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Dissociation of Recombinant Prion Protein Fibrils into Short Protofilaments: Implications for the Endocytic Pathway and Involvement of the N-Terminal Domain

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Abstract

Fibril dissociation is necessary for efficient conversion of normal prion protein to its misfolded state and continued propagation into amyloid. Recent studies have revealed that conversion occurs along the endocytic pathway. To better understand the dissociation process, we have investigated the effect of low pH on the stability of recombinant prion fibrils. We show that under conditions that mimic the endocytic environment, amyloid fibrils made from full length prion protein dissociate both laterally and axially to form protofilaments. About 5% of the protofilaments are short enough to be considered soluble and contain ~100–300 monomers per structure; these also retain the biophysical characteristics of the filaments. We propose that protonation of His residues and charge repulsion in the N-terminal domain trigger fibril dissociation. Our data suggest that lysosomes and late endosomes are competent milieus for propagating the misfolded state not only by destabilizing the normal prion protein, but by accelerating fibril dissociation into smaller structures that may act as seeds.

The normal cellular form of prion protein (PrP^C) is a monomeric glycoprotein that is tethered to the plasma membrane via its glycosylphosphatidylinositol anchor.¹ It is expressed in all mammals and avian species with high expression in the central nervous system, especially on the presynaptic membrane.^{2, 3} Natively folded PrP^C is characterized by a large unstructured N-terminal domain (residues 23–89), a hydrophobic region within residues 90–124, and a well-ordered C-terminal domain (residues 125–228) composed of 3 α -helices and 2 short anti-parallel β -strands. A short flexible segment of 4 amino acids at the C-terminus links to the GPI anchor,^{4–7} and there are two sites of glycosylation located within the structured domain.

Conversion from PrP^C to its misfolded conformer is associated with a group of neurodegenerative diseases known as prion diseases.^{8, 9} This conformer is called PrP^{Sc}, where “Sc” denotes scrapie, the ovine disease. Unlike PrP^C, PrP^{Sc} is an insoluble, partially proteinase-K resistant amyloid aggregate.⁷ Increasing evidence suggests that the neurotoxic entity responsible for prion disease is a soluble prefibrillar intermediate or an off-pathway

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Supporting Information Available. Thermal denaturation and ThT fluorescence of soluble protofilaments, mass spectrometry data that identify their amyloid core, and MALS determination of molar mass are included in Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

product generated intra-neuronally during PrP^C to PrP^{Sc} conversion.^{10–14} However, our understanding is limited due to the lack of a precise physical definition for these soluble intermediates.

PrP^C to PrP^{Sc} conversion occurs through the direct interaction between PrP^C and PrP^{Sc}, in which the PrP^{Sc} acts as a conformational template, causing PrP^C to misfold and extend the amyloid aggregate.^{13, 15} For efficient propagation of the aggregate, PrP^{Sc} must dissociate into smaller nuclei that can seed additional conversions in a process known as secondary nucleation^{15–17} Thus, the rate of fibril dissociation contributes significantly to the overall rate of propagation.^{18, 19}

Studies on mammalian PrP suggest that the conversion of PrP^C to PrP^{Sc} takes place both on the plasma membrane and along the endocytic pathway.^{14, 20–23} The majority of PrP^{Sc} has been shown to accumulate in the lysosomes of infected cells.²⁴ Interestingly, *Atg5*^{−/−} fibroblasts, which lack the ability to execute autophagy and then transport PrP^{Sc} aggregates into lysosomes, show significantly increased resistance to prion infection.²⁵ Together these results indicate that there must be a mechanism within the endocytic pathway to dissociate the mature PrP^{Sc} into seeds and thus continue its efficient propagation.¹³ One potential environmental contributor to dissociation is the acidic pH of lysosomes.²⁶

We have observed that the solution circular dichroism (CD) spectrum for amyloid fibrils generated from full-length recombinant hamster prion protein (rPrP23-232) can be measured only at acidic pH. As large fibrils merely scatter the CD light, we postulated that dissociation of some fibrils was occurring under acidic conditions. We therefore investigated the behavior of recombinant PrP fibrils under mildly acidic conditions that mimic the endocytic environment. We demonstrate that full length fibrils dissociate both laterally and axially to form shorter oligomeric structures we term protofilaments; ~5% of these are soluble and contain ~100–300 monomers. The protofilaments retain the characteristic intermolecular β -sheet structure of the mature fibrils. We propose that protonation of histidine residues in the N-terminal region of PrP is involved in fibril dissociation; suggesting that this region, which is intrinsically disordered in the normal cellular PrP^C, might take on structure when amyloid fibrils are formed. Our results provide evidence of the molecular events that may occur during prion propagation *in vivo*.

Experimental Procedures

The genes encoding residues 23-232 or 90-232 of golden Syrian hamster prion protein were amplified by PCR from plasmid pHaPrP²⁷, inserted into pET24, and the recombinant protein was expressed in BL21 (DE3)-Rosetta cells (Novagen). The results were confirmed by DNA sequencing (Murdock Sequencing Facility, University of Montana).

