Atomic structure and hierarchical assembly of a cross-β amyloid fibril

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The cross-β amyloid form of peptides and proteins represents an archetypal and widely accessible structure consisting of ordered arrays of β-sheet filaments. These complex aggregates have remarkable chemical and physical properties and the conversion of normally soluble functional forms of proteins into amyloid structures is linked to many debilitating human diseases, including several common forms of age-related dementia. Despite their importance, however, cross-β amyloid fibrils have proved to be recalcitrant to detailed structural analysis. By combining structural constraints from a series of experimental techniques spanning five orders of magnitude in length scale—including magic angle spinning nuclear magnetic resonance (NMR) spectroscopy with high-resolution electron density maps from cryoelectron microscopy (cryo-EM), together with data from X-ray fiber diffraction, scanning transmission electron microscopy (STEM), and atomic force microscopy (AFM) measurements. Our results reveal the molecular basis of the stability and polymorphism of these amyloid fibrils by defining at high resolution the variety of structural elements in their hierarchical self-assembly.

Results

Assembly of Individual Molecules into β-Sheet Arrays. A large number of intramolecular distance and torsion angle restraints (76 total) have been measured previously using MAS NMR methods, resulting in the high-resolution structure of the individual TTR(105–115) molecules in amyloid fibrils (20). To extend these studies to probe higher-order elements in the amyloid assemblies by identifying site-specific intermolecular restraints, we prepared eight different samples, each having a single isotopically labeled carbonyl atom per TTR(105–115) molecule at one of the residues 1107 to P113 and S115. Double quantum dipolar recoupling experiments (24) on these singly labeled samples provided eight high-precision (<0.2 Å), intrasheet 13CO–15CO distance restraints (SI Appendix, Table S1 and Fig. S24). The restraints span the entire backbone of the extended β-strand conformation adopted by TTR(105–115) peptides in fibrils (SI Appendix, Table S1 and Fig. S24) and unequivocally define a parallel, in-register arrangement of neighboring strands within the β-sheets.


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Data deposition: The structures of the TTR(105–115) amyloid fibrils have been deposited in the Protein Data Bank, www.pdb.org [PDB ID code 2m5n (Protein filaments), 2m6k (Doublet), 2m6m (Doublet), 3jzk [Quadraplet]] and the Electron Microscopy Data Bank [EMDB accession no. EMD-5590 (Doublet), EMD-2232 (Triplet), and EMD-2234 (Quadraplet)].

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Although knowledge of the intrasheet registry drastically reduces the number of permutations for the peptide molecules within the structure of the protofilaments, the number of possible intersheet arrangements is still very large (22). To address the challenge of defining this level of structural detail, we prepared two separate TTR(105–115) samples labeled uniformly with $^{13}$C and $^{15}$N isotopes at (underscored) residues YTIAALLSPYS and YTIAALLSPYS to characterize selectively the intermolecular packing of adjacent β-sheets in the fibrils. Intermolecular distance restraints from Z-filtered transferred echo double resonance (ZF-TEDOR) (25) and rotational resonance in the tilted frame width (R2TRW) (26) experiments on centrally labeled YTIAALLSPYS samples (SI Appendix, Table S2 and Fig. S2B) strongly suggest that the β-strands in a given β-sheet are antiparallel to those in the adjacent β-sheet.

To obtain further information on this crucial issue, we carried out additional ZF-TEDOR experiments using terminally labeled YTIAALLSPYS samples and observed three clearly resolved cross-peaks (Fig. 4I and SI Appendix, Fig. S3). The mixing-time dependence of the cross-peak intensities yielded three highly precise ($\pm 0.3 \AA$) intermolecular distances (SI Appendix, Table S2). This precision establishes an antiparallel intersheet stacking of β-sheets because not only do the contacts arise between residues at opposite ends of the TTR(105–115) molecule, but also the measured distances are much too short to occur either intramolecularly or within a parallel, in-register β-sheet. These measurements were further complemented by proton-driven $^{13}$C spin diffusion (PDSD) (27) experiments (Fig. 1B and SI Appendix, Fig. S4) on the YTIAALLSPYS sample; cross-peaks from these spectra provided a total of 16 $^{13}$C-$^{13}$C intersheet restraints (SI Appendix, Table S2 and Fig. S2B).

The complete set of 23 intersheet distance restraints (SI Appendix, Table S2 and Fig. S2B) reveals the specific manner in which adjacent β-sheets within the fibril are stacked in an antiparallel arrangement, thereby solving the first crucial aspect of the assembly of these multimolecular species.

