Core Sequence of PAPf39 Amyloid Fibrils and Mechanism of pH-Dependent Fibril Formation: The Role of Monomer Conformation

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Supporting Information

ABSTRACT: PAPf39, a 39-residue peptide fragment from human prostatic acidic phosphatase, has been shown to form amyloid fibrils in semen (SEVI), which increase HIV infectivity by up to 5 orders of magnitude. The sequence of the PAPf39 fibrillar core was identified using hydrogen–deuterium exchange (HDX) mass spectrometry and protease protection assays. The central and C-terminal regions are highly protected from HDX and proteolytic cleavage and, thus, are part of the fibrillar core. Conversely, the N-terminal region is unprotected from HDX and proteolytic cleavage, suggesting that it is exposed and not part of the fibrillar core. This finding was tested using two N-terminal truncated variants, PAPf39Δ1–8 and PAPf39Δ1–13. Both variants formed amyloid fibrils at neutral pH. However, these variants showed a markedly different pH dependence of fibril formation versus that of PAPf39. PAPf39 fibrils can form at pH 7.7, but not at pH 5.5 or 2.5, while both N-terminally truncated variants can form fibrils at these pH values. Thus, the N-terminal region is not necessary for fibril formation but modulates the pH dependence of PAPf39 fibril formation. PAPf39Δ1–8 and PAPf39Δ1–13 are capable of seeding PAPf39 fibril formation at neutral pH, suggesting that these variants are structurally compatible with PAPf39, yet no mixed fibril formation occurs between the truncated variants and PAPf39 at low pH. This suggests that pH affects the PAPf39 monomer conformational ensemble, which is supported by far-UV circular dichroism spectroscopy. A conceptual model describing the pH dependence of PAPf39 aggregation is proposed and provides potential biological implications.

Amyloid fibrils are well-characterized protein aggregates involved in many human diseases. Fibrils can form under a variety of conditions from many sequences, suggesting that they represent a stable generic protein conformation. The amyloid fibril structure is proposed to have a core of β strands arranged perpendicular to the fibril axis and a hydrogen bonding network parallel to the fibril axis. The fibrillar core sequence is typically composed of hydrophobic residues and is often flanked by polar or charged residues.

Amyloid fibrils are involved in neurodegenerative diseases, diabetes, amyloidosis, biofilm formation, and melanin synthesis and were more recently implicated in HIV infectivity. Sexual transmission of HIV accounts for more than 80% of new HIV-1 infections, with the highest transmission rates occurring when the infected partner is male. Semen enhances HIV infectivity, and the source of the HIV infectivity enhancement appears to be due to a number of cationic peptides that form amyloid fibrils. PAPf39, or the prostatic acidic phosphatase fragment of 39 residues (corresponding to residues 248–286 in human prostatic acidic phosphatase), is a cationic peptide that forms amyloid fibrils in semen that increase HIV infectivity by up to 5 orders of magnitude. Thus, understanding the mechanism of PAPf39 fibril formation may provide insights regarding HIV transmission via semen and lay a foundation for the development of therapeutics against this effect.

It has been established that PAPf39 fibrils have a β sheet structure, are thioflavin T (ThT) positive, and form via a nucleation-dependent elongation mechanism. Ionizable residues appear to mediate PAPf39 fibril formation, because fibril formation is dependent on pH and salt concentration. PAPf39 fibrils readily form at neutral pH in the presence of salt, but not in the absence of salt and/or at acidic pH. Thus, ionizable residues appear to be important for modulating the pH dependence of PAPf39 fibril formation.

In this work, the residues involved in the PAPf39 fibrillar core were identified using hydrogen–deuterium exchange mass spectrometry (HDXMS), which was further supported by protease protection assays. It was found that the central and C-terminal regions are part of the PAPf39 fibrillar core, while the N-terminal region is not. On the basis of these experiments, two PAPf39 variants lacking sequences outside of the fibrillar core were characterized to determine the ability of these variants to form fibrillar structures at different pH values. It was found that the N-terminal region is not necessary for fibril formation but is important for determining the pH dependence of fibril formation.

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of fibril formation. Furthermore, intermolecular charge—charge repulsion between PAPf39 N-terminal regions is not responsible for the pH dependence of fibril formation. Rather, changes in pH alter the conformation of the PAPf39 monomer causing fibril formation to be inhibited at low pH.

**EXPERIMENTAL PROCEDURES**

**Buffer Preparation.** Milli-Q water (Millipore, Billerica, MA) was used for all buffers. Phosphate-buffered saline (PBS pH 7.7) was prepared as a 10x concentrated stock solution containing 1.37 M NaCl, 27 mM KCl, 101.4 mM dibasic sodium phosphate, 17.6 mM monobasic potassium phosphate, and 0.3076 M sodium azide [0.2% (w/v)]. The stock solution was then diluted 10-fold to give a working solution of PBS pH 7.7. To generate PBS pH 5.5 and pH 2.5, the 10x PBS pH 7.7 stock was diluted 10-fold and the pH was subsequently adjusted to either 5.5 or 2.5 using hydrochloric acid. Acetate-buffered saline (ABS pH 5.5) was prepared to mimic the salt concentration of the PBS solutions as closely as possible and contained 1.8 mM acetic acid, 10.1 mM sodium acetate, 137 mM sodium chloride, 4.4 mM potassium chloride, and 30.76 mM sodium azide [0.02% (w/v)]. Sodium phosphate buffer (PB pH 7.7), at 20 mM, was prepared through the 10-fold dilution of a 10x concentrated stock solution of 200 mM PB pH 7.7 [78.85 mM monobasic sodium phosphate, 121.5 mM dibasic sodium phosphate, and 0.2% (w/v) sodium azide (pH 7.7)].

**Peptides.** The PAPf39, PAPf39Δ1−8, and PAPf39Δ1−13 peptides were synthesized using standard Fmoc chemistry at the Pennsylvania State College of Medicine Macromolecular Core Facility. The PAPf39 peptides were purified on a C18 reverse phase HPLC column (Discovery Bio Wide pore C18, 10 μm, Supelco Sigma-Aldrich, Bellefonte, PA) using a methanol gradient in the presence of 0.05% trifluoroacetic acid (TFA). For each peptide, the fractions containing the purified peptide were pooled and subjected to three cycles of lyophilization and resuspension in Milli-Q water to remove residual TFA. The molecular masses of the peptides, as determined by ExPASy’s proteomics server11 (4551.4, 3645.4, and 3103.8 Da for PAPf39, PAPf39Δ1−8, and PAPf39Δ1−13, respectively) were confirmed by mass spectroscopy (Bruker Ultraflex III MALDI TOF/TOF, Bruker Daltonics, Billerica, MA). Peptide concentrations were determined using a molar extinction coefficient of 2980 M−1 cm−1 at 280 nm.

