Amyloid Fibril Formation from Sequences of a Natural β-Structured Fibrous Protein, the Adenovirus Fiber*

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Amyloid fibrils are fibrous β-structures that derive from abnormal folding and assembly of peptides and proteins. Despite a wealth of structural studies on amyloids, the nature of the amyloid structure remains elusive; possible connections to natural, β-structured fibrous motifs have been suggested. In this work we focus on understanding amyloid structure and formation from sequences of a natural, β-structured fibrous protein. We show that short peptides (25 to 6 amino acids) corresponding to repetitive sequences from the adenovirus fiber shaft have an intrinsic capacity to form amyloid fibrils as judged by electron microscopy, Congo Red binding, infrared spectroscopy, and x-ray fiber diffraction. In the presence of the globular C-terminal domain of the protein that acts as a trimerization motif, the shaft sequences adopt a triple-stranded, β-fibrous motif. We discuss the possible structure and arrangement of these sequences within the amyloid fibril, as compared with the one adopted within the native structure. A 6-amino acid peptide, corresponding to the last β-strand of the shaft, was found to be sufficient to form amyloid fibrils. Structural analysis of these amyloid fibrils suggests that perpendicular stacking of β-strand repeat units is an underlying common feature of amyloid formation.

The aggregation of proteins and peptides into the amyloid-type, β-sheet structures is currently the subject of intense investigation by many laboratories. Amyloid fibrils derive from a wide spectrum of native proteins with no structure or sequence homology (1). Despite the diversity of sequences and folds in these proteins, the amyloid structures that can form from them are very similar. All are of the “cross-β” type with β-strands lying perpendicular to the fibril axis (2–5). However, the nature of the basic amyloid folding motif remains elusive. A number of recent papers suggest that the amyloid motif could be similar to the β-helical folding motifs described for elongated proteins such as the phage P22 tailspike and pectate lyase (6–8), and it may be that further understanding of amyloid structure and formation benefits from insights provided by the study of natural β-structured, fibrous proteins. Such fibrous proteins are often large and complex molecules that contain repetitive sequences and a high degree of structural regularity. In addition to the β-helical folds described for pectate lyase and the tailspike protein (9–11), novel triple-stranded β-structures have been described recently (12–14) for virus and bacterial fibers. However, little is known on the folding and assembly mechanisms of these natural, β-structured fibrous proteins. The P22 tailspike protein remains the most extensively studied model system providing fundamental understanding on the folding of β-helical structures (15).

The work described here focuses on folding and assembly of shaft sequences from the fiber protein of adenoviruses. These fibers protrude from the 5-fold vertices of the adenovirus virion and are the cell attachment points of the virus. They are thin fibrous trimers with an asymmetric morphology, namely an N-terminal part that binds to the viral capsid, a thin shaft, and a globular C-terminal domain that attaches to the cell receptor (16–19). Each adenovirus type 2 fiber monomer contains 582 amino acids. The shaft contains a repeating sequence motif with an invariant glycine or proline and a conserved pattern of hydrophobic residues (20). A stable fragment of the fiber (residues 319–582), containing four repeats of the adenovirus type 2 fiber shaft plus the receptor-binding head domain, has been crystallized and its structure studied to 2.4-Å resolution by x-ray crystallography (12). As depicted in Fig. 1, this study revealed a novel triple β-spiral fibrous fold for the shaft. Each sequence repeat contains an extended strand running parallel to the fiber axis, followed by a β-turn, containing a conserved glycine or proline. The turn is followed by another β-strand that runs backwards at an angle of roughly 45° to the shaft axis and is joined to the extended strand of the next repeat by a solvent-exposed loop. Within this fragment of the shaft structure, hydrogen bonds are established between the strand-loop-strand motif, which can be considered as the basic building unit at the structural level (12). The average diameter of the shaft, excluding the surface loops, is 15 Å. Unfolding studies have provided evidence that this stable domain is the last part of the fiber to unfold (21). However, relatively little is known about fiber folding and assembly, either in vitro or in vivo. The
globular head domain is thought to be essential for trimerization, as deletions or mutations in this part of the protein are known to abolish trimer formation in vivo (22, 23). Furthermore, the head domain can successfully be replaced by a foreign trimerization motif, the “foldon” domain of phage T4 fibrin (24).

