Supramolecular Structural Constraints on Alzheimer’s β-Amyloid Fibrils from Electron Microscopy and Solid-State Nuclear Magnetic Resonance†

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Received June 14, 2002; Revised Manuscript Received October 15, 2002

ABSTRACT: We describe electron microscopy (EM), scanning transmission electron microscopy (STEM), and solid-state nuclear magnetic resonance (NMR) measurements on amyloid fibrils formed by the 42-residue β-amyloid peptide associated with Alzheimer’s disease (Aβ1-42) and by residues 10–35 of the full-length peptide (Aβ10-35). These measurements place constraints on the supramolecular structure of the amyloid fibrils, especially the type of β-sheets present in the characteristic amyloid cross-β structural motif and the assembly of these β-sheets into a fibril. EM images of negatively stained Aβ10-35 fibrils and measurements of fibril mass per length (MPL) by STEM show a strong dependence of fibril morphology and MPL on pH. Aβ10-35 fibrils formed at pH 3.7 are single “protofilaments” with MPL equal to twice the value expected for a single cross-β layer. Aβ10-35 fibrils formed at pH 7.4 are apparently pairs of protofilaments or higher order bundles. EM and STEM data for Aβ1-42 fibrils indicate that protofilaments with MPL equal to twice the value expected for a single cross-β layer are also formed by Aβ1-42 and that these protofilaments exist singly and in pairs at pH 7.4. Solid-state NMR measurements of intermolecular distances in Aβ10-35 fibrils, using multiple-quantum 13C NMR, 13C–13C dipolar recoupling, and 1H–13C dipolar recoupling techniques, support the in-register parallel β-sheet organization previously established by Lynn, Meredith, Botto, and co-workers [Benzinger et al. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 13407–13412; Benzinger et al. (2000) Biochemistry 39, 3491–3499] and show that this β-sheet organization is present at pH 3.7 as well as pH 7.4 despite the differences in fibril morphology and MPL. Solid-state NMR measurements of intermolecular distances in Aβ1-42 fibrils, which represent the first NMR data on Aβ1-42 fibrils, also indicate an in-register parallel β-sheet organization. These results, along with previously reported data on Aβ1-40 fibrils, suggest that the supramolecular structures of Aβ10-35, Aβ1-40, and Aβ1-42 fibrils are quite similar. A schematic structural model of these fibrils, consistent with known experimental EM, STEM, and solid-state NMR data, is presented.

Amyloid fibrils are filamentous structures formed by a variety of peptides and proteins with disparate lengths and amino acid sequences but sharing certain morphological, tectorial, and molecular structural features (1). Interest in amyloid fibrils from the biomedical perspective stems from their involvement in amyloid diseases, including Alzheimer’s disease, type 2 diabetes, Parkinson’s disease, Huntington’s disease, and transmissible spongiform encephalopathies (2). Amyloid fibrils associated with Alzheimer’s disease are formed in vivo by the β-amyloid peptide (Aβ), a peptide with length ranging from 39 to 43 residues due to variations in the C-terminal cleavage site (3–5). Additional interest from the perspectives of biophysical chemistry and structural biology stems from the recent realization that the amyloid fibril is a stable state of many, perhaps most, polypeptides under appropriate conditions, including polypeptides that have stable globular structures as monomers or multimers and are not associated with known amyloid diseases (6, 7).

At the resolution of electron microscope (EM) images, amyloid fibrils formed by different polypeptides are remarkably similar, generally appearing as unbranched filaments with diameters of 5–10 nm and lengths exceeding 1 µm.

† Abbreviations: Aβ, β-amyloid; EM, electron microscope; NMR, nuclear magnetic resonance; Aβm,n, residues m through n of the full-length β-amyloid peptide; MQ, multiple quantum; MPL, mass per length; STEM, scanning transmission electron microscope; 1D, one dimensional; FmOC, fluorenlymethoxy carbonyl; TFA, trifluoroacetic acid; TBME, tert-butyl methyl ether; HPLC, high-performance liquid chromatography; TMV, tobacco mosaic virus; MAS, magic angle spinning; TMS, trimethylsilane; rf, radio frequency; fpRFDR-CT, constant-time finite-pulse radio frequency-driven recoupling; REDOR, rotational echo double resonance; PSL, pulsed spin locking; AFM, atomic force microscopy; fwhm, full width at half-maximum; DRAWS, dipolar recoupling with a windowless sequence; SANS, small angle neutron scattering; PEG, poly(ethylene glycol).

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Until recently, direct structural information at higher resolution has been scarce because of the inherent insoluble and noncrystalline nature of these materials. X-ray diffraction data on unaligned fibrils and aligned fibril bundles (8, 9) have shown that a principal and defining molecular structural feature of all amyloid fibrils is the presence of β-sheets spanning the length of the fibril that are formed by β-strand segments that run approximately perpendicular to the long axis of the fibril and are connected by intermolecular hydrogen bonds approximately parallel to the long axis. This orientation of β-sheets is commonly called a cross-β structure.

Recent experiments by Lansbury, Griffin, and co-workers (10–12), by Lynn, Meredith, Botto, and co-workers (13–16), and by us (17–21) have demonstrated that important molecular level structural constraints on amyloid fibrils can be obtained from solid-state nuclear magnetic resonance (NMR) measurements. These measurements require neither crystallinity nor solubility. With appropriate isotopic (e.g., 13C or 15N) labeling, solid-state NMR techniques provide quantitative constraints on the supramolecular organization of β-sheets in amyloid fibrils (11, 13–18, 20) and on the torsion angles and interatomic distances that define the peptide conformation in a fibril (11, 12, 14, 16, 18, 21). Solid-state NMR measurements also permit the characterization of the degree of structural order in amyloid fibrils and have demonstrated that amyloid fibrils contain regions in which both side chain and backbone atoms have nearly crystalline order (18, 21, 22).

An important result from solid-state NMR, originally reported by Lynn, Meredith, Botto, and co-workers (13), was the discovery that β-sheets in fibrils formed by residues 10–35 of Aβ (i.e., Aβ10–35) have an in-register parallel organization, rather than the antiparallel organization indicated for Aβ fibrils by earlier infrared spectroscopic data (23–25) and incorporated into structural models of Aβ fibrils (26–30). This result was extended by Antzutkin et al. to fibrils formed by the full-length 40-residue amyloid peptide (Aβ1–40), which were also found to have an in-register parallel organization of β-sheets by multiple-quantum (MQ) solid-state NMR (17, 31) and by 13C–15N dipolar recoupling measurements (20). In contrast, Lansbury, Griffin, and co-workers found that fibrils formed by residues 34–42 of the 42-residue form of Aβ (Aβ34–42) contain antiparallel β-sheets (11), and Balbach et al. found that fibrils formed by residues 16–22 (Aβ16–22) also contain antiparallel β-sheets (18). Taken together, these solid-state NMR data on Aβ fibrils demonstrate that the supramolecular organization of amyloid fibrils is not universal and is not uniquely determined at the level of short (e.g., seven-residue) peptide segments.

This paper presents additional constraints on the supramolecular structure of amyloid fibrils formed by Aβ10–35 and by the 42-residue variant of full-length Aβ (Aβ1–40), obtained from both EM and solid-state NMR measurements, including the first solid-state NMR data on Aβ1–42 fibrils. Figure 1 shows the sequences of the peptides Aβ10–35, Aβ10–35Scr, and Aβ1–42 examined in this work. Aβ1–42 is the form of Aβ that is normally present at the second highest concentrations (after Aβ1–40) in humans. Aβ1–42 has been shown to be the primary component of immature amyloid plaques (32, 33). Genetic mutations associated with a predisposition toward Alzheimer’s disease result in elevated Aβ1–42 levels (34). In vitro, Aβ1–42 has been shown to fibrillize more rapidly and at lower concentrations than Aβ1–40 (35, 36). It has been unclear whether the properties of Aβ1–42 reflect distinct structural features of Aβ1–42 fibrils, relative to Aβ1–40 and Aβ10–35 fibrils, especially in light of the antiparallel β-sheet organization determined for Aβ34–42 (11).

Aβ10–35 is a model system for studies of Aβ fibrils (37–39) with the important features that it retains the central hydrophobic segment (residues 17–21) and a portion of the C-terminal hydrophobic segment (residues 29–35) of Aβ1–40 and Aβ1–42. The first 10–12 residues at the N-terminus of full-length Aβ are known to be susceptible to proteolytic cleavage (40–43), and solid-state NMR measurements indicate that the N-terminal residues are disordered in Aβ1–40 fibrils (17, 20, 21). It therefore appears likely that the N-terminal residues deleted in Aβ10–35 do not play an important structural role in the amyloid fibrils. Aβ10–35Scr has the same amino acid composition as Aβ10–35, but the sequence of residues 11–20 is “scrambled” so that there are no contiguous hydrophobic residues. The hydrophobic segment spanning residues 17–21 has been shown to be important for fibrillization of Aβ10–35 (38), Aβ1–42 (44), and other Aβ peptides (45). This hydrophobic segment is destroyed in Aβ10–35Scr, while the C-terminal hydrophobic segment is preserved. Aβ10–35Scr serves as a control for EM and solid-state NMR measurements.

Data presented in this paper include (1) EM images of Aβ10–35 that demonstrate a strong dependence of fibril morphology on pH, (2) measurements of the mass per length (MPL) of Aβ10–35 fibrils by scanning transmission electron microscopy (STEM) that suggest the existence of a profilament constructed from two cross-β units (see Results for definitions of these terms), (3) EM images and MPL measurements on Aβ1–42 fibrils that also suggest a profilament constructed from two cross-β units, (4) one-dimensional (1D) solid-state 13C NMR spectra of Aβ10–35 and Aβ1–42 fibrils with 13C labels at specific sites, demonstrating characteristic changes in 13C NMR line shapes that result from amyloid fibrillization and correlate with fibril morphology and MPL, (5) MQ, 13C–15N dipolar recoupling, and 15N–13C dipolar recoupling solid-state NMR measurements on Aβ10–35 fibrils that support an in-register parallel organization of β-sheets formed by the central and C-terminal hydrophobic segments, and (6) 13C–13C and 15N–13C dipolar recoupling measurements on Aβ1–42 fibrils that support a parallel organization of β-sheets and demonstrate that the antiparallel β-sheet structure in Aβ34–42 fibrils is not retained by the C-terminal residues in fibrils formed by full-length Aβ1–42. In the final section of this paper, we summarize the
experimental EM, STEM, and solid-state NMR constraints on supramolecular structure by presenting a schematic structural model for \( \beta_{10-35} \) and \( \beta_{1-42} \) fibrils (also applicable to \( \beta_{1-40} \) fibrils) that is consistent with these constraints.

