Deposition of Monomeric, Not Oligomeric, Aβ Mediates Growth of Alzheimer's Disease Amyloid Plaques in Human Brain Preparations[†]

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Received March 29, 1999; Revised Manuscript Received June 1, 1999

ABSTRACT: Senile plaques composed of the peptide Aβ contribute to the pathogenesis of Alzheimer's disease (AD), and mechanisms underlying their formation and growth may be exploitable as therapeutic targets. To examine the process of amyloid plaque growth in human brain, we have utilized size exclusion chromatography (SEC), translational diffusion measured by NMR, and in vitro models of Aβ amyloid growth to identify the oligomerization state of Aβ that is competent to add onto an existing amyloid deposit. SEC of radiolabeled and unlabeled Aβ over a concentration range of 10^{-10}−10^{-4} M demonstrated that the freshly dissolved peptide eluted as a single low molecular weight species, consistent with monomer or dimer. This low molecular weight Aβ species isolated by SEC was competent to deposit onto preexisting amyloid in preparations of AD cortex, with first-order kinetic dependence on soluble Aβ concentration, establishing that solution-phase oligomerization is not rate limiting. Translational diffusion measurements of the low molecular weight Aβ fraction demonstrate that the form of the peptide active in plaque deposition is a monomer. In deliberately aged (>6 weeks) Aβ solutions, a high molecular weight (>100 000 M_r) species was detectable in the SEC column void. In contrast to the active monomer, assembled Aβ isolated from the column showed little or no focal association with AD tissue. These studies establish that, at least in vitro, Aβ exists as a monomer at physiological concentrations and that deposition of monomers, rather than of oligomeric Aβ assemblies, mediates the growth of existing amyloid in human brain preparations.

The formation and growth of brain amyloid lesions termed senile plaques are widely believed to be seminal events in the pathogenesis of Alzheimer's disease (AD) (*1*−*3*). The primary constituent of amyloid plaques is a 39−43 amino acid hydrophobic peptide, Aβ (*4*), which is produced as the cleavage product of a much larger protein termed the β-amyloid precursor protein (βPP) (*5*−*7*). While AD is a complex disorder that is incompletely understood, an abundance of strong circumstantial evidence supports the hypothesis that Aβ is central to the pathogenesis of AD. Although other mechanisms cannot be excluded, the available data support the hypothesis that brain amyloid is critical to the disorder. Aβ amyloid plaques are an almost invariant feature of disease (*3*, *8*), and in many studies the density of amyloid lesions at autopsy correlates well with the degree of dementia experienced by the patient in life (*9*). The Aβ peptide is toxic to neurons in culture but only in an assembled (amyloid-like) state (*10*−*17*). The pathogenic role of Aβ in AD has recently been buttressed by the observation that in some transgenic animals Aβ overproduction can lead to both the formation of amyloid plaques in the brain (*18*, *19*) and sometimes to neuronal degeneration (*20*). Most importantly, however, all known heritable forms of Alzheimer's disease associated with mutations in *APP* (the gene encoding βPP) or in genes for other proteins on other chromosomes lead to alterations in the production or amyloidogenicity of Aβ (*2*, *3*, *21*).

Although Aβ is widely believed to be an essential factor in AD, Aβ in its native unassembled form does not appear to be pathogenic. Aβ is constitutively produced by cells in

[†] This research was supported by the National Institutes of Health (Grants AG12853, AG13735, AG10123, AG12435, and AG11852) and the Veterans' Administration (Merit Review).

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[1] Abbreviations: Aβ, human β-amyloid peptide; AD, Alzheimer's disease; βPP, β-amyloid precursor protein; BPTI, bovine pancreatic trypsin inhibitor; BSA, bovine serum albumin; Ci/mmol, Curies per millimole; CSF, cerebrospinal fluid; ^{125}I-Aβ, ^{125}I-iodotyrosine^{10}-human Aβ(*1*−*40*)-OH; NMR, solution phase nuclear magnetic resonance spectroscopy; PBS, phosphate-buffered saline (10 mM phosphate, 100 mM sodium chloride, pH 7.5); PFG, pulsed field gradient; RP-HPLC, reverse phase high-performance liquid chromatography; SEC, size-exclusion chromatography.

10.1021/bi990718v CCC: $18.00
Published on Web 07/27/1999

culture (*22*), and patients who suffer from AD and unaffected individuals have approximately the same (≈nM) concentration of the peptide in cerebrospinal fluid (CSF) (*23*, *24*) although the relative levels of some Aβ isoforms can vary in AD vs control patients (*25*). Consequently, the mechanisms underlying the conversion of Aβ to amyloid may be critical to understanding the molecular etiology of the disease.