We have recently initiated a study of folding and assembly of the shaft sequences in the absence of those from the head. As part of this study, we initially synthesized a 41-amino acid peptide (PIKTKGISSGIDYNAMITKLGSGLSFDNSGAIITGNKND) corresponding to the shaft sequence that is immediately adjacent to the head. This peptide failed to assemble into the triple β-spiral conformation observed in the single crystal study of the stable domain; instead, it aggregated into fibrils similar to those associated with amyloid diseases, namely long, unbranched structures that stain with the dye Congo Red and give a characteristic x-ray fiber diffraction pattern (25). Given these observations, it seems that amino acid sequences from the adenovirus shaft may also provide a simple and well defined model system for the study of amyloid. The purpose of the studies described here was to investigate shorter amino acid sequences from the adenovirus shaft and to further characterize any tendency to form amyloid-type structures in the absence of the globular head of the native structure. To do this, we have synthesized a number of peptides that have a specific relationship to the folds of the native structure, and we studied them with a number of techniques, including electron microscopy, infrared spectroscopy, x-ray fiber diffraction, and Congo Red binding, that have come to be regarded as providing diagnostic evidence for the detection of amyloid structures. Below, we describe results from the following sub-sequences of the original 41-amino acid peptide: a 25-amino acid sequence (AMITKLGSGLSFDNSGAIITGNKND), a 12-amino acid sequence (LSFDNSGAIITGNKND), an 8-amino acid sequence (NSGAIITGNKND), a 6-amino acid sequence (GAITGNKND), and an 4-amino acid sequence (NKN). Furthermore, we have synthesized and studied a variant of the 6-amino acid peptide where the central threonine has been replaced by proline (GAIPGN). We also include infrared studies and synchrotron x-ray diffraction patterns of the 41-aa peptide that were not available at the time the original paper was published (25). We find that amyloid formation is a general tendency of the shaft sequences in the absence of the globular head, and we discuss the results in the context of the native fiber folding and assembly. Biological implications concerning the understanding of amyloid formation from repetitive sequences of disease-associated proteins are discussed.

### EXPERIMENTAL PROCEDURES

#### Chemical Reagents

The reagents for solid phase peptide synthesis were obtained from Applied Biosystems (PerkinElmer Life Sciences), except dichloromethane, which was from SDS (Solvants Documentation Synthéses, Peyrin, France), as was acetonitrile for high performance liquid chromatography. tert-Butyloxycarbonyl amino acids and resin were purchased from NeoSystem (Strasbourg, France) or Novabiochem. Ultrapure guanidine hydrochloride was purchased from ICN Biomedicals, and the dye Congo Red was purchased from Sigma. All other chemicals were of the purest grade available. D2O was from Euriso-top (CEA Group, France).

#### Peptide synthesis

C-terminal amide derivatives of the peptides were synthesized by the stepwise solid phase method using an Applied Biosystems 430A automated synthesizer as described previously (25). The 41-, 25-, 12-, and 4-amino acid peptides as well as the GAIPGN peptide were synthesized in-house; the 8- and 6-amino acid peptides were obtained from Bachem, France. The purity of the synthetic peptides was assessed by reverse phase high performance liquid chromatography, and their identity was confirmed by electrospray mass spectrometry. The experimental masses of the peptides are as follows: 2507.86 Da for the 25-aa; 1194.31 Da for the 12-aa, 730.82 Da for the 8-aa, 529.64 Da for the 6-aa, 925.32 Da for the GAIPGN peptide, and 488 Da for the 4-aa peptide. Peptide concentrations were determined by in-house amino acid composition analysis.

#### Electron Microscopy

Lyophilized peptide powders were dissolved in water at concentrations ranging from 0.5 to 20 mg/ml depending on the peptide. They were subsequently adsorbed to the clean side of a carbon film on mica and negatively stained with 1% sodium silicotungstate. Micrographs were taken with a JEOL 1200 EX electron microscope, and the magnification was calibrated with negatively stained crystals of catalase. The quality of the images was checked on an optical bench. The negatives were then digitized on a Zeiss scanner (Photodan TC) with a pixel size of 14 μm. At the level of the sample, the pixel size corresponds to 3.5 Å. For the protofilaments in the samples of the 12-aa peptide, 100 regions from one negative were selected using Xindisp (26). The images were then cut out into 128 × 128 boxes and processed by using the SPIDER image analysis package (27). The images were bandpass-filtered between 200 and 10 Å without contrast transfer function correction and normalized to the same mean and standard deviation. One image out of 100 was selected, and the rest of the images were aligned (in translation and rotation) against the selected image. The 50 best matching pictures were then averaged, and the same operation was repeated five times by using for each cycle the 50 best images. The average shown in Fig. 3C was then padded into a 512 × 512 box and Fourier-transformed, and its power spectra was calculated. The distances from the origin to the peaks were measured and converted into distance 1/Å. The way that the power spectrum is visualized with SPIDER makes that the horizontal distance in the real space is converted to horizontal distances in the Fourier space.

#### Congo Red Binding

5 μl of a 100 μM stock solution of Congo Red was added to 95 μl of peptide solution, and spectra were recorded from 400 to 600 nm. Before adding the dye, the scattering of the peptide solutions was recorded in the same wavelength range and subtracted from the spectrum of the dye in their presence. The buffer used was 10 mM Hepes, pH 7.

#### FTIR Measurements

Each lyophilized peptide was dissolved in D2O except for the 25-aa peptide that was dissolved in 10 mM deuterated ammonium acetate buffer, pH 4. Immediately after preparation of the solution, an aliquot was removed for concentration determination, and 50 μl of the peptide solution was inserted between CaF2 windows (Spectra-Tech) using a 100-μm spacer. IR spectra were recorded with a Jasco 610 Fourier transform spectrometer. Sixteen interferograms were recorded at room temperature with a resolution of 4 cm⁻¹. For each spectrum the water vapor was subtracted and baseline corrected.