The quantitative intersheet constraints between L111 and A108 and A109 and A110 (Fig. 1C, blue dashed lines), between A108 and S112 and P113 (Fig. 1C, red dashed lines), and between I107 and S112 (Fig. 1C, green dashes) establish the specificity of the even-numbered side chains. TALSYS (Fig. 1C, violet sticks), are packed against odd-numbered side chains, YIALPS (Fig. 1C, orange sticks), thus yielding an even–odd–even–odd interface between the sheets (Fig. 1C). This arrangement means that the side chains of all residues in the sequence appear both on the internal (dry) and external (wet) faces of each protofilament, and so in principle two sets of chemical shifts could be expected for each site. Indeed, some side chains do exhibit clear peak doubling, for example, I107C6, C1y1 and C1y2 and S112C0 and C0 as illustrated in SI Appendix, Fig. S5. Additionally, Y105 displays two conformations for the $^{13}$NH$_2$ and $^{13}$C0 (SI Appendix, Fig. S5). Because some side chains are more prone to dispersion than others, the chemical shift differences between the two different conformations for other cross-peaks are not resolved but contribute to the inhomogeneous broadening of the cross-peaks. For example, the linewidths of A108C9, A109C9, L110C6, and L111C5 are ~1.0–1.2 ppm. Similar doubling of some peaks has also been detected for polyglutamine (poly-Q) fibrils, although in this case this phenomenon is likely to arise from an antiparallel β-sheet structure, which is also a packing geometry leading to residues alternately pointing into (buried) and out (solvent exposed) of the fibril (28, 29). The intensities of the peaks that exhibit doubling in SI Appendix, Fig. S5 are not 1:1, unlike the peaks in poly-Q spectra (28, 29), because here, the solvent exposed side chains are more likely to be dynamic than those buried in the dry interface between the β-sheets.

A summary of the restraints used in the calculation of the protofilament structure is given in SI Appendix, Table S1 (intersheet; SI Appendix, Fig. S2A) and SI Appendix, Table S2 (intersheet; SI Appendix, Fig. S2B). The resulting set of structures, which were calculated with an average of 10 restraints per residue, has a backbone heavy atom root mean square deviation to the mean of the 20 lowest energy conformers in the NMR ensemble of 0.4 Å for the backbone and 0.7 Å for all heavy atoms (SI Appendix, Fig. S6). There are no residues with torsion angles in disallowed regions of the Ramachandran plot (SI Appendix, Table S3). Complete details concerning the structure calculations and statistics are provided in SI Appendix, SI Materials and Methods and Table S3.

**Atomic Structure of the Cross-β Protofilaments.** The results presented above define a parallel, in-register β-sheet geometry within the sheets of the TTR(105–115) fibrils (Fig. 2A and Movie S1), indicating a full complement of nine backbone–backbone hydrogen bonds (Fig. 2A) along the entire length of the molecule—i.e., involving all residues but the proline at position 113. The fact that this optimized interbackbone hydrogen-bonding network adopts this particular arrangement indicates that the intrinsically more favorable hydrogen-bonding pattern found in antiparallel β-sheets is offset by the juxtaposition of side chains with identical hydrophobic and hydrophilic character in the ordered self-assembly of these amphiphilic molecules. The in-register alignment of matching residues generates very tight packing (shape complementarity = 0.76; Fig. 2A), also maximizing favorable hydrophobic and van der Waals side-chain contacts along the long axis of the fibril (14); in addition, the structures may gain further stabilization through $\pi$–$\pi$ stacking of the tyrosine aromatic rings (10) at the termini of the TTR(105–115) molecule (Fig. 2A and B). The low degree of left-handed twist (~1°) between successive β-strands; Fig. 2B and SI Appendix, SI Supporting Equation) favored by the constituent parallel β-sheets of the fibrils is similar to the nearly flat β-sheets proposed in models of SH3 (11) and insulin (12) fibrils and limits lateral growth in the sheet–sheet direction (Fig. 2B and C), thus discriminating individual fibrils from 3D amyloid-like microcrystals (21, 22).
The structure shows that in-register, parallel β-sheets stack antiparallel to one another via a C2 axis parallel to the inter-sheet direction (Fig. 2C and Movie S1) to create a two-sheet protofilament. The structure shows that in-register, parallel β-sheets stack antiparallel to one another via a C2 axis parallel to the inter-sheet direction. The β-sheet viewed perpendicularly to the fibril axis illustrating the parallel in-register β-strands and the hydrogen bonds defining the β-sheet (yellow lines). The conformation was determined from eight [13C=O-13C=O] double quantum distance measurements and one [13C=O-13C=O] REDOR distance measurement (SI Appendix, Table S1). Cross-sectional view of the two-sheet protofilament along the peptide chain direction. There is a sheet-sheet offset of approximately one-fifth the separation of hydrogen-bonded β-strands (i.e., 0.2 × 4.67 Å = 0.93 Å) shown clearly by the interdigitation of the Y105 (orange sticks) and Y114 (violet sticks) side chains (Fig. 2F). The interprotofilament–protofilament interface viewed looking down the long axis of the fibril showing the head-to-tail packing arrangement. Interprotofilament hydrogen bonds between the terminal C=O and N–H groups and between the Y105 OH atoms and the S115 Oγ atoms are depicted as yellow dashes.

