A Molecular Model of Alzheimer Amyloid β-Peptide Fibril Formation

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Polymerization of the amyloid beta (Aβ) peptide into protease-resistant fibrils is a significant step in the pathogenesis of Alzheimer’s disease. It has not been possible to obtain detailed structural information about this process with conventional techniques because the peptide has limited solubility and does not form crystals. In this work, we present experimental results leading to a molecular level model for fibril formation. Systematically selected Aβ-fragments containing the Aβ16–20 sequence, previously shown essential for Aβ-Aβ binding, were incubated in a physiological buffer. Electron microscopy revealed that the shortest fibril-forming sequence was Aβ14–23. Substitutions in this decapeptide impaired fibril formation and deletion of the decapeptide from Aβ1–42 inhibited fibril formation completely. All studied peptides that formed fibrils also formed stable dimers and/or tetramers. Molecular modeling of Aβ14–23 oligomers in an antiparallel β-sheet conformation displayed favorable hydrophobic interactions stabilized by salt bridges between all charged residues. We propose that this decapeptide sequence forms the core of Aβ-fibrils, with the hydrophobic C terminus folding over this core. The identification of this fundamental sequence and the implied molecular model could facilitate the design of potential inhibitors of amyloidogenesis.

The polymerization of the amyloid β-peptide into protease-resistant fibrillar deposits in the brain parenchyma and vasculature is a significant step in the pathogenesis of Alzheimer’s disease (1). Compounds capable of interfering with the polymerization process thus may lead to potent therapeutics (2–7). To design such compounds, a detailed knowledge of the polymerization process at the molecular level is essential. Conventional experimental techniques alone have not been able to yield this detail. In the present work, we therefore employ both theoretical and experimental techniques to evolve a molecular model of fibril formation.

In fibrils, Aβ1 has been shown to exist in an antiparallel β-sheet conformation by x-ray diffraction and Fourier-transformed infrared spectroscopy (FTIR). Inouye and Kirschner (8) have refined their x-ray data from fibrils of Aβ11–28 by homology modeling, using the structure of β-keratin. The resulting electron density map suggested a hydrophobic core of Aβ17–20 (LVFF). A high-resolution model has not been possible because Aβ does not form crystals that are necessary for x-ray crystallography. Solid state NMR, not requiring crystals, has been used to study polymers formed by Aβ34–42, indicating a pleated antiparallel β-sheet (9). Unfortunately, solid state NMR is not suited for studies of longer peptides.

It has previously been shown that amino acid residues 16–20 in Aβ (Aβ16–20) are essential for Aβ polymerization, which is prevented by the substitution of these residues (6, 10–13). Aβ16–20 binds to the homologous region, Aβ17–21 or Aβ18–22, in Aβ and forms an antiparallel β-sheet structure (7). Peptides containing the Aβ18–20 motif, and peptides binding to this motif, prevent Aβ-fibril formation (7). However, residues 16–20 are not sufficient for polymerization. When incubated under conditions allowing polymerization of full-length Aβ, Aβ16–20 forms amorphous aggregates but not fibrils (6).

In the present study, we have focused on Aβ-fragments containing Aβ16–20 and identified the shortest fibril-forming Aβ-fragment containing this sequence as Aβ14–23. Based on substitution studies, electron microscopy (EM), molecular modeling, and the correlations of side-chain pairs found in β-sheets (14), we present a detailed structural model of fibrils formed by this sequence. This model includes interactions between sequences previously shown to interact in and to be necessary for Aβ34–42 fibril formation and should thus be of relevance also for fibrils formed from full-length Aβ. In support of this contention, deletion of the Aβ14–23 motif from Aβ16–20 renders a peptide incapable of forming fibrils.

EXPERIMENTAL PROCEDURES

Materials—Synthetic Aβ1–40 was obtained from Dr. David Teplow, the Biopolymer Laboratory at Harvard University, MA. All other peptides were purchased from Research Genetics, Huntsville, AL. The peptides were purified on a Polymer Laboratories (Church Stretton, UK) PLRP-S column (150 × 25 mm or 150 × 7.5 mm; Polymer Labs.), using a water-acetonitrile gradient with 0.1% trifluoroacetic acid. Identity and purity were verified with electrospray mass spectrometry using a Quattro triple quadrupole (Micromass, Altrincham, UK). After purification, the peptides were lyophilized and stored at −80 °C.