#### X-ray Fiber Diffraction

**Fiber Preparation**—Peptide solutions were prepared by dissolving the lyophilized powder in 10 mM ammonium acetate buffer, pH 4. Fibers were prepared by using shear alignment methods that have been described previously for nucleic acid polymers (28). A droplet of peptide solution (approximate volume 5 μl) was placed between the ends of two glass rods and left to dry. In previous work on amyloid fibers, there have been reports that the use of magnetic fields may improve sample alignment (29, 30). In this study, a number of attempts were made to improve the fiber alignment by using the high fields available in the NMR Laboratories at the Institut de Biologie Structurale in Grenoble, France. Although improved alignment was obtained for the fibers formed by the 41-aa peptide, no significant improvement was noted for the 25- and 12-aa peptides.

**Fiber Diffraction**—X-ray fiber diffraction patterns were recorded at the European Synchrotron Radiation Facility on the microfocus beamline ID13. The wavelength was 0.976 Å, and the beam was 5–10 μm in diameter. This experimental arrangement allows the study of thin amyloid fibers for which the orientation was very often better than that of the thicker fibers (as judged by optical microscopy/birefringence measurements under cross-polarized light).

Data sets on ID13 were recorded using the instrument in one of two possible modes. The “scanning” arrangement is a diffractometer configuration that is optimized for the measurement of sample intensities from the 25- and 12-aa peptides.
study of small single crystals. Both arrangements have been described (31, 32). The fibers formed by the 6- and 8-aa peptides were studied using the scanning arrangement, whereas those for the 41-, 25-, and 12-aa peptides were studied using the diffractometer arrangement. In both cases, data sets were recorded using a MAR CCD detector with a 130-nm entrance window and the following readout parameters: 2048 × 2048 pixels and 64.45 × 64.45 μm pixel size. The specimen to detector distance ranged from 57.1 to 42.8 mm. Diffraction data sets were processed and measured using CCP13 software (33) and the FIT2D package (34).

In all experiments, radiation damage was a major concern. The effect of this was minimized by recording a sequence of diffraction patterns at different positions along the length of the fiber and then adding these images together. "Blank" data sets (recorded with no sample in the

FIG. 1. Sequence of the synthetic peptides and structure of the corresponding segment within the native human adenovirus type 2 fiber. Upper panel, sequence of the various peptides. Residue numbers correspond to positions in the full-length fiber sequence as described previously (12). The last four amino acids are in italics and are not part of a repeat but form the junction between the shaft and the head domain. The first amino acid of each sequence repeat (Pro-356, Ala-372, and Ala-388) is indicated; the conserved secondary structure of the repeats is shown above with L for loop and T for β-turn. Lower panel, structure of the peptides within the native shaft segment (adapted from Ref. 25). One of the strands of the trimeric shaft is shown in black and the other two in gray. N and C termini of the segment are labeled, corresponding to residues 355 and 396, respectively; the head domain starts after residue 396. The first amino acid residue of each peptide is shown with arrows. The 41- and 25-amino acid peptides include the last four amino acids (residues 393 to 395, NKND), whereas the 12-, 8-, and 6-aa peptides stop at residue 392.
TABLE I

Solubility of the peptides

All peptides were tested at a 10 mg/ml concentration, except for the 12-aa peptide that is soluble up to 5 mg/ml. The term "solubility" refers to the capacity of the lyophilized peptide powder to dissolve physically under these conditions. Once in solution, the 25-, 12-, 8-, and 6-aa peptides aggregate and form amyloid fibrils in all of the above solvents. pH 4 corresponds to 10 mM ammonium acetate buffer; pH 7 corresponds to 50 mM sodium phosphate buffer or 10 mM Hepes buffer; acetonitrile/trifluoroacetic acid/water corresponds to the following percentages: 40% acetonitrile, 0.1% trifluoroacetic acid, 60% water.

| Peptide, 10 mg/ml | H₂O | pH 4 | pH 7 | Acetonitrile/trifluoroacetic acid/water |
|-------------------|-----|------|------|----------------------------------------|
| 25 aa             | -   | +    | +    | +                                      |
| 12 aa (5 mg/ml)   | +   | +    | +    | +                                      |
| 8 aa              | +   | +    | +    | +                                      |
| 6 aa              | +   | +    | +    | +                                      |
| GAIPIG            | +   | +    | +    | +                                      |
| 4 aa              | +   | +    | +    | +                                      |

Peptide Design and Solubility—The original 41-amino acid peptide starts at proline 356 of the full-length fiber sequence; it contains two sequence repeats and ends with a short peptide that starts at residue 372, contains one sequence repeat, and ends with a short peptide (residues 388–392) followed by four residues (393–396) belonging to a flexible linker region that connects the shaft to the globular head (Fig. 1). Based on this original 41-amino acid sequence, we synthesized a 25-mer (AMITKLGSLFDNSGAIPTGKNKND) that starts at residue 372, contains one sequence repeat, and ends with a short β-strand (residues 388–392) plus the four residues (393–396) of the flexible linker region. We have also synthesized a 12-aa acid peptide (resides 381–392, LSFDNSGAIPTG), which makes up the basic strand-loop-strand motif in the native structure. We subsequently designed an 8-aa acid peptide, comprising the loop-strand sequence from the 12-aa acid peptide (NSGAIPTG), and a 6-aa amino acid peptide, comprising the last loop residue and the strand residues (GAIPTG). We have also synthesized a 4-aa acid peptide where the central threonine of the GAIPTG sequence has been replaced by a proline (GAIPIG). The 4-amino acid peptide (residues 393–396, KNKND) that is not part of the shaft repeats but corresponds to the charged, flexible linker region that connects the shaft to the head was also synthesized.

