Biosci. Biotechnol. Biochem., **72** (8), 2170–2175, 2008

Verification of the Intermolecular Parallel β-Sheet in E22K-Aβ42 Aggregates by Solid-State NMR Using Rotational Resonance: Implications for the Supramolecular Arrangement of the Toxic Conformer of Aβ42

Yuichi MASUDA,[1] Azusa NAKANISHI,[2] Ryutaro OHASHI,[2] Kiyonori TAKEGOSHI,[2] Takahiko SHIMIZU,[3,4] Takuji SHIRASAWA,[3,5] and Kazuhiro IRIE[1,†]

[1]*Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan*
[2]*Department of Chemistry, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan*
[3]*Research Team for Molecular Biomarkers, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan*
[4]*Applied Biological Chemistry, United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan*
[5]*Department of Ageing Control Medicine, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan*

Received April 15, 2008; Accepted May 14, 2008; Online Publication, August 7, 2008
[doi:10.1271/bbb.80250]

Formation of the intermolecular β-sheet is a key event in the aggregation of 42-residue amyloid-β (Aβ42). We have recently identified a physiological and toxic conformer, the turn positions of which are slightly different from each other, in the aggregates of E22K-Aβ42 (one of the mutants related to cerebral amyloid angiopathy). However, it remains unclear whether the intermolecular β-sheet in the E22K-Aβ42 aggregates is parallel or antiparallel. We prepared an equal mixture of E22K-Aβ42 aggregates labeled at C_α and those labeled at C=O with ^{13}C, whose intermolecular ^{13}C–^{13}C distance was estimated by solid-state NMR using rotational resonance (R2). The intermolecular proximity of β-strands at positions 21 and 30 was less than 6 Å, supporting the existence of the intermolecular parallel β-sheet in the E22K-Aβ42 aggregates as well as in wild-type Aβ42 aggregates. The results also suggest that each conformer would not accumulate alternately, but form a relatively large assembly.

Key words: Aβ42; Alzheimer's disease; intermolecular parallel β-sheet; Italian mutation; solid-state NMR

Alzheimer's disease (AD) is characterized by the abnormal accumulation of 40- and 42-mer amyloid β (Aβ40 and Aβ42),[1)] the latter being far more aggregative and neurotoxic.[2)] Since the aggregation of Aβ induces neuronal death, a structural analysis of Aβ aggregates is essential for understanding the mechanism of neurotoxicity.

Aβ aggregates (fibrils) consist of a cross-β structure, in which β-strands run perpendicular to the fiber axis.[3)] These β-strands are connected to each other with hydrogen bonds and form intermolecular β-sheets. The formation of an intermolecular β-sheet is the key event in the aggregation of not only Aβ, but also other aggregative proteins such as prions in prion disease and α-synuclein in Parkinson's disease.[4)] The intermolecular β-sheet in amyloid fibrils can be either parallel or antiparallel, depending on the primary structure of the Aβ peptides. Antiparallel β-sheets have been found in the aggregates formed from short fragments of Aβ: $A\beta_{34\text{-}42}$,[5)] $A\beta_{16\text{-}22}$,[6)] and $A\beta_{11\text{-}25}$.[7)] On the other hand, β-strands in the aggregates of wild-type Aβ40 and Aβ42 are aligned parallel to each other.[8–11)]

Our proline scanning and solid-state NMR recently identified two conformers in the Aβ42 aggregates: a physiological one with a turn at positions 25 and 26, and a toxic one with a turn at positions 22 and 23 (Fig. 1A and B).[12)] We suggested that E22K-Aβ42 (Italian), one of the mutant peptides related to cerebral amyloid

† To whom correspondence should be addressed. Tel: +81-75-753-6281; Fax: +81-75-753-6284; E-mail: irie@kais.kyoto-u.ac.jp

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β; DARR, dipolar-assisted rotational resonance; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ESR, electron spin resonance; Fmoc, *N*-α-(9-fluorenylmethoxycarbonyl); FTIR, Fourier transform infrared spectroscopy; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MAS, magic angle spinning; PEG-PS, polyethylene glycol-polystyrene support; R2, rotational resonance; TFA, trifluoroacetic acid

