Apolipoprotein E Inhibits the Depolymerization of β2-Microglobulin-Related Amyloid Fibrils at a Neutral pH†

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ABSTRACT: β2-Microglobulin-related (Aβ2M) amyloidosis is a common and serious complication in patients on long-term hemodialysis, and β2-microglobulin (β2-m) is a major structural component of Aβ2M amyloid fibrils. Fluorescence spectroscopic analysis with thioflavin T and electron microscopic study revealed that Aβ2M amyloid fibrils readily depolymerize into monomeric β2-m at a neutral to basic pH. Circular dichroism analysis revealed that soon after the initiation of the depolymerization reaction at pH 7.5, the characteristic spectrum of β2-m in Aβ2M amyloid fibrils changes to resemble that of monomeric β2-m at pH 7.5. Apolipoprotein E (apoE), a representative amyloid-associated protein, formed a stable complex with Aβ2M amyloid fibrils and inhibited the depolymerization of Aβ2M amyloid fibrils dose-dependently in a range of 0−10 μM. These results showed that apoE could enhance the deposition of amyloid fibrils in vivo, possibly by binding directly to the surface of the fibrils and stabilizing the conformation of β2-m in the fibrils.

β2-Microglobulin-related (Aβ2M)[1] amyloidosis is a common and serious complication in patients on long-term hemodialysis (*1*). Carpal tunnel syndrome and destructive arthropathy associated with cystic bone lesions are the major clinical manifestations of Aβ2M amyloidosis (*2*, *3*). Several biochemical and cell biological studies have revealed that intact β2-microglobulin (β2-m) (M_r = 11 731) is a major structural component of amyloid fibrils deposited in the synovia of the carpal tunnel (*4*−*7*). The mechanism of the deposition of these Aβ2M amyloid fibrils is still unknown. Although the retention of β2-m in the plasma appears to be prerequisite, other factors, such as the age of the patient, the duration of dialysis, and the type of dialysis membrane used, may also be involved (*8*−*10*).

To understand the pathogenesis of Aβ2M amyloidosis, it is essential to study not only the mechanism of the formation of Aβ2M amyloid fibrils from β2-m in vitro but also the mechanism of the depolymerization of Aβ2M amyloid fibrils in vitro. We and other groups have proposed that a nucleation-dependent polymerization model could explain the general mechanisms of amyloid fibril formation in vitro, in various types of human as well as murine amyloidosis (*11*−*15*). This model consists of two phases, i.e., a nucleation phase and an extension phase. Nucleus formation requires a series of association steps of monomers, which are thermodynamically unfavorable, representing the rate-limiting step in amyloid fibril formation in vitro. Once the nucleus (*n*-mer) has been formed, further addition of monomers becomes thermodynamically favorable, resulting in rapid extension of amyloid fibrils in vitro. We have developed a first-order kinetic model of amyloid fibril extension in vitro, by measuring the polymerization velocity of amyloid fibrils as an indicator of the reaction, and confirmed that the extension of amyloid fibrils including Aβ2M amyloid fibrils proceeds via the consecutive association of monomeric precursor proteins onto the ends of existing fibrils (*11*, *13*, *15*). The extension of Aβ2M amyloid fibrils was greatly dependent on the pH of the reaction mixture, and the rate of extension was maximal around pH 2.5 (*15*). Recently, it was reported that the formation of Aβ2M amyloid fibrils from monomeric recombinant β2-m (r-β2-m) is also critically dependent on the pH and ionic strength of the solution, with low pH and high ionic strength favoring fibril formation (*16*). The net rate of fibril extension is equal to the sum of the rates of polymerization and depolymerization (*11*, *13*, *15*). This indicates that the depolymerization of amyloid fibrils could occur under some in vitro conditions. However, as for the issue of amyloid fibril depolymerization in vitro, only a few studies have thus far been reported (*17*−*19*).

García-García et al. (*20*) reported that β2-m in mono- and dimeric forms was solubilized in vitro from prepurified Aβ2M amyloid fibrils and crude amyloid deposits by the action of phosphate-buffered saline alone, with no need for proteases. These findings suggest that Aβ2M amyloid fibrils

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[1] Abbreviations: AA, amyloid A protein; Aβ, amyloid β-peptides; Aβ2M, β2-microglobulin-related; AL, immunoglobulin light chain-related amyloid protein; apoE, apolipoprotein E; β2-m, β2-microglobulin; CD, circular dichroism; HSA, human serum albumin; r-β2-m, recombinant β2-m; SAP, serum amyloid P component; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ThT, thioflavin T.

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could depolymerize into monomeric and dimeric β2-m at a neutral pH. However, there is disagreement about the regression of Aβ2M amyloid deposits after renal transplantation (*21*−*25*). One possible explanation is that some amyloid-associated molecules may prevent the regression of Aβ2M amyloid deposits in some in vivo situations.

Apolipoprotein E (apoE) is a cholesterol transport protein that serves as a ligand for low-density lipoprotein receptors (*26*). ApoE is colocalized to amyloid deposits in all types of systemic and localized amyloidosis thus far examined, i.e., Alzheimer's β-amyloidosis (*27*, *28*), prion amyloid disease (*27*), amyloid A protein (AA) amyloidosis (*28*−*30*), immunoglobulin light chain-related (AL) amyloidosis (*29*), transthyretin-related amyloidosis (*29*), and Aβ2M amyloidosis (*29*). Moreover, the apoE4 isoform was found to be significantly associated with late-onset Alzheimer's disease (*31*), AA amyloidosis associated with rheumatoid arthritis (*32*), and Aβ2M amyloidosis (*33*).

Although the data described above prompted many research groups to examine the effect of apoE on amyloid fibril formation in vitro, the data are somewhat controversial. Some groups found that apoE promoted and accelerated Alzheimer's β-amyloid fibril formation from amyloid β-peptides (Aβ) in vitro (*34*−*38*). On the basis of these observations, apoE has been regarded as one of the "pathological molecular chaperones" which are the "promoters" of amyloid fibril formation from various precursor proteins (*28*, *39*). In contrast, other groups reported that apoE inhibited β-amyloid fibril formation in vitro (*14*, *40*−*42*). Although all of the studies described above have been focused on the interaction of apoE and Aβ in the process of β-amyloid fibril formation in vitro, no exact assessment of the role of apoE in the process of amyloid fibril depolymerization in vitro has thus far been available.