**PAPf39 Peptide Fibril Preparation.** PAPf39 fibrils for the pH-induced fibril dissociation assays, HDXMS, and protease protection assays were prepared by dissolving dry PAPf39 peptide (2 mg/mL) in PBS pH 7.7 on ice and incubating the sample for 48 h at 37 °C with agitation. Because of the limited solubility of the variants, fibrils used for the fibril formation kinetics and seeding assays were prepared by dissolving dry peptide monomer in 3.5 mM hydrochloric acid (pH 2.5) at a concentration of 6 mg/mL. The samples were subsequently diluted 3-fold in a dilution buffer to yield a 2 mg/mL (439.4 μM full length PAPf39, 548.6 μM PAPf39Δ1−8, and 644.4 μM PAPf39Δ1−13) peptide solution. The following dilution buffers were used: 18 mM PBS pH 8.1, 18 mM PBS pH 5.88, 18 mM ABS pH 6.14, and 18 mM PBS pH 2.6. Upon dilution with one part 6 mg/mL peptide in 3.5 mM HCl and two parts dilution buffer, these buffers yielded final buffer compositions equivalent to those of PBS pH 7.7, PBS pH 5.5, ABS pH 5.5, and PBS pH 2.5, respectively. The samples were prepared on ice, and fibril formation was initiated immediately after preparation. For seeding assays, 2% (v/v) of 2 mg/mL preformed fibril (formed by incubation for 48 h with agitation at 37 °C) was added to the sample immediately before incubation under the desired conditions.

**Atomic Force Microscopy (AFM).** Atomic force microscopy (AFM) amplitude and height images were acquired using AC tapping mode in air at room temperature and humidity on a MFP-3D instrument (Asylum Research, Santa Barbara, CA), using a silicon, Al reflex coated cantilever with a tip radius of 9 nm, a resonant frequency of 70 kHz, and a spring constant of 2 N/m (AC240TS, Olympus America Inc., Center Valley, PA). AFM images were analyzed with Igor Pro MFP3D (WaveMetrics Inc., Portland, OR). AFM plates were prepared by spotting 5–25 μL of 0–20-fold diluted samples on freshly cleaved mica and incubated at room temperature for 20 min followed by a gentle wash with 4 mL of Milli-Q water to remove extra sample layers and buffer salts. The plates were allowed to dry overnight prior to imaging.

**Thioflavin T Fluorescence Measurements.** Thioflavin T (ThT) fluorescence assays were performed at 37 °C on a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon, Kyoto, Japan) using FluorEssence in a 10 mm cuvette. For the pH dependence of PAPf39 fibril dissociation, HDXMS, and protease protection experiments, ThT fluorescence assays were prepared by mixing 50 μL of sample, 60 μL of 100 μM ThT in 20 mM phosphate buffer pH 7.7, and 440 μL of PBS pH 7.7 (total volume of 550 μL). For all other ThT measurements, ThT fluorescence assays were prepared by mixing 25 μL of sample, 30 μL of 100 μM ThT in 20 mM phosphate buffer pH 7.7, and 495 μL of PBS pH 7.7 (total volume of 550 μL) to ensure that the fluorescence signal remained within the linear range of the instrument. The samples were excited at 440 nm, and the fluorescence emission intensity was collected at 482 nm for 90 s and averaged. The fluorescence intensity was corrected for fluctuations in lamp intensity by dividing the fluorescence signal by the lamp intensity. Error bars are the standard deviation of at least three independent experiments.

**Hydrogen–Deuterium Exchange Assays.** HDX was assayed using electrospray ionization liquid chromatography with mass spectrometry (ESI-LC–MS) for three samples: exchanged fibrils, fully exchanged monomer, and in-exchange monomer. For each sample, five independent experiments were performed. PAPf39 fibrils were formed by agitating 2 mg/mL PAPf39 dissolved directly in PBS pH 7.7 for 48 h at 37 °C. The presence of fibrils prior to HDX was confirmed using ThT fluorescence assays and AFM imaging, as described above. PAPf39 fibrils were pelleted via centrifugation at 14000 rpm (20200g) for 20 min at 4 °C. The supernatant was removed, and the fibril pellet was resuspended in 20 mM PB pH 7.7, 98% D2O, and 2% H2O for the exchanged fibril sample. The fully exchanged monomer was prepared by dissolving dry PAPf39 monomer in the same buffer at a concentration of 2 mg/mL. Both the fibrils and monomer were allowed to exchange for 1 h at 25 °C to generate the exchanged fibril and fully exchanged monomer samples. Importantly, fibril formation does not occur in the exchange buffer,10 but preformed fibrils are stable (see Figure 2). HDX was quenched, and the fibrils were dissociated by placing the samples on ice and diluting the sample 10-fold in 100% H2O-based 20 mM PB pH 2.3. This lowered the pH to 2.5 and gave a final PAPf39 peptide concentration of 0.2 mg/mL. These quenching conditions (pH 2.5, on ice) were chosen because the HDX rate is reduced at lower temperatures2,13 and
reaches a minimum at pH ~2.5. In addition, low pH inhibits fibril formation and dissociates PAP39 fibrils rapidly (Figures 1 and 2). Pepsin (porcine gastric mucosa, 3200–4500 units/mg, Sigma-Aldrich, St. Louis, MO) was added to the samples to a final concentration of 0.2 mg/mL from a 10 mg/mL stock solution in H2O (final D2O content of ~9.6%) to induce PAP39 peptide fragmentation. The in-exchange monomer samples were prepared by dissolving 0.2 mg/mL dry PAP39 monomer in 20 mM PB pH 2.5 and 9.8% D2O at 4 °C and adding pepsin to a final concentration of 0.2 mg/mL (final D2O content of ~9.6%). As a control, 0.2 mg/mL unexchanged PAP39 monomer in H2O-based 20 mM PB pH 2.5 was cleaved with 0.2 mg/mL pepsin and analyzed using LC–MS.

The LC–MS analysis was performed as follows. Samples (4 μL) were loaded onto a C18 reverse phase HPLC column (BioBasic-C18, Thermo Electron Corp., Waltham, MA) using a NanoFlow autosampler (Agilent, Santa Clara, CA) and eluted at 200 μL/min in a 0% (0.2% formic acid in water) to 80% (0.2% formic acid in acetonitrile) organic phase gradient over 20 min. The eluent was directly injected into the Thermo Scientific LTQ Orbitrap XL electrospray mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Mass spectra were deconvoluted to a single charge state using Xcalibur Xtract version 2.0.7. Peptide fragments were identified in the unexchanged PAP39 monomer controls using SEQUEST in Proteome Discoverer (Thermo Fisher Scientific Inc.) and manually with ExPASy’s FinPep tool. Individual peptide fragment deuterium incorporation levels were determined using two different approaches to ensure the robustness of the analysis. Both methods correct for the effects of local sequence on HDX by comparing the HDX of the PAP39 fibril to that of the monomer, which has the same local sequence effects but is a random coil and lacks the HDX protection observed in the fibrils.

In the first approach, the centroid of each peptide fragment isotopic envelope was extracted and used to calculate the percent HDX in the fibril for each peptide fragment as

\[ H = \frac{m_c - m_i}{m_M - m_i} \times 100 \]  

where \( H \) is the percent HDX for an individual peptide fragment, \( m_c \) is the centroid mass from the exchanged fibril sample, \( m_i \) is the centroid mass from the in-exchange monomer sample, and \( m_M \) is the centroid mass from the fully exchanged monomer sample.

In the second approach, the distribution of deuterium incorporation was calculated for each peptide fragment using the linear fit method described by Chik et al. A weighted average of the deuterium incorporation distribution was used to calculate the average percent HDX for each fragment in the in-exchange monomer, exchanged fibril, and fully exchanged monomer samples, assuming that the maximal number of amide protons exchanged. The HDX of the fibrils was subsequently corrected for in-exchange and back-exchange using eq 1, where the centroid masses were substituted for the corresponding percent HDX for each sample.