**MATERIALS AND METHODS**

**Peptide Synthesis and Fibrillization.** \( \beta \) peptides were synthesized on an Applied Biosystems model 433A automated synthesizer using standard fluorenlymethoxycarbonyl (FMOC) protocols with \( H \)-benzotriazol-1-yl-tetramethyluronium hexafluorophosphate activation. \( ^{13} \)C- and \( ^{15} \)N-labeled amino acids with FMOC protection were obtained from Cambridge Isotopes Laboratories and Isotec. \( \beta_{1-42} \) was synthesized on preloaded Wang resins. \( \beta_{10-35} \) and \( \beta_{10-35,\text{Scr}} \) were synthesized on Rink amide resins. Peptides were cleaved from the synthesis resins with a solution of phenol, ethanediolith and thioanisole in 95% trifluoroacetic acid (TFA) for approximately 90 min and then precipitated from the cleavage solution in cold tert-butyl methyl ether (TBME). Precipitated peptides were washed several times with cold TBME, dissolved in water, and lyophilized. Peptides were then purified by high-performance liquid chromatography (HPLC) at 55°C using a Vydac C18 column and a water/acetonitrile gradient with 0.1% TFA. The purity of \( \beta_{1-42} \) determined by electrospray mass spectrometry was 93%. For \( \beta_{10-35} \), electrospray mass spectra showed a species with mass 17 units less than expected, comprising up to 15% of the product. This species was found to be entirely absent from mass spectra obtained with matrix-assisted laser desorption ionization, which indicate >95% purity. Final yields of purified peptides were roughly 50% of the theoretical maximum for \( \beta_{10-35} \) and 15% for \( \beta_{1-42} \). After lyophilization of HPLC fractions containing purified peptide, peptides were fibrillized by dissolution in water at approximately 0.2 mM concentration for \( \beta_{10-35} \) and 0.1 mM concentration for \( \beta_{1-42} \), with the pH adjusted as necessary by dropwise addition of dilute NaOH or acetic acid and with addition of 0.01% NaN\(_3\). Peptide solutions were incubated at room temperature for 3–5 days with gentle rocking. Aliquots of incubated solutions were taken for EM measurements after 3–5 days. Amyloid fibril formation was verified by EM images of negatively stained samples and by solid-state NMR spectroscopy (see Results). Incubated solutions were lyophilized for solid-state NMR measurements. Solid-state NMR samples contained 3–10 mg of peptide. Measurements on unfibrillized peptides were performed directly on the lyophilized, HPLC-purified material, without incubation.

**Electron Microscopy.** For EM images, carbon film substrates were prepared by evaporation from a carbon rod source onto freshly cleaved mica in an Edwards Auto 306 coating system. Films were floated off in deionized water and picked up on lacy Formvar/carbon films (EM Sciences) supported on 200 mesh copper grids. Grids were glow-discharged in air prior to peptide deposition. Aliquots (5 \( \mu \)L) of incubated or fibrillized peptide solution were applied to EM grids and allowed to adsorb for 2 min. Grids were then washed 10 times by applying successive drops of deionized water and drawing off the excess liquid before negative staining by passing through two drops of 1% uranyl acetate. Excess fluid was blotted off and grids were allowed to dry in air. Transmission electron micrographs were recorded using a Philips/FEI CM120 electron microscope and Gatan GIF100 imaging filter equipped with a cooled slow-scan CCD camera. Images were acquired and processed by means of the Digital Micrograph program (Gatan).

MPL measurements were performed by scanning transmission electron microscopy (46). Aliquots of fibrillized peptide solutions were applied to grids covered by 3 nm carbon films and allowed to adsorb for 8 min. A mass calibration standard of tobacco mosaic virus (TMV) at a concentration of 0.4 \( \mu g/\mu L \) was injected into the solutions and allowed to adsorb for 2 min. Grids were then washed 10 times. After partial blotting to maintain a thin layer of water, the grids were immediately frozen by plunging into liquid ethane at −180 °C by means of a Leica KF80 freezing device. Specimens were transferred at −170 °C into a VG Microscopes HB501 field-emission STEM and were freeze-dried by warming slowly to −100 °C. After recoiling the specimens, annular dark-field images containing 1024 × 1024 pixels were acquired at an accelerating voltage of 100 kV using a 1 nm probe diameter and a current of 2 pA to provide an electron dose of approximately 10\(^5\) e/nm\(^2\). Images were quantified using the NIH IMAGE program. MPL values were determined from the STEM images as described in the Results.

**Solid-State NMR Spectroscopy.** NMR measurements were performed on Varian/Chemagnetics Infinity-400 spectrometers, operating at \( ^{13} \)C NMR frequencies of 100.4 and 100.8 MHz. Varian/Chemagnetics magic-angle spinning (MAS) probes with 3.2 mm diameter rotors were used in all measurements. One-dimensional \( ^{13} \)C NMR spectra were obtained with cross-polarization and with proton decoupling at 100–120 kHz. \( ^{13} \)C chemical shifts are referenced to an external adamantane sample, with the chemical shift of the methylene \( ^{13} \)C NMR line taken to be 38.56 ppm relative to tetramethylsilane (TMS). MQ NMR data were obtained without MAS as previously described (47, 48), using the MQ excitation pulse sequence of Suter et al. (17), with the MQ excitation pulse sequence of Suter et al. (48) to excite all MQ orders and starting with transverse \( ^{13} \)C magnetization. Radio frequency (rf) pulse lengths in the MQ excitation sequence were adjusted to maximize the amplitude of high-order MQ signals from a polycrystalline \( [^{13} \text{C}]_{-\text{L-methionine}} \) sample immediately before MQ NMR data were acquired on \( \beta \) samples. \( ^{13} \)C–\( ^{13} \)C dipolar recoupling measurements were performed with the constant-time finite-pulse radio frequency-driven recoupling (fpRFDR-CT) technique of Ishii et al. (49), at a MAS frequency of 20.0 kHz and with \( \pi/2 \) and \( \pi \) pulse lengths of 5 and 15 \( \mu s \), respectively. In the notation of Ishii et al. (49), fpRFDR-CT data were acquired with an initial \( N \) value of 64, decremented in units of 4, and with \( K = 4 \), leading to a total recoupling period of 76.8 ms and effective dipolar dephasing periods from 0 to 72 ms in increments of 4.8 ms. Proton decoupling fields during the recoupling period were 100–120 kHz. \( ^{13} \)C rf pulse amplitudes in the fpRFDR-CT sequence were optimized by maximizing the signal from a polycrystalline [\( ^{13} \text{C}_{-\text{L-alanine}} \) setup sample at \( N = 64 \). \( ^{13} \)C-detected, \( ^{15} \text{N}–^{13} \text{C} \) rotational echo double resonance (REDOR) data were acquired with the pulse sequence of Anderson et al. (50, 51) at a MAS frequency of 5.0 kHz. \( ^{13} \)C and \( ^{15} \text{N} \) pulse lengths in the REDOR dephasing period were 10.0 \( \mu s \). Pulse amplitudes were optimized by maximizing the REDOR dephasing effect on a polycrystalline [\( ^{13} \text{C}_{,^{15} \text{N}} \text{glycine} \) setup
sample. Proton decoupling fields during the dephasing period were 100 kHz.

Possible artifacts due to spectrometer instabilities were minimized in all experiments by block averaging. RF pulse sequences were actively synchronized with a MAS tachometer signal in fpRFDR-CT and REDOR experiments. Total signal acquisition times for each Aβ sample were approximately 100 h for MQ NMR data, 12–24 h for fpRFDR-CT data, and 12–36 h for REDOR data, with recycle delays of 1.5–4 s. The sensitivities of fpRFDR-CT and REDOR measurements, but not MQ NMR measurements, were enhanced by pulsed spin lock (PSL) detection (52).

Simulations of Solid-State NMR Data. MQ NMR data were simulated by numerical quantum mechanical calculations as previously described (17, 47), representing the candidate β-sheet organizations by six 13C spins (representing the 13C labels) at positions indicated in Figure 6, with three additional spins placed at random positions in a rectangular box around the labels with a volume of 15 nm3 to represent natural-abundance 13C nuclei close to the labels. D1 was set to 0.48 nm, the interchain spacing in both parallel and antiparallel β-sheets. D2 was set to 1.02 nm, the estimated distance between labeled Ala21 sites in a hypothetical in-register antiparallel β-sheet structure. Additional displacements above and below the plane of the β-sheets were set to 0.15 nm for the methyl 13C labels in simulations. MQ NMR simulations were averaged over 1024 orientations and random configurations of the three additional spins. Natural-abundance 13C nuclei that are more distant from the 13C labels also contribute to the experimental MQ NMR signals but cannot be included in the quantum mechanical simulations (which become prohibitively time-consuming for systems of more than nine spins). Experiments on unlabeled peptide samples indicate that natural-abundance 13C nuclei only contribute significantly to one-quantum and two-quantum signals (because dipole-coupled groups of more than two natural-abundance 13C nuclei are rare). To account for these contributions, quantities q1 and q2 were added to the simulated one-quantum and two-quantum signal amplitudes in Figure 8, with q1/q2 = 25, 13, and 9 for simulations at Tmøø = 4.8, 9.6, and 14.4 ms, respectively, and with q1 + q2 set to 24% of the sum of all MQ amplitudes resulting from the nine-spin numerical simulations. These values of q1/q2 represent typical ratios observed in MQ NMR measurements on unlabeled peptide samples, and the value of q1 + q2 was determined from the ratio of unlabeled aliphatic carbons to labeled sites in Aβ10–35, assuming a 1.1% natural abundance of 13C at the unlabeled sites. This approximate correction for MQ NMR signals from distant natural-abundance 13C nuclei improves the agreement between experimental and simulated one-quantum amplitudes in Figure 8 but has no effect on the interpretation of the MQ NMR data in terms of the β-sheet organization in amyloid fibrils.