Recently, utilizing mice lacking the apoE gene by gene targeting, it was found that apoE is not essential for but can promote the development of AA amyloid deposition in mice (*43*, *44*). Moreover, utilizing a transgenic mouse model of Alzheimer's disease, expressing mouse, human, or no apoE, Paul's group demonstrated that apoE has a critical and isoform-specific (E3 < E4) role in β-amyloid deposition in the mouse brain (*45*, *46*). They suggested that apoE plays at least two critical and possibly distinct roles to facilitate Aβ deposition: (1) apoE promotes the deposition of Aβ and its conversion to a fibrillar form, and (2) apoE impedes the clearance of Aβ in brain tissue, thus promoting deposition of Aβ and eventually fibrillogenesis. However, the observations could also be interpreted from the standpoint of the interaction of apoE and amyloid fibrils in the process of amyloid fibril depolymerization.

In this study, we characterized the depolymerization of Aβ2M amyloid fibrils in vitro and the role of apoE in the process of amyloid fibril depolymerization in vitro. The role of apoE and other amyloid-associated molecules in the development of amyloidosis is discussed.

EXPERIMENTAL PROCEDURES

β2-m and Other Proteins. Human r-β2-m was purchased from Oriental Yeast (Tokyo, Japan). Purified human serum apoE and recombinant apoE2, -E3, and -E4 were obtained from Chemicon International Inc. (Temecula, CA), and human serum albumin (HSA) was from Sigma (St. Louis, MO). r-β2-m (lot 45304605) was dialyzed against Milli Q water, lyophilized, then dissolved in a 50 mM NaCl solution, and centrifuged at 18500*g* for 20 min. The supernatant contained 300−450 μM r-β2-m and was stored at −80 °C. Human apoE (lots 62596069 and 19020250) and recombinant human apoE2 (lot 19050455), apoE3 (lot 19060321), and apoE4 (lot 19060320) were dissolved in 6 M urea, dialyzed against 10 mM phosphate (pH 7.5) and 140 mM NaCl for 3 h in a cold room set at 4 °C, and stocked at 4 °C. HSA (lot 78H7603) was dissolved at a concentration of 200 mg/mL in a 100 mM NaCl solution and stocked at −80 °C.

Preparation of Aβ2M Amyloid Fibrils. The original Aβ2M amyloid fibrils were purified from pooled synovial tissues, excised surgically from three patients suffering from Aβ2M amyloidosis, essentially as described elsewhere (*15*). The crude Aβ2M amyloid fibrils were isolated as a water suspension from the tissues described above, according to the method of Pras et al. (*47*). The suspension of Aβ2M amyloid fibrils was ultracentrifuged at $10^5 \times g$ for 100 min at 4 °C. Pellets resuspended in Milli Q water were applied on a discontinuous sucrose density gradient (from 50 to 60%) and then ultracentrifuged at $10^5 \times g$ for 18 h at 16 °C. Pellets were collected and dialyzed against 0.05% NaN_3 at 4 °C. These aggregates were mildly homogenized using a Teflon homogenizer and stocked at 4 °C. The purified Aβ2M amyloid fibrils were designated F0 fibrils. One milliliter of F0 suspension in an Eppendorf tube was sonicated on ice with intermittent pulses (0.6 s pulse, 0.4 s interval, output level of 2) for 3 min by an ultrasonic disruptor (UD-201, Tomy, Tokyo, Japan) equipped with a microtip (TP-030, Tomy). The sonicated Aβ2M amyloid fibrils were designated S0 seeds.

Unmodified Aβ2M amyloid fibrils composed solely of r-β2-m were formed by the repeated extension reaction using r-β2-m (*48*). The S0 seeds were added to yield a final concentration of 10 ng/μL in the reaction mixture containing 25 μM r-β2-m, 50 mM citrate buffer (pH 2.5), and 100 mM NaCl. After a 24 h incubation at 37 °C, the extended fibrils (designated F1) were collected by centrifugation at 18500*g* for 2 h at 4 °C. The pellet was washed and resuspended in an ice-cold 0.05% NaN_3 solution and then sonicated on ice for 1 min (0.6 s pulse, 0.4 s interval, output level of 2). The sonicated F1 fibrils were designated S1 seeds. F2 fibrils were obtained by the incubation of S1 seeds and r-β2-m. This algorithmic protocol was repeated 11 times, and the resulting F11 fibrils were finally obtained from S10 seeds. F11 fibrils were composed solely of r-β2-m (*48*).

Depolymerization Assay. F0, F1, and F11 fibrils were centrifuged at 18500*g* for 2 h at 4 °C. The pellets were washed and resuspended in 100 mM ice-cold NaCl. The reaction mixture was prepared on ice and contained 150 μg/mL Aβ2M amyloid fibrils, 50 mM buffer, and 100 mM NaCl. The buffers that were used were citrate-HCl (pH 1.0 and 1.5), citrate (pH 2.0−6.0), phosphate (pH 6.5 and 7.0), and Tris-HCl (pH 7.5−8.5). In some experiments, 0−10 μM apoE or 10 μM HSA was added to the reaction mixture. After being mixed by pipetting, triplicate 5 μL aliquots were subjected to fluorescence spectroscopy and 30 μL aliquots were put into oil-free PCR tubes (0.5 mL in size, code number 9046; Takara Shuzo Co. Ltd., Otsu, Japan). The reaction tubes were then transferred into a DNA thermal

cycler (PJ480, Perkin-Elmer Cetus, Emeryville, CA) set at 37 °C. Incubation times ranged between 2 min and 24 h (as indicated in each figure). The reaction tubes were not agitated during the reaction. From each reaction tube, 5 μL aliquots in triplicate were subjected to fluorescence spectroscopy, and the mean of the three measurements was determined.

