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Aggregation and Fibril Morphology of the Arctic Mutation of Alzheimer's A β peptide by CD, TEM, STEM and *in situ* AFM

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Abstract

Morphology of aggregation intermediates, polymorphism of amyloid fibrils and aggregation kinetics of the “Arctic” mutant of the Alzheimer's amyloid β -peptide, A β ₍₁₋₄₀₎(E22G), in a physiologically relevant TRIS buffer (pH 7.4) were thoroughly explored in comparison with the human wild type Alzheimer's amyloid peptide, wt-A β ₍₁₋₄₀₎, using both *in situ* atomic force and electron microscopy, circular dichroism and thioflavin T fluorescence assays. For arc-A β ₍₁₋₄₀₎ at the end of the ‘lag’-period of fibrillization an abrupt appearance of ~3 nm size ‘spherical aggregates’ with a homogeneous morphology, was identified. Then, the aggregation proceeds with a rapid growth of amyloid fibrils with a variety of morphologies, while the spherical aggregates eventually disappeared during *in situ* measurements. Arc-A β ₍₁₋₄₀₎ was also shown to form fibrils at much lower concentrations than wt-A β ₍₁₋₄₀₎: 2.5 μ M and 12.5 μ M, respectively. Moreover, at the same concentration, 50 μ M, the aggregation process proceeds more rapidly for arc-A β ₍₁₋₄₀₎: The first amyloid fibrils were observed after *ca* 72 hours from the onset of incubation as compared to approximately 7 days for wt-A β ₍₁₋₄₀₎. Amyloid fibrils of arc-A β ₍₁₋₄₀₎ exhibit a large variety of polymorphs, at least five, both coiled and non-coiled distinct fibril structures were recognized by AFM, while at least four types of arc-A β ₍₁₋₄₀₎ fibrils were identified by TEM and STEM and their mass-per-length statistics were collected suggesting supramolecular structures with two, four and six β -sheet laminae. Our results suggest a pathway of fibrillogenesis for full-length Alzheimer's peptides with small and structurally ordered transient spherical aggregates as on-pathway immediate precursors of amyloid fibrils.

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Keywords

Amyloid β -peptide; Arctic mutation; spherical aggregates; polymorphism of amyloid fibrils; AFM; TEM; STEM; mass-per-length measurements; real time growth; CD; ThT assay

1. Introduction

Alzheimer's disease (AD) is the most common age-related dementia whose hallmark is the abundance of amyloid plaques in the brain of AD patients. The principal constituent of these plaques are fibrils mainly composed of amyloid β -peptides most commonly as 40 or 42 amino acid long $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ variants. It is generally accepted that aggregation of $A\beta$ -monomers or oligomers is involved in AD pathogenesis and there is increasing evidence that specific fibrillar species and, in particular, prefibrillar intermediates (oligomers) play a central role in the neurodegeneration (Selkoe, 1995; Lansbury, 1999; Dahlgren et al., 2002; Lashuel et al., 2002; Walsh et al., 2002; Petkova et al., 2005; Lal et al., 2007; Chimon et al., 2007; Inoue, 2008; Zheng et al., 2008; Small, 2009; Ono et al., 2009; Ahmed et al., 2010; Jang et al., 2010b; Sandberg et al., 2010), but as yet the precise mechanism is unknown. The structures of $A\beta$ -monomers, dimers and oligomers have been visualized with high-resolution scanning tunneling microscopy at sufficient resolution to suggest the folding of the polypeptide chain (Losic et al., 2006). Nevertheless, different fibril morphologies can display distinguished molecular structures and neurotoxicity (Petkova et al., 2005; Petkova et al., 2002; Paravastu et al., 2008; Tycko et al., 2009; Miller et al., 2010). Supramolecular models for $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ have been obtained using structural constraints from STEM mass-per-length measurements, solid-state nuclear magnetic resonance spectroscopy (NMR) (Petkova et al., 2002; Petkova et al., 2005; Paravastu et al., 2008), as well as cryo-electron microscopy (Sachse et al., 2008; Schmidt et al., 2009). Although some features of the fibril structure obtained from these techniques are self-consistent, there are also differences in the model of fibril architecture determined from solid-state NMR and later from cryo-EM (Fändrich et al., 2011). From AFM measurements of $A\beta$ bound to model phospholipid membranes Lal and co-workers have suggested that non-fibrillar $A\beta$ oligomers may cause neuronal cell degeneration by formation of transmembrane cationic channels, which may disrupt the cellular Ca^{2+} homeostasis (Lal et al., 2007; Jang et al., 2010b). Already experiments on hypothalamic neurons (Kawahara et al., 1997) with the molecular chaperone $\alpha\beta$ -crystallin (Steger et al., 1999) and PC12 cells (Chromy et al., 2003) verified that oligomeric $A\beta$ intermediate globular assemblies are potent neurotoxins in the absence of any fibril formation. Sandberg et al., 2010, recently reported on a remarkably larger caspase-3/7 activity in a human neuroblastoma cell line, SH-SY5Y, incubated in the presence of cysteine-cysteine cross-linked model peptide $A\beta_{(1-42)}$ CC 100 kDa β -sheet oligomers, compared to amyloid fibrils. It is likely that different types of soluble amyloid oligomers have a common macroscopic structure and that they share a common mechanism of toxicity (Lashuel et al., 2002; Lashuel et al., 2003; Kaye et al., 2003). One of the most convincing pieces of experimental evidence for the ion-channel hypothesis came, however, from high-resolution atomic force microscopy (AFM) imaging of $A\beta$ monomers incorporated in liposomes and lipid membranes (Rhee et al., 1998; Lin et al., 1999; Lin et al., 2001). In those experiments it has been shown that the peptide appears in globular structures, which do not form fibrils for an extended period of time. When incorporated into reconstituted membranes, many amyloid molecules form typical channel-like structures and elicit single ion-channel currents (Quist et al., 2005). AFM has further been used to study toxic effects on cells (Kawahara et al., 1997; Bhatia et al., 2000; Zhu et al., 2000). Since the oligomeric aggregates are responsible for AD toxicity, the structure and the whole pathway of aggregation kinetics from $A\beta$ -oligomers/protolibrils to fibrils have been extensively investigated (Lansbury, 1999; Harper et al., 1997; Kirkitadze et al., 2001; Stine et al., 2003). Reviews of various

experimental studies of amyloidosis of Alzheimer's amyloid- β peptides and on supramolecular structure of amyloid fibrils can be found in a number of publications (Antzutkin, 2004; Tycko, 2006; Heise, 2008; Yang et al., 2010; Goldsbury et al., 2011; Fändrich et al., 2011).

The 'Arctic' E22G point mutation of $A\beta_{(1-40)}$ (arc- $A\beta_{(1-40)}$) and $A\beta_{(1-42)}$ (arc- $A\beta_{(1-42)}$) is a rare mutation found in a few families in northern Sweden, leading to an early onset (52–57 y. o.) of Alzheimer's disease (Nilsberth et al., 2001). A majority of mutations within the beta-amyloid region of the amyloid precursor protein (APP) gene cause inherited forms of intracerebral hemorrhage. Most of these mutations may also cause cognitive impairment, but the Arctic APP mutation is the only known intra-beta-amyloid mutation to date causing the more typical clinical picture of Alzheimer disease (Basun et al., 2008). It has been shown that the Arctic mutation carriers have lower plasma levels of $A\beta$ than normal, while this mutation accelerates both $A\beta$ oligomerization and fibrillogenesis *in vitro* (Dahlgren et al., 2002; Lashuel et al., 2003; Nilsberth et al., 2001; Paivio et al., 2004), and also *in vivo* (Nilsberth et al., 2001; Cheng et al., 2004; Englund et al., 2007). Testing "structure-toxicity" hypothesis Dahlgren et al., 2002, have found that an increase in the diameter of amyloid fibrils of arc- $A\beta_{(1-40)}$ is correlated with a significant decrease in viability of nerve cells *in vitro*.

