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The Carboxy Terminus of the β Amyloid Protein Is Critical for the Seeding of Amyloid Formation: Implications for the Pathogenesis of Alzheimer's Disease†

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ABSTRACT: Several variants of the β amyloid protein, differing only at their carboxy terminus (β1–39, β1–40, β1–42, and β1–43), have been identified as the major components of the cerebral amyloid deposits which are characteristic of Alzheimer's disease. Kinetic studies of aggregation by three naturally occurring β protein variants (β1–39, β1–40, β1–42) and four model peptides (β26–39, β26–40, β26–42, β26–43) demonstrate that amyloid formation, like crystallization, is a nucleation-dependent phenomenon. This discovery has practical consequences for studies of the β amyloid protein. The length of the C-terminus is a critical determinant of the rate of amyloid formation ("kinetic solubility") but has only a minor effect on the thermodynamic solubility. Amyloid formation by the kinetically soluble peptides (e.g., β1–39, β1–40, β26–39, β26–40) can be nucleated, or "seeded", by peptides which include the critical C-terminal residues (β1–42, β26–42, β26–43, β34–42). These results suggest that nucleation may be the rate-determining step of in vivo amyloidogenesis and that β1–42 and/or β1–43, rather than β1–40, may be the pathogenic protein(s) in AD.

Aggregation of the β amyloid protein (Figure 1) gives rise to amyloid plaque, which is characteristic of the Alzheimer's disease brain (Kosik, 1992; Yankner & Mesulam, 1991). The observation of lower levels of amyloid plaque in the brains of aged patients who are free of AD[1] symptoms suggests that the accumulation of amyloid plaque is accelerated in AD (Yankner & Mesulam, 1991). Extracellular and cerebrovascular plaque are primarily composed of variants of the 4-kDa β amyloid protein which differ only at their C-terminus (Glenner & Wong, 1984; Joachim et al., 1988; Masters et al., 1985; Miller et al., 1993; Mori et al., 1992; Prelli et al., 1988). Neuritic plaque has been found to contain the variant β1–42 (Glenner & Wong, 1984; Masters et al., 1985; Miller et al., 1993), while cerebrovascular plaque, which is more easily solubilized, reportedly contains β1–39 or β1–40 (Joachim et al., 1988; Miller et al., 1993). A recent study found that β1–40 may be the predominant protein in neuritic plaque, while β1–43 is present as a minor component (Mori et al., 1992). β amyloid protein has been found in the cerebrospinal fluid of AD patients[2] (Shoji et al., 1992) and, in comparable concentration, in age-matched control patients (Seubert et al., 1992; Shoji et al., 1992), as well as in the plasma of healthy individuals (Seubert et al., 1992). These findings suggest that β1–40 is

β protein:	H_2N-^{1}DAEFRHDSGYEVHHQKLVFFAEDVG^{26}SNKGAIIGLMVGGVVIA^{43}-CO_2H
β26-43:	AcHN-SNKGAIIGLMVGGVVIAT-CO_2H
β26-42:	AcHN-SNKGAIIGLMVGGVVIA-CO_2H
β26-40:	AcHN-SNKGAIIGLMVGGVV-CO_2H
β26-39:	AcHN-SNKGAIIGLMVGGV-CO_2H

FIGURE 1: Sequence of the β protein variant β1–43; other variants discussed herein (β1–39, β1–40, and β1–42) involve truncations at the C-terminus. The model peptides are shown below.

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[1] Abbreviations: AD, Alzheimer's disease; DMSO, dimethyl sulfoxide; FTIR, Fourier-transform infrared spectroscopy; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; PDMS, plasma desorption mass spectrometry; RPHPLC, reverse-phase high-performance liquid chromatography; TFMSA, trifluoromethanesulfonic acid; TFA, trifluoroacetic acid.

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normally produced and is soluble under certain conditions (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992). It is important to understand why this protein apparently aggregates so rapidly in AD brain.