The solubility of lyophilized peptide powders at the range of 10 mg/ml under different solvent conditions is presented in Table I. The 12-aa acid peptide is soluble up to 5 mg/ml. Once physically into solution in these solvents the 25-, 12-, 8-, and 6-aa acid peptides aggregate with time and form amyloid fibrils (see Table I). At the range of 10 mg/ml, fibril formation is accompanied by an increase in viscosity and subsequent formation of gels. At concentrations around 5 mg/ml, the 25-aa acid peptide forms rapidly (within 20 min) fibrillar aggregates that are visible with the naked eye and sediment at the bottom of the test tube.

Fibril Formation as Seen by Electron Microscopy—Fig. 2 shows electron micrographs recorded from each of the 25-, 12-, 8-, and 6-aa acid peptides. These pictures were recorded from preparations in water, although similar results were obtained for all of the solvents mentioned above. For each sample there were differences in sample preparation procedures relating to solubility and aggregation time. The electron micrographs shown here were recorded at 13 days at 1 mg/ml for the 12-aa peptide, 6 weeks at 10 mg/ml for the 8-aa peptide, and 20 mg/ml of a freshly made solution for the 6-aa peptide. For the 25-aa peptide, electron micrographs can only be taken immediately after dissolving at low concentrations (0.5 mg/ml) in a poor solvent (water in this case), because otherwise large macroscopic fibrils form very rapidly and cannot be visualized by EM. Unbranched fibrils with variable lengths can be seen for all peptides. They are often laterally associated, forming bundles and twisted ribbons. Single fibril diameters taken from 10 measurements for each peptide with an uncertainty of one pixel size (±1.75 Å) were as follows: 30 Å for the 25-aa peptide, 21 Å for the 12-aa peptide, 24.5 Å for the 8-aa peptide, and 16 Å for the 6-aa peptide. No fibrils, arrays, or amorphous aggregates have been detected in solutions of the GAIPIG peptide and of the 4-aa peptide under any condition; Fig. 2E shows a representative field from a grid where a drop of a 20 mg/ml solution of the GAIPIG peptide into water was deposited after 1 month of incubation in water.

When samples of the 12-aa acid peptide were deposited on the grid immediately after dissolving the peptide at 1 mg/ml in water, novel extended, array-like structures composed of the thinnest fibrils observed (which will be referred to as "protofilaments") were observed using low dose EM. At high electron dose, these fine structures are destroyed and are no longer visible. These arrays, which can be clearly seen in Fig. 3 (A–C), appear to be highly regular; Fourier transform analysis (Fig. 3D) reveals a regular spacing of 36 Å between the protofilaments plus a regular spacing of 30 Å along the length of the protofilament. Although no isolated protofilaments were ob-
served in these studies, higher order fibrils and twisted ribbons seem to co-exist in the same fields, indicating that protofilaments may be able to partition into all these morphologies. These polymorphic structural assemblies are reminiscent of those formed from amyloid fibrils of calcitonin and amylin (35–37). At later stages of assembly, no extended arrays of protofilaments are observed; only multistrand, flat, or twisted ribbons exist in the fields of the same solution deposited on the grid after aging for 13 days, as seen in Fig. 2B. Although it cannot be directly proven, it is tempting to imply that these later stage assemblies form from protofilaments by either “side-by-side” association or by coiling and twisting, as suggested previously for other systems (35–37). Most interestingly, in low dose studies at early time points of the 41-, 25-, 8-, or 6-amino acid peptides, no arrays of protofilaments are observed.

Congo Red Binding—Binding of the diazo dye Congo Red is a distinctive property of the amyloid fibrils and one of the main diagnostic tests for their presence. In the presence of amyloid fibrils, a spectral shift of the $\lambda_{\text{max}}$ of the bound dye from 488 to 540 nm is observed (38). The dye spectra in the presence of the 25-, 12-, 8-, and 6-amino acid peptides are shown in Fig. 4A–D, respectively; they all display the characteristic shift. In contrast, solutions of the GAIPIG peptide and the 4-aa peptide fail to induce the spectral shift of the dye (Fig. 4E and F, respectively). In addition to the shift of the $\lambda_{\text{max}}$, we consistently observed hyperchromicity of the dye in the presence of the 8-aa peptide (Fig. 4C). Fig. 4G shows the Congo Red spectra in the presence of a chimeric protein that comprises shaft sequences 319–392 of the fiber N-terminally fused to the foldon domain of phage T4 fibritin. These chimeric proteins form remarkably stable, SDS-resistant trimers (24). Within these chimeric trimers, the shaft sequences adopt the same triple $\beta$-spiral conformation as in their native context, as suggested by their crystal structure that has been solved recently (39). Several models have been proposed in order to explain the interaction of Congo Red with amyloid fibrils; however, the reasons that cause the change in the spectral characteristics of the dye are not well understood. The above results suggest that the fibrils formed by the 25-, 12-, 8-, and 6-aa peptides bind Congo Red with subsequent shifting of its $\lambda_{\text{max}}$. Absence of the spectral shift by the chimeric protein serves mainly as a negative control for this experiment. However, it also indicates that Congo Red either fails to bind to shaft fibrils or that the fibrils are not long enough to contribute to the Congo Red binding.
sequences in their native trimeric conformation, or if any interaction exists, it does not induce the characteristic spectral shift of the dye.