Fluorescence Spectroscopy. All studies were performed essentially as described elsewhere (*15*) on a Hitachi F-3010 fluorescence spectrophotometer. Optimum fluorescence measurements of Aβ2M amyloid fibrils were obtained at the excitation and emission wavelengths of 455 and 485 nm, respectively, with the reaction mixture containing 5 μM thioflavin T (ThT) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 50 mM glycine-NaOH buffer (pH 8.5) (*15*). Fluorescence was measured immediately after the mixture was made and was averaged for the initial 5 s.

Determination of the Size of β2-m Oligomers Depolymerized from Aβ2M Amyloid Fibrils. To determine the size of the β2-m oligomers depolymerized from Aβ2M amyloid fibrils to the supernatant, we first used Ultrafree-MC centrifugal filter units (Millipore Corp., Bedford, MA). The reaction mixture contained 150 μg/mL F11 fibrils, 100 mM NaCl, and 50 mM citrate (pH 2.5) or Tris-HCl (pH 7.5). After incubation at 37 °C for 24 h, the protein concentration and ThT fluorescence of the reaction mixture were measured. Then the reaction mixture was centrifuged at 18500*g* for 20 min at 4 °C. After the protein concentration of the supernatant was measured, 200 μL of the supernatant was centrifuged with 100 000, 30 000, and 10 000 nominal molecular weight limit (NMWL) filter unit devices [Biomax-100 high-flux (UFC3BHK25), Biomax-30 high-flux (UFC3BTK25), and Biomax-10 high-flux (UFC3BGC25), respectively; Millipore] at 5000*g* for 20 min at 4 °C. After the centrifugation, the protein concentration of each filtrate was measured. The supernatant at pH 7.5 was next analyzed by size exclusion chromatography. A Superose 12 HR10/30 column (Amersham-Pharmacia Biotech) was attached to an Äkta Purifier HPLC system (Amersham-Pharmacia Biotech). One-hundred microliters of the supernatant was loaded on the column equilibrated with 100 mM NaCl and 50 mM Tris-HCl (pH 7.5) at 6 °C. The column was calibrated using molecular weight markers for gel filtration chromatography (Sigma).

Electron Microscopy of Aβ2M Amyloid Fibrils. Reaction mixtures were spread on carbon-coated grids, negatively stained with 1% phosphotungstic acid (pH 7.0), and examined under a Hitachi H-7000 electron microscope with an acceleration voltage of 75 kV.

Circular Dichroism (CD) Spectra of r-β2-m and F11 Fibrils. CD spectra of r-β2-m and F11 fibrils were recorded on a Jasco 725 spectropolarimeter (Jasco) at 25 °C. The solutions contained 293 or 150 μg/mL r-β2-m or F11 fibrils, 100 mM NaCl, and 50 mM phosphate (pH 7.5) or acetate (pH 2.5). Five consecutive readings at a bandwidth of 1 nm, a response of 2 s, and a resolution of 0.2 nm were taken from each sample and averaged, baseline-subtracted, and noise-reduced. For measurements in the far-UV region (195−250 nm), the CD signal was recorded in a 1 mm path length quartz cell. For measurements in the near-UV region (250−350 nm), the CD signal was recorded in a 1 cm path length quartz cell. Results are expressed in terms of mean residue ellipticity (θ). For measurements of the changes of the CD spectra during the depolymerization of F11 fibrils, the CD spectra of 150 μg/mL F11 fibrils in 100 mM NaCl (pH 4.0−5.0) were first recorded. Then, 1 M phosphate buffer (pH 7.5) was added to the quartz cell to yield a final concentration of 50 mM, and the cell was gently shaked. The CD spectra of the solution were recorded 2 min, 30 min, 2 h, and 24 h after the addition of the phosphate buffer.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Western Blotting. SDS−PAGE of F11 fibrils incubated with apoE was performed by the method of Schägger and Jagow with 10% acrylamide (*49*). F11 fibrils incubated with apoE were centrifuged at 18500*g* for 20 min at 4 °C, and the pellet was resuspended in 100 mM ice-cold NaCl. This suspension was then centrifuged at 18500*g* for 20 min at 4 °C, and the pellet was resuspended in 100 mM ice-cold NaCl and centrifuged again. The pellet was dissolved in a sample buffer containing 8 M urea, 2% SDS, 1% β-mercaptoethanol, and 25 mM Tris-HCl (pH 6.8) and boiled at 100 °C for 5 min. After being electrophoresed, gels were transferred to Immobilon membranes with a blotting buffer containing 25 mM Tris-glycine (pH 8.3) and 20% (v/v) ethanol at 10 V overnight at 4 °C, using the Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc., Hercules, CA). After being blotted, membranes were washed with a washing buffer containing 0.1% Tween-20 and 20 mM Tris-HCl (pH 7.6), blocked with the buffer containing 5% skim milk and 0.1% Tween 20 at room temperature for 1 h, and stocked at −20 °C. Membranes were then incubated at room temperature for 1 h with rabbit primary antibodies, i.e., anti-human β2-m (DAKO, Glostrup, Denmark) diluted 1:1000 or anti-human apoE (DAKO) diluted 1:300. Membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody diluted 1:2000 at room temperature for 1 h, washed with a washing buffer, and visualized with 3,3′-diaminobenzidine.

Other Analytical Procedures. Protein concentrations of r-β2-m, Aβ2M amyloid fibrils, apoE, and HSA were determined by the method of Bradford (*50*) with a commercial protein assay kit (model 500-0001, Bio-Rad Laboratories, Inc.). r-β2-m in PBS (1.5 mg/mL) was used as the standard for r-β2-m and Aβ2M amyloid fibrils, while 1 mg/mL bovine serum albumin was used for HSA and apoE. The one-way analysis of variance, post-hoc test by Fischer's LSTD was used for statistical analysis.