Recombinant hamster prion proteins (rPrP23-232 or rPrP90-232) were expressed and purified using minor modifications of published protocols.^{27, 28} The purity of rPrP was confirmed by SDS-PAGE on a PhastSystem (GE Healthcare, Inc). Stocks of purified protein were stored in 6 M GdnHCl, 0.1 M phosphate, pH 8.0. Protein concentration was determined spectrophotometrically in 6 M GdnHCl using $\epsilon_{280} = 61,025 \text{ M}^{-1} \text{ cm}^{-1}$ for rPrP23-232 and $24,345 \text{ M}^{-1} \text{ cm}^{-1}$ for rPrP90-232, as calculated using the ProtParam program on the ExPASy web server.²⁹

The α -helical, PK-sensitive monomeric form of rPrP (α -monomer), which is structurally similar to PrP^C but lacks the GPI anchor and glycosylation,³⁰ was formed by extensively dialyzing the stock solution against 20 mM acetate pH 4.5. We also prepared a previously characterized octameric conformer that is highly enriched in β -sheet structure, which is

termed the β -oligomer.^{28, 31} The secondary structures of the α -monomer and β -oligomer were confirmed using circular dichroism (CD).

Generation of the initial PK-resistant amyloid-like fibrils was done by diluting the α -monomer three-fold into denaturant fibrillation buffer to give a final concentration of ~1 mg/mL rPrP in 15 mM phosphate, 0.5 M guanidine (GdnHCl), 1.2 M urea, pH 6.5.^{32–34} Samples were incubated at 37 °C under continuous orbital shaking at 600 rpm in 1.5 ml siliconized Eppendorf tubes, and fibrils were collected by centrifugation at 15,000 g for 10 min. The amyloid nature and morphological characteristics of the fibrils were confirmed by ThT fluorescence assay³⁵ and transmission electron microscopy (TEM), respectively.

To test the effects of low pH on the stability of rPrP fibrils, ~300 μ M rPrP fibrils were incubated in 20 mM acetate pH 4.5 at 37 °C for 3 days, with continuous rotation at 8 rpm to prevent fibrils from settling. Ultracentrifugation was done in a TLA-55 rotor at 120,000g for 2 h.

Prion conformers were isolated, analyzed, and sized by asymmetric flow field flow fractionation (AF4) on an AF2000 instrument (PostNova, Inc), equipped with in-line UV-Vis and 7-angle light scattering detectors.^{28, 36} Samples were loaded in one 20–30 μ l injection, focused for 4 min with a crossflow of 3.5 ml/min on a regenerated cellulose membrane (5-kDa MWCO), then eluted at a channel flow of 1.5 ml/min and a crossflow of 2.5 ml/min for 30 min. The crossflow was then linearly decreased to 0.1 ml/min over 10 min, then held constant for 30 min.^{37, 38} The molar mass and radius of gyration (R_g) of the protofilament peak were determined from the light scatter using PostNova software³⁹ and evaluated using the Berry method.^{40, 41}

R_g of the soluble protofilaments was also estimated from transmission electron microscopy (TEM) images using the standard formula for the moment of inertia of a rigid, solid cylinder and the mean width and length.⁴² TEM was done at the Electron Microscopy Facility of the University of Montana. A 5 μ L aliquot of sample (20 – 50 μ M) was cast on a Formvar coated copper grid and allowed to adsorb for 3 – 30 min in a constant humidity chamber. The grid was then rinsed with distilled water and stained with 2% (w/v) uranyl acetate for 30 sec before briefly washing again with water. Varying the staining time did not change the results. After air drying, the sample was viewed using a Hitachi H-7100 instrument at 75 kV at standard magnifications of 20,000 \times and 100,000 \times .³⁵ Fibril dimensions were determined using Image J software (NIH, Bethesda, MD); between 30 and 450 individual structures were measured per image; widths and lengths are reported as mean \pm SD; average lengths are reported as the geometric mean.

Circular dichroism (CD) spectra were recorded on a Jasco 810 spectrophotometer equipped with a Peltier temperature controller. Parameters were: 1 mg/mL rPrP; path, 0.01 cm; response time, 4 s; 5 spectra averaged.⁴³ Protein secondary structure analysis was performed using the DICHROWEB server and the CDSSTR program^{44, 45} with Reference Dataset #3.

FT-IR spectroscopy was performed using a Thermo Nicolet NEXUS 670 spectrometer with continuous nitrogen purge. Samples were exchanged into D₂O buffer and analyzed in a demountable liquid cell fitted with two CaF₂ windows separated by a 50 μ m Teflon spacer. Spectra were obtained from 128 cumulative scans from 1400 to 2000 cm^{-1} with 2 cm^{-1} resolution and corrected by scaled subtraction of a water vapor spectrum until the region from 1900 to 1750 cm^{-1} no longer showed a negative lobe.^{46, 47}

Samples for PK digestion were adjusted to 2 mg/mL in either 15 mM phosphate pH 6.5 or 20 mM acetate pH 4.5. PK digestion was performed for 2 h at 37°C at PK:rPrP ratio of 1:10. Digestion products were separated by SDS-PAGE on a PhastSystem using high density gels

(GE Healthcare Life Sciences) and analyzed vs. the migration of peptide standards (MW-SDS-17S, Sigma). For MALDI-ToF/ToF analysis, in-gel tryptic digestion was also performed.⁴⁸ Data were processed using mMass software.⁴⁹

The kinetics of fibril formation from the α -monomer was monitored under de novo (unseeded), and seeded conditions, where the seeds were soluble protofilaments purified as described in the next paragraph. No denaturants were used. Conversion was carried out at 37°C in a 96-well plate (Corning #3651) with a total reaction volume of 0.2 mL/well containing 50 μ M of α -monomer in 20 mM acetate, ± 0.1 M NaCl, pH 4.5 + 10 μ M ThT; at this concentration ThT has a negligible effect on the kinetics of fibril formation.⁵⁰ Plates were sealed and incubated at 37 °C in the SpectraMax M2^e Microplate Reader (Molecular Devices). The fluorescence emission intensity at 488 nm was recorded hourly (λ_{ex} , 445 nm) after briefly shaking the plate.

