Seeding Specificity in Amyloid Growth Induced by Heterologous Fibrils*

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Over residues 15–36, which comprise the H-bonded core of the amyloid fibrils it forms, the Alzheimer’s disease plaque peptide amyloid β (Aβ) possesses a very similar sequence to that of another short, amyloidogenic peptide, islet amyloid polypeptide (IAPP). Using elongation rates to quantify seeding efficiency, we inquired into the relationship between primary sequence similarity and seeding efficiency between Aβ-(1–40) and amyloid fibrils produced from IAPP as well as other proteins. In both a solution phase and a microtiter plate elongation assay, IAPP fibrils are poor seeds for Aβ-(1–40) elongation, exhibiting weight-normalized efficiencies of only 1–2% compared with Aβ-(1–40) fibrils. Amyloid fibrils of peptides with sequences completely unrelated to Aβ also exhibit poor to negligible seeding ability for Aβ elongation. Fibrils from a number of point mutants of Aβ-(1–40) exhibit intermediate seeding abilities for wild-type Aβ elongation, with differing efficiencies depending on whether or not the mutation is in the amyloid core region. The results suggest that amyloid fibrils from different proteins exhibit structural differences that control seeding efficiencies. Preliminary results also suggest that identical sequences can grow into different conformations of amyloid fibrils as detected by seeding efficiencies. The results have a number of implications for amyloid structure and biology.

One of the major unanswered questions of amyloid fibril formation is the specificity with which primary sequence determines both amyloidogenicity and the details of amyloid structure. The observation that many globular proteins can be induced to generate amyloid fibrils, albeit in some cases only under non-native conditions, has led to the notion that the amyloid core region (3), since all polypeptides regardless of sequence share the polypeptide backbone. Amyloid stability, however, appears to be derived from a combination of forces, including but not limited to H-bonding, much in the manner of how globular proteins are stabilized (4). In addition, mutations associated with modest changes in side chain hydrophobicity, charge, etc., have been observed to have significant impact on fibril growth kinetics and stability (5–10). These considerations raise an interesting conundrum; if side chain packing contributes significantly to amyloid stability, how is it possible that so many proteins whose sequences evolved to fold into stable, globular structures (including many that contain little or no β-sheet structure in the native state) can be equally well accommodated into the β-sheet-rich amyloid folding motif? Does amyloid fibril formation follow the same or different folding rules compared with other collapsed states of polypeptide sequences?

One measure of packing specificity in the amyloid fibril is the efficiency with which fibrils composed of one protein sequence can act as seeds for the elongation of another amyloidogenic polypeptide. The ability of the folded structure at the growth point of the amyloid fibril to serve as a template for the recruitment of an incoming monomer can be likened to the abilities of domains and subdomains of globular proteins to pack together with high complementarity and specificity. Although such “cross-seeding” between two different amyloidogenic proteins has been described (11–14), the data are often qualitative and/or difficult to assess quantitatively due to limitations in our understanding of the amyloid assembly mechanism. For example, it is well known that the addition of a fibril seed can eliminate or reduce the lag time in the spontaneous, nucleation-dependent generation of Aβ fibrils from rigorously disaggregated monomers (15, 16). However, it is not clear how to quantitatively interpret reductions in lag time or to compare one fibril to another with respect to their relative abilities as seeds to reduce lag times. Furthermore, some previous experimental explorations of seeding and cross-seeding may also have been compromised by the use of monomeric protein preparations that contained small but significant amounts of aggregate seeds.

Cross-seeding ability is not only of interest in terms of the structural aspects of amyloid fibril formation. Although the mechanisms by which amyloid fibril formation is initiated in vivo are still being worked out, it is very likely that, once these...
initial seeds are formed, fibrils grow in vitro through elongation by monomer additions (17). The ability of amyloid fibrils to be efficiently elongated in vitro has impact on a number of biological aspects of amyloid function. These include the questions of species and strain specificity in yeast (18, 19) and mammalian (20) prions, the feasibility of the recruitment-sequestration mechanism (21, 22), and the ability of short poly-Gln sequences to influence the nucleation of long poly-Gln repeats in diseases such as Huntington’s disease, the specificity of intracellular protein aggregation (24), and the better understanding of the mechanism of amyloid enhancement factor in the classic mouse amyloidosis model (25). Promiscuous cross-seeding among amyloid fibrils would suggest the possibility that one amyloid disease might influence another. For example, because the vast majority of type II diabetes patients have pancreatic amyloid deposits composed of islet amyloid polypeptide (26) and because IAPP and Aβ share striking sequence similarity (see “Results”), it might be possible that cross-seeding of Aβ amyloid formation by IAPP fibrils is the mechanism by which diabetics appear to exhibit an elevated risk for developing AD (27).

To test this latter hypothesis, we studied the abilities of Aβ and IAPP fibrils to act as seeds for the elongation reactions of Aβ and IAPP monomeric peptides. We used the pseudo-first order elongation kinetics in two separate assay modes as measures of relative seeding ability. To put these results into context we also surveyed a broad spectrum of amyloid fibrils as seeds for Aβ elongation. The results show that in general seeding of fibril elongation is highly specific, being exquisitely sensitive to point mutations at certain positions in the amyloidogenic peptide. IAPP fibrils prove to be very poor seeds for Aβ-(1–40) elongation, suggesting that heterologous seeding of amyloid formation probably does not influence AD risk in diabetics.