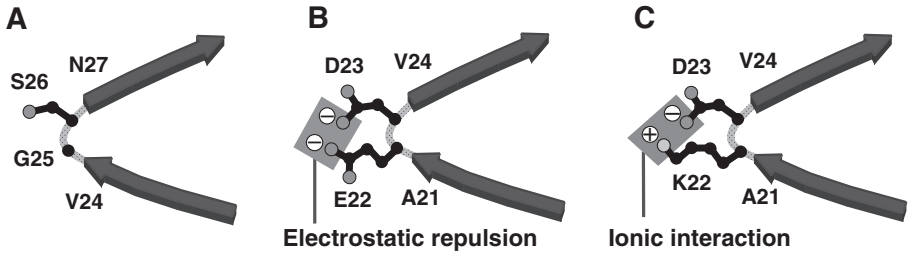

Fig. 1. Structural Models of Aβ42 Aggregates Proposed by Systematic Replacement with Proline and Solid-State NMR.[12]
A, Physiological conformer in wild-type Aβ42 aggregates. B, Toxic conformer in wild-type Aβ42 aggregates. C, Toxic conformer in E22K-Aβ42 aggregates. In E22K-Aβ42 (C), the ionic interaction between Lys-22 and Asp-23 would promote the formation of a turn structure at this position. On the other hand, electrostatic repulsion between Glu-22 and Asp-23 might suppress the formation of a turn at this position in wild-type Aβ42 (B).

angiopathy, had stronger aggregative ability and neurotoxicity than wild-type Aβ42 because ionic interaction between Lys-22 and Asp-23 could promote the formation of a turn at positions 22 and 23 to increase the ratio of the toxic conformer (Fig. 1C).[13] Thus, the establishment of a precise structural model for the E22K-Aβ42 aggregates is essential to understand the etiology of AD. However, it remains unclear whether the intermolecular β-sheet in the E22K-Aβ42 aggregates is parallel or antiparallel, and how each conformer assembles in relation to the other. To clarify these aspects, we evaluated the intermolecular distance between β-strands in the E22K-Aβ42 aggregates by solid-state NMR, using rotational resonance (R2).[14]

Materials and Methods

General. The following analytical and spectroscopic instruments were used: ^{1}H- and ^{13}C-NMR in a solution, Bruker AVANCE 400 (ref. TMS); FAB-MS, JEOL JMS-600H (matrix: glycerol); peptide synthesizer, Pioneer™ (Applied Biosystems, Foster City, CA, USA); HPLC, Waters 600E multisolvent delivery system with a 2487 UV dual wavelength absorbance detector (Waters, Milford, MA); matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS), Voyager-DE PRO and 4700 Proteomics Analyzer (Applied Biosystems); transmission electron microscope, H-7500 (Hitachi, Ibaraki, Japan); solid-state NMR spectrometer, Chemagnetics Infinity NMR spectrometer and Chemagnetics 3.2 mm magic angle spinning (MAS) probe. HPLC was carried out in a Develosil-packed column ODS-UG-5 (20-mm internal diameter × 150 mm; Nomura Chemicals, Seto, Japan).

L-Alanine (1-^{13}C) and L-alanine (2-^{13}C) were purchased from Taiyo Nippon Sanso Corporation (Tokyo, Japan). HATU,[15] *N*-α-(9-fluorenylmethoxycarbonyl) (Fmoc) amino acids, Fmoc-Ala-polyethylene glycol-polystyrene support (PEG-PS) resin, and *N*,*N*-diisopropylethylamine (DIPEA) were from Applied Biosystems. *N*,*N*-Dimethylformamide (DMF), trifluoroacetic acid (TFA), 1,2-ethanedithiol, thioanisole, *m*-cresol, and diethyl ether (peroxide-free) were purchased from Nacalai Tesque (Kyoto, Japan). Piperidine was obtained from Sigma.