In vitro systems offer a reductionist approach to model the complex process of amyloid formation and growth under well-controlled conditions, where critical features of the process can be elucidated. At high concentrations (μM to mM), synthetic Aβ assembles into fibrils that are morphologically and tinctorially similar to AD brain amyloid (*26*). This process of nascent Aβ oligomerization into amyloid-like fibrils has been shown to be nucleation dependent and follow higher order kinetic dependence (≈5th−15th order) on soluble Aβ concentration (*27*, *28*). In contrast, amyloid growth by addition of Aβ from solution (Aβ deposition) onto an existing amyloid deposit in the form of amyloid plaques in preparations of unfixed AD cortex (*29*−*31*) or a synthetic amyloid template (*32*) is independent of nucleation and follows first-order kinetic dependence on soluble Aβ concentration. Thus, comparison of the biochemical properties of de novo amyloid formation (Aβ nucleation and aggregation) and growth of preexisting amyloid (Aβ deposition) indicates that the processes are distinct in vitro and in vivo (*33*).

Although some features in the primary sequence and conformation of Aβ that are critical in supporting the formation (*34*−*44*) and growth (*29*, *30*, *32*, *45*−*47*) of amyloid deposits have been elucidated, the biochemical mechanisms underlying these processes are not fully understood. One critical question that remains to be addressed is the Aβ oligomerization state capable of deposition onto a preexisiting amyloid template. Although the process of Aβ deposition onto preexisting amyloid follows first-order kinetic dependence on soluble Aβ concentration (*31*, *32*), indicating that soluble Aβ oligomerization is not rate limiting prior to template docking, the base oligomerization state of the Aβ in solution under such conditions has not been established. There are at least three possible deposition mechanisms where solution phase assembly is not rate limiting. Aβ may be capable of depositing onto preexisting amyloid as a monomer. Alternatively, Aβ may exist as a stable oligomer (e.g. a dimer), where the oligomers deposit without further assembly. Further, a subset of Aβ molecules may be preassembled oligomers in solution and only these oligomers are competent to deposit. To distinguish between these possibilities, we have determined the oligomerization state of Aβ in solution and examined deposition kinetics of Aβ using solutions of known assembly state.

METHODS AND MATERIALS

Synthetic Peptides. Synthetic Aβ(*1*−*40*) was purchased from Quality Controlled Biochemicals (Hopkinton MA) (lot no. 03013612) with a purity of >95%. Peptides were characterized by reverse phase HPLC (RP-HPLC), matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI TOF-MS), and amino acid analysis (*48*) and gave satisfactory results in all cases. All experiments were performed using Aβ(*1*−*40*). While amyloid or preamyloid composed of Aβ(*1*−*42*) or other forms may selectively form early in AD, Aβ(*1*−*40*) was selected because it is the most common form of Aβ in cerebrospinal fluid.

Radioiodination. Peptides were radioiodinated as previously described (*29*, *49*). Briefly Aβ(*1*−*40*) was radiolabeled at tyrosine 10 by oxidative iodination using $Na^{125}I$ and chloramine T. The radiolabeled Aβ was purified by reverse-phase high performance liquid chromatography (RP-HPLC) to essentially quantitative specific activity (about 2000 Ci/mmol; 10^9 dpm/μg) and was stored at −20 °C in the eluted HPLC mobile phase. 2-Mercaptoethanol (0.5%) was added to all stock tracer solutions to prevent oxidation during storage. Under these conditions, no evidence of oxidation, degradation, or aggregation was observed over 1 month of storage. The radiolabeled Aβ peptides produced by this method (^{125}I at tyrosine 10, methionine 35 as native thioether) have been found to behave indistinguishably from unlabeled Aβ in a wide range of experiments including those used to monitor the assembly and aggregation of the peptide (*49*−*51*).

