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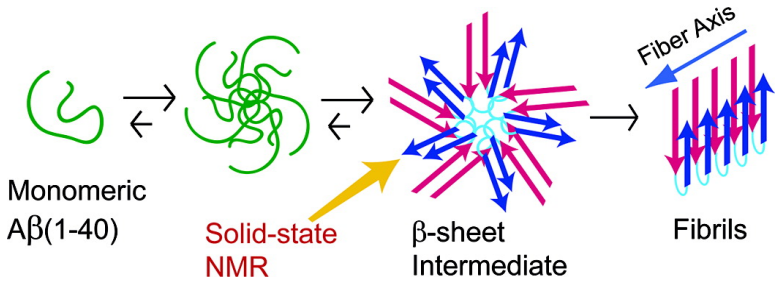


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# Capturing Intermediate Structures of Alzheimer's β-Amyloid, Aβ(1−40), by Solid-State NMR Spectroscopy

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Amyloid fibrils of Alzheimer's β-amyloid peptides (Aβ) ranging from 39 to 43 residues are primary components of senile plaque of Alzheimer's disease (AD).[1] As widely known, in vivo and in vitro, unstructured monomeric Aβ self-assembles into fibrils, in which β-sheets are layered.[2] Since the aggregated Aβ exhibits neurotoxicity, the fibrils have been a major suspect of AD.[1] On the other hand, increasing evidence suggests that early-stage aggregates of Aβ exist in fibril formation and that these diffusible intermediates are more toxic than fibrils and may be responsible for AD.[3] Recent micro imaging studies revealed morphological variants for Aβ intermediates,[4−7] but structural information on the amyloid intermediates has been very limited.[3,6] In particular, despite recent progress,[8] it has been an intractable problem to obtain site-resolved structures of amyloid intermediates because their noncrystalline and unstable nature has hampered traditional methods for structural biology such as solution NMR and X-ray crystallography.[3]

In this study, we demonstrate an approach to capture structures of amyloid intermediates by solid-state NMR (SSNMR), which has provided structural information on thermally stable amyloid fibrils.[9−11] One fundamental yet unsolved structural problem on Aβ is to define at which stage of the protein misfolding the β-sheet formation is introduced. On the basis of a kinetic model proposed by Zagorski et al,[12] we hypothesize that β-sheet formation precedes supramolecular assembly into fibrils. For Aβ(1−40), we identify formation of soluble intermediates involving β-sheets (β-sheet intermediates) using fluorescence with thioflavin T (ThT), as an indicator of β-sheet-rich aggregates.[13] Then we demonstrate that it is feasible to characterize site-resolved structures of the freeze-trapped Aβ intermediates by SSNMR.

In Figure 1a, the blue squares show incubation-time ($t$) dependence of ThT fluorescence for a 100 μM Aβ(1−40) solution at 37 °C. As previously demonstrated, the fluorescence increased in a sigmoidal manner after a lag time,[14] indicating formation of β-sheet-rich fibrils. To detect soluble intermediate species involving β-sheets, we performed a quick centrifugation (15 min, 13.2 × $10^3$ $g$) for an aliquot (∼1 mL) of the Aβ solution and measured the ThT fluorescence for the supernatant collected at various $t$ as shown by the red circles in Figure 1a. By electron microscopy (EM), we confirmed that quick centrifugation removes "insoluble" species such as fibrils and protofibrils[3] (data not shown). The red curve in (a) clearly shows relatively weak fluorescence at $t$ = 9−11 h, indicating formation of soluble β-sheet intermediates. However, the concentration of these intermediates estimated from the florescence was low (<30 μM), and the intermediates are stable only for 2−3 h; both factors limit further analysis.

On the basis of previous studies, we reduced the temperature to slow fibril formation and stabilize the intermediate.[3,7,12] Figure 1b shows the incubation-time dependence of the ThT fluorescence for 100 μM Aβ(1−40) at $T$ = 4 °C. In this condition, the fluorescence for the soluble component (red) was significantly increased to 80−86% of that for the mixture (blue). Importantly, the intermediate