**EXPERIMENTAL PROCEDURES**

**Materials—**Synthetic wild-type Aβ-(1–40) and Aβ-(1–42), the “Arctic” mutant (E22G), some proline mutants, and a Cys–1 analog of Aβ-(1–40) were all obtained via custom syntheses from the Keck Biotechnology Center at Yale University. The 1–30 fragment of the immunoglobulin light chain Len and oxidized, full-length, C-terminal-amidated IAPP were also obtained from the Keck Center. Aβ-(1–40) proline mutants F4P, R6P, and L17P were obtained from the Stanford University Protein and Nucleic Acids Biotechnology Facility. Recombinant human immunoglobulin κ light chain variable domain (JTO5) was a gift of Dr. Jonathon Wall (University of Tennessee Graduate School of Medicine), and Aβ-(25–35) was a gift of Charles Murphy (University of Tennessee Graduate School of Medicine). Amyloid-like aggregates of synthetic Glu35 and Glu36 polyglutamine peptides (28) were obtained from Tina Richey (University of Tennessee Graduate School of Medicine). Amyloid fibrils of β2-microglobulin were gifts of Susan Jones and Sheena Radford (University of Leeds). Fibrils of the yeast prion protein Ure2p (29) were gifts of Kimberley Taylor and Reed Wickner (National Institutes of Health). Bovine collagen and bovine serum albumin (essentially fatty acid free) were purchased from Sigma. N-terminal-biotinylated Aβ-(1–40) was prepared by alkylating a Cys–1 analog of Aβ-(1–40) (Keck Biotechnology Center custom synthesis) with PEO (polyethylene oxide)-iodoacetyl biotin (Pierce). All other chemicals were of analytical grade.

**General Methods—**The concentrations of disaggregated peptides were estimated from peak areas of the A225 absorbance trace in analytical HPLC of aliquots of peptide solutions using standard curves generated from peptide standards calibrated by amino acid composition analysis, as described previously (30). Reverse phase HPLC was carried out on a ZORBAX SB-C3 column (Agilent Technologies) with a 1–51% (v/v) acetonitrile gradient in 0.05% aqueous trifluoroacetic acid (Pierce). ThT fluorescence measurements (31) of reaction samples were carried out by diluting aliquots of the reaction into PBS containing 10 μM ThT. Thioflavin T fluorescence was then monitored by excitation at 450 nm and fluorescence emission at 482 nm. Unless otherwise indicated, all quantitative experimental results shown are from measurements done in triplicate. Error bars in the figures represent S.D.

**Preparation of Soluble, Disaggregated Peptides, and Sonicated Fibril Seeds—**Wild-type and mutant Aβ peptides were treated to remove aggregates as described (30, 32). Thus, synthetic lyophilized powder was exposed to sequential applications of trifluoroacetic acid and hexafluoroisopropanol and evaporated under an argon stream, trace volatile solvents were removed under high vacuum, and the peptide residue was dissolved in 2 mM NaOH followed immediately with the addition of a 10× PBS buffer to generate 1× PBS plus 0.05% sodium azide (PBSA). To remove any residual aggregates these solutions were centrifuged (51,500 × g, 17 h, 4 °C), and the upper ½ of the supernatant was carefully removed and analyzed for Aβ concentration by HPLC and as described above (generally about 50%). Solutions were immediately to conduct kinetics experiments (described below) or to make fibrils. Fibrils were made by spontaneous fibril assembly from disaggregated monomeric peptide as described previously (33). For some less stable proline mutants of Aβ-(1–40), fibril formation reactions were conducted at higher concentrations of monomeric peptide (4). Fibril formation reactions were monitored by ThT fluorescence and by analysis of centrifugation supernatants by HPLC.

Human IAPP was solubilized and disaggregated using a 1:1 mixture of trifluoroacetic acid/hexafluoroisopropanol (34) as previously described (33). Disaggregated IAPP (about 30 μg) was used immediately in elongation kinetics assays (described below) or to make fibrils as described previously (33). JTO5 fibrils were prepared as previously described (35). Preparation of reduced and alkylated ovalbumin (ovalbumin-RA) was described previously (33).

To improve seeding potency (11) and potentially normalize fibril size all aggregates were sonicated with a probe sonicator on ice for 2.5 min in 30-s bursts in PBSA containing 1 mM dithiothreitol (a precaution against precipitation; however, we have no evidence of precipitation occurs during sonication in the absence of dithiothreitol). The fact that the series of mutated Aβ fibrils yields results consistent with what is known about Aβ fibril structure (see “Results” and “Discussion”) suggests that the sonication step does produce fibrils with very similar average molecular weights, which can therefore be assumed to have approximately the same number of growth sites per weight of fibril. Recent experimental titration of fibril growth sites further supports this assumption. Initial IAPP aggregates from spontaneous aggregation experiments consist mainly of spheroidal aggregates and smaller amounts of amyloid fibrils and amorphous material (see “Results”). Except for collagen and reduced and alkylated ovalbumin, other aggregates prepared for use as seeds exhibited typical amyloid fibril structure in electron micrographs as well as typical ThT responses (data not shown).

