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The Japanese Mutant A β (Δ E22-A β _{1–39}) Forms Fibrils Instantaneously, with Low-Thioflavin T Fluorescence: Seeding of Wild-Type A β _{1–40} into Atypical Fibrils by Δ E22-A β _{1–39}

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Abstract

The Δ E693 (Japanese) mutation of the β -amyloid precursor protein leads to production of Δ E22-A β peptides such as Δ E22-A β _{1–39}. Despite reports that these peptides do not form fibrils, here we show that, on the contrary, the peptide forms fibrils essentially instantaneously. The fibrils are typical amyloid fibrils in all respects except that they cause only low levels of thioflavin T (ThT) fluorescence, which, however, develops with no lag phase. The fibrils bind ThT, but with a lower affinity and a smaller number of binding sites than wild-type (WT) A β _{1–40}. Fluorescence depolarization confirms extremely rapid aggregation of Δ E22-A β _{1–39}. Size exclusion chromatography (SEC) indicates very low concentrations of soluble monomer and oligomer, but only in the presence of some organic solvent, e.g., 2% (v/v) DMSO. The critical concentration is approximately 1 order of magnitude lower for Δ E22-A β _{1–39} than for WT A β _{1–40}. Several lines of evidence point to an altered structure for Δ E22-A β _{1–39} compared to that of WT A β _{1–40} fibrils. In addition to differences in ThT binding and fluorescence, PITHIRDS-CT solid-state nuclear magnetic resonance (NMR) measurements of Δ E22-A β _{1–39} are not compatible with the parallel in-register β -sheet generally observed for WT A β _{1–40} fibrils. X-ray fibril diffraction showed different *D* spacings: 4.7 and 10.4 Å for WT A β _{1–40} and 4.7 and 9.6 Å for Δ E22-A β _{1–39}. Equimolar mixtures of Δ E22-A β _{1–39} and WT A β _{1–40} also produced fibrils extremely rapidly, and by the criteria of ThT fluorescence and electron microscopic appearance, they were the same as

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ASSOCIATED CONTENT

Supporting Information. Sample chromatograph for the RP-HPLC assay of thioflavin T concentration, calibration curve for the RP-HPLC assay of thioflavin T concentration, calibration curves for the mass of WT A β _{1–40} and Δ E22-A β _{1–39}, sample chromatograph for the separation and quantitation of Δ E22-A β _{1–39} and WT A β _{1–40} in mixtures, and HPLC analysis of concentrations of Δ E22-A β _{1–39} and WT A β _{1–40} remaining in solution, in fibrillizing mixtures of the two peptides. This material is available free of charge via the Internet at <http://pubs.acs.org>.

fibrils made from pure $\Delta E22-A\beta_{1-39}$. X-ray diffraction of fibrils formed from 1:1 molar mixtures of $\Delta E22-A\beta_{1-39}$ and WT $A\beta_{1-40}$ showed the same D spacings as fibrils of the pure mutant peptide, not the wild-type peptide. These findings are consistent with extremely rapid nucleation by $\Delta E22-A\beta_{1-39}$, followed by fibril extension by WT $A\beta_{1-40}$, and “conversion” of the wild-type peptide to a structure similar to that of the mutant peptide, in a manner reminiscent of the prion conversion phenomenon.

A small percentage of familial forms of Alzheimer’s disease are caused by point mutations in the β -amyloid precursor protein (β -APP) within the sequence of the common forms of $A\beta$ peptides. Of these, most occur at residues 21–23, including the Dutch (E22Q), Flemish (A21G), Italian (E22K), Arctic (E22G), and Iowa (D23N) mutants.^{1–6} Wild-type (WT) $A\beta_{1-40}$ fibrils contain two parallel, in-register β -sheets, linked by an ordered, non- β -sheet bend region.^{7–10} These mutations may fall near the boundary between the N-terminal β -sheet and the bend region. One might expect, therefore, that such mutations could have profound effects on either the aggregation kinetics, the structure of aggregates, or both.

Tomiya et al. reported a new mutation in this part of β -APP, which gives rise to $A\beta$ peptides missing E22, such as $\Delta E22-A\beta_{1-39}$.^a In marked contrast to both WT and most of the point mutant forms of $A\beta$ mentioned above, however, $\Delta E22-A\beta$ peptides were reported not to form amyloid fibrils.¹¹ This conclusion was based primarily on three sets of findings: negative Pittsburgh B brain scan, absence of thioflavin T (ThT) fluorescence, and absence of fibrils in electron micrographs of synthetic $\Delta E22-A\beta$. As the proband with this mutation is alive, it is not known whether neuritic plaques are present in the brain. These negative findings, however, go to the heart of a controversy in this field. Whereas the amyloid cascade hypothesis^{12,13} holds that formation of fibrils is a source of neuro- and cytotoxicity of protein aggregates, more recent studies have highlighted the importance of small, soluble oligomers of $A\beta$ and other aggregating proteins.^{14–16} The apparent inability of $\Delta E22-A\beta$ to form fibrils would lend support to the notion that fibrils are not the main neuro- and/or cytotoxins, and may even be innocuous or protective.¹⁷

Here, we show that contrary to previous reports, $\Delta E22-A\beta$ forms fibrils and does so “instantaneously”, within the limits of several assays. These fibrils are typical amyloid, except for their apparently low level of thioflavin T binding and fluorescence. (As discussed below, these variables depend on which assay is used.) In addition, X-ray diffraction and preliminary solid-state NMR, among other data, suggest that the fibril structure differs from those of previously reported WT $A\beta$ fibrils. Finally, we show below that when $\Delta E22-A\beta$ and WT $A\beta$ are mixed in a disaggregating solvent and then transferred to buffer, the resulting fibrils are mutant-like, suggesting that the mutant peptide nucleates rapidly and templates the wild-type peptide, converting it to an alternate structure in a prion-like fashion.

^aThis term refers to the analogue of WT $A\beta_{1-40}$. Although it is sometimes termed $\Delta E22-A\beta_{1-40}$, it has only 39 amino acids, and therefore, we prefer to call it $\Delta E22-A\beta_{1-39}$. This sequence of this peptide is given in the Materials and Methods.

MATERIALS AND METHODS

Peptide Synthesis and Purification

Peptides were synthesized on an Applied Biosystems (Foster City, CA) 433A synthesizer essentially as described previously.¹⁸ Briefly, peptides were synthesized at a 0.25 mmol scale, using Fast-Moc chemistry with *N*-hydroxybenzotriazole (HOBt) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Peptides International) as coupling reagents and an Fmoc-Wang resin. Coupling times were extended by ~3.3-fold; in addition, the following residues were doubly coupled: R5, S8, V11, V18, V24, G25, S26, and N27 (residue numbers refer to wild-type A β or the corresponding residues in Δ E22-A β ₁₋₃₉). Peptides were cleaved from resin when the resin was stirred in 94% trifluoroacetic acid (TFA), 2.5% water, 2.5% ethanedithiol, and 1% triisopropylsilane (all v/v) in a closed flask for 2 h at room temperature and purified by reverse phase HPLC. Using these techniques, we synthesized and purified Δ E22-A β ₁₋₃₉ and WT A β ₁₋₄₀ using the standard techniques described above: NH₂-DAEFRHDSGY¹⁰ EVHHQKL VFF²⁰ AEDVGSNKGAI³⁰ IGLMVGGVV⁴⁰-COOH and NH₂-DAEFRHDSGY¹⁰ EVHHQKL VFF²⁰ ADVGSNKGAI³⁰ IGLMVGGVV³⁹-COOH, respectively. Peptides were checked for purity by analytical reverse phase (C18) HPLC and MALDI-TOF mass spectrometry, as described previously.²⁰

