Published in final edited form as:

J Mol Biol. 2016 January 29; 428(2 0 0): 274–291. doi:10.1016/j.jmb.2015.06.008.

C-terminal threonine reduces A β ₄₃ amyloidogenicity compared with A β ₄₂

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Abstract

A β ₄₃, a product of the proteolysis of the amyloid precursor protein APP, is related to A β ₄₂ by an additional Thr residue at the C-terminus. A β ₄₃ is typically generated at low levels compared with the predominant A β ₄₂ and A β ₄₀ forms, but it has been suggested that this longer peptide might have an impact on A β aggregation and Alzheimer's disease that is out of proportion to its brain content. Here we report that A β ₄₂ and A β ₄₃ both spontaneously aggregate into mature amyloid fibrils *via* sequential appearance of the same series of oligomeric and protofibrillar intermediates, the earliest of which appears to lack β -structure. In spite of the additional β -branched amino acid at the C-terminus, A β ₄₃ fibrils have fewer strong backbone H-bonds than A β ₄₂ fibrils, some of which are lost at the C-terminus. In contrast to previous reports, we found that A β ₄₃ spontaneously aggregates more slowly than A β ₄₂. In addition, A β ₄₃ fibrils are very inefficient at seeding A β ₄₂ amyloid formation, even though A β ₄₂ fibrils efficiently seed amyloid formation by A β ₄₃ monomers. Finally, mixtures of A β ₄₂ and A β ₄₃ aggregate more slowly than A β ₄₂ alone. Both in this A β ₄₂/A β ₄₃ co-aggregation reaction and in cross-seeding by A β ₄₂ fibrils, the structure of the A β ₄₃ in the product fibrils is influenced by the presence of A β ₄₂. The results provide new details of amyloid structure and assembly pathways, an example of structural plasticity in prion-like replication, and data showing that low levels of A β ₄₃ in the brain are unlikely to favorably impact the aggregation of A β ₄₂.

Abstract

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Keywords

Amyloid; polymorphism; cross-seeding; hydrogen-deuterium exchange; weight-normalized ThT

INTRODUCTION

The accumulation of amyloid- β (A β) peptides in extracellular plaques is one of the main pathological characteristics of Alzheimer's disease (AD) ¹. A β peptides are generated from the transmembrane amyloid precursor protein (APP) through sequential cleavage by β -secretase and γ -secretase enzymes ¹. The varying lengths of A β C-termini that have been isolated ² have been attributed to details of the γ -secretase processing pathway, which generates A β peptides by sequential cleavage at every three to four residues starting with the A β_{48} /A β_{49} initial APP cleavage products ^{3,4}. Two major forms of A β , A β_{40} and A β_{42} , exist in AD brain, with A β_{42} being the more toxic and faster aggregating of the two ². The absolute levels of A β_{42} and the ratio of A β_{42} to A β_{40} , for example as influenced by some familial AD mutations, are key factors in determining neuronal toxicity and AD risk ^{2,5,6}.

At the same time, evidence has been presented showing the co-existence in the AD brain of longer A β species, including A β_{43} , A β_{46} , A β_{48} , and A β_{49} ^{7,8}. In particular, A β_{43} has been detected in plaque cores obtained from occipital and frontal cortex in both sporadic and familial AD cases ^{9,10} and is also present in cored ("cotton wool") neuritic plaques ¹¹ and in diffuse plaques ⁷. Longer A β species have also been detected in transgenic mouse ¹²⁻¹⁴ and cell culture ³ models. Some FAD associated mutations in the presenilin genes ^{15,16} have been shown to increase the generation of longer A β species including A β_{43} ¹⁷. Being derived from cleavages within the transmembrane segment of APP ², longer A β peptides tend to be more hydrophobic than shorter versions and, being rich in β -branched amino acids with high propensities for β -structure ¹⁸, might be expected to be even more prone to make β -sheet rich aggregates. There have been only limited studies, however, on the solution and assembly properties of longer A β species either alone or in mixture with the more common A β species. A β_{46} has been reported to spontaneously aggregate much more rapidly than A β_{42} and A β_{40} ¹⁹, and mature fibrils of A β_{46} were reported to seed elongation of A β_{42} and A β_{40} ²⁰. A β_{43} in isolation has been reported to aggregate at similar ²¹ or faster ^{22,23} rates compared with A β_{42} , and, when present in 10% the molar amount of A β_{42} , to not alter aggregation kinetics ²¹. Altered aggregation properties, either in isolation or a mixture, might prove to be responsible for the reported elevated toxicity of A β_{43} in a transgenic mouse model ²². This mechanism seems especially feasible given the recent report that A β_{43} is deposited into brain aggregates ahead of A β_{42} in a mouse model ²⁴.

Although much is known about the self-assembly of amyloidogenic peptides, there have been relatively few studies detailing assembly kinetics and amyloid structures from mixtures of amyloidogenic peptides, and most of these focus on interactions between A β_{40} and A β_{42} ²⁵⁻³⁰. Furthermore, while

Here, based on studies of the comparative properties of A β_{42} and A β_{43} , we report how a single amino acid addition can influence amyloid structure and properties in some surprising ways. We found that the added C-terminal Thr residue in A β_{43} slows aggregation rates compared with A β_{42} , but does so without altering the assembly pathway, as defined by the

progression through a rich series of assembly intermediates. The slower fibrillogenesis of A β ₄₃, in isolation or in admixture with A β ₄₂, in addition to a stark inability of A β ₄₃ fibrils to seed fibril formation by A β ₄₂, casts doubt on any substantial toxicity-enhancing role for A β ₄₃ related to its fibril forming properties. The C-terminal Thr of A β ₄₃ also impacts amyloid structure, including the loss of several strong backbone H-bonds in comparison to A β ₄₂ fibrils. We also describe some unusual features of seeded elongation in this system, including a lack of reciprocal cross-seeding between the A β ₄₂ and A β ₄₃ peptides, and the generation of a novel, polymorphic fibrillary state of A β ₄₃ that is generated in response to A β ₄₂ seeding.

RESULTS

Progression of amyloid assembly states by A β ₄₂ and A β ₄₃ peptides

Assembly intermediates have been implicated as toxic species that contribute to AD pathology³⁵⁻³⁸, but the number of such intermediates in the pathway and their individual structures and stabilities are incompletely understood. Since different toxicities of A β ₄₂ and A β ₄₃ might be due to altered progression through these various assembly states, we focused on careful characterization of these intermediates and how they wax and wane during the spontaneous formation of mature amyloid fibrils from solutions of the monomer. We analyzed the aggregation kinetics of freshly disaggregated A β ₄₂ and A β ₄₃ monomers at a 10 μ M starting concentration (Methods).

We monitored amyloid assembly by following reactions both by the loss of monomer from solution and by a novel “aggregate weight-normalized” ThT intensity (Methods) (Fig. 1a). The drop in monomer concentration is a measure of total sedimentable (i.e., detectable by the sedimentation assay) aggregates produced, regardless of their morphology. The weight-normalized ThT intensity (obtained by dividing the gross ThT intensity by the weight fraction of starting A β converted into sedimentable aggregates) is a measure of the average β -content of the aggregate pool at each time point. Thus, in contrast to the standard use of ThT, in which time-dependent intensity increases are interpreted as increases in the amount of β -aggregates, a rise in the weight-normalized ThT intensity comes about because of an enhanced average β -content within the existing aggregates, regardless of the extent of the aggregation reaction. In situations where there is effectively only one aggregated species (such as the end of the reaction) the weight normalized ThT value is characteristic of the polymorphic state of the aggregate. Previously we showed that structurally different polymorphic forms of mature A β ₄₀ fibrils exhibit different weight-normalized ThT intensities³⁹, and it was recently suggested that such intensity variations are due to differences in the number of ThT binding sites (normally considered to be in β -sheet⁴⁰) in an aggregate⁴¹.