The nine amino acid peptide β34–42, corresponding to a portion of the hydrophobic C-terminus of β1–42 (Figure 1), was shown to form extremely stable amyloid fibrils (Halverson et al., 1990). This discovery led to our proposal that this sequence may be involved in the initiation of amyloid formation (Halverson et al., 1990). Studies of synthetic β proteins have confirmed the qualitative differences in the solubility properties of β1–39 and β1–42, which had been originally observed for naturally derived amyloid deposits. In both cases, the higher molecular weight species (β1–42) is reported to be less soluble (Barrow et al., 1993; Barrow & Zagorski, 1991; Burdick et al., 1992). Thus the hydrophobic C-terminal sequence of the β protein seems to be a critical determinant of solubility, although the charged N-terminal sequence may modulate solubility in a pH-dependent manner (Hilbich et al., 1991; Zagorski & Barrow, 1992).

The experimental results presented herein suggest that the C-terminus of the β protein affects the rate of amyloid formation (kinetic solubility) rather than the stability of the amyloid (thermodynamic solubility). We have studied the kinetics and thermodynamics of amyloid formation from both model peptides (β26–39, β26–40, β26–42, and β26–43) and full-length β amyloid protein variants (β1–39, β1–40, and β1–42). We have demonstrated seeding of kinetically soluble variants (β26–39, β26–40, β1–39, and β1–40) with peptide fibrils derived from the kinetically insoluble variants (β26–42, β26–43, and β1–42). These results may have important implications for the pathogenesis of AD. We propose that the relative concentrations of the β protein C-terminal variants, rather than the total β protein concentration, may be a critical determinant of the rate of in vivo amyloidogenesis.

MATERIALS AND METHODS

Synthesis and Purification of Peptides. The peptides β26–39, β26–40, β26–42, and β26–43 were synthesized on the PAM resin using standard (BOC/benzyl) synthetic protocols. Peptide–resin was deprotected with TFMSA/dimethyl sulfide/TFA (1:3:5) at 0 °C for 2 h, and the resin was washed with dichloromethane and dried in vacuo. The peptide was removed from the resin with TFMSA/thioanisole/*m*-cresol/TFA (1:1:1:10) at 0 °C for 1 h, and the supernatant was precipitated and washed with diethyl ether. The peptides β26–39 and β26–40 were dissolved in DMSO and purified by RPHPLC (purity >90% by isocratic elution). The peptides β26–42 and β26–43, due to their insolubility in water, were purified by gel permeation HPLC in hexafluoro-2-propanol (HFIP) (Hendrix et al., 1992). Peptides were analyzed by PDMS and produced the expected parent ion.[3] The purity of each peptide based on PDMS and amino acid analysis was ≥90%. In order to determine peptide ratios, PDMS was calibrated to account for possible differences in ionization efficiencies. Known β26–39/42 and β26–40/43 mixtures (determined by weight and/or amino acid analysis) were analyzed by PDMS. The observed intensities of the respective parent ion peaks reflected the calculated ratio, indicating that these peptides ionized with similar efficiency. Fragments observed in the PDMS spectrum confirmed the sequence of each peptide from the C-terminus to Lys28.

β1–39 was prepared by a fragment coupling route and purified by gel permeation HPLC in HFIP by methods analogous to those reported previously (Hendrix et al., 1992). Amino acid analysis and analytical RPHPLC were consistent with a purity of ≥90%. Analysis by laser desorption MS gave the molecular ion ($M + H^+ = 4229$) and a small amount (ca. 20–30% by MS) of a single peptide impurity (MW = 4172) corresponding to the deletion of a glycine residue from β1–39. β1–40 was purchased from Sigma (80% pure; our RPHPLC and MS analyses were consistent with this estimate). The synthesis of β1–42 was reported previously (Hendrix et al., 1992). However, the first stage of the deprotection procedure was modified (dimethyl sulfide–HF–*p*-cresol, 5:2:1), resulting in elimination of the benzylated impurity; this peptide was used in the kinetic aggregation studies. β1–42 containing the benzylated impurity was used in the seeding experiments (Hendrix et al., 1992).

