C-Terminal PEG Blocks the Irreversible Step in β-Amyloid(10-35) Fibrillogenesis

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β-Amyloid, the major component of plaques found in the brains of patients with Alzheimer's disease (AD),[1] is a 40-43 amino acid proteolytic fragment of the β-amyloid precursor protein,[2] which rapidly and irreversibly associates into insoluble fibrils.[3] While generalized models exist for the mechanisms of aggregation and the structure of the final fibrillar products,[4] these peptides have proven to be intractable for detailed structural and mechanistic studies. Here we report a synthetic strategy that makes fibril formation freely reversible and use this derivative to characterize the steps in fibrillogenesis.

The Aβ peptide contains a hydrophilic N-terminus, a central hydrophobic region (aa 17-21) and a long and very hydrophobic C-terminus (aa 29-43). Aβ(10-35) constitutes the central core amino acids, retains the domain structure (hydrophobics in bold), and is competent to add to bona fide plaques from brains of patients with AD.[5] Solid-state NMR experiments characterized the central domain of the fibrils as an extended parallel β-sheet with each residue in register across the strand.[6] This structure predicts clustering of the hydrophobic C-termini in apposition, an orientation deemed to be critical to the water solubility of the fibril. The C-terminal PEG-derivatized peptide **1** was prepared for solution phase studies to be carried out in conjunction with the solid-state NMR studies of Aβ(10-35).

^{10}YEVHHQK**LVFFA**EDVGSNK**GAIIGL**^{35}**M**-NH-(CH$_2$CH$_2$O)$_x$-OH **1**

Compound **1** was prepared using standard FMOC protocols on PAP Tenta-Gel purchased from Rapp Polymer.[7] The last 10 residues were double coupled to give an overall crude coupling yield of 96%. Cleavage by TFA and deprotection yielded a linear poly(ethylene glycol) 3000 covalently bound to the carboxyl terminus of the peptide. **1** was a single peak by RP-HPLC, a single band by SDS−PAGE and showed a modal molecular weight of 6212 Da (2900 peptide + $\bar{M}_w$ = 3000 PEG) by MALDI-TOF mass spectroscopy. Purity was established by CNBr cleavage of the poly(ethylene glycol) block at M35, giving a single peak on RP-HPLC with a mass of 2856.6 Da.

Solutions of **1** between pH 3 and 7 were easily prepared and maintained. In contrast, under similar ionic strength (<1 mM salt) and peptide concentrations, Aβ(10-35) formed gels or precipitated above pH 5.5. However both peptides bound the dye Congo Red and exhibited the characteristic apple-green birefringence of the amyloid fibrils.[8] These fibrils were further analyzed by side chain cross-linking with tissue transglutaminase, which couples Q15 and K16 side chains in Aβ.[9] Both **1** and Aβ(10-35)[6] yielded similar banding "ladders" from monomer to hexamer on SDS−PAGE, suggesting that the fibrillar structure of **1** and Aβ(10-35) were structurally related to each other and to the native Aβ fibril.

The increased solubility of **1** greatly simplified the ^{1}H NMR analyses.[10] The ^{1}H NMR of **1** gave well-resolved signals with sharp lines at pH 3.6 that began to broaden only above pH 6. The 2D spectra were quite similar to those reported previously for Aβ(10-35),[5] including a pH-dependent transition from an unstructured peptide at low pH to a more ordered one as indicated by strong NOESY cross-peaks to aromatic side chain protons. However, for both **1** and Aβ(10-35), even at pH 5.6, there was a notable absence of the downfield shift of the Cα proton resonances diagnostic for β sheets[11] and only a weak β-signature by CD. When the pH was raised above 6, the lines broadened with a loss of signal intensity, consistent with the formation of larger aggregates. In the presence of 0.1 M sodium phosphate at pH 7.0, the signal intensity was further diminished.