Electron Microscopy

Peptides were dissolved in neat DMSO and then diluted into sodium phosphate (pH 7.40) or Milli-Q purified water so that the final peptide concentration was 10 or 100 μ M and the DMSO concentration was 2% (v/v). The solutions were allowed to aggregate at 37 °C. At various times (described in Results), aliquots of the fibril slurry were examined by electron microscopy by applying the slurry to glow-discharged, 400-mesh, carbon-coated support films. Because of matting of the Δ E22-A β ₁₋₃₉ fibrils, to obtain larger sample sizes for measuring mean diameters, we sonicated slurries for 30 min using a Branson ultrasonic cleaner and then examined them via electron microscopy as described. Measurements of ~40 single Δ E22-A β ₁₋₃₉ fibrils suggested that sonication did not cause a change in mean fibril diameter; in addition, for WT A β ₁₋₄₀ fibrils, which do not form matted tangles as much as the mutant peptide, sonication did not alter mean fibril diameter. Samples were stained with 0.1% (w/v) uranyl acetate. Excess solution was wicked off with a paper tissue, and the grids were air-dried. Micrographs were imaged on an FEI Tecnai F30st-STEM microscope at magnifications of 15000 $\times$, 49000 $\times$, and 98000 $\times$. The CCD camera of the microscope added another 1.4 $\times$ magnification to all images. Fibril diameters were measured using the freeware program Irfanview, with both freehand and straight-line selection tools. Fibril morphologies were compared against those of several other fibril preparations of different peptide stocks before final measurement of the dimensions.

Congo Red Binding

The Congo Red binding assay was performed essentially as described previously.¹⁸ Briefly, an aliquot of peptide solution containing 50 μ g of peptide was added to 1 mL of a 3 μ M solution of Congo Red in 100 mM sodium phosphate (pH 7.40). The solution was incubated for 15 min at room temperature, and then the absorbance was measured from 400 to 600 nm.

Thioflavin T Fluorescence

We started fibrillization reactions by dissolving the lyophilized peptide, either WT-A β ₁₋₄₀ or Δ E22-A β ₁₋₃₉, in neat DMSO so that the final DMSO concentration would be 2% (v/v). Peptide concentrations were typically 10 or 100 μ M. Peptide concentrations were determined from the absorbance at 274.6 nm, using the extinction coefficient for tyrosine of

1420 M⁻¹ cm⁻¹. Peptides were incubated at 37 °C in 10 mM sodium phosphate (pH 7.40) containing 0.01% (w/v) NaN₃. Fluorescence measurements were performed using a Hitachi F-2000 fluorescence spectrophotometer as described previously,^{19–21} which is based on the assay described by LeVine.^{22,23} In short, a 5 µL aliquot of slurry containing fibrils was added to 1 mL of a 10 µM thioflavin T solution in 10 mM sodium phosphate (pH 7.40). The solution was mixed vigorously by vortexing, and the fluorescence signal was then averaged for 10 s with the excitation and emission wavelengths set to 446 and 490 nm, respectively. The slit width was 1 mm.

Subsequently, we examined whether the apparently low ThT fluorescence of ΔE22-Aβ_{1–39} could be due, in part, to inaccessibility of the dye to binding sites on the tangled, matted fibril cords. To address this question, we performed ThT fluorescence assays by an alternate method,²⁴ in which 10 µM ThT was incorporated into the fibrillization reaction mixture. That is, fibrillization reactions were performed in stirred or unstirred fluorescence cuvettes and were initiated by dilution of Aβ peptides in neat DMSO into 10 mM sodium phosphate (pH 7.40) containing 10 µM ThT, so that the final DMSO concentration was 2% (v/v) and the peptide concentration 100 µM. The progress of the reaction was measured by periodically monitoring the fluorescence (same λ_{ex} and λ_{em} described above).

ThT Binding

Initial observations (see Results) indicated that ΔE22-Aβ_{1–39} forms fibrils, but with very low ThT fluorescence. To determine whether ΔE22-Aβ_{1–39} fibrils bind ThT, we developed an HPLC-based assay of ThT binding that does not rely on ThT fluorescence. For this assay, fibrils of wild-type Aβ_{1–40} and ΔE22-Aβ_{1–39} were formed by incubating 100 µM and 1 mM solutions of each peptide at 37 °C in 10 mM sodium phosphate (pH 7.40) with 0.01% NaN₃ (NaP), for ~2 weeks, until mature fibrils had formed; 150 µL of 100 µM ThT [in 10 mM sodium phosphate (pH 7.40)] was added to various volumes of these peptide slurries and the volume adjusted with the same buffer to 1.5 mL. Thus, the final concentration of ThT was 10 µM. Various volumes of the fibril slurry were incubated in 10 mM ThT for 2 h at 37 °C, after which the samples were centrifuged for 2 h at 100000g using the Beckman Airfuge. The top third of the supernatant was then removed, and the ThT concentration was assayed by injecting an aliquot with a known volume (generally 50 or 100 µL) onto a C18 reverse phase HPLC column. The sample was eluted using the following gradient: 5 min at 0% B, followed by a gradient from 100% A and 0% B to 55% A and 45% B over 55 min [where A is a 100:0.1 (v/v) water/TFA mixture and B is a 100:0.1 (v/v) methanol/TFA mixture]. The effluent was monitored at 430 nm. A sample chromatograph is shown in Figure 1A of the Supporting Information. The concentration of ThT was measured by integrating the peaks using Hewlett-Packard Chemstation to obtain the mass of ThT in the injected volume. A calibration curve (Figure 1B of the Supporting Information) was constructed by injecting various known volumes of a 10 mM solution of ThT. As shown in the figure, areas of the peaks were proportional to the mass of ThT in the injected sample.

For the purposes of comparing macroscopic binding of ThT to WT Aβ_{1–40} and ΔE22-Aβ_{1–39} fibrils, we analyzed data, using the simplest possible scheme, in which ThT in solution forms a complex with binding sites on the fibrils

where C_x is a complex between ThT and a binding site on the fibril, S_f denotes free sites on the fibril for ThT, and T_f is the free (unbound) ThT. C_x = T_b = bound ThT, and K_d is therefore

$$K_d = (S_f T_f) / Cx = S_f (10 - T_b) / T_b \quad (2)$$

In addition, $S_f = S_t - S_b = S_t - T_b$, where S_t is the total number of binding sites for ThT added and S_b is the number of sites on the fibril to which ThT is bound (occupied sites). Because the number of binding sites per peptide is not known a priori, we measured an apparent K_d ($K_d^{\text{app}} = nK_d$, where n is the number of sites per peptide). This yields the following quadratic equation:

$$K_d^{\text{app}} = (P_t - T_b)(10 - T_b) / T_b \quad (3)$$

from which K_d^{app} can be obtained. In addition, the number of sites per peptide can be estimated from the K_d^{app} , because at this concentration, $T_b = T_f = 5 \mu\text{M}$ and $n = K_d^{\text{app}} / 5 \mu\text{M}$. Although the binding of ThT to A β and other fibrils is complex and the binding sites are heterogeneous,^{25–27} we use this phenomenological treatment solely to compare WT and mutant A β fibrils.

Critical Concentration (C_t) of WTA β_{1-40} and $\Delta\text{E22-A}\beta_{1-39}$

For amyloid-forming peptides, a critical concentration (C_t) can be defined as the concentration of peptide remaining in solution at equilibrium, which can be approached by either formation of fibril at infinite time or dissolution of preformed fibrils, also at infinite time.²⁸ In practice, this parameter is estimated by extrapolation to infinite time from the fit of rate equations to experimental data; i.e., $P = (P_0 - P_{\text{eq}}) \times \exp(-kt) + P_{\text{eq}}$, where P is the soluble peptide concentration, P_0 is the initial soluble peptide concentration, P_{eq} is the soluble peptide concentration at equilibrium, k is the apparent first-order rate constant, and t is the time.