Size Exclusion Chromatography. Superdex Peptide and Superdex 75 high-resolution (HR) grade columns (1 × 30 cm) were purchased prepacked from Pharmacia (Amersham Pharmacia Biotech, Piscataway, NJ) and run in series to extend the molecular weight range for effective fractionation. Samples were run in a mobile phase PBS (10 mM sodium phosphate, 100 mM NaCl, 0.02% NaN_3, pH 7.5) at a flow rate of 0.75 mL/min. The columns were equilibrated with mobile phase for at least 30 min prior to each sample run and were periodically cleaned with 0.1 M NaOH. The column system was calibrated using 200 μL samples of amino acid, peptide, and protein standards detected by UV absorbance at 280 nm (UV_{280}). Blue dextran was run to determine the void volume of the column series, and pyridine was used to mark the passage of a column series volume. Unlabeled Aβ(*1*−*40*) samples were dissolved in PBS from dry lyophilized material to the final experimental concentrations of 230, 200, 100, and 10 μM and centrifuged at 100 000*g* overnight to remove particulates. Immediately prior to column loading, all samples were briefly recentrifuged (15 000*g*, 10 min) and a 200 μL aliquot of the supernatant was injected onto the column series. ^{125}I-Aβ(*1*−*40*) was diluted with PBS to the appropriate concentration from stock (specific activity ≈ 2000 Ci/mmol). For experiments involving the simultaneous running of both labeled and unlabeled Aβ, the stock ^{125}I-Aβ was diluted in PBS then added immediately to lyophilized unlabeled Aβ(*1*−*40*) to yield the desired concentrations of labeled and unlabeled Aβ. Unlabeled Aβ(*1*−*40*) was detected by UV_{280} for loading concentrations of 10 μM and above. For very low Aβ concentration (≤1 nM) experiments, radiolabeled Aβ was used to facilitate detection. For concentrations ranging from 1 nM to 100 μM, an Aβ specific immunoassay (*50*, *51*) was used to detect and quantify Aβ(*1*−*40*) in eluted column fractions. For time course experiments, 200 μM Aβ(*1*−*40*) was prepared as described above and stored at 4 °C. The 200 μL aliquots of the sample were sized at time = 0, 2 days, 2 weeks, and 1 month. Aβ was monitored by UV detection and/or immunoassay of eluted fractions. All SEC experiments were performed at room temperature.

Aβ Deposition Experiments. Radiolabeled Aβ deposition experiments were performed at room temperature using a

modification of the method described (*31*). Briefly, slide mounted sections of unfixed AD and normal (non-AD) cortex were allowed to preincubate for 30 min in PBS containing BSA (0.1% w/v) and protease inhibitors. Radiolabeled Aβ was obtained by pooling fractions (from a single SEC run) that correspond to the elution point of low molecular weight (unassembled) Aβ. The fractionated Aβ was then immediately diluted in PBS (containing BSA and protease inhibitors) to the desired concentration and incubated with slide-mounted cortex sections for 3 h. The time interval from fraction collection to deposition experiment was less than 30 min. Following the incubation period, the sections were thoroughly washed and dried and then analyzed by autoradiography (*29*−*31*). For kinetic experiments, size exclusion fractionated ^{125}I-Aβ was prepared as described above and aliquoted to flexible 96-well assay plates and mixed with AD cortex homogenate. Following incubation, bound and free tracer were separated by centrifugation and washing. Bound tracer was quantified by γ-counting. Time course measurements of Aβ deposition were made at each of four Aβ concentrations. A log−log plot of deposition rate vs Aβ concentration was used to determine kinetic dependence on soluble Aβ concentration. The process of Aβ deposition is specific as all D-Aβ (*47*), an N-terminal Aβ fragment Aβ-(*1*−*28*) (*45*), or an Aβ peptide with a point substitution F19T (*46*) are incapable of stable deposition. Further, while fibrils of Aβ(*1*−*40*) can serve as a template for Aβ deposition, similar preparations of Aβ(*40*−*1*) (*32*), scrambled Aβ (*32*), or all D-Aβ (*47*) do not act as templates for deposition Aβ-(*1*−*40*) (*23*, *24*).

Translational Diffusion. To determine the oligomerization state of Aβ in aqueous solution, translational self-diffusion coefficients ($D_{25,w}$) were determined using pulsed field gradient (PFG) solution phase NMR for Aβ solutions, prior to and following SEC, using the method described by Lee and co-workers (*52*). Briefly, data were collected on a Varian UNITY*plus* 500 MHz NMR instrument equipped with a 5 mm ULTRA*linear* PFG probe using a modification (*52*) of the pulse sequence of Gibbs and Johnson (*53*) to improve solvent suppression. Data were acquired at 25 °C in the PBS used for SEC experiments. To provide a lock signal, D_2O (final concentration 10%) was added to the samples. Gradient strength was calibrated against the self-diffusion coefficient of water, and using this method the coefficient for a well-characterized protein standard, lysozyme, was determined and found to be consistent with published values (*54*). A set of well-characterized synthetic peptides of known molecular weight and oligomerization state (*52*) were used as standards for all diffusion constant determinations of Aβ samples. A 200 μM Aβ solution was analyzed by SEC, and the resulting low molecular weight fraction was isolated and examined using translational diffusion measurements. Following the diffusion measurements, the sample was reanalyzed by SEC. In addition, experiments were performed where the sample was analyzed first by translational diffusion and then fractionated by SEC. The low molecular weight fraction was then isolated and subjected to a second round of diffusion measurements. For each diffusion constant determination, three separate measurements were made. In all cases the differences between the measurements was less than 0.5%. Because Aβ(*1*−*40*) adopts a nonnative coil conformation in water solution (*45*, *55*), measured Aβ diffusion coefficients were compared to expected Aβ monomer and dimer values predicted from diffusion coefficients of a series of molecular weight standards of the same structural genre (*52*).

