Analysis of the Secondary Structure of β-Amyloid (Aβ42) Fibrils by Systematic Proline Replacement*

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Amyloid fibrils in Alzheimer’s disease mainly consist of 40- and 42-mer β-amyloid peptides (Aβ40 and Aβ42) that exhibit aggregative ability and neurotoxicity. Although the aggregates of Aβ peptides are rich in intermolecular β-sheet, the precise secondary structure of Aβ in the aggregates remains unclear. To identify the amino acid residues involved in the β-sheet formation, 34 proline-substituted mutants of Aβ42 were synthesized and their aggregative ability and neurotoxicity on PC12 cells were examined. Prolines are rarely present in β-sheet, whereas they are easily accommodated in β-turn as a Pro-X corner. Among the mutants at positions 15–32, only E22P-Aβ42 extensively aggregated with stronger neurotoxicity than wild-type Aβ42, suggesting that the residues at positions 15–21 and 24–32 are involved in the β-sheet and that the turn at positions 22 and 23 plays a crucial role in the aggregation and neurotoxicity of Aβ42. The C-terminal proline mutants (A42P-, I41P-, and V40P-Aβ42) hardly aggregated with extremely weak cytotoxicity, whereas the C-terminal threonine mutants (A42T- and I41T-Aβ42) aggregated potently with significant cytotoxicity. These results indicate that the hydrophobicity of the C-terminal two residues of Aβ42 is not related to its aggregative ability and neurotoxicity, rather the C-terminal three residues adopt the β-sheet. These results demonstrate well the large difference in aggregative ability and neurotoxicity between Aβ42 and Aβ40. In contrast, the proline mutants at the N-terminal 13 residues showed potent aggregative ability and neurotoxicity similar to those of wild-type Aβ42. The identification of the β-sheet region of Aβ42 is a basis for designing new aggregation inhibitors of Aβ peptides.

Alzheimer’s disease (AD) is neuropathologically characterized by the progressive deposition of amyloid fibrils in the brain parenchyma and cortical blood vessels (1). This deposition mainly consists of 40- and 42-mer peptides (Aβ40 and Aβ42) generated from amyloid precursor protein by two proteases, β- and γ-secretase (2, 3). Aβ42 plays a pivotal role in the pathogenesis of AD, because the aggregative ability and neurotoxicity of Aβ42 are considerably higher than those of Aβ40 (4). Because the aggregative ability of Aβ peptides is closely related to the neurotoxicity, precise structural information for amyloid fibrils is indispensable for understanding the molecular mechanisms of AD and related folding diseases and for developing new medicinal leads using the inhibitory activity of amyloid fibril formation.

Previous studies on Aβ fibrils showed that Aβ aggregates mainly consist of intermolecular parallel β-sheet (5–10). However, the technical barriers to using x-ray crystallography or solution NMR have hampered its structural determination in high resolution (10). Solid-state NMR spectroscopy is a fairly reliable approach to elucidating the structure of amyloid fibrils. In fact, solid-state NMR analysis on the Aβ40 aggregates has been reported recently (7, 9). However, there are few reports on the structure of Aβ42 aggregates that are more important in AD, possibly because efficient synthesis of Aβ42 with 14 hydrophobic and bulky amino acid residues at the C terminus is quite difficult (11). The weak point of solid-state NMR is that it requires a series of 13C- and/or 15N-labeled Aβ peptides at different positions in large quantity (20–30 mg).

