AMYLOID β-PROTEIN FIBRILLOGENESIS

DETECTION OF A PROTOFIBRILLAR INTERMEDIATE

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Fibrillogenesis of the amyloid β-protein (Aβ) is a seminal pathogenetic event in Alzheimer’s disease. Inhibiting fibrillogenesis is thus one approach toward disease therapy. Rational design of fibrillogenesis inhibitors requires elucidation of the stages and kinetics of Aβ fibrillogenesis. We report results of studies designed to examine the initial stages of Aβ oligomerization. Size exclusion chromatography, quasielastic light scattering spectroscopy, and electron microscopy were used to characterize fibrillogenesis intermediates. After dissolution in 0.1 M Tris-HCl, pH 7.4, and removal of pre-existent seeds, Aβ chromatographed almost exclusively as a single peak. The molecules composing the peak had average hydrodynamic radii of 1.8 ± 0.2 nm, consistent with the predicted size of dimeric Aβ. Over time, an additional peak, with a molecular weight >100,000, appeared. This peak contained predominantly curved fibrils, 6–8 nm in diameter and <200 nm in length, which we have termed “protofibrils.” The kinetics of protofibril formation and disappearance are consistent with protofibrils being intermediates in the evolution of amyloid fibers. Protofibrils appeared during the polymerization of Aβ-(1–40), Aβ-(1–42), and Aβ-(1–40)-Gln22 peptides associated with both sporadic and inherited forms of Alzheimer’s disease, suggesting that protofibril formation may be a general phenomenon in Aβ fibrillogenesis. If so, protofibrils could be attractive targets for fibrillogenesis inhibitors.

Fibrillar amyloid plaques in the cerebral parenchyma and vasculature are a cardinal neuropathologic feature of Alzheimer’s disease (AD)1 (1). Plaques are composed predominantly of insoluble fibers of the amyloid β-protein (Aβ) (2). Aβ is a normal component of the plasma and cerebrospinal fluid, occurring as a soluble 40- or 42-residue peptide (3, 4). Thus, a normal component of the plasma and cerebrospinal fluid, occurring as a soluble 40- or 42-residue peptide (3, 4). Thus, a central question in the etiology of AD is the mechanism(s) by which these soluble Aβ molecules are converted into plaque-associated fibers (5). This question is particularly relevant because Aβ fibers, unlike nonfibrillar Aβ, are neurotoxic in vitro and are associated with damaged neuropil in vivo (6).

These observations suggest that inhibiting fiber formation would be an effective approach toward AD therapy. However, if these efforts are to succeed, fiber formation must be understood at the molecular level.

Aβ fibrillogenesis is a nucleation-dependent polymerization process (7, 8). The kinetics of this type of process is controlled by two key parameters, nucleation rate and elongation rate. Past studies of the kinetics of Aβ fibrillogenesis, utilizing techniques including turbidity, sedimentation, and thioflavine T binding, could only provide information on the appearance of high molecular weight aggregates (7, 9) or the disappearance of soluble peptide (10–13). Neither rate constants nor structures of fibrillogenesis intermediates could be determined by these approaches. In contrast, the technique of quasielastic light scattering spectroscopy (QLS) is particularly well suited for resolving individual stages of polymerization processes and for examining polymerization kinetics (14). QLS was used initially to monitor late stages of Aβ fibrillogenesis (15, 16). Recently, however, a model system was developed for the highly reproducible growth of Aβ fibers (8). This allowed QLS monitoring of polymer size during all phases of fibrillogenesis and determination of the rates of Aβ fibril nucleation and elongation (8).

The QLS approach is less useful for studying small structures, such as Aβ monomers and oligomers, when they exist in mixtures with larger polymers. Because these prefibrillar intermediates are potential targets for fibrillogenesis inhibitors, it is important to characterize them. One method for doing so is SDS-PAGE (17–20). However, since many Aβ polymers are SDS-labile, interpreting SDS-PAGE studies of Aβ polymerization is problematic. Size exclusion chromatography (SEC) is an attractive alternative to SDS-PAGE because it fractionates on the basis of molecular weight and is performed using nondenaturing and nondisaggregating buffers. SEC has been used to study the aggregation state of Aβ in solution (20–23), but its potential in kinetic studies has not been exploited. We report here the use of SEC, coupled with QLS and electron microscopy, to characterize the temporal evolution and structures of oligomeric intermediates in the pathway of Aβ fibrillogenesis.