Simulations of fpRFDR-CT data in Figure 9c were also carried out by numerical quantum mechanical calculations, assuming six 13C spins in a linear chain and averaging over 512 orientations. These simulations were performed at the average Hamiltonian level, assuming the average dipole–dipole Hamiltonian given by Ishii et al. (49). Average Hamiltonian calculations for fpRFDR-CT data include effects due to finite π pulses (which give rise to homonuclear dipolar recoupling in the limit of high-speed MAS) but treat the π/2 pulses in the fpRFDR-CT sequence as instantaneous rotations. Simulations of REDOR data in Figure 10a were also performed at the average Hamiltonian level, including a spin system of one 13C nucleus and two 15N nuclei with only heteronuclear dipole–dipole couplings. REDOR simulations were averaged over 4096 orientations.

Contributions of Background Signals to REDOR and fpRFDR-CT Data. 13C NMR signals from natural-abundance 13C nuclei and from residual unifibrillated peptide molecules contribute to both REDOR and fpRFDR-CT data. Because NMR signals in these measurements were detected under PSL (52), signals from the 13C-labeled sites were not fully resolved from natural-abundance signals from unlabeled sites. Comparisons of NMR peak areas in 13C MAS spectra of Aβ10–35 fibrils obtained with and without PSL indicate that all carbonyl (both labeled and unlabeled), all carboxyl, and all aromatic carbon sites (total of 55 sites) contribute to the REDOR data on Aβ10–35 fibrils in Figure 10a. All methyl (both labeled and unlabeled) sites and approximately nine other natural-abundance aliphatic carbon sites (total of 26 sites) contribute to the fpRFDR-CT data in Figure 9a.

The REDOR data plotted in Figure 10 can be expressed as

\[
\Delta S/S_0 = \frac{(S_{0,1f,0} + S_{0,na,d}) - (S_{1f,0} - S_{na,d})}{S_{0,1f,0} + S_{0,na,d}} \left(\frac{S_{0,1f,0} + S_{0,na,d}}{S_{tot}}\right)
\]

or

\[
\Delta (S/S_0) = \delta S_0 F
\]

\[
S_{tot} = S_{0,1f,0} + S_{0,1u,0} + S_{0,na,d} + S_{0,na,u}
\]

where \(S_{1f,0}\) is the 13C NMR signal from labeled sites in fibrillized peptides, \(S_{1u,0}\) is the signal from labeled sites in unfibrillized peptides, \(S_{0,na,d}\) is the signal from natural-abundance sites that are sufficiently close to 15N-labeled sites to be dephased by 15N–13C couplings, and \(S_{na,u}\) is the signal from natural-abundance sites that are more distant from 15N labels. Subscripts \(x = 1\) and \(x = 0\) indicate signals with and without 15N pulses. \(\Delta (S/S_0)\) is the REDOR data that would be obtained in the absence of signal contributions from undeveloped natural-abundance 13C and from undeveloped 13C labels in unfibrillized peptides. \(F\) is the scaling factor that relates data for this ideal case to the actual experimental data. Given the 1.1% natural abundance of 13C, given that Aβ10–35 contains 55 carbonyl, carboxyl, and aromatic sites, and given that 50% of the Aβ10–35 molecules in the REDOR measurements in Figure 10a contain a single 13C label, we calculate \(S_{1f,0} + S_{1u,0})/S_{tot} = 0.46\) and \((S_{0,na,d} + S_{na,u})/S_{tot} = 0.54\). To determine the fraction of natural-abundance 13C signal that is dephased under the experimental conditions in Figure 10a, separate REDOR measurements were performed on an Aβ10–35 fibril sample with a single 15N label at F20 and no 13C labels (data not shown). In these measurements, 18 ± 4% of the 13C signal was dephased. Taking into account the 1:1 mixing of 15N-labeled and 13C-labeled peptides in Figure 10a, we estimate \(S_{0,na,d} \approx 0.09(S_{0,na,d} + S_{na,u})\). Assuming that approximately 20% of the Aβ10–35 molecules are unfibrillized, so that \(S_{1f,0} \approx 0.80(S_{1f,0} + S_{1u,0})\), we then calculate \(F \approx 0.41\). This estimate for \(F\) is in excellent agreement with the results in Figure 10a, in which a scaling factor of 0.43 is applied to simulations (which do not include signal contribu-
ions from un fibrillized peptides and from natural-abundance $^{13}$C) in order to optimize the fit to experimental data.

Experimental data in fpRFDR-CT measurements can be expressed as the sum of four contributions as in eq 2, but with $S_{0,\text{nat}}^{0,\text{u}}$ and $S_{0,\text{nat}}^{0,\text{d}}$ referring to signals from natural-abundance $^{13}$C nuclei that are in the proximity of or distant from other $^{13}$C nuclei, respectively. The quantity $G \equiv (S_{0,\text{u}}^{0,\text{u}} + S_{0,\text{d}}^{0,\text{u}})/S_{\text{tot}}$ then represents the fraction of $^{13}$C signal that does not decay to zero in experimental fpRFDR-CT data.

For the data in Figure 9a, with the conditions stated above, $(S_{0,\text{u}}^{0,\text{u}} + S_{0,\text{d}}^{0,\text{u}})/S_{\text{tot}} \approx 0.78$ and $(S_{0,\text{u}}^{0,\text{d}} + S_{0,\text{d}}^{0,\text{u}})/S_{\text{tot}} \approx 0.22$. Again assuming that approximately 20% of peptide molecules are un fibrillized and that 90% of the natural-abundance signal does not dephase in fpRFDR-CT experiments, we calculate $G \approx 0.35$. This estimate is in good agreement with the experimental result that approximately 38% of the $^{13}$C signal does not decay in Figure 9a.

Figure 9b reports the results of fpRFDR-CT experiments on A$\beta_{1-42}$ fibrils with $^{13}$C labels at both the Leu34 carbonyl and the Ala21 methyl carbon sites. In analogy to the case of A$\beta_{10-35}$ described above, a total of approximately 41 carbon sites, with one $^{13}$C label, contribute to the methyl fpRFDR-CT data. A total of 84 carbon sites, with one $^{13}$C label, contribute to the carbonyl fpRFDR-CT data. For the methyl data, $(S_{0,\text{u}}^{0,\text{u}} + S_{0,\text{d}}^{0,\text{u}})/S_{\text{tot}} \approx 0.69$ and $(S_{0,\text{u}}^{0,\text{d}} + S_{0,\text{d}}^{0,\text{u}})/S_{\text{tot}} \approx 0.31$. For the carbonyl data, $(S_{0,\text{u}}^{0,\text{u}} + S_{0,\text{d}}^{0,\text{u}})/S_{\text{tot}} \approx 0.52$ and $(S_{0,\text{u}}^{0,\text{d}} + S_{0,\text{d}}^{0,\text{u}})/S_{\text{tot}} \approx 0.48$. Assuming that approximately 30% of A$\beta_{1-42}$ molecules are un fibrillized and that 90% of the natural-abundance signal does not dephase in fpRFDR-CT experiments, $G \approx 0.35$ for the methyl data and $G \approx 0.48$ for the carbonyl data. These estimates are in good agreement with the experimental result that approximately 30% of the methyl signal and approximately 50% of the carbonyl $^{13}$C signal do not decay in Figure 9b.

RESULTS

Electron Microscopy of A$\beta_{10-35}$ Fibrils. Figure 2 shows EM images of negatively stained A$\beta_{10-35}$ and A$\beta_{10-35,\text{Scr}}$ samples after incubation at pH 3.7 or 7.4. The pH value of 3.7 was chosen because this was the un adjusted pH after dissolution of lyophilized, purified A$\beta_{10-35}$. At pH 3.7 (Figure 2a,b), A$\beta_{10-35}$ forms apparently single filaments with quite uniform morphology and with diameters of 6.6 $\pm$ 0.6 nm. At pH 7.4 (Figure 2c–f), A$\beta_{10-35}$ fibrils display substantially more heterogeneous morphologies. These fibrils appear to be twisted pairs of single filaments with varying periodicities of twist. Fibril diameters vary from 5.5 $\pm$ 1.0 nm at the narrowest points to 10.5 $\pm$ 1.0 nm at the widest points. The fibrils at pH 7.4 have a tendency to aggregate laterally. The dependence of fibril morphology on pH seen in these EM images correlates with solid-state NMR and STEM data presented below. Dependence of fibril morphology on pH has been reported previously for A$\beta_{10-35}$ (16). Twisted morphologies are commonly observed in EM and atomic force microscopy (AFM) images of A$\beta$ (53–55) and other amyloid fibrils.

Under similar incubation conditions at pH 7.4, A$\beta_{10-35,\text{Scr}}$ appears not to form well-ordered fibrils. Instead, non fibrillar aggregates are observed in EM images of negatively stained samples (Figure 2g,h). The absence of visible amyloid fibrils of A$\beta_{10-35,\text{Scr}}$ is consistent with previous evidence that the central hydrophobic segment of A$\beta_{10-35}$ plays an important role in the stabilization of the amyloid fibril structure (38, 45). The EM results for A$\beta_{10-35,\text{Scr}}$ correlate with solid-state NMR data presented below.