The conformational transitions during aggregation of 25–30 μM $A\beta_{(1-42)}$, $A\beta_{(1-40)}$, arc- $A\beta_{(1-40)}$ and other mutations of $A\beta$ have been studied in glycine buffer at pH 7.5 by Teplow and co-workers with circular dichroism (CD) (Kirkitaдзе et al., 2001). At a few days from the onset of aggregation the content of the α -helix secondary structure has increased at the expense of the initially predominantly random-coil conformation. Then, after reaching a maximum, the fraction of the α -helical component decreases with time, while the β -sheet component increases. The formation of amyloid fibrils has been detected by electron microscopy in the same samples over the same time intervals (Kirkitaдзе et al., 2001). Subsequently, arc- $A\beta_{(1-40)}$ aggregates have been studied by Lashuel et al., 2003, by electron microscopy. It has been observed that for this mutation both morphology and size distributions of $A\beta$ protofibrils are different compared with those for wt- $A\beta_{(1-40)}$. Several morphologies of arc- $A\beta_{(1-40)}$ aggregates have been identified: (i) relatively compact spherical particles with a diameter of roughly 4–5 nm; (ii) annular pore-like protofibrils; (iii) large spherical particles of diameter 18–25 nm; and (iv) short filaments with a chain-like morphology (Lashuel et al., 2003). The conversion of arc- $A\beta_{(1-40)}$ protofibrils to fibrils proceeds more rapidly than that for protofibrils formed in mixed solutions of wt- $A\beta_{(1-40)}$ /arc- $A\beta_{(1-40)}$, which has been explained by a kinetic stabilization of the protofibrils in the course of co-incubation of arc- $A\beta_{(1-40)}$ with wt- $A\beta_{(1-40)}$ (Lashuel et al., 2003). Both conformations and the size distribution of arc- $A\beta_{(1-40)}$ aggregates have been also studied by Gräslund and co-workers, who have shown that protofibrils of this mutation are relatively stable oligomers with a large distribution of sizes ranging from 100 kDa (~25-mer) to 3000 kDa (~700-mer) with predominantly β -sheet secondary structural motifs (Paivio et al., 2004).

Molecular dynamics simulations of protofibrils of both arc- $A\beta_{(1-40)}$ and wt- $A\beta_{(1-40)}$ using a coarse grain model have revealed remarkable differences in the putative structure of oligomers formed by these two peptides (Fawzi et al., 2008). Despite an extensive number of above mentioned reports on arc- $A\beta_{(1-40)}$ several important issues are still left unexplored: (i) details of the morphological differences in polymorphs of fibrils of arc- $A\beta_{(1-40)}$, which grow and coexist in the same sample; (ii) mass-per-unit length of these fibrils, which is an important constraint needed for developing (yet unknown) supramolecular models for arc- $A\beta_{(1-40)}$ fibrils; (iii) structure and stability of transient aggregation intermediates; (iv) critical concentrations for arc- $A\beta_{(1-40)}$ fibril formation in different buffers at conditions

close to physiological; (v) interaction of arc-A $\beta_{(1-40)}$ with phospholipid membranes; (vi) cellular toxicity of specific aggregation intermediates and different polymorphs of arc-A $\beta_{(1-40)}$ amyloid fibrils.

In the present work aggregation and polymorphism of the human arc-A $\beta_{(1-40)}$, is further studied *in vitro* using a variety of experimental techniques and compared with the behavior of the wild type A $\beta_{(1-40)}$ (wt-A $\beta_{(1-40)}$) at similar conditions. Here, we visualize in detail aggregation events of both wt-A $\beta_{(1-40)}$ and arc-A $\beta_{(1-40)}$ with atomic force microscopy imaging, and complement these studies with both CD and ThT analyses. Furthermore, we present a systematic TEM, STEM and *in situ* AFM investigation of the detailed morphology of A β -fibrils in TRIS buffer solutions and studies by AFM of structure of A β -oligomers and *in-situ* growth of amyloid fibrils adsorbed on a mica sample surface. To develop further the “structure-toxicity” concept, the precise measured EM and AFM structural parameters of different oligomers and polymorphs of arc-A $\beta_{(1-40)}$ fibrils can be employed for development of supramolecular models for these systems using structural constraints from solid-state NMR and other spectroscopic techniques and correlated further with data from neurotoxicity assays.

2. Materials and methods

2.1 Sample preparation

Peptides (wt-A $\beta_{(1-40)}$ and arc-A $\beta_{(1-40)}$) were synthesized, purified by HPLC and lyophilized (with special precautions to avoid formation of pre-aggregates) as previously described by (Antzutkin et al., 2000; Antzutkin et al., 2002; Antzutkin, 2004). A buffer solution was prepared dissolving 10 mM TRIS, 5 mM EDTA, 10 mM KCl and 0.01 wt% NaN₃ in doubly distilled water. pH of the initial buffer solution (100 mL, pH 8.75) was adjusted with 0.55 mL 0.1 M HCl(aq) and later (fine adjustments) with 0.05 M NaOH(aq) to pH 7.4. 50 μ M aqueous stock solutions of peptides were prepared in Eppendorf 1.5 mL plastic test tubes by a gentle mixing of powdered peptides into the buffer. Other concentrations (<50 μ M) were achieved by diluting stock solutions with the buffer. All solutions were kept at room temperature in the course of experiments (ca 293 K).

AFM samples were prepared by depositing a droplet of the peptide solution (usually around 15 μ L) directly onto a freshly cleaved mica surface and leaving it to incubate in a small container for approximately 15 minutes. The samples were then gently rinsed with excess buffer and transferred to the AFM microscope for imaging. Approximately 40–50 μ L of the buffer solution were added to fill the AFM fluid cell.

When interpreting results one should appreciate that the aggregation process of A β is highly dependent on handling factors including: (i) purification and storage of the peptides; (ii) pre-treatment with hexafluoroisopropanol or trifluoroethanol prior to sample incubation; (iii) with or without ultrasound sonication to dissolve peptides, and with or without sample agitation; (iv) salt concentration and buffer composition and (v) pre-freezing of the peptide solutions in liquid nitrogen. Therefore, experimental results reported by different groups are generally difficult to directly compare. In this study the conditions were chosen to be as reproducible as possible in all the experiments to facilitate the comparison of wt-A $\beta_{(1-40)}$ and arc-A $\beta_{(1-40)}$. The peptides were synthesized, purified, and lyophilized directly after freezing, in liquid nitrogen, of the collected HPLC fraction of the monomeric form of peptides in water/acetonitril solution and stored under the same conditions (at -22°C). To remove peptide pre-aggregates, which may affect aggregation kinetics, a standard pretreatment TFA/TFE protocol was used prior lyophilization of the samples. Thereafter, the peptides were gently dissolved, without sonication to avoid facilitation of secondary structural transitions in the peptides (Filippov et al., 2010), in the same freshly prepared

buffer and incubated in the same type of plastic vials without additional agitation. We also found that the pre-freezing of freshly prepared stock buffer solutions of both wt-A β ₍₁₋₄₀₎ and arc-A β ₍₁₋₄₀₎ in liquid N₂ and then thawing the samples for kinetic experiments, changes dramatically the whole aggregation pathway of the peptides leading, for example, to formation of worm-like 'protofilaments' and amorphous nonspherical aggregates previously observed by other groups (see Fig. S2 in Supporting Information). Therefore, we tend to consider these structures as the result of specific *in vitro* preparation protocols and prefer to work only with the peptide solutions freshly dissolved in TRIS buffer at room temperature. We also avoided ultrasound sonication of peptide solutions since even a short-term sonication (from 30 seconds to 2 minutes) leads to a formation of peptides aggregates with a distinct secondary structure depending on the solvent used (Filippov et al., 2010).

2.2 Transmission Electron Microscopy and Scanning Transmission Electron Microscopy

For imaging by electron microscopy, arc-A β ₍₁₋₄₀₎ peptide was first dissolved in TRIS buffer to a final concentration of 50 μ M and fibrils allowed to grow at room temperature without agitation for at least 72 hours. Negatively stained fibrils were then prepared by applying 4 μ L aliquots of the fibrillized peptide solution onto thin carbon substrates supported by lacey carbon films on 300 mesh copper grids. After adsorption for 1 min, the grids were blotted off, washed with several successive drops of deionized water, and counter-stained with 1% uranyl acetate. Excess liquid was removed and the grids dried in air. Specimens of unstained fibrils for mass-per-length (MPL) measurements were prepared by applying 4 μ L aliquots of the peptide solution onto ultrathin carbon support films (< 5 nm in thickness). After adsorption for 1 min, excess liquid was blotted off and the grids washed with several drops of deionized water. Tobacco mosaic virus (TMV) was then co-adsorbed onto the carbon films to serve as an internal mass calibration standard. Grids were finally blotted off, washed, and allowed to dry in air.