Secondary Structure of the Peptides in Their Fibrillar State as Determined by FTIR—FTIR is a powerful method that allows the study of the secondary structure of protein aggregates in suspension, as well as in gel and solid states (40). By using this technique, we followed the evolution of the secondary structure of peptide solutions with time. The recorded spectra at the amide I region are presented in Fig. 5. All peptides were insoluble under these conditions and has therefore been dissolved in deuterated acetate buffer, pH 4. For the 41-amino acid peptide (Fig. 5A), a band at 1645 cm$^{-1}$ characteristic of random coil is present in the first spectrum recorded (5 min after dissolving and placing in the cell holder). This band converts over time to a band at 1618 cm$^{-1}$; bands in the 1620 cm$^{-1}$ region are typically assigned to intermolecular $\beta$-sheet structure associated with amyloid fibrils (41, 42). These kinetics level off at about 15 h. It is interesting to note that the spectra taken at various times present an isosbestic point, indicating a two-state interconversion from random coil to intermolecular $\beta$-sheet structure. Observation of the second, much weaker component typical of $\beta$-structure (around 1680 cm$^{-1}$) is hidden by a peak because of the presence of trifluoroacetic acid. Residual trifluoroacetic acid is present as a result of the purification procedure in most peptides (except for the 12-aa one). For the 25-aa peptide, a strong peak at 1622 cm$^{-1}$ is observed in the first recorded spectrum (Fig. 5B), indicating that this peptide rapidly forms amyloid fibrils, in agreement with EM observations and the appearance of visually perceptible fibrils during solubility tests. There is essentially no evolution of the spectrum over 18 h, except for a minor shift of the peak toward 1620 cm$^{-1}$. For the 12-amino acid peptide, the random coil peak is smaller in the first recorded spectrum; a strong peak at 1626 cm$^{-1}$ is observed (Fig. 5C). One minor peak at 1680 cm$^{-1}$ (at the early stages of the fibril formation. However, the time-dependent changes suggest that additional structural adjustment takes place over time; this structural adjustment might involve a $\beta$-sheet rearrangement or conversion of $\beta$-turns to $\beta$-sheet

![FTIR spectra of the different peptides dissolved in D$_2$O (10 mM deuterated ammonium acetate buffer, pH 4, for the 25-aa peptide). The evolution of spectra is shown as a function of time. The dashed lines correspond to the first recorded spectrum for each peptide, after dissolving and placing in the cell holder. A, 41-aa peptide; first recorded spectrum (dashed line) and spectrum recorded after 5 (thin solid line) and 44 h (thick solid line); B, 25-aa peptide; first recorded spectrum (dashed line) and spectrum recorded after 19 h (thick solid line), C, 12-aa peptide; first recorded spectrum (dashed line) and spectrum recorded after 32 h (thick solid line), D, 8-aa peptide; first recorded spectrum (dashed line), spectrum recorded after 1 h 30 min (thin solid line), and after 20 h (thick solid line), E, 6-aa peptide; first recorded spectrum (dashed line) and spectrum recorded after 6 days (thick solid line), F, GAIPIG peptide; first recorded spectrum (dashed line) and spectrum recorded after 1 month of incubation (thick solid line). Peptide concentration was 10 mg/ml for the 41- and 25-aa peptides, 5 mg/ml for the 12-aa peptide, and 20 mg/ml for the 8- and 6-aa peptides as well as for the GAIPIG peptide. In most of the peptides residual trifluoroacetic acid is present as a result of the purification procedure and gives a band in the region of 1670 cm$^{-1}$, marked “TFA.” RC, random coil.