The difference between **1** and Aβ(10-35) was most obvious in the CD spectra. Figure 1A shows a dramatic concentration

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(10) Lyophilized peptide, 1 and 5 mM, was dissolved in 10% D_2O/H_2O, pH adjusted with NaOH/HCl, diluted to 600 μL, and centrifuged for 15 min at 14 000×*g*. ^{1}H NMR data were collected at 500 MHz (Unity INOVA) and solvent and polymer signals were presaturated. 2D ^{1}H NMR spectra were collected at 25 or 10 °C, with 256 FIDs of 2 to 4K data points, 32 to 128 scans per FID, and a spectral width of 5000 Hz. The chemical shifts were referenced to DSS, 0.0 ppm.

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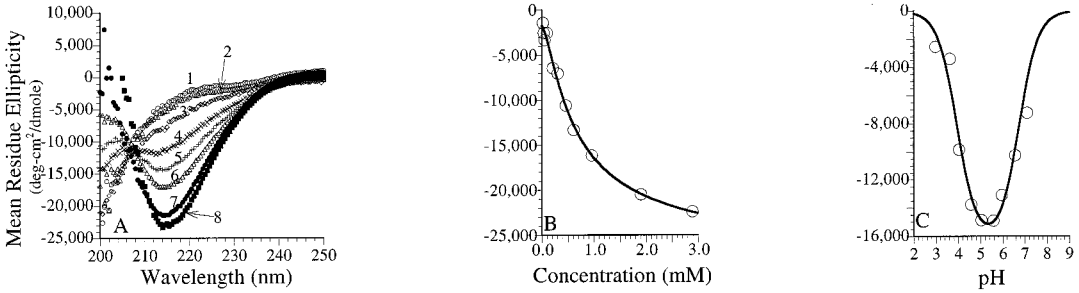

Figure 1. Concentration-dependent CD of **1** (A), with $[\theta]_{217}$ fit to both [**1**] (B) and pH (C).

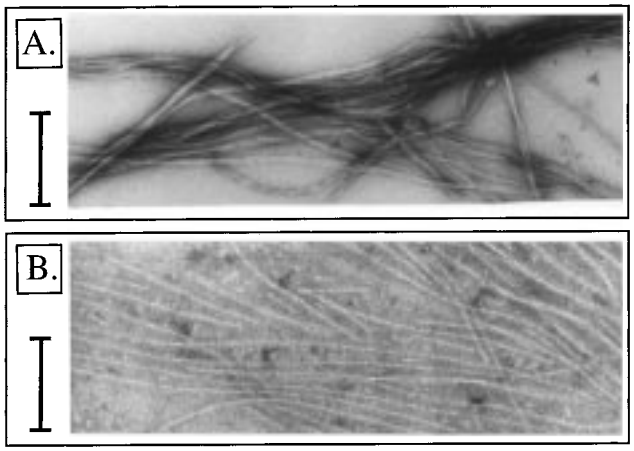

Figure 2. EM of (A) Aβ(10-35) and (B) **1**, each prepared at pH 7.4. Scale 200 nm.

induced transition from coil to β structure for **1** in ~0.3 M salt. Curve fitting analyses of the concentration-induced transition, Figure 1B, identified a reversible monomer−oligomer equilibrium. Consistent with the enzymatic cross-linking experiments above, the best fit for the CD data was obtained with a hexameric or heptameric aggregation state. Light scattering experiments[12] within the same concentration range showed reversibility − raising the pH to 7 induced aggregation to $\bar{M}_w > 2.5 \times 10^5$ over several hours, but this aggregation was rapidly reversed ($\bar{M}_w \approx 7 \times 10^3$) when the pH was lowered below 3.6.

This pH dependency was quite dramatic when monitored by CD (Figure 1C).[12] As the pH was increased, there was an increase in the β character, up to pH 5.6; at higher pH, $-[\theta]_{217}$ decreased, probably due to the formation of the much larger aggregates detected by electron microscopy (Figure 2). The two apparent p*K* values, 3.9 and 7.0, would be consistent with side chain−side chain interactions stabilizing β sheet aggregates.

