The Tottori (D7N) and English (H6R) Familial Alzheimer Disease Mutations Accelerate Aβ Fibril Formation without Increasing Protofibril Formation

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Received for publication, August 28, 2006, and in revised form, November 6, 2006
Published, JBC Papers in Press, December 14, 2006, DOI 10.1074/jbc.M608220200

A subset of Alzheimer disease cases is caused by autosomal dominant mutations in genes encoding the amyloid β-protein precursor or presenilins. Whereas some amyloid β-protein precursor mutations alter its metabolism through effects on Aβ production, the pathogenic effects of those that alter amino acid residues within the Aβ sequence are not fully understood. Here we examined the biophysical effects of two recently described intra-Aβ mutations linked to early-onset familial Alzheimer disease, the D7N Tottori-Japanese and H6R English mutations. Although these mutations do not affect Aβ production, synthetic Aβ(1–42) peptides carrying D7N or H6R substitutions show enhanced fibril formation. In vitro analysis using Aβ(1–40)-based mutant peptides reveal that D7N or H6R mutations do not accelerate the nucleation phase but selectively promote the elongation phase of amyloid fibril formation. Notably, the levels of protofibrils generated from D7N or H6R Aβ were markedly inhibited despite enhanced fibril formation. These N-terminal Aβ mutations may accelerate amyloid fibril formation by a unique mechanism causing structural changes of Aβ peptides, specifically promoting the elongation process of amyloid fibrils without increasing metastable intermediates.

Alzheimer disease (AD) is characterized pathologically by deposition of the amyloid β-protein (Aβ) in the form of amyloid plaques in the brain (1). Aβ is produced through proteolytic cleavage of the amyloid β-protein precursor (APP) through sequential cleavages by two proteases, β- and γ-secretase (2, 3). A subset of AD cases is inherited in an autosomal-dominant manner. Three genes, APP and presenilins 1 and 2, have been linked to familial AD (FAD) (4–6). Missense mutations in presenilin 1, presenilin 2, or APP generally increase the proportion of the 42-residue form of Aβ, Aβ(1–42), relative to the 40-residue form of the peptide, Aβ(1–40). Aβ(1–42) is the species initially deposited in the brain in AD and has been shown to aggregate more readily than Aβ(1–40) (7, 8). A Swedish APP double mutation increases the overall production of Aβ by enhancing β-secretase cleavage of the Aβ N terminus.

In contrast, the pathological phenotypes caused by mutations that alter amino acid residues within the Aβ sequence are variable, and the underlying pathogenetic mechanisms are not fully understood. Recent data support the notion that intra-Aβ amino acid substitutions affect peptide self-association. For example, the Arctic mutation that causes an E22G substitution and early-onset FAD enhances the formation of protofibrils (9) and fibrils of Aβ in vitro (10) and the deposition of Aβ in vivo in the brains of transgenic mice (11, 12). The Dutch (E22Q), Italian (E22K), or Iowa (D23N) mutations, linked to hereditary cerebral hemorrhage with amyloidosis or AD with severe amyloid angiopathy, have been shown to enhance the formation of protofibrils or fibrils from Aβ in vitro (10, 13–15) and produce parenchymal or vascular Aβ deposits in the brains of transgenic mice (16, 17). Taken together, the intra-Aβ mutations appear to enhance the deposition of Aβ by increasing the propensity of Aβ to aggregate.

In vitro studies have revealed that fibril formation of Aβ is a complex process in which nucleation of assembly is the rate-limiting step (18, 19). Following nucleation, rapid monomer addition results in fibril elongation. In addition to classic nucleation-dependent polymerization pathways, a variety of metastable intermediates, including paranuclei, protofibrils, and Aβ-derived diffusible ligands, have been described (13, 20–23). Paranuclei and protofibrils appear to have a precursor-product relationship (22). The product protofibrils are flexible structures of diameter ~5 nm and lengths not exceeding ~150 nm (13, 21). Protofibrils are isolable in the high molecular weight fraction by
size exclusion chromatography (13) and are potent neurotoxins (24, 40).

