPrP state of amyloid fibrils mimic the conformation of PrPSc? With respect to PK resistance, Am*-rPrP is comparable with the PK-resistant form of PrPSc. In the amyloid fibrils, the 16-kDa band resisted PK digestion under solvent conditions that are normally used for PK treatments of scrapie brain homogenates (incubation for 1 h at 37 °C at neutral or slightly basic pH) (39, 40). Furthermore, both the Am*-rPrP fibrils generated in vitro and PrPSc have a similar unfolding pattern. Caughey and co-workers (41) demonstrated that the central region of PrPSc (residues 90–143) acquires a PK-sensitive conformation under partially denaturing conditions (in 2 or 3 M GdnHCl), whereas the C-terminal part remains PK-resistant. In our studies, the same region adopted a PK-sensitive conformation upon exposure of Am*-rPrP fibrils to GdnHCl (Fig. 5). Partial denaturation of PrPSc was found to be fully reversible at <3 M GdnHCl (41, 42). Similarly, renaturation of the Am*-rPrP form was effective only when the fibrils were exposed to ≤3 M GdnHCl.

Different strains of transmissible spongiform encephalopathy are characterized by distinct PK cleavage sites, most of which have been identified within residues 79–103. We found that the new PK-resistant core in the Am*-rPrP state starts at residue 97. This result confirms our former studies in which the 16-kDa fragment was shown to contain the epitopes for Fab fragment P (residues 96–105), Fab fragment D18 (residues 133–157), and Fab fragment R1 (residues 225–230) (25). Notably, the bovine spongiform encephalopathy strain of PrPSc has a PK cleavage site identical to that of Am*-rPrP (43). This site seems to be unique for the bovine spongiform encephalopathy strain and is not shared by other prion strains (43). This similarity between Am*-rPrP produced via thermal annealing in vitro and the bovine spongiform encephalopathy strain of PrPSc is quite remarkable, considering that the bovine spongiform encephalopathy strain appears to emerge through a rendering process consisting of high temperature treatment. In our previous studies, the amyloid fibrils were shown to have limited infectivity in their Am-rPrP conformation (44). One could hypothesize that a long latency in developing prion disease after inoculation of the amyloid fibril may simply reflect additional time required for the transformation of Am-rPrP into the Am*-rPrP conformation in vivo. It would be interesting to test whether prion disease develops faster upon inoculation of the Am*-rPrP form of the fibrils compared with the Am-rPrP form.

Are there any cellular cofactors that might assist in the transition to the PK-resistant form? Several classes of polyanions, including sulfated glycans (45), RNA (46, 47), and DNA (48), were shown previously to stimulate cell-free conversions of cellular PrP into PK-resistant forms (45, 47) or conversions of α-PrP into the β-sheet rich isoform (48). RNA- or heparan sulfate-stimulated conversion reactions occur only in the presence of PrPSc seeds. In contrast to the PrPSc-dependent reactions described previously (49–51), the in vitro conversion protocol introduced in our work does not require seeding with PrPSc. Therefore, our system has more parallels with sporadic formation of prions, rather than with prion diseases acquired through transmission. Our findings do not support the hypothesis that polyanions such as RNA and sulfated glycans are involved in the sporadic conversion of cellular PrP into PK-resistant forms. In contrast, both classes of polyanions attenuate the annealing reaction. However, the transition into the Am*-rPrP state can be achieved in brain homogenate even in the absence of detergents. Some yet unknown cellular factor may play the role of Triton X-100 in assisting with the annealing process. Triton X-100 was shown previously to stimulate dimerization among members of the Bcl-2 family via a mechanism similar to domain swapping (52). In the absence of detergent, Bax fails to form homodimers or heterodimers in vitro. However, formation of both homodimers and heterodimers occurs in a cellular environment (52). Because lipids destabilize PrP fibrillar structure (53), they may replace Triton X-100 in brain homogenate and facilitate transformation of the Am-rPrP to Am*-rPrP state. Lipids are known to be permanent components of prion rods purified from scrapie brains (54).

Taken together, these data demonstrate that, instead of denaturation, high temperatures induce substantial rearrangement in PrP fibrils, leading to the extension of PK-resistant cores. Because amyloid fibrils undergo further temperature-dependent annealing, it is possible that apparently mature amyloid fibrils may not represent the thermodynamically most stable state. These studies provide new insight into the complex features of prion polymerization and highlight the annealing process as an essential step in maturation of fibrils. Notably, a process of pressure-induced structural reorganization of amyloid fibrils similar to temperature-induced annealing was reported recently for fibrils produced from β2-microglobulin (55). After treatment with high pressure, the reorganized fibrils of β2-microglobulin have a more tightly packed structures (55). Whether or not annealing is a general step in the evolution of amyloid structures remains to be elucidated in future studies.

Acknowledgment—We thank Pamela Wright for editing the manuscript.

REFERENCES
1. Carrell, R. W., and Lomas, D. A. (1997) Lancet 350, 134–138
2. Prusiner, S. B. (2001) N. Engl. J. Med. 344, 1516–1526
3. Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G., and Dobson, C. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3590–3594
4. Ramirez-Alvarado, M., Merkel, J. S., and Regan, L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8979–8984
5. Yutani, K., Takayama, G., Goda, S., Yamagata, Y., Makis, S., Namba, K., Tsunasawa, S., and Ogashara, K. (2000) Biochemistry 39, 2769–2777
6. Guijarro, J. L., Sunde, M., Jones, J. A., Campbell, I. D., and Dobson, C. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4224–4228
7. Dobson, C. M. (2002) Trends Biochem. Sci. 27, 329–332
8. Chapman, M. R., Robinson, L. S., Pinkner, J. S., Roth, R., Heuser, J. I., Hammar, M., Normark, S., and Hultgren, S. J. (2002) Science 295, 851–855
9. Icomomidou, V. A., Vriend, G., and Hamodrakas, K. (2000) FEBS Lett. 479, 141–145
10. Berson, J. F., Theos, A. C., Harper, D. C., Tenza, D., Raposo, G., and Marks, M. S. (2003) J. Cell Biol. 161, 521–533