Preparation of protected amino acids labeled with ^{13}C. Fmoc derivatives of L-alanine (1-^{13}C) and L-alanine (2-^{13}C) were synthesized as reported previously.[13,16] The yields were 86% for *N*-Fmoc-L-alanine (1-^{13}C) and 87% for *N*-Fmoc-L-alanine (2-^{13}C). The structures were confirmed by ^{1}H-NMR, ^{13}C-NMR, and FAB-MS measurements.

Synthesis of E22K-Aβ42 peptides. Synthesis of the E22K-Aβ42 peptides was performed in a stepwise manners on 0.1 mmol of Fmoc-Ala-PEG-PS resin by the Pioneer™ instrument with the Fmoc method, as reported previously.[13,17–19] The coupling reaction was carried out with the Fmoc amino acid (0.4 mmol), HATU (0.4 mmol), and DIPEA (0.8 mmol) in DMF for 30 min. E22K-Aβ42 labeled at Ala-21 and that labeled at Ala-30 with ^{13}C were prepared in different ways. E22K-Aβ42 labeled at C_α of Ala-21 with ^{13}C and that labeled at C=O of Ala-21 with ^{13}C were respectively synthesized. In the synthesis of an equal mixture of E22K-Aβ42 labeled at C_α of Ala-30 and that labeled at C=O of Ala-30 with ^{13}C, the Fmoc amino acid at Ala-30 was applied as an equal mixture of *N*-Fmoc-L-alanine (1-^{13}C) and *N*-Fmoc-L-alanine (2-^{13}C). After each coupling reaction, the Fmoc group at the N-terminus was removed with 20% piperidine in DMF.

After completing the chain elongation, the peptide resin washed with DMF and CH_2Cl_2 was treated with a cocktail containing TFA, *m*-cresol, ethanedithiol, and thioanisole for final deprotection of the side chains and cleavage from the resin. After shaking at room temperature for 2 h, the crude peptide precipitated by diethyl ether was purified by HPLC under alkaline conditions, as reported previously.[13,18,19] Lyophilization gave corresponding E22K-Aβ42, the purity of which was confirmed by HPLC as >98%. The yields of the peptides were 5–8%. The synthesized peptide exhibited satisfactory mass spectral data by MALDI-TOF-MS (Supplemental Figs. 1–3; see the *Biosci. Biotechnol.*

Biochem. Web site) for E22K-Aβ42 labeled at C_α of Ala-21 with ^{13}C (MH^+, average molecular mass; observed, 4515.58; calculated, 4515.16), E22K-Aβ42 labeled at C=O of Ala-21 with ^{13}C (MH^+, average molecular mass; observed, 4514.55; calculated, 4515.16), and an equal mixture of E22K-Aβ42 labeled at C_α of Ala-30 and that labeled at C=O of Ala-30 with ^{13}C (MH^+, average molecular mass; observed, 4515.30; calculated, 4515.16).

Fibril formation of Aβ42 labeled with ^{13}C. An equal mixture of E22K-Aβ42 labeled at C_α of Ala-21 and that labeled at C=O of Ala-21 with ^{13}C was dissolved in 0.1% NH_4OH at 250 μM. After a 10-fold dilution with 50 mM sodium phosphate containing 100 mM NaCl at pH 7.1, the resulting peptide solution (25 μM, pH 7.4) was incubated at 37 °C under quiescent conditions for 48 h. After centrifugation at 21,000 *g* and 4 °C, and subsequent washing with distilled water, the resulting aggregates (fibrils) were dried *in vacuo*.

An equal mixture of the E22K-Aβ42 aggregates labeled at C_α of Ala-30 and those labeled at C=O of Ala-30 with ^{13}C was prepared by the same method.

Transmission electron micrographs of negatively stained preparations of the fibrils formed by Aβ42 labeled with ^{13}C. Fibrillation of the E22K-Aβ42 peptides was confirmed by electron microscopy (Fig. 2). The incubation conditions were the same as those used for preparing the samples for solid-state NMR. Each E22K-Aβ42 peptide was dissolved in 0.1% NH_4OH at 250 μM. After a 10-fold dilution with 50 mM sodium phosphate containing 100 mM NaCl at pH 7.1, the resulting peptide solution (25 μM, pH 7.4) was incubated at 37 °C for 48 h. After centrifugation, the supernatant was removed from the pellets. The deposits were suspended in distilled water by vortex mixing. The resulting suspensions were applied to a 400-mesh collodion-coated copper grid (Nissin EM, Tokyo, Japan) and dried in air, before being negatively stained for 2 min with 2% uranyl acetate. Formation of the fibrils was examined with an H-7500 electron microscope.