Thermodynamic Solubility. Peptides (20–200 nmol) were dissolved in DMSO (50 μL) and added to an aqueous buffer (950 μL, 100 mM NaCl and 10 mM NaH_2PO_4, titrated to pH 7.4 with 1 M NaOH; the same buffer was used in all aggregation experiments). The mixture was stirred for at least 3 days (agitation greatly increased the aggregation rate). The aggregated peptide was removed by centrifugation (2150*g* for 5 min), and the supernatant was filtered through Millipore GV 0.22-μm filters. An internal standard (Pro or Arg) was added to the filtrate, and after concentration, the residue was hydrolyzed (6 N HCl, 110 °C, 24 h). Amino acid content and concentration were determined by the Waters Picotag amino acid analysis method. Due to the low solubility of these peptides and the difficulties in removing buffer salt, there was considerable variability in the quantitative amino acid analysis. Therefore, conservative concentration ranges are reported herein.

Kinetic Aggregation Studies. Stock solutions were prepared of each peptide in HFIP, and the concentration was determined by amino acid analysis as described above. Aliquots (20–200 nmol in HFIP) were dried, dissolved in DMSO (50 μL), and added to an aqueous buffer (950 μL). At various intervals, the fibrils were suspended by briefly vortexing each tube, and the turbidity was measured at 400 nm as described previously (Come et al., 1993; Jarrett & Lansbury, 1992). β1–39, β1–40, and β1–42 were followed at an initial concentration of 20 μM (Figure 3A), while β26–39, β26–40, β26–42, and β26–43 were studied at 200 μM (Figure 3B). Turbidity data from two to three identical samples were averaged to provide the final data (error in each data point = 10%).

Seeding Experiments. β1–40 (Figure 4A), β26–39, and β26–40 were seeded with preformed fibrils of β1–42, β26–42, and β26–43, respectively. A solution of β1–42 (20 μM) or β26–42/43 (200 μM) was stirred for 3 days and sonicated for 30 min to produce amyloid fibrils. A portion (10%) of the preformed fibrils (2 nmol of β1–42 or 20 nmol of β26–42/43 in 100 μL) was added to a supersaturated solution (900 μL) of β1–40 (20 μM) or β26–39/40 (200 μM). β26–39 and β26–40 were also seeded in situ with β26–42 and β26–43, respectively (Figure 4B shows β26–40 + β26–43). For example, β26–40 (200 nmol) and β26–43 (20 nmol) were mixed in HFIP. The samples were dried and dissolved in DMSO (50 μL), and the solution was added to buffer (950

[2] Several proteins of ca. 4 kDa, each of which is cross-reactive with antibodies directed toward an epitope at the N-terminus of the β protein, have been identified in cell culture media, CSF, and plasma (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992). One of these was identified as β1–40 by mass spectrometry (Seubert et al., 1992). The other cross-reactive proteins have not been identified.

[3] PDMS: ($M + H^+$) β26–39, 1358.3 (calculated 1358.8); β26–40, 1457.5 (1457.8); β26–42, 1642.0 (1641.9); β26–43, 1743.0 (1743.0).

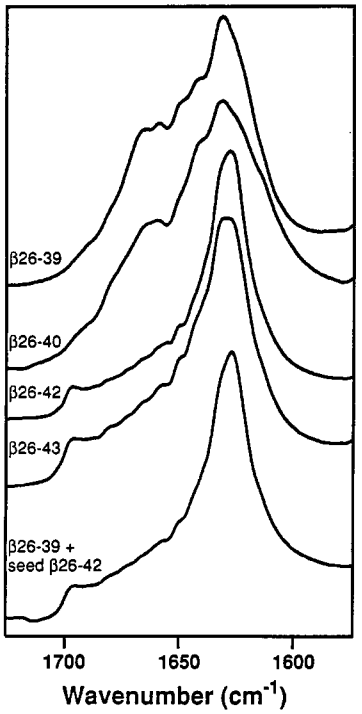

FIGURE 2: FTIR spectra (amide I region only, normalized absorption) of the peptide amyloid fibrils and fibrils grown from a seeding experiment.

μL). In each case, the turbidity was followed as described above.