For the A β ₄₂ aggregation reaction at a starting concentration of 10 μ M, the monomer concentration drops over 50% within the first several hours, then continues to decline more slowly over the next three days, finally reaching apparent equilibrium at about 100 hrs (Fig. 1a, •). The weight-normalized ThT signal produced by aliquots of this reaction correspondingly rise rapidly in the initial 24 hours, reaching a plateau within 60 hrs to a relatively high yield of ThT fluorescence of $\sim 2.2 \times 10^5$ fluorescence units per μ g (Fig. 1a,

○). The steady increase in weight-normalized ThT signal over the first 24 hrs suggests a transition through one or more intermediates with steadily increasing levels of ThT-sensitive β -structure. Further detail is provided by EM (Fig. 2) and DLS (Fig. 3) analyses of reaction time points. After 1 hr, when 10% of the $A\beta_{42}$ molecules have already been converted to sedimentable aggregates (Fig. 1a, ●), DLS reveals a broad distribution of particles centered at ~ 10 nm in hydrodynamic radius (Fig. 3b), and this is consistent with EM images showing lightly staining particles in the same size range (Fig. 2a). After 2 hrs, when about 30% of the $A\beta_{42}$ molecules have been converted to aggregates (Fig. 1a, ●), most particles take up slightly more stain (Fig. 2b) and many have more clearly defined boundaries (Fig. 2c). By 8 hrs, when about 60% of $A\beta_{42}$ molecules are aggregated, EM analysis shows two types of oligomeric intermediate: narrow, positively stained filaments (Fig. 2d) and globular and wormlike negatively stained aggregates (Fig. 2e). At this point light scattering is high and the DLS data can no longer be deconvoluted. Between 8 and 12 hrs, the total amount of sedimentable $A\beta$ remains at $\sim 60\%$ and the aggregate population remains mostly negatively stained globules and worms, although an occasional negatively stained fibril can also be detected (Fig. 2f). By 24 hrs, when $\sim 75\%$ of the starting $A\beta_{42}$ is aggregated (Fig. 1a, ●), the reaction mixture is dominated by mature, negative-stained amyloid fibrils (Fig. 2g). By this point the weight-normalized ThT signal has risen to near maximum (Fig. 1a, ○), consistent with amyloid fibrils with maximized β -sheet content. Based on the sizes of these aggregates, all of the assembly intermediates described here would typically be characterized in the amyloid literature as “soluble” aggregates, while only the mature amyloid fibrils, which in suspension are measurably turbid, would be classified as “insoluble”.

The $A\beta_{43}$ assembly reaction proceeds through very similar structures to $A\beta_{42}$ intermediates, but does so over an extended time course. After 1 hr, when $\sim 7\%$ of the $A\beta_{43}$ molecules are aggregated, EM shows poorly staining oligomers (Fig. 2h) similar to 1 hr- $A\beta_{42}$ oligomers (Fig. 2a). After 4 hrs, when $\sim 14\%$ of $A\beta_{43}$ molecules are aggregated (Fig. 1a, ■), oligomers remain relatively poorly resolved but take up somewhat more stain (Fig. 2i), similar to some 2 hrs- $A\beta_{42}$ assemblies (Fig. 2b). By 12 hrs, when about 30% of $A\beta_{43}$ molecules are aggregated (Fig. 1a, ■), oligomers have more highly resolved boundaries (Fig. 2j), like some 2 hrs- $A\beta_{42}$ aggregates (Fig. 2c). Beginning at 16 hrs, when $\sim 40\%$ of $A\beta_{43}$ molecules are aggregated, and extending to 24 hrs, when about 45% are aggregated (Fig. 1a, ■), the reaction mixture is composed entirely of thin, positively stained filaments (Fig. 2k) identical to 8 hrs- $A\beta_{42}$ oligomers (Fig. 2d). These filaments have average particle sizes in DLS at 20 hrs of ~ 75 nm in hydrodynamic radius (Fig. 3d). Between 25 hrs and 40 hrs, neither the percentage of $A\beta_{43}$ that is aggregated nor the weight-normalized ThT signal has changed very much (Fig. 1a), but aggregate morphology changes dramatically, now being dominated by a mix of negatively stained worm-like aggregates either in isolation (Fig. 2l) or coating newly observed amyloid fibrils (Fig. 2m). From 45 hrs to 90 hrs, the amount of aggregated $A\beta_{43}$ increases from $\sim 45\%$ to $\sim 90\%$, the weight normalized ThT increases from the intermediate plateau value of 0.6×10^5 to a final value of 1.5×10^5 fluorescence units per μg (Fig. 1a), and the reaction mixture transforms to uniform mature amyloid by EM (Fig. 2n).

The EM and weight normalized ThT data for the $A\beta_{43}$ reaction reveal a number of important trends that are mirrored in the faster-moving $A\beta_{42}$ assembly reaction. First, the steady rise in weight-normalized ThT signal during the first 10 hrs of the $A\beta_{43}$ assembly reaction is

associated with a change in aggregate morphology from lightly staining, poorly resolved oligomers (Fig. 2h,i) with no apparent β -content to better-resolved oligomers (Fig. 2j) and narrow, positively stained filaments (Fig. 2k) that exhibit similarly elevated β -contents. A similar set of transitions occurs in the early part of $A\beta_{42}$ assembly ending at about 8 hrs. Interestingly, the rates of increase and final magnitudes of weight normalized ThT signals for this early phase of the assembly process are essentially identical for $A\beta_{42}$ and $A\beta_{43}$ (Fig. 1b). Thus, both peptides feature initial, very fast formation of a non- β oligomer, followed by transformation over ~ 10 hrs into a β -oligomer.

Second, the point where amyloid structure is nucleated, as defined by the time of first appearance of amyloid fibrils, occurs for $A\beta_{43}$ at ~ 40 hrs, when about 50% of $A\beta_{43}$ remains monomeric (Fig. 1a, ■). In contrast, nucleation of amyloid structure in the $A\beta_{42}$ assembly reaction occurs at ~ 12 hrs, when about 40% of the $A\beta_{42}$ molecules are monomeric (Fig. 1a, •). Although narrow filaments (Fig. 2 d,k) are populated in both peptide reaction mixtures leading into the appearance of mature amyloid, both reaction mixtures at these times also contain other structures, including monomers, making it impossible from our data to determine the species within which nucleation occurs.

Third, nucleation of amyloid does not necessarily equate to a rapid increase in aggregation kinetics. Rather, both for $A\beta_{42}$ (Fig. 1a, •) and $A\beta_{43}$ (Fig. 1a, ■), the early steps of assembly exhibit relatively rapid increases in aggregate weight, while amyloid generation rates after nucleation are relatively slow by comparison. The relatively rapid early aggregation steps before amyloid nucleation appear to take place *via* non-nucleated assembly reactions⁴²⁻⁴⁴.

Thus, these two highly related $A\beta$ peptides assemble into amyloid fibrils by similar mechanistic pathways involving similar intermediates. The markedly slower amyloid growth by $A\beta_{43}$ appears to be primarily due to a delay in amyloid nucleation, from ~ 12 hrs for $A\beta_{42}$ to ~ 45 hrs for $A\beta_{43}$. In contrast, the two peptides exhibit similar kinetics in the subsequent elongation phase leading into homogeneous suspensions of amyloid. Both $A\beta$ species utilized for these comparisons were purified products of solid phase synthesis. Although aggregation kinetics can vary depending on the synthetic source and/or level of purity of the peptides being studied^{19, 45}, the relative aggregation rates of the chemically synthesized $A\beta_{42}$ and $A\beta_{43}$ peptides shown in Figure 1 are qualitatively replicated by the same peptides obtained by recombinant expression (Supplemental Figure 1).