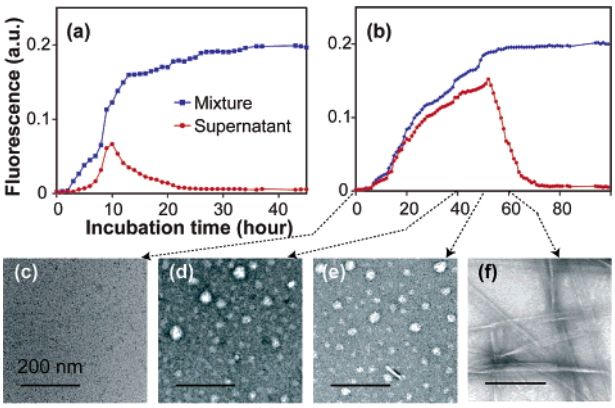


***Figure 1.*** (a, b) Incubation-time dependence of ThT fluorescence for 100 μM Aβ(1−40) solution at (a) 37 °C and (b) 4 °C for the sample gently mixed before sampling (mixture: blue) and the supernatant sample separated by centrifuging an aliquot of the Aβ solution (supernatant: red). The buffer contained 5 mM NaCl, 10 mM phosphate, and 0.02% $NaN_3$. The pH was at 7.4 ± 0.1. (c−f) Incubation-time dependence of EM images for the Aβ solution used for (b), which was sampled at $t$ = (c) 0 h, (d) 40 h, (e) 48 h, and (f) 60 h. Spherical intermediates were observed in (d, e). See SI for details.

was stable for a much longer period (20 h ≤ $t$ ≤ 52 h). We confirmed that the fluorescence at 40 and 48 h diminishes after filtration with a 50-kDa molecular-weight-cutoff (MWCO) filter. Hence, relatively large aggregates (>10mer) should be responsible for the β-sheet formation.

Figure 1c−f shows the incubation-time dependence of EM images for the Aβ(1−40) solution sampled at $t$ = (c) 0 h, (d) 40 h, (e) 48 h, and (f) 60 h. The images in (d, e) demonstrate that spherical intermediates with diameters of 15−30 nm exist as a predominant species at $t$ = 40 and 48 h, while no visible structures were observed in (c) at $t$ = 0 h. The intermediate in (d, e) has spherical morphology similar to those of previously reported Aβ aggregates such as *ADDL*,[7] *amylospheroid*,[5] and *β-amy ball*.[15] In diameter, the spherical species in (d, e) is comparable to the spheroid (8−16 nm), rather than ADDL (∼5 nm) or amy ball (20−200 μm). However, it was reported that the amylospheroid exhibits no ThT florescence, suggesting that their spheroid does not contain β-sheets.[5] Therefore, the spherical β-sheet intermediate is likely to be a new intermediate species.

In Figure 1f at $t$ = 60 h, fibrils became a predominant species; this is matched by the reduced fluorescence for the soluble component (red) at $t$ > 52 h in (b). The bundled and twisted morphologies of the fibrils obtained in the refrigerated condition in (f) are similar to that of Aβ fibrils obtained at room temperature.[10] Hence, we consider that the reduced temperature stabilized the intermediates, resulting in slower fibrillization, but did not alter the misfolding pathway significantly. From the above evidence, we identify the β-sheet intermediate as a key kinetic intermediate preceding formation of protofibrils and fibrils of Aβ(1−40).


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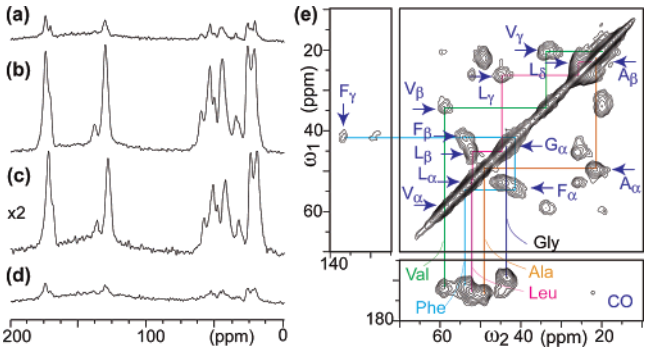


***Figure 2.*** (a−d) $^{13}C$ CPMAS spectra for lyophilized A$\beta$(1−40) that was sampled after incubation time of (a) 0 h, (b, d) 52 h, and (c) A$\beta$(1−40) fibrils. The sample for (d) was obtained by filtering the sample with a 50-kDa-MWCO filter before lyophilization. The fibrils were collected by centrifuge. The A$\beta$(1−40) peptide was uniformly $^{13}C$- and $^{15}N$-labeled at Val18, Phe19, Ala21, Gly33, and Leu34. The spectra acquired with 512 scans were displayed in a common scale except for (c) scaled by two. (e) 2D $^{13}C/^{13}C$ correlation SSNMR spectrum obtained with a fpRFDR sequence using a 1.6 ms mixing time for the intermediate sample used in (b). See SI for details and assignment in (e).

