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Amyloid Protofibrils

Solid-State NMR Spectroscopic Investigation of Aβ Protofibrils: Implication of a β-Sheet Remodeling upon Maturation into Terminal Amyloid Fibrils**

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Aβ(1–40) and Aβ(1–42) constitute the major fibril-forming peptide in Alzheimer's disease (AD).[1] Monomeric Aβ(1–40) is largely unstructured,[2] while its aggregation into amyloid fibrils induces the formation of β-sheet structure. Mature amyloid fibrils (MAFs) represent the end products of fibril formation. These fibrils possess a long, straight, and highly regular morphology (Figure 1 A). The β-sheet structure of MAFs has been characterized by solid-state nuclear magnetic resonance (ssNMR) spectroscopy and other techniques.[3,4]

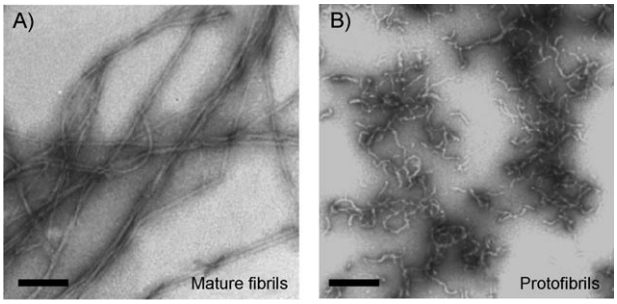

Figure 1. Electron micrographs (scale bars: 200 nm) of Aβ(1–40) in 50 mM HEPES (pH 7.4) and 50 mM NaCl incubated at 37 °C for 72 h in the absence (A) or presence of B10AP (B). Molar ratio 10:1 (Aβ/B10AP). HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid.

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The formation of these terminal fibril states is preceded by several intermediates (see Figure S1 in the Supporting Information),[5–7] but detailed structural information on these intermediates is scarce. In the present study, we have used ssNMR spectroscopy to determine the β-sheet structure of one such fibrillation intermediate—protofibril (PF). Our data reveal structural details of the Aβ(1–40) amyloidogenic pathway and illuminate conformational transitions associated with specific steps of this process.

PFs are major cytotoxic Aβ species and candidate structures for the causative agent of AD.[8] PFs were identified as the earliest fibrillar aggregates within the Aβ amyloidogenic pathway.[5] Their curvilinear, irregular morphology differs considerably from MAFs.[5,6] PFs do not interact well with Congo red and thioflavin T dyes.[6] Nevertheless, PFs incorporate significant amounts of β-sheet structure, as demonstrated by infrared spectroscopy and X-ray diffraction studies.[9]

The current analysis is facilitated by the stabilization of otherwise metastable PFs with the antibody-derived fusion protein B10AP (see Figure S1 in the Supporting Information).[9] A set of eight Aβ(1–40) peptides with various isotopic labeling schemes was obtained from chemical synthesis. The ^{15}N and ^{13}C labels cover 30 residues from all structural regions of the peptide (see Figure S2 in the Supporting Information). These peptides were used to prepare separate samples of B10AP-stabilized Aβ(1–40) PFs in vitro. The morphology of the PFs and the sample homogeneity were confirmed by transmission electron microscopy (TEM), which showed that nonfibrillar aggregates and MAFs are effectively absent (Figure 1 B). ^{13}C CP-MAS (CP = cross-polarized, MAS = magic-angle spinning) NMR spectra and two-dimensional ^{13}C-^{13}C correlation experiments were recorded to monitor the NMR signals of the labeled amino acids in Aβ (see Figure S3 in the Supporting Information). The line widths of the NMR signals was typically between 1.5 and 3.0 ppm. All $^{13}C\alpha$ and $^{13}C\beta$ signals could be assigned unambiguously (see Table S1 in the Supporting Information).