Results

Acidic Conditions Induce Protofilament Formation

Negative stain TEM images revealed that fibrils formed under partially denaturing conditions from either N-terminally truncated rPrP90-232 or full length rPrP23-232 consisted of long, unbranched structures. These were typically well over 1000 nm in length, with widths of 27 ± 2 nm and 27 ± 3 nm (mean \pm SD) (Fig. 1), respectively. Both fibrils displayed twists, with a periodicity of about 180 nm for rPrP90-232 and 250–360 nm for rPrP23-232. This suggests that the fibrils are composed of at least two thinner strands, as has previously been described,^{32, 51} and which we will refer to as protofilaments. The amyloid nature of these fibrils was further confirmed by high ThT fluorescence (Supplementary Fig. S1).

To determine whether the acidic environment of endosomes might encourage fibril dissociation, rPrP90-232 and rPrP23-232 fibrils were resuspended in pH 4.5 buffer for 3 days. TEM showed that the morphology of the rPrP90-232 fibrils was unaffected by the decrease in pH; no changes in twist, width, or length were noted (Fig. 1, A→D). In contrast, the rPrP23-232 sample was dramatically different. TEM revealed that at low pH the fibrils were changed into a population of structures of various lengths that were devoid of twist (Fig. 1 E→H). We then used ultracentrifugation to isolate the smallest of these structures, which were recovered from the supernatant and deemed soluble according to the standards of Hjelmeland and Chrambach.⁵² About 5% of the original rPrP23-232 fibrils were present in the supernatant (Fig 1, I). The lengths of these soluble structures ranged from 20 to 150 nm (mean = 65 nm). We also noted an approximate 50% decrease in width: 15 ± 2 nm (supernatant) and 14 ± 2 nm (pellet) and a lack of twist for both the soluble and insoluble fractions (Fig. 1, I, J).

Composition of the Protofilament Amyloid Core

To compare the composition of the amyloid cores of the protofilaments with those of their parent fibrils, we performed proteinase K (PK) digestion. Full length PrP fibrils were digested at pH 6.5 and also after the fibrils had been resuspended in pH 4.5 buffer. In the pH 4.5 experiments, PK digestion was performed immediately after the change in pH and again 72 hours later. TEM imaging indicated that at 72 hours, the sample has mostly dissociated into protofilaments of varying lengths. This pre-incubated sample was further processed by centrifugation to remove the majority of the insoluble protofilaments prior to PK digestion.

The results were then analyzed by SDS-PAGE (Fig. 2, top). At pH 6.5 (lane 1), the most intense PK-resistant band is ~5.5 kDa, although bands at 5 and 7 kDa are also visible. Lane 2 shows the results of the sample digested immediately after the pH was lowered to 4.5. A

triplet of bands at 7, 9, and 12 kDa are detected. This shift of the PK-resistant PrP bands with pH is attributed to the pH dependence of PK specificity.⁵³ The same triplet was also present in the pre-incubated sample (lane 3), although the band intensities were slightly different.

For control purposes, PK digestion of truncated rPrP90-232 fibrils was also performed (Fig. 2, bottom). Conditions were identical to those in full-length PrP except that the centrifugation step was omitted from the sample that was pre-incubated, since no protein could be found in the supernatant. As expected, the 72-hour pre-incubation had no effect on the PK-resistance, as the truncated fibrils do not dissociate at low pH. There were slight variations in the band intensities, but overall the PK-resistance of truncated fibrils was similar to that of full-length PrP.

For each gel lane in Fig. 2, the peptides bands were excised, digested with trypsin and analyzed by mass spectrometry to map the composition of each fragment (Supplementary Table S1 and Fig. S2). No peptides from residues 23 – 137 were detected in any PK-resistant fragment. This confirmed that protofilament formation did not involve refolding of all or part of the N-terminal domain into a PK-resistant β -sheet structure. The data in Table S1 show that the 12, 9, and 7 kDa peptide fragments begin at residues 137, 149 and 157, respectively. There was little difference in the MS data collected for identical masses derived from different gel lanes.

The identity of the 7 kDa peptide (~residues 157–220) is consistent with the amyloid core detected by hydrogen/deuterium exchange mass spectrometry⁵⁴ and site-directed spin labeling electron paramagnetic resonance spectroscopy⁵⁵ for human rPrP90-232. We attribute the presence of larger peptide fragments in these samples to the inability of PK to fully access non-amyloid regions of PrP.⁵⁴ PK-resistant, non-specific aggregates have been shown to form after the N-terminal domain of amyloid PrP is cleaved and/or partially digested.³² It is believed that the exposure of hydrophobic residues causes non-specific aggregation that impedes PK digestion. Therefore, the presence of larger PK-resistant peptides noted in Fig. 2 does not mean that the amyloid core of the protofilaments has extended beyond the 7 kDa β -sheet core identified for recombinant PrP fibrils by other techniques.⁵⁵

Overall, it appears that fibrils and protofilaments possess similar global architectures in their amyloid core regions; there is no evidence that protofilament formation involves reorganization of the N-terminal domain into an amyloid state.