The percent HDX of each peptide fragment was used to calculate the average percent HDX protection for each residue in the PAP39 sequence. To test the robustness of the analysis, two averaging algorithms, weighted and unweighted, were used. In the weighted method, the calculated percent HDX of each peptide fragment, for which non-zero HDX was observed in the fully exchanged monomer, was used in a modified version of the equation described by Damo et al.

\[ HX_i = 100 - \left( \frac{\sum_{j=1}^{n_i} h_j}{\sum_{j=1}^{n_i} m_j} \right) \]  

where \( HX_i \) is the average percent HDX protection for residue \( i \), \( n_i \) is the number of peptide fragments containing residue \( i \), \( h_j \) is the percent HDX for a peptide fragment \( j \) containing residue \( i \), and \( m_j \) is the number of exchangeable backbone amide sites in peptide fragment \( j \). In the unweighted averaging algorithm, the average percent HDX protection is calculated as:

\[ HX_i = 100 - \left( \frac{\sum_{j=1}^{n_i} h_j}{n_i} \right) \]  

where \( HX_i \) is the percent HDX protection for residue \( i \), \( n_i \) is the number of peptide fragments containing residue \( i \), and \( h_j \) is the percent HDX for a peptide fragment \( j \) containing residue \( i \). Again, only peptide fragments for which exchange was observed in the fully exchanged monomer were used in the calculations because peptide fragments with no HDX in the fully exchanged monomer sample contain no protection information. Four combinations of the calculations given above (the centroid or linear method in eq 1 with weighted or unweighted averaging in eq 2 or 3, respectively) were used to determine the PAP39 fibril HDX protection profiles. Errors were calculated as the standard deviation of five independent experiments. All combinations yielded quantitatively similar results (Figure S1E of the Supporting Information); however, only the profile calculated from the centroid method with weighted averaging is shown in the main body of the paper.

Although an abundance of PAP39 fragments was generated from cleavage between residues 16 and 17, many peptide fragments overlap this cleavage site, and no major changes are observed in the HDX protection profile near this cleavage site (Figure 3 and Figure S1 of the Supporting Information). This suggests that the observed HDX protection profile is not simply a result of the cleavage pattern of the PAP39 peptide. Furthermore, to ensure that uneven sequence coverage between the N- and C-terminal regions of PAP39 (Figure S1F of the Supporting Information) does not affect the HDX protection profile, values from random subsets of sequences were used to calculate the HDX protection profile. The subsets were chosen such that approximately equal numbers of sequences covered each residue. Importantly, the HDX protection profiles generated in this analysis were similar to the HDX protection profile calculated when all sequences were used, confirming that the HDX protection profile is not a result of the sequence coverage. Furthermore, a jackknife analysis was used to confirm that the HDX protection of a single peptide fragment does not determine the HDX protection profile. These analyses demonstrated that the calculated HDX protection profile represents consensus HDX behavior and is not due to HDX fluctuations in a single peptide fragment or a subset of fragments.

**Protease Protection Assays.** Protease protection assays were performed using two proteases with different sequence specificities: thermolysin (Bacillus thermoproteolyticus rokko, type X, 50–100 units/mg, Sigma-Aldrich) and trypsin (bovine pancreas, type XI, ≥6000 units/mg, Sigma-Aldrich). The cleavage reaction was initiated by mixing PAP39 monomer
or fibrils with protease at a 1000:1 PAPf39 monomer:protease mass ratio (in PBS pH 7.7 and 0.8 mM CaCl2) followed by incubation at 37°C for 10 min, at which time formic acid was added to a final concentration of 0.4% (v/v) to quench the reaction and dissociate the fibrils. Samples were flash-frozen with liquid nitrogen and stored at −80°C until they were used. The cleaved samples were separated and analyzed by LC−MS, in a manner similar to the procedure described in the previous section.

Peptide proteolytic fragment peaks and retention times were identified manually using predicted peptide cleavage fragments generated by Protein Prospector MS-Digest (University of California, San Francisco, CA). Peptide cleavage fragments were identified with Xcalibur, using mass and retention time pairs, and the area of the elution peak for a single peptide was calculated to determine the relative amount of peptide produced by proteolytic cleavage. The area of the peptide elution peak varied linearly with peptide concentration as determined by analyzing PAPf39 monomer controls at different concentrations, confirming that this method can be used to determine the relative amount of peptide in a sample. In this analysis, only primary peptide proteolytic fragment sequences (i.e., containing only one cleavage site) were used because it is not known if the fibrillar structure is altered as a result of the first cleavage event. A cleavage site was assigned to each primary sequence, and the elution peak area of the peptide proteolytic fragment in the monomer was divided by the elution peak area of the peptide proteolytic fragment in the fibril to determine the fold protection of a particular cleavage site in the fibrils, as compared to the monomer. Importantly, this calculation normalizes for the effect of the local sequence on the frequency of proteolytic cleavage. The average protection for a cleavage site was determined from the protection values calculated from both the N- and C-terminal primary sequence fragments in three independent sample replicates.

Far-UV Circular Dichroism. Far-UV CD spectra were recorded on a Jasco-715 spectropolarimeter at 10°C from 190 to 260 nm in a 1 mm light path length cylindrical quartz cuvette. PAPf39, PAPf39Δ1–8, and PAPf39Δ1–13 peptide samples were prepared at a concentration of 10 mg/mL in 3.5 mM HCl pH 2.5 and then diluted to 0.2 mg/mL in CD buffer (1 mM sodium borate, 1 mM sodium citrate, 1 mM sodium phosphate, and 10 mM NaCl) at pH 7.7, 5.5, or 2.5. For each condition, spectra for three independent replicates were averaged and the reported errors are one standard deviation of the mean. For each replicate, four wavelength scans (three accumulations each) were averaged. The ellipticity of the peptide solution (θ) was corrected by subtracting the buffer baseline and converted to molar ellipticity using the equation Θ = (θM)/(10lc), where M is the peptide molecular mass, l is the light path length in centimeters, and c is the peptide concentration in milligrams per milliliter.

Figure 1. Sequences of PAPf39, PAPf39Δ1–8, and PAPf39Δ1–13 peptides (A) and effect of pH on fibril formation as monitored by ThT fluorescence (B–E) and AFM imaging (F–Q). Black, red, and green bars depict data for the PAPf39, PAPf39Δ1–8, and PAPf39Δ1–13 peptides, respectively. AFM images for each peptide were taken after incubation for 48 h in different buffers (specified for each row). Scale bars on the AFM images are 500 nm.
RESULTS

pH Dependence of PAP39 Fibril Formation and Dissociation. It is known that PAP39 fibrils form in PBS pH 7.7 in the presence of agitation, but not in the absence of agitation, at salt concentrations below ~100 mM, and/or at low pH (i.e., pH 2.5, 2% acetic acid). Four residues in the PAP39 sequence change ionization state between pH 7.7 and 2.5 and may be responsible for the pH dependence of PAP39 fibril formation (Figure 1A). There are two histidine residues (unperturbed side chain pK_a of ~6.5) at positions 3 and 23 that change from neutral to positively charged and two glutamic acid residues (unperturbed side chain pK_a of ~4.3) at positions 7 and 19 that change from negatively charged to neutral between pH 7.7 and 2.5. To determine which of these residues are responsible for the pH dependence of fibril formation, PAP39 fibril formation was tested at pH 5.5. Assuming no pK_a shifts, at pH 5.5, histidine residues are positively charged while glutamic acid residues remain neutral, thus allowing the possible effects of histidine residues versus glutamic acid residues to be separated. In these experiments, 2 mg/mL PAP39 peptide in PBS pH 7.7, PBS pH 5.5, ABS pH 5.5, or PBS pH 2.5 was incubated with agitation at 37°C. Both PBS pH 5.5 and ABS pH 5.5 were used for incubation because phosphate does not buffer well at this pH and there may be differences in fibril formation in acetate versus phosphate buffer.