**Solution Phase Aggregation Assays—**Aggregation kinetics studies were conducted using relatively high concentrations of peptide using modifications of the procedure described by Naiki and Nakakuki (36) and Naiki and Gejyo (37). A typical solution phase assay was carried out by sonating (unshaken) a solution of 50–30 μg of freshly disaggregated Aβ or IAPP in PBSA in a water bath at 37 °C. For seeded reactions, solutions were preincubated to reach temperature, then seeded with 10–70 μg/ml aggregate at time 0. Reactions were followed by removing aliquots of gently mixed suspensions and measuring ThT fluorescence. For each time point, an aliquot was removed from each of three independent samples and diluted into PBSA containing ThT. Because the ThT signal varies linearly with fibril mass for any given amyloid fibril, the signal can be used as a measure of the completeness of the fibril formation reaction and, when appropriately plotted, will yield values for the pseudo-first order rate constant (37).

**Solid Phase, Microtiter Plate-Based Aggregation Assays—**Progress curves for polypeptide aggregation were also determined using a solid phase microtiter plate assay modified from previously described methods (38, 39). N-terminal-biotinylated Aβ-(1–40) (see “General Methods” under “Experimental Procedures”) was disaggregated, dissolved at a low micromolar concentration in PBS buffer, separated into aliquots, snap-frozen in liquid nitrogen, and stored at −80 °C. Other aggregates were applied to the microplate wells by incubating 100 μl/well of a 1 μg/ml suspension of aggregate (i.e. 100 ng of aggregate/ well) in PBSA for 18 h at 37 °C in activated high binding microtiter plates (EIA/RIA Plates, Costar, Atlanta, GA). The wells were then washed twice with PBSA containing 0.05% Tween 20 (Fisher) (assy buffer) in the standard manner, centrifuged, and reactions were carried out throughout this experiment. To ensure that aggregation rates are not significantly affected by variable efficiencies of aggregate adhesion to the plastic, we...
confirmed good immobilization by recovering unbound protein from the wells as part of the wash protocol and estimating the amount of non-adsorbed protein using a protein assay (33). In all cases more than 95% of the aggregate remained on the plastic. Wells were blocked with 1% BSA in PBSA (blocking buffer) for 1 h at 37 °C, then 100 μl of assay buffer was added into each well, and the plate was sealed with an adhesive overlay.

For the assay, the microtiter plate was pre-equilibrated at 37 °C. The kinetics data were collected by determining individual time points in reverse temporal order. For each replicate series of each analysis on the plate, a 100-μl aliquot of a 25 nm solution of biotinyl-αβ in assay buffer was added to a well by first removing the assay buffer in which the well was stored. This constitutes the longest time point of the kinetics run. The plate was resealed and returned to 37 °C incubation. At the appropriate time, the plate was again removed, and the storage buffer was removed from the wells designated for this time point and replaced with fresh aliquots of biotinyl-αβ solution. This process was repeated until the last incubation, constituting the earliest time point, was completed. After the last incremental incubation for the kinetics the plate was removed from 37 °C incubation and washed with assay buffer, 100 μl was added per well of a 1:1000 dilution of a europium-streptavidin conjugate (PerkinElmer Life Sciences) in blocking buffer containing 0.05% Tween 20, and the plate was incubated at room temperature for 1 h. The plate was then washed 3 times, and europium was released from the microplate well surface using 100 μl/well of chelation buffer (Enhancement Solution, PerkinElmer Life Sciences). After 10 min europium was measured by time-resolved fluorometry (40) in a Victor2 1420 Multilabel Counter (PerkinElmer Life Sciences) using the programmed parameters for europium. fmol of europium were determined from a standard curve and converted to fmol of biotin using the stated europium content of the commercial streptavidin reagent.

Congo Red Staining and Birefringence of IAPP Aggregates—Congo red staining of aggregates was carried out using a modified version (Sigma: procedure HT60) of the protocol of Puchtel et al. (41). For each experiment 20 μl of freshly prepared IAPP aggregates (about 0.2 mg/ml) were adsorbed to a glass microscope slide overnight at 37 °C and then Congo red-stained. Birefringence was determined with an Olympus microscope (20×) equipped with a polarizing stage.

Electron Micrographs—Aggregates (0.1–0.4 mg/ml) were adsorbed onto carbon and Formvar-coated copper grids and then negatively stained with 0.5% uranyl acetate. Stained samples were examined and photographed in a Hitachi H-800.

RESULTS

Sequence Similarity between αβ and IAPP—The αβ peptide is derived from the proteolytic processing of the cross-membrane region of the receptor-like, type I cross-membrane protein amyloid-β protein precursor (42). IAPP, or amylin, is derived from the proteolytic processing of a propeptide and appears to play a role along with insulin in the regulation of glucose metabolism (43). Despite the absence of a larger sequence homology between their precursor proteins and no obvious relationship in known functions, the mature peptides αβ and IAPP have very similar sequences, as shown in Fig. 1. After introduction of a 1-residue gap in αβ to maximize the alignment, the 2 peptides overall share 25% sequence identity and 50% sequence similarity (Fig. 1). When the overlap region is confined to that portion of the αβ sequence, residues 15–37, known to be involved in the H-bonded core region of the amyloid fibril (4, 44), sequence identity jumps to 39%, and sequence similarity jumps to 65%. Finally, when the amyloid core region of the WT αβ sequence is compared with the sequence of the S20G (αβ position 25) single point mutant of IAPP, associated with both early onset diabetes (45) and enhanced amyloidogenicity (46, 47), the extents of identity and similarity climb further to 43 and 70%, respectively.