FTIR Spectroscopy. Amyloid fibrils formed as described above were spread on a CaF_2 plate and dried in vacuo, washed with H_2O, and redried. Spectra were measured on a Perkin-Elmer 1600 series FTIR spectrophotometer and smoothed (Figure 2). Seeded fibrils were made by in situ seeding and collected after 8 h. Fibrils were pelleted by centrifugation, washed with fresh buffer, and repelleted. These fibrils were spread on a CaF_2 plate and treated as above. After spectra were recorded, the sample was scraped off the plate, and the ratio of β26–39 to β26–42 was determined by PDMS.

RESULTS

FTIR Spectroscopy Indicates Subtle Structural Differences between the Model Peptides. The peptides β26–39, β26–40, β26–42, and β26–43, which were designed to model the effects of the C-terminus on amyloidogenesis, formed amyloid fibrils which were visualized by electron microscopy (data not shown). The fibrils were morphologically indistinguishable. The fibrils produced FTIR spectra which were dominated by a low-frequency absorption band (ca. 1630 cm^{-1}), usually assigned as antiparallel β sheet (Figure 2) (Krimm & Bandekar, 1986; Lansbury, 1992). The FTIR spectra of β26–42 and β26–43 were virtually superimposable and clearly different from those of the truncated variants (β26–39 and β26–40), which contained a significant amount of absorption due to non-β structures (1650–1680 cm^{-1}). The structural differences between the two types of fibrils parallel differences in aggregation kinetics which are discussed below. FTIR spectra of amyloid fibrils derived from in situ seeding of β26–39 with β26–42 closely resembled those of pure β26–42 fibrils, rather than β26–39 fibrils (Figure 2). However, PDMS analysis indicates that these fibrils are ca. 70% β26–39.

The C-Terminus Has a Minor Effect on Thermodynamic Solubility. After supersaturated solutions were stirred to drive the aggregation process to equilibrium, the thermodynamic solubilities of model peptides β26–39, β26–40, β26–42, and β26–43 were measured. Comparable values were obtained: β26–39, 5–10 μM; β26–40, 2–7 μM; β26–42, 0.3–1 μM; and β26–43, 1–3 μM. The β protein variants also had similar thermodynamic solubilities: β1–39, 2–5 μM; β1–40, 6–9 μM; and β1–42, 4–15 μM. The ranges indicate the experimental limitations of amino acid analysis.