PAP39 fibril formation occurred in PBS pH 7.7, as previously observed, but did not occur in PBS pH 5.5, ABS pH 5.5, or PBS pH 2.5. This was shown by increases in ThT fluorescence intensity and the observation of fibrils in AFM images for the PBS pH 7.7 incubations as a function of time, but not for the PBS pH 5.5, ABS pH 5.5, or PBS pH 2.5 samples (Figure 1B–I).

Fibril formation at pH 5.5 and 2.5 may be limited if acidic pH disfavors conformations in the monomer capable of nucleation or elongation, or if the fibrillar structure of PAP39 is not stable under acidic conditions. To determine if PAP39 fibrils are stable at low pH, the dissociation of PAP39 fibrils formed in PBS pH 7.7 was monitored using ThT fluorescence assays and AFM imaging. Dissociation assays were performed at room temperature in different buffers. Corresponding AFM images can be found in Figure S2 of the Supporting Information. (B) Kinetics of pH-induced dissociation of preformed PAP39 fibrils as monitored by light scattering at pH 2.8. The time point of HCl addition is denoted with an arrow.

Figure 2. Effect of pH on PAP39 fibril dissociation. (A) ThT fluorescence intensity of preformed PAP39 fibrils after 24 h at room temperature in different buffers. Corresponding AFM images can be found in Figure S2 of the Supporting Information.

Because PAP39 fibrils that formed at pH 7.7 appear to rapidly dissociate at pH 2.5, the kinetics of PAP39 fibril dissociation was probed at low pH (Figure 2B). In these assays, PAP39 fibril dissociation was induced by adding hydrochloric acid to reduce the pH to 2.8 and the kinetics of dissociation was monitored by light scattering. The light scattering intensity of the PAP39 fibrils remained high until acid was injected, after which the light scattering intensity rapidly decreased to the buffer baseline, indicating fibril dissociation (Figure 2B). Under these conditions, fibril dissociation was complete in 10 s.

PAP39 Fibril Core Sequence. To gain insight into the location of ionizable residues within the PAP39 fibrillar structure, we used two different experimental assays, hydrogen–deuterium exchange mass spectrometry (HDXMS) and protease protection (PP), to identify the residues that form the PAP39 fibrillar core.

HDXMS was used to probe the fibrillar core of the PAP39 fibrils. In these experiments, the incorporation of deuterium into the in-exchange monomer, exchanged monomer, and exchanged fibrils was measured by LC–MS and used to determine the percent HDX protection over the sequence of the fibril. The monomer and fibril samples were exchanged in a deuterium-based buffer (PB pH 7.7), where fibril formation does not occur but preformed fibrils are stable (Figure 2A). Following HDX, the samples were rapidly quenched by placing the samples on ice and diluting the sample 10-fold in 100% H_2O-based 20 mM PB pH 2.3, which leads to rapid dissociation of the fibrils (Figure 2B). The in-exchange sample was prepared by dissolving lyophilized monomer directly into ice-cold buffer to match the final composition of the quenching conditions. All samples were cleaved with pepsin and immediately loaded for analysis by LC–MS. Figure S1 of the Supporting Information shows the mass spectra of a fragment with a monoisotopic mass of 2790.5 Da covering residues 17–39. Importantly, the exchanged monomer sample contains significantly more deuterium than the exchanged fibril sample, and the in-exchange monomer sample contains very little deuterium (Figure S1 of the Supporting Information). The mass shifts due to deuterium incorporation are large enough to calculate the net percent HDX of the PAP39 fibrils and to

significantly differ between the agitated and stationary samples (Figure S2 of the Supporting Information).

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determine the percent HDX protection (see Experimental Procedures for details). The percent HDX was calculated for each fragment using both the centroid and linear fit methods (see refs 15–17 and eq 1). The percent HDX values for all fragments with observable HDX in the fully exchanged monomer (for sequence coverage, see Figure S1 of the Supporting Information) were used in weighted and unweighted averaging algorithms (see eqs 2 and 3, respectively) to calculate the average percent HDX protection over the PAPf39 sequence. Both the centroid and linear fit methods combined with the weighted and unweighted averaging algorithms yielded quantitatively similar results (for comparison, see Figure S1 of the Supporting Information). The results of the average percent HDX protection per residue calculated from the centroid method and the weighted averaging algorithm are shown in Figure 3.

Figure 3. PAPf39 fibril core sequence determination by HDXMS and protease protection assays. The bar graph shows the sequence-averaged percent protection of PAPf39 fibrils from HDX. Bars are the average protection calculated from five independent experiments, and error bars are one standard deviation of the mean. The arrows above and below the PAPf39 sequence show the results of the protease protection assays using thermolysin (Therm) and trypsin (Tryps). White, pink, and red arrows indicate <5-, 5–15-, and >15-fold protection, respectively, from proteolytic cleavage at the indicated cleavage site. The protease protection values are calculated from the average protection for both N- and C-terminal primary fragments from three independent experiments. See Experimental Procedures for more details on these experiments and Figure S5 of the Supporting Information for numerical values from the protease protection assays.

The results of HDXMS experiments show that the level of HDX protection of residues 1–7, in the N-terminal region of PAPf39, is low (~33%). The level of HDX protection increases for residues 7–13 and reaches a plateau at approximately 60% for residues 14–33. In the C-terminal region, comprised of residues 34–39, the level of HDX protection further increases to approximately 85% (Figure 3). This suggests that the N-terminal region, from residue 1 to 7, is not part of the fibrillar core. Conversely, the increased level of HDX protection in the central region, between residues 14 and 33, suggests that it is involved in the PAPf39 fibrillar core. The high level of protection observed in the C-terminal region, between residues 34 and 39, indicates that this region may play an important role in the PAPf39 fibrillar core. This agrees with the previous observations that residues 13–18 in the central region of the peptide can form amyloid-like fibrils22 and that the removal of C-terminal residues 36–39 reduces the extent of fibril formation and also with sequence-based predictions of the amyloidogenic regions in the PAPf39 peptide (see Figure S1 of the Supporting Information). The observed features in the HDX protection profile do not appear to be an experimental artifact because there is good sequence coverage and extensive sequence overlap between different peptide fragments and each residue is present in multiple peptide fragments (Figure S1 of the Supporting Information). The quantitatively similar results of the four methods used to calculate the HDX protection profile also demonstrate the robustness of the analysis of the experimental data (see Experimental Procedures and Figure S1 of the Supporting Information).