Systematic replacement with proline in peptides is a reliable and rapid method for predicting the secondary structure, especially β-sheet and turn (12). Prolines are rarely present in β-sheet, whereas they are easily accommodated in a variety of turns, for example, as a Pro-X corner (where X is a variable amino acid residue) (13). Quite recently, Williams et al. (14) have investigated a systematic proline replacement of Aβ40, showing that the residues 15–21, 24–28, and 31–36 are likely to include the β-sheet portions of the fibril and that the residues at positions 22, 23, 29, and 30 probably occupy turn positions among these β-sheet elements. This conclusion does not contradict our preliminary investigation using proline-substituted Aβ42 at positions 19–26 (15). However, such systematic proline replacement should be carried out with Aβ42, the aggregative ability and neurotoxicity of which are especially high. DMF, N,N-dimethylformamide; HATU, N-(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide; HPLC, high pressure liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MTt, 3-(4,5-diethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; Th-T, thioflavin-T; Fmoc, N-(9-fluorenyl)methoxycarbonyl.

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‡ The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid.
high (4). Our continuous efforts to synthesize a series of Aβ42 derivatives with proline replacement (Fig. 1) led to the proposal of a new structural model of Aβ42 aggregates. This is a full report on the aggregative ability and neurotoxicity of a series of proline-substituted Aβ42 mutants.

**EXPERIMENTAL PROCEDURES**

**General**—The following spectroscopic and analytical instruments were used: Pioneer™ peptide synthesizer (Applied Biosystems, Foster City, CA); HPLC, Waters 600E multi solvent delivery system with 2487 UV dual λ absorbance detector, and Waters 625 LC system with 486-UV tunable absorbance detector and 741 data module; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Applied Biosystems Voyager-DE PRO); thioflavin-T (Th-T) fluorescence (SPECTRA Max GEMINI XS-TR, Molecular Devices, Ashiya, Japan); a microplate reader (MP-AIII, TOYO, Tokyo, Japan); and electron microscope (Hitachi H-7500). MALDI-TOF MS was measured as reported previously (16, 17). HPLC was carried out on a Develosil packed column, ODS-UG-5 (20-mm inner diameter × 150- and 6.0-mm inner diameter × 100 mm) (Nomura Chemicals, Seto, Japan).

HATU (N-[[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene]-N-methylmethylammonium hexafluorophosphate N-oxide) (18), piperidine, Fmoc amino acids, Fmoc-Val-polystyrene glycopheryl-polystyrene support resin, and DIPEA (N,N-diisopropylethylamine) were purchased from Applied Biosystems. N-N-Dimethylformamide (DMF), trifluoroacetic acid, 1,2-ethanethiol, thioanisole, m-cresol, and diethyl ether (peroxide-free) were purchased from Nacalai Tesque (Kyoto, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Applied Biosystems. Rat pheochromocytoma PC12 cells were obtained from Riken Cell Bank and were cultured as reported previously (15, 21, 22). For experimental purposes, near-confluent cultures of the cells were plated at ~104 cells/100 μL well fresh culture medium in a 96-well tissue culture plate coated with collagen and incubated at 37 °C in 5% CO2 before the experiments.

**RESULTS**

**Synthesis of Aβ Derivatives—**Each Aβ derivative was synthesized in a stepwise fashion on 0.1 mmol of preloaded Fmoc-Val-PEG-PS (for WT-Aβ42) using the Fmoc method as reported previously (15, 19–22). The coupling reaction was carried out using Fmoc amino acid (0.4 mmol), HATU (0.4 mmol), and DIPEA (0.8 mmol) in DMF for 30 min. The coupling reaction was carried out using Fmoc amino acid (0.4 mmol), HATU (0.4 mmol), and DIPEA (0.8 mmol) in DMF for 30 min. After completion of the chain elongation, each peptide resin washed with 20% piperidine in DMF. After centrifugation at 15,000 rpm in an Eppendorf microcentrifuge at 37 °C for 4, 8, 16, 24, 48, or 72 h. Three microliters of each Aβ solution was then analyzed by HPLC (4.5 mmol) with 50 amino acid residues with a continuous flow-type peptide synthesizer. Each solution of Aβ derivatives (0.1% NH4OH) sterilized by a filter (0.22 μm) was diluted with 0.1% NH4OH at concentrations ranging from 0.12 to 120 μM. Each solution of Aβ derivatives of 10 μL of the resultant solutions was incubated at 37 °C for 4, 8, 16, 24, 48, or 72 h. Each Aβ derivative of 10 μL of the resultant 30% solution was incubated at 37 °C for 4, 8, 16, 24, 48, or 72 h.