Figure 2 A shows the $^{13}C\alpha$ and $^{13}C\beta$ chemical shifts, which are particularly informative about the secondary structure, as differences from random-coil values ("secondary shifts").[10] In this diagram, all the residues apart from Gly show negative Cα and positive Cβ secondary shifts when they are in a β-sheet structure, while shifts in the opposite directions are typical for α helices.[11] Values close to zero indicate a random-coil structure. In addition, the secondary structure of the backbone was evaluated by using a database for chemical shift and sequence homology (TALOS),[12] which

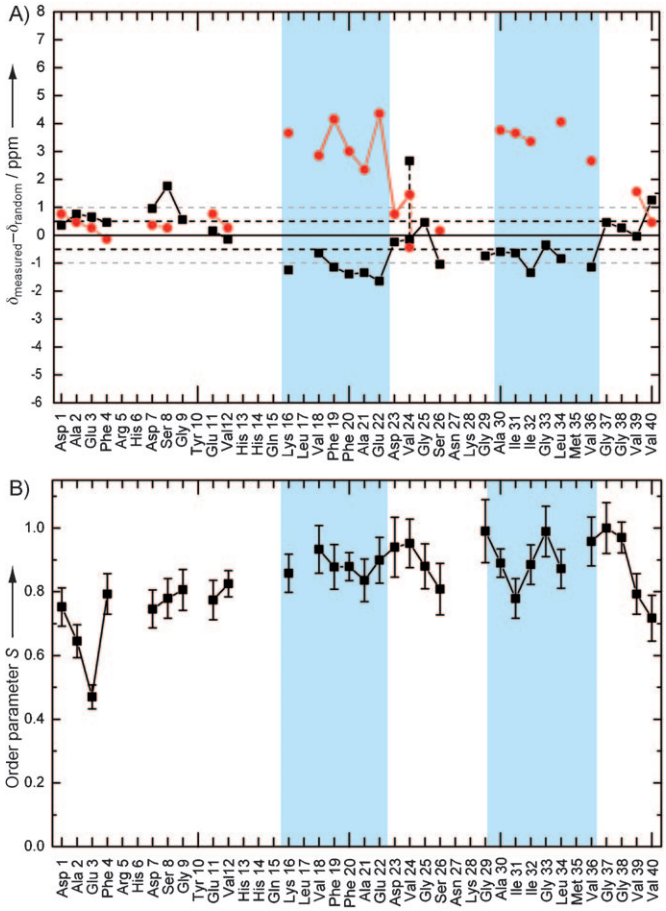

Figure 2. A) Secondary ^{13}C MAS NMR isotropic chemical shift values for Cα (black squares) and Cβ (red circles) for B10AP-stabilized Aβ(1–40) PFs. Data are given as the difference of a measured chemical shift for a specific amino acid to chemical shifts of random coils taken from the literature. Regions that are in agreement with a β-sheet secondary structure are highlighted in blue. Line widths are given in Figure S7 in the Supporting Information. B) Plot of the order parameter *S* for B10AP-stabilized PFs of Aβ(1–40) determined from quantitative measurement of the $^{13}C\alpha$-^{1}H dipolar couplings. Blue regions refer to the β-sheet structures of the Aβ PFs determined in this study (see Table S2 in the Supporting Information).

provides information about the dihedral torsion angles Ψ and Φ. On the basis of the combined secondary shift and TALOS data, the analyzed Aβ PFs feature residues 16–22 and 30–36 in a β-sheet conformation (see Table S2 in the Supporting Information). The Ψ-Φ pairs of these two β strands, as obtained by TALOS, have a narrow distribution: They occur mostly on the right side of, though in proximity to, the diagonal of the Ramachandran plot (see Figure S4 in the Supporting Information). These properties indicate a highly regular β-sheet structure with a low twist angle.

Further information about the β-sheet regions is provided by measurement of the strength of the ^{1}H-^{13}C dipolar couplings. These couplings are partially averaged by molecular motions; therefore, they are informative about the structural dynamics, as expressed by the order parameter *S*. Rigid structural motifs present order parameters *S* close to 1, while *S* values close to 0 indicates high mobility. Thus, order parameters were used previously to distinguish between the rigid cross-β core of fibrils and those peptide regions that are outside this core and are highly flexible.[13]

B10AP-stabilized PFs show relatively high order parameters (> 0.8) within the β-strand regions (Figure 2B), while the measured *S* values for the N and C termini are below 0.8. B10AP-stabilized PFs lack regions of very high mobility ($S <$ 0.4), which indicates that residues 1–12 and 23–26 do not represent thermally fluctuating chains, although their chemical shifts correspond to a random-coil structure (Figure 3A).