Incubation of Peptides—The peptides were dissolved at 200 μM in 50 mM Triss-buffered saline (TBS) (150 mM NaCl), pH 7.4 and incubated at 37 °C for 3 days. In control experiments, the peptides were initially dissolved in 2 volumes of 50 mM Tris, pH 10, to ensure starting solutions...
free from possible seeds. After 3 min, 1 volume of 50 mM Tris-HCl containing 450 mM NaCl was added to give a final pH of 7.4 and a concentration of 150 mM NaCl. No differences in the results were observed using this protocol.

**Thioflavine T Fluorescence Assay**—The incubated samples were vortex mixed, aliquots were withdrawn and mixed with 10 μM ThT, and the fluorescence was measured. The binding capacity correlated to fibril formation; all peptides except the three shortest were found to form fibrils as detected by EM (Fig. 2).

**Electron Microscopy**—The incubated samples were centrifuged at 20,000 g for 20 min, and the supernatants were aspirated. The pellets were sonicated for 5 min in 100 μl of water and 8 μl of these suspensions were placed on grids covered by a carbon-stabilized formvar film. Excess fluid was withdrawn after 30 s, and the grids were negatively stained with 3% uranyl acetate in water. The stained grids were then examined and photographed in a JEOL 100CX at 60 kV.

**Gel Electrophoresis**—Polyacrylamide gradient gels, 5–18%, were used in a Tris-Tricine buffer system. Supernatants (20,000 g) from the incubations were subjected to the ThT assay as described in the legend to Fig. 1. The nonapeptide Aβ15–23 (QKLVFFAED) was found to be the most potent ThT binder. Interestingly, this peptide formed thin flakes but no fibrils as detected by EM.

**FIG. 1.** ThT binding to Aβ peptide sequences containing the Aβ binding motif KLVFF. Peptides with the indicated sequences were incubated at a concentration of 200 μM in TBS, pH 7.4, for 3 days. The samples were vortex mixed, aliquots were withdrawn and mixed with 10 μM ThT, and the fluorescence was measured. The binding capacity correlated to fibril formation; all peptides except the three shortest were found to form fibrils as detected by EM (Fig. 2).

**FIG. 2.** Aβ13–23 forms fibrils. After 3 days of incubation, the peptides shown in Fig. 1 were centrifuged at 20,000 × g, and the supernatants were aspirated. The pellets were resuspended in water, placed on grids and negatively stained with 3% uranyl acetate. Examination of the stained samples revealed, in all cases except for the three shortest peptides, fibrils similar to those formed by full-length Aβ. The micrograph shows fibrils formed by the 11-residue peptide HHQKLVFFAED. Bar = 100 nm.

**FIG. 3.** ThT binding to truncated variants of Aβ13–23. Truncated variants of Aβ13–23 were subjected to the ThT assay as described in the legend to Fig. 1. The nonapeptide Aβ15–23 (QKLVFFAED) was found to be the most potent ThT binder. Interestingly, this peptide formed thin flakes but no fibrils as detected by EM.

**FIG. 4.** A decapeptide is the shortest fibril-forming peptide. N- and C-terminal truncated variants of the shortest fibril-forming peptide found in the first experiment were incubated and examined by EM as described in the legend to Fig. 2. A, the shortest fibril-forming sequence was found to be the decapeptide Aβ14–23 (HQKLVFFAED). B, only a few fibrils, ultrastructurally different from the fibrils formed by Aβ14–23 and full-length Aβ, were observed after incubation of the decapeptide Aβ13–22 (HHQKLVFFAED). The nonapeptide Aβ15–23 (QKLVFFAED) and the octapeptide Aβ15–23 (KLVFFAED) (C) formed thin flakes. Shorter peptides did not form ordered structures. Bars = 100 nm.

**FIG. 5.** ThT binding to variants of Aβ14–23 and to Aβ1–42 devoid of the Aβ14–23 sequence. Substituted and truncated variants of the shortest fibril-forming sequence, Aβ24–23, and a variant of Aβ1–42 in which residues 14–23 were deleted (Aβ1–42D14–23) were assayed for ThT binding as described in the legend to Fig. 1.

were sonicated for 5 s in 100 μl of water and 8 μl of these suspensions were placed on grids covered by a carbon-stabilized formvar film. Excess fluid was withdrawn after 30 s, and the grids were negatively stained with 3% uranyl acetate in water. The stained grids were then examined and photographed in a JEOL 100CX at 60 kV.