Initial observations suggested that C_t for $\Delta\text{E22-A}\beta_{1-39}$ might be at or below the limits of detection of many commonly used protein assays, such as the microBCA assay (limit of detection of $\sim 0.5 \mu\text{g/mL} = 0.12 \mu\text{M}$ $\Delta\text{E22-A}\beta_{1-39}$). This was subsequently confirmed. For this reason, we analyzed protein concentrations by first hydrolyzing the peptide to “amplify the signal” of protein and then using a sensitive fluorescence assay, i.e., fluorescamine assay. Peptides were hydrolyzed for 16 h at 100 °C in 0.2 mL of 6 M HCl. The solution was lyophilized, after which the powder was dissolved in 1 mL of 0.2 M sodium borate (pH 9.00) for the fluorescamine assay. Fluorescamine was freshly dissolved to a final concentration of 0.1 mg/mL in acetone; 0.15 mL of this solution was added to the hydrolyzed protein solution. After the solution had been vortexed, fluorescence was measured with a λ_{ex} of 390 nm and a λ_{em} of 475 nm.

The mass of the protein present in the sample was calculated from a calibration curve constructed from aliquots of authentic WT A β_{1-40} and $\Delta\text{E22-A}\beta_{1-39}$ [masses of peptide between 0 and 0.625 μg of peptide (from 0 to 250 μL of peptide stock solutions at 2.5 $\mu\text{g/mL}$) that had undergone the same hydrolysis and subsequent treatment, as described above]. Figure 2 of the Supporting Information shows the calibration curves for WT A β_{1-40} (○) and $\Delta\text{E22-A}\beta_{1-39}$ (●), which are essentially super-imposable. In both cases, the curves were nonlinear and could be fit to the equation of a straight line plus a hyperbola: for WT A β_{1-40} , $F = 95.14 + 1.44 \times 10^{12} \times M + 229.2 \times M / (8.10 \times 10^{-12} \times M)$, where F is the fluorescence and M is the number of moles of peptide.

Size Exclusion Chromatography

Wild-type A β_{1-40} and $\Delta\text{E22-A}\beta_{1-39}$ were freshly disaggregated by dissolving them in neat DMSO for ~ 1 h immediately prior to chromatography. Solutions were centrifuged at 14300g for 20 min, before samples were injected onto the column. Peptides were injected onto the

column either in neat DMSO at a peptide concentration of 100 μM or after being diluted to a final concentration of 10 or 100 μM with 10 mM sodium phosphate (pH 7.40) [with 0.1% (v/v) NaN_3]. The column was a Superdex Peptide 10/300 column (GE Healthcare). The eluant was 10 mM sodium phosphate (pH 7.4) containing 2% (v/v) DMSO. When the same buffer without DMSO was used as the eluant, none of the $\Delta\text{E22-A}\beta_{1-39}$ emerged from the column, presumably because it precipitated onto the column matrix. Chromatography was performed using an Agilent model 1100 HPLC system. The flow rate was 0.45 or 0.5 mL/min. The temperature was $\sim 22^\circ\text{C}$, and the effluent was monitored at 220, 230, and 274 nm. Apparent molecular weights (MW^{app}) were estimated from a calibration curve constructed using the following standards: horse myoglobin (16951.5), cytochrome *c* (12372), E7-peptide dimer (8431), aprotinin (6512), E7-peptide (4215.5), vitamin B_{12} (1350), and glycine (75) (molecular weights in parentheses). E7-peptide is a synthetic peptide from the E7 protein of human papillomavirus type 16. We synthesized it using standard “Fast-Moc” Fmoc chemistry, as described above, for purposes other than the studies in this paper; the molecular weight of the monomer was determined by MALDI-TOF mass spectrometry. The calibration curve using these peptides is contained in the Supporting Information of ref 29.

X-ray Diffraction

X-ray diffraction data were obtained for fibrils of WT $\text{A}\beta_{1-40}$ and $\Delta\text{E22-A}\beta_{1-39}$, essentially as described previously.²¹ Briefly, fibrils formed as described above were washed by dialysis against distilled water, centrifuged, and placed into 0.7 mm glass capillaries (Charles Supper). Prior to obtaining X-ray diffraction data, the capillary tubes containing fibrils were placed onto the surface of a 11.7 T NMR magnet for 5 days, which achieved partial alignment of fibrils (see also refs 30 and 31). Synchrotron data were collected at Argonne National Laboratory, Advanced Photon Source beamline BioCAT, on a CCD-based X-ray detector (PCCD 168080/Aviex LLC). Other conditions and methods of analysis were as described previously.¹⁹

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra were recorded using an Aviv (Lakewood, NJ) model 202 spectropolarimeter with a temperature-controlled cuvette holder. Samples were disaggregated in 20 μL of neat hexafluoro-2-propanol (HFIP) and then diluted to a concentration 100 μM in 10 mM phosphate buffer (pH 7.4). Diluted samples were added to a 0.1 cm path length cell (Starna), and five scans were obtained from 280 to 180 nm at intervals of 1 nm with a 1 s averaging time. The temperature of the cuvette holder was 25°C . The bandwidth was 1 nm. Data were collected in the form of sample dynode and sample raw ellipticity signal. Buffer signals were subtracted from each scan, and all scans were zeroed by subtracting the mean of ellipticity values between 255 and 260 nm for all scans of each peptide.

Fluorescence Depolarization

To obtain a measure of peptide aggregation that is independent of thioflavin T binding or fluorescence, we measured fluorescence anisotropy as a function of time. To begin the experiment, we added a solution containing 0.04 or 0.4 mg of either WT- $\text{A}\beta_{1-40}$ or $\Delta\text{E22-A}\beta_{1-39}$ in 20 μL of neat DMSO to 980 μL of 0.01 M sodium phosphate (pH 7.40) and 0.01% (w/v) NaN_3 . Final peptide concentrations were either 10 or 100 μM . The solutions were gently inverted to mix the solution, and fluorescence spectra were recorded immediately thereafter, with a delay of ~ 1 min. In some experiments, we made mixtures of WT $\text{A}\beta_{1-40}$ and $\Delta\text{E22-A}\beta_{1-39}$ by mixing peptides in DMSO prior to adding the peptides to aqueous buffers, generally 0.01 M sodium phosphate (pH 7.40) containing 0.01% (w/v) NaN_3 . In these experiments, as in the previous experiments, the final concentration of DMSO was 2% (v/v) and the final total peptide concentration was either 10 or 100 μM .

The samples were excited at 275 nm, for the single intrinsic Tyr fluor in either peptide (both peptides have one Tyr residue and no Trp residues). The samples were excited first with the presample polarizer in the vertical (0°) position; emission spectra were then recorded, with the postsample polarizer first in the vertical (0°) and then the horizontal (90°) position. Next, samples were excited with the presample polarizer in the horizontal (90°) position; emission spectra were then recorded, with the postsample polarizer first in the vertical (0°) and then the horizontal (90°) position. Anisotropy (A) is defined as follows:

$$A = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

where G is an instrument correction factor that compensates for any polarization bias of the detection system.³² G is defined as

$$G = I_{HV} / I_{HH}$$

Spectra were recorded at intervals of 5 min from 0 to 60 min. Measurements of anisotropy were taken at the peak of the emission spectrum, ~307 nm; fluorescence values at 307 nm were smoothed by interpolating the values from 300 to 315 nm.

Solid-State NMR

Peptides containing single ^{13}C labels were synthesized for solid-state NMR studies as follows: $\Delta\text{E22-A}\beta_{1-39}$ containing $1\text{-}^{13}\text{C-V36}$, $\Delta\text{E22-A}\beta_{1-39}$ containing $1\text{-}^{13}\text{C-V18}$, and $\text{WT-A}\beta_{1-40}$ containing $1\text{-}^{13}\text{C-V12}$. Peptides were synthesized and purified as described above. Fibril slurries were made as described above; salts were removed by dialysis against Milli-Q water, using dialysis tubing with a molecular weight cutoff of 500, followed by three cycles of centrifugation of water at 14300g for 30 min in siliconized Eppendorf tubes and resuspension of the fibrils in Milli-Q water. The final pellet was flash-frozen and lyophilized, and the lyophilized samples (approximately 5 mg each) were packed into 3.2 mm magic angle spinning (MAS) rotors with Teflon spacers to contain the samples in the center of the NMR solenoid coil. Solid-state NMR measurements were taken at 9.4 T (100.4 MHz ^{13}C NMR frequency), using three-channel 3.2 mm MAS probes (Varian) and Varian InfinityPlus spectrometer consoles. To assess distances between ^{13}C labels in adjacent peptide molecules, we performed homonuclear dipolar recoupling experiments using the PITHIRDS-CT recoupling technique.³³ Measurements of ^{13}C - ^{13}C dipole-dipole couplings were performed at 9.4 T and a 20.00 kHz MAS frequency. Radiofrequency (Rf) pulses were actively synchronized with an MAS tachometer signal.

