The Solvent Protection of Alzheimer Amyloid-β-(1–42) Fibrils as Determined by Solution NMR Spectroscopy*

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Alzheimer disease is a neurodegenerative disorder that is tightly linked to the self-assembly and amyloid formation of the 39–43-residue-long amyloid-β (Aβ) peptide. Considerable evidence suggests a correlation between Alzheimer disease development and the longer variants of the peptide, Aβ(1–42/43). Currently, a molecular understanding for this behavior is lacking. In the present study, we have investigated the hydrogen/deuterium exchange of Aβ(1–42) fibrils under physiological conditions, using solution NMR spectroscopy. The obtained residue-specific and quantitative map of the solvent protection within the Aβ-(1–42) fibril shows that there are two protected core regions, Glu1-Gly25 and Lys28-Ala42, and that the residues in between, Ser26 and Asn27, as well as those in the N terminus, Asp1-Tyr10, are solvent-accessible. This result reveals considerable discrepancies when compared with a previous investigation on Aβ-(1–40) fibrils and suggests that the additional residues in Aβ-(1–42), Ile41 and Ala42, significantly increase the solvent protection and stability of the C-terminal region Lys28-Ala42. Consequently, our findings provide a molecular explanation for the increased amyloidogenicity and toxicity of Aβ-(1–42) compared with shorter Aβ variants found in vivo.

Protein assemblies in the form of amyloid fibrils are today linked to a group of ~20 different syndromes, of which Alzheimer disease (AD),2 as well as various forms of prion disorders, are among the most well known. Amyloids, in general, are composed of predominantly β-sheet structures, where the β-strands are arranged perpendicular to the fibrillar axis into a common so-called cross-β pattern (1–4). AD is a neurological disorder presenting itself as progressive dementia. The pathology is tightly linked to the aggregation of a 39–43-residue-long peptide fragment denoted amyloid-β (Aβ) derived as a result of proteolytic processing of the considerably larger amyloid precursor protein. Aggregated Aβ peptides are found in AD brains in the form of diffuse and senile plaques as well as in cerebrovascular tissues. Considerable experimental evidence suggests an important role of Aβ-(1–42/43) in the progression of AD, because it represents the main constituent of the first deposits found in the course of disease development (5, 6) and if overproduced may result in early onset AD (7, 8) (for a recent review, see Ref. 9). The mechanisms by which a cytotoxic effect is exerted in vivo and the reasons why a pathologic self-aggregation is induced in certain individuals are complex and at present not completely understood. Prevention of Aβ assembly therefore constitutes a considerable therapeutic challenge, where an increased understanding regarding the properties of amyloid, and the pathways leading to its formation, is of utmost importance.

Because of the generic quaternary structure and the large size of amyloid structures, elucidation of their architecture provides a complicated problem, where traditional methods, such as crystal diffraction and solution NMR, are not readily applicable. However, two recent crystallographic studies on fibrous micro-crystals, grown from peptides with either seven or twelve residues, have revealed many interesting structural details about the cross-β spine of fibrils (10, 11). Solid-state NMR performed on dried fibrils provides an alternative to the above mentioned techniques and has been used extensively to investigate the structure of Aβ amyloid. Studies on Aβ-(10–35), Aβ-(1–40), and Aβ-(1–42) suggest an arrangement where fibrils are formed by extended parallel β-strands arranged into two sheets (12–14). The results suggest a fibrillar core involving residues Val12, Val24 and Ala30-Ala42 and a loop spanning between residues Val24-Ala30 (15). The structural restraints obtained from these solid-state NMR studies suggest a model where parallel β-sheets are organized in exact register (16). Other models have been proposed (9), among them β-helical (17) and nanotube (18) models.