**Gel Electrophoresis**—Polyacrylamide gradient gels, 5–18%, were used in a Tris-Tricine buffer system. Supernatants (20,000 × g) from
the incubated peptides were mixed with Laemmli sample buffer and loaded, without boiling, onto the gel. Rainbow markers (Amersham, Little Chalfont, UK) were used to estimate molecular weights. After 4 h at 70 mA, the gels were stained with Coomassie Brilliant Blue or were silver stained.

**Molecular Modeling**—The molecular simulations were performed using the Insight/Discover 2.9.7 program suite (Biosym/MSI, San Diego, CA). The simulations were performed in vacuum, with the dielectric constant set to unity. Default values were used for all other parameters. Steepest-descent and conjugate gradient minimization schemes were used to optimize the putative structures, with the backbones fixed in $\beta$-sheet conformations generated by the Biopolymer module of the program suite.

**RESULTS**

**Identification of the Shortest Fibril-forming Sequence in $A\beta$**—A set of peptides with the $A\beta^{16-20}$ sequence systematically extended at both ends, were synthesized (Fig. 1). The peptides were incubated for 3 days under conditions allowing polymerization of full-length $A\beta$. Following incubation, an aliquot of the samples was mixed with 10 $\mu$M ThT, and the fluorescence was measured (Fig. 1). ThT shifts excitation and emission maxima upon binding to amyloid fibrils and has therefore been used to monitor amyloid fibril formation (15, 16). The incubates were then centrifuged at 20,000 $\times$ g, and the sedimented material was negatively stained and examined by EM. The undecapeptide HHQKLFFAED ($A\beta^{13-23}$) was the shortest peptide in this series capable of forming amyloid fibrils similar to those of full-length $A\beta$ (Fig. 2). Moreover, all peptides longer than 11 residues formed similar fibrils (not shown). The shorter peptides bound ThT considerably less efficiently than the longer, fibril-forming peptides (Fig. 1). When supernatants from the samples were analyzed by gel electrophoresis, all fibril-forming peptides displayed bands corresponding to dimers and/or tetramers, whereas no such species were detected in samples containing nonfibril-forming peptides.

One N- and one C-terminally truncated variant of $A\beta^{13-23}$ were synthesized and studied under the same conditions as above. The N-terminal truncated variant ($A\beta^{14-23}$) bound more...
ThT than the C-terminally truncated variant (Aβ13–22) did (Fig. 3). EM showed that deletion of the N-terminal His did not affect the tendency to form fibrils. However, the deletion of the C-terminal Asp impaired fibril formation (Fig. 4, A and B). Gel electrophoretic analysis of the supernatants revealed in both cases a band at 4–5 kDa, suggesting that the peptides formed stable tetramers. To investigate whether the Aβ14–23 sequence could be further truncated in the N terminus and still form fibrils, we analyzed nona- to hexapeptides with an intact C terminus as above. The nonpeptide bound ThT most efficiently, the octapeptide showed intermediate binding, whereas the hepta- and hexapeptide displayed only weak ThT binding (Fig. 4C), but none formed fibrils. Hence, the minimum sequence that was able to form amyloid fibrils was Aβ14–23. No fibrils were formed when Aβ14–23 was incubated in TBS, pH 9, indicating that protonation of His was necessary for fibril formation.

In addition to the symmetric extensions around the Aβ16–20 motif described above, we also examined a few peptides that extend at either end of this sequence, viz., Aβ11–20, Aβ12–21, and Aβ16–25. None of these peptides formed fibrils. Aβ11–20 and Aβ12–21 gave rise to rigid rods (up to about 200 × 20 nm), whereas Aβ16–25 mainly produced larger diffuse flakes (up to about 500 × 100 nm) and short fibrillar fragments (usually <50 nm in length). These findings confirm the notion that Aβ14–23 is the minimum Aβ sequence giving rise to amyloid fibrils.

To investigate further the mechanism of fibril formation, substituted and truncated variants of Aβ14–23 were synthesized (Fig. 5). Although several of the peptides were found to bind ThT, some even more than the nonsubstituted decapeptide (Fig. 5), only AQQKLVFDEA formed fibrils with any similarities to Aβ-fibrils (Fig. 6). To investigate whether the identified central minimum fibril-forming motif is necessary for Aβ-fibril formation, a peptide corresponding to Aβ1–42 without the Aβ14–23 sequence, Aβ1–42(A14–23), was synthesized and incubated as above. This peptide bound ThT (Fig. 5) and formed small aggregates but no fibrils (Fig. 7). Hence, we conclude that the Aβ14–23 sequence is not only sufficient but also necessary for Aβ-fibril formation.