Analytical Reverse Phase HPLC Separation of Soluble $\Delta\text{E22-A}\beta_{1-39}$ and WT $\text{A}\beta_{1-40}$ from Cofibrillizing Mixtures

Experiments in which a 1:1 (molar) mixture of $\Delta\text{E22-A}\beta_{1-39}$ and WT $\text{A}\beta_{1-40}$ in neat DMSO is transferred into aqueous buffer [10 mM sodium phosphate (pH 7.40) with 0.01% NaN_3 (NaP)] for fibrillization of the mixture will be presented below. We developed an analytical reverse phase HPLC assay to determine the concentration of each peptide remaining in solution at various times; 100 μL aliquots of the mixed fibril slurries were centrifuged using a Beckman Airfuge at 100000g for 1 h. The top 30 μL of the supernatant was injected onto a Zorbax C18 analytical HPLC column and chromatographed isocratically using a 68:32 (v/v) A/B mixture, where A is 0.1% (v/v) TFA in water and B is 0.1% (v/v) TFA in acetonitrile. The column was heated to 60 °C. The column effluent was monitored by absorbance at 210 nm. A calibration curve was constructed from aliquots from stock

solutions, to contain known masses of each peptide (concentrations determined by UV spectroscopy, as described above). Figure 3A of the Supporting Information shows a typical chromatographic separation of Δ E22-A β ₁₋₃₉ and WT A β ₁₋₄₀, using this isocratic solvent system. The two peptides elute as clearly separated peaks, which were integrated using Hewlett-Packard Chemstation to obtain the mass of each peptide.

Experimental data were analyzed by a nonlinear least-squares fit to the equation of a first-order approach to equilibrium, $P = P_{eq} + (P_0 - P_{eq}) \times \exp(-kt)$, where P is the peptide concentration at various times, P_0 is the initial peptide concentration (50 μ M), and P_{eq} is the peptide concentration at equilibrium. Because the centrifugation step took 1 h, this was added to the time for kinetic analysis.

¹H-¹⁵N HSQC NMR spectra of Δ E22-A β ₁₋₃₉ and WT A β ₁₋₄₀

The rapid fibrillization by Δ E22-A β ₁₋₃₉ raised the question of whether this peptide had been fully disaggregated by being dissolved in DMSO. We addressed the question of whether the peptide is fully disaggregated, within experimental limits of detection, by obtaining ¹H-¹⁵N HSQC NMR spectra of Δ E22-A β ₁₋₃₉ dissolved in DMSO-*d*₆. Natural abundance ¹H-¹⁵NHSQC spectra³⁴ were recorded on a Varian Unity Inova NMR spectrometer equipped with a cryogenically cooled “Cold Probe”, operating at a ¹H frequency of 601 MHz and a ¹⁵N frequency of 61 MHz. The HSQC experiment was conducted with the standard Varian pulse sequence gNhsqc; 128 transients with 4096 complex points were signal-averaged over a proton sweep width of 9615.4 Hz in the direct dimension. The acquisition time in the direct dimension was 0.43 s; 128 complex points were taken in the indirect (¹⁵N) dimension over a sweep width of 1944 Hz, giving an acquisition time of 0.066 s. Quadrature detection in the indirect dimension was achieved via the echo-antiecho method. With a recycle delay of 1 s, the total experiment time was approximately 9 h.

RESULTS

Δ E22-A β ₁₋₃₉ Forms Fibrils Very Rapidly, but with Low Thioflavin T Fluorescence

We assayed fibrillization of Δ E22-A β ₁₋₃₉ and WT A β ₁₋₄₀ by ThT fluorescence (Figure 1). Peptides were first dissolved in neat DMSO and then diluted to the appropriate concentrations, typically 100 μ M, using NaP, so that the final DMSO concentration was 2% (v/v). Attempts to dissolve Δ E22-A β ₁₋₃₉ directly in aqueous media without DMSO or other organic solvents did not produce measurable quantities of peptide in solution. After peptides had been dissolved in DMSO and transferred to buffer, they were incubated at 37 °C under quiescent conditions. Although Δ E22-A β ₁₋₃₉ induced little ThT fluorescence, in agreement with previous reports,¹¹ a small amount of ThT fluorescence above the background appeared “instantaneously” and remained essentially constant during a 2 week incubation. This contrasts with the well-known pattern for unseeded WT A β ₁₋₄₀ fibrillization (Figure 1), in which ThT fluorescence rises rapidly only after a lag period of ~2–3 days. The latter kinetic pattern has been attributed to a nucleation—polymerization mechanism of fibril formation.^{35–37}

Immediately upon transfer of a solution of Δ E22-A β ₁₋₃₉ in neat DMSO into NaP, a visible precipitate appeared. This slurry was examined by electron microscopy; the slurry was also examined after incubation for 7 days at 37 °C. In the case of WT A β ₁₋₄₀, formation of a precipitate required incubation for several days at 37 °C, which is consistent with the ThT fluorescence data. Electron microscopy of the Δ E22-A β ₁₋₃₉ precipitates revealed numerous, abundant fibrils, both immediately after introduction of the peptide into buffer (Figure 2A–C) and after incubation for 7 days at 37 °C (Figure 2D–F); the appearance of the fibrils did

not change upon incubation. The Δ E22-A β ₁₋₃₉ fibrils have a slightly irregular edge. Only occasional fibrils show an apparent “twist”; more fibrils appear as flat ribbons with longitudinal striations. Fibrils of Δ E22-A β ₁₋₃₉ had a strong tendency to supertwist into matted, thick, ropelike helical bundles. Diameters of WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉ fibrils were measured (Figure 2I). Δ E22-A β ₁₋₃₉ fibrils had a slightly smaller mean diameter (9.44 ± 2.56 nm) than WT A β ₁₋₄₀ fibrils (11.0 ± 2.44 nm).

One method of obtaining single, unmatted Δ E22-A β ₁₋₃₉ fibrils for measuring diameters was to form the fibrils at low peptide concentrations. As shown in Figure 4 of the Supporting Information, fibrils were present even when the initial concentration was as low as 1 μ M.

¹H-¹⁵N HSQC NMR Spectra of Δ E22-A β ₁₋₃₉ and WT A β ₁₋₄₀

The very rapid formation of Δ E22-A β ₁₋₃₉ fibrils raises the question of whether DMSO completely disaggregates the lyophilized peptide. To address this question, we obtained ¹H-¹⁵N HSQC spectra of Δ E22-A β ₁₋₃₉ in DMSO-*d*₆, reasoning that if monomers and an aggregated soluble species were both present in DMSO, there would be more than one peak per amide for some of the amides in the peptide. As shown in Figure 5 of the Supporting Information, the spectrum of Δ E22-A β ₁₋₃₉ contains 39 peaks, consistent with 37 of the 38 backbone amides in the peptide, plus the two amide side chains for the one Asn and one Gln in the peptide. WT A β ₁₋₄₀ gave a similar ¹H-¹⁵N HSQC spectrum, containing 40 peaks, again consistent with one peak per amide. These results suggest that only a single species of each peptide exists in solution, either a monomer or, if oligomeric, a single species in which all molecules are in a symmetrical arrangement. By this criterion, Δ E22-A β ₁₋₃₉ is as fully disaggregated by DMSO as WT A β ₁₋₄₀.