To gain information about the structural and dynamical properties of fibrils in an aqueous environment, a novel method using solution NMR spectroscopy in combination with hydrogen/deuterium (H/D) exchange was developed (19, 20). This technique relies on the partial solvent protection of amide protons, either as a result of their involvement in hydrogen bonds between β-strands within the fibril or as a result of solvent exclusion. In aqueous solutions, amide protons located at the exterior of the fibril are more accessible to solvent compared with amide protons buried within the fibril interior, and consequently these will experience a higher hydrogen exchange rate. Through exchange of the surrounding water by D2O, followed by a rapid conversion of the fibrils into a monomeric NMR-detectable state, the H/D exchange pattern of the amyloid can be measured indirectly using solution NMR. The method pinpoints the fibrillar core in a residue-specific and quantitative manner and gives a view of the fibrillar dynamics in solution. This approach has therefore turned out to be a powerful complement to solid state measurement and has offered a more complete picture of the fibrillar properties of the amyloid in several cases (19–25).

This study utilizes H/D exchange experiments and solution NMR spectroscopy to probe, for the first time, the fibrillar core of amyloid fibrils derived from Aβ-(1–42). The obtained solvent protection pattern identifies two well protected regions, a result that differs considerably from a similar study on Aβ-(1–40) (21). The additional residues, Ile41 and Ala42, appear to significantly stabilize the fibril assembly, in
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...particular the C-terminal parts of the peptide. These results provide a molecular explanation to the increased amyloidogenicity of Aβ-(1–42) compared with the shorter variants of Aβ found in vivo.

EXPERIMENTAL PROCEDURES

NMR Spectroscopy and Resonance Assignment of Aβ-(1–42)—Isotope-enriched chemicals were purchased from Cambridge Isotope Laboratories. According to a newly established procedure, uniformly 15N-labeled Aβ-(1–42) was produced recombinantly in Escherichia coli. A typical NMR sample contained 1 mM recombinant Aβ-(1–42), 80% 1H, 1.3, 3.3-hexafluoropropanol-d2 (HFIP)/20% H2O (v/v), and 150 mM NaCl buffered to pH 3.0 using d3-acetate and d6-acetic acid. NMR experiments were carried out at 15 °C on a Bruker AVANCE spectrometer operating at 600 MHz (proton frequency) and equipped with a 5-mm triple resonance, pulsed field z-gradient cryoprobe. Recorded homonuclear two-dimensional clean total correlation spectroscopy and heteronuclear two-dimensional 15N-HSQC, as well as three-dimensional 15N-DIPSI-HSQC and 15N-nuclear Overhauser effect spectroscopy-HSQC experiments, were processed using NMRPipe (26) or XWINNMR (Bruker Biospin) software. The sequence-specific backbone resonance assignment of Aβ-(1–42) was carried out in Amsig for Windows (27) following the standard strategy for 15N-labeled proteins.

H/D Exchange of Aβ-(1–42) Fibrils—The fibrillar solution was prepared by incubating a sample of ~1 mM 15N-labeled Aβ-(1–42) in double distilled H2O containing 50 mM NaCl at 37 °C for 10–12 days during slow agitation. The resulting gel-like solution was diluted 30 times using D2O, pH 7.0. To ensure an essentially complete removal of H2O, the washing procedure was repeated once. These two samples were subsequently incubated in D2O for 20 and 120 min, respectively, which includes the duration for the buffer exchange procedure. The third sample contained fully protonated fibrils and served as a control. The pelleted fibrils in the three samples were rapidly dissolved into monomers in 80% HFIP/20% D2O (v/v) and 150 mM NaCl, as a control. The pelleted fibrils in the three samples were subsequently incubated in D2O for 1300 s and at low temperatures, amide proton exchange rates could be minimized. Furthermore, an induction of secondary structure with an increasing concentration of NaCl up to a nearly saturated solution of ~150 mM salt (data not shown). The observed 15N-HSQC spectrum of the Aβ-(1–42) peptide in this HFIP/water solution and 150 mM NaCl, pH 3.0, displayed a well dispersed spectrum with an optimal separation of peaks at 15 °C, a temperature at which the hydrogen exchange rate is relatively slow.