RESULTS

Depolymerization of Aβ2M Amyloid Fibrils at a Neutral pH. As shown in Figure 1, the ThT fluorescence of F0, F1, and F11 fibrils incubated at pH 7.5 and 37 °C decreased immediately after the initiation of the reaction. At 24 h, the fluorescence of F0, F1, and F11 fibrils was 70, 50, and 6% of the initial fluorescence, respectively. This decrease in ThT fluorescence at a neutral pH was also observed in other buffers, i.e., 50 mM phosphate (pH 7.5), 100 mM NaCl and 10 mM HEPES (pH 7.4), and 140 mM NaCl (data not shown). As shown in Figure 2, the decrease in ThT fluorescence was greatly dependent on pH. At pH $<$5.0, most or all of the initial fluorescence was preserved at 24 h, while at pH $>$6.0, most or all of the initial fluorescence was lost. Electron microscopic study revealed that most of the F11 fibrils in the reaction mixture depolymerized at 24 h (compare panels A and B of Figure 3).

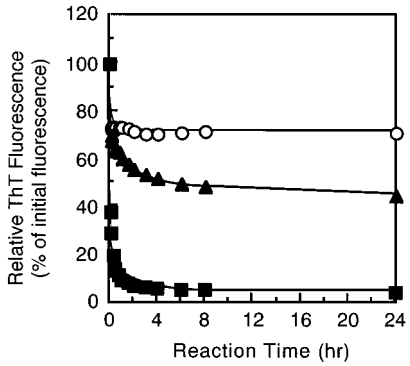

FIGURE 1: Depolymerization of Aβ2M amyloid fibrils at a neutral pH. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 150 μg/mL F0 (○), F1 (▲), or F11 fibrils (■). The reaction was initiated by shifting the temperature to 37 °C, as described in Experimental Procedures. At each incubation time, the reaction mixture was analyzed by fluorescence spectroscopy as described in Experimental Procedures. Each point represents the average of three independent experiments. Error bars are not indicated for simplicity.

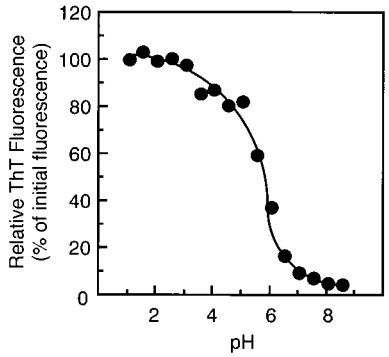

FIGURE 2: Effect of pH on the depolymerization of Aβ2M amyloid fibrils. The reaction mixture containing 150 μg/mL F11 fibrils, 100 mM NaCl, and 50 mM buffer [citrate-HCl (pH 1.0 and 1.5), citrate (pH 2.0−6.0), phosphate (pH 6.5 and 7.0), or Tris-HCl (pH 7.5−8.5)] was incubated for 24 h at 37 °C and analyzed by fluorescence spectroscopy as described in Experimental Procedures. Each point represents the average of three independent experiments. Error bars are not indicated for simplicity.

We determined the size of the β2-m oligomers depolymerized from Aβ2M amyloid fibrils to the supernatant. As shown in Figure 4A, when F11 fibrils were incubated at pH 2.5 for 24 h, most of the proteins in the reaction mixture moved to the pellet fraction after centrifugation. This indicates that most of the F11 fibrils did not depolymerize during the reaction. On the other hand, when F11 fibrils were incubated at pH 7.5 for 24 h, most of the proteins in the reaction mixture remained in the supernatant fraction after centrifugation (Figure 4B). This indicates that most of the F11 fibrils depolymerized during the reaction. After the preliminary size fractionation of the supernatant by the Ultrafree-MC centrifugal filter units, the protein concentration of the filtrates passed through 100 000 and 30 000 nominal molecular weight limit (NMWL) filter unit devices was ∼80 and ∼50% of that of the reaction mixture, respectively. This may indicate that most of the β2-m oligomers depolymerized from Aβ2M amyloid fibrils to the supernatant at pH 7.5 are less than 10-mer and at least half of them are monomers or dimers. It should be noted that these filtration experiments are semiquantitative and the Bradford assay used in this study may not accurately quantify

