Aromaticity of Phenylalanine Residues Is Essential for Amyloid Formation by Alzheimer’s Amyloid β-Peptide

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The abnormal aggregation of amyloid β-peptide (Aβ) is central to the pathogenesis of Alzheimer’s disease, the major form of dementia. Aromatic π–π interactions have been suggested to play a crucial role in the aggregation of not only Aβ, but also other amyloidogenic proteins. In this study, each or all phenylalanine (Phe) residues at the 4th, 19th, and 20th positions of Aβ-(1–40) were substituted by hydrophobic cyclohexylalanine (Cha), which is sterically similar to Phe, but lacks π-electrons, to reveal effects of interactions involving π-electrons on the aggregation of Aβ both in aqueous solution and GM1-containing membranes. We found that each Cha substitution significantly inhibited fibril formation by Aβ, indicating a pivotal role of aromatic interactions. Furthermore, the Aβ analog with three Cha residues effectively retarded the fibrillation of the wild-type Aβ.

Key words Alzheimer’s disease; amyloid β-peptide; phenylalanine; cyclohexylalanine; aromatic interaction; amyloid formation

Alzheimer’s disease, a progressive neurodegenerative disease, is the major form of dementia. A pathological hallmark of the disease is the deposition of senile plaques in the brain, the main component of which is fibrillar amyloid β-peptide (Aβ). Aβ is mainly composed of 40–42 amino acid residues and is produced by the enzymatic cleavage of the single membrane-spanning amyloid precursor protein by β- and γ-secretases. Since aggregated Aβ (soluble oligomers and insoluble fibrils) shows neurotoxicity, it is highly important to understand how essentially soluble Aβ is self-assembled under pathological conditions.1,2)

Aromatic π–π interactions have been suggested to play a crucial role in fibril formation by not only Aβ, but also other amyloidogenic proteins because aromatic residues occur markedly in amyloid-forming proteins and short fragment peptides containing aromatic residues inhibit amyloidogenesis by parent proteins.3) In principle, side chain interactions are important for fibril formation because they are arranged in a highly ordered fashion within fibrillar structures. Aromatic residues also appear to be important for peptide–lipid interactions. We have shown that Aβ amyloidogenesis occurs on clusters of monosialoganglioside GM1 in neuronal membranes, leading to neurotoxicity.4) Molecular dynamic simulations suggested that CH–π and/or OH–π interactions are involved in the binding of Aβ to GM1.5)

In this study, each or all Phe residues at the 4th, 19th, and 20th positions of Aβ-(1–40) were substituted by cyclohexylalanine (Cha) (Table 1), which is sterically similar to Phe but lacks π-electrons (Fig. 1), to reveal the effects of interactions involving π-electrons on the aggregation of Aβ both in aqueous solutions and GM1-containing membranes. The introduction of Cha does not weaken hydrophobic interactions because Cha is more hydrophobic than Phe.6) We found that each Cha substitution significantly inhibited fibril formation by Aβ, indicating a pivotal role of aromatic interactions. Furthermore, the Aβ analog with three Cha residues (Cha3, Table 1) effectively retarded Aβ fibrillation, suggesting that small peptides containing Cha could be promising candidates for disease modulators.

Experimental

Aβs  Aβ-(1–40) (wt) was produced as ubiquitin extensions and purified as described in detail elsewhere.7) Aβ-(1–40) variants (Cha3, 4Cha, 19Cha, and 20Cha) were synthesized by the standard 9-fluorenylmethoxycarbonyl (Fmoc)-based method on a Fmoc-Val-NovaSynTGA resin (Millipore, Billerica, MA, U.S.A.). The purity (>95%) and identity of the peptides were confirmed by analytical reverse phase HPLC and matrix-assisted laser desorption ionization mass spectrometry (MALDI). Monomeric Aβ was obtained by size exclusion chromatography (SEC). The peptide was dissolved in 6 M guanidine hydrochloride, sonicated for 5 min, centrifuged at 10000×g for 5 min. The resulting solution was injected into a Sephacryl S-300 HR column (GE Healthcare Bio-Science, NJ, U.S.A.) and purified as described in detail elsewhere.