RESULTS

Selecting a Suitable Solvent for Dissolution and NMR Analysis of Aβ-(1–42) Fibrils—The ability to quickly convert the fibrils into a monomeric state is essential for NMR detection of protected amide protons within the fibril. During this process, the H/D exchange pattern trapped by the secondary structures of the fibrils is transferred to and must be preserved in the monomeric state. By performing the experiments at a pH value of ~3 and at low temperatures, amide proton exchange rates could be minimized. Furthermore, an induction of secondary structure in the monomer considerably reduced the amide proton exchange rates via the formation of new hydrogen bonds. We found that a mixture of 80% HFIP/20% water (v/v) at pH 3 had the capability of rapid dissolution of Aβ-(1–42) fibrils. In a previous investigation, performed under very similar conditions, the peptide formed a well structured conformation with two α-helices, comprising residues Ser28-Gly33 and Lys28-Gly38 and a bend centered at position Ser26-Asn27 (31). Circular dichroism measurements showed a further increase of α-helical structure with an increasing concentration of NaCl up to a nearly saturated solution of ~150 mM salt (data not shown). The observed 15N-HSQC spectrum of the Aβ-(1–42) peptide in this HFIP/water solution and 150 mM NaCl, pH 3.0, displayed a well dispersed spectrum with an optimal separation of peaks at 15 °C, a temperature at which the hydrogen exchange rate is relatively slow.

NMR Resonance Assignment of Aβ-(1–42) in Solution—Sequence-specific resonance assignment turned out to be straightforward with few overlapping resonances. Most resonances were assigned via a...
sequential walk between the backbone amide protons in the nuclear Overhauser effect spectroscopy spectrum or via sequential or medium range nuclear Overhauser effect contacts, characteristic for α-helices. All 41 backbone amide resonances (residue 2–42) were identified. Our data are generally in agreement with the previously reported assignment and structure of Aβ-(1–42) performed in HFIP (31).

**Fibril Formation and Atomic Force Microscopy Analysis of Aβ-(1–42)**—Fibril formation was induced by dissolving ~1 mM 15N-labeled Aβ-(1–42) in double distilled H2O containing 50 mM NaCl, followed by incubation at 37 °C for 10–12 days during slow agitation. The initially non-viscous solution acquired a gel-like appearance, and the presence of fibrils was verified using atomic force microscopy (Fig. 1). The fibrils formed were of varying length usually exceeding 1 μm in length and with an average height corresponding to 3.5 nm. A repetitive pattern of nodules could also be observed, indicating a twist of the fibril with an average pitch of ~110 nm.

**H/D Experiments and Determination of Protection Factors of Aβ-(1–42) Fibrils**—The fibrillar material was easily collected from the gel-like fibril solution by centrifugation. H/D exchange was carried out by resuspension and incubation of the fibrillar pellet in D2O, followed by re-collection of fibrils using centrifugation. This procedure was repeated once to efficiently remove water (residual water, ~0.1% (v/v)) and non-aggregated material. After a total exchange period of either 20 or 120 min, the fibrils were converted into NMR-detectable monomers by dissolution in 80% HFIP/20% D2O (v/v) and 150 mM NaCl at pH 3.0. H/D exchange in the monomeric state was monitored by recording a series of 15N-HSQC spectra over time. Fig. 2A shows a spectrum of the fully protonated peptide, whereas the spectra in Fig. 2, B and C, were recorded 12 and 196 min, respectively, after dissolution of the H/D-exchanged fibril material. The observed signal intensity of each amide resonance is determined by the level of solvent protection in the fibrillar state, the concentration of monomers, the rate for which fibrils are converted into monomers, and the H/D exchange rate in the monomeric state. By recording one-dimensional proton NMR spectra prior to each 15N-HSQC spectrum and integrating the non-exchangeable methyl region of these spectra, the dissolution rates, as well as the relative concentration between samples, could be determined (data not shown).
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FIGURE 3. Hydrogen-deuterium exchange of Aβ-(1–42). Examples of the measured signal decay for three amide groups within Aβ-(1–42) as a result of post-trap exchange with the surrounding D2O. Open squares, Ile31; filled diamonds, Ile39; open rings, Val40.