A protease protection (PP) assay was used to support the HDX protection trends.23–25 In this assay, monomer and fibril samples were cleaved under limiting proteolysis conditions using thermolysin or trypsin. The primary fragments (peptides generated by one cleavage of PAPf39) were identified by LC–MS, and their relative amounts were determined by integrating the elution peaks. The concentration of a cleavage product was assumed to correlate with the frequency of cleavage at a particular site and was used to calculate the level of protection of a cleavage site in the fibril versus the monomer (the area of peptide fragment elution peak in the monomer divided by the area of the peptide fragment elution peak in the fibril). The proteolytic cleavage protection varies throughout the PAPf39 sequence (Figure 3) and appears to be independent of the protease used. There is <5-fold protection in the N-terminal region (residues 1–9) and >5-fold protection for residues 14–39 (in the central and C-terminal regions). The validity of the PP results is supported by the presence of both N- and C-terminal fragments for each cleavage site and the agreement of the protection trend for both the N- and C-terminal fragments. The observed PP pattern is not a result of the protease specificity or cleavage site preference, because the frequency of cleavage in the monomer or fibril alone does not correlate with the protease protection. Finally, comparison of the fibril to the monomer normalizes for the effect of the local sequence on the frequency of proteolytic cleavage.

The PP profile can be compared to the HDXMS protection profile. These experiments provide two independent measures of the PAPf39 fibrillar core and appear to be in good qualitative agreement (Figure 3). Importantly, both the HDXMS and PP profiles suggest that the central and C-terminal regions are involved in the PAPf39 fibrillar core while the N-terminal region is not.

Fibril Formation in PAPf39 Variants. The hydrogen–deuterium exchange and protease protection of the central and C-terminal regions of PAPf39 suggest that they are involved in the fibrillar core (Figure 3). Conversely, the lack of protection in the N-terminal region suggests that it is not in the fibrillar core and is probably part of an unstructured region in the PAPf39 fibril. Thus, removing the N-terminal region should not affect fibril formation and may even enhance fibril formation because this unstructured region contains many ionizable residues. To test this hypothesis, two PAPf39 variants, PAPf39Δ1–8 and PAPf39Δ1–13, corresponding to the PAPf39 peptide with the first 8 and 13 residues truncated, respectively (Figure 1A), were characterized. These variants were chosen because only the highly unprotected residues were removed in PAPf39Δ1–8 while PAPf39Δ1–13 has additional residues with intermediate protection removed.

Figure 1B compares the kinetics of fibril formation in PBS pH 7.7 of full length PAPf39 with those of PAPf39Δ1–8 and PAPf39Δ1–13 as monitored by ThT fluorescence assays. The kinetics of fibril formation for the variants followed sigmoidal curves, suggesting that the fibrils form via a nucleation-dependent elongation mechanism, similar to that for the
formation of fibrils by full length PAPf3. The PAPf3 truncated variants formed amyloid fibrils more readily than the full length PAPf3 peptide, as evidenced by shorter lag times [20 ± 3, 1 ± 2, and 1 ± 2 h for PAPf3, PAPf3Δ1−8, and PAPf3Δ1−13, respectively (see Table S1 of the Supporting Information)]. Full length PAPf3 and variant fibrils were also observed using AFM imaging, and the fibrils appear to be morphologically similar for all three peptides (Figure 1F, J, N and Table S1 of the Supporting Information).

To further demonstrate that the variant fibrils are structurally similar to the PAPf3 fibrils, PAPf3Δ1−8 and PAPf3Δ1−13 fibrils formed in PBS pH 7.7 were used to seed PAPf3 fibril formation. Both variants were able to seed (induce) full length PAPf3 fibril formation, thus suggesting that the variant fibrils are structurally similar to the full length PAPf3 fibrils (Figure 4 and Figure S3 of the Supporting Information).

Furthermore, fibrils from the seeded incubations appear to have a morphology similar to that of the unseeded and seeded full length PAPf3 incubations, as shown by AFM images (Figure S3 of the Supporting Information).

**pH Dependence of PAPf3 Variant Fibril Formation.**

The results for the PAPf3 variants presented above demonstrate that the N-terminal region is not necessary for fibril formation at neutral pH. PAPf3 does not appear to form fibrils at low pH, and it is unclear which residues in the PAPf3 sequence are responsible for this effect. The PAPf3Δ1−8 and PAPf3Δ1−13 peptides lack two of four possible residues that ionize between neutral and acidic pH (Figure 1A). Each of the variants does not contain a histidine residue (H3) or a glutamic acid residue (E7), allowing the contribution of changes in ionization state of these residues to the pH dependence of PAPf3 fibril formation to be determined.

The kinetics of PAPf3Δ1−8 and PAPf3Δ1−13 fibril formation were monitored in PBS pH 7.7, PBS pH 5.5, ABS pH 5.5, and PBS pH 2.5 by ThT fluorescence measurements (Figure 1B–E) and AFM imaging (Figure 1J–Q). Full length PAPf3 formed fibrils only at neutral pH, while both PAPf3 variants formed fibrils at all tested pH values (Figure 1). The most abundant fibril heights and lengths, as determined from the AFM data, were similar within one standard deviation between different incubation conditions and variants (Table S1 of the Supporting Information), suggesting similarities in fibril morphology.

To test that PAPf3Δ1−8 fibrils formed at low pH are structurally similar to full length PAPf3 fibrils, PAPf3 in PBS pH 7.7 was seeded with PAPf3Δ1−8 fibrils preformed in PBS pH 7.7, pH 5.5, or pH 2.5. PAPf3Δ1−8 was chosen for these experiments rather than PAPf3Δ1−13 because PAPf3Δ1−8 has fewer residues removed than PAPf3Δ1−13. PAPf3Δ1−8 fibrils preformed in PBS pH 7.7, pH 5.5, or pH 2.5 all seeded full length PAPf3 fibril formation at pH 7.7 with similar efficacy, as shown by shorter lag times in the seeded samples than in the unseeded sample (Figure 4) and the presence of fibrils in AFM images (Figure S3 of the Supporting Information). Thus, PAPf3Δ1−8 fibrils preformed at all tested pH values are structurally compatible with full length PAPf3 fibrils. Although PAPf3Δ1−8 is capable of seeding PAPf3 fibril formation in PBS pH 7.7, PAPf3Δ1−8 fibrils do not seed PAPf3 fibril formation in PBS pH 5.5 or pH 2.5 (Figure 4 and Figures S3 and S4 of the Supporting Information). This suggests that PAPf3 is incapable of forming fibrils at pH 5.5 or 2.5 and that the process is not limited by nucleation.

**Mixed Peptide Fibril Characterization at pH 2.5.**

The N-terminal region of PAPf3 is necessary for the pH dependence of PAPf3 fibril formation, and removal of the first eight residues (in PAPf3Δ1−8) eliminates the pH dependence of fibril formation. This region contains one negative and three positive charges at pH 7.7 and four positive charges at pH 2.5. Intermolecular charge–charge repulsion between positively charged residues in this region of PAPf3 may inhibit fibril formation at acidic pH and also cause preformed fibrils to dissociate at acidic pH. If this is the case, mixed fibrils between PAPf3 and PAPf3Δ1−8 should be observed at a certain molar excess of PAPf3Δ1−8. This will occur when the PAPf3 N-terminal region concentration is low relative to that of the core sequence (from both PAPf3 and PAPf3Δ1−8), thus weakening the intermolecular charge–charge repulsion between PAPf3 N-terminal regions and allowing fibrils to form. To test this hypothesis, PAPf3 and PAPf3Δ1−8 were incubated together in PBS pH 2.5 at 37°C with agitation at PAPf3:PAPf3Δ1−8 molar ratios of 75:25, 50:50, 25:75, and 0:100. Fibril formation was observed at these molar ratios, but there was no evidence that PAPf3 was incorporated into these fibrils as they all contained only PAPf3Δ1−8 (see Figure S4 and Experimental Procedures of the Supporting Information). These results show that at pH 2.5 PAPf3 and PAPf3Δ1−8 do not form mixed fibrils and suggest that charge–charge repulsion between PAPf3 N-terminal regions is not responsible for inhibiting fibril formation at pH 2.5. Rather, it appears that the PAPf3 monomer does not readily adopt a conformation amenable to elongation at pH 2.5.