Two novel mutations that alter amino acid residues in the N-terminal region of Aβ recently were reported in early-onset FAD pedigrees from Tottori-Japan (D7N) (26) and England (H6R) (25). The probands in both of the families were clinically diagnosed as “probable AD.” The ages of onset of two affected sisters in the Tottori kindred were 60 and 65 years (26). The proband in the English family was diagnosed at 55 years of age (25). We hypothesized that the N-terminal region of Aβ around residues His-6 and Asp-7 was a “hot spot” of intra-Aβ mutations that facilitated Aβ deposition and the development of AD. In this study, we studied the aggregation properties of Aβ containing either the D7N or H6R substitution. We found that the substitutions selectively accelerated the elongation phase of Aβ fibril formation. Unexpectedly, the process occurred without significant formation of protofibrils.

**EXPERIMENTAL PROCEDURES**

Peptides and Reagents—Aβ(1–40) and Aβ(1–42) peptides, and analogues containing the H6R, D7N, or E22G amino acid substitutions, were synthesized using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry, purified by reversed-phase high-performance liquid chromatography, and characterized by mass spectroscopy and amino acid analysis as described (27). Peptides were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (Kanto Chemical) at a concentration of 1 mg/ml, dried, and tides were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (Wako Pure Chemical). Ethanol (TFE) was purchased from Wako Pure Chemical.

In Vitro Aβ Fibrillization and Thioflavin T Fluorescence Assays—In vitro Aβ fibrillization assays were performed as described (29). Briefly, synthetic Aβ(1–42) peptides were solubilized at a concentration of 0.1 mg/ml and incubated at 37 °C for the indicated times in a PCR thermal cycler (TaKaRa). For the analysis of the nucleation phase, synthetic Aβ(1–40) peptides were solubilized at 0.3 mg/ml and incubated at 37 °C for the indicated times. For the analysis of the elongation phase, synthetic Aβ(1–40) peptides were solubilized at 0.1 mg/ml in 20% (v/v) TFE and incubated at 37 °C for the indicated times. For analysis of the effects of addition of fibril seeds, Aβ(1–40) peptides were solubilized at 0.1 mg/ml in the presence of prefibrillized Aβ(1–42) that had been fragmented by sonication and incubated at 37 °C for the indicated times. After incubation, 50-μl aliquots were put immediately on ice to prevent further fibril formation, followed by addition of 500 μl of 3 μM thioflavin T (ThT; Tokyo Chemical) in 0.1 M glycine-NaOH (pH 8.5). Fluorescence levels were then assayed using an Hitachi F2500 fluorometer (λ_{ex} = 443 nm and λ_{em} = 484 nm). Placement of samples on ice does not cause structural changes or fibril dissociation, as assessed by ThT fluorescence, because samples maintained on ice for up to 7 days produced constant ThT fluorescence (data not shown).

Evaluation of the Kinetics of Fibril Elongation.—The kinetic properties of Aβ fibril formation can be described by the first-order kinetic model as in Reaction 1,

\[ [P] + [M] \xrightleftharpoons{k_1} [P]\]

where [P] is the number concentration of nuclei, [M] is the concentration of monomeric Aβ, and the constants for polymerization and depolymerization are \( k_1 \) and \( k_{-1} \), respectively (30). When \( F(t) \) represents the concentration of Aβ in the form of fibrils quantitated by ThT fluorescence for the incubation time \( t \), the model can be described as in Equation 1.

\[ F'(t) = k_2 [P][M]_0 - k_{-2} [P]\]  \hspace{1cm} (Eq. 1)

If \([M]_0\) represents the initial Aβ concentration, \([M]\) can be described as in Equation 2.

\[ [M] = [M]_0 - F(t) \]  \hspace{1cm} (Eq. 2)

The insertion of Equation 2 into Equation 1 is then arranged as in Equation 3.

\[ F'(t) = k_2 [P][M]_0 - k_{-2} [P]F(t) \]  \hspace{1cm} (Eq. 3)

When \( t \) is infinity, the reaction reaches an equilibrium between polymerization and depolymerization. Then Equation 4 is introduced,

\[ k_2 [P][M]_0 = k_{-2} [P] \]  \hspace{1cm} (Eq. 4)

giving \([M]_\infty = k_{-2} / k_2\) and if \( b = k_2 [P] \), then Equation 3 can be described as Equation 5.

\[ F'(t) = b([M]_0 - [M]_\infty) - bF(t) \]  \hspace{1cm} (Eq. 5)

where \([M]_0 - [M]_\infty\) gives the value \( F(\infty) \). Then Equation 5 can be arranged as Equation 6.

\[ F'(t) = b F(\infty) - bF(t) \]  \hspace{1cm} (Eq. 6)

Equation 6 can be also rewritten by integration as Equation 7.

\[ \log [F(\infty) - F(t)] = a - bt \quad \text{and} \quad a = \log F(\infty) \]  \hspace{1cm} (Eq. 7)

Following Equation 7, \( \log [F(\infty) - F(t)] \) is approximated by a linear function in the elongation phase. The velocity of fibrillization in the elongation phase thus is determined by the slope of this linear function, \( i.e. \) the value of \( b \). When the concentrations of seeds are equal among samples, \( b \) is also proportional to \( k_2 \) (the constant for polymerization), and then the evaluation of fibril elongation by \( b \) values is justified.