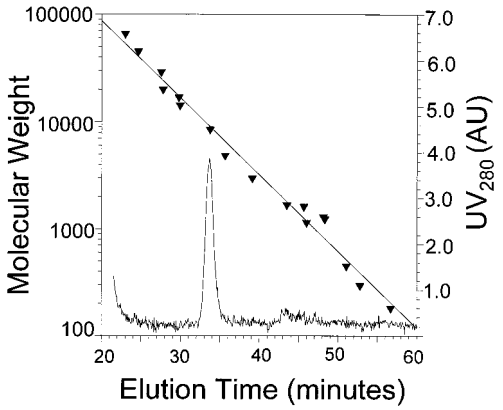

FIGURE 1: Comparison of the SEC elution of low molecular weight Aβ to molecular weight standards. Synthetic Aβ(1−40) was dissolved to yield a final concentration of 0.23 mM in PBS and sized using UV_{280} detection as described in the text. Aβ eluted (UV trace shown) with an apparent molecular weight of 8800 M_r relative to the elution positions (▼) of standards with known molecular weight run under similar conditions. Standards (and M_r) used: bovine serum albumin (66 000); ovalbumin (45 000); carbonic anhydrase (29 000); trypsin inhibitor (20 000); myoglobin (16 000); α-lactalbumin (14 200); ubiquitin (8500); synthetic peptide (4840); synthetic peptide (2980); neurotensin (1670); ranamagarin (1610); Y^0-neurokinin A (1300); uperolein (1230); Y^6-substance K (1150); 4 mer (GGYR) (450); 3 mer (YGG) (295); tyrosine (181). The elution position of the standards plotted vs log molecular weight was linear ($r^2 = 0.984$). The UV trace shown illustrates the elution position of low molecular weight Aβ relative to standards in the gel-included range of the column. The entire elution (gel-included and gel-excluded) for Aβ is shown in Figure 2.

RESULTS

Aβ Was Detected as a Single Low Molecular Weight Species. Following ultracentrifugation of freshly dissolved Aβ, >90% of the material remained in the supernatant. SEC of freshly dissolved Aβ(*1*−*40*) at a concentration of 0.23 mM indicated that the peptide eluted as a single low molecular weight species (Figures 1 and 2). Against known molecular weight standards, Aβ sized with an apparent molecular weight of 8800 M_r (Figure 1). Anti-Aβ immunoassay of eluted fractions (Figure 2a) was performed to determine if any assembled Aβ eluted in the column void, which would be obscured in UV detection by the internal marker Blue Dextran. Immunoassay using a method (*50*) shown to be capable of detecting high molecular weight Aβ assemblies as well as monomeric Aβ (*51*) indicated that, in freshly dissolved samples, no high molecular weight Aβ species eluted from the column, even in the void volume (Figure 2a). Further, the same low molecular weight species was observed regardless of the method of detection (Figure 2b). For concentrations above 10 μM, Aβ was detected by UV_{280} monitoring and invariably eluted at the same position (Figures 2−4). With Aβ detection in eluted fractions using immunoassay of unlabeled Aβ or γ counting for labeled Aβ, the elution position was negligibly variable (within one column fraction) due to finite sampling. Freshly prepared ^{125}I-Aβ (less than 1 month old) sized as a single low molecular weight species consistent with the elution of