Binding of ThT to Fibrils, Access of ThT to Binding Sites, and Congo Red Binding to Fibrils

The low, easily overlooked level of ThT fluorescence could be due to a structural difference between WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉ fibrils. (Other possibilities are considered below.) We next determined whether the relative lack of ThT fluorescence induced by Δ E22-A β ₁₋₃₉ fibrils was due to lack of ThT binding, a lack of fluorescence of bound ThT, or both. Accordingly, we developed an HPLC-based assay for ThT binding that was independent of fluorescence (see Materials and Methods). The results of these experiments (Figure 3A, for WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉ fibrils) indicate that both the apparent K_d and the number of binding sites for ThT are reduced for Δ E22-A β ₁₋₃₉ fibrils compared to those for WT A β ₁₋₄₀ fibrils. The K_d values for WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉ were 0.54 and 2.39 mM, respectively. The number of binding sites for ThT is far fewer than the number of peptide molecules, 510.5 and 117.0 for WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉, respectively. The binding sites for ThT on A β fibrils are heterogeneous;²⁵⁻²⁷ the equation is a phenomenological binding isotherm, used only to compare slurries of WT and mutant A β fibrils.

An additional potential cause of the reduction in the number and affinity of binding sites for ThT in Δ E22-A β ₁₋₃₉ fibrils is the matting of the fibrils, which could prevent access of ThT to binding sites on fibrils. This could cause an underestimate of the number of binding sites. By the commonly employed ThT fluorescence assays used in the experiments described above, aliquots of a fibril slurry are added to a solution of ThT for fluorescence measurements. Because Δ E22-A β ₁₋₃₉ forms fibrils very rapidly and by electron microscopy the fibrils are matted even at the earliest time points, we sought to determine whether the low level of ThT binding by Δ E22-A β ₁₋₃₉ fibrils could be due to the inability of ThT to penetrate fibril bundles to reach binding sites. Accordingly, we performed ThT fluorescence assays in which the dye was included in the solution of the fibrillizing peptide. As shown in Figure 6 of the Supporting Information, the development of ThT fluorescence was

essentially instantaneous. The fluorescence intensity was now some-what higher, which can be attributed to the greater fibril:ThT ratio. WT A β ₁₋₄₀ developed fibrils that gave high ThT fluorescence after 2–3 days. Again, the fluorescence intensity of WT A β ₁₋₄₀ fibrils was much higher than that of Δ E22-A β ₁₋₃₉ fibrils. The difference between WT and mutant fibrils was somewhat smaller than when ThT fluorescence was assayed from aliquots, as in Figure 1. Thus, a small part of the low ThT fluorescence of mutant fibrils is caused by the lack of access of the dye to potential binding sites. Nevertheless, an intrinsic difference remains between the two types of fibrils, by either assay.

We also tested whether Δ E22-A β ₁₋₃₉ fibrils cause the same shift in the visible spectrum of Congo Red (CR) as WT A β ₁₋₄₀ fibrils. WT and mutant fibrils caused similar spectral shifts in CR (Figure 3B). The absorbance maxima are ~517 and ~508 nm for WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉, respectively. The fact that both mutant and WT A β fibrils bind CR similarly, but not ThT, suggests that the binding sites for these two dyes do not entirely overlap.

Critical Concentrations of WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉

In this set of experiments, critical concentrations (C_T) were estimated for WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉ by incubation of 100 μ M solutions of each peptide for 3 weeks and subsequent seeding every 2.5 days with 5 μ L of a slurry of mature fibrils. The slurries were then allowed to incubate under quiescent conditions for 2 weeks. As described in Materials and Methods, the peptide remaining in solution was assayed by centrifugation of the slurry twice at 100000g, after which the peptide remaining in solution was assayed by hydrolyzing the peptide and measuring the fluorescamine-associated fluorescence. As shown in Figure 4, the fluorescamine fluorescence reached final value of 496 and 223, corresponding to C_T values of 2.89 and 0.167 μ M for WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉, respectively. Because our value for the C_T of WT A β ₁₋₄₀ was somewhat higher than that previously reported,²⁸ we considered the possibility that some of the peptide had been oxidized or hydrolyzed during the prolonged incubation. We centrifuged the slurry at 100000g using the Airfuge, isolated the supernatant, and analyzed this peptide by HPLC and MALDI-TOF mass spectrometry. HPLC showed the peptide to coelute with authentic WT A β ₁₋₄₀, and mass spectrometry revealed the usual mass for the intact peptide, without evidence of oxidation. We conclude that this small difference may be due to minor differences between our solubilization or assay procedures and those used in other laboratories.

Fluorescence Depolarization Experiments Show Very Rapid Aggregation of Δ E22-A β ₁₋₃₉

We performed fluorescence depolarization to examine the early stages of aggregation of Δ E22-A β ₁₋₃₉ by a method that is independent of ThT fluorescence. As described in Materials and Methods, solutions of Δ E22-A β ₁₋₃₉ and WT A β ₁₋₄₀ were excited at a λ_{ex} of 275 nm, and emission spectra were recorded at 5 min intervals from 290 to 350 nm. Both peptides contain a single Tyr residue and no Trp residues. Measurements of both peptides were taken at 10 and 100 μ M. The anisotropy of Δ E22-A β ₁₋₃₉ at 100 μ M could not be measured because the peptide precipitated too rapidly from solution (all fluorescence intensities decreased *pari passu*, and the signal was essentially absent after ~5 min). Figure 5 shows the fluorescence anisotropy of WT A β ₁₋₄₀ at 100 μ M and Δ E22-A β ₁₋₃₉ at 10 μ M at times up to 60 min (a similar profile is seen for WT A β ₁₋₄₀ at 10 μ M). The anisotropy of WT A β ₁₋₄₀ remains constant over the course of the experiment, indicating that no change occurs in the state of aggregation of this peptide. In contrast, Δ E22-A β ₁₋₃₉ undergoes a rapid increase in anisotropy, even at 10 μ M, indicating aggregation of the peptide within this time period. The increase in anisotropy could be fit as a first-order rate equation, with a k of 0.08 s⁻¹.

Size Exclusion Chromatography (SEC) Shows That Δ E22-A β ₁₋₃₉ Forms Oligomers but Requires Organic Solvent (DMSO) To Elute from the Column

The preceding experiments indicate that Δ E22-A β ₁₋₃₉ can form fibrils instantaneously, within the limits of the techniques described above, with no evident lag phase in the kinetics. Size exclusion chromatography (Superdex Peptide column) gives an indication of the completeness of fibrillization of Δ E22-A β ₁₋₃₉. Attempts to dissolve Δ E22-A β ₁₋₃₉ in aqueous buffer and then perform SEC never resulted in elution of peptide from the column. When Δ E22-A β ₁₋₃₉ was first dissolved in neat DMSO and then diluted with NaP to a final concentration of 100 μ M [2% (v/v) DMSO], only a miniscule peak eluted from the column, approximately in the same position as WT A β ₁₋₄₀ when the eluant was NaP (Figure 6A,B). The same experiment was attempted using only 10 μ M peptide (panels C and D of Figure 6 for WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉, respectively). Panels E and F of Figure 6 show elution profiles when the same experiment was repeated, except that the eluant also contained 2% (v/v) DMSO. Elution of anything more than a trace of Δ E22-A β ₁₋₃₉ from the column appeared to require the presence of an organic solvent such as DMSO.

WT A β ₁₋₄₀ (Figure 6A,C) elutes predominantly as a peak (~10 mL) with an apparent molecular weight (5528) consistent with a monomer or dimer. The limited resolution of the column does not allow discrimination between these species. A second peak with a smaller area elutes near the void volume (~8 mL), presumably as oligomers of the peptide. Δ E22-A β ₁₋₃₉ consistently eluted at a slightly later time (lower apparent molecular weight of 4606) than the WT peptide (Figure 6E,F). In addition, although the same quantities of WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉ were loaded onto the column, less mutant peptide eluted from it, suggesting that some of this peptide had precipitated on the column.

