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Conversion of non-fibrillar β-sheet oligomers into amyloid fibrils in Alzheimer's disease amyloid peptide aggregation ☆

Núria Benseny-Cases, Mercedes Cócera, Josep Cladera *

Unitat de Biofísica, Departament de Bioquímica i de Biologia Molecular, i Centre d'Estudis Biofísics, Facultat de Medicina, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

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Abstract

Aβ(1–40) is one of the main components of the fibrils found in amyloid plaques, a hallmark of brains affected by Alzheimer's disease. It is known that prior to the formation of amyloid fibrils in which the peptide adopts a well-ordered intermolecular β-sheet structure, peptide monomers associate forming low and high molecular weight oligomers. These oligomers have been previously described in electron microscopy, AFM, and exclusion chromatography studies. Their specific secondary structures however, have not yet been well established. A major problem when comparing aggregation and secondary structure determinations in concentration-dependent processes such as amyloid aggregation is the different concentration range required in each type of experiment. In the present study we used the dye Thioflavin T (ThT), Fourier-transform infrared spectroscopy, and electron microscopy in order to structurally characterize the different aggregated species which form during the Aβ(1–40) fibril formation process. A unique sample containing 90 μM peptide was used. The results show that oligomeric species which form during the lag phase of the aggregation kinetics are a mixture of unordered, helical, and intermolecular non-fibrillar β-structures. The number of oligomers and the amount of non-fibrillar β-structures grows throughout the lag phase and during the elongation phase these non-fibrillar β-structures are transformed into fibrillar (amyloid) β-structures, formed by association of high molecular weight intermediates.

Keywords: Amyloid; Oligomer; Fibril; Fluorescence; Infrared

Amyloid peptides Aβ(1–40) and Aβ(1–42) are the main components of the proteinaceous deposits found in central nervous systems affected by Alzheimer's disease [1–3]. Numerous 'in vitro' studies with these peptides have shown their capacity to form the so called amyloid fibrils, rich in β-structure [4]. Monitoring of the aggregation process that leads to the formation of fibrils has revealed sigmoid-shaped kinetics, usually interpreted as a consequence of a nucleation-dependent polymerization process. In such a process two distinct phases can be defined: a first, slow nucleation phase (formation of nuclei from peptide monomers) followed by a fast elongation phase during which fibrils are formed [5]. A detailed description of the molecular mechanisms that define each one of these phases is at present still lacking and different interpretations are at hand on the nature and the number of intermediates which would lead to the formation of nuclei and fibrils. Besides the existence of peptide monomers and fibrils, low (2–8 monomers) and high (20–40 monomers) molecular weight oligomers and protofibrils have been defined in the literature [6–8]. In the last few years evidence has accumulated indicating that Aβ toxicity is linked to the presence of oligomeric species rather than to mature fibrils [9,10]. This means that for a complete understanding of the cytotoxic mechanisms a full characterization of the processes by which monomers fold and oligomerize is necessary. Different studies have addressed the secondary structure characterization of oligomeric species [8,11]. Although in some

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* Corresponding author. Fax: +34 935811907.
E-mail address: josep.cladera@uab.cat (J. Cladera).

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cases structural measurements have been carried out as a function of time [8], it is not possible from the literature to establish a clear correlation between these structural kinetics and the aggregation kinetics measured using amyloid specific dyes such as Thioflavin T (ThT) or scattering techniques. Such a comparison would allow to clearly place the different aggregated species and their secondary structures on the corresponding phase (lag phase, elongation phase, plateau) of the aggregation sigmoid.

In the present paper, we have undertaken a study directed to the structural and morphological characterization of the different aggregated species that form along the process by which Aβ(1–40) aggregates into amyloid fibrils. In order to achieve this goal the aggregation kinetics have been monitored in real time using the fluorescent dye ThT. At different times corresponding to the different phases of the detected sigmoid, aliquots have been taken in order to carry out Fourier-transform infrared spectroscopy (FTIR) measurements (secondary structure determination) and electron microscopy (morphology studies). The results show that during the lag (nucleation) phase oligomers form which already contain a significant amount of aggregated non-fibrillar β-structure. Interestingly, the total amount of β-structure remains almost unchanged from half of the nucleation phase up to the end of the aggregation process. Being the main difference between these two time points a change in the fluorescence of the ThT dye (which reflects the formation of amyloid fibrils) we conclude that during the elongation phase, a transition from non-fibrillar aggregated β-structures into fibrillar β-structures by combination of globular oligomers is the main conformational change.