Figure 3 shows STEM images of unstained A$\beta_{10-35}$ fibrils formed at pH 3.7 (Figure 3a) and pH 7.4 (Figure 3c). The intensity in these STEM images is directly proportional to mass density per unit area, after subtraction of background intensity due to scattering from the carbon film. Thus, the MPL of the fibrils can be determined from the difference between the total intensity in rectangular areas of fixed length centered on apparently straight segments of fibrils and the total intensity in identical areas of background displaced slightly from the fibrils, as indicated in Figure 3. The constant of proportionality between intensity and mass density is determined from similar measurements on codeposited TMV particles in the same field, which have a known MPL of 131 kDa/nm. Multiple STEM images of A$\beta_{10-35}$ fibrils and multiple fibrils in each image were measured. The resulting data for fibrils formed at pH 3.7 are plotted as a histogram in Figure 3b, after binning in units of 1 kDa/nm. The histogram is well described by a single Gaussian peak centered at 12.8 kDa/nm, with a full width at half-maximum (fwhm) of 5.5 kDa/nm. This fwhm value is attributable to statistical Poisson noise in the STEM signal counts at each pixel and to random variations in the density of the carbon support film. MPL data for fibrils prepared at pH 7.4 are plotted as a histogram in Figure 3d, after binning in units of 2 kDa/nm. In this case, the histogram is not well described by a single Gaussian peak. Values ranging from 15 to 120 kDa/nm are observed, with the majority of counts occurring in peaks between 20 and 34 kDa/nm and between 46 and 60 kDa/nm.

A common feature of many (15, 27–30, 56, 57), but not all (26, 53, 58), structural models for amyloid fibrils is lamination of the $\beta$-sheets. In a hypothetical cross-$\beta$ structure with $\beta$-strands precisely perpendicular to the fibril axis and
with the entire peptide chain in a single extended $\beta$-strand, the MPL of a single lamina is simply the molecular mass divided by the 0.48 nm spacing between hydrogen-bonded peptide chains in a $\beta$-sheet. In the remainder of this paper, we use the term "cross-$\beta$ unit" to describe any structural component with MPL equal to the MPL of this hypothetical cross-$\beta$ lamina. By using this intentionally nonspecific term to discuss the STEM data, we mean to avoid unconfirmed assumptions about the folding of the $\beta$-sheets, the width of the $\beta$-sheets, or the presence of $\beta$-sheet lamination in the fibrils. Clearly, the MPL value alone does not uniquely determine any aspect of the fibril structure other than the number of peptide molecules per 0.48 nm displacement along the long axis of the fibril.

For A$\beta_{10-35}$, with molecular mass 2.902 kDa, the MPL of a single cross-$\beta$ unit is 6.0 kDa/nm. The data in Figure 3b strongly suggest that A$\beta_{10-35}$ fibrils formed at pH 3.7 are constructed exclusively from two cross-$\beta$ units. We refer to this fibril structure, with MPL equal to that of two cross-$\beta$ units, as the "protofilament" because this structure has the minimum MPL of all observed fibrils. The MPL histogram in Figure 3d suggests that the number of cross-$\beta$ units in fibrils formed at pH 7.4 is not unique but ranges primarily between four and nine. The broad distribution of apparent MPL values in Figure 3d is at least partly a consequence of the tendency for the fibrils to associate laterally at pH 7.4 (see Figure 2c-f) and the difficulty of distinguishing single fibrils from laterally associated fibrils in the STEM images. Fibrils formed at pH 7.4 with MPL values of approximately 24 kDa/nm may be twisted pairs of the protofilaments observed at pH 3.7.

**Figure 3**: Scanning transmission electron micrographs of unstained A$\beta_{10-35}$ fibrils prepared at pH 3.7 (a) and pH 7.4 (c). The mass per length (MPL) of amyloid fibrils is determined by integrating the image intensity in rectangular areas centered on the fibrils (rectangle 1 in panel a) and subtracting the intensity in identical areas of background (rectangle 2 in panel a). Intensities are calibrated by similar measurements on codeposited tobacco mosaic virus (TMV) particles. Histograms of MPL measurements on A$\beta_{10-35}$ fibrils prepared at pH 3.7 (b) and pH 7.4 (d) indicate a strong pH dependence. A single cross-$\beta$ sheet of A$\beta_{10-35}$ with a 0.48 nm interchain spacing would have an MPL value of 6.0 kDa/nm.

**Figure 4**: Negatively stained electron micrographs (a, b) and unstained scanning transmission electron micrographs (c, d) of A$\beta_{1-42}$ fibrils prepared at pH 7.4 and a histogram of MPL values (e) determined as in Figure 3. A single cross-$\beta$ sheet of A$\beta_{1-42}$ with a 0.48 nm interchain spacing would have an MPL value of 9.4 kDa/nm.

**Electron Microscopy of A$\beta_{1-42}$ Fibrils.** EM images of negatively stained A$\beta_{1-42}$ fibrils formed at pH 7.4 are shown in Figure 4a,b. These fibrils exhibit obvious morphological heterogeneities, with variations in the periodicity of the apparent twist, the apparent diameter, and the degree of curvature. The majority of fibrils exhibit a periodic modulation of diameter, from minimum diameters of 6 ± 1 nm to maximum diameters of 12.5 ± 2.5 nm and with periods of 100 ± 40 nm. Apparently tightly twisted, drill-shaped fibrils with diameters of 6 ± 1 nm resemble fibrils identified at an early stage of incubation in earlier EM studies of A$\beta_{1-40}$ fibrillation and classified as "type 1" by Goldsbury et al. (54). Apparently spherical aggregates of A$\beta_{1-42}$, with diameters of 10–20 nm, are also seen. Similar spherical structures have been observed previously in EM (53, 54) and AFM (59) studies of A$\beta$ fibrillation.

STEM images of unstained A$\beta_{1-42}$ fibrils are shown in Figure 4c,d. Analysis of these images as described above results in the MPL histogram in Figure 4e. The histogram for A$\beta_{1-42}$ shows a peak at 19 ± 2 kDa/nm and a tail at higher values, extending to 50 kDa/nm. Structures that are unambiguously single filaments (e.g., the A$\beta_{1-42}$ fibril indicated in Figure 4c) have MPL values of approximately
One-Dimensional Solid-State $^{13}$C NMR Spectroscopy of Amyloid Fibrils. Figure 5 shows 1D solid-state $^{13}$C NMR spectra of $\alpha_{10-35}$ fibrils prepared by incubation at pH 7.4 (a), unincubated $\alpha_{10-35}$ (b), $\alpha_{10-35}$ fibrils prepared by incubation at pH 3.7 (c), $\alpha_{10-35,Scr}$ incubated at pH 7.4 (d), and $\alpha_{1-42}$ fibrils prepared by incubation at pH 7.4 (e). All samples are in lyophilized form. Arrows indicate signals from $^{13}$C labels at the methyl carbon of Ala21 (a–c) and the carbonyl carbon of Leu34 (c). The spectrum in spectrum e indicates an MAS sideband line from the carbonyl label. Expansions of the methyl signal region are shown for spectra a–d. The dashed line in spectrum a is a fit of the methyl line shape to a sum of four Gaussian lines, centered at 21.1, 19.4, 17.4, and 15.6 ppm with widths of 1.4, 1.7, 1.8, and 1.8 ppm.

19 kDa/nm. Structures with higher MPL values are likely to be bundles of single fibrils. Given the molecular mass of 4.514 kDa for $\alpha_{1-42}$, the MPL of a single cross-$\beta$ unit is 9.4 kDa/nm. The peak in the MPL histogram therefore appears to correspond to an $\alpha_{1-42}$ protofilament constructed from two cross-$\beta$ units. Higher MPL values observed in the STEM image analysis are attributed to bundled or overlapping fibrils that are difficult to distinguish from single protofilaments due to the relatively low resolution of the STEM images. The apparent number of laminae for all measured fibrils lies primarily in the range from two to four, suggesting that some fibrils may be pairs of protofilaments. Thus, unpaired $\alpha_{1-42}$ protofilaments appear to coexist with paired protofilaments at pH 7.4, unlike the case of $\alpha_{10-35}$ where only unpaired protofilaments are observed at pH 3.7 but no unpaired protofilaments are observed at pH 7.4.

One-Dimensional Solid-State $^{13}$C NMR Spectroscopy of Amyloid Fibrils. Figure 5 shows 1D solid-state $^{13}$C NMR spectra of $\alpha$ peptides, obtained with standard MAS, cross-polarization, and high-power proton decoupling techniques to provide adequate sensitivity and high spectral resolution. Panels a and b of Figure 5 are spectra of $\alpha_{10-35}$, $^{13}$C-labeled at the methyl carbon of Ala21, after fibrillization at pH 7.4 and before fibrillation (but after purification and lyophilization), respectively. Fibrillation induces characteristic changes in the $^{13}$C NMR signals. Most strikingly, the natural-abundance aliphatic signals between 25 and 60 ppm develop sharper features, including a well-resolved peak at 58 ppm attributable to a subset of the $\alpha$-carbons in the peptide backbone. In addition, the width (fwhm) of the natural-abundance carbonyl peak at 172 ppm is reduced from 6 to 4 ppm. These changes in natural-abundance $^{13}$C NMR signals serve as an empirical indication that the majority of $\alpha_{10-35}$ molecules in the sample are in fibrils, rather than in nonfibrillar aggregates. Similar features are observed in solid-state $^{13}$C NMR spectra of $\alpha_{1-40}$ fibrils (17, 20).

Upon fibrillization of $\alpha_{10-35}$ at pH 7.4, the strong signal at 20.3 ppm arising from the labeled Ala21 methyl carbon changes from a single peak with a width of 3.2 ppm to a series of partially resolved peaks at 21.0, 19.4, and 17.4 ppm with widths of approximately 1.4, 1.7, and 1.8 ppm and areas in an approximate 3:3:2 ratio. In contrast, as shown in Figure 5c, fibrillation of the same peptide at pH 3.7 produces a single $^{13}$C NMR line for the Ala21 methyl carbon at 21.3 ppm with a width of 2.5 ppm. Incubation of $\alpha_{10-35,Scr}$ at pH 7.4 leads to a $^{13}$C NMR spectrum that strongly resembles the spectrum of unincubated $\alpha_{10-35}$, as shown in Figure 5d.