CD Spectroscopy Indicates that Δ E22-A β ₁₋₃₉ Adopts a β -Sheet Conformation “Instantly”

Δ E22-A β ₁₋₃₉ could not be dissolved into NaP from a lyophilized powder, and thus, its CD spectra could not be obtained without adding organic solvent, e.g., HFIP. Figure 7B shows the CD spectra of Δ E22-A β ₁₋₃₉ injected from neat HFIP into NaP [final HFIP concentration of 2% (v/v)] at 0 h, and 7 days later; Figure 7A shows spectra obtained under the same conditions for WT A β ₁₋₄₀. The WT peptide spectrum was consistent with “random coil” at time zero but had converted to β -sheet by day 7, consistent with ThT and other data indicating conversion to a β -sheet fibril structure. In contrast, Δ E22-A β ₁₋₃₉ immediately exhibited a β -sheet CD spectrum, and this did not change after 7 days. To assess whether the peptide was in the fluid or solid phase, CD spectra of the peptide solution (or slurry) were recorded immediately after it had been dissolved and then after centrifugation at 14300g (top 1 mL of 1.5 mL in the centrifuge tube). As shown in Figure 7C, most of the signal for WT A β ₁₋₄₀ remained in the supernatant, while for Δ E22-A β ₁₋₃₉, centrifugation caused a nearly total loss of signal, indicating that the β -sheet form of the peptide was insoluble and fibrillar, consistent with the electron microscopy data shown in Figure 2. [Similar experiments in which the peptides from DMSO were injected yielded similar results, but this solvent precludes measurements in the far-UV range (data not shown).] These results indicate that the preponderance of Δ E22-A β ₁₋₃₉ peptide giving a β -sheet was fibrillar, and not oligomers, which would have remained in the soluble phase.

Solid-State NMR Spectroscopy of Δ E22-A β ₁₋₃₉ Fibrils

Panels A and B of Figure 8 show PITHIRDS-CT data of two samples of Δ E22-A β ₁₋₃₉ fibrils, one containing a single 1 - ^{13} C label in V18 and the other with a single 1 - ^{13} C label in V36, respectively. For comparison, similar data are shown for WT A β ₁₋₄₀ fibrils containing a single 1 - ^{13} C label at V12 (Figure 8C). The decay of ^{13} C NMR signals (peak areas) with increasing dephasing time is related to the distance between ^{13} C labels, r , in adjacent peptide molecules, in a $1/r^3$ relationship. As described previously, in WT A β ₁₋₄₀ fibrils, which

contain parallel, in-register β -sheets, this distance is $\sim 5 \pm 0.2 \text{ \AA}$ for V12, V18, and V36, indicating that these residues are within the parallel β -sheets.^{7,8,38} This is not the case for the $\Delta E22$ -A β_{1-39} fibrils, which show a slower decay of the signal with increasing dephasing time, indicating that these residues are $>6 \text{ \AA}$ apart and thus are not within parallel, in-register β -sheets.

X-ray Diffraction of $\Delta E22$ -A β_{1-39} Fibrils

Panel A of Figure 9 shows the one-dimensional azimuthal plots of partially aligned $\Delta E22$ -A β_{1-39} and WT A β_{1-40} fibrils. Panels B and C of Figure 9 show a pseudocolored plot of the diffraction data for $\Delta E22$ -A β_{1-39} fibrils and WT A β_{1-40} fibrils, respectively. A diffraction pattern most consistent with a “cross- β ” structure is observed in both cases. The D spacings of the reflections differ between wild-type and mutant peptides, however. In both cases, a sharp, meridional reflection is observed at $4.7 \text{ \AA}$. At right angles, there is a broad equatorial reflection, which is at $10.4 \text{ \AA}$ for WT A β_{1-40} but at $9.6 \text{ \AA}$ for $\Delta E22$ -A β_{1-39} . This latter value is quite similar to that observed for D23N-A β_{1-40} , which contained antiparallel β -sheet fibril structures.

“Prion-like” Behavior of $\Delta E22$ -A β_{1-39}

Most patients with the $\Delta E22$ mutation of A β (i.e., the $\Delta E693$ mutation of β -APP) are heterozygotes, and likely synthesize both $\Delta E22$ -A β_{1-39} and WT A β_{1-40} . Figure 1 shows the ThT fluorescence time course obtained when a 1:1 molar solution of $\Delta E22$ -A β_{1-39} and WT A β_{1-40} is mixed in neat DMSO (both at $50 \text{ }\mu\text{M}$, hence a total peptide concentration of $100 \text{ }\mu\text{M}$) and then diluted with aqueous buffer (NaP). The mixture forms fibrils very rapidly, though there is a brief buildup period in which ThT-associated fluorescence increases in the mixture (consistent with first-order kinetics), which does not occur with pure $\Delta E22$ -A β_{1-39} . Thus, the presence of the WT peptide somewhat retards the fibrillization of $\Delta E22$ -A β_{1-39} . The final fluorescence value for the mixture obtained is identical to that obtained for pure $\Delta E22$ -A β_{1-39} , but notably, without a second phase that might be seen if the two peptides were fibrillizing in a manner independent of each other. If the WT peptide were not forming fibrils at all, one would expect the ThT fluorescence of a 1:1 mixture to be approximately half of that seen with $50 \text{ }\mu\text{M}$ pure $\Delta E22$ -A β_{1-39} . Furthermore, if the wild-type and mutant peptides were fibrillizing in a manner independent of one another, the ThT fluorescence would be the average observed for the two different types of fibrils. The fact that the fluorescence of the mixed fibrils is identical to that of pure $\Delta E22$ -A β_{1-39} fibrils suggests that the rapidly fibrillizing mutant peptide forms a seed or template upon which the wild-type peptide deposits, also in a form that binds ThT poorly (using the assay of refs 19–23). As shown in panels G and H of Figure 2, electron microscopy shows that the mixture contained abundant fibrils, and no obvious amorphous precipitate, confirming that the WT peptide is also fibrillizing.

The kinetic pattern is consistent with rapid nucleation of $\Delta E22$ -A β_{1-39} , followed by accretion of WT A β_{1-40} onto nuclei or fibrils of the mutant peptide. If this is the case, then the mutant peptide would have “converted” the wild-type peptide to a structure of fibrils that it does not ordinarily adopt, i.e., other than a parallel in-register β -sheet. We performed an HPLC analysis of the supernatant remaining at various times during the fibrillization of the mixture, to determine the concentration of each peptide. As shown in Figure 3B of the Supporting Information, the concentration of soluble $\Delta E22$ -A β_{1-39} decayed more rapidly than that of WT A β_{1-40} , though not so rapidly as that of $\Delta E22$ -A β_{1-39} alone. The kinetics were consistent with first-order kinetics, with k values of 0.08 and 0.29 h^{-1} for WT A β_{1-40} and $\Delta E22$ -A β_{1-39} , respectively. Note that at the earliest measurable time point, more than half of the $\Delta E22$ -A β_{1-39} had already precipitated, whereas essentially all of the WT A β_{1-40} was still in solution. After fibrillization for 2 weeks, the supernatant contained $12.1 \text{ }\mu\text{M}$

soluble $\Delta E22-A\beta_{1-39}$ and $32.6 \mu M$ WT $A\beta_{1-40}$. Both of these values are considerably higher than the critical concentrations, suggesting that the two peptides may be able to form a soluble mixed complex or several different types of complexes, i.e., mixed dimers or oligomers.

To test the hypothesis further that $\Delta E22-A\beta_{1-39}$ had converted WT $A\beta_{1-40}$ to an alternate structure, we performed X-ray diffraction of the mixed fibrils. As shown in Figure 9C, the mixed fibrils also showed a cross- β pattern, but with D spacings essentially identical to those of the pure $\Delta E22-A\beta_{1-39}$ fibrils, and clearly distinct from those of WT $A\beta_{1-40}$ fibrils, i.e., a sharp, meridional reflection at $4.7 \text{ \AA}$ and a broad equatorial reflection, which is at $9.6 \text{ \AA}$. In other words, the D spacings were those of the more rapidly fibrillizing species and were not means of the two sets of values. These results agree with the ThT fluorescence results in suggesting that the rapidly fibrillizing $\Delta E22-A\beta_{1-39}$ fibrils served as a seed or template for the WT peptide, “converting” the latter to some structure other than the usual parallel, in-register β -sheet.