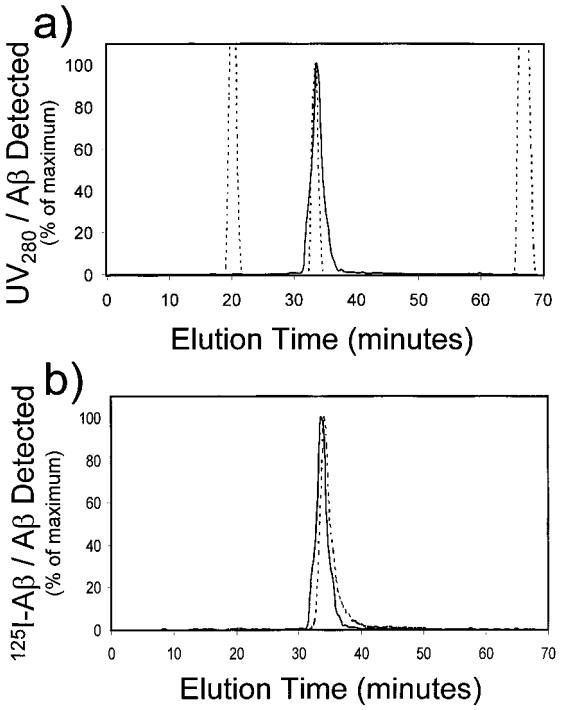

FIGURE 2: Multiple methods of Aβ detection yield similar SEC results. Unlabeled Aβ was detected using UV_{280} detection (dotted line) and immunoassay (solid line) (a). In the UV_{280} trace, the first off-scale peak (20 min) represents blue dextran used to determine the void volume of the column series. The second off-scale peak (68 min) represents pyridine and is used to mark the low molecular weight end of the column series. Radiolabeled Aβ tracks unlabeled Aβ (b). The radiolabeled peptide at a concentration of 10^{-10} M eluted (dotted line) as a single species in a similar position to unlabeled Aβ detected by immunoassay (solid line). Aβ eluted in this peak represents a 75% recovery of the total material injected. The elution position of the labeled material was not effected by the presence of excess unlabeled Aβ (10^{-4} M) in the sample (not shown). Similarly, unlabeled Aβ(1−40) eluted in the same position in the presence and absence of trace amounts of labeled Aβ (not shown).

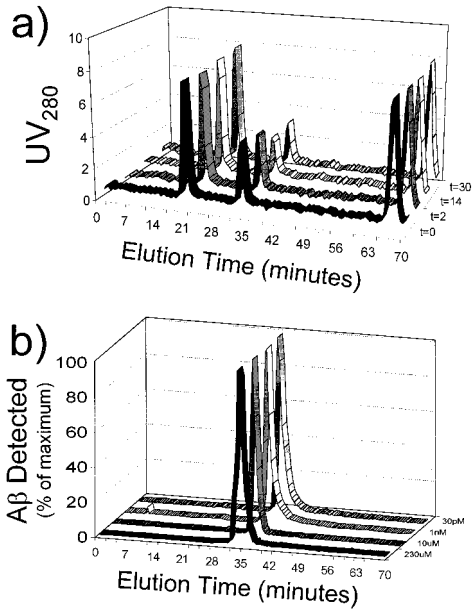

FIGURE 3: Aβ sized as a single species of low molecular weight over a wide range of concentrations (b) and storage times (a). (a) 200 μM Aβ(1−40) was prepared in PBS, incubated at 4 °C, and analyzed by SEC at times 0, 1, 2, 14, and 30 days. The elution position of Aβ detected by UV_{280} remained constant at all times tested. At time 14 and 30 days, the amount of Aβ eluting (by peak integration) at this position appeared to decrease by about 20% relative to time zero while the internal standards (≈20 and ≈68 min) remained constant with time (b). SEC of freshly dissolved Aβ samples at 230 μM, 10 μM, 1 nM, and 30 pM indicated that the low molecular weight species observed in Figures 1 and 2 predominated over the entire (7 orders of magnitude) concentration range tested. Detection was by anti-Aβ immunoassay for unlabeled peptide (230 and 10 μM) and γ-counting for radiolabeled (1 nM and 30 pM) samples.

unlabeled material. After correction for the delay between the UV monitor and the fraction collector, synthetic Aβ and ^{125}I-Aβ eluted as indistinguishable (within one fraction) peaks. Further, Aβ detected by immunoassay eluted at the same position (within one fraction) as ^{125}I-Aβ detected by γ counting.

To examine the stability of the low molecular weight Aβ species, we examined the effect of concentration and sample age on Aβ elution (Figure 3). At 200 μM and 4 °C, Aβ was detectable only as a single low molecular weight species that eluted in the same position for at least 1 month (Figure 3). After 2 weeks, the quantity of Aβ from the sample detectable by UV decreased by about 20% (Figure 3), but its position remained consistent with the earlier time points (Figure 3a). Analysis showed that, over long periods of time, there was a small increase in the void volume peak. This increase roughly corresponded to the decrease in the signal of the low molecular weight Aβ species at times 2 weeks and 1 month and is likely due to slow assembly (not shown). A similar void peak could be detected when Aβ was prepared without the ultracentrifugation step and incubated at room-temperature overnight (not shown). Further, over a concentration range of 3×10^{-11}−2.3×10^{-4} M, the same low molecular weight species was universally present as the major species. For concentrations between 10^{-10} and 10^{-4} M, the low molecular weight species was the only one detectable in the column elute. The species with an apparent molecular weight of 8800 M_r was the only peak of detectable Aβ in unaged samples. Synthetic Aβ was recovered at >65% and ^{125}I-Aβ was recovered at >70% from SEC. Quantitative (100%) recovery of injected Aβ is not expected as the peptide has been reported to adhere nonspecifically to a variety of surfaces (*46*).