Purification and Size Determination of Soluble Protofilaments

We used asymmetric flow field-flow fractionation (AF4) and multi-angle light scattering to further purify and analyze the soluble protofilaments of rPrP23-232 that were isolated by ultracentrifugation (Fig. 3A). Small amounts of α -monomer and β -oligomer were present in the supernatant, eluting at 6.8 and 20.9 min, respectively. Their identities were confirmed by circular dichroism (CD),^{28, 31} shown in Fig. 3B. The majority of the sample eluted under low crossflow at 42.6 min. The molar mass elugram (Supplementary Fig. S3) revealed this peak had significant polydispersity with the centroid at 5.8 MDa, which is equivalent to ~250 monomeric subunits. The radius of gyration (R_g) for the species eluted at the centroid is 29.5 nm. This is consistent with the R_g value of 27 nm that we estimated from the TEM images. This value was calculated using the average radius of 7 nm and a mean particle length of 65 nm (Fig. 1I) and treating the protofilaments as rigid cylinders. This confirms that the soluble protofilaments observed by TEM are the main constituent purified by AF4 from the supernatant.

As expected, no peaks were observed in the supernatant of low pH treated rPrP90-232 fibrils (Fig. 3A). Re-injection of the 42.6 min peak after storage for an additional 3 days showed no significant changes. SDS-PAGE analysis confirmed that the purified protofilaments contain full length rPrP; no degradation products were observed (data not shown). These results demonstrate that the soluble protofilaments are quite stable under acidic conditions. Axial dissociation of the long, insoluble protofilaments to form short, soluble structures reached a steady state maximum after ~ 2 days incubation at pH 4.5 (Fig. 3C).

The pH Dependence of Protofilament Formation

To confirm the pH-dependent nature of dissociation, we assessed the stability of rPrP23-232 fibrils after incubation at pH 6.5, 5.5, and 4.5 for 3 days. AF4 analysis (Fig. 3D) showed that soluble protofilaments are also formed at pH 5.5, although the centroid of the protofilament peak shifted to a slightly higher apparent molecular weight (43.7min, 8.5 MDa, ~370 monomers) compared with the results obtained at pH 4.5. No short protofilaments were formed at pH 6.5. These data confirm that fibril dissociation and the size distribution of the products are pH dependent, and that rPrP23-232 fibrils are stable under neutral conditions. The amyloid nature of the soluble protofilaments was confirmed by their resistance to thermal denaturation and the ThT fluorescence assay (Supplementary Fig. S1). The dissociation of mature fibrils into protofilaments occurred in the presence and absence of 0.1 M NaCl, although the dissociation into short protofilaments took longer when salt was included (see Supplementary Fig. S4).

Circular Dichroism Spectroscopy

The CD spectrum of the soluble protofilament fraction (42.6 min peak) is shown in Fig. 3B, along with that of α -monomer and β -oligomer. The protofilament signal is markedly different from both the α -monomer and the β -oligomer. A single negative peak centered at 216 nm was associated with a rather large MRE value ($-12,000 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}\cdot\text{residue}^{-1}$). Secondary structure analyses indicate that the soluble protofilaments have considerably less α -helix and more β -sheet in comparison with the α -monomer (Table 1).^{44, 45} Although the overall shapes and MRE values of the protofilaments and the β -oligomer are quite different, the predicted secondary structure content is surprisingly similar. However, CD spectroscopy often misestimates β -sheet content due to the significant overlap of the β -sheet absorption band with that from α -helices. We could not obtain a solution CD spectrum of fibrils at neutral pH for direct comparison with the protofilaments, probably because they scatter too much light. The solution CD spectrum of similar fibrils has been previously published,⁵⁶ but these fibrils were in an acidic buffer and thus the reported CD signal may actually be that of dissociated, soluble protofilaments.

FT-IR Spectroscopy

We compared the secondary structure of the soluble protofilaments with that of their parent rPrP23-232 fibrils using FT-IR spectroscopy, Fig. 3E. Unlike CD, FT-IR spectroscopy may be used for both solution and solid samples and is better suited for the characterization of β -sheet structure. Taking into account the limitations of both techniques, the FT-IR results are reasonably consistent with the results obtained from CD. The high cross- β sheet content of protofilaments is evident in the strong single amide I band centered at 1621 cm^{-1} (solid red line) which is shifted to slightly higher frequency as compared with the recombinant fibrils (1619 cm^{-1} , solid black line). A shoulder at 1628 cm^{-1} in the fibril spectrum is absent in the protofilament spectrum. The amide I bands previously assigned to turns and loops (1663 cm^{-1} and 1676 cm^{-1})^{32, 56, 57} were less substantial in protofilaments as compared with fibrils, possibly indicating some unfolding of these structures. Overall, the spectrum of protofilaments is quite similar to the parent fibrils.