EXPERIMENTAL PROCEDURES

Chemicals and Solvents

Chemicals were obtained from Sigma and, unless otherwise stated, were of the highest purity available. Solvents were HPLC grade and were obtained from Fisher. Water was double-distilled and deionized using a Milli-Q system (Millipore Corp., Bedford, MA).

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§ The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid β-protein; QLS, quasielastic light scattering spectroscopy; PAGE, polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; HPLC, high performance liquid chromatography; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; RIA, radioimmunoassay; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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Peptides

Peptides were synthesized as described by Lomakin et al. (8). Crude Aβ-(1–40) was purified by reverse phase HPLC using a Vydac phenyl column (22 × 250 mm) and a linear gradient of 28–56% B over 50 min (A: 0.1% (v/v) trifluoroacetic acid; B: 70% (v/v) acetonitrile (CH_3CN), 0.09% (v/v) trifluoroacetic acid) at a flow rate of 20 ml/min. Aβ-(1–42) was purified by using a PLRP-S column (25 × 150 mm) and a linear gradient of 0–80% B over 50 min (A: 50 mM Tris-HCl, pH 9.1, 0.1% trifluoroacetic acid; B: 50% (v/v) formic acid, pH 9.1, containing 54% (v/v) CH_3CN and 6% (v/v) 2-propanol) at a flow rate of 15 ml/min (24). Following purification, Aβ-(1–42) was dialyzed against 15 mM ammonium hydroxide in 40% (v/v) CH_3CN, lyophilized, and converted to its trifluoroacetic acid salt by dissolution in 100% trifluoroacetic acid. Peptide mass, purity, and quantity were determined by a combination of matrix-assisted laser desorption/ionization-tandem mass spectrometry (MALDI-MS/MS) and analytical HPLC, and respective native amino acid analysis, respectively. Purified peptides were aliquoted, lyophilized, and stored at −20 °C until used. Aβ-(40–1) was generously provided by Dr. Dennis Selkoe (Brigham & Women’s Hospital and Harvard Medical School).

SEC

Columns and Buffers—A description of columns and buffers is found in Table I. Superdex, Superose and TSK columns were attached to a Rapid-LC system consisting of a HPX-50C pump, a Rheodyne 7161 injector, and a Dynamax UV-1 detector. Columns were eluted at a flow rate of 0.5 ml/min and peptides detected by UV absorbance at 254 nm. Sephadex G-50 (superfine) media were prepared using either 1 × 30 cm or 1 × 50 cm borosilicate Econo-columns (Bio-Rad, Hercules, CA) and eluted at 0.17 ml/min using an LKB Microperpex S peristaltic pump. Peptides were detected by UV absorbance at 254 nm using a Pharmacia UV-1 single path monitor. Each experiment was performed at two or 3 times. Pre-packed columns were washed with 50% (v/v) CH_3CN, lyophilized, and converted to its trifluoroacetic acid salt by dissolution in 100% trifluoroacetic acid. Peptide mass, purity, and quantity were determined by a combination of matrix-assisted laser desorption/ionization-tandem mass spectrometry (MALDI-MS/MS) and analytical HPLC, and respective native amino acid analysis, respectively. Purified peptides were aliquoted, lyophilized, and stored at −20 °C until used. Aβ-(40–1) was generously provided by Dr. Dennis Selkoe (Brigham & Women’s Hospital and Harvard Medical School).

RESULTS

Electrophoresis

SDS-PAGE was carried out on 16.5% Tricine gels as described by Schagger and Von Jagow (27). Aliquots of each chromatographic fraction (20 µl) were mixed with 3 × SDS sample buffer (10 µl) and boiled for 5 min immediately prior to electrophoresis. Gels were silver-stained using the Bio-Rad silver stain kit and photographed.

Oligomerization of Aβ-(1–40)—To monitor oligomerization of Aβ, an analytical method is necessary that can resolve monomeric Aβ and its oligomers under non-denaturing, non-disaggregating conditions. We reasoned that SEC would be appropriate for this purpose. Initial analysis of presumably monomeric Aβ revealed a single peak with a molecular weight of 10,000 (Table I). This unexpectedly high molecular weight suggested that Aβ might not be monomeric or was partially excluded from the column matrix, indicating a nonideal analyte-matrix interaction.

