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Aβ40 has a subtle effect on Aβ42 protofibril formation, but to a lesser degree than Aβ42 concentration, in Aβ42/Aβ40 mixtures

Shana E. Terrill-Usery [1], Benjamin A. Colvin [1], Richard E. Davenport, Michael R. Nichols*

Department of Chemistry and Biochemistry, Center for Nanoscience, University of Missouri, St. Louis, USA

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ABSTRACT

Recent findings suggest that the senile plaques in Alzheimer's disease may contain soluble amyloid-β peptide (Aβ) fibril precursors along with insoluble fibrils. These soluble Aβ species, including oligomers and protofibrils, have been well-studied *in vitro* and are formed via non-covalent self-assembly of Aβ monomers. While both 40- and 42-residue forms of Aβ are observed in the human body, the majority of the Aβ aggregation work has been conducted on Aβ42 or Aβ40 separately, with relatively few investigations of mixtures. In order to study the effect of different combinations of Aβ40 and Aβ42 on protofibril formation, mixtures of either dry solid peptide, or purified Aβ40 and Aβ42 monomer solutions were mixed together and protofibril/monomer distributions were quantified. Increases in the Aβ42/Aβ40 ratio increased protofibril formation but the presence of Aβ40 in the mixed Aβ solutions had a significant negative impact on protofibril formation compared to equivalent solutions of pure Aβ42. Protofibril size was less affected, but β-sheet structure increased with protofibrils formed from higher Aβ42/Aβ40 ratio solutions. Direct measurement of Aβ42/Aβ40 ratios by C-terminal-selective ELISA found very little Aβ40 incorporated into protofibrils. The cumulative data emphasizes the critical importance of Aβ42, yet establishes Aβ40 as a regulator of Aβ42 aggregation.

1. Introduction

Alzheimer's disease (AD) is the most prevalent type of dementia in older adults and is caused by progressive neurodegeneration in the hippocampus and the amygdala in affected patients [1]. As of 2013, AD was the sixth leading cause of death in the United States. It has been estimated that 5.3 million Americans are currently living with AD and in the coming years the number will increase significantly due to the aging baby boom generation [2,3]. Furthermore, it is estimated that the number of people currently living with dementia worldwide (46.8 million) is expected to double in 20 years [4], Two pathological hallmarks, neurofibrillary tangles (NFTs) and senile (amyloid) plaques, still dominate the biochemistry of AD [5]. NFTs are intraneuronal deposits of insoluble filamentous aggregates of tau, a microtubule-associated protein. Senile plaques are extracellular deposits in the brain parenchyma of insoluble amyloid-β protein (Aβ) fibrils. Clinicopathologic studies have shown that the clinical progression of AD correlates closely with the density and distribution of abnormally-folded tau NFTs in the brain; while the initial cause of the disease appears to be linked to Aβ aggregation and accumulation [6].

Aβ is a collection of secreted peptide fragments produced by β- and γ-secretase-catalyzed proteolytic cleavage of the amyloid precursor protein (APP). The cleavage yields Aβ fragments 37–43 amino acids in length (reviewed in Refs. [7–10]). The two most commonly-studied fragments are Aβ40 and Aβ42. Both peptides are found circulating normally in the blood and cerebrospinal fluid with Aβ40 at much higher levels than Aβ42 [11,12]. Even though the two isoforms differ by only two amino acid residues, their biophysical properties are very different. It is widely recognized that Aβ42 is more fibrillogenic than Aβ40 [13] and this property has many ramifications on the disease process. In early-onset familial AD, mutations in the APP gene surrounding the γ-secretase cleavage site alter Aβ metabolism by increasing the total Aβ amount and the Aβ42/Aβ40 ratio [14–16]. While the circulating levels of Aβ are dominated by Aβ40, senile plaques consist overwhelmingly of Aβ42

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β protein; aCSF, artificial cerebrospinal fluid; DLS, dynamic light scattering; HFIP, hexafluoroisopropanol; R_H, hydrodynamic radius; R_g, root mean square radius; MALS, multi-angle light scattering; SEC, size exclusion chromatography; ThT, thioflavin T.