**Mitochondrial Reductase Assay**—WT-Aβ42: DAEFRHDSGYEVHQQKVFFAEVDGSNKGAIILMVGVGVIA

**EXPERIMENTAL PROCEDURES**

**General**—The following spectroscopic and analytical instruments were used: Pioneer™ peptide synthesizer (Applied Biosystems, Foster City, CA); HPLC, Waters 600E multi solvent delivery system with 2487 UV dual λ absorbance detector, and Waters 625 LC system with 486-UV tunable absorbance detector and 741 data module; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Applied Biosystems Voyager-DE PRO); thioflavin-T (Th-T) fluorescence (SPECTRA Max GEMINI XS-TR, Molecular Devices, Ashiya, Japan); a microplate reader (MP-AIII, TOYO, Tokyo, Japan); and electron microscope (Hitachi H-7500). MALDI-TOF MS was measured as reported previously (16, 17). HPLC was carried out on a Develosil packed column, ODS-UG-5 (20-mm inner diameter × 150- and 6.0-mm inner diameter × 100 mm) (Nomura Chemicals, Seto, Japan).

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**Synthesis of Aβ Derivatives—**Each Aβ derivative was synthesized in a stepwise fashion on 0.1 mmol of preloaded Fmoc-Val-PEG-PS (for WT-Aβ42) or Fmoc-Ala-PEG-PS (for Aβ42 derivatives) resin by Pioneer™ using the Fmoc method as reported previously (15, 19–22). The coupling reaction was carried out using Fmoc amino acid (0.4 mmol), HATU (0.4 mmol), and DIPEA (0.8 mmol) in DMF for 30 min. After each coupling reaction, the N-terminal Fmoc group was deblocked with 20% piperidine in DMF.

After completion of the chain elongation, each peptide resin washed with DMF and CH2Cl2 was treated with a mixture containing trifluoroacetic acid, m-cresol, ethanethiol, and thioanisole for final deprotection and cleavage from the resin. After 2 h of shaking at room temperature, the crude peptide precipitated by diethyl ether was purified by HPLC under alkaline conditions as reported previously (15, 19–22). Lyophilization gave a corresponding pure Aβ peptide, the purity of which was confirmed by HPLC (>98%). Each purified peptide exhibited satisfactory mass spectrometric data. The difference between the calculated and theoretical molecular mass was less than one mass unit.

**Sedimentation Assay for Fibril Formation—**Each Aβ derivative was dissolved in 0.02% NH4OH at 250 μM. After a 10-fold dilution by 50 mM sodium phosphate containing 100 mM NaCl at pH 7.4, the resultant peptide solution (25 μM) was incubated at 37 °C for 4, 8, 16, 24, or 48 h. After centrifugation at 15,000 rpm in an Eppendorf microcentrifuge at 4 °C for 10 min, 25 μL of the supernatant was then analyzed by HPLC as reported previously (15, 21, 22). The area of the absorption at 220 nm was integrated and expressed as a percentage of the control. Molar concentration of soluble Aβ was integrated and expressed as a percentage of the control.

**Th-T Fluorescence Assay—**Each Aβ derivative was dissolved in 0.02% NH4OH at 250 μM. The peptide solution (25 μM) diluted with the phosphate buffer solution described above (pH 7.4) was incubated at 37 °C for 4, 8, 16, 24, 48, or 72 h. Three microliters of each Aβ solution was then analyzed by HPLC (4.5 mmol) with 50 amino acid residues with a continuous flow-type peptide synthesizer (Pioneer™) using HATU (18) as an effective coupling reagent for Fmoc chemistry. After final deprotection and cleavage from the resin followed by purification using HPLC in the alkaline condition (CH3CN, 0.1% NH4OH), each Aβ derivative was successfully synthesized in a highly pure form as reported previously (15, 19–22). The total yields of the Aβ derivatives synthesized in this study were consequently between 2 and 36%, indicating that the average coupling yield of each condensation step was 95–97.5%. Their molecular weights were confirmed by MALDI-TOF MS, and their purity was determined by HPLC analysis (>98%).