Because accurate molecular weight determination was critical for the proper interpretation of our experiments, we determined whether the chromatographic behavior of Aβ varied depending on column matrix or elution conditions (Table I). When Aβ-(1–40) from the same peptide lot was dissolved in phosphate-buffered saline and chromatographed on Superdex 75 (dextran-agarose), Superose 12 (agarose), or TSK (silica) matrices, the relative molecular weight of Aβ varied from 10,000 to 18,000. Similarly, when Aβ-(1–40) was dissolved in 0.1 M Tris-HCl, pH 7.4, and then analyzed, molecular weights of 9,000–15,000 were observed. Other solvent conditions resulted in Mr values ranging from 5,000 to 10,500. One explanation for the variations in Mr was nonideal chromatographic behavior of Aβ. This was confirmed in experiments in which the elution buffer was modified by the addition of ethylene glycol, an agent used to inhibit solute-column interactions. Under these conditions, a concentration-dependent decrease in Aβ Mr from 10,000 to 6,000 was seen. We also found that Mr values varied depending on the calibration standards used. Thus, for consistency, all columns were calibrated using the same five standards.

In each experiment, Aβ-(1–40) chromatographed as a single peak in the included volume of the column, but because of its nonideal behavior, the oligomerization state of Aβ within these peaks was unclear. If noncovalent dimers composed these peaks, then treating Aβ with strong denaturants or solvents prior to chromatography might disaggregate these complexes and reduce the Mr. In fact, pretreatment of Aβ with SDS-PAGE sample buffer, MeSO_4, HFIP, or 90% formic acid, had no effect on its Mr. In addition, Aβ-(40–1), the “reverse” peptide of Aβ-(1–40) and one that does not polymerize readily, co-eluted with the radiotracer experiments. Fractions were frozen at −20 °C until assayed. Aβ-(1–40) content in each fraction was determined by RIA (26).

QLS

Fractions from SEC were collected directly into cuvettes and analyzed within 1 min of elution. QLS was carried out essentially as described (8). Measurements were performed at 25 °C using a Langley Ford model 1097 autocorrelator and Coherent argon ion lasers (model Innova 90 or Innova 90-plus) operated at 514 nm.

Electron Microscopy

To examine insoluble material, pellets of Aβ were suspended in a small volume of buffer and prepared as above.
Aβ-(1–40) was chromatographed on five different size exclusion media, as described under “Experimental Procedures.” For each study, the appropriate column was equilibrated with at least three column volumes of elution buffer and then calibrated with five molecular weight standards: avian ovalbumin (44,000); equine myoglobin (17,000); equine cytochrome C (12,384); bovine aprotinin (6,500); and vitamin B₁₂ (1,350). Standard curves were constructed by regression analysis and used to determine the molecular weights of analytes. Each condition yielded one peak, which decreased in size over 24 h. A small void peak appeared after 24 h of incubation in some experiments.

### TABLE I

| Column | Separation range (kDa) | Elution buffer | Aβ \( M_r \) | Void peak | Correlation coefficient \( r^2 \) |
|--------|------------------------|----------------|-------------|-----------|-------------------------------|
| Superdex 75 (1 × 30 cm) | 3–70 | PBS | 10,000 | – | 0.98 |
| | | PBS, 20% sucrose | 10,500 | – | 0.98 |
| | | PBS, 20% MPD | 9,000 | – | 0.96 |
| | | PBS, 20% ethylene glycol | 8,000 | ± | 0.98 |
| | | PBS, 50% ethylene glycol | 6,000 | ± | 0.98 |
| | | 0.1 M Tris-HCl, pH 7.4 | 15,000 | – | 0.81 |
| Superdex 200 (1 × 30 cm) | 10–600 | 0.1 M Tris-HCl, pH 7.4 | 14,000 | ± | 0.77 |
| Superose 12 (1 × 30 cm) | 1–300 | 0.1 M Tris-HCl, pH 7.4 | 15,000 | ± | 0.79 |
| | | PBS | 12,000 | ± | 0.92 |
| TSK G2000sw (0.7 × 30 cm) | 5–100 | 0.1% trifluoroacetic acid | 8,000 | – | 0.85 |
| | | PBS | 18,000 | ± | 0.58 |
| G50sf (1 × 28 cm) | 1.5–30 | 0.1 M Tris-HCl, pH 7.4 | 9,000 | – | 0.98 |
| G50sf (1 × 28 cm) | 1.5–30 | TBS | 8,000 | – | 1.0 |
| G50sf (1 × 47 cm) | 5–100 | TBS | 7,500 | – | 0.98 |
| G50sf (1 × 47 cm) | 5–100 | 50 mM ammonium acetate, pH 7.4 | 5,000 | – | 0.98 |

*MPD, 2-methyl-2,4-pentanediol; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TBSE, 0.02 M Tris-HCl, pH 7.4, containing 0.1 M NaCl and 50 μM EDTA.