Overall Architecture of the Mature Fibrils. Because fibrils are typically composed of between two and six protofilaments (12), a vast number of interprotofilament packing arrangements are possible, for example, as observed in Aβ fibrils (16, 17, 19, 32). Although variations in intrasheet and intersheet stacking within protofilaments give rise to molecular-level polymorphism (15), interprotofilament arrangements represent an ultrastructural polymorphism (33) that can result in vast numbers of fibrils with distinct morphologies, where the nature of the interactions are unlikely to affect significantly the chemical shifts of any of the residues (12). Knowledge of the atomic structure of the protofilaments from the MAS NMR analysis, however, provides clues as to the possible higher-order packing interfaces. We therefore have combined single-particle and helical cryo-EM approaches (11, 16) to determine the overall structure of the complete fibrils formed by TTR (105–115). The fibrils are observed to be twisted ribbons with regular cross-over distances ranging from 850 to 1,000 Å (Fig. 3A–C, and SI Appendix, Fig. S1 and Table S4). Three prominent fibril types were identified (Fig. 3A–C) with distinct widths between crossovers (84 Å, Fig. 3A and D; 121 Å, Fig. 3B and E; and 154 Å, Fig. 3C and F). All fibrils have a width at crossover of ~37 Å and thus a nearly rectangular cross-section (Fig. 3D–F). The widths between crossovers correspond to integer multiples of the peptide chain length (~38 Å × 2, 3, or 4) and indicate the presence of multiple protofilaments (11, 19).

The internal packing of the protofilaments is revealed by examination of cross-sections through the 3D fibril reconstructions. Although the three fibril morphologies differ in the dimensions of the long side of their cross-sections (Fig. 3D–F), they are remarkably similar in other respects. In particular, they are all composed of two elongated, high-density regions separated by a well-defined region of low density (~13 Å wide at 1σ above the mean density) that extends to the slightly open cross-sectional ends (Fig. 3D–F). The high-density regions are ~12 Å in width (at 1σ above the mean density) and correspond to the paired β-sheets of individual protofilaments (11, 12, 16, 19) arranged in a linear fashion. The electron density maps all display twofold symmetry (Fig. 3D–F), which was not imposed during image processing, about the long axis of the fibril. This axial symmetry relates the paired β-sheet density regions to each other so that all of the fibrils described here are left-handed, double-layered helical ribbons with a hollow core (Fig. 3A–F).
Because the cryo-EM maps strongly suggest that the fibrils formed by TTR(105–115) are composed of integer multiples of linearly connected protofilaments, we sought to identify the interprotofilament packing interface. Low-angle X-ray experiments on TTR(105–115) fibrils (34) indicate an equatorial repeat of 37 ± 2 Å, which closely matches the length of the TTR(105–115) peptide in an extended β-strand conformation (38 Å; Fig. 2A); this observation suggests that the protofilaments are stacked in a periodic manner in the peptide chain direction. We therefore isotypically labeled the termini of the peptide [(⁵⁷⁷N-Y105 and ¹³⁷CO-S115) and performed rotational-echo double-resonance (REDOR) (35) experiments to probe backbone-to-backbone contacts. The observation of a strong dipolar coupling between S115–¹³⁷CO=NH,²⁻⁷⁻⁻⁻Y105, corresponding to an interprotofilament distance of 3.57 ± 0.06 Å, indicates the presence of head-to-tail contacts between adjacent protofilaments (Fig. 1D). This result is crucial in determining the relative orientations of individual protofilaments within the overall fibril topology (Fig. 1D).