Electron microscopy was performed in a Tecnai TF 30 electron microscope (FEI Company) operating at 300 kV and equipped with a Schottky field-emission electron source. Images of negatively stained specimens were acquired in an Ultrascan charge-coupled device (CCD) camera (Gatan). Dark-field STEM images of unstained fibrils were recorded using a Model 3000 in-column high angle annular dark-field detector, Fischione. The pixel size of the STEM images was 0.86 nm and the associated electron dose approximately 2400 e nm⁻². The NIH IMAGE program (see ref. Rasband) was used to measure MPL of the fibrils according to standard procedures described in the literature (Thomas et al., 1994; Müller and Engel, 2006; Sousa and Leapman, 2007).

2.3 Atomic force microscopy and data analysis

AFM images were recorded using a Nanoscope II (Digital Instruments/Veeco, Santa Barbara, CA) with a custom design for tapping mode in fluids (Hellberg and Norlin, 2003). Dedicated electronics are used to detect the amplitude of the preamplified deflection sensor signal. The amplitude is compared with the user defined amplitude setpoint and the difference is sent as the error signal to the regular feedback loop of the Nanoscope II. The time constant of this amplitude detection system is 0.25 ms, which approximately corresponds to 2.5 oscillations of the tapping cantilever under normal imaging conditions. This setup has shown to provide gentle imaging. All imaging, with recording of 400 $\times$ 400 pixel images, were conducted in the buffer solution at room temperature and the standard Veeco fluid cell was used without the silicone O-ring to avoid load on the piezotube scanner and to minimize drift in the AFM imaging. Standard or oxide-sharpened commercially available, 100 μ m long, Si₃N₄ cantilevers, with integrated tips (Digital Instruments) and a nominal spring constant, of 0.32 N/m, were used. These cantilevers were driven close to their resonance frequencies at ~9–11 kHz in the aqueous buffer. Typical scan rates were 1–3

scan lines per second, i.e. a typical acquisition times for a complete AFM image were between 1 and 6 minutes.

The Nanoscope III software (version 4.23R2 or offline version 5.12, Digital Instruments) was used for image visualization and manual measurements. The presented AFM images are raw data, which eventually were flattened by removal of the background. Otherwise, no image filtering or other manipulation was applied to the data except for the image in Fig. 9A, for which a gentle low-pass filter was used. The grayscale, of the top view AFM images presented herein, reflects the height of the surface (brighter means higher). IGOR Pro (Wavemetrics, Lake Oswego, OR) were used with procedures developed in our laboratory, to semi-automatically extract volume, statistics and height distributions of A β aggregates and fibrils. First, the aggregates were found in the image by a threshold technique. The best fitting two-dimensional plane was removed, a simple flatten operation was applied and a slight low-pass filter was used. A histogram of surface heights was extracted and heights above the surface background were considered as candidate aggregates. Moreover, a morphological particle analysis algorithm was applied to recognize aggregates as particles when their areas were greater than 75 nm². Individual heights and volumes were evaluated from the simple flattened original image. For each particle/aggregate, a plane is fitted to the surrounding background, of size fifteen times the area of the aggregate. The height of the particle is evaluated in each point as the distance between this plane and the aggregate surface. Accordingly, the aggregate height is defined as this distance at the maximum height of the aggregate. Typically, 200–600 aggregates in an image are used to form an aggregate height histogram.

2.4 Circular dichroism experiments and analysis

Secondary structures of peptides in solutions were analyzed by circular dichroism (CD). A spectropolarimeter (Jasco J-720, USA) equipped with a quartz cell (Hellma, Germany) with a sample volume of 370 μ L and an optical path of 1 mm was used. CD measurements were carried out in a wavelength range of 190–250 nm. Each sample was measured between eight and 32 times, and the CD spectra were averaged.

2.5 Thioflavin-T fluorescence monitoring of A β fibril formation

Solutions of wt-A β ₍₁₋₄₀₎ and arc-A β ₍₁₋₄₀₎ peptides (ca 70 μ L) were added to the standard polystyrene spectroscopic cuvette containing 1 mL of 5 μ M ThT solution (50 mM Tris, pH 7.4). Fluorescence intensity was measured using USB2000 Fiber Optic Spectrometer (Ocean Optics, Inc., USA) configured for fluorescence measurements, using a laser for excitation (440 nm) and detecting fluorescence at 482 nm. Control solutions of wt-A β ₍₁₋₄₀₎ and arc-A β ₍₁₋₄₀₎/TRIS without ThT were used as references for background signals. Integral intensities of ThT fluorescence corrected for the background signals were plotted as a function of time from the onset of incubation of peptides in the buffer solutions. These plots were indicators of the formation of amyloids rich in cross- β sheet structures exposed to the outer surface of aggregates.

3 Results and Discussion

3.1 Aggregation kinetics of arc-A β ₍₁₋₄₀₎ and wt-A β ₍₁₋₄₀₎: ThT fluorescence and Circular Dichroism measurements

Figure 1 shows results of the thioflavin-T (ThT) fluorescence assay for both arc-A β ₍₁₋₄₀₎ and wt-A β ₍₁₋₄₀₎ peptides incubated at 50 μ M in TRIS buffer solutions at pH 7.4 and room temperature (ca 293 K) without agitation: An 'S'-shaped dependence of fluorescence with time, after a lag period of ca 70 hours, was found for arc-A β ₍₁₋₄₀₎. This type of dependence is typical for self-catalytic processes such as aggregation. After reaching its maximum (at ca

120 hours) the ThT fluorescence gradually decreased to *ca* 60% of its maximum intensity. The growth in intensity of the ThT fluorescence is correlated with the growth of amyloid fibrils detected by AFM at the same time points of the sample incubation (AFM data will be discussed in detail below). The gradual decrease in intensity of the ThT fluorescence after 120 hours of sample incubation is also correlated with clumping and clustering of polymerized amyloid fibrils (also detected by AFM), which may lead to a decrease in surface area of fibrils exposed to the ThT dye. For 50 μ M solutions of wt-A β ₍₁₋₄₀₎ in TRIS under similar experimental conditions only a slight change in the ThT fluorescence intensity was detected during first 7 days of the sample incubation.

Figures 2A and 2B show CD spectra of wt-A β ₍₁₋₄₀₎ and arc-A β ₍₁₋₄₀₎ peptides, respectively, obtained immediately after this peptide was dissolved in the TRIS buffer and then at different time points from the onset of the sample incubation. Changes in the shape of CD spectra for both wt-A β ₍₁₋₄₀₎ and arc-A β ₍₁₋₄₀₎ indicate changes in secondary structure of this peptide upon incubation in the TRIS buffer. For wt-A β ₍₁₋₄₀₎ CONTIN analysis of the CD spectra was employed using DICHROWEB interactive software (Whitmore and Wallace, 2004). Results of this analysis are shown in Fig. 2C. During the first two days of incubation wt-A β ₍₁₋₄₀₎ is in the random coil conformation. But after three days from the onset of incubation of wt-A β ₍₁₋₄₀₎ its secondary structure has already a large α -helical content, which reaches its maximum at day five when even β -structures (both β -sheets and β -turns) are present in the peptide conformation up to *ca* 20%.

In the case of arc-A β ₍₁₋₄₀₎ CD spectra are different from these of wt-A β ₍₁₋₄₀₎ at all times of the sample incubation studied here. The shape of CD spectra of arc-A β ₍₁₋₄₀₎ suggests a mixture of α -helix and β -structures. Unfortunately, the automated CONTIN analysis of the CD spectra using DICHROWEB interactive software (Whitmore and Wallace, 2004) failed to provide unambiguous deconvolutions of CD spectra into components corresponding to different secondary structures in the peptide. This is because of a high UV absorption and noise in the spectral region from 190 to 200 nm. However, the observed ellipticity at 217 nm in the CD spectra decreases substantially in the course of sample incubation. We choose this parameter (ellipticity₂₁₇) to quantify a relative change in the content of β -sheet structure following previously described methods (Paivio et al., 2004). A time dependence of ellipticity₂₁₇ for arc-A β ₍₁₋₄₀₎ is shown in Fig. 2D. This behavior correlates well with ThT data for aggregation of arc-A β ₍₁₋₄₀₎ discussed above (see Fig. 1): Almost no changes in ellipticity₂₁₇ of the peptide were detected during the first two days from the onset of the sample incubation, while prominent changes in this parameter were observed after day three suggesting an increase in the content of β -structures until day five, when this parameter has reached its minimum and did not change substantially further.