Structural properties of homogenous aggregation reaction products

In addition to weight-normalized ThT signal, the structures of the final fibrils were assessed by EM, FTIR, hydrogen-deuterium exchange, and C_r values⁴⁶. Species instability and sample heterogeneity precluded obtaining interpretable data on most oligomeric intermediates. The exception is the major aggregated $A\beta_{43}$ species co-existing with monomers in the 25-40 hrs time frame. These positively stained, thin filaments were found to possess a substantial amount of β -structure, as evidenced by their weight-normalized ThT signal of 0.6×10^5 fluorescence units per μg aggregate (Fig. 1a).

The final amyloid product of $A\beta_{42}$ incubation consists of typical unbranched, straight fibrils that appear to be composed of two or more strands of individual filaments each about 6 nm

in diameter (Fig. 2g). A typical version of these fibrils consists of two aligned filaments not featuring any twist (Fig. 2g). The C_T value of these fibrils, a measure of stability equal to the concentration of monomer after the reaction has reached equilibrium^{46, 47}, is 0.11 ± 0.01 μM (Table 1). The characteristic weight-normalized ThT value for $\text{A}\beta_{42}$ fibrils is 2.5×10^5 fluorescence units per μg (Fig. 1a).

The final amyloid product of $\text{A}\beta_{43}$ incubation is similar in gross appearance to $\text{A}\beta_{42}$ fibrils, exhibiting structures consisting of bundles of two or more filaments (Fig. 2n). The $\text{A}\beta_{43}$ filaments appear to be somewhat narrower, measuring 4-5 nm in diameter. The C_T value of $\text{A}\beta_{43}$ fibrils is slightly higher (i.e., less stable) than $\text{A}\beta_{42}$ fibrils, at 0.20 ± 0.02 μM (Table 1), and the weight-normalized ThT value is substantially lower, at 1.5×10^5 (Fig. 1a). Like $\text{A}\beta_{42}$ fibrils, $\text{A}\beta_{43}$ fibrils exhibit a dominant β -sheet band in an FTIR comparison that otherwise shows minor, difficult to interpret differences (Supplemental Figure 2).

We used hydrogen-deuterium exchange monitored by mass spectrometry (HX-MS)^{48, 49} to glean information on highly stable H-bonded structure in the β -sheet cores of the mature amyloid fibrils. The number of exchangeable backbone amide hydrogens that remain protected by stable secondary structure even after an overnight exposure to D_2O is characteristic of a particular $\text{A}\beta$ polymorph³⁹. We obtained exchange information linked to both global structure and local structure at peptide C-termini.

Results of the global exchange protection analysis are summarized in Table 2 and are listed in terms of the number of amide protons exchanged. All other things being equal, a smaller number of exchanged protons corresponds to a greater amount of protective secondary structure. Previously we reported that $\text{A}\beta_{40}$ amyloid grown in PBS at 37 °C exhibits exchange of 17.1 amide protons when analyzed by the methods used here³⁹. In contrast, we report here that $\text{A}\beta_{42}$ amyloid fibrils grown in the same way experience exchange of 14.9 protons (Table 1). Considering the presence of two additional amino acids in $\text{A}\beta_{42}$, and hence two additional backbone amide hydrogens potentially capable of exchange, the HX-MS suggests a net gain of approximately 4 amide hydrogens in the $\text{A}\beta_{42}$ β -sheet H-bonding network compared with the $\text{A}\beta_{40}$ fibrils. In contrast, $\text{A}\beta_{43}$ amyloid exhibits net incorporation of 18.2 deuteriums (Table 2). Since the added Ala42-Thr43 amide proton of $\text{A}\beta_{43}$ is very likely not H-bonded in the fibrils (see below), this represents for $\text{A}\beta_{43}$ fibrils a net loss of about 2 of the protective H-bonds that exist in $\text{A}\beta_{42}$ fibrils.

Further insight into the molecular source of these differences in exchange protection comes from an analysis of the deuterium content of the C-terminal pepsin fragments of these $\text{A}\beta$ molecules (cleavage after Leu34) after exchange of the amyloid fibrils. The ESI-MS spectrum of $\text{A}\beta_{35-40}$ cleaved from protonated $\text{A}\beta_{40}$ monomer shows, as expected, a major peak in the $z = +1$ spectrum at 561 Da, plus several small peaks at higher mass/charge due to the presence of natural abundance isotopes (Fig. 4a, blue curve). In contrast, the spectrum of this fragment cleaved from fully deuterated monomer (Fig. 4a, black curve) shows a complex pattern of seven peaks whose centroid is shifted to higher mass/charge by the presence of deuterated backbone amide groups in the fragment. When the same pepsin fragment is isolated from $\text{A}\beta_{40}$ fibrils that were exposed to overnight H/D exchange, the mass spectrum (Fig. 4a, red curve) superimposes almost perfectly with the spectrum of the

fragment from deuterated monomer (black curve). This indicates that the backbone amide groups of this C-terminal fragment are almost completely unprotected against exchange in A β ₄₀ amyloid fibrils, consistent with an absence of highly stable β -sheet involving the A β C-terminus as previously reported by our lab^{50, 51} and other labs⁵²⁻⁵⁴. Thus, of the 5 amide protons in the C-terminal fragment of A β ₄₀ beginning with Met35, only 0.9 are protected (Table 3). This is in very good agreement with a previous analysis of A β ₄₀ fibrils by a completely different in-line proteolysis/exchange method⁵¹ that gave a value of 1.1 protected amides out of the 5 available.

In contrast to this result, the analysis of A β ₄₂ amyloid fibrils shows a pattern in the ESI-MS spectrum of the A β ₃₅₋₄₂ fragment (Fig. 4b, red curve) that is highly similar to the fragment from protonated monomeric A β ₄₂ (Fig. 4b, blue curve), and substantially different from the fragment from deuterated monomeric A β ₄₂ (Fig. 4b, black curve). This indicates that the C-terminus of A β ₄₂ is almost entirely protected (Table 3) by being incorporated into stable β -sheet in the amyloid fibril state, a result that contrasts with the A β ₄₀ result and which is consistent with previous reports^{54, 55}.

Interestingly, analysis of A β ₄₃ amyloid fibrils produces an intermediate result between those for A β ₄₀ and A β ₄₂ fibrils (Fig. 4c). Here, the ESI-MS spectrum of A β ₃₅₋₄₃ released from A β ₄₃ amyloid exposed to exchange (red curve) is significantly shifted to higher mass from the spectrum from protonated monomers (blue), but at the same time is not shifted as much as the spectrum from deuterated monomers (black). Analysis of the centroids of these data shows that of the 8 exchangeable protons in the A β ₃₅₋₄₃ fragment, 5.8 are protected from exchange in the fibril. Although the data do not specify which 2 amide protons are exposed to exchange, it is reasonable to postulate that they are in the last amide group (connecting Ala42 and Thr43) and perhaps the penultimate amide group. This suggests that the added Thr residue not only does not extend the β -sheet containing the C-terminus, it modestly compromises the stability of the β -sheet involving the residues it shares with A β ₄₂.

Previously we reported a C_r value for A β ₄₀ fibrils, grown under the same conditions as employed here, of $\sim 1 \mu\text{M}$ ⁴⁷. As discussed above, the addition of Ile-Ala to make A β ₄₂ decreases this C_r by an order of magnitude, while the further addition of Thr43 to make A β ₄₃ leads to a slight increase in C_r (Table 1). As previously found for a series of A β ₄₀ polymorphs³⁹, the data on A β ₄₀, A β ₄₂ and A β ₄₃ fibrils grown by spontaneous aggregation in PBS at 37 °C without stirring indicate a correlation of increasing stability (that is, decreasing C_r values; Table 1)) with increasing protective backbone H-bonds (Tables 2 and 3).