**Fig. 1.** Size exclusion chromatography of Aβ. Aβ-(1–40) was dissolved in 0.1 M Tris buffer at a concentration of 2 mg/ml and chromatographed on a Superdex 75 column, as described under “Experimental Procedures.” The gel-included peak elutes at ~12.4 ml, while the gel-excluded peak elutes at ~7.2 ml. Elution positions of molecular weight standards are indicated by arrows. Molecular masses are indicated in kDa.
of these columns as well, suggesting that the Aβ multimers had molecular masses in excess of 100 kDa, or that they behaved anomalously on SEC. Experiments using Aβ-(1–40) obtained following dissolution in 0.1 M Tris-HCl and centrifugation for 3 min at 17,000 × g (A); the gel-included fraction obtained following SEC of the supernatant from A (B); apotinin (6,500 Da) (C); buffer alone (D); soluble Aβ-(1–40) following dissolution in 0.1 M Tris-HCl, incubation at room temperature for 48 h, and centrifugation for 3 min at 17,000 × g (E); and the gel-excluded fraction obtained by SEC of Aβ-(1–40) (F) prepared as in E. Each Rh distribution was normalized to 100% intensity. In A, E, and F, scattering intensity comes predominately from polymeric and aggregated Aβ. In B, C, and D, scattering from the buffer (Rh < 0.2 nm; data not shown) accounts for the remaining scattering, and signals at 35 nm are not peptide-related, since they are present in buffer alone. Comparison of the magnitudes of intensities among experiments is not meaningful.

Size Determination of Aβ—To estimate the actual sizes of Aβ particles in the peaks obtained by SEC, QLS was used to measure the average diffusion coefficient of the particles. Particle size is expressed as the radius of a sphere with an identical diffusion coefficient. This quantity is termed the hydrodynamic radius (Rh) (for a review, see Ref. 28) and is conceptually equivalent to a Stoke’s radius in gel permeation chromatography. For spherical particles, RH = r, where r is the geometric radius. For a fibril, RH depends on the length, diameter, and flexibility of the particle. RH is always less than the radius of the sphere circumscribed around the particle.

Immediately after dissolution in Tris-HCl and centrifugation (17,000 × g, 3 min), but prior to SEC, soluble Aβ-(1–40) had an average Rh of ~40–200 nm (Fig. 2A). Because scattering intensity is proportional to molecular weight, monomers and small oligomers can be difficult to detect in the presence of larger polymers and aggregates. However, by fractionating the Aβ mixture using SEC, we observed a gel-included fraction, with an extrapolated molecular weight of 15,000, which had an average Rh of 1.8 ± 0.2 nm (Fig. 2B). Geometric considerations predict that a 4,331-Da peptide in dimeric form will have a Rh = 1.5–2.1 nm. For comparison, QLS analysis of apotinin (6,500 Da) fractionated by SEC yielded an average Rh of 1.6 ± 0.6 nm (Fig. 2C). These data argue that Aβ existed as a dimer2 in the gel-included fraction, not as a trimer or tetramer as suggested by SEC.

We next sought to determine the size(s) of the Aβ oligomers in the gel-excluded fraction. To produce sufficient material for analysis, Aβ-(1–40) was incubated for 48 h prior to chromatography. QLS analysis of the pre-SEC supernatant (17,000 × g) revealed a broad distribution of particles sizes (range 4 to 100 nm) (Fig. 2E). Comparison of this distribution with that obtained from Aβ immediately after dissolution (Fig. 2A) shows that particles of intermediate size form during fibrillogenesis. Dimers were not resolved clearly in this experiment due to the predominance of larger Aβ oligomers/polymers, as discussed above. QLS analysis of the gel-excluded peak showed that the majority of intermediate particles ranged in size from ~10 to 50 nm. For a solution of noninteracting rods with diameters of 8 nm, this range corresponds to lengths of 30–500 nm. These lengths would be significantly smaller if rod-rod interactions were occurring. On the other hand, if the rods were flexible, the lengths would be even larger.