3.2 A β -peptides assemble via distinctly shaped intermediate aggregates to fibrils and the process is most rapid for arc-A β ₍₁₋₄₀₎

Samples for AFM investigations were prepared on the mica surface from the peptide test-tube solution (50 μ M, TRIS buffer, pH 7.4) at distinct times after the onset of the aggregation reaction. Initially, the AFM images of the freshly dissolved A β peptides revealed small aggregates of a variable size (see Figs. 3A and 4A). The images indicate that both wt-A β ₍₁₋₄₀₎ and arc-A β ₍₁₋₄₀₎ have globular structures but that their sizes are widely distributed. Their distribution in height (diameter) indicates more frequent sizes at 3–4 nm. The surfaces are uniformly covered with the non-fibrillar, rather featureless, peptide aggregates. Figures 3 and 4 show examples of low magnification AFM images at a few other time points for wt-A β ₍₁₋₄₀₎ and arc-A β ₍₁₋₄₀₎, respectively.

In the case of wt-A β ₍₁₋₄₀₎, no sign of ordered structures is revealed after 55 h of incubation (Fig. 3A). Figure 5 shows profiles and the measured aggregate heights at different times

compiled into histograms. The profiles are measured along the dashed lines in the inset images of Figs. 3 and 4. Each histogram represents height measurements of 200–600 aggregates in images of scan size 2 μm . Besides a significant naturally occurring tip broadening of surface features, it was discovered that the AFM tip sometimes appears contaminated with peptides resulting in a multi apex tip. However, the AFM height measurements are assumed to be rather independent of these imperfections. The height distribution of the grains of wt-A $\beta_{(1-40)}$ is fairly wide at the initial period of incubation, <145 h (Figs. 5A and 5B). Only slightly more order (a sharper distribution of heights) can be noticed after 6 days (145 h, Fig. 5B) and still, no fibrillar structures are seen. Up to this time point, there are two dominating heights of the aggregates, 3.0 ± 1.1 (average $\pm \sigma$) and 6.7 ± 1.0 nm as determined from Gaussian curve fits to the height distributions in Fig. 5B. From both ThT fluorescence (no binding of the dye) and CD measurements for wt-A $\beta_{(1-40)}$ (see Figs. 1, 2A and 2C) we can conclude that these spherical aggregates have predominantly α -helical structure (>70 %). However, on day seven, more distinct localized and ordered nucleation aggregates of wt-A $\beta_{(1-40)}$ appear and an initial fibril formation is observed (172 h, Figs. 5C and 3C). Since that, the transition to more ordered amyloid aggregates (fibrils) is almost fully completed in about 50 hours (219 h, Fig. 3D). The appearance of initial fibrils on day seven has been verified a few times in independent experiments with wt-A $\beta_{(1-40)}$ peptide solutions (50 μM , TRIS buffer, pH 7.4). The ultrastructure of the aggregates at this stage is uniform and distinctly shaped. Cross-section analysis of individual aggregates reveals a fairly flat and only slightly curved uppermost aggregate surface (data not shown), further confirmed by the use of tip-deconvolution software. However, we denoted the aggregates as “spherical aggregates” (SA) since the spherical shape has been found in other studies of similar structures (Goldsbury et al., 2000; Huang et al., 2000; Antzutkin et al., 2002). The aggregate heights are 6.6 ± 0.9 nm as shown in Fig. 5C. Occasionally small fibrils are found with lower heights than the spherical aggregates. Hence, it can be concluded that the fibrils are not simply assembled by the addition of these aggregates as suggested in some previous studies. A smaller number of very small nanometer-sized aggregates are also found to co-exist with the spherical aggregates. The number of SA decreases continuously with time as long fibrils develop. In other words, the reduction of SA correlates with the appearance of amyloid fibrils. Figure 3D shows the situation after 219 h (9 days). Long fibrils but only a minor number of spherical bodies are seen. After another 44 days, SA has completely disappeared and we only observed long mature fibrils (data not shown).

The time dependent assembly of arc-A $\beta_{(1-40)}$ shows similarities with the aggregation process of the wild type peptide. Even at high concentrations (50 μM), arc-A $\beta_{(1-40)}$ fibrils were formed only after prolonged incubation and the build up of fibrils with a lag period reminds a typical phase transition. However, there are a few very important differences, evident when comparing images in Figs. 4 and 3. Initially, for arc-A $\beta_{(1-40)}$ small oligomers/aggregates 1.4 – 5 nm in height were observed (see Figs. 4A and 5D). After 24 hours (Fig. 4B), there are no ordered structures of spherical aggregates and their height is widely distributed as shown in the histograms of Fig. 5E. The distinct localized and ordered spherical aggregates already begin to appear after 48 hours (not shown). The height profiles of individual aggregates, more convincingly reveal a spherical shape than in the experiments on the wt-peptide described above. Figure 4C shows the initial fibril formation, which starts on day three from the onset of incubation of arc-A $\beta_{(1-40)}$. Note that most of the initial fibrils are much higher (brighter contrast) than the spherical aggregates. The formation of a homogeneous population of SA with a distinct structure takes place in between 24 and 72 hours. When SA were completely formed, at 72 hours, the distribution of aggregate heights becomes narrowest with a Gaussian fit to the histogram in Fig. 5F yielding 3.1 ± 0.8 nm. After this time point, the fibril growth is rapid and the population of the spherical aggregates diminishes in a few hours. After 8 days we observe fibrils that have aggregated into large

plaque-like fibril clusters (data not shown). AFM data presented here for arc-A $\beta_{(1-40)}$ agree well with both, an increase in ThT fluorescence (Figure 1) and with an increase in ellipticity₂₁₇ in CD spectra after day 3, both suggesting formation of amyloid fibrils (and/or SA) rich in the cross- β sheet structure. AFM measurements also reveal a lag time of *ca* 70 hours for formation of arc-A $\beta_{(1-40)}$ amyloid fibrils at these experimental conditions. In contrast to our observations of a homogeneous-size population of transient SA forming from arc-A $\beta_{(1-40)}$, it is known from other studies that arc-A $\beta_{(1-40)}$ ‘protofibrils’ are rather stable β -sheet containing aggregates with a heterogeneous-size distribution that form rapidly compared with wt-A $\beta_{(1-40)}$ (Paivio et al., 2004).

When comparing the aggregation of the two peptides, we find that the distinct localized and ordered spherical aggregates (Figs. 5C and 5F) appear for both the wild type peptide (6.6 nm in height) and the Arctic mutation (3.1 nm in height) but substantially different in size and temporal occurrence. Interestingly, it appears that the dimensions of the two peptide SAs differ more than two-fold, unless the aggregates really not have spherical shape and acquire different orientation on the surface. Nevertheless, it takes longer time for wt-A $\beta_{(1-40)}$ than for arc-A $\beta_{(1-40)}$ before the intermediate SA are formed and the polymerization into fibrils starts. Both wt-A $\beta_{(1-40)}$ and arc-A $\beta_{(1-40)}$ assemble to fibrils with a height larger than the individual aggregates. The so-called protofibrils or protofilaments (single filaments of uniform morphology) are widely accepted terms for intermediate aggregates/fibrils that interact with each other to form more complex morphologies such as twisted pairs and coiled structures (Harper et al., 1997; Gregory et al., 1998; Harper et al., 1999; Blackley et al., 2000; Lashuel et al., 2002; Antzutkin et al., 2002; Ding et al., 2002; Lashuel et al., 2003; Modler et al., 2003; Nichols et al., 2005). We did not detect any obvious protofibrils for samples prepared at room temperature; only for samples pre-frozen in liquid nitrogen were the ‘worm’ like protofilaments detected (data not shown). Therefore, the protofibrils did not constitute a necessary step in fibrillogenesis in our study when the fibrils were assembled in test tubes, but most probably are artifacts of the pre-freezing type of sample preparation protocols widely used in many groups working with amyloids. In our additional experiments aggregation of wt-A $\beta_{(1-40)}$ solutions in TRIS were frozen and stored at either -70 or -22°C . Protofibrils were formed at day 3 in samples prepared with these solutions (see Fig. S2 C in Supporting Information). Therefore, it cannot be excluded that protofibrils may form *in vivo* at specific conditions (but, of course, different from low temperature storage in the frozen state) and they putatively may also play a role in AD development.

