

Conformational transition state is responsible for assembly of microtubule-binding domain of tau protein

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Abstract

In the brains of Alzheimer's disease patients, the tau protein dissociates from the axonal microtubule and abnormally aggregates to form a paired helical filament (PHF). One of the priorities in Alzheimer research is to clarify the mechanism of PHF formation. Although several reports on the regulation of tau assembly have been published, it is not yet clear whether *in vivo* PHFs are composed of β -structures or α -helices. Since the four-repeat microtubule-binding domain (4RMBD) of the tau protein has been considered to play an essential role in PHF formation, its heparin-induced assembly propensity was investigated by the thioflavin fluorescence method to clarify what conformation is most preferred for the assembly. We analyzed the assembly propensity of 4RMBD in Tris-HCl buffer with different trifluoroethanol (TFE) contents, because TFE reversibly induces the transition of the random structure to the α -helical structure in an aqueous solution. Consequently, it was observed that the 4RMBD assembly is most significantly favored to proceed in the 10–30% TFE solution, the concentration of which corresponds to the activated transition state of 4RMBD from a random structure to an α -helical structure, as determined from the circular dichroism (CD) spectral changes. Since such an assembly does not occur in a buffer containing TFE of <10% or >40%, the intermediate conformation between the random and α -helical structures could be most responsible for the PHF formation of 4RMBD. This is the first report to clarify that the non-native α -helical intermediate in transition from random coil is directly associated with filament formation at the start of PHF formation.

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Microtubules (MTs) play an important role in the maintenance of cell shape, cell division, axonal transport, secretion, and receptor activity. Microtubule-associated proteins (MAPs) are believed to be important for MT formation and stabilization [1]. The tau protein, one of the neuronal MAPs in mammalian brain, binds to MTs through the three- or four-repeat MT-binding domain (MBD) located in the C-terminal half [2]. Water-soluble tau protein in Alzheimer's disease patients was shown to bind to MTs with lower affinity than the normal tau and to aggregate into an insoluble structure

called paired helical filament (PHF) [3]. Since these aggregates are toxic to neurons owing to the damage they cause to the cell interior, it is important to find a means of preventing this pathological aggregation.

Concerning the PHF formation of the tau protein, several insights have accumulated in recent years. As examples, (i) the conformation of tau changes from a random structure to a higher-order structure with PHF formation [4], (ii) the core moiety of PHF is composed of MBD, which promotes PHF formation *in vitro* [5,6], and (iii) this assembly process can be enhanced by the oxidation of SH groups [6] or by polyanions such as heparin [7–9]. However, the starting mechanism of the self-assembly is still far from being fully understood.

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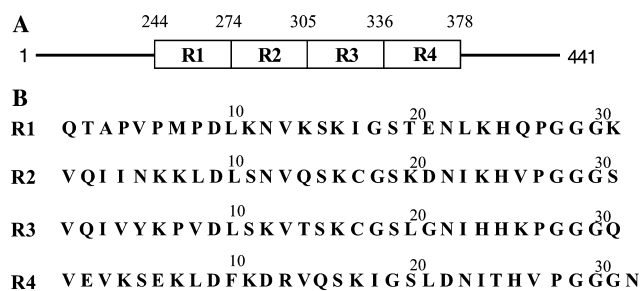


Fig. 1. Schematic of 4RMBD moiety in the entire human tau protein (A) and the amino acid sequence of each repeat (B). The regions from the first to the fourth repeat fragments in 4RMBD (A) are named R1–R4, respectively. The numbering of the amino acid residues in (A) refers to the longest isoform of the human tau protein (441 residues).

It is not even clear yet whether *in vivo* PHFs are composed of β -structures or α -helices.