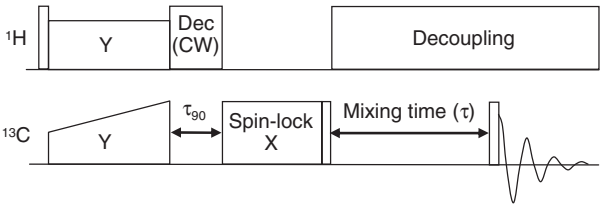

Fig. 3. Pulse Sequence for the 1D R2 Experiments.

Solid-state NMR experiments. Solid-state NMR experiments were performed at 9.4 T (100 MHz for ^{13}C) with a Chemagnetics Infinity NMR spectrometer and Chemagnetics 3.2 mm MAS probe at room temperature. The ^{13}C chemical shifts were calibrated in ppm relative to TMS by taking the ^{13}C chemical shift for the methine carbon nucleus of solid adamantane (29.5 ppm) as an external reference standard.

To evaluate the ^{13}C–^{13}C distance, 1D rotational resonance (R2)[14] experiments were conducted, the pulse sequence being shown in Fig. 3. Pulse sequence parameters for the R2 experiments were as follows: MAS speed = 12,540 and 12,650 Hz, variable amplitude cross polarization contact time = 0.5–2.0 ms, pulse delay = 2 s, two pulse phase-modulated ^1H decoupling power = 120 kHz, t_1 increment = 44 μs, and mixing time (τ) = 0–100 ms.

Results and Discussion

To verify the parallel arrangement of the intermolecular β-sheet, the proximity of the β-strands at more than two positions must be determined (Fig. 4A). Our previous study involving the systematic replacement of Aβ42 with proline suggested that alanine residues at

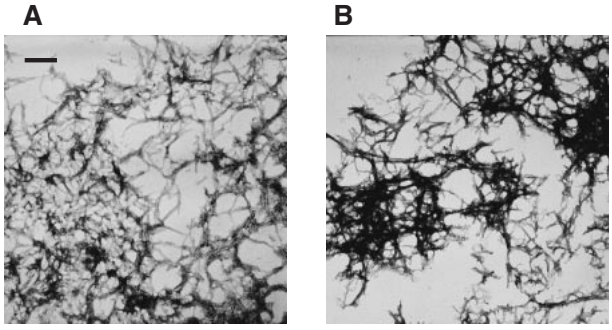

Fig. 2. Transmission Electron Micrographs of Negatively Stained Preparations of Fibrils.

A, Equal mixture of E22K-Aβ42 aggregates labeled at C_α of Ala-21 and that labeled at C=O of Ala-21 with ^{13}C. B, Equal mixture of E22K-Aβ42 aggregates labeled at C_α of Ala-30 and that labeled at C=O of Ala-30 with ^{13}C. Scale bar = 200 nm.

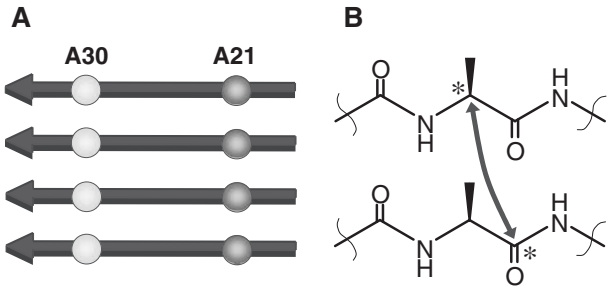

Fig. 4. Estimation of the Intermolecular Distance between β-Strands by the R2 Experiments.