This striking similarity introduces the questions of whether these two highly amyloidogenic peptides use their shared sequences in similar ways to engage the amyloid folding motif and the extent to which they might thereby exhibit specificity in amyloid formation as measured by their sensitivities to heterologous seeding. The hypothesis that the shared sequence element is involved in the core structure of amyloid fibrils from both peptides is supported by the sharp reduction in amyloid growth observed when proline residues are substituted within this region in both αβ (4) and IAPP (48, 49). Given these facts it is not unreasonable to suppose that a growing αβ amyloid fibril might be able to relatively easily accommodate IAPP molecules into the fibril structure.

It should be stressed that, although the sequence similarities discussed above are in a structural sense undeniable, we infer no significance to this similarity outside of the context of amyloid formation. That is, it is highly unlikely that these sequences share a common precursor in evolution, and there is no reason to believe that their common sequence elements are associated with any similarity in the normal function of these polypeptides.

Growth of IAPP and αβ Amyloid by Spontaneous, Nucleation-dependent Polymerization and by Homologous Seeding—The spontaneous, nucleation-dependent amyloid formation reaction by IAPP is complicated by the existence of two amyloid-related products. The first-formed product in the unseeded reaction consists of clusters of spheroidal bodies (Fig. 2A) that, despite their non-fibrillar morphologies, exhibit in our hands a number of other amyloid-like features. These include the ability to be labeled with the amyloid-selective (41, 50) dyes Congo red (Fig. 2, G and H) and thioflavin-T (see below). Their structural relationship to the classic amyloid fibril is also suggested by their response to sonication, which generates from these globular aggregates clusters of small fibrils (Fig. 2, E–F). Although related phenomena have been reported previously (51), the exact relationship between the aggregates shown in Fig. 2A and previously described aggregates is not clear, as discussed below.

The kinetics of formation of these initial, globular aggregates from rigorously disaggregated IAPP exhibit a profile similar to that found for many amyloid fibrils, consisting of an initial lag phase followed by a rapid growth phase terminating at a plateau where fibrils and monomers exist at equilibrium (Fig. 3). Like amyloid fibrils, the growth of these globular aggregates can be conveniently monitored by their ability to bind to the dye thioflavin T and alter its fluorescence properties (Fig. 3). Unlike many other amyloid aggregation reactions, however, the deve-
Development of these ThT-positive structures is very aggressive, so that even after a rigorous disaggregation protocol designed to eliminate all traces of seeds from the peptide (see "Experimental Procedures"), the lag phase is only 2 h. Other workers find essentially no lag phase on incubation of IAPP at neutral pH and describe an initial, amorphous product that appears to be somewhat different than the one described here in that it gives a relatively low ThT signal that has been attributed to small amounts of fibrils in the preparation (51); these differences may have to do with differences in disaggregation protocols and/or incubation buffers.

Interestingly, both globular and fibrillar IAPP aggregates are equally effective seeds for IAPP elongation, as judged by ThT kinetics, in that they eliminate the 2-h lag phase of the unseeded reaction when added at 8% by weight of total monomeric IAPP in the reaction (Fig. 3). As judged by the morphologies of the products of elongation, however, different IAPP seeds produce different results. The elongation of IAPP seeded with fibrillar IAPP aggregates (Fig. 2, E–F) produces what appears to be a homogenous fibrillar product (Fig. 4A). In contrast, the elongation of IAPP seeded by spheroidal IAPP aggregates (Fig. 2A) generates a mixture of aggregate types, including some fibrils. Interestingly, some grids in the EM of this aggregated product exhibit structures that give the appearance of amyloid fibrils growing out of the spheroidal aggregate seeds (Fig. 4F), but further studies would be required to confirm this interpretation.

In contrast to IAPP, rigorously disaggregated Aβ-(1–40) incubated at 50 μM in PBS at 37 °C typically exhibit a lag phase of 3–7 days before aggregation begins (16). Seeding such reactions with preformed Aβ amyloid fibrils reduces or eliminates this lag time in a dose-dependent manner (16). Fig. 5 shows that a solution of 30 μM Aβ-(1–40) under these conditions exhibits no indication by ThT fluorescence of fibril formation after 2 days of incubation. However, when an equivalent solution is seeded with 10 μg/ml Aβ-(1–40) fibrils (~8% by weight of the monomeric Aβ-(1–40)), the reaction exhibits rapid fibril growth with no lag phase.

**Heterologous Seeding of Aβ and IAPP Fibril Formation**

Because seeding effects on the length of the lag phase are qualitative, non-linear with dosage and cannot be easily modeled mathematically, we chose to compare homologous and heterologous seeding efficiencies where feasible by comparing the pseudo-first order rates of fibril elongation reactions seeded at levels sufficient to eliminate the lag phase. Such elongation reactions are conceptually simple, with rates dependent on the concentration of monomers, the concentration of growing ends of fibrils, and a second order rate constant characteristic of the

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**FIG. 2. Micrographs of IAPP aggregates.** A–F, electron micrographs of IAPP aggregates from spontaneous growth of unseeded IAPP solutions. A, initial aggregate formed within 6 h. B–D, product of sonication of the initial aggregate. E and F, product of incubation of initial aggregate in PBS at 37 °C for 2 weeks. Where multiple images are shown for the same preparation (B–D), it reflects the range of image types represented among the grids; correspondingly, when only one image is shown for a preparation, only one image type was observed. G and H, polarized light microscopy of Congo red-stained IAPP aggregates. Fresh IAPP aggregates, stained and visualized as described under "Experimental Procedures," before (G) and after (H) sonication.