The ThT fluorescence levels of samples are then fit to a sigmoidal curve, and \( F(\infty) \) was calculated by using KaleidaGraph version 3.6 (\( r^2 > 0.95 \)). Using calculated \( F(\infty) \), the data of ThT fluorescence levels were re-plotted in a semi-logarithmic plot, and \( b \) values were estimated by using Microsoft Excel 2004 for Mac (\( r^2 > 0.95 \)).

Isolation and Quantitative Analysis of Protofibrils and LMW Aβ Drugs—To monitor protofibril formation, Aβ(1–42) peptides were dissolved at a concentration of 0.2 mg/ml and incubated for the indicated times at 30 °C. Following incubation, the solution was centrifuged at 17,000 × g for 5 min, and then the supernatants were fractionated by size exclusion chromatography on a Superdex 75 column (GE Healthcare Bio-Sciences) attached to a series 1100 high-performance liquid chromatography (HPLC) system.
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Graph (Hewlett-Packard) at a flow rate of 0.5 ml/min, as described (13). Protophilibrils and LMW Aβ were detected and recovered at elution times of ~16 min and ~27 min, respectively, by UV absorbance at 214 nm.

**Negative Stain Electron Microscopy**—Samples were spread on 400-mesh collodion-coated grids, negatively stained with 2% (w/v) phosphotungstic acid, pH 7.0 (Wako Pure Chemical), and viewed in an electron microscope (JEOL 1200EXII), as described (29). The length and diameter of fibrils were determined by averaging those of 150 fibrils viewed in three independent visual fields.

**DNA Constructs, Cell Culture, Transfection, Immunoblot Analysis, and Quantitation of Aβ**—A cDNA encoding human APP695 was subcloned into a mammalian expression vector pcDNA3 (Invitrogen), as described (8). H6R and D7N mutations were introduced into the human APP695 cDNA by in vitro site-directed mutagenesis using the following oligonucleotides: H6R, 5’- GATGCAGAATTCACGTAATCAGG-3’ (forward) and 5’-GGTGTAGGTGACGCATTCTACGATC-3’ (reverse); D7N, 5’-GCAAGATCCTGGACATAACTCTCGGAATGATTTGG-3’ (forward) and 5’-CATATCCGGAGTTAGTGTGCAGATTCGAC-3’ (reverse). A rabbit polyclonal antibody N1D against the N terminus of Aβ(1–5) was produced by T. C. S., as described (31). Mouse monoclonal antibodies BAN50, BNT77, BA27, and BC05 were generated against synthetic peptides Aβ(1–16), Aβ(11–28), Aβ(1–40), and Aβ(35–43), respectively, as described (32–34). A mouse monoclonal antibody 22C11 was purchased from Chemicon International. SDS-PAGE was performed as described (35). The immunoblots were visualized using Immunostar reagent (Wako Pure Chemical) and LAS-1000 plus (Fujifilm). The expression levels of APP were quantitated by densitometry using Image Gauge (Fujifilm). Mouse N2a neuroblastoma cells were cultured as described (8), and stable transformants were generated using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Expression of transfected proteins was enhanced by treatment with 10 mM butyric acid for 24 h prior to harvesting cells or culture supernatants. Two-site ELISAs that specifically detect the C terminus of Aβ were used as described (8). Culture media were collected after 24-h incubation and subjected to BNT77/BA27 or BNT77/BC05 ELISAs. The levels of secreted Aβ were normalized by the expression levels of APP. ELISA data were statistically analyzed by analysis of variance using StatView-J 4.11.