The head-to-tail, interprotofilament packing interface (Fig. 2C) is very closely matched (shape complementarity = 0.83) and appears to be stabilized by dipole–dipole interactions between the N and C termini of peptide chains in neighboring protofilaments (SI Appendix, Fig. S7) and by four hydrogen bonds between the terminal C=O and N–H groups (Fig. 2C) and two tyrosine (OH—OH) hydrogen bonds (Fig. 2C).

**Atomic-Level Structures of the Fibril Polymorphs.** The reconstructed cross-sectional density maps are fully consistent with the NMR-derived protofilament structure discussed above (SI Appendix, Fig. S8) and can accommodate perfectly four, six, or eight of the two-sheet protofilaments interconnected in a head-to-tail fashion (Fig. 4A–C). Side-chain details are only present in exceptionally high-resolution electron density maps (3.3–4 Å) (36), which is not the case here because the resolution of the three maps is ~11 Å (SI Appendix, Table S4 and Fig. S9). As a result of the slightly curved cross-section of the protofilaments (Fig. 2C), the presence of a twofold symmetry axis (Fig. 3D–F), and the nanometer resolution (SI Appendix, Table S4) of the 3D reconstructions, there is a common structural arrangement that gives the best fit to the set of electron density maps (up to 7% less atoms outside the electron density maps at 1.0σ threshold) and on average more than seven times more interfacial contact area (with no steric clashes; Fig. 4D–F) than other alternative structures. This structural arrangement involves a pair of oppositely directed, linear stretches of two (Fig. 4A and SI Appendix, Fig. S10A and Table S3), three (Fig. 4B and SI Appendix, Fig. S10B and Table S3) and four (Fig. 4C and SI Appendix, Fig. S10C and Table S3) protofilaments with the even-numbered residues (TALSY) lining the outer surface of the fibrils and the odd-numbered residues (YIALPS) buried in the hollow core (Fig. 4D–F). Therefore, different numbers of otherwise identical protofilaments (12) are able to self-assemble into a generic structural arrangement.

To obtain an independent estimate of the number of peptides contained in the fibril cross-sections, we performed STEM mass-per-length (MPL) measurements on an ensemble of fibrils (Fig. 5A). The distribution can be described by three Gaussian curves peaking at 9.7 ± 1.2, 15.9 ± 1.2, and 15.9 ± 1.2 peptides per 4.67-Å layer of the different types of fibrils. These MPL measurements are in good agreement with the cryo-EM fibril reconstructions (Fig. 4A–C), which have cross-sections with twofold symmetry (Fig. 3D–F) to accommodate 8 (Fig. 4A), 12 (Fig. 4B), or 16 (Fig. 4C) peptides—i.e., 4, 6, or 8 two-sheet protofilaments—respectively. As an additional validation, we simulated the X-ray diffraction pattern of the fibril cross-sections and found that the resulting peaks closely match the primary and secondary reflections in the experimental diffraction pattern (34) (Fig. 5B).

The cryo-EM maps of the three fibrils are characterized by a nearly constant ~16-Å backbone-to-backbone low-density region between the high-density regions of paired β-sheets (Figs. 3D–F and 4A–C). Such a separation suggests that this interface may contain structured water involved in a hydrogen-bonded network with the exposed side chains (Fig. 4D–F) and water molecules. The dimensions of the cavity (Figs. 3D–F and 4A–C) can accommodate the two sets of exposed β-sheet side chains (~9 Å), leaving a 2 × 3.4 Å (the diameter of a water molecule) ~7 Å layer for water molecules to occupy (Fig. 4D–F). Moreover, the cavity dimension matches very closely the size of the wet interfaces in amyloid-like microcrystals in which crystal contacts result in bilayer of water molecules that can be observed crystallographically (21). Molecular dynamics simulations (SI Appendix, SI Materials and Methods) suggest that a single layer of water molecules becomes bound to each of the slightly hydrophilic protofilament surfaces (SI Appendix, Fig. S11).