* Corresponding author. Department of Chemistry and Biochemistry, University of Missouri, One University Boulevard, St. Louis, Missouri 63121, USA.
E-mail address: nicholsmic@umsl.edu (M.R. Nichols).

[1] Denotes an equal contribution by the first two authors.

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[17]. Aβ deposits in the leptomeningeal vessels also have a greater percentage of Aβ42 [18]. Furthermore, studies in mouse models, human presenilin-expressing mouse fibroblast lines, and human plasma indicate that the total Aβ concentration may not be as important as the Aβ42/Aβ40 ratio for plaque formation [14,19–21]. These observations, along with other findings, support the critical, and detrimental, role of Aβ42 in AD pathogenesis [22]. In fact, the Aβ42 level in cerebrospinal fluid (CSF) is one component of an important biomarker test for AD patients that also includes CSF tau and amyloid imaging [23]. The reduction in circulating plasma and CSF Aβ42 is believed to be due to aggregation and deposition of Aβ42 in the brain prior to onset of AD symptoms [24,25].

Much of the detailed knowledge about Aβ aggregation kinetics and mechanisms has been acquired through *in vitro* studies using synthetic peptides. These studies have been invaluable for understanding the process by which Aβ undergoes non-covalent self-assembly in solution and identifying the conditions that modulate this process. There is general agreement that unstructured monomeric Aβ42 and Aβ40, at sufficient concentration, will nucleate and begin to form soluble oligomers [26,27] and protofibrils with significant β-sheet content [28–30]. The initial nucleation step, conformational pathway, and subsequent oligomerization can be influenced by pH, ionic strength, temperature, and agitation [28,31–33]. Many of the soluble Aβ species ultimately progress to insoluble fibrils, which are indistinguishable from those isolated from brain tissue [34]. Although fibril-bearing senile plaques are the most visible Aβ pathological feature, the failure of insoluble Aβ load to correlate strongly with memory loss [35] significantly increased the interest in soluble Aβ aggregates for their role in neurodegeneration [36,37].

As discussed above, numerous *in vivo* investigations have established that the Aβ42/Aβ40 ratio is an important factor in AD pathology. Despite this information, most studies utilize only one form of Aβ. Although fewer in number, there have been several reports on Aβ aggregation kinetics and mechanisms in Aβ42/Aβ40 mixtures with most suggesting that Aβ40 inhibits Aβ42 aggregation. For example, the inclusion of monomeric Aβ40 suppressed both Aβ42 monomer and protofibril conversion to fibrils and failed to promote Aβ42 fibril elongation [38]. Other reports have demonstrated shorter lag times and/or faster aggregation rates as the Aβ42/Aβ40 solution ratio increased [39,40]. It has been postulated that Aβ40 inhibits Aβ42 aggregation by preferentially binding to Aβ42 aggregates thereby preventing further polymerization [39]. A contrasting theory maintains that Aβ40 potentially delays Aβ42 aggregation through “non-productive” interactions [41]. The findings that a minor increase in the solution Aβ42/Aβ40 ratio increases neurotoxicity [40,42] emphasizes the importance of understanding the aggregation dynamics of Aβ42/Aβ40 mixtures and the impact on Aβ structure and biological activity.

The current study examined the effect of varying Aβ42/Aβ40 ratios on protofibril formation. Protofibrils are a well-characterized member of an ever-broadening class of soluble Aβ aggregates. Their formation *in vivo* may play a role in AD pathogenesis making them an important potential therapeutic target [43]. The investigation revealed marked changes in both the extent of protofibril formation and the character of the protofibrils as the Aβ42/Aβ40 ratio changed. The findings demonstrated profound effects by Aβ42 on the initial nucleation phase, which is closely coupled to rapid protofibril formation.