Electron micrographs[13] verified the formation of long fibrils. As shown in Figure 2, both **1** and Aβ(10-35) form long filaments at pH 7.0, but two distinguishing characteristics were apparent. First, uranyl acetate stain did not penetrate the fibers of **1**, consistent with a model in which the PEG coats the surface of the fiber. Second, while the fibers prepared from Aβ(10-35) readily self-associate laterally into bundles (Figure 2a), the fibrils of **1** are separate and rarely if ever self-associate (Figure 2b). This finding is consistent with a model in which the PEG block of **1** is located on the surface of the fibril, such that longitudinal fibril growth proceeds, but lateral fibril−fibril self-association, a critical irreversible step in fibrillogenesis, is retarded.

From the above data, the following scheme for fibrillogenesis of **1** is proposed:

$$U_{\text{unstructured}} \rightleftharpoons \beta_{\text{aggregate}} \rightleftharpoons (\beta_{\text{fibril}})_{\text{sol}} \rightarrow (\beta_{\text{fibril}})_{\text{precip}}$$

At low peptide concentrations, low pH, and/or low ionic strength, the peptide block of **1** is unstructured (U) and can be observed by NMR and CD spectroscopy. As the pH or concentration is raised, the peptide self-associates to form the oligomeric $\beta_{\text{aggregate}}$, possibly a hexamer. $\beta_{\text{aggregate}}$ readily forms a soluble fibril, $(\beta_{\text{fibril}})_{\text{sol}}$, which contains a classic β signature by CD, can be observed by EM, and is perhaps similar to the previously described protofibril.[14] The lateral aggregation of linear fibrils into $(\beta_{\text{fibril}})_{\text{insol}}$, so readily seen with the unmodified peptide, is inhibited by the C-terminal addition of PEG.

These data also argue that the core of the fibril formed with **1** and Aβ(10-35) are similar, and the cross-linking data suggest that both resemble the aggregates formed by Aβ. Significant evidence now exists to suggest that the early steps in fibril formation by Aβ are reversible,[4h−m] but are difficult to analyze because of irreversible precipitation. We argue, therefore, that **1** represents a congener of the pathogenic Aβ peptide which forms fibrils in a freely reversible manner. By this model, fibril formation with **1** by the addition of small oligomeric aggregates, e.g., hexamers, represents a thermodynamic step function where small changes in concentration and/or pH are transduced into the highly favorable energetics of fibrillogenesis. Moreover, the addition of PEG shields the hydrophobic domain of the peptide near the C-terminus and inhibits irreversible fibril−fibril association, placing specific limits on the structure of the peptide in these fibrils. Therefore, these studies suggest a strategy to define the energetics and possibly even aid in the development of other specific inhibitors of different phases of fibrillogenesis.

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Supporting Information Available: Curve fitting analyses, characterization of **1** (Figure 1S), Congo Red staining of **1** (Figure 2S), SDS−PAGE analysis of tTG cross-linking experiments (Figure 3S), and NOESY spectra of **1** (Figure 4S) (6 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(12) A Wyatt Technologies Dawn Laser photometer in batch mode was used for light scattering, and ~450 points were averaged using 5 detectors at angles 90−132.2°. Molecular weight was calculated by Debye plots using ASTRA v 4.5 as a weight average molecular weight ($\bar{M}_w$). A 1.14 mg/mL solution of **1** showed no detectable change in $\bar{M}_w$ at pH 3.0 for 12 h. Upon adjustment above 6.5, the $\bar{M}_w$ increased over 300 min. At this point, an identical solution of **1** bound Congo Red. At 350 min, the pH was adjusted back to <3.6 with HCl, and the $\bar{M}_w$ dropped to the basal reading over 40 min.

(13) Peptides were dissolved in citrate/phosphate buffer containing 0.1% (w/v) NaN_3, centrifuged for 15 min at 14 000×*g* and maintained for 4 days at room temperature. Each sample was applied to a glow discharge 400 mesh carbon-coated support film, followed by staining with 1% uranyl acetate. Micrographs were obtained with a Philips EM 300 at magnifications of 45 000×.

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