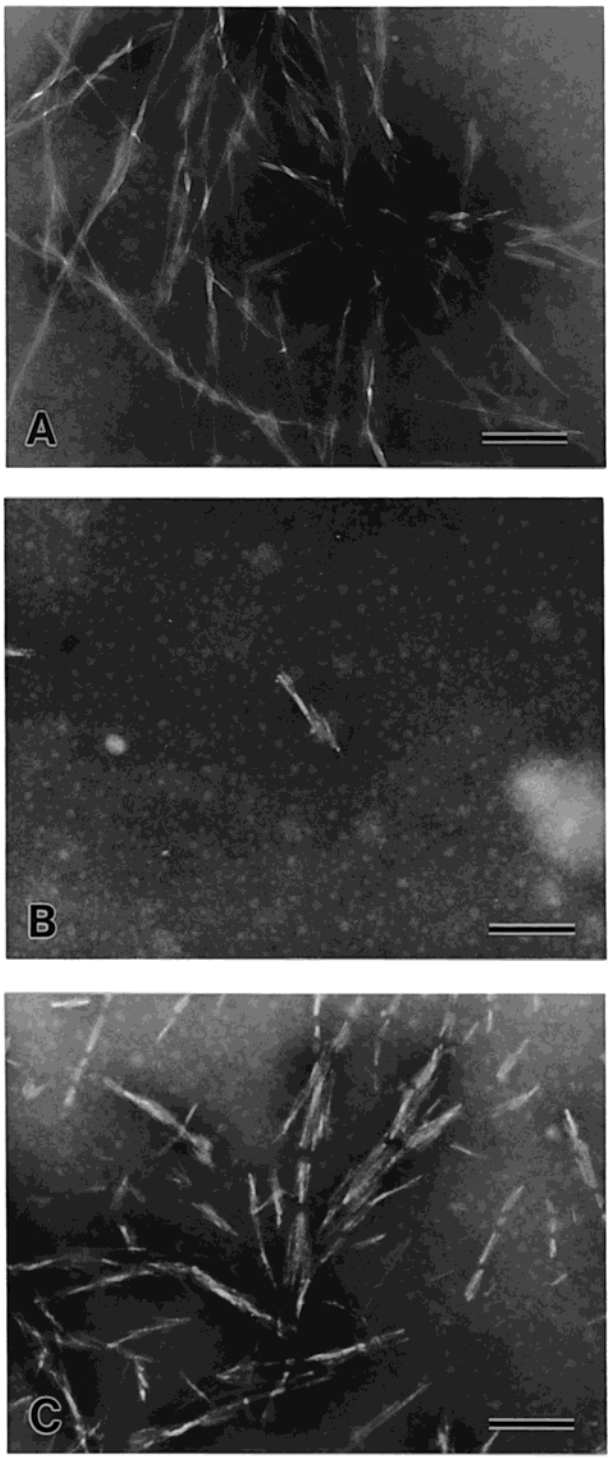

FIGURE 3: Electron micrographs of depolymerized Aβ2M amyloid fibrils. The reaction mixture containing 150 μg/mL F11 fibrils, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0 or 10 μM apoE was incubated at 37 °C for 24 h and prepared for electron microscopy as described in Experimental Procedures: (A) F11 fibrils before depolymerization, (B) F11 fibrils incubated with no apoE, and (C) F11 fibrils incubated in the presence of 10 μM apoE. The bars are 250 nm long.

the protein concentration of the mixture containing monomeric to oligomeric r-β2-m and Aβ2M amyloid fibrils. Therefore, we next performed the size exclusion chromatography of the supernatant at pH 7.5. As shown in Figure 4C, the supernatant at pH 7.5 eluted as a single peak corresponding to monomeric r-β2-m (11 500). This may

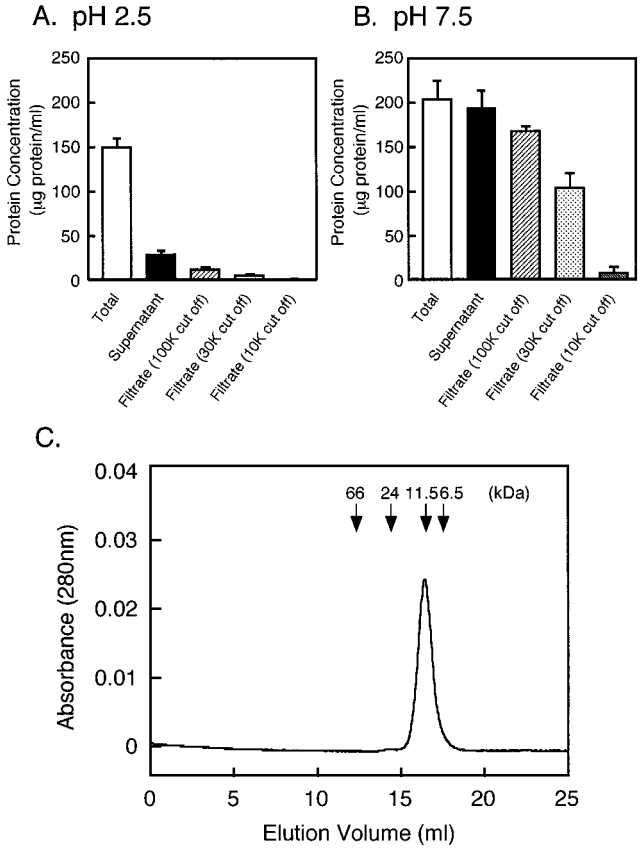

FIGURE 4: Determination of the size of the β2-m oligomers depolymerized from Aβ2M amyloid fibrils. The reaction mixture contained 150 μg/mL F11 fibrils, 100 mM NaCl, and (A) 50 mM citrate (pH 2.5) or (B) Tris-HCl (pH 7.5). After incubation at 37 °C for 24 h, the reaction mixture was centrifuged and the size fractionation of the supernatant was performed as described in Experimental Procedures. Protein concentrations of the reaction mixture, supernatant, and size-fractionated filtrates were measured. Each column represents the average of three independent experiments. The error bars indicate standard derivations. The supernatant at pH 7.5 was subjected to size exclusion chromatography (C) as described in Experimental Procedures. Arrows indicate the peak elution point of molecular weight markers.

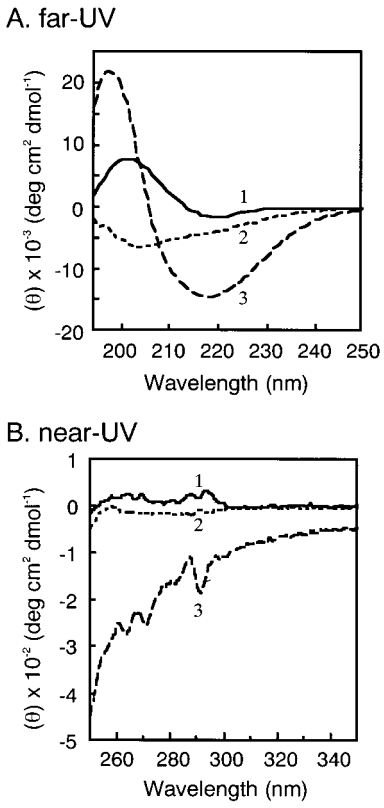

FIGURE 5: (A) Far- and (B) near-UV CD spectra of r-β2-m and F11 fibrils. In each spectrum, the reaction mixture contained 293 μg/mL r-β2-m (1 and 2) or F11 fibrils (3), 100 mM NaCl, and (1) 50 mM phosphate (pH 7.5) or (2 and 3) acetate (pH 2.5).

clearly indicate that most of the β2-m oligomers depolymerized from Aβ2M amyloid fibrils to the supernatant at pH 7.5 are monomers.