Taken together, our SEC and QLS data indicate that the gel-included peak contained dimeric Aβ, the gel-excluded peak contained a distribution of oligomers, and the pellet obtained prior to chromatography was composed of higher molecular weight polymers and aggregates.

Morphology of Aβ Oligomers—To determine the morphology of the Aβ species observed in the SEC experiments, electron microscopy was performed. For both Aβ-(1–40) and Aβ-(1–42), no structures were detected in the gel-included fractions, while the 17,000 × g pellets obtained prior to chromatography contained a mesh of fibers 6–10 nm in diameter (Fig. 3, A and B). These fibers were indistinguishable from those found in senile plaques (29). In addition, particularly in the case of Aβ-(1–42), short fibrils were seen associated with the longer fibers. Generally, one end of each fibril appeared to adhere to the side of a fiber, while the other end was free. Similar fibrils were found in the void volume peaks, where they appeared as short, curly fibrils 6–10 nm in diameter and 5–160 nm in length, on average (Fig. 3, C and D). We have termed these structures “protofibrils” to distinguish them from the mature, amylloid-type fibers found in the pellets.

Chromatographic Analysis of Seedless Aβ—Low molecular weight oligomers (larger than dimers) were not detected in the prior SEC experiments. Oligomerization could be thermodynamically unfavorable or be suppressed kinetically due to rapid consumption of precursor by growing fibrils. Fibril formation would be accelerated by the presence of pre-existing aggregates (seeds) in the starting material. Freshly prepared solutions of Aβ-(1–40) did, in fact, contain high molecular weight species that might act as seeds (Fig. 2A). To determine if rapid fibril formation affected Aβ oligomerization, we sought to eliminate seeds prior to our experiments by dissolving Aβ in HFIP, lyophilizing the solution, dissolving the lyophilizate in Me2SO, and then filtering the solution through a filter containing 20-nm pores. The chromatographic behavior of peptides “deseeded” in this way did not differ from that of peptides prepared without deseeding (data not shown). An included peak was observed initially, which was replaced over time by a peak in the void volume of the column. These data suggest that formation of stable low molecular weight oligomers is a relatively unfavored process.

High Sensitivity Monitoring of Aβ Polymerization—Our re-

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2 For simplicity, Aβ within the gel-included peak will be referred to as “dimeric.” However, the QLS data alone do not eliminate the possibility that Aβ within this peak is monomeric.
sults suggested that, if present, steady state levels of oligo
ermic fibril precursors would be extremely low, making their
detection by UV absorbance difficult. For this reason, two sen-
sitive methods were utilized to monitor Aβ polymerization: 1)
radioiodinated peptide was used as a tracer in mixtures with
unlabeled peptide; and 2) SEC fractions were assayed for Aβ
content by RIA. Radioiodinated Aβ-(1–40) (125I-Aβ-(1–40); 100
pM final concentration) was mixed with unlabeled Aβ (1 mg/ml)
in 0.1 M Tris-HCl, pH 7.4, chromatographed on a Superdex 75
column, and detected in column fractions by scintillation count-
ing. At 0 h, a single peak was observed coincident with unla-
beled Aβ (Fig. 4). After incubation at room temperature for 8 or
24 h, an additional peak of radioactivity was detected in the
void volume (Fig. 4; at ~7.4 ml). The radioactivity and UV
absorbance of this peak increased at the same rate over time,
suggesting that the labeled and unlabeled peptides polymer-
ized in an analogous manner.

In the above experiment, given that ~33,000 cpm of Aβ were

**FIG. 3.** Morphology of Aβ polymers fractionated by size exclusion chromatography. Electron microscopy was performed on prechro-
matography pellets of Aβ-(1–40) (A) and Aβ-(1–42) (B) and on the void peaks of Aβ-(1–40) (C) and Aβ-(1–42) (D). Scale bar, 100 nm.