A, Possible alignment of β-strands in the parallel β-sheet. B, Intermolecular labeling of C_α and C=O of the Ala residue in a parallel β-sheet with ^{13}C. Asterisks show carbon atoms labeled with ^{13}C. Double-headed arrows show possible dipole-dipole interactions reintroduced under the R2 conditions.

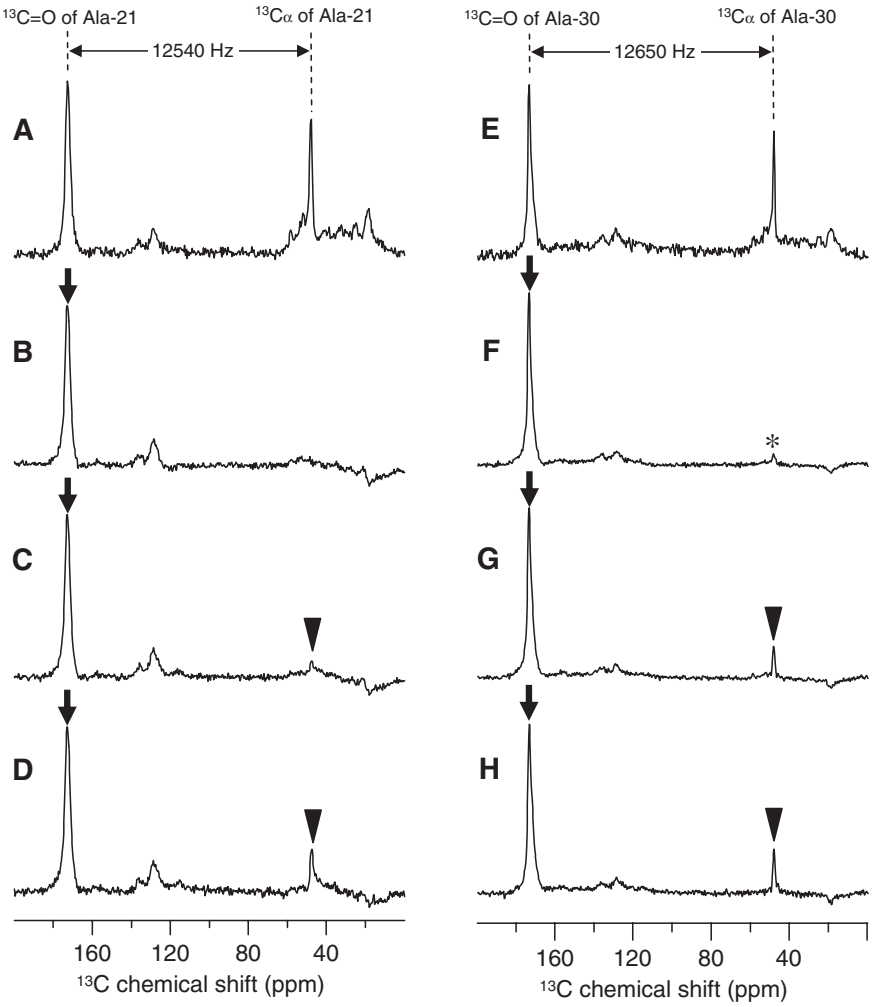

Fig. 5. Structural Analysis of the Intermolecular β-Sheet in E22K-Aβ42 Aggregates Using R2.

1D ^{13}C CP-MAS spectrum (ns = 1,024) (A) and 1D R2 spectra (ns = 10,800) (B–D) of an equal mixture of E22K-Aβ42 aggregates labeled at C_α of Ala-21 and those labeled at C=O of Ala-21 with ^{13}C (12.3 mg/tube). The mixing times were (B) 0 ms, (C) 13 ms, and (D) 100 ms. 1D ^{13}C CP-MAS spectrum (ns = 1,024) (E) and 1D R2 spectra (ns = 10,800) (F–H) of an equal mixture of E22K-Aβ42 aggregates labeled at C_α of Ala-30 and those labeled at C=O of Ala-30 with ^{13}C (9.9 mg/tube). The mixing times were (F) 0 ms, (G) 20 ms, and (H) 100 ms. The signal with the asterisk shows the spinning side band of ^{13}C=O. Magnetization transfer from ^{13}C=O (arrow) to $^{13}C_\alpha$ (arrowhead) by the R2 effect was observed at both Ala-21 and Ala-30.

positions 21 and 30 were included in the β-sheet region.[19] In this study, we examined the proximity between the β-strands at these positions using solid-state NMR.