Table 1. The Amino Acid Sequences of the Peptides Used

| Designation | Sequence<sup>a</sup> |
|-------------|----------------------|
| wt          | DAEFRHDSGY<sup>a</sup>EVHHQKLFF<sup>a</sup>AEDVGSNKGA<sup>a</sup>IIGLMVGVV<sup>a</sup> |
| Cha3        | DAEXRHSGY<sup>a</sup>EVHHQKLXX<sup>a</sup>AEDVGSNKGA<sup>a</sup>IIGLMVGVV<sup>a</sup> |
| 4Cha        | DAEXRHSGY<sup>a</sup>EVHHQKLFF<sup>a</sup>AEDVGSNKGA<sup>a</sup>IIGLMVGVV<sup>a</sup> |
| 19Cha       | DAEXRHSGY<sup>a</sup>EVHHQKLXX<sup>a</sup>AEDVGSNKGA<sup>a</sup>IIGLMVGVV<sup>a</sup> |
| 20Cha       | DAEXRHSGY<sup>a</sup>EVHHQKLFX<sup>a</sup>AEDVGSNKGA<sup>a</sup>IIGLMVGVV<sup>a</sup> |

<sup>a</sup> Substituted residues are shown in bold. ‘X’ denotes cyclohexylalanine (Cha).

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U.S.A.) previously equilibrated with phosphate buffered saline (PBS) buffer containing 0.0125% (w/v) NaN₃, and eluted at a flow rate of 0.5 mL/min. The system was kept at 4°C and had been washed with 0.5 M NaOH and subsequently pretreated with an excess of bovine serum albumin (BSA) to block nonspecific binding of proteins. The peak attributed to monomeric Aβ was collected, and its peptide concentration was determined from UV absorbance at 275 nm.

**Liposomes** GM1 from bovine brains, N-acyl-α-sphingosine-1-phosphocholine from bovine brains (SM), and cholesterol were purchased from Larodan (Solna, Sweden), Olbracht Sardary Research Laboratories (Toronto, Canada), and Sigma (St. Louis, MO, U.S.A.), respectively. Small unilamellar vesicles (SUVs) were prepared as reported previously. GM1, cholesterol, and SM were dissolved in a chloroform–methanol (1:1, v/v) mixture, chloroform, and ethanol, respectively. The concentrations of GM1, cholesterol, and SM were determined at least in triplicate by a resorcinol–hydrochloric acid method, a cholesterol oxidase method [Free Cholesterol E-Test kit by Wako (Osaka, Japan)], and a phosphorus assay, respectively. Lipids were mixed at a molar ratio of 1:1:1, and the solvent was removed by evaporation in a rotary evaporator. The residual lipid film, after drying under a vacuum overnight, was hydrated with PBS containing 0.0125% (w/v) NaN₃ at 50°C and vortex mixed to produce multilamellar vesicles, which were subsequently sonicated under a nitrogen atmosphere for 9 min (3 min×3 times) using a probe-type sonicator.

**Fig. 1.** The Chemical Structures of (a) Phenylalanine and (b) Cyclohexylalanine Side Chains

**Fig. 2.** Binding of the Peptides to GM1-Containing Vesicles

Each peptide (50 µM) was incubated with GM1–SM–Cholesterol (1:1:1) vesicles ([GM1]=500 µM) for 10 min at 37°C. The concentration of the peptide in the supernatant after ultracentrifugation was determined by HPLC. Experiments were carried out in duplicate (means±S.D.). * p<0.05.