**FIG. 4.** High sensitivity size exclusion chromatography of Aβ-(1–40). 125I-Aβ-(1–40) was added to a freshly prepared solution of Aβ-(1–40)
(1 mg/ml) and fractionated on a Superdex 75 column after 0, 8, and 24 h of incubation. The 125I-Aβ-(1–40) content of the fractions was determined
by scintillation counting. The chromatograms shown are representative of results from three independent experiments.
used, oligomers representing as little as 1% of the initial protein mass could have been detected. Asymmetry in the void and dimer peaks, indicated by trailing and leading shoulders, respectively, mirrored the behavior of the unlabeled peptide (cf. Figs. 1 and 4). RIA analysis of Aβ-(1–40) SEC fractions produced using the same protocol as above yielded results similar to those presented in Fig. 4 (data not shown). Experiments were also performed at much lower Aβ concentrations by using tracer alone; however, below 10⁻⁵ M, recovery of ¹²⁵I-Aβ-(1–40) from the column was poor, probably due to nonspecific adsorption to the column matrix and associated glass and plastic surfaces (22, 30). Taken together, these data suggest that although oligomeric intermediates do exist, they do not accumulate to a significant degree during the polymerization process.

Kinetics of Protofibril Formation—As a first step to determining the key primary structural elements of Aβ that control the kinetics of dimer and protofibril utilization, comparative polymerization studies were done using Aβ-(1–42), Aβ-(1–40), and Aβ-(1–40) containing the substitutions Phe<sup>19</sup> → Pro (31), Ala<sup>21</sup> → Gly (32), or Glu<sup>22</sup> → Gln (33). Peptides were dissolved in Tris-HCl, pH 7.4, at concentrations of 1 mg/ml, incubated for up to 24 h, and then their polymerization states were analyzed by SEC on a Superdex 75 column.

Immediately after dissolution, all samples produced a single chromatographic peak with a retention time equivalent to that of dimeric wild type Aβ-(1–40). The level of the Aβ-(1–40) peak declined by ~20% over 24 h, while the levels of the Aβ-(1–40)-Pro<sup>19</sup> and Aβ-(1–40)-Gly<sup>21</sup> peaks declined <10% (Fig. 5A). Aβ-(1–42) and Aβ-(1–40)-Gln<sup>22</sup> peaks decreased rapidly initially, with a ~40% decrease within 1 h, followed by a slower decrease down to 20–30% of initial levels by 24 h (Fig. 5A). The inverse was observed for the protofibril peaks (Fig. 5B). The levels of protofibrils produced by Aβ-(1–40) and Aβ-(1–40)-Gly<sup>21</sup> increased ~15 and ~5%, respectively, over the first 24 h. No protofibril peak was detected at 24 h in experiments with Aβ-(1–40)-Pro<sup>19</sup>. In contrast, the levels of protofibrils formed by Aβ-(1–42) and Aβ-(1–40)-Gln<sup>22</sup> increased rapidly within the first hour. Peak levels of protofibrils were seen after 8 h for Aβ-(1–42) and after 1 h for Aβ-(1–40)-Gln<sup>22</sup>, after which these levels declined. The continuous decline in levels of the included peak, coupled with the transitory appearance of protofibrils, is consistent with the protofibril being an intermediate in Aβ fibrillogenesis.

SDS-PAGE of Aβ Oligomers—SDS-stable oligomers have been detected previously in media from cultured cells secreting Aβ (34). To determine the SDS stability of the oligomers observed during SEC, peak fractions were analyzed by SDS-PAGE. Interestingly, dimer, protofibril (void), and fiber (pellet) fractions produced during polymerization of Aβ-(1–40) all produced a single 4-kDa band (Fig. 6A). To identify trace levels of oligomers, ¹²⁵I-Aβ-(1–40) was incubated with Aβ-(1–40), and the resulting products were characterized autoradiographically following gel electrophoresis. A single 4-kDa band was observed in this experiment as well (data not shown). Electrophoresis of SEC fractions of Aβ-(1–42) produced a different pattern (Fig. 6B). Void peak fractions produced five bands, corresponding in mass to monomeric through pentameric Aβ (Fig. 6B, lanes 2–4). The dimer peak of Aβ-(1–42) ran as a single 4.5-kDa band (Fig. 6B, lanes 5 and 6). Prechromatography pellets (see "Experimental Procedures"), which contained fully formed Aβ fibers, produced the same five bands as did the void peak fractions, plus an occasional gel-excluded band (Fig. 6B, lane 1). These data show that SDS disaggregates Aβ fibers and protofibrils down to monomers, in the case of Aβ-(1–40), or to monomers and low molecular weight oligomers, in the case of Aβ-(1–42).