To evaluate the intermolecular distance, we used rotational resonance (R2)[14] which is a reliable and convenient method for selectively detecting dipole-dipole interactions. In this experiment, the MAS speed was adjusted to the difference between the chemical shifts of the two ^{13}C spins of interest. Under the R2 condition, the magnetization transfer within the ^{13}C spins was driven by the reintroduction of the dipole-dipole interaction. In previous studies with R2 experiments, magnetization transfer between spatially remote carbons up to 6 Å was generally detected.[5,20,21] In the parallel β-sheet model (Fig. 4B), the intermolecular distance between C_α and C=O in the main chains would be proximal (4–6 Å). To solely detect the intermolecular dipole-dipole interaction accurately, we prepared aggregates of an equal mixture of E22K-Aβ42 labeled at C_α and that labeled at C=O with ^{13}C (Fig. 4B). E22K-Aβ42 peptides labeled with ^{13}C were prepared by solid-phase Fmoc synthesis, as reported previously.[13,17–19] Labeled E22K-Aβ42 (25 μM) aggregated completely at 37 °C in phosphate-buffered saline (pH 7.4) for 48 h. Typical fibril formation was confirmed by transmission electron microscopy (Fig. 2). After centrifuging and subsequent washing with distilled water, the aggregates were dried *in vacuo* and subjected to solid-state NMR measurement.

We applied the 1D R2 experiment (Fig. 3) to detect the magnetization transfer with high sensitivity. Briefly, we maintained one of the ^{13}C magnetizations by a spin-lock pulse until the other magnetization of interest had disappeared. After the required mixing time, the disappeared signal would be recovered by magnetization

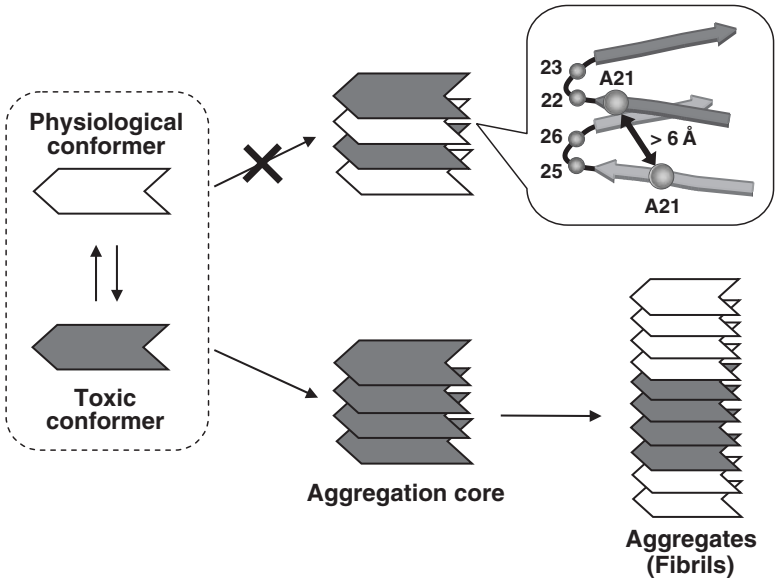

Fig. 6. Possible Supramolecular Structures of the Aβ42 Aggregates.

transfer from the remaining magnetization through dipole-dipole interaction.