**Fig. 3.** Aggregation of wt

The wt peptide (50 µM) was incubated in (a, b) PBS or (c, d) with GM1–SM–cholesterol (1:1:1) vesicles ([GM1]=50 µM) for various periods at 37°C. (a, c) Peptide aggregation was monitored by a Th-T assay (closed circles) and soluble peptide concentration (open squares). (b, d) The soluble fractions were analyzed by SEC. The left and right arrowheads indicate the elution times for 150 (void) and 4.4 kDa markers (FITC-dextran), respectively.
Tomy UD-201 (Tokyo, Japan). Metal debris from the titanium tip of the probe was removed by centrifugation. The concentration of vesicles was determined based on the concentration of GM1 because the lipid composition of vesicle preparations was very close to the expected value within a 10% error margin. The vesicles have been characterized in terms of size and lamellarity.11)

Aggregation of Aβ

The Aβ solution was diluted to 50 µM and left to aggregate at 37°C with or without SUVs (Aβ–GM1=1:1) without agitation. The aggregation was monitored by a Thioflavin-T (Th-T) assay, circular dichroism (CD), centrifugation, and SEC for 48 h.

Th-T Assay

Amyloid formation was monitored with the amyloid-specific dye Th-T.12) The sample (final Aβ concentration, 0.5 µM) was added to a 5 µM Th-T solution in 50 mM glycine buffer (pH 8.5). Fluorescence at 490 nm was measured at an excitation wavelength of 446 nm on a Shimadzu RF-5300 spectrometer (Kyoto, Japan) with a cuvette holder thermostatically controlled at 25°C. The averaged blank intensity (SUV solution or buffer) was subtracted. Statistical analysis was performed using ANOVA (n=3).

CD Spectra

CD spectra were measured at 37°C on a Jasco J-820 apparatus (Hachioji, Japan), using a 0.5 mm path length quartz cell to minimize the absorbance due to buffer components. The precise path length (0.538 mm) was determined by the absorbance of cytochrome c (pH 7.0) at 406 nm as a standard. Four scans were averaged for each sample. The averaged blank spectra (SUV solution or buffer) were subtracted.

Centrifugation and SEC

Each sample was centrifuged at 10000×g for 10 min to remove insoluble components including fibrils. The peptide concentration in the supernatant was determined by analytical HPLC, which was performed using a COSMOSIL 5C18-AR-II Packed Column (4.6 mm×150 mm) (Nacalai Tesque, Kyoto, Japan) eluted with a linear gradient of CH3CN (25 to 50% over 30 min) in 0.1% aqueous trifluoroacetic acid at a flow rate of 1 mL/min. The peptide was detected by measuring the UV absorbance at 220 nm.

The supernatant was also fractionated with a Sephacryl S-300 HR column with an exclusion limit of 150 kDa. The column was eluted with PBS containing 0.0125% NaN3 (w/v) at a flow rate of 0.5 mL/min and the Aβ peptide was detected by measuring UV absorbance at 220 nm.

Membrane Binding Aβ was mixed with SUVs ([Aβ]=50 µM, Aβ–GM1=1:10) in PBS and equilibrated for 10 min at 37°C. The free Aβ monomer and membrane-bound Aβ were separated by ultracentrifugation at 200000×g and 37°C for 3 h, and the peptide concentration in the supernatant was determined by analytical HPLC.

Fig. 4. Aggregation of Cha3

The peptide (50 µM) was incubated in (a, b) PBS or (c, d) with GM1–SM–cholesterol (1:1:1) vesicles ([GM1]=50 µM) for various periods at 37°C. (a, c) Peptide aggregation was monitored only by the soluble peptide concentration because the Cha-containing peptides exhibited unusual Th-T signals (see text and Fig. S1). Slight variations in concentration were due to independent sample preparations at each time point. (b, d) The soluble fractions were analyzed by SEC. The left and right arrowheads indicate the elution times for 150 (void) and 4.4 kDa markers (FITC-dextran), respectively.
Results

Membrane Binding The effects of the Cha mutations on the binding of Aβ to GM1-containing membranes were examined by a combination of ultracentrifugation and reverse-phase HPLC (Fig. 2). About 70% of wt bound to liposomes under the conditions examined. Among the Aβ variants, only 4Cha exhibited a significantly reduced binding compared to wt ($p=0.024$). However, the difference was rather small.