Mixed peptide aggregation kinetics

A major motivation of this work was to test the hypothesis that A β ₄₃ present in sub-stoichiometric amounts might influence the aggregation properties of A β ₄₂. To test the effect on aggregation kinetics, we tested a mixture of an 8:1 molar ratio of A β ₄₂ to A β ₄₃, chosen to approximate the ratio reported in some AD brain material⁹. Crucially, these reaction mixtures were prepared in such a way that both peptides are entirely monomeric when they first encounter each other in PBS buffer (Methods). We monitored reactions of 10 μM A β ₄₂ and 1.25 μM A β ₄₃, both in mixture and alone, plotting the data as the percent of monomer

remaining at various incubation times (Fig. 1d). As already shown in Figure 1a, the aggregation kinetics of 10 μM $\text{A}\beta_{42}$ ($\circ$) are relatively fast. Consistent with the study shown in Figure 1a, the 1.25 μM $\text{A}\beta_{43}$ reaction (Fig. 1d, $\square$, $t_{1/2} \approx 150$ hrs) aggregates relatively slowly. In contrast, when mixed, $\text{A}\beta_{42}$ and $\text{A}\beta_{43}$ aggregate at essentially the same rate on a percentage basis (Fig. 1d, $\text{A}\beta_{43}$, $\blacksquare$; $\text{A}\beta_{42}$, $\bullet$). Thus, in mixture 10 μM $\text{A}\beta_{42}$ aggregates more slowly than it does in isolation (Fig. 1d, $\circ$), while 1.25 μM $\text{A}\beta_{43}$ aggregates more rapidly than it does in isolation (Fig. 1d, $\square$). Therefore, in contrast to expectations and to the original hypothesis, the presence of a small amount of $\text{A}\beta_{43}$ actually retards spontaneous amyloid assembly by $\text{A}\beta_{42}$, rather than enhancing it. These data are a strong suggestion that these highly sequence-related peptides are somehow interacting in the mixed reaction.

One way in which this implied interaction might occur is by co-assembly into a single mature fibril. Although there is no evidence for this by EM morphology (Fig. 5) or FTIR spectra (Supplemental Fig. 2), other data is consistent with (but do not prove) formation of a mixed fibril. Thus, in the homogeneous assembly reactions, we obtained a C_r for $\text{A}\beta_{42}$ of 0.11 ± 0.01 μM and for $\text{A}\beta_{43}$ a value of 0.20 ± 0.02 μM (Table 1). In contrast, the fibril product of the mixed reaction exhibits essentially identical C_r values for the two peptide components of the fibrils, with a value for $\text{A}\beta_{42}$ of 0.067 ± 0.005 μM and for $\text{A}\beta_{43}$ of 0.075 ± 0.007 μM (Table 1). Thus, co-assembly with $\text{A}\beta_{42}$ has altered the concentration of $\text{A}\beta_{43}$ monomer in equilibrium with fibrils by a factor of about 3-fold compared to the value from homogeneous fibrils. If the mixed reaction product were a mixture of homogeneous $\text{A}\beta_{42}$ and $\text{A}\beta_{43}$ fibrils, we would expect the measured C_r of $\text{A}\beta_{43}$ to be the same whether or not $\text{A}\beta_{42}$ was present.

HX-MS analysis also indicates structural differences for $\text{A}\beta_{43}$ in mature fibrils from homogeneous vs. mixed reactions. We found that the number of deuteriums exchanged into $\text{A}\beta_{42}$ in the fibril product from the mixed reaction (15.3 ± 0.2) is essentially identical to the deuteriums exchanged into homogeneous $\text{A}\beta_{42}$ fibrils (14.9 ± 0.3) (Table 2). In sharp contrast, the number of deuteriums exchanged into $\text{A}\beta_{43}$ in the mixed reaction fibril product (16.5 ± 0.3) is about 2 lower than the amount of exchange into the fibrils from a homogeneous $\text{A}\beta_{43}$ assembly reaction (18.2 ± 0.2) (Table 2). Thus, the HX-MS data for the mixed fibrils (Table 2) mirror exactly the C_r data on the mixed fibrils (Table 1). Interestingly, this global increase in $\text{A}\beta_{43}$ protected structure is not due to any changes in the C-terminus, as shown by segmental exchange analysis (Fig. 4d, Table 3).

Cross-seeding analysis

One of the hypotheses we are testing for how a small amount of $\text{A}\beta_{43}$ in the brain might influence $\text{A}\beta_{42}$ deposition is the suggestion that $\text{A}\beta_{43}$ fibrils form rapidly and then subsequently efficiently seed $\text{A}\beta_{42}$ deposition. In order to be able to observe any influence of seeding on the assembly kinetics of these very rapidly spontaneously aggregating $\text{A}\beta$ peptides, it is necessary to use relatively low (5 μM) concentrations of monomer (to slow spontaneous aggregation) and relatively high levels (10% by weight) of seeds (to drive efficient seeded elongation). We found, as expected, that the initial aggregation rate of monomeric $\text{A}\beta_{42}$ plus $\text{A}\beta_{42}$ fibrils (Fig. 6a, $\bullet$) is greatly enhanced compared with the spontaneous aggregation of monomeric $\text{A}\beta_{42}$ alone (Fig. 6a, $\circ$). Surprisingly, however, we

found that the same level of A β ₄₃ fibrils has very little impact on the aggregation kinetics of 5 μ M A β ₄₂ (Fig. 6a, ■). At the same time, the fibrils from the 8:1 A β ₄₂:A β ₄₃ mixed peptide assembly reaction (Fig. 1d) proved as effective as homogeneous A β ₄₂ fibrils at seeding A β ₄₂ monomer aggregation (Fig. 6a, ▲). These results contrast with the results of mirror image cross-seeding reactions with A β ₄₃ monomer. In this experiment (Fig. 6b), all three amyloid fibrils, including those of A β ₄₂, are equally effective in the seeding of A β ₄₃ monomer aggregation. While the conditions used in these experiments are far from physiological, we expect that seeding of A β ₄₂ monomers by A β ₄₃ fibrils would be even less efficient at physiological concentrations.

Structural (Table 2) and stability (Table 1) analyses of the fibril products of these seeded elongation reactions reveal some interesting subtleties. As expected³⁹, for both A β ₄₂ and A β ₄₃, the fibril products of *self*-seeding are identical in structure to the fibril products of spontaneous assembly. For example, exchanged hydrogens (Table 2) and C_r values (Table 1) for A β ₄₃ are 18.2 hydrogens and 0.20 μ M for spontaneous assembly and 18.3 hydrogens and 0.20 μ M for self-seeded assembly. *Cross*-seeded reaction products exhibit more complex relationships, however. As expected based on the absence of a kinetic effect of A β ₄₃ fibril seeds on A β ₄₂ monomer assembly kinetics (Fig. 6a), we found that the structural parameters for the (ineffectively) A β ₄₃-cross-seeded product are indistinguishable from those of spontaneous A β ₄₂ fibrils (Tables 1 and 2). More interesting are the products of the opposite orientation cross-seeding reaction. In both exchanged hydrogens and C_r values, the fibrils produced when A β ₄₃ monomers are seeded with A β ₄₂ fibrils (15.9 and 0.115, respectively) are substantially different from self-seeded A β ₄₃ fibrils (18.3 and 0.20, respectively). In fact, the parameters are much more like those for spontaneously assembled A β ₄₂ fibrils (14.7, 0.09). As in the case of the A β ₄₃ component of the mixed reaction fibrils, the additional HX protection gained in the A β ₄₃ fibrils obtained by A β ₄₂ seeding appears to not be derived from any change at the C-terminus, since it exhibits the same degree of protection as seen in A β ₄₃ fibrils from spontaneous aggregation of pure A β ₄₃ monomers (Fig. 4d, Table 3). Thus, the fibril product of the seeding of A β ₄₃ monomers with A β ₄₂ fibrils appears to partially replicate A β ₄₂ fibril structure, but not at the C-terminus, where it retains the structural preferences found in homogeneous A β ₄₃ fibrils. That is, the structural features of this polymorph are partially influenced by A β ₄₂ fibril seed structure, and partially by tendencies intrinsic to the A β ₄₃ monomer.