The aggregate dimensions of A β SA measured here are in the same range as the ones previously measured by other groups. For example, Parbhu et al., 2002, have found that A $\beta_{(1-42)}$ forms two types of aggregates: initially small aggregates (1.5–2.5 nm) and, as fibrillization began, also larger aggregates (4–5 nm). The Arctic variant of fragments A $\beta_{(12-28)}$ has shown to give aggregates with heights about 3 nm (Peralvarez-Marín et al., 2009). Under different preparation conditions Stine et al., 2003, have observed aggregates of A $\beta_{(1-42)}$ peptides with a size of *ca* 1 nm and at longer times of incubation between 2 and 4 nm. The latter AFM measurements have been performed, however, on dried samples with a stiff cantilever for tapping mode. Naturally, such measurements may yield lower aggregate heights due to protein structure collapse and sample deformations. Inversely, tapping mode images in fluid possibly slightly overestimate the heights due to adhesion forces. A small type of aggregates from soluble A $\beta_{(1-40)}$ oligomers has been shown with CD to have a large content of β -sheet structures (Huang et al., 2000). Such smaller-sized β -sheet SA can be invoked to explain some of the differences between the height histograms of the two peptides in Figure 5. Larger self-aggregated A $\beta_{(1-40)}$ spheres, such as SA identified by Huang et al., 2000, are toxic *in vitro* for cell cultures, whereas aggregates with a smaller diameter and fibrils, are supposedly less toxic (Hoshi et al., 2003). The occurrence of SA, coexisting with amyloid fibrils, in samples of arc-A $\beta_{(1-40)}$ has first been observed at

physiological conditions with TEM by Lannfelt and co-workers (Nilsberth et al., 2001) and later in aqueous solutions of this peptide at both pH 3.8 and 7.4 (Antzutkin, 2004). Similar spherical aggregates coexisting with amyloid fibrils in similar sample preparations have been also observed for wt-A β ₍₁₋₄₂₎ (Goldsbury et al., 2000; Huang et al., 2000; Blackley et al., 2000; Antzutkin et al., 2002). The AFM investigation described in this work is complimentary to previous studies in the sense of showing the very apparent phase transformation in both arc-A β ₍₁₋₄₀₎ and wt-A β ₍₁₋₄₀₎ aggregation cascades. On the basis of previously reported CD measurements of Teplov and co-workers it can be suggested that, preceding fibril formation, wt-A β ₍₁₋₄₀₎ may form spherical A β -intermediates with a large content of α -helical secondary structure (>30 %) (Kirkittadze et al., 2001). In the latter study, the α -helical component was observed to be most pronounced after eight days of incubation at a concentration of 25–30 μ M wt-A β ₍₁₋₄₀₎ in a glycine buffer at pH 7.5. Thereafter, the α -helical component decreased and disappeared after two weeks of the sample incubation, while the β -component increased upon transformation of intermediates into amyloid fibrils as observed by TEM. In our study performed at a higher concentration of peptides (50 μ M) and using TRIS buffer instead of glycine buffer, a large content of α -helices was observed for wt-A β ₍₁₋₄₀₎ already after 3 days (Figure 2C). In turn, for arc-A β ₍₁₋₄₀₎ β -sheet component was growing sharply after day 2 (see Fig. 2D), i. e. before the growth of fibrils visible only at day 3 (see Fig. 4C). Therefore, it is reasonable to suggest that SA of arc-A β ₍₁₋₄₀₎ with distinct diameters 3.1 ± 0.8 nm (Fig. 5F), which are formed right before the growth of fibrils, have β -sheet structural fragments. The latter structural motifs most probably contain β -hairpins, as identified in A β ₍₁₋₄₀₎ complexes with Z α β ₃ affibodies, whose structure was recently solved by liquid state NMR (Hoyer et al., 2008).

Our AFM results can be explained by, and supports, the model of key kinetic intermediate spherical aggregates in amyloid β -peptide fibrillogenesis. SA are likely intermediates or A β -oligomers of particular importance as a link to the neurotoxicity. This would favor indirectly, the hypothesis that A β peptides are potent to form ion channels in lipid bilayers and neurons (Arispe et al., 1993). The ion channel hypothesis has lately gained much additional experimental support from AFM studies (Lin et al., 1999; Bhatia et al., 2000; Zhu et al., 2000; Lin et al., 2001; Parbhu et al., 2002) and suggests a dysregulation of Ca²⁺ homeostasis as a key element of AD neurotoxicity. Soluble globular oligomers of A β and other peptides and protein fragments are toxic to cells (Lin et al., 1999; Bhatia et al., 2000; Zhu et al., 2000; Lin et al., 2001; Chromy et al., 2003; Kaye et al., 2003; Sandberg et al., 2010). Furthermore, since α -helix secondary structure is a common structure for transmembrane proteins in the membrane region, it is tempting to believe that the aggregates, i.e. the larger SA, might in fact form possible ion channels in biomembranes. The size and shape of the spherical bodies allows interaction with membranes. However, recent experimental data and theoretical modeling implicate channels with β -sheet structural fragments (Chimon and Ishii, 2005; Singer and Dewji, 2006; de Planque et al., 2007; Jang et al., 2010a). Nevertheless, more experiments, for example solid-state NMR and further AFM studies on arc-A β ₍₁₋₄₀₎ intermediates with phospholipid membranes, are needed to resolve these issues. Supramolecular structure of SA of sonicated wt-A β ₍₁₋₄₀₎ solutions has been examined by solid-state NMR measurements, which have revealed similarities with the structure of amyloid fibrils (Chimon and Ishii, 2005; Chimon et al., 2007). Solid-state NMR measurements on solid samples prepared from non-sonicated buffer solutions of arc-A β ₍₁₋₄₀₎ are currently in progress in our laboratory.

3.3 Arc-A β ₍₁₋₄₀₎ spontaneously assembles into fibrils at lower concentrations than wt-A β ₍₁₋₄₀₎

We compared the influence of wt-A β ₍₁₋₄₀₎ and arc-A β ₍₁₋₄₀₎ peptide concentrations on fibrillogenesis in TRIS buffer solution (pH 7.4). The solutions of peptides were prepared a

long time, several weeks, before the experiments in order to assure fully developed “mature” fibrils. Several samples, with different peptide concentrations, were prepared for each peptide. We observed significant differences in concentration effects on fibrillization between the two peptides. Incubation time for samples shown in Figure 6 was >3 weeks (>500 h). At high wt-A $\beta_{(1-40)}$ concentrations (50 μ M), mica surfaces are covered almost solely of amyloid fibrils. At the lower 12.5 μ M concentration, fibrils occur at a considerably less extent (see Fig. 6A). At a concentration of 4.2 μ M only a very few short fibrils can be observed (data not shown). Figure 6B shows that no fibrillization of wt-A $\beta_{(1-40)}$ have occurred at concentrations as low as 2.5 μ M. For this sample AFM images were also obtained after 10 weeks (> 1700 h) of incubation and neither SA nor amyloid fibrils were detected in this sample, thus, suggesting that the critical concentration of fibril formation is > 4 μ M for wt-A $\beta_{(1-40)}$ in TRIS buffer at pH 7.4 and room temperature. As shown in Figs. 6C and 6D, the critical concentration for fibrillization in TRIS buffer is lower for arc-A $\beta_{(1-40)}$. Long rigid fibrils at all peptide concentrations were observed in our experiments, the lowest being 2.5 μ M. Hence, the critical peptide concentration for fibrillization of arc-A $\beta_{(1-40)}$ is lower than 2.5 μ M, i.e. at least twice below than that for the wild type A $\beta_{(1-40)}$. Note that for samples with a low concentration of peptides both fibrils and SA were detected for wt-A β_{40} and Arc-A β_{40} even after a few weeks of incubation.