**FIG. 3. IAPP aggregate growth with and without seeding.** Disaggregated IAPP (~30 μM) was incubated without (○) or with (■ and △) sonicated aggregates (10 μg/ml, or 8% by weight of monomer in solution). A, spheroidal IAPP aggregates as seeds (○); B, fibrillar IAPP aggregates as seeds (■); C, pseudo-first order kinetics plots of seeded elongation data from A and B.
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Amyloidogenic peptide sequence and reaction conditions (37). If secondary nucleation events are negligible under the typically heavily seeded, rapidly completed reactions, the concentration of fibril-growing ends does not change significantly during the course of the reaction, and the reaction appears to be pseudo-first order, with the unchanging concentration of fibril ends incorporated into the pseudo-first order rate constant.

To assess the relative seeding efficacies of various fibrils on IAPP elongation, we added equal weight concentrations (10 μg/ml) of amyloid fibrils of IAPP (i.e. bona fide fibrils from an aged sample; see above), Aβ-(1–40), and Aβ-(1–42) to disaggregated monomeric IAPP. As can be seen in Fig. 6A, all three amyloid fibrils are equally effective at eliminating the normal 2-h lag phase of this reaction. Fig. 6B shows the pseudo-first order rate plot for the growth of amyloid fibrils as assessed by the increase in the thioflavin T signal, and Table I summarizes the rate constants derived from the kinetics analysis. Table I shows that Aβ-(1–40) and Aβ-(1–42) fibrils give essentially identical rate constants in seeding IAPP elongation. Fig. 6 and Table I also show that both Aβ fibrils as well as the initial, globular IAPP aggregates are all quite similar to authentic IAPP fibrils in their abilities to seed IAPP elongation. These data might suggest that the strong sequence similarity between Aβ and IAPP yields fibrils of very similar structure and, hence, seeding properties. However, as will be seen below, such equipotent seeding efficiencies are unusual even for fibrils from peptides differing by only one amino acid and may, therefore, be a special case for IAPP aggregation.

Fig. 5 shows the abilities of various aggregates to seed elongation of Aβ-(1–40). In contrast to the potent ability of Aβ-(1–40) fibrils at 10 μg/ml (7% by weight) to seed Aβ-(1–40) elongation, globular IAPP aggregates (Fig. 2A) are completely inert at stimulating Aβ-(1–40) elongation, even at a concentration of 70 μg/ml (about 50% by weight of the solution phase Aβ). Although perhaps not surprising in isolation given the nonfibrillar morphology of the aggregate, this result was somewhat unexpected based on the potent ability of the same IAPP nonfibrillar aggregates to seed IAPP elongation (Fig. 3A). Fig. 5 also shows that IAPP amyloid fibrils (Fig. 2, E–F) are good seeds of Aβ-(1–40) elongation, albeit much less efficient than are Aβ fibril seeds for IAPP elongation (Fig. 6). The pseudo-first order kinetics plots in Fig. 5B show that a concentration of IAPP fibrils equal to that of Aβ-(1–40) fibrils is much less...
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![Fig. 6. Time course (A) and pseudo-first order kinetics plots (B) of IAPP fibril growth without (C) and with (A, D) seeding. Disaggregated IAPP (30 µM) was seeded with sonicated amyloid fibrils of IAPP (□), Aβ(1–40) (▲), or Aβ(1–42) (●).](image)

**Table I**

| Aggregate as seed | Rate constant | Relative seeding efficiency |
|------------------|---------------|----------------------------|
| IAPP fibrils     | 0.804 ± 0.05  | 100                        |
| IAPP spheroids   | 0.866 ± 0.08  | 120                        |
| Wild-type Aβ(1–40) | 0.940 ± 0.18 | 117                        |
| Wild-type Aβ(1–42) | 1.01 ± 0.05  | 126                        |

Values are from pseudo-first order fits of the data shown in Figs. 3 and 6.

Aggregates from incubation of initial IAPP aggregates for 2 weeks at 37 °C, as described under “Results.”

Initial aggregates from spontaneous IAPP aggregation, as described under “Results.”

Efficient than the Aβ fibrils at supporting elongation; the ratio of these rates at identical weight concentrations of seeding fibrils gives a relative efficiency of IAPP seeds, compared with Aβ(1–40) seeds, of 2.2% (Table II). Although quite low, the Aβ elongation rate for IAPP seeding is clearly above the background when collagen, a fibrous protein exhibiting quite different secondary structure from amyloid fibrils, is used as a seed. As expected, Fig. 5B also shows that seeding efficiency increases as the mass of IAPP fibril seeds increases; although compensated by the non-linearity (see “Discussion”) of the kinetics in the more heavily seeded experiment, the initial rates of these two reactions, which differ 5-fold in seed concentration, differ themselves by about 5-fold, as expected.