Raising the pH of the protofilaments to 7.0 caused an immediate shift of the β -sheet amide I band from 1621 cm^{-1} to 1619 cm^{-1} , and the shoulder at 1628 cm^{-1} appeared, becoming more defined after 72 hours incubation at pH 7.0 (Figure 3E, dashed blue and dotted green lines). Overall the data suggest that the minor differences in the β -strand structure between protofilaments at acidic pH and fibrils are pH-dependent and largely reversible. However, we did not observe any re-assembly of protofilaments into twisted or parallel fibrils by TEM; instead, clumps of aggregates were formed when the pH was raised (Fig. 3F). In regions of the TEM image where individual structures could be isolated and measured, the widths of the structures were $\sim 14\text{ nm}$, resembling protofilaments and not fibrils.

Overall, the biophysical and spectroscopic data suggest the main structural characteristics of amyloid fibrils are preserved in the soluble protofilaments.

Ability of Protofilaments to Accelerate Conversion of α -Monomeric PrP

Typical fibril formation shows a characteristic nucleation-dependent pattern with three kinetic phases: the initial lag phase is followed by a steep log phase and then a final plateau. It has been well-established that in the presence of pre-formed fibril seeds, conversion under partially denaturing conditions is accelerated with a dramatically shortened lag phase.³² We examined whether this also held true for pre-formed protofilament seeds under physiologically relevant conditions, i.e. in the absence of denaturants.

Propagation experiments were performed using conformers of rPrP23-232, in 20 mM acetate, 0.1 M NaCl, pH 4.5 to approximate the environment of late endosomal vesicles. A direct comparison between fibrils and protofilaments is not possible since fibrils dissociate into protofilaments of varying lengths in less than 24 h at pH 4.5. Similarly, protofilaments form amorphous aggregates at neutral pH.

The ratio of soluble protofilaments (seeds) to α -monomer was varied from 0 – 2 % (Fig. 4A). The unseeded control samples showed little change even after 3 weeks, but all seeded experiments followed a typical nucleation-dependent polymerization mechanism. Adding more protofilament seeds proportionally shortened the incubation times and increased both the rate of the log phase kinetics and the final plateau levels of ThT fluorescence intensity. However, overall the lag phase was significantly elongated (days) as compared with reactions carried out in the presence of denaturants (hours).³²

After 3 weeks the products from the 2% seeded reaction were examined by TEM imaging (Fig. 4B). There were considerable amounts of small particles present in the sample. This was confirmed by AF4 (Fig. 4C, top trace), which detected both β -oligomer (retention time 20.9 min) and ThT-binding high molecular weight aggregates that were larger than the seeds themselves (broad peak with retention time $\sim 46\text{ min}$). Control experiments showed that this peak results from conversion of the α -monomer, as the seeds themselves (retention time 42.6 min) are undetectable at the highest concentration used (bottom trace).

The TEM image in Fig. 4B also indicated that some highly elongated, unbranched structures had also formed in the reaction, with an approximate width of $34 \pm 5\text{ nm}$. This width matches that of the parent fibrils produced under partially denaturing conditions (Fig. 1E) and not that of the protofilament seeds.

Consistent with previous reports⁵⁸, α -monomers are quite stable in the absence of salt, even when 2% seeds are added (Fig 4C, dotted magenta trace). The destabilizing effect of salt is notable in the unseeded control (dashed green trace), where the peak at 6.8 min has decreased and larger products appear at longer retention times. The major product is the β -

oligomer (peak at 20.9 min). Small amounts of non-ThT binding aggregates are also formed, which result in the elevated baseline at later elution times.

Discussion

Mechanism of Fibril Dissociation into Protofilaments

The current study demonstrates that full-length rPrP fibrils dissociate when they are exposed to mildly acidic conditions. As judged from microscopy imaging (Figure 1), dissociation occurs in two dimensions: lateral and axial. Lateral dissociation involves the disassembly of mature fibrils into constituent protofilaments, while axial dissociation further divides the protofilaments along the fibrillar axis into smaller entities of variable lengths. To our knowledge, the ability of mature amyloid fibrils to undergo dissociation into protofilaments under mildly acidic conditions has not been previously reported. The pH conditions used here are similar to those found in late endosomal and lysosomal vesicles. Kinetic analysis shows that the protofilaments preserve the ability to propagate *in vitro* (Figure 4) and thus may be of physiologic relevance, as our conditions mimic those of late endocytic and lysosomal vesicles.

Fibril dissociation is necessary for efficient propagation of the prion state. Two possible mechanisms for fibril dissociation have been proposed in the literature: mechanical stress-induced dissociation and chaperone-mediated dissociation.^{21, 59} The results from this study suggest another possible mechanism; fibril dissociation into protofilaments induced by low pH. The lack of dissociation in N-terminally truncated fibrils (rPrP90-232) at low pH indicates that the N-terminal region of PrP is important to the dissociation process. In full-length hamster PrP, this region (residues 23-140) is quite basic, containing 0 Asp, 0 Glu, 4 Arg, 7 Lys, and 7 His; this pattern is highly conserved among mammalian PrPs. His residues have a pKa of ~ 6.8 and thus are the only residues present that could account for the observed pH effect. We hypothesize that protonation of His residues in a domain already enriched with positively charged residues leads to charge repulsion, which causes the dissociation of fibrillar superstructures into protofilaments. Conformational perturbations induced by charge repulsion in residues 90 – 140 (contains 3 of the conserved His) have been reported in studies of human truncated rPrP90-231, with an associated pKa of 5.3.⁶⁰ Given that we did not observe dissociation of N-terminally truncated fibrils (hamster rPrP90-232) into protofilaments, we postulate that the four additional His residues found in residues 23-89 play an important role in acid-induced fibril dissociation. Additional studies to test this hypothesis are underway.