**RESULTS**

**D7N and H6R Mutant Aβ(1–42) Show Enhanced Fibril Formation in Vitro**—To determine the effects of D7N and H6R substitutions on fibril formation, we used a well characterized assay of β-sheet formation, ThT binding (36, 37). Prior to doing so, we confirmed that equal amounts of fibrils formed by each peptide bound comparable levels of ThT (data not shown). We found that the H6R, D7N, and E22G Aβ(1–42) peptides exhibited accelerated fibril formation compared with wild-type (wt) peptide (Fig. 1).

The Elongation Phase, but Not the Nucleation Phase, of Aβ(1–40) Fibril Formation Is Accelerated by D7N or H6R Substitution—To further determine the effects of D7N and H6R mutations on Aβ fibrillization, we analyzed the nucleation and elongation phases of the process using Aβ(1–40) (Fig. 2A). The rapidity with which Aβ(1–42) assembled precluded similar analyses with this alloform. We evaluated the nucleation rate by monitoring the time lag prior to the rapid rise in ThT fluorescence (38). The E22G peptide formed fibrils most rapidly with no lag phase. In contrast, wt, H6R, and D7N peptides displayed significantly longer lag phases. wt Aβ(1–40) had a lag phase of ~225 h, determined by extrapolation of a line fitted to the linear portion of the ThT binding curve to the baseline of the binding curve (Fig. 2B). Both H6R and D7N peptides had slightly longer lag periods, of ~238 h and ~247 h, respectively (Fig. 2B). To determine if these altered assembly rates correlated with altered fibril morphologies, we performed negative-stain electron microscopy after 338 h. Each of the peptides formed straight fibrils of ~3.5- to 4-nm diameter. No significant difference in the shape was observed (Fig. 2C).

We next studied the effects of the substitutions on fibril elongation. To eliminate fibril nucleation from our consideration of elongation kinetics, we monitored fibril formation in Aβ solutions to which had been added preformed Aβ(1–42) fibril seeds (29). For the Tottori and Arctic peptides, the lag phase was eliminated. For the English and wt peptides, the lag phase was diminished by greater than two orders of magnitude (cf. Figs. 2B and 3A). Data from the linear and plateau regions of the progress curves were incorporated into a first-order kinetic model to determine elongation rates (“b” values; see “Experimental Procedures”) (30), which were 8.94 × 10^-2 h^-1 (wt), 3.30 × 10^-1 h^-1 (H6R), 1.44 × 10^-1 h^-1 (D7N), and 2.62 × 10^-1 h^-1 (E22G) (Fig. 3B). We further examined the rates of fibril elongation by inclusion of 20% (v/v) TFE in the assembly reactions. TFE has been shown to facilitate the conformational conversion of random coil conformers of Aβ into α-helix forms that rapidly assemble into fibrils (29). In the presence of TFE, fibril formation and elongation of Aβ(1–40) were observed immediately. The elongation rates were found to be 2.59 × 10^-2 h^-1 (wt), 5.04 × 10^-2 h^-1 (H6R), 1.07 × 10^-1 h^-1 (D7N), and 2.44 × 10^-1 h^-1 (E22G), again supporting the notion that the
H6R, D7N, and E22G mutations accelerate the elongation phase of Aβ fibril formation (supplemental Figs. S1, A and B).

H6R or D7N Mutant Aβ Exhibit Accelerated Fibril Formation without Accumulation of Protofibrils—During the fibrillogenesis of Aβ in vitro, protofibrillar intermediates form (13, 21). Enhanced protofibril formation has been linked to the Arctic form of AD (9). To determine whether D7N or H6R mutations affect the formation of protofibrils, we monitored Aβ assembly using size exclusion chromatography. Two peaks, one at ~16 min corresponding to protofibrils and one at ~27 min corresponding to low molecular weight (LMW) Aβ, were observed (data not shown). We simultaneously measured the ThT fluorescence of the samples after incubation, but prior to centrifugation (total fraction), and the supernatant after centrifugation (supernatant fraction). A previous report showed that protofibrils bound ThT with slightly lower affinity compared with fibrils (40), indicating that the ThT fluorescence of total fraction represents the sum of protofibrils and fibrils, whereas that of supernatant fraction reflects the amount of protofibrils. Thus, the difference between the ThT levels of total fraction and supernatant fraction accounts for the amount of fibrils.