To examine the site-resolved intermediate structures, we trapped the intermediates by quick freezing and subsequent lyophilization and then performed structural analysis by SSNMR. Figure 2a,b shows 1D $^{13}C$ CPMAS spectra of A$\beta$(1−40) peptides trapped at (a) $t = 0$ h and (b) 52 h, which correspond to the monomers and the $\beta$-sheet intermediates, respectively. Uniformly $^{13}C$- and $^{15}N$-labeled amino acids were introduced in selected residues in the hydrophobic core (Val18, Phe19, Ala21) and C-terminal (Gly33, and Leu34). In fibrils, the two regions are in $\beta$-sheet conformations and connected by a loop region.[10] Interestingly, the spectral intensities in (a) are significantly lower than those in (b), although the same amount of the peptide (~4.5 mg) was used for (a) and (b). From additional experiments (see Supporting Information (SI)), we confirmed that signals for the unstructured A$\beta$ sample at $t =$ 0 h were not observed by the existence of dynamics or for other unspecified reasons. In contrast, in (b) the structures of A$\beta$ peptides appear well ordered. Our ThT fluorescence analysis for rehydrated A$\beta$ showed that lyophilization induces little additional $\beta$-sheet formation or aggregation (see SI). It is also known that various proteins including A$\beta$ in fibrils retain their structures after lyophilization.[9,10,16] Figure 2c shows a $^{13}C$ CPMAS spectrum for the A$\beta$(1−40) fibril sample. The lower intensity for (c) is attributed to the fact that only about 2.2 mg of the peptide was collected for the fibril sample. Surprisingly, the spectra in (c) and (b) are alike. This indicates that the $\beta$-sheet intermediate and the fibril have similar structures at these labeled sites.

To confirm that there is little contribution from monomers or small oligomers in (b), we filtered the A$\beta$ solution sampled at $t =$ 52 h with a 50-kDa MWCO filter and performed 1D $^{13}C$ CPMAS on the lyophilized filtrate as shown in (d). The spectrum in (d) yielded only weak signals, which are consistent with the results in (a). Hence, we conclude that the SSNMR spectrum in (b) represents the existence of a well-ordered intermediate species.

Figure 2e shows the 2D $^{13}C/^{13}C$ correlation SSNMR spectrum obtained with the fpRFDR sequence[17] and signal assignment for the intermediate sample that was used for Figure 2b. All the line widths are relatively sharp (2.3−3.3 ppm) except for slightly broader $C_\beta$ resonances of Val and Ala (~3.5 ppm), considering that the system is noncrystalline and the line widths include Gaussian broadening of 0.9 ppm and broadening due to $^{13}C-^{13}C$ $J$ couplings (~0.5 ppm). It was found that there were no missing one-bond cross-peaks in the spectrum. These results suggest that the A$\beta$(1−40) structures at the intermediate stage are well ordered in the hydrophobic core and the C-terminal regions. The slightly broader signals for the side chains may be associated with heterogeneity in side-chain packing or structural polymorphology.[10] The chemical shifts of the intermediates are quite similar to those for the fibrils including side-chain resonances (see SI). Site-specific secondary structural analysis based on $^{13}C_\alpha$, $^{13}C_\beta$, and $^{13}CO$ shifts by the TALOS software[18] suggested $\beta$-sheet structures for all the labeled sites, except for Gly33. For Gly33, 8 out of 10 suggested sets of dihedral angles are for $\beta$-sheets or close to those for $\beta$-sheets.

To our knowledge, the present work demonstrates the first example of site-resolved structural analysis for amyloid intermediate species. This study also reveals that structurally homogeneous $\beta$-sheet intermediates are formed prior to fibril formation in the misfolding pathway of A$\beta$(1−40). Details of the molecular and supramolecular structures of this intermediate will be studied in our future work. The SSNMR-based method presented here can also open an avenue for analyzing intermediates of various amyloid proteins and other A$\beta$ intermediates,[3] if coupled with different optical spectroscopic probes such as antibody markers to detect structural changes.

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**Supporting Information Available:** Details of sample preparation and experimental conditions, fluorescence analysis for rehydrated lyophilized samples, complete refs 7 and 12, and signal assignment from Figure 2e. This material is available free of charge via the Internet at http://pubs.acs.org/.

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