Aggregation of wt Aggregation of wt in PBS or in the presence of SUVs was monitored by a Th-T assay, CD, centrifugation, and SEC. Th-T specifically binds to amyloids, so is often used to detect them. In PBS, wt formed amyloids after a lag time of 12 h (Fig. 3(a)) with conformational changes from a random coil structure (a minimum at around 197 nm) to a β-sheet rich structure (a minimum at around 216 nm) (Fig. 7(a)). At the same time, the soluble Aβ concentration was dramatically decreased by aggregation (Fig. 3(a)). The SEC experiments indicated that the soluble fraction was composed of monomers (Fig. 3(b)). In contrast, these changes occurred without a lag time in the presence of SUVs, suggesting that membrane-bound Aβ served as a seed for amyloid formation (Figs. 3(c) and 7(e)). Again, the soluble fraction contained only monomers (Fig. 3(d)). Note that the gradual disappearance of the void peak was due to the co-sedimentation of SUVs with fibrils.

Aggregation of Cha3 To understand the effects of π electrons of the Phe residues on the amyloid formation by Aβ, the aggregation of the triple mutant Cha3 was examined first. The Th-T assay could not be used because the fluorescence intensity was unstable in Aβ variants, although the reason for this was not clear (Fig. S1). In a striking contrast to wt, Cha3 did not aggregate for at least 48 h in solution. All peptide molecules were soluble (Fig. 4(a)) and monomeric (Fig. 4(b)). CD spectra essentially did not change (Fig. 7(b)). Similar results were observed in the presence of liposomes (Figs. 4(c, d) and 7(f)), although the void peaks (SUVs+soluble aggregates) appeared to slightly increase with incubation time. These results indicated that the capability of amyloid fibril formation by Aβ was nullified by the triple mutation at least during a 48 h-incubation.

Aggregation of Single Mutants In order to determine which Phe residue is most important in amyloid formation, the aggregation of monosubstituted Aβ variants, 4Cha (Fig. S2), 19Cha (Figs. 5 and 7(c, g)), and 20Cha (Fig. S3) were examined. 19Cha behaved similarly to Cha3 in PBS. The peptide remained soluble, monomeric (Figs. 5(a, b)), and with random structures (Fig. 7(c)) for 48 h. In the presence of GM1-containing membranes, most of 19Cha was soluble and monomeric (Figs. 5(c, d)), although its conformation slightly changed during the incubation period (Fig. 7(g)). Similar results were obtained for 4Cha (Fig. S2) and 20Cha (Fig. S3) in solution and liposomes.

Cha3 as Modulator The possibility of Cha3 as a modulator for wt aggregation was examined. The wt peptide (final 50 μM) was coincubated with an equal concentration of Cha3 at 37°C. The total Aβ concentration was doubled compared to the other experiments. Soluble aggregates (>30 mer) were formed with conformation changes from a random coil to...
Fig. 6. Inhibition of wt Aggregation by Cha3
A mixture of wt (final 50 \( \mu \)M) and Cha3 (final 50 \( \mu \)M) was incubated in (a, b) PBS or (c, d) with GM1–SM–cholesterol (1:1:1) vesicles ([GM1]=50 \( \mu \)M) for various periods at 37°C. (a, c) Peptide aggregation was monitored by the soluble peptide concentrations of wt (closed circles) and Cha (open squares). (b, d) The soluble fractions were analyzed by SEC. The left and right arrowheads indicate the elution times for 150 (void) and 4.4 kDa markers (FITC-dextrans), respectively.