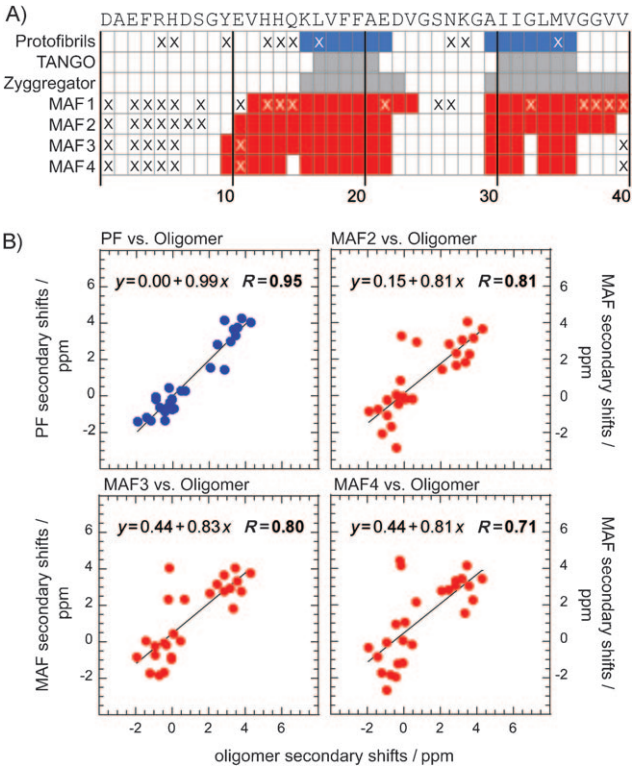

Figure 3. Comparison of PFs with MAFs, oligomers, and theoretical predictions. A) Residues in β strands (shaded boxes) as determined by ssNMR spectroscopy for PFs (blue, this study) and different MAFs (red; 1,[4] 2,[16] 3, and 4[14]), or predicted with TANGO[17] and Zyggregator (gray).[17,18] X: residue not analyzed in the respective study. B) Correlation of the Cα and Cβ secondary shifts of Aβ(1–40) oligomers,[19] B10AP-stabilized PFs, and three preparations of MAFs.[14,16] Only 15 positions are reported for oligomers. In the case of multiple reported chemical shifts, the closest values were considered. In the MAF4 sample, the dominant shift series is shown.

These data imply that the respective regions have stable conformations, although neither α helical nor β sheet in nature. The relatively high structural stability at the N terminus of the peptide (Figure 2B) may arise from the binding of B10AP, which is consistent with the unusual increase in the *S* values at residues Asp1 and Ala2 compared with that of Glu3 (Figure 2B).

Direct comparisons with previously measured chemical shifts for other Aβ(1–40) conformers are complicated by different isotopic labeling patterns and the fact that multiple chemical shift values have been reported for the same labeled

^{13}C nucleus.[4,14] While multiple chemical shift values are consistent with the substantial structural polymorphism of typical MAF samples,[6,15] such heterogeneity appears less problematic for the currently analyzed PFs. Only Val24 shows two chemical shift values (see Table S1 in the Supporting Information).

Detailed analysis of the chemical shift data of B10AP-stabilized Aβ(1–40) PFs and previously published values obtained with Aβ(1–40) MAFs reveals the presence of β structure at very similar positions of the peptide sequence (see Figure S5 in the Supporting Information). However, MAFs seem to recruit more residues into their β strands than do PFs (Figure 3A). This fact is especially evident in the first β strand, which starts in different MAF samples at residues 10–12. By contrast, PFs evidently comprise residues Glu11 and Val12 in a non-β conformation (Figures 2A, 3A), which indicates that their first β strand does not start before residue 13.

Theoretical prediction of the β-sheet segments with the programs TANGO and Zyggregator are consistent with the short β strands of PFs (Figure 3A). TANGO predicts β strands at residues 17–21 and 31–36,[17] while Zyggregator predicts a β-sheet conformation at residues 15–23 and 30–40.[18] These comparisons imply that PFs stabilize the β strands that are intrinsically most favorable and, therefore, predictable by theory.

Further, structural differences between PFs and MAFs are evident in the intermediate region (residues 23–26), which defines the connection of the β strands (see Figure S5 in the Supporting Information). Early studies suggested that MAFs stabilize this region in a β-arch conformation,[20] but recent cryo-TEM reconstructions of mature Aβ(1–40) fibrils at 8–10 Å resolution raised concerns regarding these proposals.[21] Substantial deviations are also seen at Gly33, which occurs within the C-terminal β strand. Gly33 shows secondary shifts from $+3$ to $+5$ ppm in MAFs and of -0.3 ppm in PFs (see Figure S5 in the Supporting Information). In conclusion, all the current data indicate that PFs and MAFs possess a remodeled β-sheet structure. This notion is further supported by the different interactions of PFs and MAFs with Congo red and thioflavin T dyes, and the profound morphological differences seen by TEM.[5,6]