CD Analysis of the Depolymerization of Aβ2M Amyloid Fibrils. To evaluate the conformational changes of β2-m during the polymerization and depolymerization of Aβ2M amyloid fibrils, we performed CD analysis of β2-m and F11 fibrils at both pH 7.5 and 2.5. As shown in Figure 5A, the far-UV CD spectra of β2-m at pH 7.5 exhibited a positive peak at 200 nm and a negative peak at 221 nm. The near-UV CD spectra of β2-m at pH 7.5 exhibited characteristic positive peaks at 261, 264, 269, 281, 287, and 293 nm (Figure 5B). These data indicate that β2-m at pH 7.5 is rich in β-sheet conformation and has a compact tertiary structure. On the other hand, the far-UV CD spectra of β2-m at pH 2.5 exhibited only a negative peak at 205 nm (Figure 5A). The near-UV CD spectra of β2-m at pH 2.5 exhibited only a slightly negative ellipticity (Figure 5B). These data indicate that β2-m at pH 2.5 has lost much of the secondary and tertiary structure observed at pH 7.5. The far-UV CD spectra of F11 fibrils at pH 2.5 exhibited a positive peak at 198 nm and a negative peak at 219 nm (Figure 5A). The near-UV CD spectra of F11 fibrils at pH 2.5 exhibited a negative ellipticity different from those of β2-m both at pH 7.5 and 2.5 (Figure 5B). The interpretation of these two spectra will be discussed later.

As shown in Figure 6, changes in the far- and near-UV CD spectra were observed during the depolymerization reaction of F11 fibrils at pH 7.5. Even after incubation for 2 min, both far- and near-UV CD spectra of the reaction mixture changed greatly and then approximated those of β2-m at pH 7.5. However, a significant difference was observed in both far- and near-UV CD spectra between β2-m at pH 7.5 and F11 fibrils at 24 h (compare spectra 5 and 6 in Figure 6). This difference may be due to the presence of oligomeric β2-m molecules and short Aβ2M amyloid fibrils in the reaction mixture at 24 h (see Figures 3 and 4).

ApoE Inhibits the Depolymerization of Aβ2M Amyloid Fibrils at a Neutral pH. As shown in Figure 7A, during incubation at pH 7.5, the depolymerization of F11 fibrils was inhibited dose-dependently by the presence of apoE. Moreover, an electron microscopic study revealed that a significant amount of F11 fibrils remains in the reaction mixture at 24 h (Figure 3C). Although the fibrils became shorter than the fibrils before depolymerization, they maintained the typical filament structure with a diameter of ~10 nm (compare panels A and C of Figure 3). As shown in Figure 7B, 10 μM HSA did not exhibit a significant inhibitory effect on the depolymerization of F11 fibrils. We observed no significant difference in the inhibitory activity among three apoE isoforms (E2, E3, and E4) (data not shown).

We extensively washed F11 fibrils remaining in the reaction mixture after incubation for 24 h with apoE (Figure

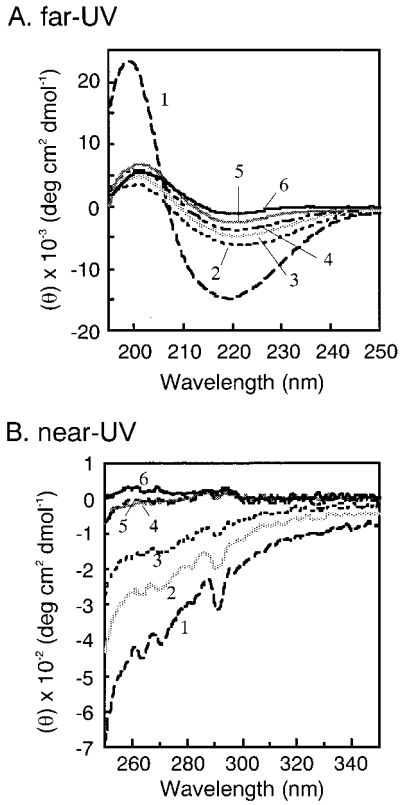

FIGURE 6: Time course of the CD spectra during the depolymerization of F11 fibrils. After the CD spectra (1) of 150 μg/mL F11 fibrils in 100 mM NaCl (pH 4.0−5.0) were recorded at 25 °C, the reaction mixture was adjusted to pH 7.5 and the CD spectra of the mixture were recorded 2 min (2), 30 min (3), 2 h (4), and 24 h (5) after the pH shift as described in Experimental Procedures. The CD spectra (6) of 150 μg/mL r-β2-m in 50 mM phosphate (pH 7.5) and 100 mM NaCl were also recorded.

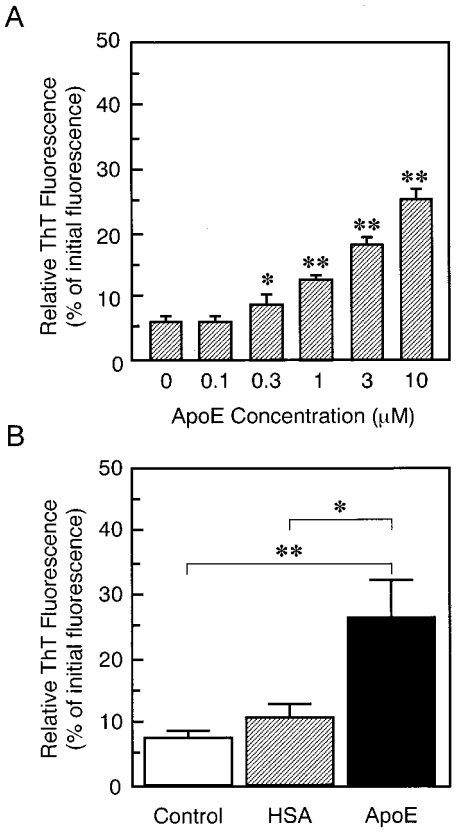

FIGURE 7: Effect of human serum apoE and HSA on the depolymerization of Aβ2M amyloid fibrils. The reaction mixture containing 150 μg/mL F11 fibrils, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0−10 μM apoE (A) or 10 μM apoE or HSA (B) was incubated for 24 h at 37 °C and analyzed by fluorescence spectroscopy as described in Experimental Procedures. Each column represents the average of three independent experiments. The error bars indicate standard derivations. (A) One asterisk denotes $p < 0.001$, and two asterisks denote $p < 0.0001$ versus 0 μM apoE. (B) One asterisk denotes $p < 0.0002$, and two asterisks denote $p < 0.0001$ (one-way ANOVA, post-hoc test by Fischer's LSTD).