**Far-UV Circular Dichroism.**

The results described above demonstrate that the N-terminal region is responsible for the pH dependence of fibril formation and that the pH dependence is not determined by intermolecular charge–charge repulsion of the N-terminal regions of PAPf3. Furthermore, because PAPf3 fibril formation can be seeded with PAPf3Δ1−8 at pH 7.7 but not at pH 5.5 or 2.5, it appears that fibril elongation is deficient at low pH in PAPf3. Thus, it is possible that the pH alters the PAPf3 monomer conformation to inhibit fibril formation, while pH has little effect on PAPf3Δ1−8 or PAPf3Δ1−13. To test this hypothesis, far-UV CD spectra of
PAPf39, PAPf39Δ1−8, and PAPf39Δ1−13 were obtained at pH 7.7, 5.5, and 2.5. Even though the spectra overall appear to have relatively small amplitudes around 220 nm, there are noticeable differences. The PAPf39 molar ellipticities at pH 5.5 and 2.5 are similar in this range, while the ellipticity at pH 7.7 has a lower signal, suggesting more secondary structure (Figure 5). Furthermore, the molar ellipticities of PAPf39Δ1−8 and PAPf39Δ1−13 do not vary with pH and have similar magnitudes, which are comparable yet somewhat larger than the molar ellipticity of PAPf39 at pH 7.7. These results suggest that truncated variants contain more secondary structure than PAPf39. This difference is particularly large at pH 5.5 and 2.5 where PAPf39 does not form fibrils while both PAPf39Δ1−8 and PAPf39Δ1−13 do.

**DISCUSSION**

This work aimed to determine which residues form the PAPf39 fibrillar core and to understand the pH dependence of PAPf39 fibril formation. The results of the HDXMS and protease protection assays suggest that the central and C-terminal regions of PAPf39 are part of the fibrillar core while the N-terminal region is not. The N-terminal region modulates the pH dependence of PAPf39 fibril formation because PAPf39 fibrils form at pH 7.7 but not at pH 5.5 or 2.5, while N-terminal truncation variants, PAPf39Δ1−8 and PAPf39Δ1−13, form fibrils at all tested pH values. From these experiments, it is evident that the PAPf39 N-terminal region is not necessary for fibril formation but modulates the pH dependence of fibril formation.

There are two mechanisms that may account for the pH dependence of PAPf39 fibril formation. Modulating the pH to increase the net charge of the N-terminal region of PAPf39 may prevent monomer association through intermolecular charge−charge repulsion or alter the conformational ensemble of the monomer to inhibit fibrillation. The first mechanism can be ruled out for two main reasons. First, PAPf39 does not form fibrils when incubated with high concentrations of PAPf39Δ1−8 monomer or fibrils at pH 2.5. If charge−charge repulsion between PAPf39 N-terminal regions were responsible for the pH dependence of fibril formation, then decreasing the concentration of the N-terminal region while keeping the concentration of the core sequence constant would reduce this effect and allow fibril formation to occur. However, because PAPf39 and PAPf39Δ1−8 do not form mixed peptide fibrils when the monomers are incubated together (PAPf39Δ1−8 forms fibrils without incorporating PAPf39), charge−charge repulsion between PAPf39 N-terminal regions is not responsible for the pH dependence of fibril formation. Importantly, PAPf39Δ1−8 fibrils formed at all tested pH values seed PAPf39 fibril formation at neutral pH, verifying that PAPf39Δ1−8 fibrils are proficient at seeding and that the PAPf39 and PAPf39Δ1−8 fibril structures are compatible. Second, the ionic strength of the buffer does not appear to affect fibril formation at pH 2.5. If charge−charge repulsion were responsible for the pH dependence of fibril formation, then the salt concentration would affect fibril formation, because ions can shield charge−charge interactions. However, PAPf39 does not form fibrils in 2% acetic acid in the absence of...

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**Figure 5.** pH dependence of far-UV CD ellipticity for PAPf39 (●), PAPf39Δ1−8 (▲), and PAPf39Δ1−13 (■). Average values of molar ellipticity between 215 and 225 nm are shown. See the Supporting Information for full CD spectra and the results of the secondary structure deconvolution analysis (Figure S6 of the Supporting Information).

**Figure 6.** Conceptual model of the pH dependence of PAPf39 fibril formation and dissociation. Yellow, red, and blue regions represent the N-terminal, central, and C-terminal regions of the PAPf39 peptide, respectively. Peptides lacking the yellow N-terminal region represent the variant peptides. The background color indicates the pH conditions (green, orange, and red for pH 7.7, 5.5, and 2.5, respectively). The cartoon representation of the fibrils is inspired by the X-ray structure of Protein Data Bank entry 3PPD. The depicted monomer structures intend to illustrate changes in the overall conformational ensemble.
The pH dependence of PAP39 fibril formation can be explained by conformational changes in the PAP39 monomer (second mechanism), which is shown as a conceptual model in Figure 6. The model proposes that the PAP39 monomer conformational ensemble changes between pH 7.7 and 5.5, making it capable of forming amyloid fibrils at pH 7.7 but not at pH 5.5 or 2.5. The conformational ensemble of the truncated variants, however, is not significantly altered by pH and is able to form fibrils at all tested pH values (Figure 6). These assertions are supported by the pH dependence of the far-UV CD spectra of the PAP39, PAP39Δ1–8, and PAP39Δ1–13 monomers (Figure 5). The spectra suggest that the PAP39 monomer contains more secondary structure at pH 7.7 than at pH 5.5 or 2.5. The PAP39Δ1–8 and PAP39Δ1–13 monomer spectra, however, do not vary with pH and indicate that the truncation variants contain more secondary structure than PAP39, particularly at pH 5.5 or 2.5. It also appears that the amount of monomer secondary structure is inversely correlated with the fibril formation lag time, because the PAP39Δ1–8 and PAP39Δ1–13 monomers contain more structure and have shorter lag times than PAP39. Thus, alterations in the pH change the ionization state of residues in the N-terminal region, which alters the PAP39 monomer conformational ensemble, causing pH-dependent fibril formation. Similarly, the N-terminal region also appears to modulate PAP39 fibril dissociation at low pH because PAP39 fibrils formed at neutral pH dissociate at acidic pH (Figure 2), while PAP39Δ1–8 and PAP39Δ1–13 fibrils do not. Dissociation may occur because of conformational changes in the peptide subunits, which initiates the dissociation of monomeric units from the fibril ends.