Extensive AFM imaging, which has a much higher contrast than single cryo-EM images, of TTR(105–115) samples (Fig. 5C) revealed the presence of a low population (~3% of the total fibril dataset) of singlet fibrils, or “filaments,” with a uniform height of 38.7 ± 4.4 Å (Fig. 5C). The dimensions of this species match perfectly to the predicted height profile of a pair of protofilaments separated by the constant ~16 Å (backbone to backbone) water cavity (overall dimensions 38 × 43 Å; SI Appendix, Fig. S12). We also saw no evidence of the number of protofilaments per fibril varying along the length of a single TTR(105–115) fibril (37). Rather, it is clear from the cryo-EM images (Fig. 3) and 3D reconstructions (Fig. 4), AFM images, and STEM MPL measurements of TTR(105–115) fibrils (Fig. 5) that there are three main subsets of the fibril population—namely, the doublet, triplet, and quadruplet fibrils (Fig. 6 and SI Appendix, Figs. S13–S15, and Movies S2, S3, and S4), a helical symmetry was imposed on to the NMR/cryo-EM atomic cross-sections to generate longer fibril segments (Fig. 6, SI Appendix, Figs. S13–S15, and Movies S2, S3, and S4). The fit of four,
Discussion

An atomic-resolution structure of a cross-β amyloid fibril and its constituent self-assembling subunits has proved elusive to structural biology. Such a structure is of great interest, because the thermodynamic and kinetic properties of amyloid fibrils, as well as their frequently adverse effects on cellular homeostasis leading to disease, are linked to the details of their morphological features (40). In this study, we have resolved each of the structural elements in a cross-β amyloid fibril to atomic resolution, thus revealing the structural basis for the hierarchical organization of these aggregates. The architecture of the fully assembled amyloid fibrils is not strongly dependent on sequence because the constituent β-strands within the core of the fibril are linked by a vast array of interbackbone hydrogen bonds (Fig. 2, 4, and Movie S1), which are main-chain interactions common to all polypeptides (23). This generic character is evident in the marked similarity in appearance (17), dimensions (12), X-ray diffraction patterns (3), material properties (5), kinetics (31), thermodynamic stabilities (6), and dye-binding properties (41) of fibrils formed by peptides and proteins varying widely in sequence and native conformation.

The nature of the side-chains does, however, modulate the details of the fibrillar scaffold by determining which regions of the sequence self-assemble to form the intermolecular packing arrangement within, and between, the constituent β-sheets of the fibril core (1, 2). Although side chains influence which protofilament structures are adopted under a given set of experimental conditions, ultrastructural polymorphism is also a common feature of amyloid fibrils (1, 2). This variability at the intra- and interprotofilament levels provides an explanation of different fibril polymorphs characteristic of distinct clinical subphenotypes, in analogy to the strains of prions (42).

The ability to understand the hierarchical organization of cross-β amyloid fibrils (Fig. 5D) reveals how this structure...
represents a template that many amino acid sequences can adopt (23). Thus, although in longer sequences additional residues will undoubtedly affect the overall assembly of the fibrils and the nature of interactions between protofibrillaments (11), the basic structural elements (Fig. S2D) remain closely similar (12, 17, 43). In this respect, understanding the formation and molecular pathology of the cross-β fibrils formed by other peptides and proteins involves the study of variations on a theme common to most polypeptide chains (23). Indeed, both polar and nonpolar (22), and even homopolymeric (44) sequences can adopt the cross-β form of polypeptide states. By contrast, the β-helical structure of HET-s(218-289) fibrils (18) which represents a functional yeast prion, is stabilized by specific interactions selected through evolution and therefore not accessible to most amino acid sequences. The structures presented here (Fig. 6, SI Appendix, Figs. S13–S15, and Movies S2, S3, and S4) have instead the widely accessible cross-β geometry commonly associated with misfolding disorders such as Alzheimer’s disease and type II diabetes (1, 2).

Conclusions

We have determined the atomic-resolution structure of a cross-β amyloid fibril and two of its associated polymorphic variants. Our approach has enabled us to dissect each distinct level of the structural hierarchy of the fibrils at atomic resolution and to identify the packing interactions that drive the self-assembly and ultimately stabilize these persistent filamentous aggregates. The results that we have presented illustrate the increasing potential for amyloid polymorphism with the ascending hierarchy of fibrillar structures, a phenomenon that we expect to be common for many other peptides and proteins.

Methods

Amyloid fibrils were prepared by dissolving TTR(105–115) in a 10% (vol/vol) acetonitrile/water solution (adjusted to pH 2 with HCl) at a concentration of 15 mg/mL. The samples were incubated for 2 d at 37 °C followed by incubation for 14 d at room temperature (20). The samples were routinely characterized by TEM, and great care was taken to ensure that the morphologies of the fibrils studied by NMR were identical to those examined by cryo-EM and the other techniques (X-ray diffraction, AFM, and STEM). Full methods are available as SI Appendix, SI Materials and Methods.

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