shown). Our results showed that >94% of the total material was dissolved prior to the first recorded spectrum, and a correction was made by fitting a single exponential with a rate constant of 0.0015 min\(^{-1}\). As a consequence of the H/D exchange, the intensity of the amide resonances will decline with time (Fig. 2). The signal decay rates of each individual amide resonance were obtained by fitting the corrected signal intensities to the equation under “Data Analysis and Fitting.” An extrapolation to time zero of fibril dissolution gives the signal intensity \(I_0\) of each residue in the fibrillar state. Fig. 3 illustrates the curve fit of the post-trap decay for residues Ile31, Val39, and Ile40. This treatment enables identification of solvent-protected residues within the fibril and facilitates quantification of the protection level. The protection factors of individual residues are determined by calculating the ratio of the initial signal intensities obtained from samples pre-incubated in D2O and a fully protonated reference sample. Fig. 4 shows the obtained protection factors, which will be discussed below.

To obtain a reliable H/D protection pattern, one must discriminate between the H/D amide proton exchange occurring as a result of the preceding incubation in D2O and H/D amide proton exchange occurring as a result of an unprotected position within the monomeric state. Therefore, the recorded series of \(^{15}N\)-HSQC spectra, which monitor the H/D exchange in fully protonated fibrils (no pre-incubation in D2O) serves as a highly important reference. The result showed that 35 of 41 possible amide protons can be used for probing the solvent protection pattern of the fibril. Residues Ala2, His6, Asp7, Ser8, His14, and Asp23 experienced an H/D exchange rate within the monomeric state, which is too fast to enable detection and thereby makes their level of protection within the fibril unattainable. This lack of protection is because of the solvent-exposed positions of these residues in the monomeric fold, either in the flexible N-terminal tail or, as in the case of Asp23, its position close to a turn.

The solvent protection pattern of the fibrils was determined from fibrillar samples that were pre-incubated in D2O for 20 or 120 min (Fig. 4). By using two different pre-incubation times, valuable information about the solvent exchange rates and structural stability of the fibrils could be obtained. Our results showed that, after a pre-incubation of 20 min, a total of 28 amides were observed, showing a protection pattern essentially covering two regions of the fibril. Both regions showed a slightly bell-shaped protection curve, where the first covered residues Glu1–Gly25, except for residues His14 and Asp23, and the second region covered residues Lys28–Ala32 with a very high degree of protection. Residues Glu1, Phe3, Arg5, Gly9, Tyr10, Ser26, and Asn27 were all confirmed to be unprotected within the fibrillar state (Fig. 4). After 2 h of pre-incubation in D2O, the protection pattern was essentially the same for a majority of residues. However a slight decrease of protection was observed in the flanking residues of the first region (residues Glu11–Gly25), and one residue, Val12, was now completely exchanged. Fig. 5 shows the obtained protection factors mapped onto our model of the Aβ-(1–42) fibril.

DISCUSSION

The Aβ peptide is the main protein component of plaques found in patients with the neurodegenerative disorder Alzheimer disease. Production of Aβ is the result of proteolytic digestion of the significantly larger amyloid precursor protein resulting in a length of the peptide corresponding to 39 – 43 residues. Most of the amyloid precursor protein mutations that ultimately result in early onset of AD have now been linked to an increased concentration of Aβ peptides either via an enhanced expression (6) or through an increased proteolytic processing (32, 33). The aggregation propensity among Aβ fragments of different length differs significantly, and AD has moreover been linked to an increased proportion of longer variants, in particular the highly amyloidogenic 42-residue form (7, 8). Senile or neuritic plaques in AD brains are further enriched with the Aβ-(X-42/43) variants, which also represent the initial deposits found in vivo (5). A recent study suggests a pivotal role for Aβ-(1–42) concerning development of parenchymal and vascular amyloid deposition in mice (34). Currently, the increased aggregation propensity for the longer variants, in particular Aβ-(1–42), has not been explained on a molecular level.