Plotting the reported Cα and Cβ secondary shifts of PFs against reported data on oligomers[19] produces a much higher correlation coefficient ($R = 0.95$) than the correlation between oligomers and MAFs (Figure 3B). Similar results are obtained when the Cα and Cβ secondary shifts of oligomers, PFs, and MAFs are correlated separately. At positions where PFs and MAFs show significant differences, as determined by the present study (Val12, Gly25, and Gly33), oligomers are more closely related to PFs than to MAFs (see Figure S6 in the Supporting Information). These results are especially remarkable because PFs and MAFs possess common overall structural properties, such as a linear morphology and significant interactions with the fibril-specific B10 antibody fragment.[8] While it remains to be established whether all PFs, including those that form in the absence of any stabilizing agent, match the characteristics described here for B10AP-stabilized PFs, our data argue that PFs should instead be considered as elongated oligomers, which is also consistent with recent atomic force microscopy data on Aβ protofibrils and oligomers.[23] Moreover, a preparation of Aβ protofibrils featuring ring-labeled Phe19 and uniformly labeled Leu34 and Gly38 showed interactions only between Phe19 and Leu34, but not with Gly38 (see Figure S8 in the Supporting Information), as observed previously with Aβ(1–42) oligomers and fibrils.[22]

Figure 4 summarizes the structural implications of the current findings for the mechanism of Aβ(1–40) fibrillation. Upon oligomerization, the initially disordered Aβ peptide

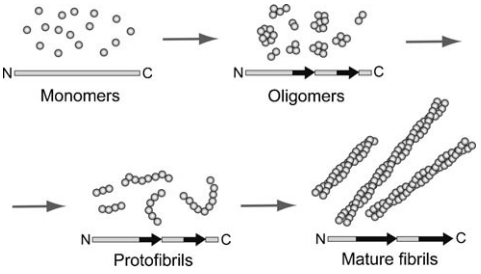

Figure 4. Aβ fibrillation and β-sheet structure (black arrows) of the different states.

stabilizes β-sheet segments according to its structural predilection, as determined by theory and experiment. These β-sheet segments enable PF outgrowth, as shown previously through mutagenic analysis of Aβ(1–40) PFs.[24] As the fibrils mature, the β-sheet structure becomes remodeled so that further residues can be incorporated into the β strands (Figure 3A). Associated with these rearrangements, residues 23–26 and Gly33 present revised conformations and have different chemical shifts (Figure 3B), and new and MAF-specific interactions are enabled. As a result, thermodynamically more stable MAFs can prevail.

The current work is also relevant for Aβ(1–42) amyloid fibrillation, because of the common features of these two peptides. Infrared spectroscopy, dye binding,[25] and ssNMR spectroscopy[22] indicate that Aβ(1–42) oligomers must also undergo substantial structural alterations upon conversion into terminal MAFs. However, it is not known whether this transition also occurs at the PF to MAF stage. Moreover, further work will be required to determine the structures of the involved states at atomic resolution and to reveal the PF-specific contacts which need to be broken upon conversion into MAFs.

Experimental Section

Aβ(1–40) peptides with different labels were synthesized by standard solid-phase synthesis according to the 9-fluorenylmethoxycarbonyl (Fmoc) protocol (see Figure S2 in the Supporting Information). B10AP-stabilized PFs were prepared in 1 mL buffer (50 mM HEPES, pH 7.4, 50 mM NaCl) containing 4 mg mL^{-1} labeled Aβ(1–40) and B10AP at 10:1 molar ratio (Aβ:B10AP). After incubation of the sample for 3 days (37 °C), B10AP-stabilized protofibrils were recovered by ultracentrifugation (100 krpm, 2 h, 4 °C, TLA120.2 Rotor, Beckman Optima TLX centrifuge). The pellet was lyophilized,

rehydrated with 50 wt% H_2O, and homogenized by freezing the sample in liquid nitrogen and thawing it at 37 °C. TEM confirmed that the PF morphology was not affected by this procedure. TEM samples were prepared by applying 5 μL droplets from the sample after 1:10 dilution with pure water onto a carbon film (floating carbon method), counterstained with 2% (w/v) uranyl acetate, and analyzed with a Zeiss 900 electron microscope (80 kV).

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