These solution phase-seeding experiments indicate a surprising lack of reciprocity in the cross-seeding reactions. Although Aβ(1–40) and both types of IAPP aggregates are essentially equally effective seeds for IAPP elongation, only Aβ(1–40) is an efficient seed for Aβ(1–40) elongation. IAPP fibrils exhibit 2.2% of the seeding efficacy of Aβ fibrils on a weight basis, and the initial IAPP globular aggregates exhibit no detectible seeding ability despite being excellent seeds for IAPP aggregation. This lack of reciprocity cannot be attributed to differences in the average molecular weights of the fibrils used as seeds, since the same fibril preparations were used in all experiments. The key to this lack of reciprocity may be related to the different degrees to which the structures of aggregation products resemble those of the aggregation seeds (Figs. 2 and 4). The tendency of IAPP to form an unusual, initial aggregation product complicates the interpretation of these results and suggests that the apparent lack of reciprocity in seeding may be a special case mechanistically linked to the diverse morphologies of IAPP aggregates.

Important subtleties of the elongation kinetics shown in Fig. 5 are (a) the modest upward curvature in the pseudo-first order plots for Aβ(1–40) elongation seeded by IAPP fibrils, where dramatic upward curvature might have been expected, and (b) the significantly higher ThT value at the plateau of the 50 µg/ml IAPP-seeded reaction versus the Aβ-seeded reaction. These issues will be considered under “Discussion.”

**Seeding Efficiencies Quantified in a Microtiter Plate Elongation Assay**—The solution phase, ThT-based assay described above is robust but relatively material-intensive. We explored the use of a microtiter plate elongation assay, adapted from previously described assays (38, 53), as a convenient alternative for comparing seeding efficiency. In this assay a small amount of fibrils immobilized non-covalently on the microtiter plate well serve as a seeding support for elongation of an N-terminal-biotinylated derivative of Aβ(1–40). The amount of biotin immobilized via Aβ elongation is quantified by time-resolved fluorescence of europium ions introduced via a complex with streptavidin; this assay approach has been well documented in application to polyglutamine aggregation (53). Fig. 7 shows the results of using this assay on immobilized Aβ(1–40) and IAPP fibrils. Panel A shows that although the homologous elongation reaction of Aβ(1–40) is quite efficient, the elongation of solution phase Aβ onto immobilized IAPP fibrils is almost negligible. Panel B shows an expanded scale version of the weak responders from Panel A. It can be seen that despite their low response, IAPP fibrils also give a linear response of fmol of IAPP immobilized per unit time. Remarkably, the relative seeding efficiency of IAPP fibrils, as determined by a comparison of the initial rates of elongation in the microplate assay, is very similar to their relative efficiency in the solution phase ThT assay (1.1% *versus* 2.2%, Table II). Similar good agreement was also obtained for other amyloid fibrils as well as a collagen control to assess the stimulatory ability of non-amyloid fibrous proteins when used as seeds and assayed by both methods (Table II). This good agreement supports the contention that both assays monitor the same fundamental process and suggests that the microplate assay can be used to routinely determine relative seeding efficiencies.

Encouraged by the excellent agreement between these widely different assay approaches, we used the more convenient microplate assay to assess the ability of a wider range of amyloid fibrils to serve as a seeding support for Aβ(1–40) elongation. First, we studied Aβ(1–40) elongation on a series of Aβ mutant fibrils, including amyloid from Aβ(25–35), Aβ(1–42), the E22G Arctic (54) mutant of Aβ(1–40), and a series of single point mutants of Aβ(1–40) substituted with proline. All the fibrils were sonicated before plating on microplate wells, and it was confirmed that >95% of the fibrils stuck to the wells. Table II includes the results of these elongation reactions compared with wild-type Aβ(1–40) fibrils. The results indicate that even single point mutants of an amyloidogenic peptide can produce fibrils that are measurably impaired in their abilities to support elongation by the WT peptide, suggesting a signifi-
The modest seeding ability of the E22G mutant for WT Aβ-(1–40) studies as a surrogate for full-length Aβ-(1–40) titration plate wells by biotinylated Aβ-(1–40) ably controlled by the amyloid conformational preferences of conformation. change its seeding specificity and, hence, presumably its point mutation in an amyloidogenic peptide can substantially elongation is a dramatic demonstration that even a single fold of amyloid fibrils (IAPP amyloid fibrils), and collagen (ovalbumin). In fact, with two exceptions, all of the fibrils tested almost indistinguishable from the background rates seen for the non-amyloid protein aggregates collagen and denatured ovalbumin. In fact, with two exceptions, all of the fibrils tested exhibit seeding efficiencies of about 1% or less compared with that of Aβ-(1–40) fibrils. The exceptions are the amyloid-like aggregates grown from polyglutamine peptides (28) associated with Huntington’s disease and other expanded CAG repeat diseases (55). The ability of these aggregates to do tolerably well as seeds for Aβ-(1–40) elongation contrasts with their lack of sequence homology with Aβ and with the overall lack of complexity in the polyglutamine sequence. We can only surmise that the efficacy of these aggregates to seed Aβ-(1–40) fibrils stems from some coincidental similarity in the details of the folds that the Aβ and polyglutamine peptides assume in forming their respective amyloid fibrils. If this hypothesis is correct, it would suggest that amino acid sequence effects on the specificity of cross-seeding reactions may be mediated by the way that side-chain packing within the fibril controls details of the folding of the polypeptide backbone (rather than by the way that side chains in the fibril directly interact with side chains in the in-coming monomer).