### CONCLUDING REMARKS

PAP39 was first identified as a main component of the semen-derived enhancer of viral infection (SEVI). It forms amyloid fibrils in semen that increase HIV infectivity by up to 5 orders of magnitude.3 The pH dependence of PAP39 fibril formation and dissociation has interesting biological implications because the pH of semen is markedly different than the pH of the vaginal tract.7 Because PAP39 fibrils do not fully dissociate within 24 h at pH 5.5, fibrils formed in semen (average pH of 7.7±0.2) may be stable enough to enhance HIV infectivity in the vaginal tract, which has a pH of ~5.2, ranging between 3.6 and 7.7,17 even if fibrils do not form at this pH. Furthermore, the HDX and protease protection assays suggest that the N-terminal region of PAP39 is exposed (not part of the fibrillar core) and therefore may interact with HIV virus particles to facilitate transmission. Interestingly, it has been shown that cationic residues are essential for PAP39 fibril-mediated enhancement of HIV transmission,28 and it is possible that the cationic residues in the N-terminal region are responsible for enhancing HIV transmission.

### ASSOCIATED CONTENT

#### Supporting Information

Additional experimental procedures, one table reporting fibril morphology, and four figures providing additional details of ThT fluorescence and AFM experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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#### Notes

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### ABBREVIATIONS

ABS, acetate-buffered saline; AFM, atomic force microscopy; CD, circular dichroism; ESI-LC−MS, electrospray ionization liquid chromatography and mass spectrometry; HIV, human immunodeficiency virus; HDX, hydrogen−deuterium exchange; HDXMS, HDX mass spectrometry; HPLC, high-performance liquid chromatography; LC−MS, liquid chromatography and mass spectrometry; PAP39, prostatic acidic phosphatase fragment of 39 residues (residues 248−286 of human prostatic acidic phosphatase); PAP39Δ1–8, N-terminally acetylated truncated variant of PAP39 with the first 8 residues removed; PAP39Δ1–13, N-terminally acetylated truncated variant of PAP39 with the first 13 residues removed; PB, phosphate buffer; PBS, phosphate-buffered saline; PP, protease protection; SEVI, semen-derived enhancer of viral infection; TFA, trifluoroacetic acid; ThT, thioflavin T.

### REFERENCES


CORE SEQUENCE OF PAPF39 AMYLOID FIBRILS AND THE MECHANISM OF pH DEPENDENT FIBRIL FORMATION: THE ROLE OF MONOMER CONFORMATION

Kinsley C. French and George I. Makhatadze

Supplementary Information

Experimental Procedures

Peptide Identification and Concentration Determination in Mixed Peptide Fibrils

The PAPf39 and PAPf39Δ1-8 peptides (439.4 µM total concentration) were incubated together in PBS pH 2.5 at 37°C for 84 hours in molar ratios of 0:100, 25:75, 50:50, 75:25, and 100:0 of PAPf39 to PAPf39Δ1-8. ThT fluorescence intensity measurements and AFM amplitude images were taken after 72 hours of incubation. The concentration of each peptide in the pellet (fibrillar peptide) and supernatant (soluble peptide) of the incubations was determined by liquid chromatography-mass spectrometry (LC-MS). The mixed peptide incubations were pelleted via centrifugation at 14,000 rpm (20,200 x g) for 20 minutes at 4°C. The supernatant was removed and analyzed by LC-MS. The fibril pellet was resuspended in an equal volume of 8 M urea, 0.1 M sodium phosphate, 0.1 M Tris, pH 8. Pellets were dissolved for 36 hours at room temperature, centrifuged at 14,000 rpm (20,200 x g) for 20 minutes at 20°C to remove any undissolved pellet, and analyzed by LC-MS. Although a substantial amount of the pellet was dissolved by the urea buffer, as shown by the large peptide signal in the MS, the pellets did not completely dissolve, because a small clear pellet was visible after resuspension in urea followed by centrifugation. The PAPf39 and PAPf39Δ1-8 peptide concentrations in the pellet and supernatant fractions were determined by LC-MS as described in the main text. Peptide elution peaks were identified manually in the Xcalibur software, using mass and retention time pairs and the elution peak areas were calculated to determine the relative amount of peptide eluted. Fresh peptide monomer samples (unfibrillated and unfractionated) were used as controls to determine the elution peak area of the peptides prior to incubation. Importantly, the elution peaks of the unfibrillated monomer varied linearly with the peptide concentration.

Fibrils formed in the mixed 50:50, 25:75, and 0:100 PAPf39 to PAPf39Δ1-8 samples, as indicated by both ThT fluorescence measurements (Figure S4A) and AFM images taken at 72 hours (Figure S4B-F). Although there was no significant ThT fluorescence in the 75:25 PAPf39 to PAPf39Δ1-8 sample (Figure S4A), a small number of fibrils were observed in some AFM images (Figure S4C). As expected, no fibrils were observed in the 100:0 PAPf39 to PAPf39Δ1-8 sample (Figure S4A-B).

Although fibrils form in mixed PAPf39 and PAPf39Δ1-8 incubations at pH 2.5, the fibrils are composed solely of PAPf39Δ1-8 and PAPf39 is not incorporated in the fibrils. To demonstrate this, the relative amounts of PAPf39 and PAPf39Δ1-8 in the fibril pellet (fibrillar material) and supernatant (unfibrillated material) of mixed fibril incubations were determined using LC-MS (Figure S4G-H). Monomeric controls with no fibrils were analyzed to determine the amount of peptide present prior to incubation and fractionation. Importantly, the peptide elution peak area varied linearly with the peptide concentration, demonstrating that the peptide elution peak area correlates directly with the peptide concentration. For the mixed PAPf39 and PAPf39Δ1-8 incubations, the PAPf39 elution peak area in the supernatant was within error of the unfibrillated monomeric controls (Figure S4G). This indicates that PAPf39 was not incorporated.
into the fibrils since the concentration of non-fibrillar peptide is equivalent to the initial concentration of peptide present in the incubations. Analysis of the redissolved fibrillar pellet suggests that there is little to no PAPf39 in the fibrils (Figure S4G). Although there is a slight increase in the amount of PAPf39 in the pellet when the percent of PAPf39 incubated with PAPf39Δ1-8 is low, the increase is not significant and is most likely due to residual supernatant in the pellet. Conversely, the PAPf39Δ1-8 concentration in the supernatant significantly decreases in fibrillated samples, suggesting that PAPf39Δ1-8 is in the fibrillated fraction (Figure S4H). In the 75:25 PAPf39 to PAPf39Δ1-8 sample, fibrils were not observed by ThT fluorescence and very few fibrils were observed by AFM. Thus, the PAPf39Δ1-8 concentration in the supernatant is equivalent to the initial PAPf39Δ1-8 concentration, indicating little to no fibril formation. However, in the 50:50, 25:75, 0:100 PAPf39 to PAPf39Δ1-8 supernatant, the PAPf39Δ1-8 concentration decreases significantly (Figure S4H), suggesting that the fibrils are composed of PAPf39Δ1-8. Indeed, PAPf39Δ1-8 is present in the redissolved fibrillar pellets (Figure S4H), as shown by increases in the elution peak area of PAPf39Δ1-8 for higher percentages of PAPf39Δ1-8. The amount of PAPf39Δ1-8 in the redissolved fibrillar pellet is not as high as the monomeric peptide control, possibly because the fibril pellet did not completely dissolve in 8 M urea. These results show that at pH 2.5 PAPf39 and PAPf39Δ1-8 do not form mixed peptide fibrils. Fibrils that form are composed of PAPf39Δ1-8 with no detectable PAPf39 incorporation.