Fig. 7. Secondary Structures of the Peptides during Aggregation as Monitored by CD
Each peptide (50 \( \mu \)M) was incubated in (a–d) PBS or (e–h) with GM1–SM–cholesterol (1:1:1) vesicles ([GM1]=50 \( \mu \)M) for various periods at 37°C and CD spectra were measured. Peptides: (a, e), wt; (b, f), Cha3; (c, g), 19Cha; (d, h) wt+Cha3 (50 \( \mu \)M each).
β-sheet structures after a 12 h lag time in PBS (Fig. 6(b) and 7(d)) and without a lag time in the presence of SUVs (Fig. 6(d) and Fig. 7(h)). However, the soluble concentration of wt did not greatly decrease (Figs. 6(a, c)) and a major part was monomeric (Figs. 6(b, d)). These results suggested that Cha3 is a promising modulator of Aβ amyloidogenesis.

Discussion

The roles of aromatic interactions in amyloidogenesis by full-length Aβ-(1–40) have not been reported, although substitutions by Ala for Phe19 and Phe20 were reported to destabilize fibrils by 1.5 and 0.8 kcal/mol, respectively.\(^9\) Similar results were obtained for Cys mutations.\(^{10,11}\) In these experiments, not only aromatic, but also hydrophobic, interactions were lost at the same time. We used hydrophobic Cha to evaluate the effects of aromatic interactions without deteriorating hydrophobic interactions. Not only triple, but also even single, Cha substitution completely inhibited Aβ aggregation in PBS at least for 48 h (Figs. 4, 5, 7, S2, and S3). Besides interchain interactions, Phe19 has been suggested to form a hydrophobic core with Leu17, Ile32, Leu34, and Val36\(^6\) or with Gln15, Leu17, Ala21, Ile31, Met35, and Val39,\(^7\) stabilizing the internal quaternary contacts. Phe20 has been proposed to be involved in contacts between protofilaments.\(^8\) It is interesting that a Cha mutation at Phe4, which is in the disordered region Asp1–Tyr10,\(^9\) also nullified Aβ aggregation. Taking into consideration the observation that disulfide bond formation between A2C Aβ-(1–40) hampered mature fibril formation,\(^7\) the N-terminal disordered region also plays an important role in fibrilization.

In contrast to the solution-phase aggregation, Cha substitutions could not completely inhibit, but rather just retard Aβ aggregation in GM1-containing liposomes (Figs. 4, 5, 7, S2, and S3). This was not due to altered affinity for membranes (Fig. 2). Although molecular dynamic simulations suggested the involvement of the aromatic residues (three Phe residues and Tyr10) in the interaction of Aβ with the GM1 oligosaccharide via CH–π and/or OH–π interactions,\(^5\) their contributions to the total binding energy appear to be trivial. We have proposed that amyloid fibrils formed in membranes (membrane fibrils) contain antiparallel β-sheets and are more toxic than fibrils formed in the aqueous phase,\(^9,10\) which are solely composed of in-resister parallel β-sheets.\(^7\) The present results suggest that aromatic interactions are also important for the formation of this unique membrane fibril. Indeed, we observed exciton couplings between Phe residues in membrane-mediated amyloidogenesis.\(^5\)

The short peptide Aβ-(16–20) corresponding to the central hydrophobic core\(^10\) and its analogs\(^11\) bind to and inhibit fibrillization by Aβ-(1–40). The presence of the aggregation incompetent Cha3 also retarded fibril formation by wt and the mixture retained soluble aggregates after 48 h (Figs. 6, 7), indicating that Cha3 binds to wt. This was supported by the facts that 1) the soluble concentrations of both types of Aβ were slightly reduced with prolonged incubation in a similar fashion (Figs. 6(a, c)) and 2) Cha that conformed to random structure alone was involved in the formation of β-sheets in the presence of wt (Figs. 7(d, h)). Cha probably interacted with wt at different positions from the Phe sites and inhibited π–π interactions necessary for fibril formation, resulting in the accumulation of soluble aggregates. Short peptides containing Cha may be useful for modulating Aβ aggregation and thus be candidates for preventing or treating Alzheimer’s disease.

Conflicts of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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