It is of interest to note that ThT binds not only to amyloid-like fibrils, but also to other peptide aggregates (Figs. 4C, 6, E–F, and 7). Thus, when the ThT assay is used to monitor fibril formation, other techniques such as EM are a valuable complement to confirm the nature of the aggregates formed.

**Molecular Modeling**—It is known (7) that radiolabeled KKLVFDEA binds efficiently only to Aβ-fragments containing the KLVFF or LVFFA motif. From this observation and the above-mentioned fact that full-length Aβ adopts an antiparallel β-sheet conformation in fibrils, it is natural to posit an antiparallel β-sheet conformation also for the oligomeric decamers Aβ14–23, with the LVFF residues paired with hydrophobic groups. Additionally, the strength of a salt bridge is increased.

![Image](Molecular Model of Amyloid β-Peptide Fibril Formation)
by a factor of approximately 80 (the dielectric constant of water) when water is driven out of a hydrophobic environment. In a water-free environment like the interior of a fibril, salt bridges are thus energetically comparable with covalent bonds. The correct model should therefore involve a maximum of salt bridges and hydrogen bonds. Additional evidence for the importance of salt bridges arises from the observation of structural transitions in Aβ-fibril assembly as pH is varied, suggesting a strong dependence upon electrostatic interactions (17). In this latter study, fibrils were formed only at pH values where the His and Asp/Glu side chains are ionized. The twin requirements of hydrophobic overlap and maximal salt bridges limit the allowed pairings to two possibilities.
or a combination of these. Dimer “I” is formed with a maximum of intermolecular hydrogen bonds and salt bridges between Asp and His, whereas there is maximal interaction between hydrophobic side chains and salt bridges between Glu and Lys in dimer “II.” We subjected Dimer I to molecular modeling and energy minimization (Fig. 8A). Adding these dimers to each other in a β-sheet conformation would give a fibril with unpaired lysines on one side and unpaired glutamates on the other side. However, if the dimers are added to each other with an offset of one, Lys and Glu could form ion pairs (note that sequences 2 and 3 are aligned as in Dimer II).

1  DEAFFVLKQH  2  HQKLVVFAED  Dimer II
3  DEAFFVLKQH  4  HQKLVVFAED  Tetramer I

An energy-minimized model of such a tetramer is shown in Fig. 8B. Dimer after dimer (or tetramer after tetramer) could be added in this way, building a protofibril (Fig. 8C). On the basis of this model and NMR data from studies of Aβ13–35 indicating a turn-strand-turn motif in residues 13–24 (18), and assuming that the hydrophobic C terminus of Aβ forms an intramolecular β-sheet, we propose a model for Aβ-fibril formation (Fig. 8D). The N terminus, considered not to be necessary for amyloidogenesis because N-terminal truncated variants of Aβ are found in amyloid plaques (19), is not shown. In our model the core is made up from the fibril-forming decapeptide, and the C terminus of Aβ folds alternately above and below this core, minimizing the hydrophobic surface exposed to water. The diameter of the protofibril in our model is 4 nm, consistent with the data obtained with atomic force microscopy (20). Two or more such protofibrils could then twist around each other to exclude hydrophobic surfaces and form an amyloid fibril.

DISCUSSION

The KLVFF-sequence (Aβ16–20) is the region in Aβ that most efficiently binds to Aβ, and this sequence is necessary for fibril formation (6). Aβ16–20 binds to the homologous region (Aβ17–21 or/and Aβ18–22) in Aβ in an antiparallel manner (7), and it is likely that this interaction exists also in the fibrils. The pentapeptide Aβ16–20 does not form fibrils, and thus, also amino acid residues flanking this region are important for Aβ-fibril formation. To delineate a structural model for Aβ-fibril formation, we identified the shortest fibril-forming Aβ-sequence containing Aβ16–20. EM examination of systematically selected peptides showed this sequence to be HQKLVVFAED (Aβ14–23). Molecular models of a dimer, a tetramer, and an oligomer of Aβ14–23, where the charged residues form ion pairs and the hydrophobic residues form a hydrophobic core, were energy-minimized in a molecular modeling program (Fig. 8, A–D).