![X-ray fiber diffraction patterns recorded for the 41-aa (A), 25-aa (B), 12-aa (C), 8-aa (D), and 6-aa peptides (E), showing the diffraction features normally associated with the cross-$\beta$-structure: a 4.7-Å meridional reflection and equatorial diffraction at ~10 Å. For the 8-aa peptide, two additional reflections at 9.11 and 18.5 Å are shown.](http://www.jbc.org/)

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structure. It is possible that the first recorded spectrum could correspond to the protofilaments observed by EM, and the final one to amyloid fibrils. A small shift of the 1626 cm$^{-1}$ peak toward 1622 cm$^{-1}$ over time is observed for this peptide. For the 8-aa peptide, a shoulder is observed at 1620 cm$^{-1}$ in the first spectrum recorded (Fig. 5D) that subsequently evolves into a peak, with kinetics leveling off at about 20 h. Finally, for the 6-aa peptide, the appearance of the signal at 1620 cm$^{-1}$ is much slower, with kinetics evolving at the time scale of days (Fig. 5E). No change in the spectrum of the GAIPG peptide was observed; in Fig. 5F, the first recorded spectrum is shown alongside the spectrum recorded after 1 month of incubation. A shoulder centered around 1612 cm$^{-1}$ is observed, possibly because of proline-induced turns, that does not evolve with time. Similarly, no evolution of the random coil signature was observed after 1 month of incubation for the 4-aa peptide (not shown). These results are in agreement with the absence of fibrils observable by electron microscopy and the absence of Congo Red binding. Furthermore, it was impossible to draw fibrils observable by electron microscopy and the absence of Congo Red staining, has become one of the main indicators of amyloid fibrils observable by electron microscopy and Congo Red binding. Furthermore, it was impossible to draw fibrils observable by electron microscopy and the absence of Congo Red staining, has become one of the main indicators of amyloid fibrils. A small shift of the 1626 cm$^{-1}$ peak toward 1622 cm$^{-1}$ over time is observed for this peptide. For the 8-aa peptide, a shoulder is observed at 1620 cm$^{-1}$ in the first spectrum recorded (Fig. 5D) that subsequently evolves into a peak, with kinetics leveling off at about 20 h. Finally, for the 6-aa peptide, the appearance of the signal at 1620 cm$^{-1}$ is much slower, with kinetics evolving at the time scale of days (Fig. 5E). No change in the spectrum of the GAIPG peptide was observed; in Fig. 5F, the first recorded spectrum is shown alongside the spectrum recorded after 1 month of incubation. A shoulder centered around 1612 cm$^{-1}$ is observed, possibly because of proline-induced turns, that does not evolve with time. Similarly, no evolution of the random coil signature was observed after 1 month of incubation for the 4-aa peptide (not shown). These results are in agreement with the absence of fibrils observable by electron microscopy and the absence of Congo Red binding. Furthermore, it was impossible to draw fibrils observable by electron microscopy and the absence of Congo Red staining, has become one of the main indicators of amyloid fibrils observable by electron microscopy and Congo Red binding. Furthermore, it was impossible to draw fibrils observable by electron microscopy and the absence of Congo Red staining, has become one of the main indicators of amyloid fibrils.

X-ray Fiber Diffraction—The recorded x-ray fiber diffraction patterns are shown in Fig. 6. In general they show features out to a resolution of ~3–5 Å, although in the meridional direction sharp Bragg reflections are visible to a d-spacing of 1.6 Å. In this work we have found no evidence to suggest that the fibers studied do not have a cylindrically symmetric texture of the type that has been observed for some magnetically aligned samples (43).

All of the diffraction patterns show the main features of a “cross-β” diffraction pattern (44). This type of diffraction pattern, along with evidence from electron microscopy and Congo Red staining, has become one of the main indicators of amyloid-type structures (45). Each pattern in Fig. 6 has characteristic reflections at ~4.7 Å on the meridian and strong equatorial diffraction in the region from ~10 to 11 Å (as a guide, specific reflections in this region of the diffraction pattern are indicated in Fig. 6). The sharp 4.7-Å meridional reflection is assumed to arise from the periodicity of hydrogen-bonded β-strands oriented perpendicular to the fiber axis, and the diffraction in the region of ~10–11 Å is presumed to relate to the stacking of these sheets perpendicular to the fiber axis (45).

The meridional data recorded for the five peptides are summarized in Table II. In addition to the reflection recorded at ~4.7 Å, each pattern shows a clear meridional reflection at ~2.4 Å and another weaker one at ~1.6 Å, as shown for the 12-aa peptide in Fig. 7. For each of the recorded diffraction patterns, these reflections have similar profiles and all are assumed to derive from the amyloid structure. With the exception of the 8-aa peptide (discussed below), no reflections are seen in the region of ~9.4 Å. In previous work on amyloid, the presence of a reflection corresponding to this periodicity has been taken to indicate a cross-β structure in which successive β-strands are arranged in an anti-parallel rather than a parallel fashion (46, 47). However, the absence of such a reflection does not necessarily imply that the structure is composed of parallel β-strands, because the reflection may be too weak to be observable. The diffraction patterns recorded for the 8-aa peptide show two sharp reflections at 9.11 and 18.05 Å. Although these could reflect the existence of a larger axial repeat distance, the reflections themselves are heavily arced within a diffraction ring that joins with an equatorial feature at the

![Fig. 7. Fiber diffraction pattern recorded from an amyloid fiber of the 12-aa peptide, showing the meridional reflections observed at 2.40 and 1.59 Å as well as at 4.69 Å. As indicated in Table II, similar observations were made for all of the five peptides for which lower resolution diffraction patterns are shown in Fig. 6. For clarity, the center of the diffraction pattern has been attenuated so that these could reflect the existence of a larger axial repeat distance.](http://www.jbc.org/)