3C) and analyzed them by Western blotting. We observed two bands of β2-m (12 000 and 24 000) and several bands of apoE, including intact apoE (34 000) (lane c in panels A and B of Figure 8). Moreover, we observed a 46 000 band, which is not observed in the lanes of β2-m, F11 fibrils, or apoE alone (compare lanes a−c in panels A and B of Figure 8). This band may represent an apoE−β2-m complex, since this is positive for both anti-human β2-m and anti-human apoE antibodies, and the size of this band (46 000) is equal to the sum of the molecular weights of β2-m (12 000) and intact apoE (34 000). This complex seems to be very stable, since it is observed even after the dissolution in SDS sample buffer and subsequent boiling (see Experimental Procedures).

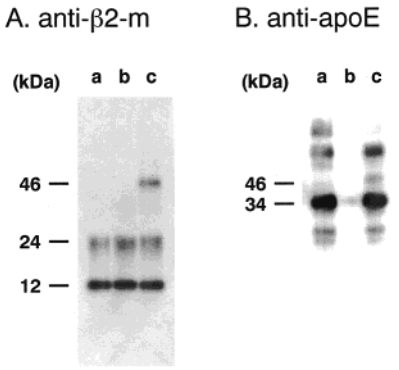

FIGURE 8: Western blotting of F11 fibrils incubated with apoE and remaining in the mixture. One milligram of protein of each sample was electrophoresed, transferred to Immobilon membranes, and immunostained for anti-human β2-m (A) and anti-human apoE (B) as described in Experimental Procedures: lane a, r-β2-m (A) and human apoE (B); lane b, F11 fibrils; and lane c, F11 fibrils incubated with 10 μM apoE for 24 h at 37 °C and washed twice as described in Experimental Procedures.

DISCUSSION

pH-Dependent Transformation between β2-m and Aβ2M Amyloid Fibrils. In this section, we first consider the mechanism of the pH-dependent and reversible transformation between β2-m and Aβ2M amyloid fibrils. We previously showed that the extension of Aβ2M amyloid fibrils proceeds via the consecutive association of monomeric β2-m onto the ends of existing fibrils, and the rate of extension was maximal at pH ~2.5 (*15*). McParland et al. (*16*) showed that the formation of Aβ2M amyloid fibrils from monomeric r-β2-m is also critically dependent on the pH and ionic strength of the solution, with low pH and high ionic strength favoring fibril formation. They also showed that titration of one or more residues with a pK_a of 4.7 destabilizes native β2-m and generates a partially unfolded species retaining a significant secondary structure and having residual, nonnative tertiary structure. Our CD analysis also indicated that

β2-m at pH 2.5 has lost much of the secondary and tertiary structure observed at pH 7.5 (Figure 5). Aβ2M amyloid fibrils at pH 2.5 exhibited the characteristic CD spectra clearly different from those of monomeric β2-m at both pH 7.5 and 2.5 (Figure 5). Because β2-m is in the fibrillar form rather than the monomeric form, light scattering and some other factors might influence aspects of the CD spectra. Thus, we cannot estimate the precise secondary and tertiary structures of β2-m in Aβ2M amyloid fibrils from the CD experiments whose results are presented here. Goldsbury et al. (*51*) examined the temporal changes in the far-UV CD spectra, ThT fluorescence, and fibril morphology during the polymerization reaction of human amylin into amyloid fibrils. The far-UV CD spectra of amylin amyloid fibrils were quite similar to the spectra of Aβ2M amyloid fibrils at pH 2.5 and exhibited a positive peak at 198 nm and a strong negative peak at 218 nm. While they noted that their data should be treated as qualitative numerical estimates only rather than precise percentages of secondary structures, they estimated the secondary structure of amylin in the fibrillar form to be β-sheet/α-helical structure. Fezoui et al. (*52*) examined the temporal changes in the far-UV CD spectra, Congo red binding, and fibril morphology during the polymerization reaction of Aβ into β-amyloid fibrils. The far-UV CD spectra of β-amyloid fibrils also exhibited a negative peak at 218 nm, and the secondary structure of Aβ in β-amyloid fibrils was estimated to be rich in β-sheet structure. It is generally accepted that amyloid precursor proteins change their conformation and polymerize into amyloid fibrils with cross-β pleated sheet organization (*53*). Therefore, it may be reasonable to consider that β2-m incorporated into Aβ2M amyloid fibrils at pH 2.5 is rich in β-sheet structure and has secondary and tertiary structures different from those of monomeric β2-m at both pH 7.5 and 2.5.

In this paper, we showed that Aβ2M amyloid fibrils readily depolymerize into monomeric to oligomeric β2-m at a neutral to basic pH (Figures 1, 2, and 4). Moreover, CD analysis revealed that soon after the initiation of the depolymerization reaction at pH 7.5, the characteristic spectrum of β2-m in Aβ2M amyloid fibrils changes and then approximates that of monomeric β2-m at pH 7.5 (Figure 6). This indicates that β2-m depolymerized from Aβ2M amyloid fibrils reversibly refolds to its original conformation at pH 7.5. A few mechanisms could be considered for the depolymerization of Aβ2M amyloid fibrils at a neutral pH. First, the rate of depolymerization of β2-m from the fibril ends would become much faster than the rate of polymerization of β2-m onto the fibril ends (Figure 9A). At an acidic pH where the rate of Aβ2M amyloid fibril extension is maximal, the rate of depolymerization is much slower than the rate of polymerization in the extension reaction (*15*). These pH-dependent change in the rates of polymerization and depolymerization in the extension reaction would be critical to the pH-dependent polymerization and depolymerization of Aβ2M amyloid fibrils. Second, although less likely, Aβ2M amyloid fibrils would depolymerize at a neutral pH, by partial unfolding of β2-m in the fibrils, subsequent shearing of the fibrils, and the depolymerization of β2-m from the fibril ends.