3.4 Polymorphism of arc-A $\beta_{(1-40)}$ fibrils

The ultrastructure of amyloid fibrils was studied and characteristic fibril heights, lengths and shapes were measured in the AFM images. Figure 7 shows representative morphologies of both wild type and Arctic A $\beta_{(1-40)}$ fibrils. The measurements are summarized together with the characteristic peptide SA dimensions in Table 1. All wild type fibrils in our experiments are found to coil in a left-handed sense as has been reported also by Goldsbury et al., 2000, who used EM with metal shadowing to demonstrate the handedness of the A $\beta_{(1-40)}$ amyloid twist. The wt-A $\beta_{(1-40)}$ fibril heights are uniform. Surprisingly, the distance between the repeating twists, i.e. the axial crossover spacing (CoS), vary significantly, also within the same fibril. Measurements indicate larger CoS probabilities at approximately 110 and 170 nm repeats, but the data is not statistically sufficient to classify different types of wt-A $\beta_{(1-40)}$ fibrils using this parameter. One example of such fibril is shown in Fig. 7A. It resembles the ‘quiescent’ type of wt-A $\beta_{(1-40)}$ amyloid fibrils with C₃ symmetry: A structural model of these fibrils based on both structural constraints from solid-state NMR and mass-per-length STEM measurements has been recently suggested by Paravastu et al., 2008.

Arctic A $\beta_{(1-40)}$ fibrils exhibit a variety of distinct morphologies, significantly different from the wt-A $\beta_{(1-40)}$ fibrils. We classify at least five different distinct arc-A $\beta_{(1-40)}$ fibril polymorphs, two non-coiled and three coiled, as exemplified in Figs. 7B – 7H. The height profiles along the fibril axis (schematically illustrated in Fig. 7I) are shown in Fig. 7J for all these distinct types of amyloid fibrils. With an incubation period of a few days, the flat non-coiled fibrils, with lengths limited to a few hundred nanometers, are widely observed but they do not appear in samples older than about ten days. They are denoted *Type 1* and *Type 2*, based on their different heights as shown in Table 1. The different heights are possibly from different conformations but could also reflect different orientations on, or interactions with, the mica surface. The coiled fibrils are classified as *Type 3* – *Type 5* according to differences in their CoS, which generally is shorter than for the wild type fibrils. The crossover spacing in arc-A $\beta_{(1-40)}$ fibrils ranges from 30 to 88 nm (Table 1). Our measurement of crossover spacing are consistent with previous measurements on A $\beta_{(1-40)}$ obtained by three-dimensional reconstructions from cryo-EM (Meinhardt et al., 2009), which gave values in the range 65–163 nm, with wider fibrils tending to have longer crossover distances.

Interestingly, as for the wild type, all arc-A $\beta_{(1-40)}$ fibrils on the hydrophilic mica surface are left-handed as exemplified in Figs. 7E – 7H. This observation finds experimental support in the literature by others (Goldsbury et al., 1999; Jarvet et al., 2003; Moreno-Herrero et al., 2004).

TEM and STEM studies also showed diversity in arc-A $\beta_{(1-40)}$ fibril morphology. At least four major types of fibrils were identified in images of negatively stained specimens (Fig. 8A), with fibrils of type I and II being the most abundant. These fibril types correspond to *Type 1* and *Type 2* observed by AFM (see Fig. 7). Type III is coiled and similar to *Type 3* and *Type 4* in AFM images, but on parameters of thickness and homogeneity it better corresponds to *Type 3* observed by AFM. Note, however, that morphological differences between *Type 3* and *Type 4* are rather minor. Type V fibrils in TEM images are not coiled, but they differ apparently from types I and II because of size in the normal direction to the fibril long axes. It may correspond to *Type 5* fibrils identified by AFM, but with a somewhat different pitch, which may depend on either wet (AFM) or dry (TEM) conditions of measurements (compare Figs. 8A(V) with 7H). To perform mass per length (MPL) measurements on the four different fibril morphologies illustrated in Fig. 8A, we also recorded dark-field STEM images from unstained specimens (Fig. 8B and 8C). In these images, intensity integrated along the length of the fibrils can be directly converted to values of MPL through a calibration with co-deposited TMV, whose MPL is known (131 kDa/nm). For each fibril type, MPL data was plotted as a histogram and fitted to a Gaussian function, resulting in well-fitted curves centered at 19.7 (± 2.9), 37.1 (± 3.2), 37.0 (± 4.2) and 54.2 (± 4.8) kDa/nm for fibrils I, II, III and V, respectively (Fig. 8D), where the standard deviation of the measurements are indicated in parenthesis. These values, when divided by the estimated MPL of 9.02 kDa/nm for a single molecule of arc-A $\beta_{(1-40)}$ (Molecular Weight = 4.26 kDa) in a cross- β sheet structure, yield the number of peptide molecules per 0.48-nm repeat of the fibrils: 2.18, 4.10, 4.11 and 6.01 for fibrils I, II, III and V, respectively. In these results, deviations in the number of arc-A $\beta_{(1-40)}$ molecules from integer numbers are probably not real but due instead to measurement errors caused by impurities (e.g., denatured peptides) and/or residual buffer associated with the fibrils. We note that under negative staining fibrils of type II and V appear to be higher order bundles of single protofilaments (type-I (TEM) or *Type 1* (AFM) fibrils). MPL values reveal specifically that 2 and 3 protofilaments of type I associate to form fibrils of type II and V. We also observed occasionally the presence of wider bundles constructed by more than three protofilaments, but these were too few to generate histograms of MPL.

3.5 Real time studies of arc-A $\beta_{(1-40)}$ fibril growth

Finally, the arc-A $\beta_{(1-40)}$ fibrillization growth kinetics was studied *in situ*. The stock solution originally incubated for 73 hours in a plastic tube without additional agitation, was then transferred on the mica surface and fresh buffer was added to fill up the liquid cell. Polymerization of several fibrils, both coiled and non-coiled, were followed with the AFM in real-time, at room temperature, during eight hours of growth. Figure 9 shows one panel of such sequential AFM images. During continuous imaging, the time laps between successive AFM images was 5–10 minutes. The time zero, in this figure, represents a reference time when the AFM sample was prepared (73 h) and the time indicated in each image is the time lag, at which the AFM image was captured, independently of the scan direction (up/down). Initially, few and short fibrils, as the two fibrils in Fig. 9A, are observed together with a large number of spherical aggregates. In the figures 9B – 9I one can follow the growth of fibrils, while the decrease in number of SA on the mica surface during polymerization of arc-A $\beta_{(1-40)}$ into fibrils is apparent. Note, that originally *ca* 20 μ L of the peptide solution was incubated on mica surface for a short time and then fresh buffer was added to fill the whole cell. Eventually, some aggregates were taken by the buffer flow and then could fell

down back on mica surfaces during AFM measurements. However, based on aggregate volume measurements in the images, we do not expect any major supply of peptides from the buffer solution. Therefore, to a good approximation fibrils are grown from the peptide molecules (or smaller structural domains) originally present in SA.