The differences in Ala21 methyl $^{13}$C NMR signals of $\alpha_{10-35}$ fibrils prepared at pH 3.7 and 7.4 correlate with the differences in the corresponding EM images (Figures 2 and 3). Observation of a single $^{13}$C NMR line suggests a single structural environment for Ala21 in the pH 3.7 fibrils, consistent with the homogeneous morphology in the EM images and the observation of a single peak in the MPL histogram. Observation of several partially resolved lines suggests several distinct structural environments for Ala21 in the pH 7.4 fibrils, consistent with the more heterogeneous and complex morphology in the EM images and the broad distribution of MPL values. The similarity between $^{13}$C NMR spectra of incubated $\alpha_{10-35,Scr}$ and unincubated $\alpha_{10-35}$ is consistent with the lack of visible amyloid fibrils in EM images of $\alpha_{10-35,Scr}$.

Figure 5e shows the 1D $^{13}$C MAS NMR spectrum of $\alpha_{1-42}$ after fibrillation at pH 7.4, with $^{13}$C labels at the methyl carbon of Ala21 and the carbonyl carbon of Leu34 and with a $^{15}$N label at the amide nitrogen of Val40. The signal from natural-abundance $\alpha$-carbons (48–60 ppm) resembles that of well-fibrillized $\alpha_{10-35}$. The carbonyl $^{13}$C label exhibits a single peak at 172.2 ppm with a width of 2.5 ppm. This relatively narrow carbonyl line indicates a high degree of structural order at Leu34. The methyl $^{13}$C label exhibits a single peak at 19.0 ppm with a width of 5.5 ppm. The morphological heterogeneity seen in the EM images of this $\alpha_{1-42}$ sample may be associated with a larger degree of structural heterogeneity at Ala21 than at Leu34.

Multiple-Quantum $^{13}$C NMR Measurements on $\alpha_{10-35}$ Fibrils. As previously demonstrated for $\alpha_{1-40}$ and $\alpha_{16-22}$ fibrils (17, 18), $^{13}$C MQ NMR spectroscopy (31, 47) can be used to probe the supramolecular organization of $\beta$-sheets in amyloid fibrils. As depicted in Figure 6a, if a single $^{13}$C-labeled site is contained in a $\beta$-strand segment that forms a fully parallel and in-register $\beta$-sheet in the fibrils, the $^{13}$C labels in a single $\beta$-sheet lamina form a nearly linear chain with internuclear spacings of approximately 0.48 nm. If the labeled site is contained in an antiparallel $\beta$-sheet in the fibrils, as in Figure 6b, the labels form a zigzag pattern with internuclear distances that exceed 0.48 nm and depend on the position of the labeled site within the $\beta$-strand segment. In a hypothetical dimeric or trimeric parallel $\beta$-sheet, as in
Figure 6: Candidate β-sheet organizations in amyloid fibrils considered in the analysis of multiple-quantum (MQ) NMR data: fully parallel (a), antiparallel (b), dimeric (c), and trimeric (d). Arrows represent peptide chains, with arrowheads at the C-termini. Open circles represent 13C labels introduced at a single carbon site in each chain. The internuclear spacing $D_1$ is approximately 0.48 nm in all β-sheets. The distance $D_2$ depends on the position of the label in the peptide chain in antiparallel structures.

Figure 6c,d, the 13C labels occur as pairs or triplets with a 0.48 nm internuclear distance and with greater distances between pairs or triplets.

The structures in Figure 6 can be distinguished experimentally by solid-state NMR measurements that probe the strengths of nuclear magnetic dipole–dipole couplings among the 13C labels, as represented by the dipole–dipole coupling constants $d = \gamma^2 \hbar/2 \pi R^3$, where $R$ is the internuclear distance, $\gamma$ is the nuclear magnetogyric ratio, and $\hbar$ is Planck’s constant. At $R = 0.48$ nm, $d = 69$ Hz. In a MQ NMR measurement, NMR transitions in which groups of $n$ nuclear spins flip simultaneously in the magnetic field of the spectrometer are excited and detected, using a MQ excitation sequence of length $\tau_{MQ}$. For an $n$-quantum signal to be observable, at least $n$ nuclear spins must be linked by dipole–dipole couplings of strength $d < \tau_{MQ}^{-1}$; i.e., they must be sufficiently close in space. Thus, for $\tau_{MQ} \sim 14$ ms, at a qualitative level one expects to detect only one-quantum signals in an antiparallel β-sheet, two-quantum signals in a dimeric parallel β-sheet, three-quantum signals in a trimeric parallel β-sheet, and $n$-quantum signals with $n > 3$ in a fully parallel β-sheet.

Figure 7 shows experimental MQ NMR spectra of fibrillized $\alpha\beta_{10-35}$ (Figure 7a,b for pH 7.4 and 3.7, respectively), incubated $\alpha\beta_{10-35,Scr}$ (Figure 7c), and unfibrillized $\alpha\beta_{10-35}$ (Figure 7d). In all cases, the peptides are 13C-labeled at the methyl carbon of Ala21 (same samples as in Figure 5a–d), and data are shown for $\tau_{MQ} = 4.8, 9.6, and 14.4$ ms. For $\alpha\beta_{10-35}$ fibrillized at either pH value, MQ NMR signals up to and including four quantum are observed above the noise level at $\tau_{MQ} = 14.4$ ms. According to the qualitative expectations outlined above, these MQ NMR data suggest a parallel organization of β-sheets in $\alpha\beta_{10-35}$ fibrils. Additionally, the similarity of MQ NMR signal amplitudes in Figure 8 indicates that the organization of β-sheets is not dependent on pH, despite the differences in fibril morphology, MPL, and 1D 13C NMR spectra reported above. In contrast, only very weak three-quantum signals and no detectable four-quantum signals are observed in MQ NMR spectra of unfibrillized $\alpha\beta_{10-35}$ and incubated $\alpha\beta_{10-35,Scr}$. At all values of $\tau_{MQ}$, two-quantum signals in MQ NMR spectra of unfibrillized $\alpha\beta_{10-35}$ and incubated $\alpha\beta_{10-35,Scr}$ are significantly weaker relative to the one-quantum signals than in MQ NMR spectra of fibrillized $\alpha\beta_{10-35}$. Two-quantum signals in Figure 7c,d are attributable to dipole–dipole couplings between 13C labels and natural-abundance 13C nuclei.

Quantitative analyses of MQ NMR data for $\alpha\beta_{10-35}$ fibrils are accomplished by comparison with numerical simulations. Figure 8 compares experimental MQ signal amplitudes with simulations for the four β-sheet organizations depicted in Figure 6. Experimental one-quantum amplitudes contain significant contributions from natural-abundance 13C nuclei, and data are shown for $\tau_{MQ} = 4.8, 9.6, and 14.4$ ms. For $\alpha\beta_{10-35}$ fibrillized at either pH value, MQ NMR signals up to and including four quantum are observed above the noise level at $\tau_{MQ} = 14.4$ ms. According to the qualitative expectations outlined above, these MQ NMR data suggest a parallel organization of β-sheets in $\alpha\beta_{10-35}$ fibrils. Additionally, the similarity of MQ NMR signal amplitudes in Figure 8 indicates that the organization of β-sheets is not dependent on pH, despite the differences in fibril morphology, MPL, and 1D 13C NMR spectra reported above. In contrast, only very weak three-quantum signals and no detectable four-quantum signals are observed in MQ NMR spectra of unfibrillized $\alpha\beta_{10-35}$ and incubated $\alpha\beta_{10-35,Scr}$. At all values of $\tau_{MQ}$, two-quantum signals in MQ NMR spectra of unfibrillized $\alpha\beta_{10-35}$ and incubated $\alpha\beta_{10-35,Scr}$ are significantly weaker relative to the one-quantum signals than in MQ NMR spectra of fibrillized $\alpha\beta_{10-35}$. Two-quantum signals in Figure 7c,d are attributable to dipole–dipole couplings between 13C labels and natural-abundance 13C nuclei.
FIGURE 8: Comparison of experimental MQ NMR amplitudes for \( \alpha \beta_{10-35} \) fibrils, obtained from peak areas in Figure 7, with simulated amplitudes for the four candidate \( \beta \)-sheet organizations in Figure 6. Experimental and simulated MQ amplitudes are normalized to the two-quantum amplitudes. Amplitudes are plotted on a logarithmic scale to permit assessment of three- and four-quantum amplitudes. Uncertainties in all experimental measurements due to noise in the MQ NMR spectra are approximately ±0.06 (2\( \sigma_{\text{rms}} \)).

Simulations give comparable agreement with experimental MQ amplitudes. The fully antiparallel and the dimeric \( \beta \)-sheet simulations yield substantially lower three- and four-quantum amplitudes and are clearly excluded by the experimental MQ data at both pH values. Thus, at a quantitative level, the MQ NMR data support a parallel organization of \( \beta \)-sheets in \( \alpha \beta_{10-35} \) fibrils at both pH values, extending over at least three successive peptide chains.

The parallel \( \beta \)-sheet simulations in Figure 8 assume in-register alignment of \( \alpha \beta_{10-35} \) chains, meaning that residue \( k \) of one chain is hydrogen bonded to residues \( k - 1 \) and \( k + 1 \) of a neighboring chain. Additional simulations were performed for a putative out-of-register parallel \( \beta \)-sheet structure in which the hydrogen-bonding pattern is shifted by one residue, so that residue \( k - 1 \) of one peptide chain is hydrogen bonded to either residues \( k - 2 \) and \( k \) or residues \( k \) and \( k + 2 \) of a neighboring chain. On the basis of molecular modeling of \( \beta \)-sheets, nearest-neighbor distances between methyl \( ^{13} \)C labels in the shifted structure were taken to alternate between 0.70 and 0.77 nm. Simulations for the shifted parallel \( \beta \)-sheet structure (not shown) yielded weaker three- and four-quantum signals, relative to the two-quantum signals, than simulations for either the in-register parallel \( \beta \)-sheet structure or the trimeric \( \beta \)-sheet structure. Comparison with the experimental data excludes out-of-register parallel \( \beta \)-sheet structures for \( \alpha \beta_{10-35} \) fibrils at both pH values.