**DISCUSSION**

The initial motivation for these studies was the confluence of the striking sequence similarity between IAPP and Aβ-(1–40)
and a reported relationship between type II diabetes (with its associated pancreatic deposition of IAPP amyloid) and disease risk in AD (27). However, despite the strong sequence similarity between these peptides, we find that IAPP amyloid is a very poor seed for Aβ(1–40) elongation in vitro. It is not clear, however, what level of efficiency in the heterologous seeding of Aβ elongation by IAPP might be significant in terms of an elevated risk of Aβ amyloid plaque formation and AD onset. Although, based on the results presented here, one expects that most cases of heterologous seeding between peptides of dissimilar sequence will be relatively inefficient, it is also true that almost all of the fibrils in the cross-seeding of Aβ elongation in Table III exhibit finite seeding potentials that exceed the background levels of control proteins collagen and denatured albumin. Because the nature of seeded polymerization is that very small initial effects can have devastating consequences over time, it is difficult to gauge whether or not a particular, low cross-seeding efficiency might be potentially significant biologically; we believe that the work described here provides the theoretical framework and experimental tools to begin to address such questions. In any case, the apparent linkage between diabetes and AD may be related to other molecular interactions, such as the potential role for insulin-degrading enzyme in controlling Aβ flux, steady state concentration, and therefore aggregation (56).

Amyloid fibrils grown from purified peptides and proteins in vitro are remarkably similar, in both electron micrographs and x-ray fiber diffraction, to amyloid fibrils extracted from human tissue (57). At the same time, it is well known that amyloid fibrils in tissue contain a number of other molecules besides the major constituent protein. Despite the similar appearance of fibrils grown in the absence of these other molecules besides the major constituent protein. Despite the similar appearance of fibrils grown in vitro, it is possible that amyloid fibrils in tissue are functionally distinct from fibrils grown in vitro from the pure, major constituent protein. This is relevant to the work described here, since we attempt to draw biological conclusions from studies of such artificial fibrils. We think it is justified to do this, since amyloid deposits in authentic disease tissue from both Alzheimer’s disease (38) and Huntington’s disease brains are capable of carrying out elongation reactions in vitro when provided with appropriate monomeric building blocks. At the same time, amyloid fibrils grown in vitro may not be identical in structure and/or function to naturally derived amyloid in some other respects.

The surprising potency of Aβ fibrils as seeds for IAPP aggregation is interesting from two points of view. First, we found no other cases in the survey reported here in which an aggregate of a peptide of less than 98% sequence identity was essentially equipotent with WT fibrils for seeding amyloid growth by WT monomer. Second, the strong seeding by Aβ fibrils represents a case of non-reciprocity in heterologous seeding (although the significance of this observation is uncertain given the plethora of aggregation products observed in IAPP aggregation and the extremely aggressive nature of even the unseeded reaction). Perhaps IAPP is a special case in that, as a very aggressive amyloid forming peptide, it requires a less structurally homologous fibril to initiate polymerization and has a number of avenues available through which it can form amyloid-like products.

Our studies on cross-seeding within the family of Aβ peptides show the sensitivity of seeding efficiency to small sequence changes and suggest a number of biological points. Of those amyloid fibrils compared by the microtiter plate assay listed in Table II, the Aβ fibril with the poorest ability to seed Aβ(1–40) elongation is Aβ-(25–35). It seems very unlikely that this short peptide, which contains within its 11 amino acids only 5 residues likely to exist in H-bonded β-sheet in the fibril and which lacks another major segment involved in β-sheet formation (4), could be capable of making fibrils that share any significant structural or shape relationship to 1–40 fibrils (outside of a generic amyloid fold). In fact, the relative seeding efficiency of these fibrils is about 5% that of WT Aβ(1–40) fibrils (Table II), about the same efficiency as the better seeding fibrils from non-homologous peptides listed in Table III. In contrast, fibrils of biologically relevant Aβ analogs Aβ-(1–42) (59) and Aβ(1–40) E22G (54) exhibit seeding efficiencies in the 20–40% range compared with WT Aβ(1–40). The former is significant since elevated levels of the 1–42 peptide are thought to be more relevant to AD disease risk than high levels of the 1–40 peptide (59). The latter is significant since this peptide, associated with the Arctic familial mutation responsible for early onset AD (54), exhibits similar or even greater specificity with respect to WT Aβ(1–40) in protofibril and fibril formation occurring spontaneously in vitro from 1:1 mixtures of these peptides (60).

The studies reported here on the abilities of amyloid fibrils from various point mutants of Aβ strongly suggest that seeding specificity has less to do with primary sequence similarities and more to do with shape, i.e. the details of the conformation of the amyloid fibril, and in particular of the network of H-bond donors and acceptors at the fibril growing end. Apparently, fibrils from single point mutants of Aβ can differ from WT Aβ in this required shape almost as much as fibrils from unrelated peptides. The proline replacement analogs of Aβ(1–40) studied here were shown elsewhere (4) to fall into two classes, (a) the F4P and R6P mutants, which form fibrils with WT-like stabilities and hydrogen exchange properties, and (b) the L17P, G33P, and L34P mutants, which form fibrils only with much reduced stabilities and which exhibit measurably different hydrogen exchange protection associated with small but significant structural perturbations in their amyloid fibrils. The results in Table II mirror these classes, with F4P and R6P mutant fibrils exhibiting seeding abilities in the range of WT Aβ(1–40) fibrils, while the L17P, G33P, and L34P mutant fibrils exhibit significantly lower seeding efficiencies. In particular, L34P fibrils exhibit a relative seeding efficiency less than 10%, in the same range as a polyglutamine fibril (Table III). Thus, point mutants can make fibrils that are strong or weak at seeding WT Aβ elongation, depending on how much the structure of the mutant fibrils deviates from the structure of WT fibrils.

