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Nuclear magnetic resonance evidence for the dimer formation of beta amyloid peptide 1–42 in 1,1,1,3,3,3-hexafluoro-2-propanol

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ABSTRACT

Alzheimer's disease involves accumulation of senile plaques in which filamentous aggregates of amyloid beta (Aβ) peptides are deposited. Recent studies demonstrate that oligomerization pathways of Aβ peptides may be complicated. To understand the mechanisms of Aβ(1–42) oligomer formation in more detail, we have established a method to produce ^{15}N-labeled Aβ(1–42) suited for nuclear magnetic resonance (NMR) studies. For physicochemical studies, the starting protein material should be solely monomeric and all Aβ aggregates must be removed. Here, we succeeded in fractionating a "precipitation-resistant" fraction of Aβ(1–42) from an "aggregation-prone" fraction by high-performance liquid chromatography (HPLC), even from bacterially overexpressed Aβ(1–42). However, both Aβ(1–42) fractions after 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) treatment formed amyloid fibrils. This indicates that the "aggregation seed" was not completely monomerized during HFIP treatment. In addition, Aβ(1–42) dissolved in HFIP was found to display a monomer–dimer equilibrium, as shown by two-dimensional ^{1}H–^{15}N NMR. We demonstrated that the initial concentration of Aβ during the HFIP pretreatment altered the kinetic profiles of Aβ fibril formation in a thioflavin T fluorescence assay. The findings described here should ensure reproducible results when studying the Aβ(1–42) peptide.

Alzheimer's disease (AD) is a common cognitive disorder found predominantly in older people. The major pathological observations of AD patients are the extracellular deposition of amyloid beta (Aβ peptides [1] and intracellular deposition of the tau protein [2] in brain tissue. A large body of research shows that Aβ plays a key role in AD. Aβ peptides, which consist of 39 to 43 residues, constitute a major component of the senile plaques [1,3]. Senile plaques are polymorphous Aβ deposits containing amyloid fibrils that were observed in the brains of AD patients and people without AD. Amyloid fibrils are insoluble fibrous aggregates and are sensitive to thioflavin T (ThT) fluorescence. Although amyloid fibrils are a major risk factor, recent research has led to an expanded hypothesis that other soluble and non-fibrillar Aβ oligomers with toxicity (e.g., globulomers [4], amyloid β-derived diffusible ligands [5], amylospheroids [6–8]) may be important in determining whether a person suffers from AD. These different Aβ oligomers are based around observations that mature fibrils are less soluble in aqueous environments and have low toxicity in vitro [9,10]. Currently, the absence of atomic resolution structures of these Aβ oligomers hinders our understanding of Aβ physicochemistry and AD pathology.

The first important step in the mechanistic study of Aβ amyloid fibril formation and oligomerization is the preparation of peptide material with high physical homogeneity. Two major methods for the production of Aβ(1–42) peptide monomers are used: recombinant expression in bacteria and chemical synthesis (particularly

Abbreviations used: AD, Alzheimer's disease; Aβ, amyloid beta; ThT, thioflavin T; NMR, nuclear magnetic resonance; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; IB, inclusion body; HPLC, high-performance liquid chromatography; MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; 3D, three-dimensional; TEM, transmission electron microscopy; 2D, two-dimensional.

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solid-phase synthesis). Bacterial expression systems provide a cost-effective method to introduce stable isotopes when compared with chemical synthesis approaches. Preparation of an isotopically labeled Aβ(1–42) peptide is particularly important for nuclear magnetic resonance (NMR) experiments.

Simmons and coworkers and Soto and coworkers have reported independently that sample handling and conditions can influence the morphology of the Aβ peptide [11,12]. In addition, bacterially prepared Aβ is more prone to precipitation than preparation using chemical synthesis [13,14]. These reports suggest that slight differences in the sample-handling process or a trace contamination of an aggregation "seed" during purification steps could alter the kinetics of Aβ oligomerization and result in a distinct morphology of the Aβ peptide (e.g., spheric oligomers, fibrils, non-fibrillar precipitates). The seed is considered as a small invisible trace of Aβ oligomer that enhances aggregation [15]. The seed enhances fibril formation and non-fibril Aβ aggregation. The contaminated seed is difficult to remove by any purification process and is a critical issue for Aβ researchers because "seed-free," monomeric, and highly homogeneous Aβ peptide materials, especially Aβ(1–42), are required for Aβ experiments. We addressed this problem using two approaches: (i) establishing a method for the separation of the seed-free Aβ peptide and (ii) assessing a method that reverts Aβ aggregates to monomer species.

Stine and coworkers reported that dissolving lyophilized Aβ(1–42) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), which is known to be a strong α-helix inducer [16,17], induces monomerization of Aβ aggregates [18]. Thus, exposure of Aβ to HFIP (HFIP treatment) is widely used for sample preparation. However, there have been several reports that self-association still occurs when high concentrations of Aβ(1–42) are dissolved in HFIP [19–21]. Thus, we speculate that HFIP treatment might not always guarantee a pure Aβ(1–42) monomer population. If so, it is unclear what concentration of Aβ(1–42) in HFIP is suitable to monomerize Aβ(1–42), although a final Aβ(1–42) concentration of 1 mM in HFIP was often used in these reports [19–21].