Protofilament Structure and Seeding Capability—Some of the protofilaments were small enough to be considered soluble (Figures 1 and 2). These have an average length of about 65 nm and an apparent molecular weight of ~5.8 MDa (~250 monomeric subunits). The soluble protofilaments retain the hallmark characteristics of full-length amyloid fibrils and share the same amyloid core. Small but largely reversible pH-dependent differences in the β -sheet core structure were noted by FT-IR. The protofilaments have a unique CD spectrum compared with other soluble forms of PrP.

At pH 4.5 in the presence of 0.1 M NaCl and α -monomer, protofilaments are efficient seeds (Figure 4). The propagated products are mostly elongated protofilaments, though a few fibrils are detectable after several weeks. This fits with our initial observations that the yield of soluble protofilaments from parent fibrils was higher when salt was omitted from the acidic buffer. It is likely that the presence of counter-ions in the buffer shields the large positive charge on the N-terminal domains of individual protofilaments, permitting some fibrils to be formed when there is an excess of α -monomer present. As previously reported,

destabilization of the α -monomer in the presence of NaCl also facilitates the conversion process.⁵⁸

Physiologic Relevance of pH-Induced Protofilament Formation—A number of studies have proposed that the endocytic pathway, including endosomes and lysosomes, is important for the PrP^{Sc} conversion *in vivo*.^{14, 20–23} Although most efforts have been devoted to illustrate the destabilizing effect of these acidic vesicles on the global structure and thermodynamic stability of PrP^C,^{61–63} less attention has been paid to its impact on mature amyloids. We show here that in addition to destabilizing α -monomers and thus facilitating primary nucleation, low pH may also contribute to fibril dissociation, a secondary nucleation event that is necessary for efficient conversion and maintenance of the prion state.⁶⁴ Results from the present *in vitro* studies offer direct evidence that at low pH, mature fibrils can dissociate both laterally and axially into protofilaments, which are then able to recruit and convert α -monomer. Assuming that a similar dissociation mechanism occurs *in vivo*, protofilaments of PrP^{Sc} formed in the lysosome might be excellent seeds for the propagation of PrP^C during its normal recycling from the cell surface via the endocytic pathway.²⁰

In summary, we provide experimental evidence that mildly acidic conditions resembling those of late endosomal and lysosomal vesicles are sufficient to dissociate rPrP23-232 fibrils laterally and axially. The dissociation is believed to involve charge repulsion of protonated His residues in the N-terminal domain. The protofilaments thus formed retain amyloid characteristics and are competent seeds for propagating new prions. Thus, late endocytic and lysosomal vesicles are sites of efficient prion propagation not only because they destabilize the α -monomer, but because they accelerate the dissociation of fibrils into protofilament seeds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and Textual Footnotes

AF4	asymmetric flow field flow fractionation
CD	circular dichroism
MRE	mean residue ellipticity
PK	proteinase K
PrP	hamster <u>P</u> rion <u>P</u> rotein
PrP^C	normal cellular form
PrP^{Sc}	infectious misfolded form

rPrP23-232	full-length recombinant PrP
rPrP90-232	N-terminally truncated recombinant PrP
TEM	transmission electron microscopy
ThT	Thioflavin T

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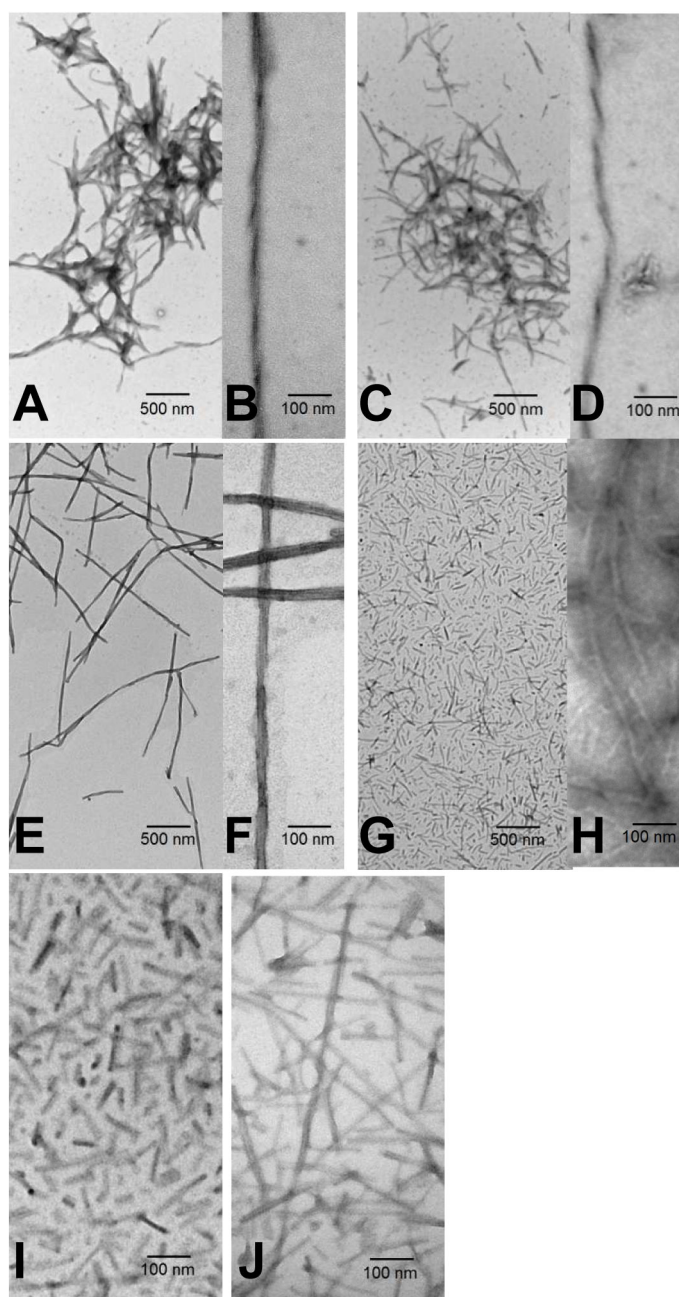