Aβ-(1–40) elongation

| Aggregate/polymer used as seed | Rate (mol h⁻¹) | Relative seeding efficiency |
|------------------------------|---------------|-----------------------------|
| Aβ-(1–40)                    | 7.50 ± 1.1    | 100                         |
| IAPP                         | 0.086 ± 0.01  | 1.1                         |
| Polyglutamine Gh30            | 0.019 ± 0.001 | 0.25                        |
| Polyglutamine Gln50           | 0.44 ± 0.01   | 5.9                         |
| Ure2p                        | 0.28 ± 0.01   | 3.7                         |
| Polyglutamine Gln20           | 0.069 ± 0.001 | 0.92                        |
| β2 microglobulin              | 0.056 ± 0.002 | 0.56                        |
| Collagen                      | 0.004 ± 0.001 | 0.1                         |
| Ovalbumin-RA                  | 0.009 ± 0.003 | 0.12                        |

Note: Initial rates from elongation of biotinylated Aβ-(1–40) on microplates coated with fibrils.

Mature amyloid fibrils obtained by incubating initial spheroidal aggregates for 2 weeks at 37 °C.
This astounding sensitivity of fibril seeding efficiency to single point mutations suggests that polypeptide chains do not sacrifice their exquisite capabilities for folding specificity when they undergo alternative assembly into amyloid fibrils. In fact, it would appear possible that even protein mutants that fold into very similar structures in the native state may well be capable of folding into functionally distinct and discriminatory amyloid fibrils.

This plasticity in amyloid structure is also suggested by our interpretation of some of the details of the IAPP fibril-seeded Aβ elongation reaction (Fig. 5). From much experience using the ThT assay to monitor amyloid growth, we know that the signal strength at the reaction end point is quite reproducible for Aβ fibrils grown from Aβ seeds. The other unexpected aspect of Fig. 5 is the persistently substantially different, presumably influenced by the nature of the seed. The other unexpected aspect of Fig. 5 is the persistence of the low elongation rate for Aβ elongation seeded by IAPP fibrils. In an elongation of monomer A seeded by fibril B, one might have expected that, as more and more monomer A incorporates into the growing ends of fibril B, the fibril ends will eventually take on the character of A fibrils in all respects, including their intrinsic homologous seeding rate. Thus, in cross-seeded reactions one might have expected to see a nonlinear pseudo-first order kinetics curve, with a slow start followed by a surge to a new, much faster rate identical to that of the homologously seeded reaction. In fact, there is a small amount of upward curvature in the plot in Fig. 5B for Aβ elongation seeded by 50 μg/ml IAPP fibrils; however, the corresponding slope for Aβ elongation seeded by 50 μg/ml Aβ fibrils would be 5 times the slope shown for the Aβ-seeded Aβ elongation in Fig. 5. Clearly the cross-seeded reaction, by the time it reaches completion, has not achieved anything close to this projected rate. This suggests that over the entire cross-seeded elongation reaction, the elongation rate changes little from the initial rate, which was initially established by the unique conformation of the IAPP fibrils (at the same time, the slight but significant upward curvature never observed in homologously seeded reactions) may be consistent with a trend toward such a conformational switch as the heterologously seeded fibrils continue to grow. Thus, both the ThT amplitude and the elongation kinetics suggest that Aβ fibrils propagated from an IAPP seed are conformationally different from Aβ fibrils grown from Aβ seeds.

If confirmed by further experiments, these results from IAPP fibril seeding of Aβ-(1–40) elongation suggest that a single peptide sequence is capable of forming multiple amyloid conformations depending on the seeding fibril and perhaps other variables. Such behavior would be consistent with the recently described ability of certain yeast prion proteins to make amyloid fibrils that exhibit different seeding capabilities depending on the details of how the seeding fibrils are generated (18, 61) and, more broadly, could provide the underlying structural basis for species barriers and strain phenomena in prion infectivity (18–20, 61).

The studies described here reveal unanticipated subtleties in the conformations of amyloid fibrils as measured by their cross-seeding efficiencies. Although the wide ability of unrelated polypeptides to grow into amyloid fibrils might suggest that the forces driving amyloid formation are not very discriminating, our data suggest that amyloid exhibits the same degree of structural specificity as found for the folding of evolved, globular protein structures. We believe that further studies of the kind described here will reveal much about the structures and biological properties of amyloid fibrils.

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Note Added in Proof—An article (Janson, J., Laedtke, T., Parisi, J. E., O’Brien, P., Petersen, R. C., and Butler, P. C. (2004) Diabetes 53, 474–481) recently appeared showing that, while patients with Alzheimer’s disease do not appear to be statistically at risk of developing diabetes, patients with type II diabetes do have an increased risk of developing Alzheimer’s disease. It is interesting, and possibly relevant, that these epidemiological trends appear to be recapitulated by the cross-seeding effects reported here, in which IAPP fibrils are inefficient at seeding Aβ elongation, while Aβ fibrils are very efficient at seeding IAPP elongation. The Aβ peptide is produced throughout the body, and it is conceivable that small amounts of Aβ fibrils might be produced outside the brain, especially in patients with elevated fibril levels in the brain.

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