Low Molecular Weight Aβ Species Is Competent of Deposition without Further Assembly. To examine whether the low molecular weight Aβ species was capable of depositing onto preexisting amyloid, we examined radiolabeled Aβ isolated from SEC fractions in an in vitro model of amyloid growth. In deliberately aged (>6 weeks old) tracer solutions a high molecular weight ^{125}I-Aβ species was clearly separable from the low molecular weight Aβ species. The low molecular weight Aβ species was competent of deposition onto preexisting amyloid in the absence of the assembled species (Figures 4 and 6). Low molecular weight Aβ (Figure 4a) deposited onto preexisting amyloid plaques in unfixed preparations of AD cortex (Figure 4b), but essentially no focal association with non-AD cortex (Figure 4c) was observed. The site for radiolabeled Aβ deposition in AD cortex has been shown to correspond to the location of anti-Aβ immunopositive preexisting brain amyloid (*29*, *30*). In contrast, the high molecular weight Aβ species isolated from the column void region, the elution position

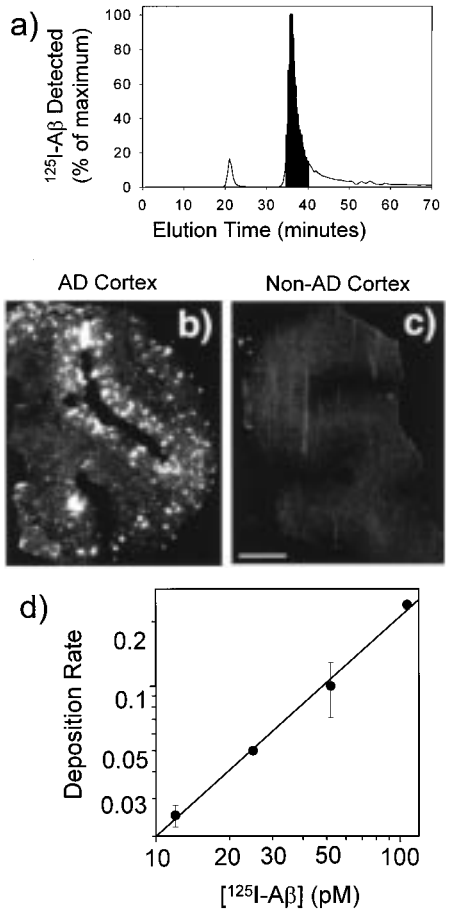

FIGURE 4: Low molecular weight Aβ species is deposition competent. (a) An apparent high molecular weight ^{125}I-Aβ assembly (elution time ≈22 min) was detectable and separable from the low molecular weight ^{125}I-Aβ species (elution time ≈35 min) in deliberately aged (>6 weeks) peptide solutions. To examine the ability of the low molecular weight Aβ species to add to existing amyloid deposits, SEC fractions containing the low molecular weight ^{125}I-Aβ species (shaded fractions) were isolated and diluted in PBS to yield a final ^{125}I-Aβ concentration of 200 pM. Slide-mounted sections of unfixed AD and non-AD cortex were allowed to incubate with the low molecular weight ^{125}I-Aβ species for 2 h. Shown are dark-field autoradiograms produced from film exposure of the AD (b) and non-AD (c) cortex sections following incubation and washing. Light areas indicate the sites and intensities of ^{125}I-Aβ deposition onto the tissue section. Scale bar = 3.5 mm. Freshly prepared ^{125}I-Aβ stocks that do not contain the high-molecular weight ^{125}I-Aβ assembly were also deposition competent (not shown). Higher MW Aβ from fractions of the void volume elution peak showed little to no focal binding to AD and non-AD cortex (not shown). (d) Deposition of the low molecular weight Aβ species is not rate-limited by solution phase assembly. The isolated low molecular weight ^{125}I-Aβ species was diluted in PBS to yield a final ^{125}I-Aβ concentration of 12 (●), 25 (■), 52 (▲), and 107 (▼) pM and incubated with AD cortex homogenates for 1, 2, and 3 h. At each time point, the amount of ^{125}I-Aβ deposited at each concentration was quantified as described in the text. Rates of ^{125}I-Aβ deposition were calculated from the linear slopes ($r^2 > 0.92$) of time course data and plotted on a log−log scale vs ^{125}I-Aβ concentration. The slope of the log−log plot of rate vs concentration was linear ($r^2 = 0.996$) with slope 1.02 ± 0.05, indicating that deposition of the low molecular weight ^{125}I-Aβ species was first order in soluble ^{125}I-Aβ concentration. Deposition of freshly prepared ^{125}I-Aβ taken from stocks without SEC purification is also first order in soluble ^{125}I-Aβ concentration (not shown).

of Aβ assemblies detectable by electron microscopy (*51*), showed little or no focal binding to either AD or non-AD cortex sections (not shown).