2. Materials and methods

2.1. Aβ preparation

Aβ42 and Aβ40 peptides were obtained from W.M. Keck Biotechnology Resource Laboratory (Yale School of Medicine, New Haven, CT) in lyophilized form, individually dissolved in 100% hexafluoroisopropanol (HFIP) (Sigma-Aldrich, St. Louis) at 1 mM, separated into aliquots in sterile microcentrifuge tubes, and evaporated uncovered at room temperature overnight in a fume hood. The following day, the aliquots were vacuum-centrifuged to remove any residual HFIP and stored in desiccant at −20 °C. Preparation of Aβ42/Aβ40 dry peptide mixtures was accomplished by resuspending Aβ42 and Aβ40 aliquots separately in 100% HFIP and mixing the two solutions in the desired amounts to form either pure Aβ42 or Aβ40 or molar Aβ42/Aβ40 ratios of 4:1, 1:1, and 1:4. The volumes used to prepare the planned dry peptide ratios were calculated to yield a total Aβ concentration of 200 μM upon reconstitution. Briefly, Aβ42 and Aβ40 (1 mg) were resuspended in 100% HFIP to a concentration of 1 mM. Ratios of 4:1, 1:1, and 1:4 Aβ42/Aβ40 were prepared by mixing selected volumes of each Aβ/HFIP into three separate tubes. The volumes used were 160 μL Aβ42 (0.72 mg) and 40 μL Aβ40 (0.17 mg) for 4:1; 100 μL Aβ42 (0.45 mg) and 100 μL Aβ40 (0.43 mg) for 1:1; and 40 μL of Aβ42 (0.18 mg) and 160 μL Aβ40 (0.69 mg) for 1:4. The Aβ42/Aβ40 HFIP mixtures were evaporated overnight in a fume hood uncovered. Vacuum centrifugation was used to remove any remaining HFIP and samples were stored in a desiccant at −20 °C.

2.2. Size exclusion chromatography

Dry Aβ42/Aβ40 peptide mixtures were dissolved in 50 mM NaOH to yield a 2 mM solution followed by dilution to 200 μM Aβ in prefiltered artificial cerebrospinal fluid (aCSF, 15 mM $NaHCO_3$, 1 mM Na_2HPO_4, 130 mM NaCl, 3 mM KCl, pH 7.8) and incubation for 30 min at 25 °C to allow protofibril formation. Protofibrils and monomers were separated as previously described [44]. The Aβ solutions were centrifuged at 18,000 *g* for 10 min and the supernatant was fractionated using size exclusion chromatography (SEC) on a Tricorn Superdex 75 10/300GL column (fractionation range 3–70 kD, GE Healthcare) attached to an AKTA FPLC system (GE Healthcare). Bovine serum albumin (Sigma) was routinely run each day to prevent non-specific binding of Aβ to the column matrix. Aβ was eluted at 0.5 mL min^{-1} in aCSF and 0.5 mL fractions were collected and immediately placed on ice. Aβ concentrations were determined in-line by UV absorbance using an extinction coefficient for Aβ of 1450 cm^{-1} M^{-1} at 280 nm. For aggregation of Aβ42/Aβ40 monomer mixtures directly isolated by SEC, solutions were diluted with aCSF to 40 μM total Aβ, supplemented with 0.05% sodium azide (NaN_3) to prevent contamination, and quiescently incubated at 25 °C for 25 days with numerous measurements during the incubation. For aggregation of Aβ42/Aβ40 monomer mixtures prepared from separately SEC-purified Aβ42 and Aβ40 monomers, solutions were mixed together at different ratios to a total Aβ concentration of 40 μM in aCSF buffer and incubated at 37 °C without disturbance for 24 h. Following an 18,000 *g* spin, supernatants were further separated on SEC in-line with light scattering.

2.3. Dynamic light scattering

Hydrodynamic radius (R_H) measurements were made as previously described [44]. Measurements were taken at room temperature with a DynaPro Titan instrument (Wyatt Technology, Santa Barbara, CA). Samples (30 μl) were placed into a quartz cuvette and light scattering intensity was collected at a 90° angle using a 5-s acquisition time. Particle diffusion coefficients were calculated from auto-correlated light intensity data and converted to R_H with the Stokes-Einstein equation using Dynamics software (version 6.12.0.3). Histograms of percent intensity vs. R_H were generated by