We suggest that the polymerization starts with the formation of dimers (Fig. 8A) which in turn form tetramers (Fig. 8B) and that dimers and/or tetramers are added to form oligomers (Fig. 8C). The existence of dimers and tetramers (modeled in Fig. 8, A and B) is supported by gel electrophoresis which showed that all fibril-forming peptides also formed dimers and/or tetramers. Likewise, a recent study by Garce-Rodriguez (21) shows that Aβ exists as a stable dimer even at low concentrations, and circular dichroism studies show a conformational change from random coil to β-sheet in association with fibril formation (22, 23). NMR studies of the solution structure of Aβ have been complicated by the fact that Aβ readily forms aggregates at the concentrations necessary for the use of this technique. However, the Aβ10–35 fragment has been studied by NMR in a solution free from detergents and organic solvents. This study shows a turn-strand-turn motif for residues 13–24 (18), and Chou and Fasman (24) analysis indicates a β-turn in the region of amino acids 25–29 of Aβ. These observations are both in agreement with the proposed model (Fig. 8D).

X-ray studies of fibrils formed by Aβ11–28 suggest that residues 17–20 (LVFF) form a hydrophobic core in these fibrils (8), and Phe-Phe interactions are favored in β-sheets. In accordance with the proposed model, Asp-His pairs, and especially Glu-Lys pairs, are frequently found in antiparallel β-sheets (14). Moreover, the pH-dependence of fibril formation shows the importance of salt bridges, and interactions between His and Glu or Asp have been suggested to be involved in Aβ-fibril formation (17). The extreme stability of the β-sheet structure formed by a peptide with the sequence AAKEKAKEAEKAK (25) and thermodynamic studies (26) also demonstrate the strength of charge complementarity and salt bridges between Glu and Lys. The polar Gln is located in a polar environment between the Asp-His and Glu-Lys pairs in our model. Although Gln does not seem to form any strong interactions with these pairs, no fibrils but high concentrations of dimers were formed when this residue was deleted from Aβ14–23. In this case, both the Asp-His and the Glu-Lys pairs can be formed within one dimer, offering an explanation to the absence of fibrils.

The length of the C terminus of Aβ is an important kinetic determinant for polymerization. Aβ1–42 polymerizes faster than Aβ1–40 (27), but amino acids 41 (Ile) and 42 (Ala) are (in contrast to residues 16–20) not necessary for fibril formation. The increased polymerization rate can be explained by increased hydrophobic interaction, first by the formation of a longer intramolecular β-sheet in the C terminus and second by the folding of this sheet over the core of the dimer (Fig. 8D). Another example of how increased hydrophobicity leads to a higher rate of fibril formation is the Glu-22 to Gln mutation in Aβ found in families with hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-D) (28). Gln, as well as Glu, interacts favorably with Lys, and the loss of a salt-bridge can be compensated by increased hydrophobic interactions.

A few short Aβ peptides not containing the whole Aβ14–23 sequence have previously been shown to form fibrils. However, in all cases except for Aβ15–28 (17), these fibrils were morphologically distinct from those formed by the full-length peptide and by Aβ14–23. Fibrils formed by Aβ22–35 (29) and Aβ26–35 (30) were thin and flexible, whereas Aβ24–42 (30) displayed a thicker, rope-like structure similar to the alanine-substituted decapeptide shown in Fig. 6E. Although a model by Kirschner et al. (31) is superficially similar to the one proposed here, the specific residue pairings differ. In particular, residue His-13 is important in their model, whereas it proved less essential for fibril formation in the present study and is not included in our fundamental decapeptide. Their model also involves only a single residue pairing, rather than the alternating alignments (Dimers I and II) of the present structure. Moreover, the Lys-Phe pairing of the Kirschner model is significantly less frequent in β-sheets than the Lys-Glu pairing incorporated into our model (14).
A large variety of proteins form amyloid fibrils structurally similar to those formed by Aβ (32). Short sequences from several such proteins have been shown to form fibrils. Examples include the decapeptide SNPNGAILSS derived from the prion protein (35). Neither the octapeptide FNNGNCFIL derived from gelsolin (34), and the octapeptide AGAAAAAGA derived from the prion protein (35). Neither of these fragments nor Aβ has a net charge, and it is possible that exposed hydrophobic side chains and appropriate pairing of extant charges and dipoles in a sequence of about 10 residues are sufficient for fibril assembly. The present model may be of general relevance for fibril formation, and the strategy we used to identify functionally important sequences could be applied to delineate the fibril-forming motifs in other amyloidogenic proteins.

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