The antiparallel \( \beta \)-sheet simulations in Figure 8 also assume an in-register alignment, meaning that residue \( 10 + k \) of one chain is hydrogen bonded to residue \( 35 - k \) of a neighboring chain. A shifted antiparallel structure in which residue \( 10 + k \) is hydrogen bonded to residue \( 32 - k \) would be consistent with the MQ NMR data on Ala21-labeled \( \alpha \beta_{10-35} \) fibrils but is ruled out by the additional solid-state NMR data described below.

\( ^{13} \)C–\( ^{13} \)C Dipolar Recoupling Measurements on \( \alpha \beta_{10-35} \) and \( \alpha \beta_{1-42} \) Fibrils. Additional solid-state NMR measurements were performed to test the \( \beta \)-sheet structure in the C-terminal hydrophobic segment of \( \alpha \beta_{10-35} \) and in both hydrophobic segments of \( \alpha \beta_{1-42} \). These measurements employed the fpRFDR-CT technique developed by Ishii et al. (49). In fpRFDR-CT measurements on \( ^{13} \)C-labeled samples, the \( ^{13} \)C NMR signal intensity under high-speed MAS conditions is measured as a function of the effective dipolar dephasing time \( \tau_{\text{D}} \) during which homonuclear dipole–dipole couplings are switched on by a recoupling pulse sequence. The rate of decay of the NMR signals with increasing \( \tau_{\text{D}} \) serves as a measure of the strength of dipole–dipole couplings and hence \( ^{13} \)C–\( ^{13} \)C distances. We have recently used fpRFDR-CT measurements to demonstrate an in-register parallel \( \beta \)-sheet organization in \( \alpha \beta_{1-40} \) fibrils, with parallel alignment from Gly9 through Val39 (20).

Figure 9a shows experimental fpRFDR-CT data for fibrillized and unfibrillized samples of \( \alpha \beta_{10-35} \), \( ^{13} \)C-labeled at the methyl carbon of Ala30. \( ^{13} \)C NMR signals of the methyl \( ^{13} \)C NMR signal at the methyl carbon of Ala30. Decay of the methyl \( ^{13} \)C NMR signal with increasing dipolar dephasing time in the fibrillized sample indicates the presence of intermolecular \( ^{13} \)C–\( ^{13} \)C dipole–dipole couplings. (b) Dipolar recoupling data for \( \alpha \beta_{1-42} \) fibrils prepared at pH 7.4. Squares represent data for a \( ^{13} \)C label at the methyl carbon of Ala30, circles at the carbonyl carbon of Leu34. Circles represent data for a \( ^{13} \)C label at the methyl carbon of Ala30. Decay of the methyl \( ^{13} \)C NMR signal at the methyl carbon of Ala21. Error bars indicate the root-mean-squared noise (\( \sigma_{\text{rms}} \)) in the experimental spectra. (c) Simulated dipolar recoupling curves for linear chains of \( ^{13} \)C nuclei with the indicated internuclear distances.
the maximum value of $r_D$ in these experiments, indicating the absence of measurable $^{13}$C--$^{13}$C couplings. The fibrillized sample exhibits a significant signal decay on the time scale of 40 ms. Figure 9b shows experimental data for fibrillized $\alpha\beta\beta_{10-35}$, $\alpha$C-labeled at the methyl carbon of Ala21 and the carbonyl carbon of Leu34 and $^{15}$N-labeled at Val40 (same sample as in Figure 5e). $^{13}$C NMR signals from both labeled sites, measured in separate fpRFDCT experiments in which the $^{13}$C rf carrier frequency was set to either the methyl or the carbonyl NMR frequency, decay significantly on the time scale of 40 ms. Figure 9c shows simulated fpRFDCT decay curves for linear chains of $^{13}$C nuclei with internuclear spacings ranging from 0.38 to 0.58 nm. The time scale for decay of $^{13}$C NMR signals in the experimental data for fibrillized $\alpha\beta\beta_{10-35}$ and $\alpha\beta_{1-42}$ matches the time scale in simulations for internuclear spacings of approximately 0.48 ± 0.05 nm, consistent with in-register parallel $\beta$-sheet structures for both peptides at the labeled sites.

In experimental fpRFDCT data (Figure 9a,b), the NMR signals decay to 30–50% of their initial amplitudes. In simulations (Figure 10c), the signals decay nearly to zero. As demonstrated quantitatively in Materials and Methods, this difference between experiments and simulations is due to experimental signal contributions from natural-abundance $^{13}$C nuclei and from $^{13}$C labels in residual un fibrillated peptides, which would decay only at larger values of $r_D$.

$^{13}$N--$^{13}$C REDOR Measurements on $\alpha\beta_{10-35}$ and $\alpha\beta_{1-42}$ Fibrils. Figure 10a shows data obtained with the REDOR technique (50, 51) for fibrils prepared at pH 7.4 from an equimolar mixture of $\alpha\beta_{10-35}$ molecules labeled with $^{13}$C at the carbonyl site of Phe19 or with $^{15}$N at Phe20. In a $^{13}$C-detected, $^{13}$N--$^{13}$C REDOR experiment, one measures the amplitude of the $^{13}$C MAS NMR signal as a function of a heteronuclear dipolar dephasing time $T_{RED}$ during which a rotor-synchronized rf pulse train is applied to $^{13}$C nuclei only ($S_0$ signal) or to both $^{13}$C and $^{15}$N nuclei ($S_1$ signal). As usual, the REDOR data are plotted as $\Delta S/S_0 \equiv (S_0 - S_1)/S_0$ to cancel out dependences on $T_{RED}$ due to effects other than $^{13}$C--$^{13}$N couplings. In Figure 10a, a significant buildup of $S\Delta S/S_0$ is observed on a time scale of 30 ms. As shown in Figure 10b, in an in-register parallel $\beta$-sheet structure, the carbonyl group of Phe19 is hydrogen bonded to the amide group of Phe20, leading to distances $r_1 \approx 0.41$ nm and $r_2 \approx 0.56$ nm between the $^{13}$C label and $^{15}$N labels on neighboring peptide chains, with an angle of approximately $150^\circ$ between $r_1$ and $r_2$. Because the REDOR sample was fibrillized from a 1:1 mixture of $^{13}$C-labeled and $^{15}$N-labeled $\alpha\beta_{10-35}$ molecules, we expect 25% of the $^{13}$C labels to be coupled to one $^{15}$N label at $r_1$, 25% of the $^{13}$C labels to be coupled to one $^{15}$N label at $r_2$, 25% of the $^{13}$C labels to be coupled to $^{15}$N labels at both $r_1$ and $r_2$, and 25% of the $^{13}$C labels to have no nearby $^{15}$N labels. The simulated REDOR curve in Figure 10a, calculated as the sum of curves for these four cases, shows a buildup of $\Delta S/S_0$ on the same time scale as the experimental data for $\alpha\beta_{10-35}$ fibrils.

The simulated REDOR curve in Figure 10a was scaled by a factor of 0.43 to optimize the fit to the experimental values. This scaling factor accounts for contributions to the experimental REDOR data from natural-abundance $^{13}$C background signals and from an estimated 20% of the $\alpha\beta_{10-35}$ molecules that are in nonfibrillated aggregates and therefore do not participate in the parallel $\beta$-sheet structure.
fibrils formed by the Aβ34–42 fragment investigated by Lansbury, Griffin, and co-workers (11). The antiparallel β-sheet structure determined for Aβ34–42 fibrils is depicted in Figure 10c. In this structure, Leu34 of each Aβ34–42 chain is hydrogen bonded to Val40 of a neighboring chain, leading to 13C–15N distances of approximately 0.41 nm and coupling strengths of approximately 44 Hz. The experimental REDOR data (Figure 10a) indicate that 13C–15N couplings are much weaker than predicted for the antiparallel structure. The data indicate ΔS/R0 < 0.05 at τRED = 40 ms, which implies that fewer than 15% of the fibrillized Aβ1–42 molecules participate in the antiparallel β-sheet structure (based on REDOR simulations for the antiparallel structure and taking into account signal contributions from natural-abundance 12C and unfibrillized peptides as described above).

### DISCUSSION

**Interpretation of Structural Constraints from Solid-State NMR.** In loose analogy to the usual description of globular protein structures, the levels of structure in an amyloid fibril may be termed primary, secondary, tertiary, and quaternary, with primary structure referring to the amino acid sequence, secondary structure referring to the conformation of individual peptide molecules, tertiary structure referring to the supramolecular organization of peptide molecules within one cross-β unit (as defined above), and quaternary structure referring to the organization of cross-β units within a fibril. With these definitions, the EM data presented above primarily place constraints on quaternary structure, while the solid-state NMR data place constraints on secondary and tertiary structure.

Constraints on secondary structure come from the 13C NMR chemical shifts at isotopically labeled carbonyl and β-carbon sites in one-dimensional MAS NMR spectra (Figure 5). The empirical correlation of 13C NMR chemical shifts with protein secondary structure has been established from both solid-state NMR (18, 22, 60, 61) and liquid-state (62–64) NMR data. Observed chemical shifts from fibrillized Aβ samples δobs and random coil chemical shifts δ coil are summarized in Table 1. The secondary shifts Δδ ≡ δobs − δ coil are systematically positive for alanine methyl carbons and negative for carbonyl carbons. These secondary shifts are consistent with β-strand peptide backbone conformations at all labeled sites in both hydrophobic segments of Aβ35–10 and Aβ1–42 fibrils.