**Aggregative Ability and Neurotoxicity of the Proline-substituted Aβ42 Mutants at Positions 15–32—**We focused at first on the residue 22 that frequently mutates in cerebral amyloidosis.
Angiopathy such as E22Q (Dutch) (25) and E22K (Italian) (26) and investigated the proline-substituted Aβ42 mutants at positions 15–32. Their aggregative ability was estimated by the two methods, the sedimentation assay (HPLC analysis after centrifugation of the Aβ solution) and the Th-T fluorescence assay. Since Williams et al. (14) used thermodynamic stabilities of the Aβ40 fibrils to obtain its structural information, we examined the thermodynamic stabilities of several Aβ42 mutants that were reported as a preliminary communication (15). Fig. 2C shows the critical concentration (C_r) defined as the molar concentration of soluble Aβ42 peptides present at equilibrium along with the aggregative ability estimated by the sedimentation assay (Fig. 2A) and the Th-T fluorescence assay (Fig. 2B), which were generally correlated with each other. Fairly good correlation was observed between the kinetics and thermodynamics data. E22P-Aβ42 with rapid aggregation kinetics showed a lower C_r value (0.42 μM) compared with wild-type Aβ42 (0.85 μM). On the other hand, V18P-, F19P-, and F21P-Aβ42 with slower aggregation kinetics exhibited higher C_r values (15, 17, and 17 μM, respectively). It is noteworthy that the C_r value of wild-type Aβ42 (0.85 μM) determined by us is almost equal to that of wild-type Aβ40 (0.90 μM) reported by Williams et al. (14). Since Aβ42 aggregated much faster than Aβ40 as shown in Fig. 2, A and B, aggregation kinetics rather than thermodynamics seems to be more important in Aβ42. Thus, we adopted the kinetics data to deduce the secondary structure of Aβ42 in its aggregates.

As shown in Fig. 3A, all of the proline-substituted Aβ42 mutants at positions 15–32 with the exception of E22P-Aβ42 hardly aggregated after 8 h of incubation. Only the data of the sedimentation assay are shown, because the HPLC data are more reliable than the Th-T fluorescence data as reported previously (22). Similar results were obtained after 16 and 24 h of incubation with the exception of V18P-, F19P-, G25P-, and A30P-Aβ42 that showed slow but significant aggregation kinetics (data not shown). It is quite noticeable that only E22P-Aβ42 aggregated faster than wild-type Aβ42.

The neurotoxic effects on the PC12 cells of these Aβ42 mutants were examined by MTT assay to confirm whether their aggregation reflects the pathological aggregation of wild-type Aβ42. The MTT assay consists of the conversion of MTT to colored formazan by mitochondrial reductase and serves as an indirect measurement of cell proliferation and viability. After a 48-h incubation with each Aβ42 derivative, formazan formation was measured at 600 nm in a concentration range of 0.01–10 μM. Because wild-type Aβ42 inhibited ~50% formazan formation at 10−6.5 M, the formazan formation in the presence of 10−6.5 M each proline-substituted Aβ42 peptide was measured simultaneously to estimate precisely the relative neurotoxicity (Fig. 3A). The results showed that only E22P-Aβ42 potently inhibited the formazan formation at 10−6.5 M. The IC₅₀ value of inhibition of the formazan formation by E22P-Aβ42 was 0.084 ± 0.011 μM, whereas that of wild-type Aβ42 was 0.97 ± 0.18 μM. Although only D23P-Aβ42 showed significant neurotoxicity at 10−6.5 M (IC₅₀ = 1.3 ± 0.26 μM), other Aβ42 mutants were almost inactive at 10−6.5 M.