All of the results described above may indicate a reversible change in the conformation of β2-m in the pH-dependent cycle of the polymerization and depolymerization of Aβ2M amyloid fibrils. An acidic condition suitable for the polymerization of Aβ2M amyloid fibrils may be favorable for the maintenance of the characteristic conformation of β2-m in Aβ2M amyloid fibrils, while quite unfavorable for the maintenance of the compact conformation of β2-m. On the contrary, the neutral to basic condition suitable for the depolymerization of Aβ2M amyloid fibrils may be favorable for the maintenance of the compact conformation of β2-m, while quite unfavorable for the maintenance of the characteristic conformation of β2-m in Aβ2M amyloid fibrils. Interestingly, the transitional pH of these two conditions is around the calculated isoelectric point (pI) of β2-m (6.07). Titration of one or more residues of β2-m and the subsequent conformational change seem to be critical for the transformation between β2-m and Aβ2M amyloid fibrils.

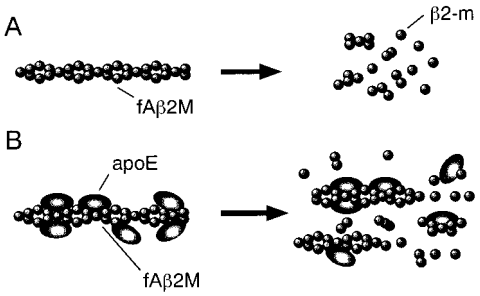

FIGURE 9: Hypothetical model of the depolymerization reaction of Aβ2M amyloid fibrils and the inhibitory effect of apoE on the depolymerization reaction. (A) Aβ2M amyloid fibrils composed solely of β2-m depolymerize into monomeric to oligomeric β2-m at a neutral pH, possibly by the accelerated depolymerization of β2-m from the fibril ends. (B) ApoE inhibits the depolymerization of Aβ2M amyloid fibrils, possibly by binding directly and specifically to the surface of the fibrils and stabilizing the characteristic conformation of β2-m in the fibrils.

Roles of ApoE and Other Amyloid-Associated Molecules in the Deposition of Amyloid Fibrils in Vivo. We showed that apoE forms a stable complex with Aβ2M amyloid fibrils and inhibits the depolymerization of Aβ2M amyloid fibrils dose-dependently (Figures 3, 7, and 8). From these results, it may be reasonable to consider that apoE inhibits the depolymerization of Aβ2M amyloid fibrils, possibly by binding directly and specifically to the surface of the fibrils and stabilizing the conformation of β2-m in the fibrils (Figure 9B).

Recently, utilizing mice lacking the apoE gene by gene targeting, apoE was found to promote the development of AA amyloid and Alzheimer's β-amyloid deposition in mice (*43*−*46*). Although apoE has been postulated to promote the deposition of AA and Aβ and its conversion to a fibrillar form, the observations could also be interpreted on the basis of the model we propose here; i.e., apoE binds tightly to the surface of amyloid fibrils deposited in the tissue and inhibits the depolymerization of the fibrils by stabilizing the characteristic conformation of AA and Aβ in the fibrils. Castaño et al. (*54*) reported that apoE carboxyl-terminal fragments form the tight complexes with AA and AL purified from patients with familial Mediterranean fever and primary amyloidosis. Tight association of apoE with amyloid fibrils seems to be a general phenomenon occurring in vivo in several types of amyloidosis.

We found no significant difference in the inhibitory activity among three apoE isoforms (E2, E3, and E4) (data not shown). Although we have no clear interpretation for this finding, the synergistic effect of apoE4 with other

unknown factors may contribute to the persistence of the deposition of Aβ2M amyloid fibrils in vivo.

Aβ2M amyloid deposits contain not only apoE but also many other amyloid-associated molecules, e.g., glycosaminoglycans (*55*), serum amyloid P component (SAP) (*56*), α2-macroglobulin, and other plasma proteinase inhibitors (*56*). There are many possibilities for the roles of these molecules in the deposition of amyloid fibrils in vivo. First, these molecules may promote the deposition of amyloid proteins (e.g., AA and AL) and its conversion to a fibrillar form in vivo. Second, these molecules may protect amyloid fibrils from the proteolysis in vivo. Utilizing mice lacking the SAP gene by gene targeting, Togashi et al. (*57*) found that SAP enhances induction of murine AA amyloidosis, and Tennent et al. (*58*) reported that SAP prevents proteolysis of AA and AL amyloid fibrils as well as Alzheimer's β-amyloid fibrils in vitro (*58*). Finally, as suggested in the paper presented here, these molecules may bind tightly to the surface of amyloid fibrils in vivo and inhibit the depolymerization of the fibrils by stabilizing the characteristic conformation of amyloid proteins in the fibrils. As shown in Figure 1, the fluorescence of F0, F1, and F11 fibrils decreased to 70, 50, and 6% of the initial fluorescence, respectively, after incubation for 24 h. F11 fibrils are composed solely of r-β2-m (*48*). On the other hand, Western blotting analysis revealed that F0 fibrils are associated with SAP and apoE (*48*). These findings may explain the difference in the depolymerization rates between F11 and F0 fibrils.

Interference with the binding of apoE, SAP, and other molecules to amyloid fibrils in vivo may be an attractive therapeutic objective. If these molecules were detached from the surface of amyloid fibrils, regression of the amyloid deposits would be promoted not only by the action of several proteases but also by the depolymerization of β2-m from the fibril ends. The experimental system described here should be useful in searching for the drugs protecting the binding of amyloid-associated molecules to the fibrils. Related experiments are ongoing in our laboratory.

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