Materials and methods

Synthetic Aβ(1–40) [Cl^- · DAEFRHDSGYEVHHQKLV FFAEDVG SNKG AIIGLMVGGVV] was purchased from JPT (Germany). Stock solutions were prepared by dissolving the peptide (250 μM) in 10 mM deuterated HEPES buffer, pD 8, containing 0.02% NH_3 (the peptide shows no tendency to aggregation at alkaline pH [12]). After sonication the peptide was diluted to 90 μM (the concentration at which the aggregation experiments were carried out), and the solution was stored at −20 °C until needed. Thioflavin T (ThT) was purchased from Sigma Chemical Company and dissolved (8 mM) in 10 mM deuterated HEPES pD 7.4.

Fluorescence measurements. Aβ(1–40) aggregation kinetics were monitored using the dye ThT, which fluorescence is dependent on the formation of amyloid aggregates. Fluorescence measurements were carried out in a SLM-Aminco 800 spectrofluorometer. Excitation and emission wavelengths were set at 450 and 490 nm, respectively. Fluorescence measurements were performed at 37 °C upon continuous and gentle stirring. Peptide concentration was 90 μM. ThT concentration was 35 μM. At different time aliquots (40 μl) were taken from the 2.2 ml fluorescence cuvette for infrared spectroscopy and electron microscopy measurements.

Infrared spectroscopy. Fourier-transform infrared spectra were recorded at 37 °C on a FTIR Mattson Polaris spectrometer equipped with a liquid nitrogen cooled mercury–cadmium–telluride (MCT) detector, at a nominal resolution of 2 cm^{-1}. The spectrometer was continuously purged with dry air (dew point lower than −60 °C). The sample (30 μl) was deposited between two CaF_2 windows separated by a 50 μm teflon spacer. One-thousand scans were averaged for each infrared spectrum. In order to obtain the infrared spectrum of the peptide, a spectrum of the solvent was subtracted from that of the sample (peptide + solvent).

Transmission electronic microscopy. Ten microliters of sample from the fluorimeter cuvette were placed for 10 min on a copper grid with a carbon surface and dried with Watman paper. The sample was stained with 2% (w/v) Uranyl acetate for 2 min and dried. Transmission electron micrographs were obtained using a Hitachi H-7000 (75 kV) microscope.

Results

ThT fluorescence measurements

The ThT fluorescence variation of two independent suspensions of Aβ(1–40) in Hepes buffer at pD 7.4 as a function of time is shown in Fig. 1A. The experimental data points reveal the typical amyloid aggregation curves with a lag phase in which no fluorescence variation is detected (nucleation phase), followed by the so called elongation phase, in which the ThT fluorescence increase denotes the formation of amyloid fibrils. The clear existence of a lag phase denotes the absence of amyloid fibrils in our initial preparations. As shown by Hortschansky et al. [13], the reproducibility of the fluorescence curves and the duration of the lag phase are dependent on the peptide concentration. In the present study a peptide concentration of 90 μM has been used in order to ensure a good reproducibility. Moreover such a peptide concentration has made possible the acquisition of infrared (FTIR) spectra and electron micrographs using aliquots taken from the fluorimeter cuvette at different times (indicated with arrows in Fig. 1A). The observed lag times do compare well with those reported by Hortschansky et al. [13] for a similar peptide concentration. However, other studies [8] report much slower aggregation processes. The analysis of the bibliography clearly shows that the duration of the aggregation process is highly dependent on whether or not filtration or other equivalent methods are used in order to eliminate already aggregated species in the sample. In the present study no such methods have been used as part of the protocol followed for sample preparation (see Materials and methods).

Fourier-transform infrared spectroscopy (FTIR) measurements

The amide I region of the infrared spectra corresponding to the different aliquots taken from one of the kinetics measured in Fig. 1A is shown in Fig. 1B. The amide I is dominated by two main features. A band centered at around 1626 cm^{-1}, indicative of the existence of intermolecular, aggregated β-structures, and a broader band centered around 1645 cm^{-1}, which can be assigned to a mixture of unordered and helical structures [14–16]. Both bands are detected very early into the aggregation process (∼2 h) and its relative intensity, although it seems to vary a bit, does not greatly change with time. Together with the spectra corresponding to the different times along the aggregation process, the Aβ(1–40) spectrum at pD 8.0 is