Constraints on tertiary structure come from 13C MQ NMR, fpRFDR-CT, and REDOR data. The observation of significant three- and four-quantum NMR signals in MQ NMR spectra of Aβ10–35 fibrils prepared at either pH 7.4 or pH 3.7 (Figure 7) and the quantitative comparison of the experimental MQ signal amplitudes with simulations (Figure 8) indicate that 13C labels introduced at the methyl carbon of Ala21, at the C-terminal end of the central hydrophobic segment, occur in groups of three or more with internuclear spacings less than approximately 0.55 nm. This result is consistent with an in-register parallel β-sheet organization of the central hydrophobic segment but is inconsistent with in-register antiparallel, dimeric parallel, or out-of-register parallel β-sheet organizations.

It is especially interesting that the experimental MQ NMR data for Aβ35–10 fibrils are nearly identical at pH 7.4 and 3.7, despite the qualitative differences in the 1D 13C NMR spectra (Figure 5a,c) and the EM data (Figures 2 and 3). Electrostatic interactions are expected to be different at pH 3.7 and 7.4 due to pH-dependent side chain charges (see below). Differences in quaternary structure, but not tertiary structure, may then be due to electrostatic interactions.

The fpRFDR-CT data indicate distances of 0.48 ± 0.05 nm between 13C labels at Ala30 methyl carbons in Aβ10–35 fibrils (Figure 9a) and between 13C labels at both Ala21 methyl carbons and Leu34 carbonyl carbons in Aβ1–42 fibrils (Figure 9b). REDOR data on Aβ10–35 fibrils (Figure 10a) are consistent with hydrogen bonding between the carbonyl group of Phe19 and the amide group of Phe20. Thus, at least four distinct solid-state NMR techniques, i.e., DRAWS (dipolar recoupling with a windowless sequence) (13, 14, 16, 65), MQ NMR, fpRFDR-CT, and REDOR, applied to samples with labels at multiple distinct sites all support the same in-register parallel β-sheet organization in Aβ10–35 fibrils, making it rather unlikely that an unforeseen experimental artifact could explain the solid-state NMR results. REDOR measurements on Aβ1–42 fibrils, designed to test for an antiparallel β-sheet organization in the C-terminal hydrophobic segment analogous to the antiparallel organization reported for Aβ34–42 fibrils (11), give a negative result (Figure 10a) and are also consistent with a parallel β-sheet organization.

The chemical shift, fpRFDR-CT, and REDOR data on Aβ1–42 fibrils reported above represent the first secondary and tertiary structural constraints on Aβ1–42 fibrils from solid-state NMR. These constraints, which are consistent with solid-state NMR data on Aβ1–40 fibrils (17, 20), support the view that the structure of Aβ1–42 fibrils is not significantly different from that of Aβ10–35 fibrils, apart from the addition of two hydrophobic residues at the C-terminus. We find no evidence that the association of elevated Aβ1–42 levels with Alzheimer’s disease (34) and the higher amyloidogenicity (35) of Aβ1–42 is attributable to differences in the supramolecular structure of Aβ40 and Aβ1–42 fibrils.

Perutz et al. have recently proposed a schematic model for the supramolecular organization of β-sheets in Aβ1–42 fibrils that includes both parallel and antiparallel alignments of neighboring peptide chains. This model is inconsistent with the observation of intermolecular distances of approximately 0.48 nm between 13C labels at Ala21 and Leu34 in Aβ1–42 fibrils (Figure 9b) and also inconsistent with solid-state NMR data on Aβ1–40 fibrils (17, 20).

It must be emphasized that our solid-state NMR data do not imply that the entire Aβ10–35 or Aβ1–42 Peptide backbone adopts a β-strand conformation in the amyloid fibrils, nor

| Table 1: 13C NMR Chemical Shifts at Labeled Sites in Aβ Fibrils |
|-------------------|-------------------|-------------------|
| Fiber sample      | 13C-labeled site  | δobs (ppm)ν       | δcoil (ppm)ν |
|                   |                   |                   |
| Aβ10–35, pH 7.4   | Ala21 methyl      | 21.0, 19.4, 17.4  | 17.4          |
| Aβ10–35, pH 7.4   | Phe19 carbonyl    | 171.6             | 174.1         |
| Aβ10–35, pH 7.4   | Ala30 methyl      | 19.7              | 17.4          |
| Aβ10–35, pH 3.7   | Ala21 methyl      | 21.3              | 17.4          |
| Aβ1–42, pH 7.4    | Ala21 methyl      | 19.0              | 17.4          |
| Aβ1–42, PH 7.4    | Leu34 carbonyl    | 172.2             | 175.9         |

ν Exponentially observed chemical shift relative to TMS in 1D MAS NMR spectra, based on external polycrystalline adamantane reference at 38.56 ppm. Uncertainty approximately 0.2 ppm. δ Random coil chemical shift, taken from Wishart et al. (76) and adjusted to TMS reference by subtraction of 1.7 ppm.

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do they prove the existence of in-register intermolecular hydrogen bonding (as opposed to in-register alignment) along the entire length of the peptide. In fact, approximately the first 10 residues at the N-terminal end of $\alpha$-1-40 and $\alpha$-1-42 appear structurally disordered in the fibrils, as indicated by solid-state NMR data (17, 20, 21) and by the observation of proteolytic digestion of these residues in vivo (40, 41, 43) and in vitro (42). In addition, solid-state NMR data on $\alpha$-1-40 fibrils, including both $^{13}$C chemical shifts and measurements of backbone $\phi$ and $\psi$ torsion angles (21), indicate a non-$\beta$-strand backbone conformation in residues 23–29 in $\alpha$-1-40 fibrils. Nonetheless, all solid-state NMR data support a parallel alignment of adjacent peptide chains, with interchain spacings of approximately 0.5 nm, along the entire structurally ordered regions of $\alpha$-10-35, $\alpha$-1-40, and $\alpha$-1-42. The tertiary structure adopted by these peptides in the amyloid fibrils may be due to the simple fact that an in-register parallel alignment maximizes hydrophobic contacts within a single cross-$\beta$ unit (17, 18, 20).

**Interpretation of Structural Constraints from Electron Microscopy.** Although the secondary and tertiary structures of all $\alpha$ fibrils appear very similar under the conditions examined in this work, the quaternary structures revealed by EM and STEM measurements differ significantly. $\alpha$-10-35 fibrils prepared at pH 3.7 appear as single filaments with an apparently uniform diameter of 6.6 ± 0.6 nm in EM images. STEM measurements indicate a homogeneous MPL value equal to twice the MPL of a single cross-$\beta$ unit. It should be noted that an $\alpha$-10-35 molecule would be approximately 8.8 nm in length if the entire peptide adopted a fully extended $\beta$-strand conformation. Thus, the observed $\alpha$-10-35 fibril diameter suggests a backbone conformation that includes a bend or other non-$\beta$-strand conformation. In contrast, $\alpha$-10-35 fibrils prepared at pH 7.4 exhibit a periodic modulation in diameter between 5.5 ± 1.0 and 10.5 ± 1.0 nm. The minimum diameter is approximately equal to the diameter of pH 3.7 fibrils, while the maximum diameter is slightly greater than the length of a single $\alpha$-10-35 molecule in a fully extended $\beta$-strand conformation. The smallest MPL values determined from STEM measurements are approximately four times the MPL of a single cross-$\beta$ unit. These results suggest that the minimal pH 7.4 fibril is a twisted pair of pH 3.7 fibrils. The pH 3.7 fibrils may then be considered the protofilament of $\alpha$-10-35 fibrils, with two protofilaments comprising a single pH 7.4 fibril. Higher apparent MPL values for $\alpha$-10-35 fibrils prepared at pH 7.4 may arise from lateral association of fibrils.

EM images of $\alpha$-1-42 fibrils prepared at pH 7.4 show a heterogeneity of morphology, with some fibrils exhibiting a uniform diameter of 6.0 ± 1.0 nm and others exhibiting a periodically modulated diameter ranging from a minimum of 6.0 ± 1.0 nm to a maximum of 12.5 ± 2.5 nm. The length of an $\alpha$-1-42 molecule in a fully extended $\beta$-strand conformation would be approximately 14.3 nm. The length after subtraction of a 10-residue disordered segment at the N-terminus would be approximately 10.9 nm. These observations suggest that the secondary structure of fibrillized $\alpha$-1-42 also includes non-$\beta$-strand conformations. MPL values determined from STEM images range primarily between the MPL of two cross-$\beta$ units and the MPL of four cross-$\beta$ units. It therefore appears that the quaternary structure of $\alpha$-1-42 fibrils may be quite similar to that of $\alpha$-10-35 fibrils, i.e., that the $\alpha$-1-42 protofilament is constructed from two cross-$\beta$ units and that fibrils with higher MPL are primarily pairs of protofilaments. Under the conditions of our experiments on $\alpha$-1-42, single protofilaments coexist with paired protofilaments as well as larger fibril bundles.

In the collection of MPL data for Figure 4e, an attempt was made to select fibrils that were single filaments, rather than pairs or higher order assemblies of filaments. For this reason, the histogram does not accurately indicate the ratio of the number of protofilaments to the number of fibrils with higher MPL. The absence of a pronounced peak at approximately 38 kDa/nm should not be interpreted as an absence of paired protofilaments.

**Relation to Other Structural Studies of $\alpha$ Fibrils.** $\alpha$-10-35 fibrils have been studied extensively by Lynn, Meredith, Botto, and co-workers (13–16), using solid-state NMR measurements, EM images, small-angle neutron scattering (SANS), enzymatic cross-linking, and chemical derivatization, especially attachment of poly(ethylene glycol) (PEG) chains at the C-terminus of the peptide (66–68). Solid-state NMR measurements using the DRAWS technique (13, 14, 16) to measure intermolecular $^{13}$C–$^{13}$C dipole–dipole couplings strongly support an in-register parallel alignment of peptide chains within a cross-$\beta$ unit in $\alpha$-10-35 fibrils prepared at pH 7.4, spanning the sequence from Val12 through Leu34. DRAWS data on $\alpha$-10-35–PEG fibrils also support an in-register parallel alignment (15). The data of Lynn, Meredith, Botto, and co-workers on $\alpha$-10-35 were the first solid-state NMR measurements to show that $\alpha$ fibrils contain parallel $\beta$-sheets. Our solid-state NMR data on $\alpha$-10-35 and $\alpha$-1-42 fibrils presented above, as well as earlier MQ NMR and fpRFDR-CT data on $\alpha$-1-40 fibrils (17, 20), are in agreement with the work of Lynn, Meredith, Botto, and co-workers. The solid-state NMR data reported above extend this work by showing that the parallel $\beta$-sheet structure in $\alpha$-10-35 fibrils is retained at pH 3.7, that the parallel organization persists over at least three successive peptide chains within a $\beta$-sheet, and that $\alpha$-1-42 fibrils have the same parallel $\beta$-sheet organization. In general, our results substantiate the view that $\alpha$-10-35 is a relevant model of full-length $\alpha$ peptides.

