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Stability of early-stage amyloid-β(1–42) aggregation species ☆

Kelley A. Coalier, Geeta S. Paranjape, Sanjib Karki, Michael R. Nichols *

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

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ABSTRACT

Accumulation of aggregated amyloid-β protein (Aβ) is an important feature of Alzheimer's disease. There is significant interest in understanding the initial steps of Aβ aggregation due to the recent focus on soluble Aβ oligomers. In vitro studies of Aβ aggregation have been aided by the use of conformation-specific antibodies which recognize shape rather than sequence. One of these, OC antiserum, recognizes certain elements of fibrillar Aβ across a broad range of sizes. We have observed the presence of these fibrillar elements at very early stages of Aβ incubation. Using a dot blot assay, OC-reactivity was found in size exclusion chromatography (SEC)-purified Aβ(1–42) monomer fractions immediately after isolation (early-stage). The OC-reactivity was not initially observed in the same fractions for Aβ(1–40) or the aggregation-restricted Aβ(1–42) L34P but was detected within 1–2 weeks of incubation. Stability studies demonstrated that early-stage OC-positive Aβ(1–42) aggregates were resistant to 4 M urea or guanidine hydrochloride but sensitive to 1% sodium dodecyl sulfate (SDS). Interestingly, the sensitivity to SDS diminished over time upon incubation of the SEC-purified Aβ(1–42) solution at 4 °C. Within 6–8 days the OC-positive Aβ42 aggregates were resistant to SDS denaturation. The progression to, and development of, SDS resistance for Aβ(1–42) occurred prior to thioflavin T fluorescence. In contrast, Aβ(1–40) aggregates formed after 6 days of incubation were sensitive to both urea and SDS. These findings reveal information on some of the earliest events in Aβ aggregation and suggest that it may be possible to target early-stage aggregates before they develop significant stability.

1. Introduction

An early event in the progression of Alzheimer's disease (AD) is the accumulation of aggregated amyloid-β protein (Aβ) which appears to precede tau neurofibrillary tangle formation [1]. Proteolytic cleavage of the amyloid-β precursor protein results in the production of two predominant forms of Aβ monomer, (1–40) and (1–42) [2]. Aβ aggregation manifests itself in different forms, the classical dense core neuritic plaques found in the brain parenchyma [3] as well as soluble aggregated species in the AD cortex [4]. While there is significant debate regarding the most deleterious aggregated form of Aβ, there is a general agreement on the important role of Aβ(1–42) relative to Aβ(1–40) in AD. The additional two hydrophobic amino acid residues on Aβ(1–42) give the peptide a significantly increased propensity for aggregation [5] and many of the genetic mutations that cause early-onset AD increase the ratio of Aβ(1–42) relative to Aβ(1–40) [6]. Furthermore, neuritic plaques consist overwhelmingly of Aβ(1–42) [7] and in vitro studies indicate that Aβ(1–42) forms a greater variety of oligomeric species [8].

In vitro studies of Aβ aggregation have provided significant kinetic and structural information on the process by which unstructured monomers noncovalently self-assemble into higher-order oligomeric [9,10], protofibrillar [11–13], and fibrillar [14] states. It is well known that protofibrils and fibrils contain substantial β-sheet structure [15,16] although less is known about the structure of early aggregation species such as low molecular weight oligomers.

Studies of Aβ aggregation are frequently limited by the capability or sensitivity of particular techniques. Early investigations utilized turbidity [5] or retention of insoluble filtrate [17] to monitor Aβ fibril formation but this method only evaluated the advanced, insoluble, stages of aggregation. Thioflavin T (ThT) fluorescence can be used to detect the formation of soluble Aβ aggregates but its effectiveness is dependent on the concentration, size, and extent of fibrillar structure as ThT does not bind oligomeric Aβ as well as fibrils [18]. Conventional microscopy methods (atomic force and electron) have provided exceptional macrostructure analysis of protofibrils and fibrils but have been less effective at imaging lower-order oligomeric Aβ species. Given the interest in soluble oligomeric and protofibrillar Aβ species, it remains an important objective to understand some of the early events in Aβ aggregation. One strategy that appears to display significant sensitivity is the development and use of conformation-specific antibodies. These antibodies have been shown to detect particular aggregated species in solution and in human tissue samples and