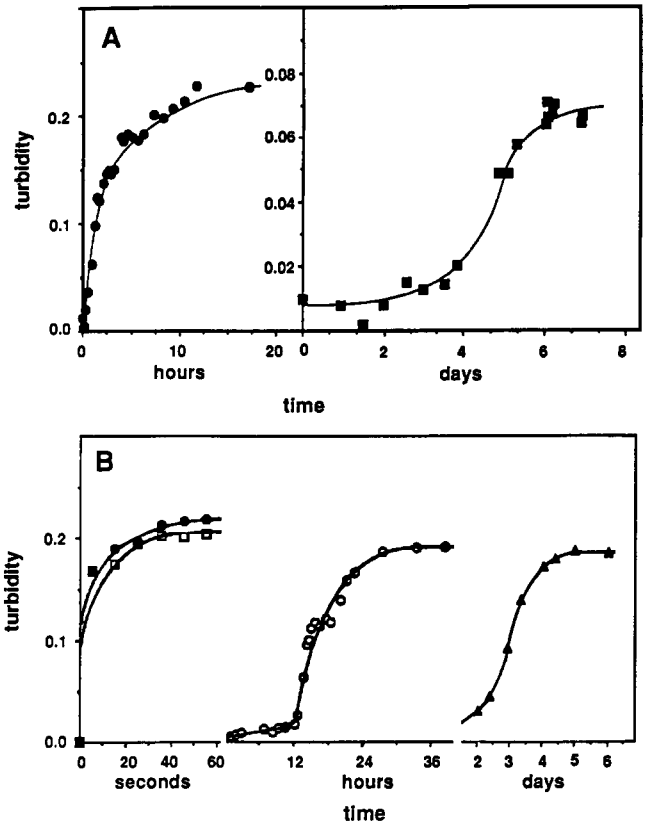

FIGURE 3: Time course of formation of amyloid fibrils from supersaturated solutions of synthetic β1–39 and β1–42 ([protein] = 20 μM, panel A) and the four model peptides ([peptide] = 200 μM, panel B). The observed difference in the final turbidity may reflect differences in fibril association. Panel A shows aggregation of β1–39 (■) vs β1–42 (●). A 20 μM solution of β1–40 showed no increase in turbidity over 8 days in two trials (Figure 4A). Panel B shows β26–39 (▲), β26–40 (○), β26–42 (□), and β26–43 (●). Note the discontinuous and nonlinear *x* axis. Lines were drawn by hand.

The C-Terminus Has a Dramatic Effect on the Kinetic Solubility. Supersaturated solutions of β26–42 and β26–43 (200 μM, ca. 100-fold supersaturated) were unstable, and amyloid formation was instantaneous (Figure 3B). However, supersaturated solutions of β26–40 and β26–39 at equal concentration (ca. 40-fold supersaturated) demonstrated significant kinetic solubility. Amyloid formation by β26–40 and β26–39 was detected after 12 h and 2 days, respectively (Figure 3B). More dilute solutions of β26–42 and β26–43 (40 μM, ca. 20-fold supersaturated) also aggregated immediately while more concentrated solutions of β26–39 (500 μM, ca. 75-fold supersaturated) demonstrated kinetic solubility for 24 h (a 500 μM solution of β26–40 aggregated immediately). The β protein variants β1–39, β1–40, and β1–42 showed a similar correlation between truncation at the C-terminus and kinetic solubility (Figure 3A). A ca. 4-fold supersaturated solution of β1–39 or β1–40 developed no detectable turbidity for several days, while a solution of β1–42 of identical concentration aggregated immediately.

Amyloid Formation Can Be Nucleated by Trace Amounts of the Seed. Addition of β26–42 amyloid fibrils (20 μM) to a supersaturated solution of β26–39 (200 μM) led to immediate

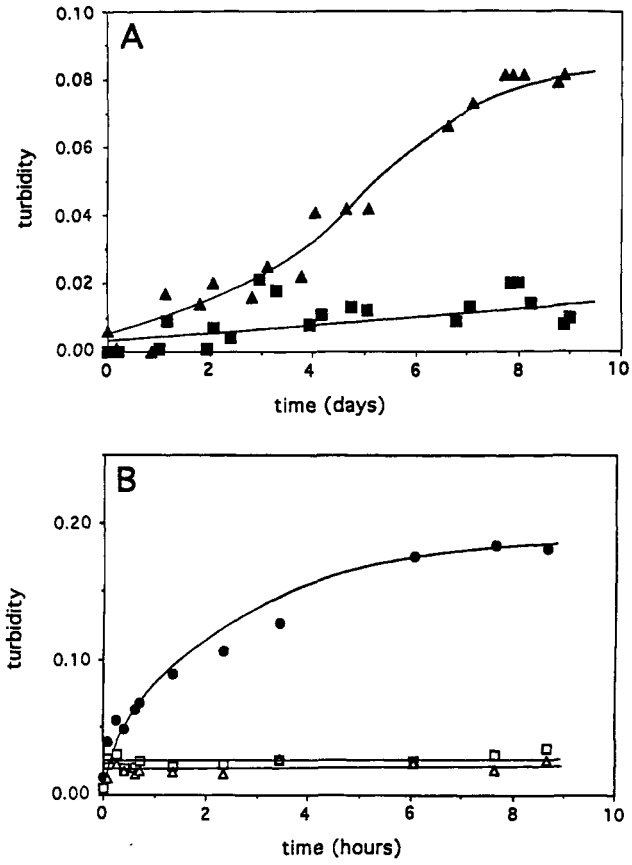

FIGURE 4: Seeding of amyloid formation by the kinetically soluble peptides β1–40 (panel A) and β26–40 (B) with low concentrations of the seed peptides β1–42 (A) and β26–43 (B), respectively (■ = 20 μM β1–40, ▲ = 20 μM β1–40 + ≤2 μM β1–42). The seed concentration of β1–42 is at or below its thermodynamic solubility. In panel B, three samples were compared: the supersaturated solution of the kinetically soluble peptide β26–40 (△, 200 μM), the seed peptide β26–43 at low concentration (□, 20 μM), and a solution containing both (●, [β26–40] = 200 μM and [β26–43] = 20 μM). Lines were drawn by hand.