DISCUSSION

Accumulating evidence supports the hypothesis that Aβ fibrillogenesis is a seminal pathogenetic event in AD (35, 36).
Inhibiting or reversing fibrillogenesis is thus a logical therapeutic strategy. The goal of the experiments reported here was to characterize, structurally and kinetically, the initial stages of Aβ fibrillogenesis to identify targets for fibrillogenesis inhibitors. We have discovered a potential target, the amyloid protofibril, which appears to be an important intermediate in Aβ fibrillogenesis. This discovery, and the unique constellation of methods used to achieve it, have implications both for our understanding of Aβ fibrillogenesis and for the methodological approaches used to study Aβ polymerization.

In our SEC studies of Aβ-(1–40) polymerization, a single peak was observed following dissolution of lyophilized peptide (Table I). The $M_r$ of this peak varied from 5 to 18 kDa, depending on the solvents and columns used, corresponding to monomeric to tetrameric Aβ. These molecular weight estimations and, in fact, those of other groups using SEC (20–23, 37, 38) were based on calibration curves derived using soluble peptides and proteins specially selected for their ideal chromatographic behavior. However, we found that Aβ, under nondenaturing conditions, did not chromatograph ideally. This behavior is not uncommon for amphipathic peptides, such as Aβ, which may undergo both electrostatic and/or hydrophobic interactions with the column matrix (39). For example, nonideal chromatographic behavior has recently been reported for both the nonamyloid component of AD amyloid (40) and melittin (41). Therefore, to be able to estimate accurately the molecular weights, and thus the oligomerization states, of Aβ using SEC, new standardization methods had to be utilized.

QLS is a particularly powerful and appropriate technique for monitoring the sizes of protein polymers in solution (for a review, see Ref. 28). By coupling SEC with QLS, the oligomerization state of Aβ within different chromatographic fractions could be estimated directly, rapidly, and noninvasively. We found that Aβ-(1–40) and Aβ-(1–42) exhibit predominantly three forms in aqueous solution: dimer, protofibril, and fiber. Metastable oligomers (dimer $< M_r <$ protofibril) were detected using highly sensitive radiochemical and immunological assays; however, none accumulated in significant quantities. Aβ-(1–40) and Aβ-(1–42) have been reported to form stable dimers (20, 23) as well as high molecular weight polymers (20), although these latter structures were not characterized. Our analysis of protofibril fractions by QLS revealed a $R_h$ distribution of 10–50 nm. Assuming Aβ forms rods with an 8-nm average diameter, and recognizing that in polydisperse populations of polymers the larger particles skew the $R_h$ distribution toward higher values, the QLS data were consistent with protofibrils 30–500 nm in length. These estimates were consistent with results of electron microscopy, which revealed curly fibrils 6–10 nm in diameter and ranging in length up to 200 nm.

An important question arising from the discovery of the protofibril was where it lay in the pathway of Aβ fibrillogenesis. In an effort to answer this question, SEC was used to study the stages and kinetics of Aβ fibrillogenesis. In the cases of Aβ-(1–42) and Aβ-(1–40)-Glu22, dimers predominated initially, and then a time-dependent decrease in dimer levels was paralleled by an increase in protofibrils. Finally, protofibril levels declined as well. Radiochemical analyses of SEC fractions showed that only two discrete Aβ species, dimer and protofibril, accumulated during fibrillogenesis. However, trailing and leading shoulders associated with protofibril and dimer peaks, respectively, appeared during fibrillogenesis. In addition, an elevated base line was observed between the protofibril and dimer peaks. These data are consistent with a polymerization process in which Aβ oligomerizes through a series of short lived intermediates to form protofibrils, which act as centers for the growth of mature fibers. A similar pathway has been proposed by Harper et al. (42) based on studies of Aβ polymerization monitored by atomic force microscopy.