As shown in Fig. 5A–D, the intermolecular magnetization transfer from $^{13}C{=}O$ to $^{13}C_\alpha$ was observed at Ala-21, and the signal intensity became larger in proportion to increasing mixing time. Clear cross-peaks were observed between $^{13}C{=}O$ and $^{13}C_\alpha$ in the 2D R2 spectrum with a mixing time of 50 ms (Supplemental Fig. 4; see the *Biosci. Biotechnol. Biochem.* Web site). These data suggest that the intermolecular distance between the main chains at Ala-21 was smaller than 6 Å. Similar R2 effects were obtained between $^{13}C{=}O$ and $^{13}C_\alpha$ at Ala-30 (Fig. 5E–H, Supplemental Fig. 5; see the *Biosci. Biotechnol. Biochem.* Web site); this indicates that the main chains between the β-strands at Ala-30 were also close to each other. These data strongly support the parallel alignment of the intermolecular β-sheet in the E22K-Aβ42 aggregates. This is the first report applying R2 in combination with mosaic labeling (Fig. 3B) to verify the intermolecular parallel β-sheet.

We have previously reported that E22K-Aβ42 aggregates contained physiological and toxic conformers, the turn positions of which were slightly different from each other (Fig. 1).[12] Although the present results revealed the existence of intermolecular parallel β-sheets in the E22K-Aβ42 aggregates, the positional relationship between the two conformers remains unclear. If the two conformers accumulated alternately (Fig. 6, upper model), the intermolecular distances between the β-strands at Ala-21 and Ala-30 would be greater than 6 Å. However, the intermolecular dipole-dipole interactions observed in this study were strong (Fig. 5), indicating that each conformer formed a relatively large assembly consisting of an intermolecular parallel β-sheet in the aggregates (Fig. 6, lower model). Since the aggregation velocity of the toxic conformer was much faster than that of the physiological conformers, the toxic conformers would form a nucleus, resulting in elongation of the physiological conformers (Fig. 6, lower model).

Acknowledgment

This research was partly supported by grant aid for scientific research A (no. 18208011 for K.I.) and for the promotion of science for young scientists (no. 18.3327 for Y.M.) from the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese government. We thank Dr. H. Fukuda of the Institute of Medical Science at Tokyo University for the MALDI-TOF-MS measurements.

References

1) Selkoe, D. J., Cell biology of protein misfolding: the examples of Alzheimer's and Parkinson's diseases. *Nat. Cell Biol.*, **6**, 1054–1061 (2004).
2) Davis-Salinas, J., Saporito-Irwin, S. M., Cotman, C. W., and Van Nostrand, W. E., Amyloid beta-protein induces its own production in cultured degenerating cerebrovascular smooth muscle cells. *J. Neurochem.*, **65**, 931–934 (1995).
3) Serpell, L. C., Alzheimer's amyloid fibrils: structure and assembly. *Biochim. Biophys. Acta*, **1502**, 16–30 (2000).
4) Meredith, S. C., Protein denaturation and aggregation: cellular responses to denatured and aggregated proteins. *Ann. NY Acad. Sci.*, **1066**, 181–221 (2005).
5) Lansbury Jr., P. T., Costa, P. R., Griffiths, J. M., Simon, E. J., Auger, M., Halverson, K. J., Kocisko, D. A., Hendsch, Z. S., Ashburn, T. T., Spencer, R. G. S., Tidor, B., and Griffin, R. G., Structural model for the β-amyloid fibril based on interstrand alignment of an antiparallel-sheet comprising a C-terminal peptide. *Nat. Struct. Biol.*, **2**, 990–998 (1995).