Figure 1.

TEM images of full-length rPrP23-232 and truncated rPrP90-232 fibrils showing pH-triggered dissociation of full-length fibrils. Images taken at 20,000 \times (scale bar 500 nm) or 100,000 \times magnification (scale bar 100 nm). Top panel: truncated rPrP90-232 fibrils at pH 6.5 (A, B) and after three days storage at pH 4.5 (C, D). Middle Panel: full-length rPrP23-232 fibrils at pH 6.5 (E, F) and after three days storage at pH 4.5 (G, H). Bottom Panel: The sample in (G,H) was ultracentrifuged to separate the soluble portion (I) from the insoluble portion (J).

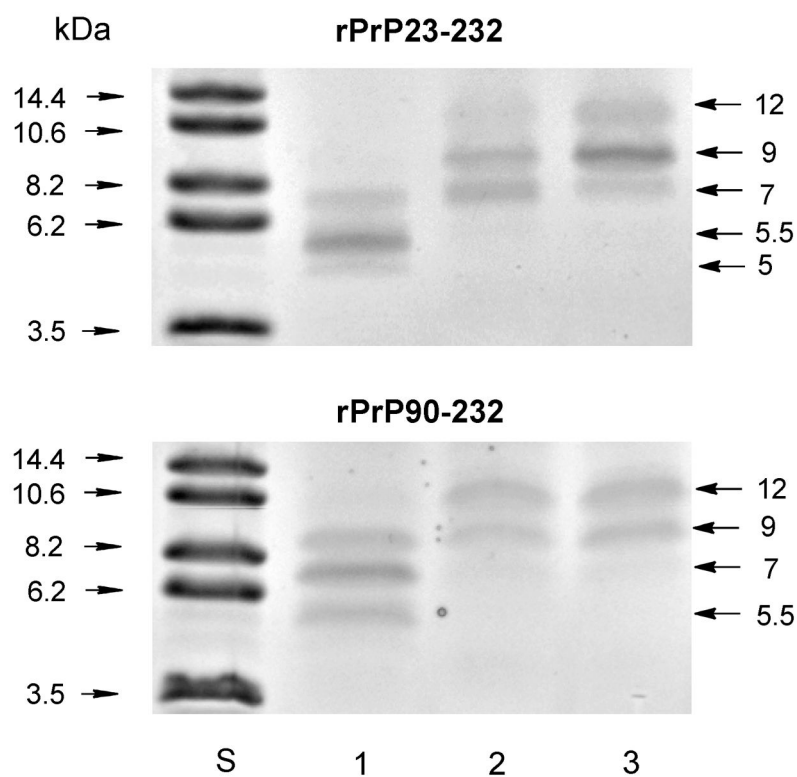
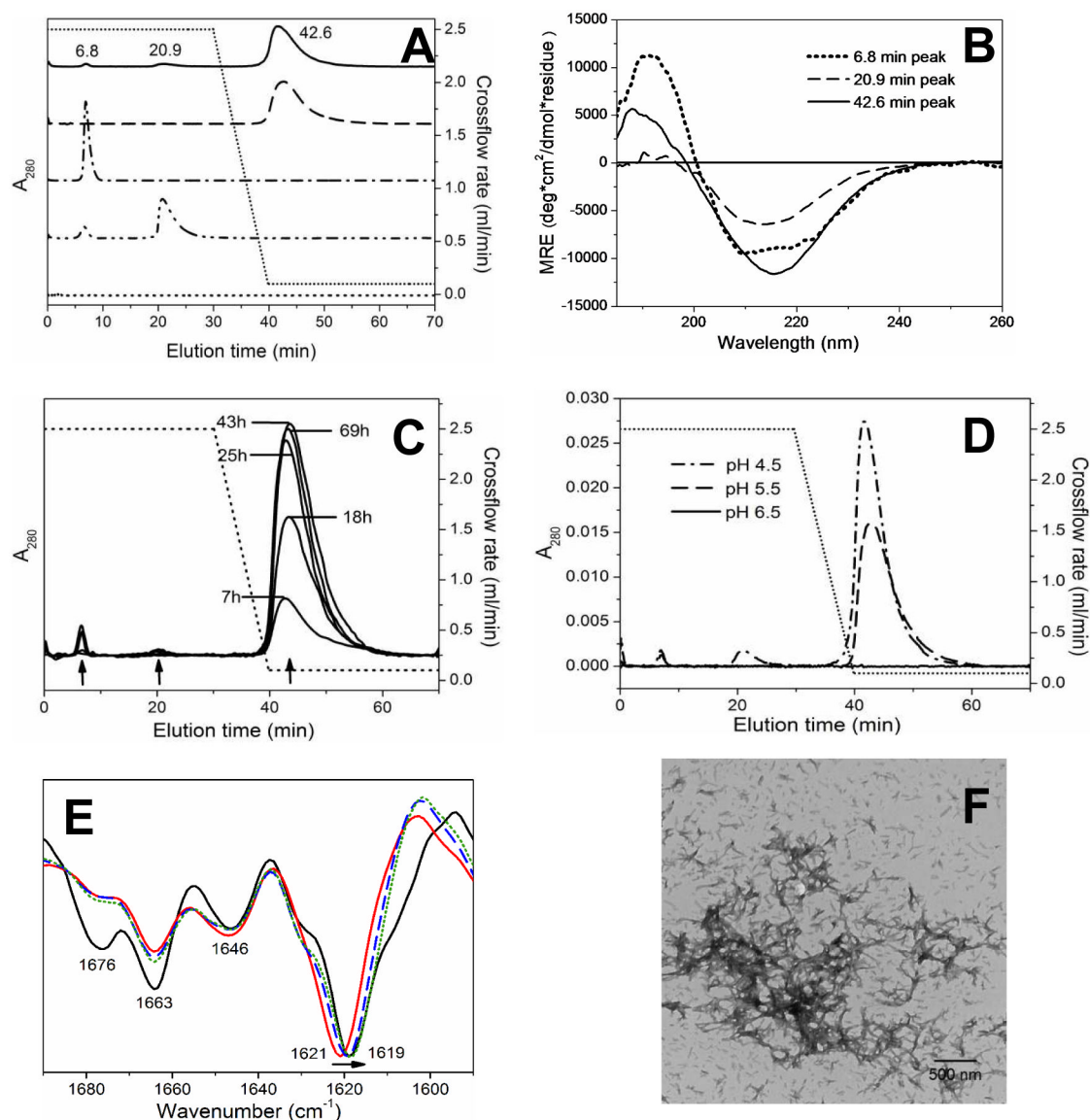