As reported previously (40), wt Aβ(1–42) rapidly formed protofibrils, which coincided with the decrease in the LMW Aβ peak (Fig. 4A and supplemental Fig. S2A). However, the formation of fibrils from wt Aβ(1–42) was not observed until 240 min (Fig. 4B and supplemental Fig. 2A). Formation of protofibrils from E22G Aβ(1–42) peptide started at 10 min, which was faster than that of wt (Fig. 4A and supplemental Fig. 2D) and was consistent with prior published results (9). In contrast, few protofibrils were formed by H6R or D7N Aβ(1–42) (Fig. 4A and supplemental Fig. 2B, C). Instead, immediate fibril formation was observed (Fig. 4B and supplemental Fig. 2B and C). To confirm these results, we studied the samples after 30-min incubation by negative-stain electron microscopy. In the supernatants of wt or E22G peptides, abundant short and curved fibrils, showing morphology of typical protofibrils, were observed (Fig. 4C). However, the supernatants from H6R or D7N Aβ peptides did not contain any observable protofibrils or fibrils, whereas the pellets contained long, straight fibrils (Fig.

FIGURE 3. The effects of H6R, D7N, and E22G mutations on the elongation phase Aβ fibrillization assayed by ThT fluorescence. A, 0.1 mg/ml of wt (circles), H6R (squares), D7N (triangles), and E22G (diamonds) synthetic Aβ(1–40) peptides were incubated in the presence of preformed seeds from corresponding type of Aβ(1–42) peptides for 0, 2, 4, 6, 8, 10, 12, 22, 34.5, and 47 h, and assayed by ThT fluorescence. The mean values ± S.D. in three independent experiments are shown. B, the semi-logarithmic graph to evaluate the elongation phase.
These data suggest that the H6R and D7N mutations enhance the fibril formation of Aβ without increasing protofibrils, whereas the E22G mutation enhances formation of both protofibrils and fibrils.

Seeds Composed of H6R or D7N Aβ Accelerate the Elongation of Aβ Fibrils More Efficiently Than wt Seeds—Based on our findings that H6R or D7N substitutions do not affect seed formation but do accelerate the elongation phase of Aβ fibril formation, we postulated that seeds made of H6R or D7N Aβ might have higher affinity for their homologous peptide monomers compared with the wt seeds, thereby leading to acceleration in elongation of the Aβ fibrils. To test this idea, we compared the abilities of seeds from wt, H6R, D7N, or E22G Aβ(1–42) to support fibril elongation. The concentration of seeds was adjusted to equal levels using ThT fluorescence in each assay, and the velocity of elongation was evaluated by b value analysis (see “Experimental Procedures”). As observed in Fig. 3A, the progress curves showed some lag periods, which were followed by linear and plateau phases. The b values in the various combinations of seeds/monomers were: 3.39 × 10⁻² h⁻¹ (wt seeds/H6R monomer), 7.71 × 10⁻² h⁻¹ (H6R seeds/H6R monomer) (Fig. 5, A and B), 4.43 × 10⁻² h⁻¹ (wt seeds/D7N monomer), 9.53 × 10⁻² h⁻¹ (D7N seeds/D7N monomer) (Fig. 5, C and D), 2.62 × 10⁻² h⁻¹ (no seeds/E22G monomer), 1.07 × 10⁻¹ h⁻¹ (wt seeds/E22G monomer), and 1.45 × 10⁻¹ h⁻¹ (E22G seeds/E22G monomer) (Fig. 5, E and F), respectively. The b values in the combination of H6R or D7N seeds/wt monomer also were higher than those of wt seeds/wt monomer (wt seed/wt monomer, 9.52 × 10⁻² h⁻¹, H6R seed/wt monomer, 1.30 × 10⁻¹ h⁻¹, D7N seed/wt monomer, 1.72 × 10⁻¹ h⁻¹). Homologous seeds always yielded the largest elongation rates compared with heterologous seeds, supporting the idea that the conformation of the seed determines the elongation rate.