Recently, we have reported on the trifluoroethanol (TFE)-induced reversible conformational transition of each repeat fragment of MBD from a random structure to an α -helical structure in an aqueous solution [10]. On the other hand, it has been reported that the fluorescence intensities of thioflavin dyes such as thioflavin S (ThS), which has been applied to stain neurofibrillary tangles (NFTs) in postmortem brains, could be used to quantify PHF formation in solution in real time [11]. Therefore, we used this assay to investigate the relationship between filament formation and four-repeat MBD (4RMBD) conformation in Tris–HCl buffer solution with different TFE contents. Here, we report on the importance of the conformational transition state on filament formation. This is the first report focusing on the conformation responsible for the MBD assembly, the result of which could provide important information on the structural behavior of the tau protein in the first step of the self-assembly.

The schematic of the full-length tau consisting of 4RMBD and each repeat construct is shown in Fig. 1, where the numbering of amino acid residues corresponds to the longest isoform of human tau having 441 residues.

Materials and methods

Chemicals and recombinant proteins. Heparin (average molecular weight = 6000) and ThS were obtained from Sigma. The gene expression and purification of His-tagged 4RMBD of human brain tau (Fig. 1) were performed according to a previous paper [12]. The purity was confirmed by SDS–PAGE analysis.

Peptide. R1–R4 peptides (Fig. 1) were synthesized by the American Peptide Company (California, USA), from which synthetic details can be obtained upon request. The respective peptides were characterized by mass spectrometry and determined to be >95.0% pure by reverse-phase HPLC. The samples (including trifluoroacetic acid as counter ion) were obtained in the lyophilized form.

CD measurements. The solution of 5 μ M 4RMBD was prepared with [10 mM Tris–HCl (pH 7.6)] and mixed with a predetermined

amount of TFE or heparin ($\leq 3.8 \mu$ M). All measurements at 25 $^{\circ}$ C were conducted with a JASCO J-820 spectrometer in a cuvette with a 2 mm path length. For each experiment under N_2 gas flow, the measurement from 190 to 250 nm was repeated eight times and summed up. Then, molar ellipticity was determined after normalizing the peptide concentration. The same experiments were performed at least three times using newly prepared samples, and their averaged values are provided. Data are expressed in terms of mean residue ellipticity $[\theta]$ in units of $\text{deg cm}^2 \text{dmol}^{-1}$.

Electron microscopy. 4RMBD and the R1–R4 peptides were adjusted to a concentration of 15 μ M and mixed with 3.8 μ M heparin using buffer A with different TFE contents. The solution was then incubated at 37 $^{\circ}$ C for 100 min. The 600-mesh copper grids were used for negative-staining electron microscopy (EM). A drop each of the protein solution and 2% uranyl acetate was placed on the grid. After 1 min, excess fluid was removed from the grids. Negative-staining EM was performed using an electron microscope (Hitachi) operated at 75 kV.

Monitoring of 4RMBD and R1–R4 aggregations by ThS fluorescence. 4RMBD and the R1–R4 peptides of 15 μ M concentration were adjusted using buffer A with different TFE contents and 10 μ M ThS dye. The aggregation was induced by adding heparin to the solution (final concentration = 3.8 μ M) and mixing with a pipette prior to fluorescence measurement. The time scan of the fluorescence intensity was carried out on a JASCO FP-6500 instrument with a 2-mm quartz cell maintained at 25 $^{\circ}$ C using a circulating water bath. The kinetics of 4RMBD aggregation was analyzed by recording the time-dependent curve of the intensity with an excitation at 440 nm and an emission at 500 nm. The excitation slit width was set at 3 nm and emission slit width was set at 10 nm. The background fluorescence of the sample was subtracted when necessary.