![Fig. 8. Fiber diffraction pattern simulated on the basis of an extended idealized version of the native shaft structure. The simulation was computed using coordinates from van Raaij et al. (12) and assumed seven repeats per turn of a left-handed helix of pitch of 88.3 Å.](http://www.jbc.org/)

**Table II**

| Peptide | d-spacings for observed meridional reflections | c axis repeat |
|---------|-----------------------------------------------|---------------|
| 41 aa   | 4.75 2.42 1.59 4.78 | A              |
| 25 aa   | 4.78 2.42 1.60 4.81 | A              |
| 12 aa   | 4.69 2.40 1.59 4.78 | A              |
| 8 aa    | 4.63 2.39 1.57 4.75 | A              |
| 6 aa    | 4.62 2.39 1.58 4.75 | A              |

**Table III**

| Peptide | 41 aa | 25 aa | 12 aa | 8 aa | 6 aa |
|---------|-------|-------|-------|------|------|
| α      | 12.50 | 15.50 | 16.10 | 17.50 | 14.60 |
| β      | 9.50  | 11.10 | 11.00 | 10.50 | 10.50 |
| γ      | 4.78  | 4.81  | 4.78  | 4.75  | 4.75  |
| α      | 90.0  | 90.0  | 90.0  | 90.0  | 90.0  |
| β      | 90.0  | 90.0  | 90.0  | 90.0  | 90.0  |
| γ      | 90.0  | 91.4  | 95.4  | 92.4  | 96.4  |
FIG. 9. Schematic showing how strand-loop elements taken from the native adenovirus structure might be exploited in the formation of amyloid fibrils. A, the strand-loop-strand corresponding to the 12 aa (residues 381–392 of the native fiber) was generated from the native fiber coordinates using RasMol (62) and re-oriented so that the strands are perpendicular to the fiber axis. B and C, two possible antiparallel stackings of this hairpin within an amyloid fibril are illustrated.
same reciprocal space radius. This diffraction feature may arise from the presence of poorly oriented material in the sample, and its significance will not be discussed further.

It is interesting to note that the fiber diffraction patterns recorded from these peptides show evidence of longer range ordering in the axial direction. This is evidenced by the presence of weak diffraction features that do not index on 4.8 or 9.6 Å layer lines, as visible for example in the region of the 2.4 Å reflection shown in Fig. 7. Long range periodicities on this scale have been reported previously and have been attributed to a helical twisting of β-sheets about the fiber axis (2). The current work indicates axial repeats in the region of ~200 Å, with some variability over the peptides studied. These features must be interpreted cautiously, because disorientation and other spot profile effects make their indexing and interpretation very difficult.

The equatorial distribution of relative intensities in these patterns varies significantly from peptide to peptide. These effects appear not to be simply attributable to changes in sampling as a result of different lattice dimensions and may relate to differences in fibril diameter and geometry. As can be seen in Fig. 6, the clarity of the equatorial data in the fibers varies quite substantially, and the indexing of the reflections and unit cell determination is not straightforward for all of the diffraction patterns. The first equatorial reflection in each of the diffraction patterns appears to have a rather different profile from those evident at higher angles and, as concluded in the work of Sikorski et al. (43), may correspond to the average separation between neighboring protofilaments. On this basis the interprotofilament distances for the 41-, 25-, 12-, 8-, and 6-aa acid peptides are 28.6, 28.6, 27.3, 25.7, and 27.9 Å, respectively. The best determined lattice parameters for each peptide have been determined from the remaining equatorial data and are shown in Table III.

**DISCUSSION**

Amyloid fibril formation implies the self-assembly of peptides and proteins into β-structured fibrous structures, irrespective of the folding that occurs within their native context. In this work, we have sought to gain an insight into amyloid formation by studying the self-assembly properties of short peptides that adopt a β-structured fibrous fold within their native context. Of the six peptides studied, five (41-, 25-, 12-, 8-, and 6-aa peptides) correspond to specific repetitive sequences from the adenovirus fiber shaft and form amyloid as probed by accepted diagnostic criteria such as morphology in electron microscopy, Congo Red binding, infrared signature, and x-ray fiber diffraction patterns. The modified 6-aa peptide that carries a proline in the place of the threonine residue (GAIPIG) and the 4-aa peptide that is not part of the shaft repeats did not form amyloid fibrils under any of the conditions tested. Thus, shaft sequences as short as 6 amino acids have an intrinsic capacity to self-assemble into amyloid fibrils; the amyloid state is accessible to these peptides under physiological conditions. In the context of the isolated peptides, our infrared results suggest a direct conversion from random coil to β-structure, when the time course of this conversion is slow enough to be followed. On the basis of the fiber diffraction results, the β-strands are assumed to be perpendicular to the fiber axis. The electron microscopy and fiber diffraction measurements suggest that the structural features of the fibrils formed from these peptides are similar to those described previously for disease-related amyloid structures. Furthermore, striking similarities are observed with fibrils formed from peptidomimetic compounds (48) and de novo designed peptides (49). It is interesting to note that our EM and fiber diffraction results are not in full agreement. One difference is the fact that the 30-Å axial periodicity noted from the electron micrographs recorded for the 12-aa peptide (Fig. 3) is not evident in the corresponding fiber diffraction pattern (Fig. 6C). We currently have no explanation for this. Although similar periodicities were not observed for the other peptides studied in this work, we are aware of the fact that such observations have been made previously in cryo-EM studies of amyloid fibrils (4). Another difference is that the protofilament diameters range between 16 and 30 Å by EM but are all fairly close to 27–28 Å by fiber diffraction. However, assuming a distance of about 3.5 Å between adjacent residues in an extended β-conformation, it is clear that for the three larger peptides (41-, 25-, and 12-aa peptides) neither set of diameters is compatible with the peptides adopting extended conformations over the width of the protofibrils. To fit within the protofibril, they should somehow be “folded”; therefore, we started considering possible folds and arrangements.