H6R and D7N Mutations Do Not Affect Aβ Secretion from Cultured Cells—Some FAD-linked mutations of APP alter its processing, increasing the total secretion of Aβ or the Aβ42/
Aβ40 ratio (32, 41–43). To examine if H6R and D7N mutations affect Aβ secretion, we stably transfected N2a cells with human APP encoding wt, H6R, or D7N forms of Aβ. We then quantitated the levels of secreted Aβ40 and Aβ42 by ELISA (33). The mutations did not affect the steady-state levels of APP on immunoblots (Fig. 6A). The levels of Aβ secreted from cells expressing H6R or D7N mutant APP normalized by the levels of APP were similar to those from cells expressing wt APP (Fig. 6B), as were the ratio of Aβ42/Aβ40 (11.3%, wt; 12.2%, H6R; 11.5%, D7N), when we used BNT77 that recognizes the mid portion of Aβ as a capture antibody. However, we did not detect the secretion of Aβ(1–40) or Aβ(1–42) from culture media of KM670/671NL+.H6R or KM670/671NL+.D7N transfecants when we used BAN50, which specifically detects the N-terminal region of human Aβ, as a capture antibody (32). In contrast, an A692G (A21G of Aβ) mutation increased the secretion of total Aβ, and T714I and V717I mutations increased the secretion of Aβ(1–42), as previously documented (data not shown) (44). We suspected if the failure of detection of H6R or D7N mutant Aβ by BAN50 might be a result of a change in epitope structure, and tested the reaction of BAN50 and other antibodies with wt and mutant Aβ peptides. On immunoblots, all the Aβ peptides were labeled by N1D and BA27 that are specific for the N or C termini of Aβ, respectively (31) (Fig. 6, D and E). In contrast, H6R and D7N peptides were not labeled by BAN50 (Fig. 6C), and they electrophoresed faster than did wt Aβ (Fig. 6, D and E). Collectively, these data support the view that the H6R and D7N mutations cause conformational changes at the N terminus of Aβ that might be related to the altered aggregation properties of the peptides (as shown above).

**Discussion**

In this study we showed that the two novel FAD-linked APP mutations at the N terminus of Aβ, D7N and H6R, accelerate the fibril elongation rate without a concomitant increase in the levels of protofibrils. The process of Aβ fibril formation can be divided into two phases, involving two energy barrier steps. The initial step is the conversion of monomeric Aβ in a soluble, native state to an aggregate nucleus, which also is termed the nucleation phase and requires the highest activation free energy (ΔG1, in Fig. 7A) among the entire process. Once aggregate nuclei are formed, polymerization of Aβ by incorporation of monomeric Aβ to the nuclei rapidly proceeds, which involves formation of metastable intermediates, e.g. paranuclei, oligomers (22), or protofibrils (13, 21). The entire process after the formation of nuclei and intermediates to the formation of amyloid fibrils is collectively referred to as the elongation phase. The conversion from protofibrils to fibrils also requires an activation free energy (ΔG2, in Fig. 7A). It is not certain which of the activation free energies, ΔG1 or ΔG2, is greater, and the actual process of amyloid formation includes multiple intermediate states. However, the presence of the “nucleation” phase and the formation of protofibrils strongly support the notion that energy barriers do exist between each state. It is difficult to rule out that some of the protein aggregation intermediates studied here may be “off-pathway” for fibril formation. However, it has been shown that Aβ protofibrils are “on-pathway” products (40), lending support to our schematic view shown in Fig. 7.

We initially compared the overall production of amyloid fibrils from Aβ(1–42)-based wt or FAD-linked mutant Aβ peptides and found that all the three mutations tested (E22G, D7N, and H6R) enhanced fibril formation. However, the rapid formation of nuclei from Aβ ending at position 42 (18) hampered further analysis of the target steps enhanced by these mutations. We therefore chose to compare the speed of seed formation by incubating Aβ(1–40)-based wt and mutant peptides and found that D7N and H6R mutations do not accelerate nucleus formation, whereas E22G mutation markedly enhances this process. We then sought to selectively examine the elongation phase by using a method to bypass the nucleation phase, the addition of mechanically disrupted fibrils as seeds. We also performed independent experiments in which 20% (v/v) TFE was included in the reaction mixtures. TFE has been shown to rapidly convert disordered Aβ monomers into
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α-helix-containing structures that rapidly assemble into fibrils (39). We found that E22G, D7N, and H6R substitutions accelerated fibril elongation. We then assessed the levels of protofibrils, which are predicted to increase during fibril formation, by size exclusion chromatography, ThT assay, and electron microscopy, and found that E22G mutant αB formed abundant protofibrils. Surprisingly, D7N and H6R mutants yielded very small steady-state levels of protofibrils compared with wt αB.