Light Scattering

Light scattering measurements at 400 nm were collected on a Fluoromax (Horiba Jobin Yvon, Kyoto, Japan) at 37°C using a 10 mm path length cuvette. For the acid induced dissociation experiments 80 μL of sample was diluted in PBS pH 7.7, stirred, and pre-incubated at 37°C for 30 minutes. To induce dissociation, hydrochloric acid (10 μL of 3.39 M HCl to give a final sample pH of 2.8) was injected into the sample cuvette, raising the final volume to 2 ml. Blank injections were performed similarly using PBS pH 7.7 instead of acid to ensure that little to no fibril dissociation occurred due to dilution. To remove noise from the signal, the average and standard deviation over a 0.5 second window centered on a given data point was calculated and values greater than two standard deviations from the average were removed. The average standard deviation for three independent repeats of the light scattering data was less than 11% of the mean.
Table S1. Lag time of fibril formation and most abundant fibril heights and lengths.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>PBS pH 7.7</th>
<th>PBS pH 5.5</th>
<th>ABS pH 5.5</th>
<th>PBS pH 2.5</th>
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<tr>
<td>Lag time (hours)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAPf39</td>
<td>20 ± 3</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PAPf39A1-8</td>
<td>1 ± 2</td>
<td>2 ± 1</td>
<td>&lt;1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>PAPf39A1-13</td>
<td>1 ± 2</td>
<td>17 ± 1</td>
<td>13 ± 4</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Fibril Height (nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAPf39</td>
<td>6.8±1.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
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<td>7.2±1.9</td>
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<tr>
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<td>170±70</td>
<td>100±55</td>
<td>170±77</td>
<td>270±100</td>
</tr>
</tbody>
</table>

1 Fibril heights and lengths were determined from AFM height images in the Igor Pro MFP3D software by drawing a line across the desired fibril to generate a two-dimensional trace of the image surface (image height over the line) and measuring fibril length or height. For each sample, at least 120 and 240 fibril lengths and heights were measured, respectively. Fibril length and height distribution histograms were calculated with bin sizes of 25 and 0.5 nm, respectively. To reduce the effects of different sample sizes on the calculated distributions, five sets of 120 or 240 randomly selected points (for length and height measurements, respectively) were used to calculate five distributions and averaged. Length and height distributions were also calculated using a kernel smoothing approach in MATLAB (MathWorks, Natick, MA). For calculations, Gaussian kernels were used with bandwidths of 50 and 0.8; and the functions were evaluated every 1 and 0.01 nm for length and height, respectively. Similar to the histogram distribution analysis, ten sets of 120 and 240 randomly selected points (length and height, respectively) were used to calculate ten density distributions, which were averaged. Each of the kernel smoothing densities was individually fit to a sum of five Gaussian distributions:

\[
f = \sum_{i=1}^{5} a_i e^{-\frac{(x-b_i)^2}{2\sigma_i^2}}
\]

where \( x \) and \( f \) are the input parameters (length or height and frequency, respectively), and \( a_i, b_i, \) and \( \sigma_i \) (amplitude, x-offset, and standard deviation of \( b_i \), respectively) are calculated using a nonlinear regression software (NLREG). The most abundant fibril heights or lengths correspond to the x-offset, \( b_i \), with the largest amplitude, \( a_i \).
Figure S1. PAPf39 fibril core sequence determination by HDXMS and sequence-based predictions of aggregation prone regions in PAPf39. Panels A-D: Mass spectra peaks corresponding to residues 17-39 of PAPf39 for the unexchanged monomer, exchanged monomer, exchanged fibril, and in-exchange monomer, respectively. Panel E: Comparison of the methods used to calculate the sequence averaged percent protection of PAPf39 fibrils from HDX. Profiles were calculated using the centroid and weighted averaging (red bars); linear fit and weighted averaging (yellow bars); centroid and unweighted averaging (blue bars); or linear fit and unweighted averaging (green bars) methods. Bars are the average protection calculated from five independent experiments and error bars are one standard deviation from the mean. Panel F: Lines indicate the HDXMS sequence coverage generated from pepsin cleavage of PAPf39. Panel G: Aggregation-prone regions as calculated by the indicated method (1-6) are highlighted in red.
Figure S2. Effect of pH on PAPf39 fibril dissociation. Panel A: ThT fluorescence intensity of preformed PAPf39 fibrils after 24 hours at room temperature in different buffers with (black bars) and without agitation (red bars). Panels B-K: AFM images were taken after 24 hours of resuspension in different buffers with and without agitation (specified for each column and row on the Figure). Scale bars on the AFM images are 500 nm.
Figure S3. Seeding of PAPf39 monomer with preformed PAPf39, PAPf39Δ1-8, or PAPf39Δ1-13 fibrils at different pH values. ThT fluorescence intensity as a function of time (panel A) and AFM images taken after 48 hours of incubation (panels B-I) for pH 5.5 no seed (black bars; panel B); pH 5.5 seed from PAPf39Δ1-8 pH 5.5 (red bars; panel C); pH 7.7 no seed (green bars; panel D); pH 7.7 seed from PAPf39Δ1-8 pH 7.7 (yellow bars; panel E); pH 7.7 seed from PAPf39Δ1-8 pH 5.5 (blue bars; panel F); pH 7.7 seed from PAPf39Δ1-8 pH 2.5 (magenta bars; panel G); pH 7.7 seed from PAPf39 pH 7.7 (cyan bars; panel H); and pH 7.7 seed from PAPf39Δ1-13 pH 7.7 (gray bars; panel I). Scale bars on the AFM images are 500 nm.
Figure S4. ThT fluorescence, AFM images, and peptide composition of mixed PAPf39 and PAPf39Δ1-8 peptide incubations in PBS pH 2.5. Panel A: ThT fluorescence intensity of the indicated mixture of PAPf39 and PAPf39Δ1-8 taken after 72 hours of incubation. Panels B-F: AFM images of 100:0, 75:25, 50:50, 25:75, and 0:100 of PAPf39 to PAPf39Δ1-8, respectively, taken after 72 hours of incubation. Scale bars on the AFM images are 500 nm. Panels G-H: Elution peak area for PAPf39 (panel G) and PAPf39Δ1-8 (panel H) assayed by LC-MS. The black, red, and green symbols represent the peptide content (elution peak area) of the un-fibrillated monomeric peptide controls, supernatants (non-fibrillar material) from the mixed peptide incubations, and redissolved pellets (fibrillated material) from the mixed peptide incubations. The black line is a linear fit of the un-fibrillated, fresh monomer controls and represents the elution peak area of the peptide prior to incubation and fractionation into the pellet and supernatant. The total peptide concentration is not conserved in these experiments because the fibril pellet did not completely dissolve in urea, causing a loss of peptide. See SI Experimental Procedures for more details on these experiments.
**Figure S5.** Numerical results of the protease protection assay.
Figure S6. Panel A. Far-UV CD spectra of PAPf39 variants at different pH values. Panel B. Results of the deconvolution analysis, using the K2D software package (7), to estimate the secondary structure content in the PAPf39 variants at different pH values.
References:


