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Constraints on the structure of fibrils formed by a racemic mixture of amyloid- β peptides from solid state NMR, electron microscopy, and theory

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Abstract

Previous studies have shown that racemic mixtures of 40- and 42-residue amyloid- β peptides (D,L-A β 40 and D,L-A β 42) form amyloid fibrils with accelerated kinetics and enhanced stability relative to their homochiral counterparts (L-A β 40 and L-A β 42), suggesting a “chiral inactivation” approach to abrogating the neurotoxicity of A β oligomers (A β -CI). Here we report a structural study of D,L-A β 40 fibrils, using electron microscopy, solid state nuclear magnetic resonance (NMR), and density functional theory (DFT) calculations. Two- and three-dimensional solid state NMR spectra indicate molecular conformations in D,L-A β 40 fibrils that resemble those in known L-A β 40 fibril structures. However, quantitative measurements of ¹³C-¹³C and ¹⁵N-¹³C distances in selectively labeled D,L-A β 40 fibril samples indicate a qualitatively different supramolecular structure. While cross- β structures in mature L-A β 40 fibrils are comprised of in-register, parallel β -sheets, our data indicate antiparallel β -sheets in D,L-A β 40 fibrils, with alternation of D and L molecules along the fibril growth direction, *i.e.*, antiparallel “rippled sheet” structures. The solid state NMR data suggest coexistence of D,L-A β 40 fibril polymorphs with three different registries of intermolecular hydrogen bonds within the antiparallel rippled sheets. DFT calculations support an energetic preference for antiparallel alignments of the β -strand segments identified by solid state NMR. These results provide insight into the structural basis for A β -CI and establish the importance of rippled sheets in self-assembly of full-length, naturally occurring amyloidogenic peptides.

Graphical Abstract

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Supporting Information

TEM images of L-A β 40 and Ac-KLVFFAE-NH₂ fibrils (Figure S1), measurements of nuclear spin relaxation times in REDOR measurements on D,L-A β 40 fibrils (Figure S2), and comparisons between REDOR data and simulations for various relative populations of intermolecular hydrogen bond registries (Figure S3). Appendix containing atomic coordinates of DFT-optimized structures of LVFFA:lvffa and IIGLM:iiglm.

Introduction

Amyloid- β ($A\beta$) peptides are intrinsically disordered peptides whose aggregated states are believed to be key neurotoxic agents in Alzheimer's disease (AD)¹. The two predominant $A\beta$ isoforms in human brain tissue, which are 40 and 42 residues in length ($A\beta$ 40 and $A\beta$ 42)², are both capable of aggregating into a variety of assemblies that are broadly classified as oligomers, protofibrils and fibrils³. The molecular structures of $A\beta$ 40 and $A\beta$ 42 assemblies, formed by conventional L-amino acids, have been studied extensively for more than 20 years^{4–28}. Recent experiments have shown that 1:1 mixtures of L- $A\beta$ and mirror-image D- $A\beta$ peptides yield fibrillar assemblies with accelerated kinetics and greater stability, suggesting that D- $A\beta$ peptides can be used to suppress the formation of non-fibrillar oligomers that may be important neurotoxic species^{29–31}. Current interest in self-assembly of racemic peptide mixtures, described in more detail below, motivates the work in this paper.

Although both L- $A\beta$ 40 fibrils and L- $A\beta$ 42 fibrils are polymorphic, meaning that they can exist in multiple distinct and self-propagating molecular structures^{3, 5, 7, 10, 17, 20, 23, 28, 32–35}, these fibrils possess certain common attributes: (i) As with all amyloid fibrils, they contain “cross- β ” motifs^{36–37}, which are β -sheets comprised of β -strand segments that run approximately perpendicular to the fibril growth direction, with their inter-strand hydrogen bonds being approximately parallel to the growth direction; (ii) As originally demonstrated by solid state nuclear magnetic resonance (NMR) and electronic paramagnetic resonance (EPR)^{4, 21, 38–39}, cross- β motifs in mature L- $A\beta$ 40 and L- $A\beta$ 42 fibrils contain in-register, parallel β -sheets, meaning that the backbone amide and carbonyl groups of residue k of one molecule are hydrogen-bonded to backbone carbonyl and amide groups of residues $k-1$ and $k+1$, respectively, of a neighboring molecule in the β -sheet; (iii) Each L- $A\beta$ 40 or L- $A\beta$ 42 molecule contains multiple β -strand segments, typically including residues 17–21, 30–32, and 34–36 (sequences LVFFA, AII, and LMV, respectively). Molecular conformations are roughly U-shaped or S-shaped in planes perpendicular to the fibril growth direction^{6–9, 17, 20, 22}, or in some cases are more intricate^{24, 26}, creating favorable interactions among sidechains of these hydrophobic β -strand segments that stabilize the overall structures; (iv) N-terminal segments, which contain multiple charged and polar residues, are conformationally disordered^{4–5, 11, 20, 22} or are located on the periphery of the cross- β fibril core^{9, 17, 26}; (v) As originally indicated by mass-per-length (MPL) measurements with electron microscopy^{3, 5, 7, 9, 39–41}, L- $A\beta$ 40 and L- $A\beta$ 42 fibrils contain two or three cross- β subunits that are related by two-fold or three-fold symmetry about the fibril growth axis, with important hydrophobic interactions between these subunits. Thus, the overall stability of the fibril structures depends on sidechain-sidechain interactions

both within and between cross- β subunits. For L-A β 40 and L-A β 42, a single cross- β subunit (sometimes called a protofilament⁴²) does not exist as a stable entity.

Partial exceptions to these structural principles have been observed, illustrating the highly complex nature of A β self-assembly. A recent study of brain-derived L-A β 40 fibrils, based on a combination of cryogenic electron microscopy (cryoEM), solid state NMR, and MPL measurements, found a polymorph with two-fold symmetry, containing molecules with extended (rather than U-shaped or S-shaped) conformations in its central cross- β layers and molecules with partially ordered β -hairpin-like conformations in its outer cross- β layers¹¹. Solid state NMR and MPL data for metastable protofibrils formed by the disease-associated Asp23-to-Asn mutant (D23N-L-A β 40) indicated U-shaped molecular conformations, but with antiparallel stacking along the fibril growth direction to produce a double-layered cross- β motif with purely antiparallel β -sheets, and with only one cross- β subunit⁸. Cross- β motifs comprised of antiparallel β -sheets have also been observed in fibrils formed by short fragments of L-A β , such as residues 34–42⁴³, 16–22⁴⁴, and 11–25⁴⁵.

In this paper, we report results from a structural study of fibrils formed by a 1:1 mixture of L-A β 40 and the mirror-image peptide D-A β 40. Recent work by Raskatov and coworkers has shown that such a racemic mixture yields fibrils with interesting physical properties and potentially important applications in the AD field^{29, 31, 46–48}. Our interest in these “D,L-A β 40 fibrils” comes from two directions. First, in light of the current understanding of homochiral L-A β fibril structures summarized above, it is intriguing to ask whether fibrils that presumably contain a 1:1 mixture of L-A β 40 and D-A β 40 are similar in structure to homochiral fibrils. The answer is not obvious, because a simple replacement of half of the L-A β 40 molecules by D-A β 40 molecules would necessarily disrupt any known fibril structure, because the positions of amino acid sidechains relative to the backbone hydrogen-bonding groups of β -sheets are dramatically different for L-amino acids and D-amino acids^{48–49}. The disposition of D- and L-A β 40 molecules within D,L-A β 40 fibrils is also unknown. Although it is tempting to assume that D and L monomers alternate along the fibril growth direction within a cross- β motif, it is conceivable that a cross- β motif constructed from dimers of D-A β 40 and dimers of L-A β 40, or a more complicated arrangement, exists. It is also conceivable that individual fibrils could contain unequal numbers of L-A β 40 and D-A β 40 molecules and/or lack reflection symmetry (in which case an equal number of fibrils would be expected to have the opposite ratio of L-A β 40 to D-A β 40 and/or a mirror-image structure).

Second, Raskatov and coworkers have shown that addition of D-A β molecules to L-A β molecules accelerates fibril formation, thereby suppressing formation of A β oligomers that have greater neurotoxicity in cell cultures^{29–30}. They introduced the term “A β chiral inactivation” (A β -CI) to describe this approach to the inhibition of A β toxicity by oligomer-to-fibril conversion^{29, 50–51}. A β -CI is related to the use of D-peptides as fibrillation inhibitors^{52–55} and amyloid-binding compounds⁵², but is distinct in that D-A β molecules are intended to enhance, rather than suppress, fibril formation by thermodynamic and kinetic effects. Raskatov and coworkers proposed that A β -CI works by formation of “rippled sheets” within D,L-A β fibrils^{30–31, 46–47}. Rippled sheets are structural motifs for racemic mixtures of polypeptide chains, introduced in 1953 by Pauling and Corey based on

theoretical considerations⁵⁶. A rippled sheet is similar to a conventional β -sheet (also called a “pleated sheet”⁵⁷), but with alternation of D and L chains. As with pleated sheets, rippled sheets can conceivably exist with either parallel or antiparallel alignments of neighboring, hydrogen-bonded chains⁵⁶.

In amyloid fibrils containing either parallel or antiparallel pleated sheets, amino acid sidechains form rows along the fibril growth direction on both faces of the sheets. In fibrils containing either parallel or antiparallel rippled sheets, amino acid sidechains would form checkerboard patterns on both faces of the sheets^{48–49}. Sidechain-sidechain interactions within rippled sheet fibrils may then be quite different from sidechain-sidechain interactions within conventional homochiral fibrils, possibly leading to different molecular structures. Evidence for rippled sheets in amyloid-like fibrils has been reported by Nilsson and coworkers⁵⁸ and Schneider and coworkers⁴⁹, for fibrils with designed amphiphilic sequences, and for Ac-KLVFFAE-NH₂⁵⁹, a fibril-forming peptide based on residues 16–22 of A β ⁴⁴. However, definitive evidence for rippled sheets in fibrils formed by racemic mixtures of longer, naturally occurring polypeptide sequences, such as A β peptides, has not yet been reported. In a study of fibrils formed by a racemic mixture of peptides representing residues 20–41 of β_2 -microglobulin, Goto and coworkers concluded that a rippled sheet structure was not possible⁶⁰. This conclusion was subsequently supported by the spectroscopic studies of Torbeev *et al.*, who also obtained evidence that racemic mixtures of full-length β_2 -microglobulin form assemblies with separate domains of L and D protein molecules⁶¹. Thus, the proposal that A β -CI works by formation of rippled sheets within D,L-A β fibrils requires experimental support from direct structural studies.

Here we present results from solid state NMR measurements on D,L-A β 40 fibrils, including measurements that identify β -strand segments and disordered segments and measurements that place constraints on β -sheet structure. Although these measurements do not fully define the structure of D,L-A β 40 fibrils, they provide evidence for antiparallel rippled sheets and for polymorphism involving several specific registries of inter-strand hydrogen bonds within the rippled sheets. Electron microscopy measurements complement the solid state NMR data and indicate the presence of three cross- β subunits within individual fibrils. Density functional theory (DFT) calculations on the β -strand segments identified by solid state NMR indicate an energetic preference for antiparallel alignments between neighboring D and L molecules. We also discuss aspects of symmetry that distinguish racemic amyloid structures from homochiral structures.

Materials and Methods

A β 40 synthesis

D-A β 40 without isotopic labels or with ¹⁵N labels at G33 and G37 positions within the A β framework was synthesized on a CEM Liberty Blue automated microwave-assisted peptide synthesizer using D-Val pre-loaded Tentagel PHB resin (Rapp Polymere). The synthesis was performed at a 0.1 mmol scale relative to resin loading. 30% piperidine (Spectrum) in dimethylformamide (DMF) was used for deprotection steps, and 1-Hydroxybenzotriazole hydrate (Oakwood Chemical) and N,N'-diisopropylcarbodiimide (DIC, Chem-Impex) were used as amino acid coupling reagents. The D-A β 40 synthesized peptide was then cleaved

and deprotected using a cocktail solution of trifluoroacetic acid (10 mL), 1, 2-diethanethiol (0.5 mL), tri-isopropylsilane (1 mL) and liquified phenol (0.5 mL). Peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) to achieve purities higher than 95% using methods reported previously.⁶² ¹⁵N-Fmoc-Gly-OH was obtained from AnaSpec.

L-A β 40 with ¹³C-labels at backbone carbonyl sites of I32 and V36 or with ¹³C-labels at backbone carbonyl sites of I31, G33, and M35 and ¹⁵N labels at L17, F19, and A21 was synthesized on a 0.1 mmol scale on a Biotage Initiator+ Alstra synthesizer, using Fmoc chemistry, DIC/OxymaPure activation, and a Fmoc-Val-NovaSyn TGA resin (Millipore-Sigma). Unlabeled residues were double-coupled with a five-fold excess of Fmoc-amino acid for 10 min at 75° C, except that Fmoc-Arg(Pbf)-OH was double-coupled for 15 min at 75° C and Fmoc-His(Boc)-OH was double-coupled for 15 min at 50° C. For isotopically labeled residues, a three-fold excess of the labeled Fmoc-amino acid was single-coupled for 20 min at 75° C, followed by single-coupling with a five-fold excess of unlabeled Fmoc-amino acid for 5 min at 75° C. After double-coupling, capping was performed with acetic anhydride/DIPEA to block the ends of unreacted chains and thereby minimize deletion impurities in the final product. Deprotection by 20% piperidine with 0.1 M 1-Hydroxybenzotriazole hydrate (Sigma-Aldrich) in DMF was performed at 75° C for 5 min. Cleavage from the synthesis resin was performed with a standard cocktail for 2.0 h. During the last 15 min of cleavage, 200 mg of tetrabutylammonium bromide was added to reduce any oxidized methionine at M35 of the A β 40 sequence. The crude peptide was precipitated in cold methyl tert-butyl ether, washed twice with methyl tert-butyl ether, and purified by preparative HPLC on a Zorbax 300SB-C3 reverse-phase column (Agilent Technologies).

A β 40 expression

For uniformly ¹⁵N,¹³C-labeled L-A β 40, a DNA insert encoding a 6His tag followed by the immunoglobulin binding domain B1 of protein G (GB1), TEV protease cleavage site, and A β 40 sequence was cloned into the pJ414 vector (ATUM) and transformed into *E. coli* BL21-DE3 cells (Agilent). The last residue (G/S) in the TEV protease recognition sequence (ENLYFQG/S) was omitted such that upon cleavage the A β 40 peptide starts with the native N-terminal Asp residue. Cells were grown in minimal medium with ¹⁵NH₄Cl and ¹³C₆-glucose as the sole nitrogen and carbon sources, respectively. The fusion protein was purified under denaturing conditions by Ni-NTA affinity chromatography. This step was followed by size-exclusion chromatography on a Superdex-75 column (Cytiva) to remove minor high molecular weight contaminants and guanidine hydrochloride and to exchange the buffer to that which is suitable for cleavage by TEV protease. TEV protease was prepared as described by Lucast *et al.*⁶³. After removal of the tag and GB1, as monitored by SDS-PAGE, L-A β 40 was collected in the flow-through from the Ni-NTA column and was further purified by reverse-phase HPLC on a POROS 20 R2 column (ThermoFisher Scientific), using a linear gradient from 10% acetonitrile/water and 0.05% TFA to 50% acetonitrile/water and 0.05% TFA over a period of 8 min at a flow rate of 4 ml/min. Aliquots of the peak fraction were lyophilized and stored at -70° C. Purity was verified both by SDS-PAGE and by electrospray ionization mass spectrometry. Before using this material to

prepare D,L-A β 40 fibrils, the lyophilized peptide was dissolved in TFA (4 mg/ml peptide concentration), then diluted 10-fold in acetonitrile/water (1:1 by volume), frozen in liquid nitrogen, and lyophilized again.

Fibril preparation

Fibrils were grown by dissolving purified, lyophilized D-A β 40 and L-A β 40 separately in 20 mM NaOH at peptide concentrations of 1.2 mM. After mixing the two solutions thoroughly, sterile-filtered phosphate-buffered saline (PBS) was added to produce a final total peptide concentration of 40 μ M. After thorough mixing, fibrils were allowed to grow quiescently at room temperature. Abundant fibrils were observed in negative-stain TEM images after 24 h, but incubation was allowed to proceed for 5 days. Fibrils were then pelleted at $220,000 \times g$ (Beckman Coulter SW40Ti rotor) for 4 h, resuspended in H₂O, pelleted again, frozen in liquid nitrogen, and lyophilized. For solid state NMR measurements, the lyophilized fibrils were packed in 1.8 mm-diameter magic-angle spinning (MAS) rotors and rehydrated by addition of 10 mM phosphate buffer, pH 7.4, containing 0.01% NaN₃. Table 1 summarizes the labeling patterns in the four samples that were prepared for solid state NMR measurements.

It is well known that L-A β fibril structures can be affected by variations in fibril growth conditions^{5, 35}. To avoid sample-to-sample variations, all D,L-A β 40 fibril samples were prepared with a single set of conditions, given above. We have not explored the effects of other conditions on D,L-A β 40 fibril structures.

L-A β 40 fibrils and Ac-KLVFFAE-NH₂ fibrils were prepared as previously described^{5, 44}. These fibrils were also lyophilized, packed in MAS rotors, and rehydrated with 10 mM phosphate buffer.

Electron microscopy and mass-per-length measurements

TEM images of negatively stained samples were obtained at 80 keV with a FEI Morgagni microscope and an Advantage HR camera (Advanced Microscopy Techniques). Grids were prepared and stained with uranyl acetate as previously described¹¹. Dark-field images of unstained D,L-A β 40 fibrils for MPL measurements were obtained with the tilted-beam technique described by Chen *et al.*⁴¹, using the Morgagni microscope and a XR-550B camera (Advanced Microscopy Techniques). Each count in the MPL histogram in Fig. 6B is the quantity $(I_F / I_{TMV,ave}) \times 131$ kDa/nm, where $I_F = F - 0.5(B_1 + B_2)$, F is the integrated image intensity within a 100 nm $\times$ 40 nm rectangle centered on a fibril, and B_1 and B_2 are integrated background intensities in rectangles on either side of the fibril. $I_{TMV,ave}$ is the average of background-subtracted, integrated intensities centered on TMV rods, averaged over 43 measurements. The MPL value of TMV is known to be 131 kDa/nm. Each count in the MPL error histogram in Fig. 6C is the quantity $\sqrt{\frac{3}{2}}[(B - B_{ave})/I_{TMV,ave}] \times 131$ kDa/nm, where B is the integrated intensity in a single background rectangle and B_{ave} is the average of B over 206 rectangles.

Solid state NMR spectroscopy

Solid state NMR measurements on fibrils with isotopic labels at specific sites were performed at 14.1 T (150.7 MHz ^{13}C NMR frequency; 60.7 MHz ^{15}N NMR frequency), using a 1.8 mm MAS NMR probe obtained from the laboratory of Prof. Ago Samoson (Tallinn University of Technology, Estonia) and either a Varian InfinityPlus spectrometer console or a Tecmag Redstone spectrometer console. Constant-time homonuclear dipolar recoupling with π pulses equal to one third of a rotor period (PITHIRDS-CT)⁶⁴ was performed at an MAS frequency of $\nu_{\text{MAS}} = 27.00$ kHz and a sample temperature of 15° C (determined from the temperature-dependent ^1H NMR frequency of water in the sample). ^1H decoupling field amplitudes were 115 kHz during ^{13}C - ^{13}C dipolar recoupling periods and 85 kHz during signal detection periods, which included pulsed spin-locking for sensitivity enhancement⁶⁵. Rotational echo double resonance (REDOR) measurements used the technique described by Anderson *et al.*⁶⁶, in which the radio-frequency (rf) pulse sequences for dephased and unde phased signals (S_1 and S_0 , respectively) differ by only one ^{15}N π pulse in the middle of the ^{15}N - ^{13}C dipolar recoupling periods. REDOR measurements used $\nu_{\text{MAS}} = 9.00$ kHz, actively rotor-synchronized 10.0 μs π pulses on the ^{13}C channel and 12.5 μs π pulses on the ^{15}N channel, 100 kHz ^1H decoupling during ^{15}N - ^{13}C dipolar recoupling periods, and sample temperatures of 4° C. Pulsed spin-lock detection with 85 kHz ^1H decoupling was used for REDOR data in Fig. 5B. Normal free-induction decay detection with 100 kHz two-pulse phase-modulated (TPPM) decoupling⁶⁷ was used for REDOR data in Fig. 5C. In all REDOR measurements, the number of scans increased linearly with the REDOR recoupling period τ_{RED} to maintain an adequate signal-to-noise level at large values of τ_{RED} . As usual, REDOR data are plotted as S/S_0 , with $S = S_0 - S_1$.

Measurements of ^{13}C T_2 in Fig. S2A used the full REDOR pulse sequence, but without pulses on the ^{15}N channel. Measurements of effective ^{13}C and ^{15}N T_1 values in Fig. S2B used pulse sequences in which longitudinal spin magnetization was created by ^1H - ^{13}C or ^1H - ^{15}N cross-polarization followed by a $\pi/2$ “flip-back” pulse. The longitudinal magnetization then evolved under a train of rotor-synchronized ^{13}C or ^{15}N π pulses (with the same XY-16 phase pattern⁶⁸ that was used in REDOR measurements) and was measured by a $\pi/2$ “read” pulse. Thus, the effective ^{13}C and ^{15}N T_1 values represent time scales over which longitudinal magnetization decays under the REDOR π pulse trains. These values are significant because values less than the relevant ^{15}N - ^{13}C REDOR dephasing times would prevent determination of ^{15}N - ^{13}C distances from the REDOR data, due to randomization of nuclear spin states and consequent attenuation of ^{15}N - ^{13}C dipole-dipole couplings.

Measurements on D,L-A β 40 fibrils with uniformly ^{15}N , ^{13}C -labeled L-A β 40 were performed at 17.5 T (187.4 MHz ^{13}C NMR frequency; 75.5 MHz ^{15}N NMR frequency), using a 3.2 mm MAS probe obtained from Dr. Peter Gor'kov (Black Fox, LLC) and a Varian InfinityPlus spectrometer console. The sample temperature was 20° C, and ν_{MAS} was 12.00 kHz. 2D ^{13}C - ^{13}C , NCACX, and NCOCX spectra in Fig. 2 were obtained with standard pulse sequences, with dipole-assisted rotation resonance (DARR) mixing periods of 25 ms (2D ^{13}C - ^{13}C and NCACX) or 50 ms (2D NCOCX), and 85 kHz TPPM decoupling during t_1 and t_2 periods. Band-selective ^{15}N - ^{13}C cross-polarization periods used 8 kHz and 20 kHz rf fields (NCACX) or 32 kHz and 20 kHz rf fields (NCOCX) on ^{13}C and ^{15}N channels,

respectively, with 4.0 ms contact times and a linear ramp of approximately 5% on the ^{13}C rf amplitude. 3D NCACX, NCOCX, and CANCEX measurements in Fig. 3 used the same conditions. Total measurement times were 5–7 days for each 3D spectrum, with 1.8 s recycle delays, time increments of 125 μs and 80 μs , and 32 and 40 complex points in indirect ^{15}N and ^{13}C dimensions, respectively. The 2D ^1H - ^{13}C INEPT spectrum in Fig. 4 was obtained with the two steps for polarization transfer set to 2.0 ms and 1.6 ms, and with 10 kHz ^1H decoupling using a train of 25-step composite π pulses⁶⁹.

^{13}C and ^1H chemical shifts are relative to DSS. ^{15}N chemical shifts are relative to liquid ammonia. All chemical shift references were determined from the carboxylate ^{13}C chemical shift of D,L-alanine powder under MAS, taken to be 179.65 ppm relative to DSS. 2D and 3D spectra were processed with NMRPipe⁷⁰ and analyzed and plotted with Sparky (available at <https://www.cgl.ucsf.edu/home/sparky/>). Chemical shifts in Table 1 were determined manually from the 3D spectra.

Simulations of PITHIRDS-CT and REDOR data

Numerical simulations of PITHIRDS-CT and REDOR data were performed with Fortran programs written specifically for this purpose. For PITHIRDS-CT simulations, the evolution of the nuclear spin density matrix under the time-dependent dipole-dipole coupling Hamiltonian for five ^{13}C spins under MAS with the full PITHIRDS-CT pulse sequence was calculated. Chemical shift anisotropy was not included. In the initial condition, ^{13}C spin polarization was limited to the central spin in a linear chain. Signals from all five spins were detected. This approach approximates the behavior of an infinite chain of spins.

REDOR simulations used formulae for the evolution of a three-spin system (one ^{13}C , two ^{15}N , simulations in Figs. 5B, 5D, and S3) or a two-spin system (one ^{13}C , one ^{15}N , simulations in Fig. 5C) under an ideal REDOR pulse sequence, assuming δ -function π pulses, no chemical shift anisotropy, no spin relaxation, and no ^{15}N - ^{15}N couplings. The dashed curve in Fig. 5B used ^{15}N - ^{13}C distances of 4.2 Å and 5.4 Å and a 162° angle between the two ^{15}N - ^{13}C internuclear vectors, representing the two strongest ^{15}N - ^{13}C dipole-dipole couplings in an antiparallel β -sheet model for Ac-KLVFFAE-NH₂ with 4+k $\leftrightarrow$ 4-k registry (*i.e.*, hydrogen bonds between V3 and F5), as determined experimentally by Balbach *et al.*⁴⁴. The dotted curve in Fig. 5B used ^{15}N - ^{13}C distances of 4.1 Å and 5.8 Å and a 155° angle, based on an ideal in-register, parallel β -sheet model with alternation of D and L molecules (Fig. 6A). The solid curve in Fig. 5B used ^{15}N - ^{13}C distances of 6.0 Å and 6.4 Å and a 107° angle, based on an ideal antiparallel β -sheet model with 33+k $\leftrightarrow$ 33-k registry (Fig. 6B). The dotted curve in Fig. 5B was scaled down by a factor of 0.7 to account for the expected 30% contribution of natural-abundance ^{13}C nuclei to the REDOR S_0 signals in measurements on $2\text{C}_L, 2\text{N}_D$ -D,L-A β 40 fibrils with a hypothetical in-register, parallel β -sheet structure. The solid curve in Fig. 5B was scaled down by a factor of 0.35 to account for the contribution of both natural-abundance ^{13}C nuclei and ^{13}C labels at V36 to the REDOR S_0 signals with a hypothetical antiparallel β -sheet structure (since only the ^{13}C labels at I32 would have significant couplings to ^{15}N labels). REDOR simulations in Fig. 5C were scaled down by a factor of 0.38 as described in the Results section.

REDOR simulations in Figs. 5D and S3 used distances and angles from the ideal antiparallel β -sheet models in Fig. 6. Simulations for carbonyl ^{13}C labels at I31, I32, G33, M35, or V36 and $33+k \leftrightarrow 32-k$, $33+k \leftrightarrow 33-k$, or $33+k \leftrightarrow 34-k$ registry were combined appropriately for comparison with experimental REDOR data. In each simulation, the two ^{15}N labels that were closest to the ^{13}C label in the β -sheet model were included. Scaling factors equal to $1 - f_{\text{nd}}$ were applied to each simulated REDOR curve before comparison with experimental data, where f_{nd} is the fraction of the total ^{13}C signal that is expected to have negligible decay from ^{15}N - ^{13}C dipole-dipole couplings during the recoupling period. Non-decaying signals are attributed to natural-abundance carbonyl ^{13}C spins, estimated to be 0.75 non-glycine carbonyl ^{13}C spins and 0.13 glycine carbonyl ^{13}C spins per D-A β 40/L-A β 40 pair (with minor corrections to account for the presence of ^{13}C labels at some carbonyl sites). χ^2 values plotted in Fig. 5D are defined as $\chi^2 = \sum_{i=1}^m (E_i - S_i)^2 / \sigma_i^2$, where $m = 17$ is the total number of data points in the REDOR measurements, E_i and S_i are experimental and simulated S/S_0 values, and σ_i is the uncertainty in E_i . Uncertainties in S/S_0 were determined from the root-mean-squared noise in the ^{13}C NMR spectra from which S_1 and S_0 values were measured, following standard error propagation methods.

DFT methods

Molecular input structures were built from Pauling-Corey coordinates using standard molecular visualization tools and subsequently geometry-optimized at the M062X/6-31G* level of theory using Gaussian09^{71–74}. Structures were confirmed as minima using frequency calculations. Energies listed are corrected for zero-point energy, basis set superposition error, and solvent contribution (water, polarizable continuum model, single point calculation). The deviation in energy difference between the LVFFA:lvffa antiparallel and parallel dimers reported here and in our past work is likely due to a combination of factors, including small conformational differences and slightly different levels of theory used⁴⁸.

Results

Fibril formation by D,L-A β 40

Fibrils were prepared by dissolving L-A β 40 and D-A β 40 separately at 1.2 mM in 20 mM NaOH, mixing the two solutions, and diluting them in PBS to a final total peptide concentration of 40 μM . After quiescent incubation overnight at 24° C, abundant fibrils were observed in negative-stain transmission electron microscope (TEM) images (Fig. 1A). Fibrils were approximately 7 ± 1 nm in width and typically 0.5–2.0 μm in length. Unlike many amyloid fibrils formed by L-amino acids^{3, 7, 10, 23, 26, 28}, the D,L-A β 40 fibrils do not show a periodic width modulation in the TEM images³⁰, suggesting the absence of either right-handed or left-handed twist about the fibril growth direction. A structure that contains equal numbers of equivalent D and L molecules is expected to be untwisted. Alternatively, if D and L molecules are structurally inequivalent within an individual fibril, equal numbers of fibrils with right-handed and left-handed twists are expected. Indistinguishable fibril morphologies were observed in TEM images after longer incubation periods and in independent sample preparations with different isotopic labeling patterns (Figs. 1B–1D).

Characterization of peptide conformation and dynamics in D,L-A β 40 fibrils

Isotopic labeling patterns of D,L-A β 40 samples are listed in Table 1. Solid state NMR measurements on U_L-D,L-A β 40 fibrils were performed to identify peptide segments that became immobilized or remained dynamic in the fibrils and to characterize the secondary structure of immobilized segments. By symmetry, conclusions drawn from NMR signals of labeled L-A β 40 molecules in these fibrils should also apply to the unlabeled D-A β 40 molecules.

Fig. 2 shows two-dimensional (2D) ^{13}C - ^{13}C , NCACX, and NCOCX spectra of the U_L-D,L-A β 40 fibrils, performed at 17.5 T and 20° C with MAS at 12.00 kHz (see Methods). The 2D spectra are poorly resolved, compared with analogous spectra of certain L-A β 40^{9, 24} and L-A β 42^{17, 22} fibrils. Linewidths in solid state NMR spectra of amyloid fibrils are variable, even for samples that are monomorphic, as the lines can exhibit heterogeneous broadening from intrinsic conformational disorder, disordered contacts between fibrils, or other sources. In the case of D,L-A β 40 fibrils, the possibility also exists that the molecular structure contains more than one inequivalent peptide conformation or structural environment and/or that the fibril samples contain structurally distinct polymorphs, as discussed below.

Three-dimensional (3D) solid state NMR measurements were performed to obtain site specific assignments of ^{15}N and ^{13}C chemical shifts. Fig. 3 shows examples of 2D planes from 3D NCACX, NCOCX, and CONCA spectra of U_L-D,L-A β 40 fibrils. Widths of resolved crosspeaks in these spectra are 1.0–1.5 ppm in the ^{13}C dimensions and roughly 2 ppm in the ^{15}N dimensions (full widths at half-maxima). Table 2 shows the assignments that could be obtained from manual analysis of the 3D spectra, spanning residues 16–22 and 29–37. Chemical shift-based predictions of backbone ϕ and ψ torsion angles⁷⁵ indicate β -strand conformations for the two assigned segments. Thus, these data indicate a strand-loop-strand motif in residues 16–37, similar to what has been observed in previous studies of L-A β 40 fibrils^{6–7} and protofibrils^{8, 12}. In D,L-A β 40 fibrils, the loop in residues 23–28 is apparently disordered.

Assignments in Table 2 were derived from crosspeaks that are relatively strong and well resolved in the 3D spectra. The spectra also contain weaker and incompletely resolved crosspeaks, which could not be assigned. Unassigned signals may arise from residues outside of the assigned segments or from fibril polymorphs with lower populations.

Fig. 4A shows a 2D ^1H - ^{13}C INEPT spectrum of the U_L-D,L-A β 40 fibrils, obtained with experimental conditions that select signals from residues that execute nearly isotropic motions on sub-microsecond time scales (see Methods). From comparisons with standard random-coil ^1H and ^{13}C chemical shifts⁷⁶, crosspeaks in this spectrum can be assigned to Ala, Ser, Val, Glu, Phe, Asp, and Arg residues, all of which occur in residues 1–12 (or in residues 1–9 and 38–40) of the A β 40 sequence. Thus, the N-terminal segment (or the N-terminal and C-terminal segments) are dynamically disordered in D,L-A β 40 fibrils. Fig. 4B summarizes the secondary structure and dynamical information from measurements on U_L-D,L-A β 40 fibrils.

Characterization of β -sheet organization in D,L-A β 40 fibrils

To obtain constraints on the organization of β -sheets in D,L-A β 40 fibrils, measurements of intermolecular ^{13}C - ^{13}C and ^{15}N - ^{13}C dipole-dipole couplings were performed on samples with ^{13}C labels at specific backbone carbonyl sites and ^{15}N labels at specific backbone amide sites (see Table 1). Measurements of ^{13}C - ^{13}C couplings in $3\text{N}_\text{L}, 3\text{C}_\text{L}$ -D,L-A β 40 fibrils, using the PITHIRDS-CT technique⁶⁴, show a slow decay of ^{13}C solid state NMR signals with increasing dipolar evolution period, consistent with ^{13}C - ^{13}C distances of approximately 7 Å (Fig. 5A). This distance corresponds to the expected intramolecular distances between ^{13}C labels at residues 31, 33, and 35 in $3\text{N}_\text{L}, 3\text{C}_\text{L}$ -D,L-A β 40, assuming that the labeled residues are in a β -strand segment as indicated by the ϕ, ψ predictions in Table 2. In contrast, PITHIRDS-CT measurements on L-A β 40 fibrils with the same labeling pattern show the more rapid signal decay expected for the 4.8 Å intermolecular distances of an in-register, parallel β -sheet (Fig. 5A; see Fig. S1 for TEM images of the L-A β 40 fibrils). These PITHIRDS-CT data show that β -sheets in D,L-A β 40 fibrils do not contain hydrogen-bonded, in-register pairs or larger groups of L-A β 40 molecules (or D-A β 40 molecules). Rather, these data are consistent with a rippled sheet structure in which L-A β 40 molecules alternate with D-A β 40 molecules, with backbone hydrogen bonds only between molecules of opposite chirality.

As shown in Fig. 6A, an in-register, parallel β -sheet structure with alternating molecular chirality would contain hydrogen bonds between backbone carbonyl groups of residue k in L-A β 40 and residue $k+1$ in D-A β 40, leading to intermolecular ^{15}N - ^{13}C distances of approximately 4.1 Å. To test for such a structure, ^{15}N - ^{13}C dipole-dipole couplings in $2\text{C}_\text{L}, 2\text{N}_\text{D}$ -D,L-A β 40 fibrils were measured with the REDOR technique⁶⁶. The build-up of normalized REDOR difference signals S/S_0 (where $S = S_1 - S_0$, with S_1 and S_0 being the ^{13}C signal amplitudes with and without net evolution under ^{15}N - ^{13}C couplings) is too slow to be consistent with 4.1 Å distances between carbonyl ^{13}C labels at I32 and V36 and ^{15}N labels at G33 and G37, respectively (Fig. 5B). These data rule out an in-register, parallel rippled sheet structure.

Since REDOR build-up can be suppressed by molecular motions that tend to average out ^{15}N - ^{13}C couplings and by ^{13}C or ^{15}N nuclear spin dephasing during the dipolar evolution period, measurements were performed on both hydrated and lyophilized $2\text{C}_\text{L}, 2\text{N}_\text{D}$ -D,L-A β 40 fibrils, leading to indistinguishable data. As additional checks, measurements of dephasing under the REDOR pulse sequence yielded dephasing times (T_2 for ^{13}C and effective T_1 for both ^{13}C and ^{15}N , see Fig. S2) that were not sufficiently short to prevent observation of a 4.1 Å ^{15}N - ^{13}C distance. Moreover, REDOR measurements on Ac-KLVFFAE-NH₂ fibrils with ^{15}N labels at V3 and ^{13}C labels at carbonyl sites of F5 produced a rapid build-up of S/S_0 under the same experimental conditions (Fig. 5B; see Fig. S1 for TEM images of Ac-KLVFFAE-NH₂ fibrils). Data for Ac-KLVFFAE-NH₂ fibrils are in quantitative agreement with simulations (dashed curve in Fig. 5B) that are based on intermolecular distances in the known antiparallel β -sheet structure of Ac-KLVFFAE-NH₂ fibrils, which includes V3-F5 hydrogen bonds⁴⁴.

An antiparallel rippled sheet structure could potentially explain the data for D,L-A β 40 fibrils in Figs. 5A and 5B. For example, as shown in Fig. 6B, an antiparallel β -sheet formed by

residues 29–37, with hydrogen bonds between residue 33+k of one molecule and residue 33-k of a neighboring molecule (*i.e.*, 33+k ↔ 33-k registry of intermolecular hydrogen bonds within the antiparallel β-sheet), would lead to nearest-neighbor intermolecular distances between I32 ¹³C labels and G33 ¹⁵N labels of approximately 6.0 Å and 6.4 Å in 2C_L,2N_D-D,L-Aβ40 fibrils, and to nearest-neighbor ¹⁵N-¹³C distances greater than 10 Å for the V36 ¹³C label. Simulations in Fig. 5B (solid curve) show that such a structure fits the experimental REDOR data reasonably well. Antiparallel β-sheets with 33+k ↔ 33-k registry do exist in D23N-L-Aβ40 protofibrils⁸. However, other rippled sheet structures could also explain the data in Figs. 5A and 5B.

As an additional constraint, REDOR measurements were performed on 3N_L,3C_L,2N_D-D,L-Aβ40 fibrils, in which the carbonyl ¹³C NMR line from the label at G33 in L-Aβ40 was resolved from the ¹³C NMR line from labels at I31 and M35. Data in Fig. 5C show a relatively rapid build-up of S/S₀ for the G33 line, consistent with the expected intermolecular distance of approximately 4.1 Å between ¹³C labels at G33 in L-Aβ40 and ¹⁵N labels at G33 in D-Aβ40 with 33+k ↔ 33-k registry. However, the maximum value of S/S₀ is only about 0.35, indicating that not all G33 carbonyl ¹³C labels have this ¹⁵N-¹³C distance. REDOR simulations in Fig. 5C are scaled by a factor of 0.38 to optimize agreement with the experimental data. Thus, it appears that our D,L-Aβ40 fibrils may contain antiparallel rippled sheets with 33+k ↔ 33-k registry, but that fewer than half of the Aβ40 molecules participate in this hydrogen bond registry.

We tested other registries, using models for antiparallel rippled sheets to determine the nearest-neighbor ¹⁵N-¹³C distances with the isotopic labeling patterns of the 2C_L,2N_D-D,L-Aβ40 and 3N_L,3C_L,2N_D-D,L-Aβ40 samples. Models for 33+k ↔ 32-k and 33+k ↔ 34-k registry are shown in Figs. 6C and 6D. No single registry resulted in acceptable agreement between all experimental REDOR data and simulations. We also considered (admittedly implausible) models with mixed registry within a single rippled sheet, for example a model with a four-molecule repeat pattern, with hydrogen bonds between G33 of molecule i and G33 of molecule i+1, L34 of molecule i+1 and G33 of molecule i+2, L34 of molecule i+2 and I32 of molecule i+3, and G33 of molecule i+3 and L34 of molecule i+4. We were unable to construct a single model, even with mixed registry, that resulted in acceptable agreement between all experimental REDOR data and simulations.

The failure of a single rippled sheet model to explain the experimental REDOR data leads us to conclude that our D,L-Aβ40 fibril samples are polymorphic, containing fibrils (or domains within individual fibrils) with different antiparallel rippled sheet structures. The experimental REDOR data are then population-weighted averages of data for the different structures. To estimate the relative populations, we performed REDOR simulations for each of the five ¹³C-labeled carbonyl sites in the 2C_L,2N_D-D,L-Aβ40 and 3N_L,3C_L,2N_D-D,L-Aβ40 samples, with 33+k ↔ 32-k, 33+k ↔ 33-k, and 33+k ↔ 34-k registries. Simulations included ¹⁵N-¹³C dipole-dipole couplings to the two nearest-neighbor ¹⁵N labels in each registry, as well as corrections for signal contributions from natural-abundance carbonyl ¹³C spins as described in the Methods section. Simulations for I32 and V36 in 2C_L,2N_D-D,L-Aβ40 fibrils and for I31 and M35 in 3N_L,3C_L,2N_D-D,L-Aβ40 fibrils were added

together, since the corresponding solid state NMR signals were not resolved in the REDOR measurements.

Fig. 5D shows the dependence of the total χ^2 deviation between experimental and simulated REDOR data on the fractions of the three registries in the simulations (see Methods for definition of χ^2). The best agreement between experiments and simulations (*i.e.*, minimum χ^2) occurs with roughly 25% 33+k $\leftrightarrow$ 32-k registry, 15% 33+k $\leftrightarrow$ 33-k registry, and 60% 33+k $\leftrightarrow$ 34-k registry. Simulations for these polymorph populations are compared directly with experimental data in Fig. S3A. Comparisons for several other choices of the polymorph populations, with larger χ^2 values are shown in Figs. S3B–S3D.

The minimum χ^2 value was not reduced when all simulated REDOR S/S_0 values were multiplied by any factor less than one. This indicates that polymorphs with large ^{15}N - ^{13}C distances simultaneously for all carbonyl ^{13}C labels in 2C_L,2N_D-D,L-A β 40 and 3N_L,3C_L,2N_D-D,L-A β 40 fibrils have negligible populations.

Our solid state NMR measurements do not place direct constraints on the β -sheet organization for residues 17–21, which form β -strands according to the chemical shift data (Table 2). The antiparallel β -sheet alignment of residues 30–36 strongly suggests antiparallel β -sheet formation by residues 17–21, which is also supported by the DFT results described below.

Mass-per-length of D,L-A β 40 fibrils

The mass-per-length (MPL) value of a single cross- β motif in an amyloid fibril is MW/d_β , where MW is the molecular weight of the peptide and $d_\beta \approx 0.48$ nm is the spacing between peptide molecules in a β -sheet, assuming that each molecule contributes one β -strand to the β -sheet. For A β 40, $\text{MW}/d_\beta \approx 9.0$ kDa/nm. The MPL value of D,L-A β 40 fibrils can be determined by a quantitative analysis of intensities in dark-field TEM images of unstained samples^{3, 39–41}, as shown in Fig. 7A. The histogram of MPL counts in Fig. 7B indicates a value of 26.6 ± 0.5 kDa/nm, indicating that each D,L-A β 40 fibril contains three cross- β subunits. The width of this histogram does not arise from variations in MPL values of individual fibrils. Rather, this width is due to noise in the dark-field images, as shown by the nearly identical width of the histogram of background fluctuations in Fig. 7C.

Results from DFT calculations

Solid state NMR measurements presented above show that residues 16–21 (KLVFFA) and 31–35 (IIGLM) of L-A β 40 and D-A β 40 participate in cross- β motifs in D,L-A β 40 fibrils. The data are consistent with an alternating, heterochiral (*i.e.*, rippled) antiparallel β -sheet structure, although with several coexisting registries.

In previous work, we used DFT calculations to study mirror-image peptide recognition leading to rippled sheet formation in model systems^{30, 46–47}. Employing similar DFT methods, we performed geometry optimizations for heterochiral dimers in either parallel or antiparallel orientation, for both LVFFA:lvffa and IIGLM:iiglm (with lower-case letters indicating D-amino acids), which are β -strand segments according to their assigned ^{13}C and ^{15}N chemical shifts (Table 2). Viable geometry-optimized low energy conformers are

shown in Fig. 8. Atomic coordinates are given in the Appendix in Supporting Information. A general preference for the antiparallel over the parallel rippled cross- β interface is noted, in agreement with the experimental data. Specifically, we find the antiparallel orientation is preferred by 6.1 kcal/mol and 3.9 kcal/mol for LVFFA:lvffa and IIGLM:iiglm, respectively.

Discussion

Summary of structural constraints

2D and 3D solid state NMR spectra in Figs. 2 and 3 provide evidence for a D,L-A β 40 fibril structure in which residues 16–37 form the cross- β core, with partial disorder or dynamics in residues 23–28. The 2D ^1H - ^{13}C INEPT spectrum in Fig. 4 shows that the N-terminal segments of A β 40, and possibly the C-termini, are dynamically disordered, executing large amplitude motions on sub-microsecond time scales. The relatively poor resolution of the 2D spectra in Fig. 2 is a likely result of internal disorder in residues 23–28 and the N- and C-terminal segments, as well as the polymorphism and symmetry considerations discussed below. Despite this disorder, the chemical shift assignments in Table 2 could be made from the 3D spectra, leading to the identification of residues 17–21 and 30–36 as β -strands.

Data in Figs. 5A and 5B rule out in-register parallel cross- β structures, either with two or more consecutive L-A β 40 molecules (or D-A β 40 molecules) in the hydrogen-bonding direction or with alternation of D-A β 40 and L-A β 40. In-register parallel structures would produce a more rapid PITHIRDS-CT signal decay for D,L-A β 40 fibrils in Fig. 5A or a more rapid build-up of REDOR signal in Fig. 5B. As an alternative, we consider antiparallel cross- β structures, similar to the structure for D23N-L-A β 40 protofibrils determined by Qiang *et al.*⁸, but with the alternation of D-A β 40 and L-A β 40 (*i.e.*, antiparallel rippled sheets). In the D23N-L-A β 40 protofibril structure (Protein Data Bank code 2LNQ), D23N-L-A β 40 molecules adopt U-shaped conformations, with β -strand conformations in residues 16–23 and 30–36 and a loop in residues 24–39. Intermolecular hydrogen-bonding along the fibril growth direction produces a double-layered antiparallel β -sheet structure, with 19+k $\leftrightarrow$ 19-k registry in the “bottom” layer and 33+k $\leftrightarrow$ 33-k registry in the “top” layer. REDOR data for G33 in Fig. 5C test for such a structure. The relatively rapid build-up of S/S_0 for G33 suggests 33+k $\leftrightarrow$ 33-k registry, but involving fewer than half of the D-A β 40 and L-A β 40 molecules. An acceptable fit to REDOR data for G33 and I31/M35 in Fig. 5C and for I32/V36 in Fig. 5B can be obtained by assuming that the D,L-A β 40 samples contain fibrils (or domains within fibrils) with 33+k $\leftrightarrow$ 32-k, 33+k $\leftrightarrow$ 33-k, and 33+k $\leftrightarrow$ 34-k registries, with populations near 25%, 15%, and 60%, respectively (Figs. 5D and S3).

MPL measurements in Fig. 7 provide strong support for an overall fibril structure comprised of three cross- β subunits. Each cross- β subunit contains antiparallel rippled sheets. Our data do not tell us whether all subunits within a single fibril have the same hydrogen bond registry.

Symmetry in D,L-A β 40 fibrils

One might expect the D,L-A β 40 fibril structure to have maximal symmetry, meaning that both D-A β 40 molecules and L-A β 40 molecules have a single conformation and structural

environment, with D and L molecules interchanged by a reflection, followed by some combination of translations and rotations of the overall structure. Maximal symmetry is observed in many experimentally determined structures for amyloid fibrils^{9, 17, 22, 24, 26}, although exceptions exist^{11, 77}.

If the D,L-A β 40 fibril structure contained only a single cross- β layer, there would be many β -sheet structures with maximal symmetry in which D and L molecules alternate along the hydrogen-bonding direction (*i.e.*, the fibril growth direction). Examples include the in-register, parallel β -sheet model and the three antiparallel β -sheet models in Fig. 6A–6D. However, real L-A β 40 and L-A β 42 fibril structures contain more than one β -strand segment, forming more than one cross- β layer^{6–9, 17, 20, 22, 24, 26}. In this case, as depicted in Fig. 6E, an in-register, parallel rippled sheet structure for D,L-A β 40 fibrils loses symmetry because sidechains of a given residue in the D-A β 40 and L-A β 40 molecules point in different directions, either towards or away from the fibril core. Similarly, as depicted in Fig. 6F, antiparallel rippled sheets with $q+k \leftrightarrow q-k$ hydrogen bonding registry, meaning that residue q of a D-A β 40 molecule is hydrogen bonded to residue q of an L-A β 40 molecule for some integer q , lose symmetry when the overall fibril structure contains more than one cross- β layer. In such rippled sheets, inter-strand hydrogen bonds exist only between even-numbered residues or between odd-numbered residues. Again, loss of symmetry results from the different directions for sidechains of a given residue in the D-A β 40 and L-A β 40 molecules.

On the other hand, antiparallel rippled sheets with $q+k \leftrightarrow q-k\pm 1$ registry, meaning that hydrogen bonds exist only between odd-numbered residues of D-A β 40 and even-numbered residues of L-A β 40 (and vice versa) can retain maximal symmetry in a structure with more than one cross- β layer, because sidechains of a given residue in the D-A β 40 and L-A β 40 molecules point in the same direction. In principle, out-of-register parallel rippled sheets, with inter-strand hydrogen bonds from residue k to residues $k-2/k$ or $k/k+2$ for odd or even k could also retain maximal symmetry. To our knowledge, out-of-register parallel β -sheets have not been observed in structural studies of any amyloid fibrils.

Our data indicate partial population of antiparallel rippled sheets with $33+k \leftrightarrow 33-k$ registry in D,L-A β 40 fibril samples. Since, D and L molecules are inequivalent within such structures, two types of fibrils (or domains within fibrils) must exist, one with I32 sidechains of L-A β 40 molecules pointing away from the fibril core, the other with I32 sidechains of D-A β 40 molecules pointing away from the fibril core (for example). The existence of two inequivalent $33+k \leftrightarrow 33-k$ structures, with equal populations, may contribute to the relatively poor resolution of 2D and 3D solid state NMR spectra of U_L-D,L-A β 40 fibrils, along with the other sources of polymorphism and disorder discussed above.

DFT molecular modeling

Using DFT computational methods, we generated the molecular structures of rippled interfaces for the hydrophobic segments LVFFA:lvffa and IIGLM:iiglm (Fig. 7), both of which show an energetic preference for the antiparallel rippled arrangement. While the preference for antiparallel alignment is consistent with the solid state NMR data, it should be kept in mind that more than one structure exists in D,L-A β 40 fibril samples, and also that different A β folds may fit to accommodate the structural elements proposed

here. Furthermore, our past work showed that some of the chiral interactions favoring rippled sheet formation are established in a trimer, but not a dimer in fibril growth dimension⁴⁷. This was attributed to steric pressure between sidechains in the homochiral (*i.e.*, pleated), but not the heterochiral (*i.e.*, rippled) trimer, which led to distortion of hydrogen-bonding networks in the former but not the latter. Future theory work considering larger segments in fibril growth direction may provide a more complete understanding of A β -CI. Interactions between A β molecules orthogonal to fibril growth direction (of which our MPL measurements suggest there are three per layer) may further fine-tune the mirror-image interactions underlying A β -CI. Theoretical studies that take those complex interactions into account would be of great interest, and it would be particularly interesting to have molecular modeling work performed, in which fibril formation is considered explicitly, so as to gain insights into kinetics of A β -CI.

Concluding remarks

Evidence for rippled sheets has been obtained in previous studies of amyloid-like fibrils formed by racemic mixtures of the designed peptides MAX1^{49, 78} and KFE8⁵⁸, as well as in fibrils formed by racemic Ac-KLVFFAE-NH₂⁵⁹. Support for coassembly of D and L molecules in these studies comes from electron microscopy^{49, 58–59}, fluorescence resonance energy transfer⁵⁸, calorimetry⁵⁸, equilibrium solubilities⁵⁹, circular dichroism⁷⁸, and rheology^{49, 78}. Support for alternation of D and L molecules within β -sheets, *i.e.*, for rippled sheets, comes primarily from isotope-edited infrared spectroscopy^{49, 58–59}, and solid state NMR^{49, 59}. Very recently, Grelich-Mucha *et al.*⁷⁹ have used infrared and fluorescence spectroscopies and molecular dynamics simulations to support an antiparallel rippled sheet structure in fibrils formed by a racemic mixture of peptides with the sequence YTIAALLSPYS (residues 105–115 of transthyretin). Homochiral YTIAALLSPYS fibrils were previously shown to contain in-register, parallel β -sheets⁸⁰.

Our results for D,L-A β 40 fibrils extend these previous studies in several important ways. First, A β 40 is a naturally occurring peptide with a higher molecular weight, more diverse amino acid composition, and greater conformational complexity than peptides in the previous studies. Our results in support of rippled sheets in D,L-A β 40 fibrils suggest that rippled sheet formation is a generic property of racemic mixtures of amyloidogenic peptides, rather than a phenomenon that might be limited to peptides with designed features or short sequences. Our results also suggest that antiparallel rippled sheets may generally be thermodynamically preferred over parallel rippled sheets, even for sequences that prefer in-register, parallel cross- β structures in homochiral samples. Moreover, our solid state NMR data show that, like many homochiral amyloid fibrils, fibrils formed by racemic mixtures of amyloidogenic peptides can exhibit molecular-level structural polymorphism, including polymorphism based on variations in hydrogen bond registry within antiparallel rippled sheets. In addition to contributing to our understanding of A β -CI and to racemic peptide assemblies in general, these structural findings may contribute to the further development of amyloid inhibitors based on D-amino acids^{52–55}.

The fact that D,L-A β 40 fibrils and L-A β 40 fibrils contain qualitatively different cross- β structures is likely to be a consequence of differences in sidechain packing and sidechain-

sidechain interactions. In homochiral fibrils, the in-register, parallel cross- β structure is favored by intermolecular interactions that depend on linear rows of like residues, including sidechain hydrogen bonds among Gln residues⁸¹, π -stacking of aromatic sidechains⁸², and hydrophobic interactions. These interactions may not be available in rippled sheets, shifting the energetic balance toward antiparallel structures. Future studies by theory and experimental methods may further elucidate the interactions that favor specific rippled sheet structures in fibrils formed by racemic mixtures of amyloidogenic peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1: Negative-stain TEM images of D,L-Aβ40 fibrils. (A,B) 3N_L,3C_L-D,L-Aβ40 fibrils after 16 h and 100 h of incubation, respectively. (C,D) U_L-D,L-Aβ40 fibrils after 120 h of incubation. In both cases, fibrils were grown under quiescent conditions in PBS at 24° C and pH 7.4 with a total peptide concentration of 40 μM. Peptides were disaggregated in 20 mM NaOH prior to mixing and dilution into PBS.

Figure 2: 2D ^{13}C - ^{13}C (A), NCACX (B) and NCOCX (C) solid state NMR spectra of U_L -D,L- $\text{A}\beta$ 40 fibrils. 1D slices above each 2D spectrum are taken at positions indicated by dashed lines. Contour levels increase by successive factors of 1.4. Crosspeak assignments were obtained from 3D solid state NMR spectra (see Fig. 3).

Figure 3:

Examples of 2D planes from 3D solid state NMR spectra of U_L -D,L- $A\beta$ 40 fibrils. ^{13}C - ^{13}C planes from 3D NCACX (A,B), NCOCX (C,D), and CANCEX (E,F) spectra are shown at ^{15}N chemical shifts of 120.5 ppm (A,C,E) and 126.6 ppm (B,D,F). 1D slices in each panel are taken at positions indicated by dashed lines. Contour levels increase by successive factors of 1.4. Assignments are shown for crosspeaks with ^{15}N chemical shifts within ± 1.5 ppm of the indicated values.

Figure 4:

(A) 2D ^1H - ^{13}C INEPT spectrum of U_L -D,L- $\text{A}\beta$ 40 fibrils, showing signals from residues that remain dynamically disordered. Residue-type assignments are based on standard random-coil chemical shifts. The 1D slice above the 2D spectrum is taken at the position indicated by the dashed line. Contour levels increase by successive factors of 1.4. (B) $\text{A}\beta$ 40 sequence, with β -strand segments in blue, additional residues with assigned solid state NMR signals in purple, and the dynamically disordered N-terminal segment in red.

Figure 5:

(A) Measurements of ^{13}C - ^{13}C dipole-dipole couplings in $3\text{N}_\text{L}, 3\text{C}_\text{L}$ -D,L-Aβ40 fibrils (blue circles) and L-Aβ40 fibrils with ^{13}C labels at carbonyl sites of I31, G33, and M35 (red triangles), using the PITHIRDS-CT solid state NMR technique. Signal amplitudes are normalized to 100 at an evolution time of zero. Error bars are uncertainties due to the root-mean-squared noise in the experimental spectra. Solid, dashed, dotted, and dash-dotted lines are numerical simulations of PITHIRDS-CT data for linear chains of ^{13}C nuclei with the indicated internuclear distances. (B) Measurements of ^{15}N - ^{13}C dipole-dipole couplings in $2\text{C}_\text{L}, 2\text{N}_\text{D}$ -D,L-Aβ40 (blue triangles and red squares for fully hydrated and lyophilized fibrils, respectively), using the ^{13}C -detected REDOR solid state NMR technique. REDOR data for Ac-KLVFFAE-NH₂ fibrils with amide ^{15}N and carbonyl ^{13}C labels at V3 and F5 are also shown (purple circles). Solid and dotted lines are REDOR simulations for hypothetical antiparallel and in-register parallel rippled sheet structures, respectively, for D,L-Aβ40 fibrils. The dashed line is a REDOR simulation for Ac-KLVFFAE-NH₂ fibrils with their known intermolecular V3-F5 hydrogen bonds. (C) REDOR data for $3\text{N}_\text{L}, 3\text{C}_\text{L}, 2\text{N}_\text{D}$ -D,L-Aβ40 fibrils. Results for the carbonyl ^{13}C signals of G33 (red squares) and I31/M35 (unresolved, blue circles) are plotted separately. Lines are two-spin REDOR simulations for the indicated ^{15}N - ^{13}C distances, scaled by a factor of 0.38. (D) Contour plot of the χ^2 deviation between experimental REDOR data for D,L-Aβ40 fibrils and simulations in which

the relative populations of antiparallel rippled sheets with three possible hydrogen bond registries were varied.

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Figure 6:

Models for β -sheets formed by residues 29–37 of A β 40 (sequence GAIIGLMVG), with alternation between D and L molecules along the intermolecular hydrogen bonding direction. (A) In-register, parallel β -sheet model. Carbon-carbon distance d_1 is 4.8 Å. Nitrogen-carbon distances d_2 and d_3 are 4.1 Å and 5.8 Å, respectively. (B) Antiparallel β -sheet model with 33+k $\leftrightarrow$ 33-k registry. Nitrogen-carbon distances d_4 and d_5 from the carbonyl carbon of G33 in L-A β 40 are 4.1 Å and 5.9 Å. Distances d_6 and d_7 from the carbonyl carbon of I32 are 6.0 Å and 6.4 Å. (C,D) Antiparallel β -sheet models with 33+k $\leftrightarrow$ 32-k and 33+k $\leftrightarrow$ 34-k registry. Distances d_8 and d_{10} are 6.4 Å, and d_9 and d_{11} are 6.0 Å. With 33+k $\leftrightarrow$ 32-k registry, d_{10} and d_{11} are distances from the carbonyl carbon of I31. With 33+k $\leftrightarrow$ 34-k registry, d_{10} and d_{11} are distances from the carbonyl carbon of M35. (E) Cartoon representation of an in-register, parallel cross- β motif comprised of alternating D and L peptides with S-shaped conformations. (F) Cartoon representation of an antiparallel cross- β motif comprised of alternating D and L peptides with U-shaped conformations.

Figure 7:

(A) Example of a dark-field TEM image of unstained D,L-Aβ40 fibrils. MPL values were determined from integrated image intensities within $100 \text{ nm} \times 40 \text{ nm}$ areas (yellow rectangles) after subtraction of integrated background intensities (green rectangles). Intensities were calibrated by similar measurements on co-adsorbed tobacco mosaic virus rods (TMV). (B) Histogram of MPL values, obtained from 163 fibril segments and 43 TMV segments in measurements in 27 dark-field images. Blue line is a best-fit Gaussian curve, centered at $26.6 \pm 0.5 \text{ kDa/nm}$ with $15.8 \pm 1.2 \text{ kDa/nm}$ full-width-at-half-maximum. Vertical green lines indicate ideal MPL values for hypothetical structures with 1–5 cross-β subunits. (C) Histogram of the expected errors in MPL values due to background noise fluctuations in individual dark-field images. Blue line is a best-fit Gaussian curve, centered at 0.0 kDa/nm with $16.6 \pm 1.0 \text{ kDa/nm}$ full-width-at-half-maximum.

Figure 8:

DFT-optimized partial structures corresponding to the rippled LVFFA:lvffa (top) and IIGLM:iiglm (bottom) in either antiparallel (left) or parallel (right) orientation. In each case, two views are shown, rotated by 90° about a horizontal line. Structures are shown in both stick and space-filling representations. Relative energies E_{rel} are given in kcal/mol.

Table 1: Isotopic labeling patterns in D,L-A β 40 fibril samples

fibril sample	labeling of L-A β 40	labeling of D-A β 40	solid state NMR measurements
U _L -D,L-A β 40	uniformly ¹⁵ N and ¹³ C	none	2D ¹³ C- ¹³ C, 2D NCACX, 2D NCOCX, 2D ¹ H- ¹³ C INEPT, 3D NCACX, 3D NCOCX, 3D CONCA
3N _L ,3C _L -D,L-A β 40	¹⁵ N at L17, F19, A21; carbonyl ¹³ C at I31, G33, M35	none	¹³ C PITHIRDS-CT
2C _L ,2N _D -D,L-A β 40	carbonyl ¹³ C at I32, V36	¹⁵ N at G33, G37	¹⁵ N- ¹³ C REDOR
3N _L ,3C _L ,2N _D -D,L-A β 40	¹⁵ N at L17, F19, A21; carbonyl ¹³ C at I31, G33, M35	¹⁵ N at G33, G37	¹⁵ N- ¹³ C REDOR

Table 2:

^{13}C and ^{15}N NMR chemical shifts for D,L-A β 40 fibrils, determined from 3D solid state NMR spectra. ^{13}C shifts are in parts per million relative to DSS. ^{15}N shifts are in parts per million relative to liquid ammonia. Predicted backbone ϕ, ψ torsion angles from the TALOS-N program are also shown in degrees, along with uncertainties reported by TALOS-N. Only predictions classified by TALOS-N as “strong” are shown

	CA	CB	CD	CG	CG1	CG2	CO	N	ϕ	ψ
K16	54.6	-	-	-	-	-	173.2	-		
L17	53.25	45.1	25.14	26.67	-	-	174.8	126.8	-110 $\pm$ 14	137 $\pm$ 10
V18	59.65	35.36	-	-	21.48	20.79	172.7	119.7	-127 $\pm$ 13	141 $\pm$ 12
F19	55.45	40.48	-	-	-	-	172.4	126.4	-106 $\pm$ 10	125 $\pm$ 7
F20	55.54	42.54	-	-	-	-	172.3	129.5	-117 $\pm$ 11	135 $\pm$ 10
A21	49.71	23.36	-	-	-	-	-	126.8	-129 $\pm$ 14	148 $\pm$ 11
E22	53.94	33.9	181.8	36.77	-	-	-	120		
G29	44.86	-	-	-	-	-	170.1	-		
A30	50.36	21.68	-	-	-	-	174.6	120.7	-129 $\pm$ 14	148 $\pm$ 10
I31	57.38	40.15	-	-	26.78	16.79	173.8	120.6	-117 $\pm$ 14	140 $\pm$ 11
I32	58.26	41.41	-	-	26.86	17.07	173.3	124.3	-129 $\pm$ 10	143 $\pm$ 8
G33	44.72	-	-	-	-	-	170.2	111.9	-159 $\pm$ 25	172 $\pm$ 19
L34	52.84	46.05	-	26.78	-	-	-	122.8	-127 $\pm$ 13	143 $\pm$ 11
M35	54.01	37.78	-	31.2	-	-	173.7	119.3	-129 $\pm$ 9	148 $\pm$ 12
V36	58.58	35.78	-	21.17	-	-	173.6	116.9	-132 $\pm$ 9	154 $\pm$ 10
G37	45.24	-	-	-	-	-	-	112.1		