DISCUSSION

While the polypeptide backbone that is common to all proteins plays a key role in organizing the generic amyloid fibril structural motif⁵⁶, side chain contacts play a critical role in stabilizing the fibril core and in determining which sequences readily form amyloid and the stabilities of the fibril products^{57, 58}. This latter discrimination amongst polypeptide sequences extends to cross-seeding. Limited studies suggest that amino acid sequences with only one or two replacements are often capable of supporting amyloid cross-seeding reactions, while more distantly related sequences are poor at cross-seeding^{59, 60}. Sequence changes outside the amyloid core residues generally have less effect on cross-seeding, and this can lead to promiscuous growth features that may have biological implications^{61, 62}. Given such effects, the existence of small amounts of highly sequence related A β processing

variants in the AD brain suggests a hypothesis that they might play an outsized role in aggregation and toxicity by interacting with and thereby influencing the fate of the major plaque peptide A β ₄₂ in the amyloid assembly pathway or in other aggregation processes. In addition, the robustness of cross-seeding and the integrity of structural replication across a series of sequence-related A β peptides are critical underlying assumptions in experiments where seeding is used to amplify biologically derived amyloids for structural studies⁶³.

In this report we probe the feasibility of a hypothesized^{24, 64} aggregation-stimulating role for the A β ₄₃ molecule, a peptide that has been reported to be present in AD neuritic plaques at levels about 10% those of their major A β component, A β ₄₂⁹. The C-terminus of A β has long been recognized as playing a key role in influencing the aggregation process⁶⁵, and the subtleties of this effect in isolation and in isoform mixtures continue to attract great interest^{21, 22, 25-30, 66, 67}. The A β ₄₀ sequence, which ends GGVV, aggregates relatively slowly into amyloid fibrils that exhibit only modestly stability⁴⁷ and an extensive β -structure that stops well-short of the peptide C-terminus, according to most⁵⁰⁻⁵⁴, but not all⁶⁸, studies. The A β ₄₂ sequence, which ends GGIVIA, aggregates much more rapidly into amyloid fibrils that are more stable and whose β -sheet core is reported to extend to the very C-terminus of the peptide^{54, 55}. Since the enhanced amyloidogenicity of A β ₄₂ is linked to a cluster of C-terminal β -branched amino acids with elevated hydrophobicity and/or β -sheet propensity¹⁸, it might be reasonable to expect that the addition of one more β -branched amino acid, Thr, into A β ₄₃ could produce a further gain in amyloidogenicity and fibril stability.

In spite of these structure-based expectations, neither of the previously hypothesized mechanisms for how trace levels of A β ₄₃ might enhance A β ₄₂ amyloidogenicity is supported by our data. Thus, the hypothetical cross-seeding mechanism is not supported, since while A β ₄₃ appears to undergo spontaneous amyloid assembly by the same mechanism as A β ₄₂ (Fig. 2), it does so significantly more slowly (Fig. 1) and, in addition, the resulting A β ₄₃ amyloid fibrils are startlingly poor at seeding A β ₄₂ amyloid formation (Fig. 6a). Likewise, the hypothetical mechanism of a co-assembly reaction with enhanced kinetics is not supported, since the aggregation kinetics of the mixed reaction are actually slower than for A β ₄₂ alone (Fig. 1d).

Previous papers report that A β ₄₃ aggregates as completely¹⁰ or more completely²² than A β ₄₂, and that small amounts of A β ₄₃ either leave unchanged⁶⁹ or enhance²² amyloid formation by either A β ₄₂ alone⁶⁹ or a mixture of A β ₄₂ and A β ₄₀²². There are a number of methodological choices that might lead to discrepancies in results. It is known that both the synthetic source of the A β ⁴⁵ and its purity with respect to synthetic side products¹⁹ can influence aggregation behavior. (In this context, however, it is worth noting that we obtained the same relatively slow A β ₄₃ aggregation for both chemically synthesized (Fig. 1a) and recombinant (Supplemental Fig. 1) peptides.) It is also known that unrecognized aggregated peptide contaminating a monomer preparation can greatly influence aggregation through seeding⁷⁰, and differences in the disaggregation protocols designed to remove these contaminating seeds, such as protocols that leave residual organic solvents (DMSO, HFIP) in the aggregation reaction buffer, might introduce additional variability. Furthermore, storage of disaggregated peptides before use can reintroduce contaminating seeds⁷¹. It also

seems possible that mixed reactions prepared from peptides that have been separately disaggregated before mixing might proceed differently from reactions prepared – as done here – with peptides that have been disaggregated together (Methods). Other methodological choices, such as starting concentration, reaction buffer, pH and temperature might also lead to disproportionate responses in a comparison of related peptides.

In addition to testing the proposed involvement of trace A β ₄₃ levels on A β ₄₂ amyloidogenicity in the brain, our results provide new insights into a surprisingly rich and previously unrecognized variety of pre-fibrillar aggregates in A β assembly. Previous studies have catalogued a number of multimers that wax and wane over the course of amyloid formation by A β peptides. Ion mobility mass spectrometry⁷² and cross-linking analysis⁷³ reveal A β ₄₂ multimers such as 12-mers early in the incubation of disaggregated monomer. Larger spherical oligomers and worm-like “protofibrils” have also long been identified as intermediates in A β ₄₂ amyloid assembly^{66, 74, 75}. While most of these oligomeric intermediates exhibit some ThT fluorescence and/or β -structure⁷⁶, there have also been hints of the existence of early oligomers in A β assembly that lack ThT binding and/or β -structure^{77, 78}.

Our data provide evidence for five distinct pre-fibrillar aggregates along the assembly reaction coordinate for both A β ₄₂ and A β ₄₃ in reactions conducted in PBS under non-stirred conditions at 37 °C. The first observed oligomers are diffuse, poorly staining assemblies (Fig. 2 a,h) that lack ThT fluorescence (Fig. 1b) and hence presumably lack β -structure. The second distinct species to appear is a more condensed, more heavily stained assembly (Fig. 2 c,j) exhibiting significant ThT fluorescence. These transform, possibly by condensation (given their similar weight-normalized ThT intensities), into the third species, which are similarly staining filamentous assemblies (Fig. 2 d,k). The fourth and fifth species to appear are, in contrast, negatively staining spherical oligomers (Fig. 2e) and protofibrils (Fig. 2l) with weight-normalized ThT intensities that are higher than the filaments but lower than mature fibrils. It is not clear which, if any, of these intermediate aggregates are the immediate precursors to amyloid nuclei. This role might be played by one of the β -aggregate intermediates described here, such as the thin, positively staining filaments (Fig. 2 d,k) that accumulate just prior to the first appearance of fibrils, or by the non- β oligomers, for example by an α -helical bundle mediated mechanism⁷⁹. Alternatively, nuclei might be formed from within the monomer pool⁸⁰ or from ephemeral small oligomers^{72, 73} not examined here. Although not the focus of this report, it is also of course interesting to speculate about which of the species catalogued in Figure 2 might play roles in AD-associated toxicity.

In contrast to our expectations for nucleated growth, nucleation of amyloid structure for both A β ₄₂ and A β ₄₃ does not result in a sharp increase in overall aggregation rates (Fig. 1a). Rather, the aggregation steps leading up to the time of amyloid nucleation, that involve formation of various oligomeric intermediates, occur much faster than the subsequent amyloid growth phase. Similar results have been observed before in the nucleation behavior of short polyglutamine peptides containing a nucleation-enhancing flanking sequence that mediates rapid initial formation of a non- β oligomer⁸¹. In such cases, amyloid formation presumably proceeds in spite of slow post-nucleation growth rates because it is far more

thermodynamically favored than the oligomeric intermediates. These counterintuitive kinetics may indeed be quite typical of spontaneous amyloid assembly, but not generally recognized in the literature because of inordinate reliance on the convenient - but β -centric - ThT assay.

