Charge Attraction and β Propensity Are Necessary for Amyloid Fibril Formation from Tetrapeptides*

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Amyloid fibrils in which specific proteins have polymerized into a cross-β-sheet structure are found in about 20 diseases. In contrast to the close structural similarity of fibrils formed in different amyloid diseases, the structures of the corresponding native proteins differ widely. We show here that peptides as short as 4 residues with the sequences KFFE or KVVE can form amyloid fibrils that are practically identical to fibrils formed in association with disease, as judged by electron microscopy and Congo red staining. In contrast, KLLE or KAAE do not form fibrils. The fibril-forming KFFE and KVVE show partial β-strand conformation in solution, whereas the non-fibril-forming KLLE and KAAE show random structure only, suggesting that inherent propensity for β-strand conformation promotes fibril formation. The peptides KFFFK or EFFE do not form fibrils on their own but do so in an equimolar mixture. Thus, intermolecular electrostatic interactions, either between charged dipolar peptides or between complementary charges of co-fibrillating peptides favor fibril formation.

Protein aggregates in the form of amyloid fibrils are found in about 20 diseases, including Alzheimer’s disease, transmissible spongiform encephalopathies, Parkinson’s disease, and type II diabetes mellitus (1). X-ray diffraction data suggest that the proteins have polymerized into a cross-β-sheet structure with the β-strands perpendicular to the fibril axis (2). The structure of amyloid fibrils is typically very similar, although the polypeptides they are formed from can be highly dissimilar in their native states. Initially, only a few peptides and proteins were considered capable of forming fibrils, although also short fragments of amyloidogenic proteins form fibrils. More recently, it has been established that even globular all-helical proteins like myoglobin, which normally do not give rise to amyloid, can be converted to fibrils if incubated under partly denaturing conditions (3). This suggests that most proteins have the potential to form amyloid fibrils.

It is not known which features cause a few specific proteins to form amyloid fibrils in vivo. Some amyloidogenic peptides and proteins harbor specific α-helices that are predicted to generate β-strands (4), and evidence that aggregation is initiated from particular regions of a polypeptide chain is accumulating (5). For example, the peptides NGAIL from islet amyloid polypeptide, HQKLVFFAED from the amyloid β-peptide (Aβ),¹ and VQIVYK from tau form fibrils and are crucial for fibril formation of the full-length peptides (6–8). Common to these sequences is the presence of hydrophobic amino acids of which at least one is aromatic. In this work, we aimed to explore the minimum requirements for fibril formation in short model peptides. The results show that peptides as short as 4 residues can form amyloid fibrils. Hydrophobic residues with high propensities for β-strand conformation and residues with complementary charges promote fibril formation, and positively and negatively charged peptides can copolymerize.

** EXPERIMENTAL PROCEDURES

**Peptide Incubations—**All peptides were purchased from Interactiva (Darmstadt, Germany) and purified by reversed-phase high pressure liquid chromatography using a C18 column and a linear gradient of water/acetonitrile supplemented with 0.1% trifluoroacetic acid. The identity of the peptides was verified with electrospray mass spectrometry. The peptides were incubated at 200 or 300 μM for 10 days at 37 °C in 50 mM phosphate buffer, pH 7.0, or in water, pH 5.0. The co-incubation of KFFFK and EFFE employed 100 μM of each peptide.

**Electron Microscopy (EM)—**After incubation the peptide solutions were centrifuged at 20,000 × g, and pelleted material was suspended in 40 μl of water by low energy sonication for 5 s. Aliquots of 8 μl were placed on EM grids covered by a carbon-stabilized Formvar film. Excess fluid was withdrawn after 30 s, and after air drying the grids were negatively stained with 1.5% uranyl acetate in water. The stained grids were examined and photographed in a Philips CM120TWIN electron microscope operated at 80 kV. Determination of fibril widths was made from high magnification micrographs (×180,000–200,000) viewed under a ×15 magnifier equipped with a graduated scale glass.

**Congo Red Staining—**Pelleted material was mixed with Congo red dissolved in Tris-buffered saline (TBS), pH 7.4, and incubated overnight. The sample was spun at 16,000 × g for 30 min. The supernatant was aspirated and the pellet was resuspended in TBS and spun at 16,000 × g. The pellet was resuspended in fresh TBS and transferred to a microscope slide and viewed under polarized light at ×200 magnification.

**CD Spectroscopy—**For analysis of secondary structure by CD spectroscopy peptides were dissolved at 300 μM concentration in sodium phosphate buffer, pH 6.0, or water. Immediately after solubilization CD spectra between 180 and 260 nm were recorded at 20 °C with 2-s response time, 2 data points/nm, and scan speed of 20 nm/min using an AVIV model 62DS spectropolarimeter (Jacksonville, NJ). To compensate for the contributions of the phenylalanine side chains to the CD spectra, the spectrum of the dipeptide FF was subtracted from the spectra of peptides containing an FF sequence. Residual molar ellipticities (θ) were calculated from peptide concentrations determined by amino acid analysis, and are expressed in kilodegrees × cm²/dmol.

**RESULTS**

To find the minimum requirements for fibril formation, we investigated the peptides KFFE, KVVE, KLLE, KAAE, KFFK, ¹The abbreviations used are: Aβ, amyloid β-peptide; EM, electron microscopy; TBS, Tris-buffered saline.
Simple Rules for Amyloid Fibril Formation

Amyloid fibril formation from a tetrapeptide. a, electron micrograph of representative fibrils formed from 300 μM KFFE after 10 days of incubation at 37 °C. Scale bar, 50 nm. b, Congo red-stained KFFE fibrils viewed under polarized light. Scale bar, 25 μm.

and EFFE. Lysine and glutamate have high β-sheet pair correlation, i.e. they are often found in neighboring strands in β-sheets (9), and KE-rich peptides form fibrils (10). Phenylalanine and valine are frequent in β-strands, whereas leucine and alanine are overrepresented in helices (4).