6) Balbach, J. J., Ishii, T., Antzutkin, O. N., Leapman, R. D., Rizzo, N. W., Dyda, F., Reed, J., and Tycko, R., Amyloid fibril formation by A$\beta_{16\text{-}22}$, a seven-residue fragment of the Alzheimer's β-amyloid peptide, and structural characterization by solid state NMR. *Biochemistry*, **39**, 13748–13759 (2000).
7) Petkova, A. T., Buntkowsky, G., Dyda, F., Leapman, R. D., Yau, W. M., and Tycko, R., Solid state NMR reveals a pH-dependent antiparallel β-sheet registry in fibrils formed by a beta-amyloid peptide. *J. Mol. Biol.*, **335**, 247–260 (2004).
8) Balbach, J. J., Petkova, A. T., Oyler, N. A., Antzutkin, O. N., Gordon, D. J., Meredith, S. C., and Tycko, R., Supramolecular structure in full-length Alzheimer's β-amyloid fibrils: evidence for a parallel β-sheet organization from solid-state nuclear magnetic resonance. *Biophys. J.*, **83**, 1205–1216 (2002).
9) Antzutkin, O. N., Leapman, R. D., Balbach, J. J., and Tycko, R., Supramolecular structural constraints on Alzheimer's β-amyloid fibrils from electron microscopy and solid-state nuclear magnetic resonance. *Biochemistry*, **41**, 15436–15450 (2002).
10) Török, M., Milton, S., Kayed, R., Wu, P., McIntire, T., Glabe, C. G., and Langen, R., Structural and dynamic features of Alzheimer's Aβ peptide in amyloid fibrils studied by site-directed spin labeling. *J. Biol. Chem.*, **277**, 40810–40815 (2002).
11) Paul, C., and Axelsen, P. H., β Sheet structure in amyloid β fibrils and vibrational dipolar coupling. *J. Am. Chem. Soc.*, **127**, 5754–5755 (2005).
12) Irie, K., Masuda, Y., Murakamia, K., Uemura, S., Ohigashi, H., Hara, H., Ohashi, R., Nakanishi, A., and Takegoshi, K., Analysis of toxic conformation of β-amyloid (Aβ42) by solid-state NMR and ESR. 49th Symposium on the Chemistry of Natural Products, Symposium Papers, 61–66 (2007).
13) Masuda, Y., Irie, K., Murakami, K., Ohigashi, H., Ohashi, R., Takegoshi, K., Shimizu, T., and Shirasawa, T., Verification of the turn at positions 22 and 23 of the β-amyloid fibrils with Italian mutation using solid-state NMR. *Bioorg. Med. Chem.*, **13**, 6803–6809 (2005).
14) Raleigh, D. P., Levitt, M. H., and Griffin, R. G., Rotational resonance in solid state NMR. *Chem. Phys. Lett.*, **146**, 71–76 (1988).
15) Carpino, L. A., 1-Hydroxy-7-azabenzotriazole. An efficient peptide coupling additive. *J. Am. Chem. Soc.*, **115**, 4397–4398 (1993).
16) Fields, C. G., Fields, G. B., Noble, R. L., and Cross, T. A., Solid phase peptide synthesis of ^{15}N-gramicidins A, B, and C and high performance liquid chromatographic purification. *Int. J. Peptide Protein Res.*, **33**, 298–303 (1989).
17) Irie, K., Oie, K., Nakahara, A., Yanai, T., Ohigashi, H., Wender, P. A., Fukuda, H., Konishi, H., and Kikkawa, U., Molecular basis for protein kinase C isozyme-selective binding: the synthesis, folding, and phorbol ester binding of the cysteine-rich domains of all protein kinase C isozymes. *J. Am. Chem. Soc.*, **120**, 9159–9167 (1998).
18) Murakami, K., Irie, K., Morimoto, A., Ohigashi, H., Shindo, M., Nagao, M., Shimizu, T., and Shirasawa, T., Synthesis, aggregation, neurotoxicity, and secondary structure of various Aβ1-42 mutants of familial Alzheimer's disease at positions 21–23. *Biochem. Biophys. Res. Commun.*, **294**, 5–10 (2002).
19) Morimoto, A., Irie, K., Murakami, K., Masuda, Y., Ohigashi, H., Nagao, M., Fukuda, H., Shimizu, T., and Shirasawa, T., Analysis of the secondary structure of β-amyloid (Aβ42) fibrils by systematic proline replacement. *J. Biol. Chem.*, **279**, 52781–52788 (2004).
20) Raleigh, D. P., Creuzet, F., Das Gupta, S. K., Levitt, M. H., and Griffin, R. G., Measurement of internuclear distances in polycrystalline solids: rotationally enhanced transfer of nuclear spin magnetization. *J. Am. Chem. Soc.*, **111**, 4502–4503 (1989).
21) Griffiths, J. M., and Griffin, R. G., Nuclear magnetic resonance methods for measuring dipolar couplings in rotating solids. *Analytica Chim. Acta*, **283**, 1081–1101 (1993).