The relatively slow aggregation of $A\beta_{43}$ and the inability of its fibrils to efficiently seed amyloid formation by $A\beta_{42}$ are presumably centered in structural differences between these peptides and their aggregates. One dramatic structural difference between fibrils is the state of the peptide C-terminus. We confirmed previous reports⁵⁰⁻⁵⁴ that the C-terminus of $A\beta_{40}$ is not involved in highly stable, protective β -structure in mature amyloid fibrils (Fig. 4, Table 3). We also confirmed reports^{54, 55} that, in contrast to $A\beta_{40}$, the entire C-terminus of $A\beta_{42}$ is involved in highly protective β -sheet network (Fig. 4, Table 3). $A\beta_{43}$ fibrils exhibit their own unique structure involving the peptide C-termini, in which two backbone amide groups – presumably at the extreme C-terminus – are not involved in protective β -structure (Fig. 4, Table 3). This structural perturbation occurs together with the loss of an additional backbone H-bond elsewhere in the $A\beta_{43}$ peptide, for a total exchange increase of about 3 from $A\beta_{42}$ fibrils to $A\beta_{43}$ fibrils (Table 2).

How does Thr43 influence the observed structural differences between $A\beta_{42}$ and $A\beta_{43}$ fibrils, and how might these differences in turn dictate the kinetics effects reported here? Like any protein folding reaction, the favorability of amyloid formation depends on the balance between monomer stability and fibril stability⁴⁷. Although Thr43 is β -branched and therefore has a favorable potential to engage in β -sheet formation, its β -potential is dampened, compared with Ile and Val¹⁸, presumably by the presence of the side chain hydroxyl group. This hydroxyl group can be expected to enhance the ability of the C-terminal Thr side chain to be exposed and solvated, rather than buried in β -sheet. This would explain the decrease in the involvement of the $A\beta_{43}$ C-terminus in protective β -sheet within fibrils (Fig. 4), which in turn is consistent with the modest stability decrease in $A\beta_{43}$ fibrils compared with $A\beta_{42}$ fibrils (Table 1). This preference for the Thr residue to be solvated and resist engaging in packed β -structure may well translate into reduced amyloid nucleation and/or elongation efficiency resulting in the observed decrease in amyloid formation kinetics compared with $A\beta_{42}$.

One of the most surprising aspects of our results is the inability of $A\beta_{43}$ amyloid fibrils to seed $A\beta_{42}$ monomers (Fig. 6a). The favorable seeding of $A\beta_{43}$ monomers by $A\beta_{42}$ fibrils (Fig. 6b) makes the poor seeding of $A\beta_{42}$ monomers by $A\beta_{43}$ fibrils (Fig. 6a) all the more mysterious. Such asymmetric cross-seeding is rare in the amyloid literature, although a similar effect was observed in studies of amyloid formation by certain tau protein derivatives⁸². We assume that the basis of this seeding defect in the experiments reported here must be in a very low efficiency by which $A\beta_{42}$ monomers add productively to the $A\beta_{43}$ fibril ends. This low efficiency may be the result of an inability of monomeric $A\beta_{42}$ C-termini to adapt to or even effectively approach the relatively “frayed ends” (Fig. 4c, Table 3) of the $A\beta_{43}$ C-termini in the amyloid structure. Additionally, the lack of reciprocity in $A\beta_{42}$ - $A\beta_{43}$ cross-seeding may reflect a high degree of pre-organization of incoming monomers in $A\beta$ amyloid elongation, as suggested by recent computational studies⁸³. Thus, even though $A\beta$ monomers are intrinsically disordered, different segments of the peptides

exhibit conformational preferences⁸⁴, and elongating fibrils may select only a subset of such conformations in productive rounds of elongation. If this is the case in A β fibril seeding, there may be a fundamental incompatibility between the apparent lack of structure at the A β ₄₃ C-terminus in the fibrils and some degree of transient structure present in the A β ₄₂ monomer. Clearly, more work is required to understand the molecular basis of asymmetric cross-seeding, which has implications both for fundamental amyloid behavior as well as for the structural rules guiding prion propagation⁸⁵.

Why does a small amount of A β ₄₃ added to A β ₄₂ significantly decrease amyloid assembly kinetics? One attractive possible mechanism is if the two peptides under these conditions were to co-assemble into a common amyloid fibril. In this case, the C-terminal Thr residue would play the same role in reducing aggregation in co-assembly as it does in homogeneous amyloid formation. Our data (Fig. 4, Tables 1 and 2) showing that structural features of A β ₄₃ differ between homogeneous fibrils and fibrils derived from A β ₄₂/A β ₄₃ mixtures are consistent with co-assembly of the two peptides into a single fibril. The change in the kinetics of monomer depletion from separate aggregation reactions to a mixed aggregation reaction (Fig. 1d) is also consistent with co-assembly into a common fibril rather than parallel assembly. All these data are indirect, however, and confirmation that the product consists of a single type of co-assembled fibril must be obtained by additional structural studies, for example by solid state NMR or FTIR.

Interestingly, the effect of the presence of A β ₄₂ on A β ₄₃ structure within fibrils formed from the two peptides aggregating together is quantitatively replicated when A β ₄₂ fibrils seed A β ₄₃ fibril growth. Thus, while A β ₄₃ fibrils from spontaneous growth or from self-seeding exhibit the exchange of about 18 protons, A β ₄₃ fibrils from a reaction efficiently seeded by A β ₄₂ fibrils (Fig. 6b) exchange only about 16 protons (Table 2). At the same time, the C-terminus of the A β ₄₃ fibrils produced by A β ₄₂ fibril seeding is unchanged compared to that of spontaneously aggregated A β ₄₃ fibrils (Table 3). This result has some implications for certain effects in prion biology, since it shows how structural details of the product of a cross-seeding reaction can be derived from a mixture of influences, with some features of the product resembling the structure of the seed, and other features being driven by structural tendencies of the monomer.

While this manuscript was under review, a new paper was published showing similar lack of reciprocal cross-seeding between A β ₄₀ and A β ₄₂, and providing an attractive structural rationale for this effect⁸⁶. The paper suggests that a salt bridge between the α -carboxylate at the A β ₄₂ C-terminus and the Lys28 side chain, that is not structurally feasible for the shorter A β ₄₀, may explain the asymmetric cross-seeding effects between these two peptides. It is likely that, if the overall structure of A β ₄₂ fibrils is retained in A β ₄₃ fibrils, the displacement of the C-terminal α -carboxylate by one residue would move it out of salt-bridging range in the latter. It is not clear that this can account for all the kinetics and structural effects reported here, but the postulated loss of salt-bridge stabilization would be expected, for example, to lead to the observed decreased stability and lost C-terminal β -structure of A β ₄₃ in both homogeneous and mixed fibrils.

While the results described here cast doubt on proposed mechanisms by which low levels of brain A β ₄₃ might impact AD risk and progression, they also reveal important new data on A β -related amyloid phenomena including the presence of an expanded menagerie of non-fibrillar assembly intermediates as well as highly specific sequence effects on amyloid structure and cross-seeding. The latter results may be particularly important in suggesting that one cannot assume that cross-seeding between highly sequence-related peptides will faithfully replicate structural details of the seed amyloid.