The present study describes how hydrogen exchange in combination with solution NMR spectroscopy was used to determine the solvent protection of Aβ-(1–42) fibrils formed under physiological pH, ion concentration, and temperature. The results suggest that the protected core of the fibril covers two regions of the Aβ-(1–42) sequence, spanning residues Glu1–Gly25 and Lys28–Ala32. In between these protected regions, two completely solvent-accessible residues, Ser26 and Asn27, are found. Moreover, residues Glu1, Phe3, Arg5, Gly9, and Tyr10 are exposed, which suggest that the N-terminal part of the peptide is solvent-accessible. As expected, the observed protection pattern of each protected region has a slightly bell-shaped appearance (Fig. 4), consistent with a decrease of solvent accessibility toward the core of the fibril. An extended pre-incubation time of the fibrils in D2O (from 20 to 120 min) caused no significant change in the protection of the core residues but affected the flanking regions to some extent, in particular Val12, which becomes completely exchanged (Fig. 4). These results agree well with those obtained in previous mass spectrometry studies, where the kinetics of the H/D exchange of backbone amide protons in Aβ-(1–40) were investigated (35, 36). The possibility of obtaining residue-specific information about quantitative solvent protection levels and dynamics of amyloid fibrils is unique for the method and was initially described in our study on a short Aβ fragment (19, 20).

Overall, the findings are in good agreement with solid-state NMR studies on Aβ-(10–35), Aβ-(1–40), and Aβ-(1–42), where an in-register parallel arrangement of residues Val12–Ala32 were proposed (12–14). Furthermore, solid-state NMR data on Aβ-(1–40) suggest that the
region comprising residues Val24-Ala30 lacks β-strand conformation (15). This supports our results indicating that Ser26 and Asn27 are completely exposed within the fibril and only partial protections of residues Gly25 and Lys28 are observed. The outcome of our investigation also supports the model of Tycko and co-workers (16). In their model, the peptide forms a so-called “cross-β unit” defined as two extended β-strands brought together via a bend in position Gly25-Gly26. In addition, parallel in-register cross-β structure is formed via intermolecular interactions, and the appropriate cross-sectional dimensions are fulfilled by juxtaposition of two cross-β units (16). Based on this model and our results, we have generated a new model for Aβ-(1–42) (Fig. 5). The overall high protection factor observed for the C-terminal part of the peptide (residues Lys28-Ala30), as compared with the region comprising residues Gln11-Gly25, is readily explained by a structural arrangement where two cross-β units associate via their hydrophobic C-terminal part, forming a less solvent-exposed core region of the fibril (Fig. 5, A and B). The width of the cross-section of the fibril model is ~4 nm, which is in accordance with our morphological data (Fig. 1). The atomic force microscopy image of Aβ-(1–42) fibrils suggests a twisted architecture and an average height between nodules measured to 3.5 nm, in good agreement with the fibril model suggested (Fig. 5C).

A comparison of our results on Aβ-(1–42) fibrils, with the results from a recent H/D exchange study on the Aβ-(1–40) fibril, show both similarities as well as interesting discrepancies. The N-terminal part, residues Asp1-Lys28 of both Aβ-(1–42) and Aβ-(1–40), displays an essentially identical solvent protection, where position Gly11 is partly protected and positions Gln15-Gly25 show a bell-shaped protection pattern. Both peptides also lack a significant solvent protection for Ser26 and Asn27, further supporting previous investigations suggesting a loop within this region of the fibril (15). However, striking discrepancies are observed in the C-terminal part of the peptides, starting from Gly29 to the C terminus. Our result suggests almost complete solvent protection at positions Gly29-Ala32 (Fig. 4), whereas the study carried out on Aβ-(1–40) is suggestive of a partly unprotected C-terminal end with only residues Lys28, Ala29, Ile30, Leu31, and Met35 significantly protected (21). Although their study utilizes a longer incubation time in D2O (25 h), a direct comparison is justified, because previous H/D exchange mass spectrometry studies on Aβ-(1–40) fibrils have shown that the bulk of solvent-accessible backbone protons exchange well before 2 h (35), and only a minor average difference is observed (0.9 protons) between 2 and 24 h (36). Similarly, the protected core of the Aβ-(1–42) fibril in our study was formed already at an incubation time of 20 min with minor changes at 2 h. Consequently, the differences observed provide strong evidence for two different fibrillar architectures in the C-terminal part of Aβ-(1–42) and Aβ-(1–40). A less structured C-terminal end of Aβ-(1–40) is further supported from studies of proteolytic fragmentation in combination with mass spectrometry (37) as well as by proline scanning of the Aβ sequence (38). Similar indications are reported in a recent solid-state NMR study, where no hydrogen bond distance information was obtained for residues Gly25, Gly26, and Gly28 (39). The highly protected C-terminal region within Aβ-(1–42) fibrils observed in the present study suggests that the addition of two C-terminal residues, Ile41 and Ala42, in a significant manner increase the structure and stability in the whole C-terminal part of the peptide, possibly acting as a molecular zipper between the cross-β units along the fibril axis. The additional protection of at least 6 residues in the C-terminal region of Aβ-(1–42) suggests that equally many intermolecular hydrogen bonds are formed, significantly shifting the equilibrium from a monomeric species toward an oligomeric assembly. Consequently, this finding provides a molecular explanation as to why Aβ-(1–42) has a stronger propensity for fibril formation. Furthermore, it offers a likely explanation regarding the suggested pivotal role for Aβ-(1–42) in the development of AD (5, 34).