Effects of the C-terminal Residues of Aβ42 on Its Aggregative Ability and Neurotoxicity—It is obvious that the C-terminal two residues of Aβ42 play a critical role in its aggregative ability and neurotoxicity. Weinreb et al. (27) proposed the “hypothesis of hydrophobic cluster,” stating that hydrophobic interaction among the side chains at the C terminus induces aggregation (Fig. 4A). In this hypothesis, Ile-41 is incorporated in the hydrophobic core formed by Leu-34 and Met-35. To confirm this, Aβ42 fibrils were obtained by centrifugation of the Aβ solution (Fig. 2A), and the Th-T fluorescence data were obtained (Fig. 2B). Both I41T- and A42T-Aβ42 aggregated rapidly similar to wild-type Aβ42. Substitution with Thr did not abolish their cytotoxic effects. The IC₅₀ values of the cytotoxicity on PC12 cells were 1.1 ± 0.11, 0.70 ± 0.10, and 0.97 ± 0.18 μM for I41T-, A42T-, and wild-type Aβ42, respectively, suggesting that hydrophobicity of the side chains at positions 41 and 42 is not requisite for the aggregative ability and neurotoxicity of Aβ42.

Because it is conceivable that the C-terminal residues participate in the β-sheet formation, a series of the proline-substituted Aβ42 mutants at positions 33–42 were synthesized and their aggregative ability and neurotoxicity were tested. As shown in Fig. 3B, the aggregative ability of L34P- and G38P-Aβ42 as estimated by the sedimentation assay was significantly higher than that of wild-type Aβ42. Both aggregated
almost completely after a 4-h incubation (only the data after an 8-h incubation is shown in Fig. 3B). G33P- and V39P-Aβ42 also showed significant aggregative ability. Although G38P- and V39P-Aβ42 showed potent neurotoxicity comparable with wild-type Aβ42, the neurotoxicity of G33P- and L34P-Aβ42 was weak regardless of its significant aggregative ability (Fig. 3B). M35P-, V36P-, G37P-, V40P-, I41P-, and A42P-Aβ42 did not aggregate, even after a 24-h incubation (data not shown). They were almost inactive in the neurotoxicity assay (Fig. 3B).

**Fig. 3.** Aggregation kinetics was estimated by the sedimentation assay after an 8-h incubation and neurotoxicity in PC12 cells estimated by the MTT assay of the proline-substituted Aβ42 mutants at positions 15–32 (A), 33–42 (B), and 3–13 (C). In the aggregation assay, similar results were obtained after a 16- or 24-h incubation except in V18P, F19P, G25P, A30P, and G33P-Aβ42, which showed moderate aggregation after a 24-h incubation (data not shown). In the neurotoxicity assay, the concentration of the proline-substituted Aβ mutants was 10−6.5 M, which is close to the IC50 value of wild-type (WT) Aβ42. All of the peptides in each group were tested simultaneously to compare relative cytotoxicity.

**Fig. 4.** Role of the C-terminal two residues. A, the hypothesis of hydrophobic cluster in Aβ fibril formation (27). B, aggregation kinetics estimated by the sedimentation assay after an 8-h incubation and neurotoxicity in PC12 cells estimated by the MTT assay of I41T- and A42T-Aβ42. In the neurotoxicity assay, the concentration of the proline-substituted Aβ mutants was 10−6.5 M, which is close to the IC50 value of wild-type Aβ42. These peptides were tested simultaneously to compare relative cytotoxicity.
Effects of the N-terminal Residues of Aβ42 on Its Aggregative Ability and Neurotoxicity—Because the C-terminal three residues play a critical role in the aggregative ability and neurotoxicity of Aβ42, the contribution of the N-terminal residues to the activities was also investigated. Aβ3–42 lacking the N-terminal two residues of Aβ42 aggregated potently with velocity quite similar to that of wild-type Aβ42 (data not shown) and showed significant neurotoxic effects. The IC₅₀ values of Aβ3–42 and wild-type Aβ42 were 0.043 ± 0.010 and 0.97 ± 0.18 μM, respectively. This indicates that the N-terminal two residues are not necessary for the aggregative ability and neurotoxicity of Aβ42. To identify the β-sheet-forming region at the N-terminal portion, proline-substituted Aβ42 mutants at positions 3–13 were synthesized and examined for their aggregative ability and neurotoxicity (Fig. 3C). All of the mutants at positions 3–13 aggregated potently and exhibited significant neurotoxic effects.