aggregation, consistent with a nucleation-dependent mechanism. Nucleation of β26–39 (and β26–40) aggregation was also observed on addition of seed fibrils of β26–40, β26–42, β34–42, and β26–43. Similarly, addition of β1–42 fibrils (2 μM, below its measured thermodynamic solubility) to a supersaturated, but kinetically stable solution of β1–40 (20 μM) led to aggregation (Figure 4A), while the control (β1–40 at 20 μM) developed little turbidity over several days. A different version of the seeding experiment produced similar results (Figure 4B). Mixing β26–40 (ca. 91 mol%) and β26–43 (ca. 9 mol%) in DMSO, followed by addition to buffer, allowed in situ generation of seed fibrils (presumably β26–43), which then nucleated aggregation of β26–40. A solution of β26–40 alone did not develop turbidity (Figure 4B). Controls showed that the increased turbidity in the seeded trials was due to β26–40.

DISCUSSION

Amyloid formation, like crystallization, exhibits nucleation-dependent kinetics (Figure 5) (Colon & Kelly, 1992; Come, et al., 1993; Jarrett & Lansbury, 1992). Nucleation-dependent kinetics result from an ordered protein polymerization mechanism and characterize "normal" biological processes such as tubulin and actin polymerization (Lindsey, 1991; Whitesides et al., 1991), as well as "abnormal" processes such as sickle cell hemoglobin polymerization (Hofrichter et al., 1974) and, possibly, prion formation (Come et al., 1993). A kinetic barrier to ordered aggregation exists, which results from the slow and thermodynamically unfavorable assembly of a nucleus [e.g., $(\beta 1\text{–}40)_n$] (Hofrichter et al., 1974; Jarrett & Lansbury, 1992). Before nucleation, a supersaturated solution is metastable, a phenomenon that we call "kinetic solubility". Once the nucleus has formed, growth of the amyloid is fast and thermodynamically favorable. There is no necessary correlation between kinetic solubility of a peptide and its thermodynamic solubility (Come et al., 1993; Jarrett & Lansbury, 1992).

The thermodynamic solubilities of model peptides β26–39, β26–40, β26–42, and β26–43 are within an order of magnitude. Supersaturated solutions of β26–42 and β26–43 were unstable, and amyloid formation was instantaneous. However, supersaturated solutions of β26–40 and β26–39 were kinetically stable. Amyloid formation was detected in these solutions after 12 h and 2 days, respectively (Figure 3B). The β protein variants β1–39, β1–40, and β1–42 also had comparable thermodynamic solubilities and showed a similar correlation between truncation at the C-terminus and kinetic solubility (Figure 3A). A supersaturated solution of β1–39 or β1–40 was stable for days, while a solution of β1–42 of comparable concentration aggregated immediately. Thus, the apparent solubility of β1–39 and β1–40 is due to a kinetic effect. This effect has important ramifications for the experimental manipulation of these proteins. Since the nucleus does not necessarily build up to detectable levels in solution, it may be impossible to distinguish a fresh solution of β1–40 from a solution which is on the verge of aggregating. Agitation of metastable supersaturated solutions could lead to immediate aggregation; therefore, they must be handled with extreme care.