IR spectra of full-length $\alpha$ fibrils (24, 25) and other $\alpha$ fibrils (23), in particular the observation of a splitting of the amide I band into a stronger band at approximately 1630 cm$^{-1}$ and a weaker band at approximately 1695 cm$^{-1}$, have been interpreted as support for antiparallel $\beta$-sheet structures. Although measurements on certain model peptides favor this interpretation (69), recent IR studies of proteins containing parallel $\beta$-sheets (70) and simulations of IR spectra (71) demonstrate that these features of the amide I band are not a unique signature of antiparallel $\beta$-sheets.

Solid-state NMR data on $\alpha$-16-22 and $\alpha$-34-42 fibrils indicate antiparallel $\beta$-sheet structures (11, 18), demonstrating that amyloid fibrils can exhibit a diversity of tertiary structures. These two $\alpha$ fragments contain the central and C-terminal hydrophobic segments of full-length $\alpha$, respectively. Apparently, the tertiary structure in amyloid fibrils is not uniquely determined by the amino acid sequence at the level of seven- or nine-residue segments. Because both $\alpha$-16-22 and $\alpha$-34-42 contain a single hydrophobic segment with a positively charged N-terminus and negatively charged C-terminus, hydrophobic contacts could be maximized in
either a parallel or an antiparallel structure, while electrostatic interactions would be expected to favor the antiparallel structure. Amyloid fibrils formed by all Aβ fragments examined by solid-state NMR to date exhibit tertiary structures that juxtapose the hydrophobic segments of neighboring peptide chains.

Analysis of SANS data on Aβ<sub>10-35</sub>–PEG fibrils at pH 5.6 yields an estimated MPL value for the peptide component alone of 31–37 kDa/nm (15), corresponding to five or six cross-β units. Although the estimated MPL value from SANS data appears roughly consistent with the MPL histogram from STEM data on Aβ<sub>10-35</sub> fibrils prepared at pH 7.4 (Figure 3d), which shows a peak in the MPL distribution at 30 kDa/nm, the interpretation of EM and SANS data on Aβ<sub>10-35</sub>–PEG fibrils given by Burkoth et al. (15) suggests that Aβ<sub>10-35</sub>–PEG fibrils represent Aβ<sub>10-35</sub> protofilaments whose association into twisted-pair fibrils is blocked by the C-terminal PEG moieties. One might therefore expect the MPL value of the peptide component in Aβ<sub>10-35</sub>–PEG fibrils to equal that of Aβ<sub>10-35</sub> fibrils prepared at pH 3.7 (Figure 3c). This apparent discrepancy between STEM and SANS determinations of MPL remains to be resolved but may be due to differences in the pH values of the two measurements (15, 68). It should be noted that the determination of MPL from STEM data is independent of any assumptions regarding the cross-sectional shape, diameter, or composition of the fibrils and is therefore a comparatively robust procedure.

Goldsbury et al. have reported a study of the time course of Aβ<sub>1-40</sub> fibril formation at pH 7.4, using EM and STEM measurements (54). An MPL histogram for fibrils formed at an early stage (called “protofibrils” by Goldsbury et al.) shows a single peak at approximately 19 kDa/nm, corresponding to two cross-β units as in the Aβ<sub>10-35</sub> and Aβ<sub>1-42</sub> protofilaments discussed above. Higher MPL values appear at a later stage, corresponding primarily to between two and four β-sheet laminae and in good agreement with our data for Aβ<sub>1-42</sub> fibrils (Figure 4e). We have also obtained STEM data on Aβ<sub>1-40</sub> fibrils similar to those of Goldsbury et al. (data not shown). Thus, it appears that both Aβ<sub>1-40</sub> and Aβ<sub>1-42</sub> predominantly form fibrils with between one and two protofilaments at pH 7.4, with the protofilament consisting of two cross-β units, while Aβ<sub>10-35</sub> predominantly forms fibrils with two or more protofilaments at pH 7.4.

Harper et al. have characterized Aβ<sub>1-40</sub> and Aβ<sub>1-42</sub> fibrils by AFM (36, 55). Although the kinetics and concentration dependence of fibrilization differ, the structural features of Aβ<sub>1-40</sub> and Aβ<sub>1-42</sub> fibrils seen in AFM images are similar. AFM images of Aβ<sub>1-40</sub> fibrils reveal the formation of twisted-pair fibrils, called “type 1 fibrils” by Harper et al. (36), from apparently single filaments. The single filaments in AFM images may correspond to the protofilaments comprised of two cross-β units discussed above. The type 1 fibrils exhibit periodic modulation in diameter (inferred from AFM tip height) between approximately 6 and approximately 9 nm and a periodicity of approximately 40 nm. These dimensions are in approximate, although not quantitative, agreement with the dimensions of apparently twisted fibrils in Figures 4 and 5.

Egnczczak et al. have reported photoaffinity cross-linking experiments on Aβ<sub>1-40</sub> in which a specific cross-link between side chains of Met35 and a L-p-benzoylphenylalanine residue substituted for Phe4 was identified and interpreted as evidence for antiparallel β-sheets in Aβ<sub>1-40</sub> fibrils (72). The source of the apparent discrepancy between this photoaffinity cross-linking result and the tertiary structure revealed by solid-state NMR is not yet understood. The possibility exists that the reported cross-links form between different fibrils or between different β-sheets within a fibril, rather than within a single β-sheet. It should be noted that Phe4 is located in the disordered N-terminal segment of Aβ<sub>1-40</sub>.

**Structural Model for Aβ Fibrils.** Experimental data obtained to date are not sufficient to determine a unique, high-resolution structural model for Aβ fibrils. In particular, the precise limits on the β-strand segments in Aβ fibrils, the backbone conformation in intervening segments, the side chain conformations, and the correct quaternary contacts remain to be determined unequivocally. Nonetheless, as a means of clarifying the structural issues and summarizing the current state of knowledge, possible structural models for Aβ<sub>10-35</sub> and Aβ<sub>1-42</sub> fibrils are depicted schematically in Figure 11.

Figure 11a shows a model for the cross-β unit that is consistent with constraints on secondary and tertiary structure from solid-state NMR. In this model, residues 1–9 are structurally disordered (and absent in Aβ<sub>10-35</sub>). Residues 10–22 (encompassing the hydrophobic segment spanning residues 17–21) and residues 30 through the C-terminus form β-strands. The two β-strand segments form in-register parallel β-sheets. Residues 23–29 form a loop or hinge between these β-sheets (but not a true β-hairpin with intramolecular
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hydrogen bonds between backbone atoms). We have recently reported NMR chemical shift and torsion angle data for \( \alpha_4 \beta_1 \) fibrils that support the secondary structure depicted in Figure 11a (21).

Figure 11b shows three possible quaternary structures for an \( \alpha_4 \beta_1 \) fibril constructed from two \( \beta_3 \) units. Other quaternary structures are possible, but these three are roughly consistent with the protofilament diameters observed in EM images and maximize the sequestration of hydrophobic residues from the surrounding aqueous medium in which the fibrils form.

Figure 11c shows one possible quaternary structure for \( \alpha_4 \beta_1 \) fibrils constructed from two protofilaments. The model in Figure 11c is roughly consistent with the dimensions in EM images of fibrils with periodically modulated diameters due to an apparent twist (Figures 2c–f and 4a,b). This particular model is also consistent with the observed pH dependence of \( \alpha_4 \beta_1 \) protofilament association (Figures 2 and 3), because potentially titratable Glu11, His13, and His14 residues are located in the interface between protofilaments. This model is also consistent with the observation that C-terminal PEG blocks protofilament association (i5, 66, 67). A model similar to that in Figure 11c may also apply to \( \alpha_4 \beta_1 \) fibrils, but with an extended C-terminal \( \beta \)-strand segment and with a conformation of the N-terminal \( \beta \)-strand that allows residues 1–9 to be located outside the structured core of the fibril.

Finally, the models in Figure 11 are variants of the laminated cross-\( \beta \) structure for amyloid fibrils that has been commonly invoked to account for X-ray diffraction (9, 56, 57) and EM (6) data. The fact that our STEM measurements indicate MPL values for \( \beta \) protofilaments that are an integral multiple of the MPL of a single cross-\( \beta \) unit (Figure 3b and 4e) is consistent with, but does not prove, a laminated cross-\( \beta \) structure containing relatively little water or other nonpeptide material (less than 10 wt %). Nonlaminated structural models have also been proposed (26, 58), reminiscent of the pacetate lyase C (73) and P.69 pertactin (74) protein structures. Both EM (75) and diffraction (53) data have been interpreted as support for a tubular structure of A\( \beta \) fibrils. Additional experimental data, including additional solid-state NMR constraints on secondary and quaternary structure, may soon establish the full details of the structure of A\( \beta \) fibrils at the molecular level.

ACKNOWLEDGMENT

We thank Jennifer Reed for assistance with peptide synthesis and purification, Dr. L. K. Pannell for mass spectrometric measurements, and Nancy W. Rizzo for electron microscopy. Simulations of NMR data were executed on the SGI Origin 2000 computer in the National Institutes of Health Center for Information Technology. Development of solid-state NMR methodology was supported by the NIH Intramural AIDS Targeted Antiviral Program.

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