Transmission Electron Micrographs of Negatively Stained Preparations of the Fibrils Formed from the Proline-substituted Aβ42 Mutants—Fibril formation of the proline-substituted Aβ42 mutants with potent aggregative ability was evaluated by transmission electron microscopy after a 48-h incubation at 37°C. N-terminal proline-substituted Aβ42 mutants at positions 3–13 and E22P-, L34P-, G38P-, V39P-, I41T-, and A42T-Aβ42 exhibited typical fibril formation, several examples of which are shown in Fig. 5. The fibrils of E22P-Aβ42 have been reported previously (22). The morphologies of these fibrils resembled each other well.

**DISCUSSION**

The aggregation of Aβ peptides is significantly related to the pathogenesis of neuronal degeneration in AD. Despite many previous studies on the structural analysis of Aβ aggregates, the precise mechanism has not yet been clarified. To obtain information on the structure of Aβ42 fibrils, we adopted the proline-scanning method proposed by Wood et al. (12). Thirty-four proline-substituted Aβ42 mutants were synthesized in high purity using the method recently established by us (19–22) and were subjected to measurements of their aggregative ability and neurotoxicity.

The aggregative ability of these mutants was estimated by the sedimentation and the Th-T fluorescence assay, which are not always correlated with each other (22), because the Th-T fluorescence can vary depending on the structure and morphology of the fibrils. However, these two assays were generally in good correlation. Only the data from the sedimentation assay are shown in Fig. 3. Among the proline-substituted Aβ42 mutants at positions 15–32, only E22P-Aβ42 aggregated more rapidly than wild-type Aβ42, whereas other proline-substituted mutants at positions 15–21 and 24–32 hardly aggregated after an 8-h incubation. Because proline has a propensity to form a β-turn structure as a Pro-X corner (13), the data strongly suggest that turn at positions 22 and 23 is a critical secondary structure in the Aβ42 fibrils. This finding demonstrates very well the high aggregative ability of the Aβ42 mutants in cerebral amyloid angiopathy (21, 22), E22Q/Aβ42 (Dutch), and E22K/Aβ42 (Italian), because Gln-Asp (Dutch) and Lys-Asp (Italian) sequences at positions 22 and 23 are more frequently found in the two-residue β-turn (13) than in Gln-Asp (wild type). Recent investigations using solid-state NMR (7–9) have indicated a parallel organization of β-sheets in Aβ40 fibrils, because the β-carbons of Ala-21 and Ala-30 are located within 5.5 Å, respectively. Thus, the present results obtained from the proline mutagenesis indicate that turn formation at positions 22 and 23 followed by intermolecular parallel β-sheet formation between positions 15–21 and 24–32, respectively, leads to the organization of Aβ42 fibrils (Fig. 6A).

Specifically, the turn position of our Aβ42 aggregation model was different from that of Petkova et al. (7), which is based on the solid-phase NMR of Aβ40 aggregates in which two residues at positions 26 and 27 adopted a bend structure. This structure seems to be reasonable, because the Ser-Asn sequence is often found in the two-residue β-turn (13). However, the aggregative ability of S26P-Aβ40 was very low (data not shown), similar to that of wild-type Aβ40, whereas E22P-Aβ40 aggregated more rapidly than wild-type Aβ40 (15), suggesting that the structure of the Aβ40 fibrils resembles that of the Aβ42 fibrils. After the completion of this study, Williams et al. (14) have reported the systematic proline replacement of Aβ40 and have shown that residues at positions 22 and 23 of Aβ40 probably occupy turn positions, supporting our conclusion. The turn at position 22 of Aβ fibrils does not contradict the solid-phase NMR data, because no NMR data at position 22 of Aβ40 were described in the study of Petkova et al. (7). Moreover, the chemical shifts for Asn-23 predicted non-β-strand Φ and ψ angles.