In this study, we have succeeded in separating a fraction containing "precipitation-resistant" (relatively seed-free) Aβ(1–42) peptide and a fraction containing precipitation-prone (probably "highly seed-contaminated") peptide, starting from bacterially expressed Aβ(1–42) peptide. During the study, we found that HFIP treatment did not completely transform both the precipitation-resistant and precipitation-prone fractions into seed-free Aβ(1–42) peptide samples. These observations suggest that HFIP treatment did not completely monomerize the seed. Accordingly, we further analyzed both the precipitation-resistant and precipitation-prone fractions of ^{15}N-labeled Aβ(1–42) in HFIP by NMR. We found that the Aβ(1–42) peptide displayed an unexpected monomer–dimer equilibrium. The dissociation constant of this monomer–dimer equilibrium and the interfacial residues for the dimer contact were determined. Finally, we demonstrated that the concentration of Aβ during the HFIP treatment altered the kinetic profiles of Aβ fibril formation, as monitored by a real-time ThT fluorescence assay.

Materials and methods

Preparation of Aβ(1–42) monomer

Aβ(1–42) peptide was expressed as a fusion protein with an N-terminal histidine × 6 tag followed by yeast ubiquitin [His$_{\times 6}$-Ub-Aβ(1–42)]. The expression vector encoding His$_{\times 6}$-Ub-Aβ(1–42) was constructed using a standard polymerase chain reaction cloning protocol with plasmid pMALc2-Aβ(1–42), a gift from Daizo Hamada (Kobe University), as a template. The ^{15}N- or ^{13}C/^{15}N-labeled recombinant proteins used for NMR experiments were expressed in *Escherichia coli* BL21(DE3) cells in M9 minimal medium at 37 °C in the presence of ^{13}C[glucose] and $^{15}NH_4Cl$ as the sole sources of carbon and nitrogen, respectively. We chose 37 °C for the cultivate temperature to obtain His$_{\times 6}$-Ub-Aβ(1–42) as inclusion bodies (IBs). This was necessary to avoid degradation of Aβ(1–42) from endogenous proteases. The harvested cells were lysed by ultrasonication in lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysozyme. The cell lysate was centrifuged to collect the pellet containing IBs. The washed IBs were solubilized in denaturing buffer containing 6 M guanidine hydrochloride and then applied to DEAE Sepharose Fast Flow (GE Healthcare UK). The solubilized inclusion body fractions were further purified using Ni Sepharose 6 Fast Flow (GE Healthcare UK) and refolded on the column by stepwise dilution of guanidine hydrochloride. The His$_{\times 6}$-Ub tag was cleaved using yeast ubiquitin hydrolase 1, and the resulting Aβ(1–42) was purified using high-performance liquid chromatography (HPLC) using a ZORBAX 300 Extend C18 column (Agilent Technologies, USA). The purified Aβ(1–42) was lyophilized, dissolved in 30% acetonitrile containing 0.1% trifluoroacetic acid (TFA), and then lyophilized again. HFIP (Kanto Chemical, Japan) was added to make final Aβ(1–42) concentrations of 1, 0.1, and 0.05 mM. Aliquots of Aβ(1–42) (50 nmol/tube) were lyophilized and stored at −20 °C until use. The purity of the Aβ(1–42) was assessed by Western blotting (data not shown) as well as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) (Bruker, Ultraflex III) using α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich, USA) as a matrix.

Real-time ThT fluorescence assay

The real-time ThT fluorescence assay was performed with a Hitachi F-7000 fluorescence spectrometer. The excitation and emission wavelengths were set to 440 and 484 nm, respectively. The lyophilized Aβ(1–42) was dissolved in 20 μl of dimethyl sulfoxide (DMSO), immediately diluted in 2 ml of 0.5 × phosphate-buffered saline (PBS), and then added to 10 μl of 1 mM ThT. Finally, the solution was transferred to a stirred cuvette, and the fluorescence intensity was measured every 5 min at 37 °C. The ThT assay by periodical sampling for longer time scale was also performed. The excitation and emission wavelengths were set to 440 and 484 nm, respectively. The lyophilized Aβ(1–42) was dissolved in 20 μl of DMSO and then immediately diluted in 2 ml of 0.5 × PBS. The solution was incubated at 37 °C with rotating. Every 150 μl of the solution was sampled at 6, 12, 18, 24, 31, 36, 42, 48, 56.5, 64, and 72 h after starting incubation, and then the sampled solution was added to 0.74 μl of 1 mM ThT. Finally, the solution was transferred to microcell and the fluorescence intensity was measured at 37 °C.

Transmission electron microscopy

The lyophilized Aβ(1–42) was dissolved in DMSO and then diluted in 0.5 × PBS to a final concentration of 25 μM. The solution was sampled two times: at 60 and 180 min after adding 0.5 × PBS. The Aβ(1–42) samples were applied onto glow-discharged, carbon-coated copper grids (Okenshoji, Japan). The samples (5 μl) were stained with 2% uranyl acetate (5 μl) and then washed three times with 2% uranyl acetate (total 10 μl). Transmission electron micrographs were recorded using a JEM1200 EX-II (JEOL, Japan) at 70 kV with Electron-Microscopic Film FG (Fujifilm, Japan).