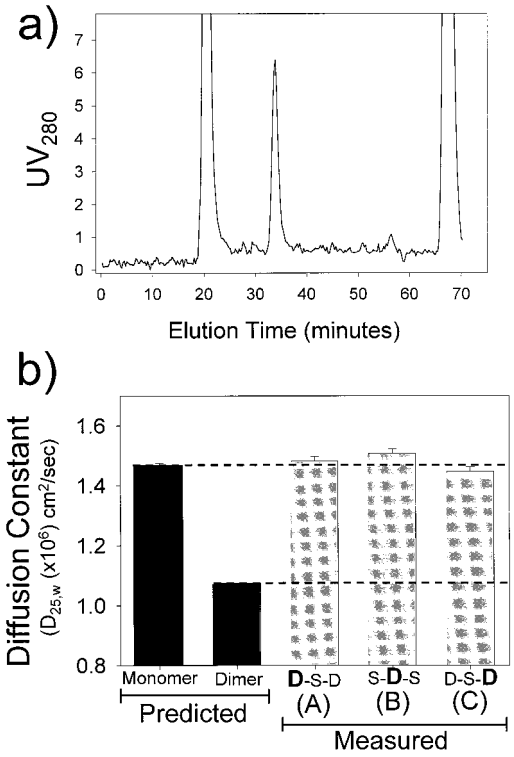

FIGURE 5: Low molecular Aβ species behaves like a monomer. To determine the oligomerization state of the low molecular weight Aβ species, translational diffusion coefficients ($D_{25,w}$) were determined. Using SEC (a) the low molecular weight Aβ fraction (elution time ≈35 min) was isolated from a 200 μM sample. The diffusion constant of low molecular weight Aβ was determined using PFG-NMR as described in the text (b). Two experiments were performed. In the first, a series of diffusion measurements was made prior to SEC [(A) **D**-S-D] and following SEC [(C) D-S-**D**]. In the second, SEC was performed first followed by diffusion [(B) S-**D**-S]. After the diffusion measurements, the low molecular weight species reeluted in the same position during SEC. All diffusion measurements were performed in triplicate. Error bars on the diffusion measurements represent the deviation between these replicate measurements. Predicted values for Aβ monomer and dimer diffusion coefficients were determined from the diffusion constants of a series of appropriate standards measured under similar conditions (*52*). In all cases, the translational diffusion of low molecular weight Aβ was consistent with that predicted for an Aβ monomer.

Low molecular weight ^{125}I-Aβ, like freshly prepared ^{125}I-Aβ not purified by size-exclusion chromatography (*29*, *31*, *33*), deposited linearly with time (not shown), with higher deposition rates observed at higher ^{125}I-Aβ concentrations (Figure 4d). Kinetic analysis (Figure 4d) showed that deposition of low molecular weight ^{125}I-Aβ followed first-order dependence on soluble Aβ concentration establishing that assembly in solution is not rate limiting for ^{125}I-Aβ deposition. Importantly, ^{125}I-Aβ isolated from fractions corresponding to the low molecular species ran in the same SEC elution position when reinjected (not shown).

Low Molecular Weight Aβ Species Behaves Like a Monomer. To determine the oligomerization state of the active low molecular weight Aβ species (Figure 5), we used SEC in combination with measurements of translational diffusion determined using solution phase NMR (*52*). Diffusion coefficients were determined before (**D**-S-D) and after (D-S-**D**) SEC and between SEC runs (S-**D**-S) (Figure 5). In all cases, the active low molecular weight Aβ species showed diffusion constants consistent with the value predicted (*52*)

for an Aβ monomer and inconsistent with the expected value for an Aβ dimer. Furthermore, SEC did not alter the oligomeric state or diffusion behavior of Aβ (Figure 5).

DISCUSSION

The formation, growth, and maturation of Aβ amyloid lesions within the AD brain are critical contributors to the molecular pathogenesis of AD and may be exploitable targets for therapeutic intervention. The present study employs SEC and NMR measurements of translational diffusion along with Aβ deposition assays to examine the importance of oligmerization state for Aβ deposition. SEC of freshly dissolved Aβ in radiolabeled and unlabeled forms over a wide range of concentration indicated that the peptide exists as a single low molecular weight species. Further, even at very high concentrations (200 μM), the low molecular weight species was stable and did not assemble to higher order oligomers for at least 2 days, and at 1 month, at least 80% of the material remained unassembled. This observation is consistent with previous reports (*51*, *52*, *56*, *57*) and strongly suggests that the thermodynamically favorable Aβ assembly into higher order assemblies (*26*, *28*) is slow in the absence of preassembled nuclei.

A critical question in the study of Aβ amyloidogenesis is the oligomerization ground state of the peptide at physiological (nM) concentrations. Although the results presented here clearly establish that a single low molecular Aβ species is present and is competent to add onto preexisting amyloid by an aggregation independent mechanism, SEC alone cannot determine the precise Aβ oligomerization state. Consistent with the observations of Soreghan and co-workers (*57*), relative to the elution of peptides and proteins of known molecular weight, Aβ (primary sequence: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV, M_r = 4331) appears to elute with an apparent molecular weight of 8800 M_r by SEC in PBS on Superdex columns. The apparent molecular weight of Aβ, however, varies significantly with chromatography conditions (*51*).