The growth rates were estimated from the AFM images with respect to the times when the AFM tip reaches the growing fibril end. The elongation of arc-A $\beta_{(1-40)}$ fibrils was dominated by growth in one of its ends exhibiting unidirectional growth, which might suggest that the internal structure of the fibril is non-symmetric in the axial growth direction. Possibly, the aggregation site acts as a nucleation site on the surface and the growth proceeds by addition of small building blocks to the growth side of the aggregate/fibril. Moreover, the elongation rates vary significantly in time and between the different polymorphs. For example, the maximum fibril growth rate of the uppermost *Type 3* fibril in Fig. 9 (fibril “a”) is at its “top” end. The lowermost end of this fibril only grew during a short time interval after the total time 4 hours. Some amount of buffer has evaporated during this experiment and new buffer was continuously added, causing some variations in peptide concentrations, which have influenced the growth rate of fibrils during these measurements. The two fibrils in Fig. 9 (fibrils “a” and “b”) have different growth rates, but their change in elongation rate follows each other, possibly reflecting the changes in local concentrations and gradients (see Fig. S1 in Supporting Information). However, we could not simply associate the addition of buffer with the variation in elongation rate. In general, common growth rates for the coiled fibrils are in the range 2.5 to 3.5 nm/min whereas the polymerization of non-coiled fibril (*Type 1*) typically proceeds at a rate of approximately 5 nm/min for fibrils in the AFM images obtained at the same time intervals. These “relative” numbers, however, are in accord with MPL measurements of fibrils discussed above: 37.0 (± 4.2) and 19.7 (± 2.9) kDa/nm for type III and I fibrils, respectively. Therefore, approximately twice as much peptide material is needed for the former type (type III), thus, giving rise to approximately a two-fold lower growth rate of these compared to type I fibrils provided that local concentrations of the peptide do not vary much at the corresponding ends of type III and I fibrils grown in the same AFM image at the same time points. It should be stressed again that these rates are measured for arc-A $\beta_{(1-40)}$ aggregates adsorbed to mica with a fresh buffer added afterwards and *not* in the original peptide solution. Growth rates measured by others in A β peptide solutions are a factor 2–6 higher (Goldsbury et al., 2005). We have, however, verified by *in-situ* AFM similar high growth rates of wt-A $\beta_{(1-40)}$ in TRIS solutions with significant peptide concentrations (data not shown). To summarize, many polymorphs of arc-A $\beta_{(1-40)}$ not only co-exist in the same sample, they grow/shrink at different rates, which is in accord with their different MPLs. Some fibrils in our study did, however, not grow at all or even decomposed (data not shown, see also Antzutkin, 2004). These fibrils, probably, were formed in the test tube solution and then precipitated onto the mica surface in such a way that both ends were not accessible for further growth.

Presumably, the different pathways originate from large variations in the internal structural build up. Different types of fibrils are in different dynamic equilibrium with A β -oligomers and other aggregation intermediates. Since A β -oligomers are believed to be potentially neurotoxic, different fibril polymorphs may indirectly correlate to different grade of neurotoxicity. Understanding the structure of the fibrils is, therefore, essential for the design of possible inhibitors. The supramolecular structure of different polymorphs of the wt-A $\beta_{(1-40)}$ and “Iowa” mutation of A $\beta_{(1-40)}$ amyloid fibrils have been studied by Tycko and co-workers using combined data from solid-state NMR and STEM (Petkova et al., 2002; Petkova et al., 2005; Paravastu et al., 2008; Tycko et al., 2009). The suggested putative models of the wt-A $\beta_{(1-40)}$ fibrils is based on a cross- β -sheet structure with peptide molecules organized in two, three or four parallel β -sheets (Petkova et al., 2002; Petkova et al., 2005; Paravastu et al., 2008). In this respect, our work supports the earlier suggested putative

structural model of wt-A $\beta_{(1-40)}$ fibrils with three (“quiescent” fibrils) folded β -sheet laminae having AFM dimensions of ca. 4.8×8.1 nm (Table 1) with a characteristic “flat-pitch-flat” type of morphology (see Figs. 7A and 7J). In the work of Paravastu et al., 2008, quiescent wt-A $\beta_{(1-40)}$ fibrils have similar dimensions in STEM images (8 nm) and somewhat smaller dimensions in TEM images (4×8 nm), which can be a result of a shrinkage of fibrils upon drying combined with artifacts of staining. It is obvious that more structural work (including solid-state NMR) is needed to elucidate the supramolecular structure of arc-A $\beta_{(1-40)}$ fibrils, which show a high degree of polymorphism comparable to this in the “Iowa” mutation of A $\beta_{(1-40)}$ (Tycko et al., 2009).

When interpreting these results, one should keep in mind that the binding of peptides to the mica surface may influence the results compared to “native” conditions or incubation of fibrils in the test tube. Moreover, the AFM probe may accelerate the fibril growth rate in AFM measurements by physical interactions or by the tapping mode, in which the tip induces acoustic oscillations in the buffer. For example, it has been shown that fibril formation is accelerated by simply shaking or agitating the peptide solution (Goldsbury et al., 2000). Bi-directional growth of wt-A $\beta_{(1-40)}$ protofibrils and human amylin has been observed by other groups (Goldsbury et al., 1999; Blackley et al., 2000). They have reported that higher-order fibrils frequently appeared blocked at one end, though their sample preparation was slightly different from that employed in our work. The present results are in agreement with earlier real-time studies on A β fibril assembly based on optical imaging (Ban et al., 2004), which showed a strong dependence on seeding conditions. Our results are also consistent with previous AFM experiments (Kellermayer et al., 2008), which showed a highly dynamic growth behavior, whereby fibrils grew in successive fast and slow phases that the authors attribute to sporadic changes in subunit structure at the growing ends of the fibrils.

3.6 An alternative A β peptide aggregation model

Previous experiments have supported the generally accepted model for a growth pathway via linkage of protofibrils, or spherical aggregates, as “building blocks” to mature fibrils. However, different fibril morphologies are known to have different underlying molecular structures (Petkova et al., 2002; Petkova et al., 2005; Paravastu et al., 2008; Tycko et al., 2009) and as other groups have noticed, an assembly pathway not related to protofibrils cannot be ruled out (Goldsbury et al., 2005). However, the fibrillization of arc-A $\beta_{(1-40)}$ observed in our study cannot be explained simply by a model of a simple linkage of protofibrils, or spherical aggregates to mature fibrils. This is because: (i) protofibrils were not observed in peptide solutions unless they had been pre-frozen; (ii) the sizes of the SA aggregates and fibril heights did not correlate; (iii) structure of many polymorphs of fibrils cannot be explained via a simple assembly of protofibrils and (iv) no linkage of SA to larger aggregates was observed in the real-time studies. Therefore, another fibrillogenesis pathway has to be invoked to explain *in-situ* AFM data presented here. A speculative process may be initiated by a surface nucleation event. The initially unstructured (random coil) small spherical aggregates are assembled in a slow process and subsequently undergo a slow, phase-transition-like, conformational change, giving rise to spherical aggregates with a distinct supramolecular structure rich in either β -hairpins (Hoyer et al., 2008) or β -sheets (Chimon et al., 2007; Chimon and Ishii, 2005) or both. It is not unlikely that these SA undergo further transformation and decompose into small aggregates/oligomers with predominantly β -sheet structures (hardly seen with AFM). The latter oligomers may assemble into amyloid fibrils with distinct cross- β structures following a model of “unzipping” of β -hairpins with simultaneous “zipping” together A β molecules into parallel β -sheets, as suggested by Härd and co-workers (Hoyer et al., 2008; Sandberg et al., 2010). The key point in such a model is that amyloid fibrils do not simply assemble from

protofibrils or from large spherical aggregates. There is a pathway where SA are the only pathway intermediates. The build up of fibrils occurs via small “building blocks” of spherical aggregates rather than from fusion together of single protofibrils or SA. There have other reports of fibril polymerization without the involvement of protofibrils. For example, Kad et al., 2001, observed growth of mature fibrils without protofibril precursors and Modler et al., 2003, and Gorman et al., 2003, have also demonstrated such possibilities. Our results are in accord with these studies.

4 Conclusions

AFM characterization of A β mutations, supported by TEM, ThT fluorescence and CD data, is valuable approach for helping to elucidate AD pathogenesis. We confirm previous results, which have reported that the Arctic mutation of the A $\beta_{(1-40)}$ peptide is more ‘aggressive’ in its tendency to aggregate than the wild type A $\beta_{(1-40)}$ peptide. Not only does the intermediate phase of spherical aggregates appear at earlier times but also fibrils polymerize more rapidly from the onset of incubation for arc-A $\beta_{(1-40)}$ than for wt-A $\beta_{(1-40)}$. In 50 μ M peptide preparations in TRIS buffer (pH 7.4), fibrils appear after day 3 for the Arctic A $\beta_{(1-40)}$ peptide, whereas for the wild type A $\beta_{(1-40)}$ they do not appear until day 7. Secondly, the mutant showed fibril formation at much lower peptide concentrations than for the wild type peptide. Finally, at late stages we detected fragmentation and clustering of arc-A $\beta_{(1-40)}$ fibrils but not for the wild type fibrils.