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β protein; GuHCl, guanidine hydrochloride; HFIP, hexafluoroisopropanol; SDS, sodium dodecyl sulfate; SEC, size exclusion chromatography; ThT, thioflavin T

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* Corresponding author at: Department of Chemistry and Biochemistry, University of Missouri-St. Louis, One University Boulevard, St. Louis, MO 63121, USA. Tel.: +1 314 516 7345; fax: +1 314 516 5342.
E-mail address: nicholsmic@umsl.edu (M.R. Nichols).

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cerebrospinal fluid [10,19,20]. One of these, OC antiserum, is able to detect certain elements of fibril structure across a broad spectrum of Aβ aggregate sizes [21]. The soluble population of the OC-positive Aβ species has been termed fibrillar oligomers and these species have been observed in thioflavin-S negative diffuse deposits in human brains [22] demonstrating the high sensitivity of OC antisera for fibrillar structural components in low molecular weight oligomers. In this study we utilized OC antisera to characterize Aβ(1–42) oligomers at their earliest formation and to highlight stark differences between Aβ(1–42) and Aβ(1–40).

2. Materials and methods

2.1. Preparation of Aβ peptides

Aβ(1–42) was obtained from W.M. Keck Biotechnology Resource Laboratory (Yale School of Medicine, New Haven, CT) in lyophilized form and stored at −20 °C. Aβ(1–40) was prepared by solid phase synthesis in the Structural Biology Core at the University of Missouri-Columbia as described previously [23]. Aβ(1–42) L34P was graciously provided by Dr. Ron Wetzel (Pittsburgh Institute for Neurodegenerative Diseases, University of Pittsburgh School of Medicine). Aβ(1–42) peptides were 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. Aβ(1–40) peptides were initially treated with 100% trifluoroacetic acid, separated into aliquots in sterile microcentrifuge tubes, and vacuum-centrifuged to dry peptide. The Aβ(1–40) samples were then dissolved in 100% HFIP, dried overnight, and vacuum-centrifuged in the same manner as the Aβ(1–42) peptides.

2.2. Size exclusion chromatography

Two different methods were used to prepare Aβ (0.5–1.5 mg) for SEC. For higher yields in the monomer fractions, lyophilized peptides were reconstituted in 10 mM NH_4OH containing 6 M guanidine hydrochloride (GuHCl). The solution was centrifuged at 18,000 *g* for 10 min with a Beckman-Coulter Microfuge 18 and the supernatant was fractionated on a Superdex 75 HR 10/30 column (GE Healthcare) in the desired elution buffer. For yields of both protofibril and monomer, lyophilized Aβ was dissolved in 50 mM NaOH to yield a 2.5 mM Aβ solution. The solution was then diluted to 250 μM Aβ in prefiltered (0.22 μm) buffer of choice, centrifuged and eluted as described above. Prior to injection of Aβ, Superdex 75 column was coated with 2 mg bovine serum albumin (BSA, Sigma) to prevent any non-specific binding of Aβ to the column matrix. Following a 1 mL loading of the sample, Aβ was eluted at 0.5 mL min^{-1} in the buffer of choice and 0.5 mL fractions were collected and immediately placed on ice. Aβ concentrations were determined by UV absorbance using an extinction coefficient of 1450 cm^{-1} M^{-1} at 280 nm. Some Aβ aggregates were prepared by incubation of SEC-purified monomer at room temperature under gentle agitation or quiescent incubation at 37 °C. SEC-purification of Aβ in different buffers including Tris–HCl pH 8.0, phosphate-buffered saline (PBS) pH 7.4, and F-12 cell culture medium (1 mM NaH_2PO_4, 14 mM $NaHCO_3$, 131 mM NaCl, 3 mM KCl, 0.003 mM $MgCl_2$, 0.3 mM $CaCl_2$, 10 mM glucose) without phenol red pH 7.4 did not alter the results obtained in the current study. We have recently demonstrated the preparation and isolation of Aβ protofibrils in the physiologically compatible F-12 cell culture medium [24].