Caution must be used, however, in interpreting these data. It is not strictly valid to compare the elution of small peptides to that of folded globular proteins for molecular weight determination. As molecular weight, shape, and solvation govern migration by size exclusion on gel-filtration columns, peptides and proteins lacking significant secondary structure would be expected to migrate with a higher "apparent" MW, making straightforward molecular weight interpretations based on elution alone particularly difficult for peptides that are only loosely folded (*58*). For example, recent studies of another amyloidogenic protein (NACP) lacking significant secondary structure indicated that this protein sized on gel-filtration chromatography with an apparent molecular weight consistent with a well-folded tetramer, although the protein sedimented as a monomer by analytical ultracentrifugation (*59*). Another amyloidogenic peptide, the SH_3 domain of phosphatidylinositol-3-kinase, similarly behaves larger than expected from molecular weight (*60*).

The observation that Aβ(*1*−*40*) exists as a nonnative collapsed coil (*55*) lacking regular secondary structure, rather than as a well-folded globular protein (e.g. BPTI or BSA), suggests that sizing chromatography may not be a reliable method for Aβ molecular weight determination. Indeed, Aβ was found to size nonideally under a wide range of conditions, with the apparent molecular weight varying between 5000 and 18 000 M_r depending on the chromatographic conditions (*51*). Further, some Aβ assemblies detectable by SDS−PAGE run anomalously in size-exclusion chromatography (*56*).

To overcome these difficulties, we used NMR measurements of translational diffusion in homogeneous solution to determine the molecular weight of the low molecular weight Aβ species. Using this method we find that the low molecular weight Aβ species isolated from SEC behaves as a monomer under near physiological conditions (pH and salt concentration), consistent with the conclusions of Lee and co-workers (*52*). This finding is also consistent with analytical ultracentrifugation experiments demonstrating that Aβ is a monomer (*61*). Further, we demonstrate that Aβ in the monomeric state is competent to deposit onto brain amyloid without solution phase assembly.

Kinetic deposition experiments using Aβ isolated by SEC indicated that deposition of the low molecular weight species followed first-order kinetic dependence on soluble Aβ concentration. Thus, monomeric Aβ is almost surely capable of adding onto and contributing to the growth of preexisting amyloid without prior self-assembly of the depositing peptide. In contrast, higher molecular weight oligomers found in aged Aβ solutions are inactive in deposition assays. This mechanism of amyloid growth by deposition of monomeric Aβ stands in sharp contrast to the mechanism of nascent amyloid or pre-amyloid formation (Aβ aggregation). Thus, in vitro and almost surely in vivo, the mechanism by which Aβ adds onto an existing amyloid or pre-amyloid template is fundamentally distinct from the mechanism by which the template originally formed.

Although the relative importance of amyloid template formation and growth to the disease state is difficult to assess, quantitative pathological observations suggest that the processes are distinct in vivo as well as in vitro (*62*). Factors that have been shown to effect Aβ nucleation in vitro also appear important for the formation of brain amyloid (*63*), suggesting that Aβ assembly may be an important early step in nascent amyloid formation. Quantitative analysis of AD (*62*) reveals a log-normal distribution of plaque size, implying growth on a porous template for Aβ deposition. Thus, increasing the mass of an existing amyloid plaque by deposition of Aβ would not necessarily increase just its diameter. Instead, the density of the deposit may increase during the maturation process. The density of mature senile plaques rather than the total number of immunopositive foci correlates with the clinical state (*3*, *8*), strongly suggesting that amyloid maturation is a key process in AD progression.

Our results, however, do not exclude that soluble Aβ oligomers may exist under some conditions or in some Aβ preparations. Fluorescence resonance energy transfer experiments on Aβ peptides modified with covalent fluorophores suggest the presence of stable low molecular weight oligomers (*64*). Further, stable Aβ oligomers that elute from SEC differently than low molecular weight Aβ have also been isolated from human brain samples (*65*) or prepared in vitro (*51*, *66*). Soluble Aβ diffusible ligands (*66*) and protofibril aggregation intermediates (*51*, *67*) have also been recently identified. The present results also do not exclude fast (on the NMR time scale) exchange between predominant mono-

mers and a trace amount of unstable dimer present at high (10^{-4} M) total Aβ concentrations; however, such an equilibrium would effectively rule out dimeric Aβ at physiological (10^{-9} M) concentrations.

The results of this study strongly argue that amyloid growth is mediated by deposition of monomeric, not oligomeric, Aβ onto a preexisting amyloid template. Targeting growth of amyloid by addition of monomeric Aβ, which requires a distinct inhibitor identification strategy from Aβ nucleation, may be a valuable therapeutic target for pharmacological intervention in AD.

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BI990718V