METHODS

Materials and General Methods

A β ₄₀, A β ₄₂ and A β ₄₃ peptides were obtained using the Fmoc chemistry from the Keck Biotechnology Center at Yale University. Wild-type A β ₄₀ and A β ₄₂ peptides were obtained pure *via* the “large scale” chemical synthesis and no further purification was done, whereas A β ₄₃ peptide was obtained in the crude state using the “small scale” synthesis option, purified by reverse phase HPLC at 65 °C¹⁹ and lyophilized as described⁸⁷. Purity and identity of the peptides was confirmed by MS. Some experiments with A β ₄₃ were duplicated using recombinant material from rPeptide, Inc. Peptides were disaggregated as described previously⁸⁸ using size exclusion chromatography column (SEC). Briefly, approximately 500 μ g of lyophilized peptide was dissolved in 500 μ L of 8 M guanidine hydrochloride (Gdn.HCl). The peptide suspension was then sonicated in a water bath for 5 mins until it completely dissolved, then injected onto the Superdex 200 GE 10/300 gel filtration column equilibrated and eluted with 1x PBS containing 0.05% sodium azide, on an Agilent 1200 isocratic HPLC system with a flow rate of 0.5 ml/min and taking 1 ml fractions. The A β monomer peak was separated from Gdn.HCl and was collected, analyzed using LC-MS for purity, and pooled. The concentration of the pool was immediately determined by analytical HPLC and diluted into PBS to obtain the desired starting concentrations. For the mixed monomer experiments, the two peptides were mixed in a per weight basis in Gdn.HCl as above, and then injected into the SEC column. All peptides were dissolved in Gdn.HCl at concentrations high enough to compensate for the dilution that occurs during SEC. All kinetics experiments were performed with freshly disaggregated peptide.

Aggregation reactions

Aggregation reactions were incubated at 37 °C under quiescent conditions and samples were withdrawn periodically, with gentle mixing, for analysis by sedimentation/HPLC and ThT as described below. The choice of 10 μ M as starting concentration was based on a compromise between a desire to make the concentration as low as possible while retaining enough peptide to satisfy the mass requirements of our measurements. Relatively low starting concentrations lead to slower kinetics that allow stepwise ensemble assembly reactions that can be conveniently monitored even with somewhat time-consuming assays. The sedimentation assay provides at each time point a measure of the division of the starting monomer into total pelletable aggregates and unreacted monomer. The ThT fluorescence assay provides a measure of total β -aggregates at each time point. Dividing this value by the fraction of starting monomer that has been converted to sedimentable aggregates generates the weight normalized ThT value – plotted in Figure 1 - that provides a measure of the

relative β -content of the total aggregates at that time point. Data points were fit in OriginPro 8.5.1, using a combination of non-linear curve fits and B-spline function, with the sole purpose of aiding visual appreciation of the data trends.

Analytical HPLC for A β concentrations

Routine determination of low A β concentrations, as required for kinetics and C_r determinations, was conducted as described ⁴⁶. Briefly, linear standard curves from 0.1 μ g to 1.0 μ g were determined separately for A β ₄₂ and A β ₄₃ of A₂₁₅ peak area vs. mass of injected peptide in reverse phase HPLC chromatography at 60 °C. These determinations are sensitive to as little as 0.1 μ g of A β . As reactions proceed toward the C_r, larger volume aliquots were injected to ensure that an interpretable peak of at least 0.1 μ g was obtained.

The A β concentrations in the stock solutions used to construct the standard curves were determined from the A₂₁₅ of an aqueous CH₃CN solution of the purified peptide using the method of Kuipers and Gruppen ⁸⁹ to calculate the extinction coefficients from amino acid compositions.

Thioflavin T fluorescence

ThT measurements were performed by transferring an aliquot of the reaction mixture containing 1.0 μ g of total A β at various time points during aggregation into a 4 mm $\times$ 4 mm cuvette containing 15 μ M ThT (Sigma) in 1x PBS (Fisher Biotech BP399-4) as described previously ⁹⁰. The fluorescence was determined using excitation wavelength at 445 nm (5 nM slit width) and emission at 489 nm (10 nm slit width) in a Fluoromax-4 spectrofluorometer (HoribaJobinYvon). To obtain the aggregate weight-normalized ThT values at each kinetics time point, the raw ThT fluorescence unit values corresponding to 1 μ g total peptide in the cuvette were divided by the mole fraction of peptide determined to be aggregated at each time point in the HPLC sedimentation assay.

The very slow (weeks) dissociation kinetics of these aggregates also shows the validity of our ThT protocol, in which aliquots of the ongoing reaction are diluted into buffer containing ThT and fluorescence data accumulated over a few minutes.

Seed preparations

Fibril seeds were prepared from assembly reactions by centrifugation and resuspension, and quantified for aggregate weight concentration by analytical HPLC, as described ⁴⁶. Fibrils were sonicated briefly just before adding to freshly disaggregated monomer obtained from SEC.

Dynamic Light Scattering

Measurements were performed at 37 °C using a DynaPro plate reader (Wyatt Technology corp). An aliquot of ~80 μ L was directly transferred from the ongoing reaction mixture at different time points to a new well of a 384 well microtitre plate to record the scattering data. Data was collected and processed to obtain the size distribution of the various molecular assemblies using the Dynamics V8 software (Wyatt Technology Corp).

Electron Microscopy

Aliquots of the reaction mixtures were taken at various time points and analyzed immediately, without further manipulations, by electron microscopy. A 5 μL sample of each reaction mixture was placed on a freshly glow discharged carbon-coated grids (Electron Microscopy Sciences, Hatfield), adsorbed for 2 min, and the excess sample blotted out with filter paper. The sample was then washed with MilliQ water, stained with 1% (w/v) uranyl acetate for 45 sec and blotted and washed again. Grids were imaged using a Tecnai T12 microscope (FEI Co, Hillsboro, Oregon) operating at 120 Kv and a magnification of 30,000x and equipped with an Ultrascan 1000 CCD camera with post-column magnification of 1.4x.

Fourier transform infrared (FTIR) spectroscopy

A β aggregates were harvested from the end points of the reaction mixture by centrifugation at 14,000 rpm in a bench-top centrifuge (Eppendorf centrifuge 5417R) for 30 min and the pellet was washed with PBS. Pellets were resuspended in PBS at a concentration of ~10 mg/mL and the spectra was obtained by placing the aggregate suspension between two polished CaF_2 windows using a BioCell module (BioTools, Inc) on an ABB Prota-2X MB3000 FTIR instrument. A total of 400 scans were collected with 4cm^{-1} resolution at room temperature and averaged for each sample. Spectra were corrected for residual buffer absorption by subtracting the spectrum of buffer alone interactively until a flat baseline was obtained between 1700 cm^{-1} and 1800 cm^{-1} . Second derivative spectra for the amide I region were calculated from the primary spectrum using PROTA software (BioTools, Inc.).

Backbone amide hydrogen-deuterium exchange (HX) mass spectrometry

Global HX and segmental HX were analyzed using an Agilent 1100 series single quadrupole electrospray ionization mass spectrometer. The analysis for global exchange was done as described previously⁹¹. The data shown here are raw data not corrected for any artifactual exchange. This semi-quantitative raw data can be compared between similar samples (see, for example,³⁹).

To obtain the electrospray ionization HX-MS data of the C-terminal pepsin fragment, we employed a modified digestion protocol using the pepsin-agarose beads (Sigma Aldrich)⁹². Briefly, the aggregates were suspended in D_2O for 24 hrs just like for global exchange analysis. Then the aggregates in D_2O were spun down (14,000 rpm, 30 min) and the pellet was dissolved in 20 μL of ice cold solution of 8M Gdn.HCl + 0.1% formic acid for 10 secs to undergo rapid dissolution. The dissolution and digestion were carried out at 4 °C to slow down artifactual exchange. The sample was quickly vortexed and 100 μL of pepsin agarose in 0.1% cold formic acid was added for 10 secs. The sample was spun down quickly (14,000 rpm, 30 secs) and the supernatant was analyzed by LC-MS at 4 °C to obtain the ESI-MS pattern. As controls, we also performed the C-terminal digestions of fully protonated and fully deuterated monomers.