Rates of dimer utilization and protofibril formation varied markedly among different Aβ species (Fig. 5). The differences observed agree qualitatively with previous reports indicating that structural elements in the central hydrophobic core (Leu17–Ala21) and at the COOH terminus of Aβ play key roles in controlling fibrillogenesis (7, 11, 43–46). For example, relative to wild type Aβ-(1–40), both Aβ-(1–40)-Glu22 and Aβ-(1–42) polymerize into protofibrils and then into fibers significantly faster. In each case, amino acid substitutions (Glu22 → Glu22) or additions (Ile51,Ala42) produce peptides of greater hydrobicity, a change that could drive aggregation through the hydrophobic effect (47). Whether significant intramolecular or intermolecular structural changes also contribute to the altered kinetics is unknown. In contrast, Aβ-(1–40)-Gly21 polymerized more slowly than did wild type peptide, while Aβ-(1–40)-Pro19 remained almost completely unpolymerized. Few protofibrils were formed by Aβ-(1–40)-Gly21 after 24 h, and none were observed in the Aβ-(1–40)-Pro19 samples. These results suggest that glycine and proline alter Aβ structure in a manner that inhibits or precludes the self-associations necessary to produce protofibrils and fibers. This could occur through destabilization of structural elements, including α-helices and/or β-strands (48, 49), required for fiber formation. This mechanism has been implicated in alterations in βPP processing induced by familial AD mutations (31, 50) and is consistent with previous findings that fibril formation requires formation of a stable β-sheet structure (11, 44, 51, 52).

In addition to SEC, denaturing gel electrophoresis (SDS-PAGE) has been used to characterize Aβ oligomers (17–20). However, on theoretical grounds (i.e., the use of the chaotrope SDS), this technique would be expected to disaggregate noncovalent complexes, limiting its usefulness in studies of protein aggregation. We examined this issue by comparing results...
obtained by SEC/QLS with those from SDS-PAGE. Fibers, protofibrils, and dimers of Aβ(1–40) all produced a single ~4-kDa band on gels (Fig. 6A). A single band was also seen with dimeric Aβ-(1–42), whereas Aβ-(1–42) protofibrils and fibers produced essentially identical ladder of low molecular weight species ranging from monomer to pentamer (Fig. 6B). These results show clearly that SDS-PAGE data do not reliably reflect the polymerization state of Aβ under native conditions. However, the technique may be of use in the characterization of factors that produce detergent-stable multimers.

**Modeling Aβ Fibrillogenesis**—The structural and kinetic analyses of Aβ fibrillogenesis presented here revealed the existence of a previously unidentified fiber intermediate, the protofibril. The data extant are consistent with a number of models of protofibril formation and conversion into fibers. Each model posits that Aβ monomers combine to form fibril nuclei (Fig. 7A, step 1), from which protofibrils emanate by linear growth (Fig. 7A, step 2). These protofibrils, in turn, give rise to the classical 6–10-nm fibers characteristic of neuritic plaques (Fig. 7A, step 3). During fibrillogenesis, Aβ dimers accumulate initially and then are consumed as protofibrils and fibers form. These dimers may be involved directly in steps 1–3 or may be in equilibrium with monomers, which could be the true building blocks for nuclei, protofibrils, and/or fibers. A dimer “reservoir” could control the monomer concentration and, therefore, the kinetics of fibrillogenesis in the way micelles control fibrillogenesis kinetics at low pH (8, 53).

A number of mechanisms may explain the protofibril–fiber transition (Fig. 7B). The simplest is end-to-end association of protofibrils. This is unlikely, however, because of the kinetic barriers associated with the diffusion and proper alignment of protofibril ends. Protofibrils could associate laterally to form “self-templates,” onto which precursors (dimers/monomers) could bind and polymerize. Alternatively, lateral association of protofibrils could accelerate end-to-end annealing by fostering proper alignment of protofibril ends. It is also possible that protofibrils are the end-products of a nonfibrillogenic pathway (Fig. 7C). In this case, protofibril dissociation would be necessary to release Aβ precursors into productive pathways of fiber formation. Experiments are currently under way to discriminate among these possibilities. Initial results suggest that protofibrils self-associate to produce fibers in the absence of low molecular weight precursors. This mechanism is supported by the failure to observe a continuous distribution of fiber lengths in size domain between protofibrils and mature amyloid fibers (42).

If protofibrils are key intermediates in Aβ fibrillation **in vitro**, as they appear to be **in vitro**, they would be attractive therapeutic targets because 1) fibrillation mediated through protofibrils might be poisoned with as little as one or two inhibitor molecules per protofibril, and 2) fibrillation inhibitors would function extracellularly. In particular, because the therapeutic levels of inhibitor in the plasma or cerebrospinal fluid would be orders of magnitude lower than those necessary for inhibitors binding to monomeric Aβ, the risk of cytotoxic side-effects would significantly decrease.

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