A consequence of the nucleation-dependent aggregation mechanism is that addition of a nucleus or "seed" to a supersaturated solution leads to rapid aggregation by circumventing the need for nucleus formation (Figure 5) (Come et al., 1993; Jarrett & Lansbury, 1992). Growth of a nucleus comprising the insoluble β protein variant β1–42 can be sustained by the kinetically soluble variant β1–40. Coincubation of the kinetically soluble model peptides (β26–39, β26–40) with small amounts of an insoluble peptide (β26–42, β26–43) resulted in immediate aggregation (in situ seeding, Figure 4B). This result suggests that the coproduction of a small amount of β1–42 or β1–43 with the normal amount of β1–40 may be adequate to seed amyloid formation in vivo. Thus, the formation of amyloid plaque may actually be seeded by trace amounts of β1–43 or β1–42 (Miller et al., 1993; Mori et al., 1992). However, the seed, rather than the major protein constituent, may determine the structure of the amyloid. The FTIR spectrum of amyloid fibrils derived from seeding β26–39 with β26–42 closely resembled that of β26–42, rather than β26–39 (Figure 2). The structure induced by seeding may have altered stability as compared to unseeded amyloid.

The structural features of the β amyloid protein C-terminus which play a role in nucleation have not been determined. Studies of the β34–42 peptide amyloid in our laboratory have elucidated a cis amide bond at the Gly37-Gly38 junction (Spencer et al., 1991). This unusual conformation may be required for nucleation of β1–42 amyloid. The structural differences between the β26–39 and β26–42 amyloid fibrils (Figure 2) may derive from the inability of the truncated variant to stabilize this conformation.

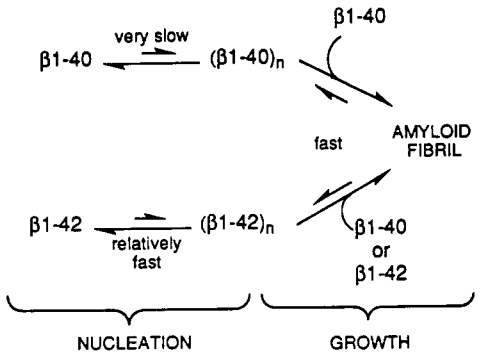

FIGURE 5: Possible scenarios for β amyloid formation in AD, based on a nucleation-dependent mechanism. Amyloid formation is very slow due to the requirement for nucleus [e.g., $(\beta 1\text{–}40)_n$] formation. The "normal" protein β1–40 demonstrates significant kinetic solubility, while β1–42 nucleates much more rapidly (Figure 3). Production of a small amount of β1–42/43 could lead to increased amyloid deposition by seeding amyloid formation (Figure 4). Alternatively, a heterologous seed (lipid, proteoglycan) could act as a template for amyloid formation by β1–40.

RAMIFICATIONS OF THE NUCLEATION-DEPENDENT MECHANISM

The studies detailed herein suggest several explanations for the accelerated in vivo deposition of amyloid which is associated with AD. First, since nucleus formation can be extremely concentration-dependent (Hofrichter et al., 1974), a slightly elevated concentration of β protein could result in a greatly increased rate of amyloid formation (Figure 5).[4] For example, a 6-fold increase in β1–40 concentration (Citron et al., 1992) would result in a 500,000-fold increase in aggregation rate (assuming an octameric nucleus). This difference corresponds to a change in lag time from 100 years to 3 h. A second possibility is that the kinetically soluble variant β1–40 is produced normally (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992) but that, in the AD brain, the insoluble variants β1–42 and/or β1–43 are produced instead (Figure 5). The possibility of altered C-terminal processing has not been investigated. In fact, coproduction of a small amount of an insoluble variant (β1–42 or β1–43) with normal levels of β1–40 may be adequate to seed amyloid formation. Detection of the seed proteins in naturally derived amyloid could be difficult, since they may be present in trace amounts (Mori et al., 1992). Thus, production of the seed β proteins (β1–42, β1–43), rather than production of the kinetically soluble variant β1–40, may be critical for the pathogenesis of AD. The mutations at codon 717 of the amyloid precursor protein which are linked to familial AD (Hardy & Higgins, 1992) may influence the specificity of the proteolytic cleavage which determines the β protein C-terminus. Consequently, future studies of the in vivo β protein distribution in its circulating and insoluble (amyloid) forms should make a point of distinguishing between the C-terminal variants.

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[4] The β protein concentration is not necessarily elevated in the CSF of AD patients (Shoji et al., 1992).

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