**Fibril Formation**—After incubation at 37 °C for 10 days KFFE and KVVE produce sedimentable amyloid fibrils (1.2–1.6 nm in width) and fibril bundles as detected by EM (Fig. 1a). Moreover, the tetrapeptide aggregates show a classical Congo red birefringence under polarized light (Fig. 1b). The tetrapeptide fibrils are indistinguishable from disease-associated amyloid fibrils in these respects. The peptides KLLK and KAAE do not form fibrils, suggesting that hydrophobic interactions are not sufficient for fibril formation. Removal of the terminal charges of KFFE by acetylatin the N terminus and amidating the C terminus significantly reduced fibril formation. Fibril formation of KFFE and KVVE was more pronounced in water than in phosphate buffer, indicating that charge attractions are important for fibril formation. The peptides KFFK and EFFE do not form fibrils when incubated individually. However, co-incubation of equimolar amounts of KFFK and EFFE produce fibrils as detected by EM (Fig. 2). Typically, these fibrils were shorter and thicker (average width, 2.5–3.0 nm) than the fibrils formed from KFFE or KVVE and were also grouped into small aggregates. Fibrils virtually identical to those shown in Fig. 1a, although less abundant, were formed also from the KFFK/EFFE mixture. Thus, heteropolymers can be formed from peptides with complementary charges.

Next, we investigated whether shorter peptides could form fibrils. The tripeptides were chosen based on optimal charge complementarity (KFE) and maximal β-strand propensity (VVF and FFF). None of these peptides formed fibrils. Therefore, we conclude that 4 residues are necessary and sufficient for fibril formation.

**Secondary Structure Analysis**—CD spectra of freshly dissolved KFFE show two minima in the 216–217 and 198–200 nm regions (Fig. 3A), diagnostic of coexisting β-strand and random structures. In contrast, non-fibrillogenic KAAE (Fig. 3B) and KLLE (data not shown) show CD spectra with one minimum at about 190 nm and a broad maximum around 215 nm, typical of an unordered structure. The comparison of the CD spectra of KVVE and KAAE (Fig. 3B) highlights the presence of β-strand structure in KVVE, evidenced by the decrease in ellipticity between 210 and 220 nm, a red shift of the short wavelength minimum, and increased ellipticity around 190 nm.

The CD spectra of the non-fibrillating KFFK and EFFE (Fig. 4) show the same features as the spectrum of KFFE. The spectrum of co-incubated KFFK and EFFE was virtually identical to the spectra of the individual peptides, suggesting that a significant part of the β-strand structure is present in the monomeric peptides.

**DISCUSSION**

Many proteins and peptides form fibrils. The determinants for fibril formation are not fully understood. Here we show that peptides as short as 4 residues can form amyloid fibrils and that β-strand structure in solution and attractive electrostatic interactions are required for fibrillogenesis. KFFE and KVVE are the shortest fibril-forming peptides known to date and could serve as models for further studies of amyloid fibril formation and structure. The similarity in fibril formation efficiency and secondary structure of KFFE and KVVE indicates that β-strand structure as such is a strong determinant of fibril formation. This suggests that the frequent occurrence of aromatic residues, in particular F, in amyloid-forming proteins is in part related to their high β-strand propensities (4). However, also the contributions from π interactions between phenylalanines in adjacent strands are probably important (11). The principles for fibril formation now proposed are in good agreement with previous results from studies of amyloid-forming peptides. For example, it has been found that changing the sequence KLVFF in Aβ (positions 16–20) to AAVFA prevents fibril formation (12), as would be predicted from the present results. Likewise, a V18A replacement in Aβ reduces the capacity to form fibrils (13), which is in excellent agreement with the different fibrillation capacity now found for KVVE and KAAE. This agreement is noteworthy, as the peptides now studied are not derived from known fibril-forming proteins but were designed from basic structural features of amino acid residues.

The fibril formation was more pronounced at low salt concentrations, indicating that charge interactions are important. The peptides KFFK or EFFE did not form fibrils when incubated alone but did so when co-incubated. Thus, the possibility of K-E interactions seems to be essential. These results and the high pair correlation of K and E in β-sheets (9) suggest that the peptides KFFE and KVVE have an antiparallel alignment in the fibrils. An energy-minimized model of KFFE in an antiparallel alignment is shown in Fig. 5.

If the present fibrils are composed of the tetrapeptides in a...
cross-β-sheet conformation, their width should be 1.4 nm (4-residue β-strand). This was found to be the case; the width of 10 measured fibrils was 1.2–1.6 nm. In general, the appearance of amyloid fibrils as observed by EM is similar, independent of size and structure of the native protein. The smallest widths in amyloid fibrils formed from 40-residue Aβ and ~350-residue transpeptidase are 3–4 nm, and for the 100-residue C-terminal fragment of the amyloid precursor protein and the ~600-residue coagulation factor XIII, 4–6-nm fibrils are found (4). The widths of the fibrils apparently do not correspond to the size of the protein of which they are composed. Possible explanations for this discrepancy include that the protein in question makes several intramolecular β-strands in the direction of the fibril and that only a fraction of the polypeptide chain is incorporated.
in the fibrils. The possibility that amyloid fibrils made from different proteins and peptides can be composed of β-strands as short as 4 residues may help to explain why attempts to find common motifs among these proteins have so far been largely unsuccessful.

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