The observed discrepancies in solvent protection for the C-terminal parts of Aβ-(1–42) and Aβ-(1–40) may require a reconsideration of how the filaments within the fibril model of Aβ-(1–40) assemble. An alternative model that better satisfies the H/D exchange results on Aβ-(1–40) is created by juxtaposing two cross-β units in such a way that their N-terminal β-strands (residues Gln15-Gly25) form the central core, whereas their C-terminal β-strands (residues Gly28-Val40) face the solvent, thereby explaining the increased accessibility of the latter residues in Aβ-(1–40) as compared with Aβ-(1–42). In such an arrangement, the two cross-β units may be oriented either parallel or anti-parallel with respect to each other. Of these, the second alternative seems preferable, as it creates favorable intermolecular electrostatic side-chain interactions between residues Lys16 and Glu22 in the core. Such an alternate lateral arrangement was previously presented together with the original model (16) but may play a much more important role than was previously assumed.

The basis for how Aβ species exert their toxic effect in vivo is a
highly toxic effect (42). Interestingly, the propensity of ADDL formation varies significantly between different forms of Aβ peptides. Although the Aβ(1–40) variant has the ability to form oligomeric species (42), formation of the highly toxic ADDLs seems to be restricted to the Aβ(1–42) variant (43, 44). Even though no structural information is available concerning ADDLs, it is likely that the peptide assembly, at least in part, shares similarities to the fibrillar state. The observation of a highly stabilized C-terminal region upon assembly of Aβ-(1–42) compared with Aβ-(1–40) may hence provide additional molecular clues to the formation of ADDLs. Interference with the most C-terminal part of Aβ-(1–42) may thereby result in the inability to form ADDLs.

In conclusion, we have determined the solvent protection pattern of fibrils from the highly amyloidogenic Aβ-(1–42) variant on a residue-specific basis, identified the fibrillar core residues, and attained information about the dynamical properties of the fibril. By comparing our result to previous studies on Aβ-(1–40), our results revealed interesting discrepancies in the core structure and suggest that Ile43 and Ala42 play an important role for stabilization of the C-terminal residues from Gly79 and forward. This finding may further provide a molecular explanation regarding their different propensity of aggregation and fibril formation and provide a novel target for drug design.

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FIGURE 5. Mapping of the observed protection factors onto a fibril model of Aβ-(1–42). The observed solvent protection factors determined for residues within Aβ-(1–42) fibrils are mapped onto our current model of the fibril. The color code interpolates between the following extremes: navy blue for complete and red for no solvent protection. Residues with no protection factors available are depicted in gray. Main-chain hydrogen bonds are directed along the fibril axis, perpendicular to the plane of the paper. A, a ball-and-stick model showing a dimer of two cross-β units taken from a cross-section of the Aβ-(1–42) fibril. Assignments are indicated in some positions with their one-letter codes. B, ribbon representation of the molecules in A, model of the fibrillar assembly. The model is based on the structural model Tycko and co-workers (6–9) proposed and was modified to include also the N- and C-terminal residues of Aβ-(1–42). Images were prepared in MOLMOL software (29).
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