Given the fact that strand and loop components are integral to the structure of the native adenovirus fiber, it is instructive to consider a possible relationship between the native and the amyloid structure. That the two structures are different is obvious from their relative dimensions and from the structural details summarized in the Introduction. However, it is further emphasized in Fig. 8, which shows a simulation of the fiber diffraction pattern from an idealized and extended fiber shaft based on crystallographic coordinates taken from the native structure. This simulated pattern is very different from the amyloid patterns shown in Fig. 6 and reflects the ~7-fold helical symmetry of the peptides in the shaft part of the native structure (12), with clear axial periodicities at 12.6, 6.3, and 4.2 Å. Despite the major differences between these two structures, it is nevertheless possible that the ability of the peptide sequences to adopt strand/loop-based conformations may be exploited in amyloid structure, although the strand and loop components would have to adopt a different arrangement with respect to the amyloid fiber axis. To illustrate this concept, Fig. 9A shows the hairpin corresponding to the 12-aa peptide generated from the native fiber coordinates and reoriented so that the two β-strands are perpendicular to the fiber axis. An amyloid fibril structure can be generated from two possible stackings of this hairpin in an antiparallel arrangement (Fig. 9B). Along the same lines, a single-stranded antiparallel arrangement can be proposed for the 8- and 6-aa peptides. In the case of the modified 6-aa peptide (GAIPIG), the absence of amyloid formation indicates that the proline residue can act as a β-sheet breaker and disrupt stacking (50). It has already been reported that the incorporation of prolines in other amyloidogenic peptides inhibits amyloid fibril formation (51); their presence in natural proteins can also disfavor the formation of aggregated structures (52). Furthermore, Pro-Gly pairs introduced into amyloid-forming polyglutamine sequences suggested that the amyloid aggregate structure could consist of alternating extended chain and turn elements (53). It is interesting to note an underlying common feature with the arrangement described in this work, i.e., an aggregate structure consisting of alternating strand and loop elements, with strands arranged perpendicular to the fibril axis.

A similar relationship between a native full-length protein and its amyloid-forming segments has been suggested by Giannetti et al. (54) who have recorded x-ray fiber diffraction data from the tau protein and from amyloid-forming peptides corresponding to sequence repeats of the native protein. For the native protein, these authors noted a 4.26-Å reflection, compatible with β-structural elements aligned neither perpendicular nor parallel to the fibril axis. However, their diffraction work on the amyloid fibrils showed strong 4.7-Å reflections compatible with a cross-β arrangement.
What then are the factors that determine the polymerization of the shaft sequences into amyloid fibrils? Given their repetitive nature, we have proposed previously (25) that these sequences need to be brought into a correct register in order to assemble into the triple β-spiral. This registration role can be played either by the native globular head or by a foreign trimerization motif as we have shown recently (24). In the absence of a trimerization domain, amyloid formation would occur by out-of-register or “strand-swapping” interactions involving hydrogen bonding between strands belonging to different peptide molecules. For some proteins that contain sequence repeats that form amyloid fibrils associated with human disease, such as α-synuclein and tau, fragmented peptides or fragments have been reported to assemble into amyloid much more readily than the full-length proteins (54–57). This led to the suggestion that a region of the full-length proteins (namely the C-terminal for the tau protein) plays a critical role in inhibiting amyloid formation (54, 58). It seems possible to us that the amyloid-forming capacity of these truncated fragments may occur through mechanisms similar to those we have described for the adenovirus fiber. Such a mechanism could explain amyloid fibril formation when parts of the C-terminal domain of these proteins are digested during intracellular degradation processes (59, 60).

In summary, the fibers of adenovirus are being used as a simplified model system for the study of the folding, assembly, and trimerization of β-sheet β-fibrous proteins both in native and in amyloid contexts. The work described in this paper has allowed the identification of short peptides as an experimentally tractable approach to study the amyloid contexts. The work described in this paper has allowed the identification of short peptides as an experimentally tractable approach to study the amyloid contexts. The work described in this paper has allowed the identification of short peptides as an experimentally tractable approach to study the amyloid contexts. The work described in this paper has allowed the identification of short peptides as an experimentally tractable approach to study the amyloid contexts.
Amyloid Fibril Formation from Sequences of a Natural β-Structured Fibrous Protein, the Adenovirus Fiber
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