DISCUSSION

Contrary to previous reports,^{11,17,39} $\Delta E22-A\beta_{1-39}$ forms amyloid fibrils extremely rapidly and with essentially complete conversion of the soluble peptide to fibrils. The fibrils have most of the hallmarks of amyloid, such as β -sheet structure as determined by CD spectroscopy and X-ray diffraction, Congo Red binding, and electron microscopic appearance, though a slight difference exists in mean fibril diameter between WT $A\beta_{1-40}$ and $\Delta E22-A\beta_{1-39}$ fibrils. Formation of fibrils and adoption of β -sheet structure are virtually instantaneous, as shown by ThT fluorescence, electron microscopy, CD spectroscopy, and fluorescence depolarization. Size exclusion chromatography indicates that $\Delta E22-A\beta_{1-39}$ can form oligomers under some conditions [e.g., when 2% (v/v) DMSO is present in the eluant], but these are short-lived and very rapidly progress toward fibril formation. The critical concentration of $\Delta E22-A\beta_{1-39}$ was ~ 1 order of magnitude lower than that of WT- $A\beta_{1-40}$ (2.89 and $0.167 \mu M$ for WT $A\beta_{1-40}$ and $\Delta E22-A\beta_{1-39}$, respectively). The value we obtained for the WT peptide was slightly higher than that obtained by O’Nuallain et al.,²⁸ presumably because of small differences in methodology. Nonetheless, our data suggest a substantial difference in the intrinsic solubilities of the two peptides.

One notable exception to the statement that $\Delta E22-A\beta_{1-39}$ forms typical amyloid fibrils is that the fibrils are associated with little ThT fluorescence, suggesting structural differences between these fibrils and those made by the WT peptide. We also showed that $\Delta E22-A\beta_{1-39}$ fibrils do bind ThT, though with a lower affinity, and with fewer apparent binding sites per mass of peptide. At least two explanations for this difference suggest themselves. First, electron microscopy of $\Delta E22-A\beta_{1-39}$ shows the presence of matted and supertwisted fibrils; these supramolecular structural features may block access of ThT to potential binding sites. When ThT is included in the solution in which fibrils are forming, a large difference between the ThT fluorescence of WT and mutant $A\beta$ remains, suggesting that the difference is due to structural differences, as well as decreased access of matted fibrils for the dye. Second, recent studies,^{25–27} from both experimental data and molecular dynamics simulations of the binding of ThT and other dyes to $A\beta$ fragments, suggest that ThT binding and fluorescence require an aromatic—hydrophobic groove spanning four consecutive β -strands. Distortion of this groove in the mutant peptide fibrils might allow some ThT binding, albeit at somewhat lower affinity, but abrogate the associated fluorescence. Thus, differences in ThT binding suggest structural differences between WT $A\beta_{1-40}$ and $\Delta E22-A\beta_{1-39}$ fibrils. Other clear indications of structural differences between these types of fibrils came from solid-state NMR measurements and the X-ray diffraction patterns of $\Delta E22-A\beta_{1-39}$ fibrils. In PITHIRDS-CT spectra, the distance between degenerate ^{13}C spins in

adjacent molecules of WT A β ₁₋₄₀ fibrils is $\sim 5.0 \pm 0.2$ Å, consistent with a parallel, in-register β -strand structure. In contrast, at two sites of Δ E22-A β ₁₋₃₉, (1-¹³C-V18 and 1-¹³C-V36), the PITHIRDS-CT spectra were inconsistent with this structure. X-ray diffraction showed a clear difference between the WT and mutant peptide fibrils: the broad equatorial reflections were at 10.4 and 9.6 Å for WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉, respectively. Further solid-state NMR work to delineate the structural features of these fibrils is ongoing.

Most patients with the Δ E693-A β -APP mutation are heterozygotes and thus, presumably, synthesize both wild-type and mutant forms of A β . Recent studies of another point mutant of the A β peptide, D23N-A β ₁₋₄₀, suggest that even fairly subtle sequence changes can lead to drastic change in fibril structure:²¹ the latter mutant forms fibrils with antiparallel β -sheet structure, in contrast to all previous observed structures of full-length A β . Here, we show that when a 1:1 solution of WT A β ₁₋₄₀ and Δ E22-A β ₁₋₃₉ was mixed in neat DMSO prior to fibrillization, the fibrils formed by this mixture had ThT fluorescence levels essentially identical to that of the mutant peptide fibrils alone. Both of the peptides were within the fibrils; a sedimentation assay of these mixtures showed that both peptides had precipitated, and electron microscopy showed fibrils and no amorphous precipitate, indicating that both peptides had formed fibrils. Furthermore, the electron microscopic appearance of the mixed fibrils was quite similar to that of the pure Δ E22-A β ₁₋₃₉ fibrils, i.e., matted and supertwisted bundles of slightly irregular fibrils. Finally, and most convincingly, X-ray diffraction of the mixed fibrils showed a pattern essentially identical to that of the pure Δ E22-A β ₁₋₃₉ fibrils and distinct from that of the WT A β ₁₋₄₀ fibrils, and, furthermore, not a mean of the two sets of values. Taken together, these results suggest that the rapidly aggregating Δ E22-A β ₁₋₃₉ peptide formed fibril seeds, which WT A β ₁₋₄₀ extended, with a structure resembling that of the mutant fibrils rather than that of typical WT A β ₁₋₄₀ fibrils. This type of “conversion” of the wild-type peptide into an atypical structure is reminiscent of the conversion of prion species, which has been observed in vitro for both yeast⁴⁰⁻⁴² and mammalian⁴³⁻⁴⁵ prion proteins, as well as for amyloids of β 2-microglobulin.^{46,47} By the same token, another rapidly fibrillizing point mutant form of A β , E22Q-A β (the Dutch mutant), has been shown to cross-seed fibril extension by the wild-type peptide.⁴⁸

Finally, these studies may have implications for the pathogenesis of early onset AD in this kindred. Two likely explanations for the negative Pittsburgh B brain scan cannot be distinguished at present. These are a lack of Δ E22-A β ₁₋₃₉ amyloid deposition in the brains of these patients and, alternatively, a lack of binding by Pittsburgh B, which is structurally homologous to ThT. The rapidity with which Δ E22-A β ₁₋₃₉ forms fibrils, however, suggests that extensive protein aggregation could occur within neurons, even leading to intracellular formation of fibrils. Indeed, Tomiyama et al. have documented a very low level of A β secretion by cells transfected with the Δ E693 mutant form of β -APP. The sparseness and transience of soluble oligomers of Δ E22-A β ₁₋₃₉ also suggest that soluble oligomeric species may not be necessary for neuronal death in all forms of Alzheimer’s disease. Recently, a transgenic mouse model of the Δ E693-A β -APP mutation has been described.⁴⁹ These mice exhibited age-dependent accumulation of intraneuronal A β , which coincided with the development of neuronal degeneration and glial cell reaction. The observation that no neuritic plaques developed should not, however, be construed to mean that only oligomers are present; fibrils developing within neurons could easily escape detection, and “fibrils” should never be equated with “plaques”. This study underscores the complexity of the pathogenesis of this disease and indicates that multiple pathogenic mechanisms may be at play.

Supplementary Material

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ABBREVIATIONS

Aβ	β -amyloid
MW^{app}	apparent molecular weight
β-APP	β -amyloid precursor protein
CD	circular dichroism
C_r	critical concentration
DMSO	dimethyl sulfoxide
HFIP	hexafluoro-2-propanol
HPLC	high-performance liquid chromatography
GBTU	2-(1 <i>H</i> -benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBT	<i>N</i> -hydroxybenzotriazole
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight
MAS	magic angle spinning
NaP	10 mM sodium phosphate (pH 7.40) with 0.01% NaN ₃
Rf	radiofrequency
RP	reverse phase
SEC	size exclusion chromatography
SS-NMR	solid-state nuclear magnetic resonance
TFA	trifluoroacetic acid
ThT	thioflavin T
WT	wild-type

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Figure 1.