2.3. Thioflavin T fluorescence measurements

Aβ solutions were assessed by ThT fluorescence as described previously [25]. Aβ aliquots were diluted to 5 μM in 50 mM Tris–HCl pH 8.0 containing 5 μM ThT. Fluorescence emission scans (460–520 nm) were acquired on a Cary Eclipse fluorescence spectrophotometer using an excitation wavelength of 450 nm and integrated from 470 to 500 nm to obtain ThT relative fluorescence values. Buffer controls did not show any significant ThT fluorescence in the absence of Aβ. All ThT fluorescence numbers are reported in relative fluorescence units.

2.4. Dot blot analysis

All steps in the dot blot assay were conducted at 25 °C and were modified from the methods previously described [26]. Briefly, 2 μL of Aβ(1–42) at the described concentrations was applied to moist nitrocellulose, allowed to stand for 20 min, and then blocked with 10% milk in PBS with 0.2% Tween 20 (PBST). Following a wash step with PBST, the membrane was incubated with OC serum (1:5000) (gift from R. Kayed, University of Texas Medical Branch, Galveston, TX) or Ab9 antibody (1:5000) (gift from T. Rosenberry, Mayo Clinic Jacksonville, Jacksonville, FL) for 1 h with gentle shaking, washed, and incubated with a 1:1000 dilution of an anti-rabbit IgG (OC) or anti-mouse IgG (Ab9) HRP conjugate (R&D Systems) for 1 h. After washing, the nitrocellulose membrane was then incubated with enhanced chemiluminescent substrate and exposed to film. The dot blot limit of detection for OC antisera was below 0.045 μg Aβ loaded on the nitrocellulose (i.e. 2 μL of a 5 μM solution) and below 0.009 μg Aβ (i.e. 2 μL of a 1 μM solution) for Ab9 antibodies (data not shown).

2.5. Stability studies

Stability assessment of OC-positive Aβ species was done by incubating SEC-purified protofibrillar or monomeric Aβ for 10 min at room temperature with either 4 M urea, 4 M GuHCl or 1% SDS. Following the incubation, 2 μL of the Aβ solution was analyzed by dual dot blots using either Ab9 or OC as the primary antibodies. For long-term SDS stability studies, an Aβ(1–42) monomer fraction was stored at 4 °C and the same fraction was assessed for SDS stability on subsequent days as described above.

3. Results

3.1. OC antibody reactivity in Aβ samples directly after SEC-purification

Aβ in lyophilized form was routinely reconstituted at basic pH followed by dilution into neutral buffers and purification by SEC. A representative elution trace is shown in Fig. 1A after separation of freshly-reconstituted Aβ(1–42) on a Superdex 75 column. This particular preparation regimen produces an excluded (void) peak containing protofibrils and a single included peak representing low molecular weight (LMW) Aβ [12]. Although LMW Aβ displays non-ideal chromatographic behavior eluting at times indicative of dimer or trimer, numerous experiments including translational diffusion measurements have demonstrated that the peak is primarily monomeric [12,27] but in rapid equilibrium with small amounts of dimers and low-n oligomers [28,29]. Our own studies have shown that Aβ(1–42) and Aβ(1–40) monomers elute at similar times [24] and multi-angle light scattering in-line with SEC of radiomethylated Aβ(1–40) determined a monomeric molecular weight [30]. Fractions from the monomer peak were assessed for ThT binding/fluorescence and also by dot blot analysis. Primary antibodies used in the dot blot procedure were Ab9, which is selective for N-terminal residues 1–16 and recognizes all conformational forms of Aβ [31], and OC antisera which recognize structural elements of fibrils but across a wide size spectrum [21]. ThT fluorescence was not present in the monomer fractions for all Aβ peptides tested (data not shown) yet an OC-positive species could be consistently observed in Aβ(1–42) monomer fractions (Fig. 1). SEC-purified Aβ(1–40) monomer fractions were not reactive with OC-antisera nor were monomer fractions