Our AFM results are consistent with a model, in which spherical aggregates serve as key kinetic intermediates in amyloid β -peptide fibrillogenesis. Spherical aggregates (containing abundant β -hairpin or/and β -sheet structures), and/or A β -oligomers, are likely intermediates of particular importance as a link to the neurotoxicity. This would favor indirectly the hypothesis that A β peptides have the potential to form ion channels in lipid bilayers and neurons. However, one cannot exclude another hypothesis that β -sheet (or/and β -hairpin) rich A β -oligomers may specifically interact with nerve cell receptors and, thus, these aggregates may trigger cells into the apoptotic cycle.

It should be stressed again that based on the present work, there may exist an alternative fibril aggregation pathway different from a simple assembly of spherical aggregates and protofilaments into fibrils. We believe that our results for this specific mutant are of more general value in understanding the basic processes and fibrillogenesis also for several other Alzheimer’s amyloid peptide mutations and for other amyloidogenic peptides and proteins.

These findings further motivate the use of high-resolution AFM in combination with TEM/STEM and biophysical methods in the future studies to further identify individual fibril morphologies, aggregation kinetics and effects of potential inhibitors of the amyloidosis even for studies on whole cell level, using also methods such as elasticity and adhesion force mapping and mechanical manipulation (Almqvist et al., 2004; Karsai et al., 2006).

Supplementary Material

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Nomenclature

Aβ	amyloid β -peptide
AD	Alzheimer's disease
wt-Aβ₍₁₋₄₀₎	wild type A β ₍₁₋₄₀₎
arc-Aβ₍₁₋₄₀₎	Arctic mutation of A β ₍₁₋₄₀₎
AFM	atomic force microscopy
TEM	transmission electron microscopy
STEM	scanning transmission electron microscopy
CD	circular dichroism
ThT	thioflavin-T

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Figure 1. Thioflavin-T fluorescence intensity at 482 nm for arc-Aβ₍₁₋₄₀₎ (filled circles) and wt-Aβ₍₁₋₄₀₎ (open circles) as a function of time from the onset of incubation of 50 μM peptide solutions in TRIS (pH 7.4).

Figure 2.

CD spectra of: (A) wt- $\text{A}\beta_{(1-40)}$ and (B) arc- $\text{A}\beta_{(1-40)}$ peptides obtained at different time (days) after onset of incubation of 50 μM peptide solutions in Tris (pH 7.4); (C) CONTIN analysis of CD spectra of wt- $\text{A}\beta_{(1-40)}$ using DICHROWEB interactive software (Whitmore and Wallace, 2004); (D) Ellipticity at 217 nm (a measure of a content of β -structures) as a function of the incubation time of arc- $\text{A}\beta_{(1-40)}$.

Figure 3.

AFM height images of wt-A $\beta_{(1-40)}$ aggregates at different time points. All images are of size 4000 nm and grayscale coded with the height from 0 (dark) to 30 nm (bright), except for the inset images, which are of size 200 nm $\times$ 200 nm, with a height scale 0–20 nm, to better visualize individual aggregates. (A) 55 h; (B) 145 h; (C) 172 h and (D) 219 h after the onset of the sample incubation. Virtual additional faint images in inserts of A and C are unavoidable artefacts from a ‘double’ AFM tip, because of aggregates of the peptide adhered to the tip.

Figure 4. AFM height images of arc-A $\beta_{(1-40)}$ aggregates at different time points. The image scales are identical to Fig. 3. (A) 2.5 h; (B) 24 h; (C) 72 h and (D) 74 h after the onset of the aggregation reaction.

Figure 5.

Aggregate heights: (A–F) are probability histograms of the aggregate heights at different times after the onset of the sample incubation. Each histogram represents height measurements of 200–600 aggregates in images of scan size 2 μm . (A–C) Height histograms of the wt-A $\beta_{(1-40)}$ aggregates at times 55, 145 and 172 h. (D–F) Height histograms of arc-A $\beta_{(1-40)}$ aggregates at times 2.5, 24 and 72 h. The smaller inset histograms are extracted from images of scan size 1 μm . The axis scaling is identical for all histograms. (G–H) Profiles along the dashed lines in the images of Figs. 3 and 4.

Figure 6. AFM height images reveal differences between wt- $A\beta_{(1-40)}$ and arc- $A\beta_{(1-40)}$ in critical peptide concentrations of fibrillization. All image sizes are 10 μm . (**A** and **B**) Wild type $A\beta_{(1-40)}$ at peptide concentrations 12.5 and 2.5 μM . (**C** and **D**) Fibrillization of Arctic $A\beta_{(1-40)}$ at peptide concentrations 6.3 and 2.5 μM . More extensive fibril formation is observed for arc- $A\beta_{(1-40)}$ despite lower concentrations of the peptide.

Figure 7.

Morphology of Aβ fibrils visualized with AFM height images. The height scale is in every image grayscale coded from 0 (dark) to 18 nm (bright). (A) A typical 'quiescent' fibril of wt-Aβ₍₁₋₄₀₎. (B–H) Polymorphism of arc-Aβ₍₁₋₄₀₎ fibrils. These fibrils are classified into the different types. The *Type 1* and *Type 2* are non-coiled fibrils and the *Type 3 – Type 5* are left-handed coiled fibrils. (I) A schematic fibril illustrating the path along the fibril axis, for which fibril cross-sections are shown in J. (J) Profiles of cross-sections of different types of both wt-Aβ₍₁₋₄₀₎ and arc-Aβ₍₁₋₄₀₎ fibrils. The profiles are offset in height for convenience, with minimum values shown as dashed lines.

Figure 8.

(A) Selection of four major fibril types I – IV of arc-A $\beta_{(1-40)}$ identified in transmission electron micrographs of negatively stained specimens. (B) Dark-field STEM image of unstained fibrils with co-deposited TMV. Fibril types I and III are present in this one field of view. White boxes show characteristic regions along fibrils, TMV and background used to calculate MPL values. (C) Major fibril types I through IV imaged in STEM. (D) Histograms of MPL measurements on the four different fibril morphologies depicted in (C). Each individual peak was fitted to a Gaussian function yielding MPL values of 19.7, 37.1, 37.0, and 54.2 kDa/nm for fibrils I through IV, respectively. The integer values above the histograms indicate for each fibril type the number of arc-A $\beta_{(1-40)}$ molecules per cross- β repeat. Scale bars, 20 nm (A, C) and 100 nm (B).

Figure 9.

Real time AFM height imaging of arc-A $\beta_{(1-40)}$ *Type 3* fibril growth on a mica surface. The panel of sequential images (A–I) shows the same $645 \times 860 \text{ nm}^2$ area at different times. The reference time (0 h 00 min) is arbitrary chosen as the time for the 73 hours old sample (incubated in the test tube) in A. The time in hours and minutes is shown in each image. The vertical height scales are equal for all images, from 0 (dark) to 18 nm (bright). All images are digitally zoomed from original images of size $2 \times 2 \mu\text{m}$ except for A, which was zoomed from a $6 \times 6 \mu\text{m}$ image and hence it has a poorer resolution. The black arrow in all images is set as a reference point, i.e. indicating the same surface spot in each image. Apparently, a number of spherical aggregates decreases as the fibrils polymerize. A virtual additional faint image in B–D and F is an artefact from an unavoidable double AFM tip because of aggregates of the peptide adhered to the tip. The fibril partly seen in the lower right corner of each image is of *Type 4*.

Summary of AFM measured dimensions of aggregates and fibrils. The abbreviation SA is used for spherical aggregates. The heights on the coiled fibrils are measured in between and on top of the crossovers, respectively.

Table 1

Aggregate:	wt-Aβ ₍₁₋₄₀₎ SA	arc-Aβ ₍₁₋₄₀₎ SA	wt-Aβ ₍₁₋₄₀₎ fibrils		arc-Aβ ₍₁₋₄₀₎ fibrils				
			Non-coiled types		Coiled types				
			1	2	3	4	5		
Height (nm)	6.6 ± 0.9	3.1 ± 0.8	4.8 ± 0.5	3.4 ± 0.3	5.0 ± 0.4	6.5 ± 0.4	7.1 ± 0.6	5.2 ± 0.7	
Crossover height (nm)	-	-	8.1 ± 0.8	-	-	8.4 ± 0.4	10.2 ± 0.9	11.1 ± 0.8	
Crossover spacing (nm)	-	-	~110, ~170	-	-	30 ± 3	40 ± 5	88 ± 10	