Thioflavin T fluorescence time course. Peptides were dissolved in neat DMSO and then diluted into aqueous buffer (NaP) so that the final peptide concentration was 100 μ M and the DMSO concentration was 2% (v/v). At various intervals, aliquots were removed from the slurry, and thioflavin T fluorescence was measured as described in Materials and Methods. For the mixtures, peptides were dissolved and mixed in neat DMSO so that the final total peptide concentration was 100 μ M and the DMSO concentration was 2% (v/v): (circles) WT $A\beta_{1-40}$, (squares) $\Delta E22-A\beta_{1-39}$, and (diamonds) 1:1 (molar) mixture of WT $A\beta_{1-40}$ and $\Delta E22-A\beta_{1-39}$.

Figure 2.

Electron micrographs of $\Delta E22-A\beta_{1-39}$ fibrils. Peptide was dissolved in DMSO and then diluted into NaP so that the peptide concentration was 100 μM and the final concentration of DMSO was 2% (v/v). A precipitate appeared instantly. Panels A–C show peptide immediately after addition of NaP to the peptide in neat DMSO. Magnifications are 15000 $\times$, 98000 $\times$, and 149000 $\times$. The images in panels A–C were taken immediately after the peptide had been transferred from neat DMSO to buffer and were viewed within 5 min of making the sample. Panels D–F are images of the same slurry after incubation for 7 days at 37 °C. Panels G and H are electron micrographs of fibrils of a 1:1 (molar) mixture of WT $A\beta_{1-40}$ and $\Delta E22-A\beta_{1-39}$. Peptides were dissolved in neat DMSO and then diluted with NaP so that

the final concentration of each peptide was 50 μM (i.e., total peptide concentration of 100 μM) and the final DMSO concentration was 2%. A precipitate formed immediately. Magnifications of 15000 $\times$ and 49000 $\times$ for panels G and H, respectively. For all images, the CCD camera added a magnification of 1.4 $\times$. (I) Histograms for $\Delta\text{E22-A}\beta_{1-39}$ and WT $\text{A}\beta_{1-40}$ fibril diameters, based on 200 and 400 diameter measurements, respectively. For both peptides, the histograms showed a single peak; means $\pm$ standard deviations were 11.0 ± 2.44 nm for WT $\text{A}\beta_{1-40}$ fibrils and 9.44 ± 2.56 nm for $\Delta\text{E22-A}\beta_{1-39}$ fibrils.

Figure 3.

(A) Binding of ThT to WT $A\beta_{1-40}$ (circles) and $\Delta E22-A\beta_{1-39}$ (squares) fibrils. Various volumes of fibril slurries (nominal peptide concentration of 1 mM) were added to a solution of ThT, as described in Materials and Methods. After an incubation period, the mixture was centrifuged and the supernatant analyzed for ThT concentration by HPLC. (B) Congo Red absorbance in the presence of WT $A\beta_{1-40}$ (circles) and $\Delta E22-A\beta_{1-39}$ (squares) fibrils; the figure also shows the spectrum of Congo Red alone (diamonds).

Figure 4.

Critical concentrations of WT Aβ₁₋₄₀ (●) and ΔE22-Aβ₁₋₃₉ (■) estimated by seeding solutions of these peptides with fibrillar seeds, as described in Materials and Methods. At various times, aliquots were removed to determine the concentration of peptide in solution, using the fluorescamine assay described in Materials and Methods. The critical concentration was estimated from a fit of the data to the equation of a first-order approach to equilibrium.

Figure 5.

Fluorescence depolarization for (circles) WT $A\beta_{1-40}$, (squares) $\Delta E22-A\beta_{1-39}$, and (diamonds) a 1:1 (molar) mixture of WT $A\beta_{1-40}$ and $\Delta E22-A\beta_{1-39}$. The line represents a nonlinear least-squares fit of the data to the first-order rate equation $A = (A_{\infty} - A_0)[1 - \exp(-kt)] + A_0$, where A , A_0 , and A_{∞} are the anisotropies at time t , time zero, and infinite time, respectively, and k is the rate constant.

Figure 6.

Size exclusion chromatography of WT Aβ₁₋₄₀ and ΔE22-Aβ₁₋₃₉ performed using a Superdex Peptide 10/300 column. For panels A–D, the peptide was initially dissolved in neat DMSO, which was diluted with NaP so that the final peptide concentration was 100 (A and B) or 10 μM (C and D). The eluant was NaP, without DMSO. For panels E and F, the peptide concentrations were 100 and 200 μM, respectively, and the samples were prepared as described for panels A–D. The eluant, however, was NaP containing 2% (v/v) DMSO: (A) 100 μM WT Aβ₁₋₄₀, (B) 100 μM ΔE22-Aβ₁₋₃₉, (C) 10 μM WT Aβ₁₋₄₀, (D) 100 μM ΔE22-Aβ₁₋₃₉, (E) 100 μM WT Aβ₁₋₄₀ (black) or ΔE22-Aβ₁₋₃₉ (blue), and (F) 200 μM WT Aβ₁₋₄₀ (black) or ΔE22-Aβ₁₋₃₉ (blue). For panels A–D, the effluent was monitored at 220

nm; for panels E and F, the effluent was monitored at 274 nm rather than 220 nm, because of the presence of DMSO in the eluant.

Figure 7.

CD spectra of WT Aβ₁₋₄₀ and ΔE22-Aβ₁₋₃₉. (A) WT Aβ₁₋₄₀ was dissolved in HFIP and then diluted to a final peptide concentration of 100 μM with NaP. Spectra were recorded immediately after dilution of the peptide into NaP ($t = 0$ days, red) and after incubation at 37 °C for 7 days ($t = 7$ days, blue). (B) ΔE22-Aβ₁₋₃₉, treated the same as WT Aβ₁₋₄₀ in panel A. (C) WT Aβ₁₋₄₀ (red) and ΔE22-Aβ₁₋₃₉ (blue) were dissolved in HFIP and then diluted with NaP as described for panels A and B. Immediately after the sample had been diluted into NaP, an initial CD spectrum was measured and the mixture was then centrifuged at 14300g, and the CD spectra of the supernatants (top 1 mL out of 1.5 mL in the centrifuge tube) were measured: (filled circles) WT Aβ₁₋₄₀ before centrifugation, (empty circles) WT

A β_{1-40} supernatant, (filled squares) Δ E22-A β_{1-39} before centrifugation, and (empty squares) Δ E22-A β_{1-39} supernatant.

Figure 8.

Raw PITHIRDS-CT data for rehydrated $\Delta\text{E22-A}\beta_{40}$ fibrils with ^{13}C labels at Val18 (A) and Val36 (B). For comparison, comparable data are provided for WT $\text{A}\beta_{1-40}$ with a ^{13}C label at Val12 (C).

Figure 9.

X-ray fiber diffraction pattern for partially aligned fibrils of $\Delta E22-A\beta_{1-39}$ and for fibrils formed from a 1:1 (molar) mixture of $\Delta E22-A\beta_{1-39}$ and WT $A\beta_{1-40}$. (A) One-dimensional azimuthal plots, showing intensity as a function of D spacing for WT $A\beta_{1-40}$ (black), $\Delta E22-A\beta_{1-39}$ (red), and the 1:1 (molar) mixture of $\Delta E22-A\beta_{1-39}$ and WT $A\beta_{1-40}$ (blue). The intensities have been normalized to the maximal intensity value of the peak of ~ 10 Å spacing. The one-dimensional azimuthal plot for WT $A\beta_{1-40}$ is from ref 19. (B) Pseudocolored plot of the X-ray diffraction pattern for $\Delta E22-A\beta_{1-39}$. (C) Pseudocolored plot of the X-ray diffraction pattern for the 1:1 (molar) mixture of $\Delta E22-A\beta_{1-39}$ and WT $A\beta_{1-40}$.