Turn formation at position 22 of Aβ peptides is closely related to the cytotoxic effects on PC12 cells. In the model proposed by Petkova et al. (7), the turn at positions 26 and 27 is stabilized by an ionic interaction between the side chains of Asp-23 and Lys-28. This conformation seems to be non-malignant, because S26P-Aβ42 and S26P-Aβ40 did not show any cytotoxicity against PC12 cells (data not shown). Mutation at position 22 of Aβ peptides by the amino acid residues that prefer turn formation would change the turn position from 26 and 27 to positions 22 and 23. This conformational change might increase the intermolecular parallel β-sheet region to enhance the aggregative ability and neurotoxicity of Aβ peptides.

It is widely accepted that Aβ42 aggregates far more potently and is cytotoxic to PC12 cells compared with Aβ40. However, there has been no persuasive explanation regarding this issue until now. Hydropobicity of the C-terminal two residues was considered to be a critical factor for the high aggregative ability of Aβ42, and the “hypothesis of hydrophobic cluster” was proposed as mentioned above (Fig. 4A) (27). However, our present results using I41T- and A42T-Aβ42 did not support this hypothesis because substitution at positions 41 and 42 of Aβ42 with the hydrophobic threonine residue did not decrease aggregative ability and neurotoxicity.

Some investigators have considered that the C-terminal hydrophobic amino acid residues are involved in the β-sheet formation (9). We also considered that the C-terminal residues adopt a β-sheet structure and examined the aggregative ability and neurotoxicity of the proline-substituted Aβ42 mutants at positions 33–42 (Fig. 3B), V40P-, I41P-, and A42P-Aβ42 hardly aggregated and were almost inactive in the assay using PC12 cells, indicating that the C-terminal three residues significantly participate in β-sheet formation. It is not known whether the β-sheet at positions 40–42 is intermolecular or intramolecular. However, this is a solid conclusion for explaining the potent aggregative ability and neurotoxicity of Aβ42. According to the proline mutagenesis of Aβ40 (14), the C-terminal residues at positions 37–40 of Aβ40 were judged to be excluded from the β-sheet structure in the Aβ40 fibril because these proline-substituted Aβ40 mutants aggregated with a potency similar to that of wild-type Aβ40. Previous spin-labeling studies using Aβ40 (28) have also suggested that the C terminus of Aβ40 is not packed in a rigid structure within the fibril. These data clearly indicate that the roles of the C terminus of Aβ42 and that of Aβ40 are quite different from each other. Tycko and co-workers (7, 9) have recently proposed a structural model for Aβ40 protofilaments consisting of the two cross-β units attached by the hydrophobic interaction between the side
chains of the C-terminal amino acid residues. However, our present data suggested that the interaction at the C terminus between the two cross-β units is not a hydrophobic interaction but a parallel or anti-parallel β-sheet. This β-sheet formation would increase the rate of fibril formation of Aβ42.

It was an unexpected result that L34P- and G38P-Aβ42 aggregated faster than the wild-type Aβ42. To examine the thermodynamic stabilities of the fibrils formed by these Aβ42
mutants, the molar concentration of soluble peptide present at equilibrium ($C_s$) was measured. The $C_s$ values of L34P- and G38P-Aβ42 were 1.0 ± 0.010 and 1.4 ± 0.087 μM, respectively, which were almost equal to those of wild-type Aβ42 (0.85 μM). Moreover, G33P- and V39P-Aβ42 also showed relatively high aggregation velocity. These results suggested the turn formation at positions 33 and 34 as well as 38 and 39 and the β-sheet formation at positions 35–37. To confirm these turn positions, the triple Aβ42 mutant (P3-Aβ42) substituted with proline residues at the three possible turn positions (22, 34, and 38) was prepared. P3-Aβ42 aggregated in the velocity equal to that of wild-type Aβ42 with a $C_s$ value of 1.0 ± 0.022 μM, supporting the three turns in the Aβ42 aggregates.