We discuss here the different behaviors of mutant αB peptides based on consideration of peptide-specific reaction coordinate diagrams (Fig. 7). As discussed above, αB peptides have to clear two energy barriers, i.e. nucleus formation from monomer (ΔG1) and fibril formation from protofibrils (ΔG2) to form fibrils. The acceleration of both nucleation and elongation in E22G may suggest that this mutation decreases both ΔG1 and ΔG2 and that the energy state of monomer and protofibril forms of E22G αB may be higher than that of wild type. CD spectra analysis of αB intermediates showing that Arctic mutant αB(1–40) increased the rate of α-helix formation relative to their wild type (45) may argue for this view. However, previous findings that E22G mutation selectively enhanced protofibril formation, keeping the rate of fibril formation comparable to that of wild type (9), may suggest an alternative possibility that ΔG2 of E22G αB is increased due to a decrease in the energy state of E22G mutant protofibrils.

In contrast, the selective acceleration of the elongation phase without accumulation of protofibrils by D7N and H6R mutations suggests that these mutations do not change ΔG, but considerably increase the energy state of D7N or H6R mutant protofibrils, causing a significant reduction in ΔG2. This may lead to production of a limited amount of nuclei (at similar levels to wt) followed by facilitation of the elongation process, which collectively leads to enhanced fibril formation without accumulation of protofibrils. In that case, D7N or H6R mutant protofibrils are formed but readily converted to fibrils, and the steady-state levels of the protofibrils are kept very low.

Bitan et al. (20, 22) examined the oligomer/paranuclei formation in the LMW αB fraction (that are smaller in size compared with protofibrils) using the photo-induced cross-linking of unmodified proteins technique and showed that E22G mutant αB(1–40) exhibits an oligomer size distribution extending to a relatively high order, whereas D7N mutant αB(1–40) displays similar distribution to wt αB(1–40) (46). This shows that oligomer formation of αB is facilitated by E22G but not by D7N mutation and suggests that D7N mutation may specifically affect the later phase of elongation, i.e. conversion of protofibrils to fibrils.

The selective acceleration of elongation may suggest that D7N and H6R mutations facilitate the self-interaction of αB peptides to pre-existing αB fibrils. What, then, are the possible mechanisms and the subdomains involved in this process? NMR studies have shown that residues 12–24 and 30–40 of αB(1–40) or residues 18–26 and 31–42 of αB(1–42) adopt a β-strand conformation and form parallel β-sheet structures through intermolecular side-chain contacts, and that Asp-23 and Lys-28 are important for the intra- and intermolecular interactions by forming a salt bridge, whereas the N-terminal region, i.e. residues 1–8 of αB(1–40) or 1–17 of αB(1–42), are disordered (47–49). These observations suggest that the disordered N-terminal region harboring His-6 and Asp-7 plays a different role from β-strand formation in amyloid fibril formation. An observation in yeast prion Sup35, that a glutamine/aspargine-rich N-terminal head domain, apart from the amyloid core region, plays an essential role in the conversion to prion state or amyloid fibrils through intermolecular contact in a head-to-head fashion (50), leads us to speculate that αB may also require the N-terminal domain for the amyloid fibril elongation through intermolecular association, along with the β-strand formation via 12–40 or 18–42. Previously Pike et al. (51) reported that N-terminal-deleted αB, such as αB(8–42) or αB(12–42), aggregated faster than wt αB(1–42) in vitro. However, they did not analyze the process of fibril elongation but only examined the extent of fibril formation, although it is still possible that the removal of the N-terminal domain causes acceleration of fibril formation as well. Our observation that the elongation of amyloid fibrils took place most efficiently in the mutant seeds, compared with the wt seed, and that the D7N or H6R mutations caused considerable changes in the conformation of αB N terminus, also suggest a mechanism of αB polymerization mediated by its N terminus, which is accelerated by these mutations.

In sum, we showed that the novel D7N and H6R mutations of αB linked to familial AD facilitate amyloid fibril formation by selectively accelerating the elongation phase, without accumulation of protofibrils. Notably, our observation may apparently contradict with the currently prevailing notion that a sustained high level of metastable intermediate forms of αB, e.g. protofibrils that are believed to be the more toxic αB species, is directly involved in the pathogenesis of AD. Further studies, including structural modeling *in vitro*, generation of transgenic mice, and especially careful postmortem examination of the affected individuals, will clarify the pathogenic significance of these mutations, as well as the roles of protofibrils and αB N-terminal portion in αB fibrillization and AD.

Acknowledgments—We thank H. Naiki for kind instruction on the αB aggregation assays and members of the laboratory for kind suggestions and discussions.

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