For the analysis of the C-terminal exchange data, peptide spectra were smoothed using Savitzky Golay method implemented in PeakFit (Systat) to identify the centroid masses of the isotopic envelope. The extent of deuterium incorporation was determined from the shift

in mass of labeled peptides relative to unlabeled peptides. To correct for deuterium back-exchange during proteolysis, HPLC separation and mass spec analysis, data from deuterated peptides was analyzed. The average level of deuterium retention for the C-terminal peptide of fully deuterated sample was around 75%. Although all pepsin digestion products, including the 1-19 and 20-34 fragments of A β , were identified in the LC-MS analysis, severe back exchange leading to deuterium retentions in the 40% range forced us to not further analyze the data from these other fragments. For the C-terminal fragments, the back-exchange-corrected extent of deuterium incorporation into the peptide was calculated as $D = \{[m - m(0\%)] / [m(100\%) - m(0\%)]\} \times N$, where D is the number of deuteriums incorporated for the peptide, m is the observed centroid mass for the experiment, m(0%) and m(100%) are the mass numbers for the non-deuterated and fully deuterated samples, and N is the total number of amide protons⁹³.

Determination of C_r values

Reported C_r values are monomer concentrations obtained from forward fibril assembly reactions after a point where values no longer changed with time. Although A β concentrations can drop substantially after very long incubations of aggregation reactions, vortexing these low apparent C_r reaction mixtures restores the higher equilibrium concentrations seen for the initial plateau⁴⁷. We interpret this as indicating that the much lower values seen at very long reaction times are representative of fibril clustering⁴⁷, so that the peptide concentrations determined after such long incubations are likely to be affected not only by details of individual fibril architecture but also by fibril clustering. To obtain data characteristic of individual fibrils, we determine the C_r at the initial plateau as follows. Reactions were set up in duplicate and aliquots of each reaction were analyzed and averaged at each time point using the analytical HPLC method described above. Spontaneous aggregation reactions were analyzed until two determinations separated by ~ 24 hrs were essentially identical, at which point the last concentration determined was reported as the C_r. For the faster developing seeded elongation reactions, determinations continued until two time points separated by 4-5 hrs were unchanged.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We acknowledge financial support from NIH grants R01 AG018416 and R01 GM099718, and preliminary experiments on the A β ₄₂ system by Indu Kheterpal and David Kaleta. We also gratefully acknowledge comments and suggestions from Seth Horne.

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HIGHLIGHTS

- The minor form A β ₄₃ has been reported to be more toxic and amyloidogenic than A β ₄₂
- However, we find that A β ₄₃ aggregates more slowly than A β ₄₂
- Furthermore, a mixture of A β ₄₃ and A β ₄₂ aggregates more slowly than A β ₄₂ alone
- A β ₄₂ fibrils seed A β ₄₃ monomers to make a new polymorph with features of both
- But A β ₄₃ fibrils don't seed A β ₄₂ monomers so are unlikely to impact plaque burden

Figure 1.

Amyloid assembly kinetics. (a) Spontaneous assembly from ~10 μM solutions of Aβ₄₂ (•, ○) and Aβ₄₃ (■, □) as monitored by loss of monomer from solution (•, ■) and by weight-normalized ThT fluorescence (○, □). (b) Kinetics of formation of ThT-positive assembly intermediates from Aβ₄₂ (•) and Aβ₄₃ (■). (c) Approach of the Aβ₄₃ amyloid-monomer equilibrium position *via* assembly (■) and *via* dissociation (□). (d) Amyloid assembly as monitored by percent monomer loss over time for 10 μM Aβ₄₂ (○, •) and 1.25 μM Aβ₄₃ (□, ■) either alone (○, □) or mixed together (•, ■).

Figure 2.

Electron micrographs of spontaneous self-assembly reactions of A β ₄₂ (a-g) and A β ₄₃ (h-n). a, 1 hr; b, 2 hrs; c, 2 hrs; d, 8 hrs; e, 8 hrs; f, 12 hrs; g, 22 hrs; h, 1 hr; i, 4 hrs; j, 12 hrs; k, 24 hrs; l, 45 hrs; m, 45 hrs; n, 90 hrs. Scale bar = 50 nm.

Figure 3. Dynamic light scattering analysis of amyloid assembly time points. (a, b) $A\beta_{42}$; (c, d) $A\beta_{43}$.

Figure 4.

Backbone H-bonding status of A β C-terminus in mature amyloid fibrils. Shown are the electrospray mass spectra of HPLC-isolated C-terminal peptides from pepsin cleavage of A β ₄₀ (a), A β ₄₂ (b) and A β ₄₃ (c) after Leu34 under the following conditions: protonated monomer (cyan); deuterated monomer (black); deuterated fibrils (red). Also shown is the degree of exchange protection in the C-terminal peptide of A β ₄₃ in mixed A β ₄₂/A β ₄₃ fibrils (dashed red line) and in fibrils made by seeding A β ₄₃ monomer with A β ₄₂ fibrils (solid red line).

Figure 5.

Electron micrographs of mature amyloid fibrils. (a) A β ₄₀; (b) A β ₄₂/A β ₄₃ 8:1 fibrils; (c) A β ₄₂ monomer seeded with A β ₄₂ fibrils; (d) A β ₄₂ monomer seeded with A β ₄₃ fibrils; (e) A β ₄₂ monomer seeded with A β ₄₂/A β ₄₃ fibrils; (f) A β ₄₃ monomer seeded with A β ₄₂ fibrils; (g) A β ₄₃ monomer seeded with A β ₄₃ fibrils; (h) A β ₄₃ monomer seeded with A β ₄₂ A β ₄₃ fibrils. Scale bar = 50 nm.

Figure 6.

Aggregation reaction kinetics of 5 μM monomers of $\text{A}\beta_{42}$ (a) and $\text{A}\beta_{43}$ (b) seeded with 10 % by weight of various amyloid fibrils. Monomer alone (open symbols); $\text{A}\beta_{43}$ seeds (■); $\text{A}\beta_{42}$ seeds (●); $\text{A}\beta_{42}/\text{A}\beta_{43}$ mixed peptide fibril seeds (▲).

Table 1Fibril stability C_r parameters from spontaneous and seeded growth reactions^a

Monomer	A β_{42} seeds	A β_{43} seeds	A β_{42} /A β_{43} seeds	Unseeded
A β_{42}	0.09 $\pm$ 0.01	0.114 $\pm$ 0.007	0.01 $\pm$ 0.01	0.11 $\pm$ 0.01
A β_{43}	0.115 $\pm$ 0.007	0.20 $\pm$ 0.02	0.125 $\pm$ 0.006	0.20 $\pm$ 0.02
A β_{42} /A β_{43}	N. D.	N. D.	N. D.	0.067 $\pm$ 0.005 0.075 $\pm$ 0.007

^aEquilibrium monomer concentrations from forward assembly reactions, in μ M. N.D. = not done.

Table 2Global fibril structural parameters by hydrogen-deuterium exchange^a

Monomer	A β ₄₂ seeds	A β ₄₃ seeds	A β ₄₂ /A β ₄₃ seeds	Unseeded
A β ₄₂	14.7 $\pm$ 0.3	15.1 $\pm$ 0.3	15.0 $\pm$ 0.3	14.9 $\pm$ 0.3
A β ₄₃	15.9 $\pm$ 0.4	18.3 $\pm$ 0.3	15.8 $\pm$ 0.2	18.2 $\pm$ 0.2
A β ₄₂ /A β ₄₃	N.D.	N.D.	N. D.	15.3 $\pm$ 0.2 16.5 $\pm$ 0.3

^a Number of backbone amide hydrogens exchanged with deuterium, uncorrected. N.D.= not done.

Table 3C-terminus HD exchange protection by amyloid structure^a

Amyloid Fibril Component	Amide hydrogens protected	Total amide hydrogens
A β ₄₀ (spontaneous)	0.9	5
A β ₄₂ (spontaneous)	6.7	7
A β ₄₃ (spontaneous)	5.8	8
A β ₄₃ (seeded by A β ₄₂)	5.8	8
A β ₄₃ (in 9:1 A β ₄₂ /A β ₄₃ fibrils)	5.9	8
A β ₄₂ (in 9:1 A β ₄₂ /A β ₄₃ fibrils)	6.7	7

^aData from centroid analysis (see Methods) of MS data shown in Figure 5.