It is noteworthy that the cytotoxicity to PC12 cells of G33P- and L34P-Aβ42 was significantly weaker than that of wild-type Aβ42, unlike that of G33P- and V39P-Aβ42. We speculated that the residue at positions 33 and 34 plays a critical role in cytotoxicity rather than aggregation. The critical amino acid residue for expressing cytotoxicity of Aβ peptides is considered to be Met-35. According to Butterfield and colleagues (29), radical formation at the sulfur atom of Met-35 is a requisite condition for Aβ42 to damage cells. The flexibility of this region would make Aβ42 a more cytotoxic species by radical transfer from Met-35 to Gly-33 as proposed.

In contrast, the N-terminal two residues were not necessary for the aggregative ability and neurotoxicity of Aβ42. Moreover, all of the proline-substituted mutants at positions 3–13 showed potent aggregative ability and neurotoxicity comparable with those of wild-type Aβ42 (Fig. 3C). This finding suggests that the N-terminal 13 residues do not adopt any solid structure such as β-sheet and α-helix. This conclusion is in good agreement with those previously reported using solid-state NMR analysis (7) and electron spin resonance spectra (28) in which the secondary structure of the N-terminal 10–15 residues of Aβ40 is not defined.

The electron microscope measurements of the proline-substituted Aβ42 mutants with potent aggregative ability clearly showed fibrillar materials (Fig. 5). Although the resolution at this level cannot distinguish fine structural differences, it is certain that the aggregates in Fig. 5 are not amorphous but are fibrils. The fibrils of the proline-substituted Aβ42 mutants were quite similar to those of wild-type Aβ42. This was also supported by the Th-T data. These mutants showed significant Th-T fluorescence, characteristic of amyloid fibrils (data not shown). These data indicated that the fibrils of the proline-substituted Aβ42 mutants adopt a tertiary structure similar to that of wild-type Aβ42.

Finally, we proposed a new aggregation model of Aβ42 on the basis of the systematic proline replacement along with a requirement for the parallel β-sheet at positions 21 and 30 (5), as shown in Fig. 6A. The most important structural feature of Aβ42 fibrils is the turn at positions 22 and 23 and the two intermolecular β-sheets on both sides (positions 15–21 and 24–32). This structure resembles several models recently proposed (7, 10, 28) with the exception of the turn position. As mentioned above, the turn formation at position 22 of Aβ42 can explain most reasonably the pathogenesis of cerebral amyloid angiopathy (Dutch and Italian mutation). The proline replacement of Aβ40 gave a similar conclusion regarding the turn position at position 22 (Fig. 6B) (14). However, the C-terminal structure in the Aβ40 aggregation model is quite different from that of Aβ42. Our proline mutagenesis data indicated that the C-terminal three residues adopt a β-sheet structure. Although it is not clear whether this β-sheet is intermolecular or intramolecular, we believe that this is an intermolecular β-sheet for binding each Aβ42 unit tightly to form fibrils because the N-terminal 13 residues are not involved in the β-sheet formation. The lack of the C-terminal intermolecular β-sheet of Aβ40 shows its fairly slow aggregative kinetics compared with that of Aβ42. The existence of turns at positions 33, 34, 38, and 39 seems to be necessary to stabilize the Aβ42 by the formation of a circular structure. The aggregation model proposed in this study (Fig. 6A) gives unique opportunities to design reasonably novel inhibitors for Aβ42 aggregation.

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