Figure 2. SDS-PAGE analysis of the PK-resistant regions of rPrP fibrils and protofilaments. Top: full-length rPrP23-232; Bottom: truncated rPrP90-232. Lane S, MW standard; lane 1, pH 6.5; lane 2, pH 4.5; lane 3, samples pre-incubated at pH 4.5 for 72 hours prior to PK digestion. For lane 3 samples, the full-length sample was centrifuged prior to digestion but the truncated sample was not.

**Figure 3.**

Biophysical characterization of rPrP23-232 protofilaments.

(A) AF4 analysis of rPrP23-232 conformers: the low-pH supernatant (—); re-injection of the 42.6 min protofilament peak 72 h after collection (---); pure α-monomer (···) and pure β-oligomer (- · - ·). The elution profile of low-pH treated rPrP90-232 fibrils (·····) is included for comparison. (B) CD spectra of the supernatant components after separation by AF4. (C) Time course of protofilament formation as monitored by AF4. (D) pH dependence of protofilament formation. (E) Second derivative FT-IR spectra of protofilaments at pH 4.5 (red —); 10 min (blue ---) and 72 h (green ·····) after raising the pH to 7.0. The spectrum of the parent fibrils at pH 7.0 is included for comparison (black, —). (F) TEM image of aggregates formed after 72 h at pH 7.0.

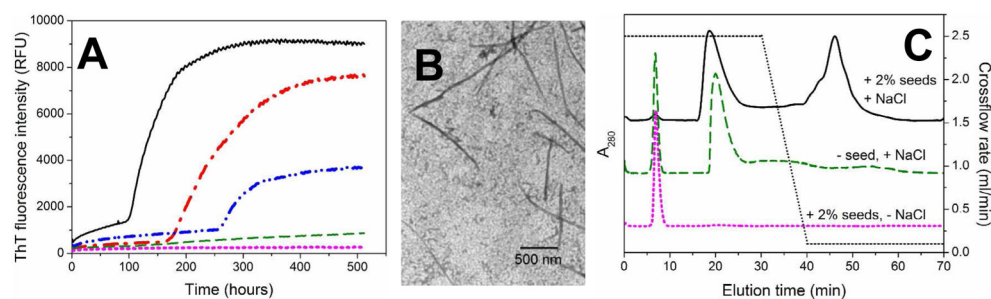


Figure 4.

Protofilament-seeded conversion of rPrP23-232 α -monomer under mildly acidic, non-denaturing conditions. (A) Kinetics as followed by the ThT fluorescence assay. Seed concentration: 2% (black —); 1% (red - - -); 0.5% (blue - · - ·); and 0% (green - - -); α -monomer + 2% seed in the absence of NaCl (magenta ·····). (B) TEM image of products formed after 3 weeks in 2% seeded reaction with NaCl. (C) AF4 analysis of the conversion products of 2% seed + NaCl (black —); 0% seed + NaCl green - - -); and 2% seed without NaCl (magenta ·····).

Table 1

Secondary-structure composition of rPrP23-232 conformers.

Secondary structure type	α -monomer	β -oligomer	Protofilaments
α -helix	23%	4%	7%
β -sheet	26%	38%	30%
Turn	21%	25%	25%
Loop/Unordered	29%	32%	37%