Results

The 4RMBD moiety of the tau protein takes a random conformation in aqueous solutions, and TFE induces the conformational transition to the α -helical structure [4,12]. To monitor the TFE-dependent conformational transition of 4RMBD, the CD spectrum was measured as a function of TFE content in buffer A (Fig. 2A). The CD spectra in the buffer with high TFE content (>40%) are indicative of the α -helical structure characterized by two negative peaks at approximately 209 and 222 nm, the conformation of which was slightly affected by pH variation, whereas those in the buffer without TFE showed a random conformation characterized by a negative peak at approximately 197 nm. The gross content of the α -helical structure could be estimated on the basis of CD ellipticity $[\theta]$ at 222 nm [13], and the α -helical structure content of MBD as a function of TFE content is shown in Fig. 2B. The heparin-induced CD spectral change of 4RMBD in buffer A containing 20% TFE is shown in Fig. 3. This figure suggests the heparin-induced conformational change of MBD from the α -helical structure to a kind of β -structure by the addition of heparin and a further structural change with reaction time.

The heparin-induced aggregation profiles of 4RMBD and its repeat fragments were monitored on the basis of the time profiles of fluorescence intensity in buffer A

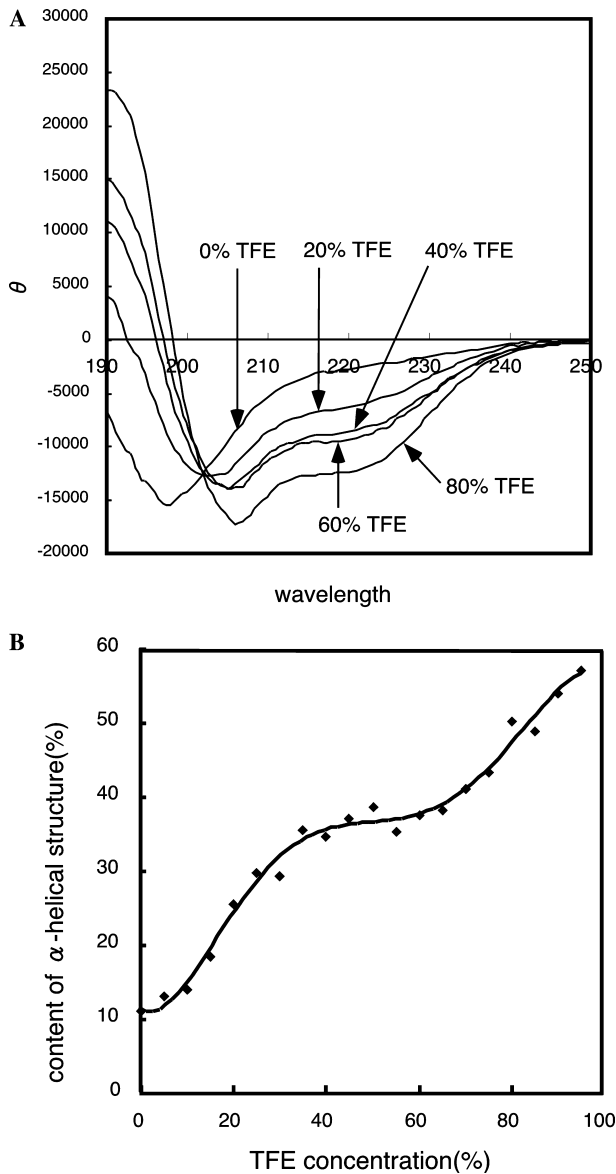


Fig. 2. (A) CD spectra of 4RMBD in buffer A with different TFE contents. Similar spectral changes were also observed for respective peptides of R1–R4 repeats. (B) Content of α -helical structure of 4RMBD as a function of TFE concentration.

with different TFE contents (Fig. 4). Consequently, it was shown that there is an optimal TFE concentration (10–30%) for 4RMBD filament formation. Similar, but not identical, TFE-dependent filament formation was also observed for the R2 and R3 peptides, although the speed of filament formation was much faster for the R3 peptide than the R2 peptide. In contrast, the R1 and R4 peptides did not show any filament formation during the experimental period despite the absence or presence of TFE. Fig. 5 shows the EM image of the heparin-induced fibrils of 4RMBD formed in buffer A containing 20% TFE. The essentially same EM image was observed for the 4RMBD prepared from buffer A without TFE,

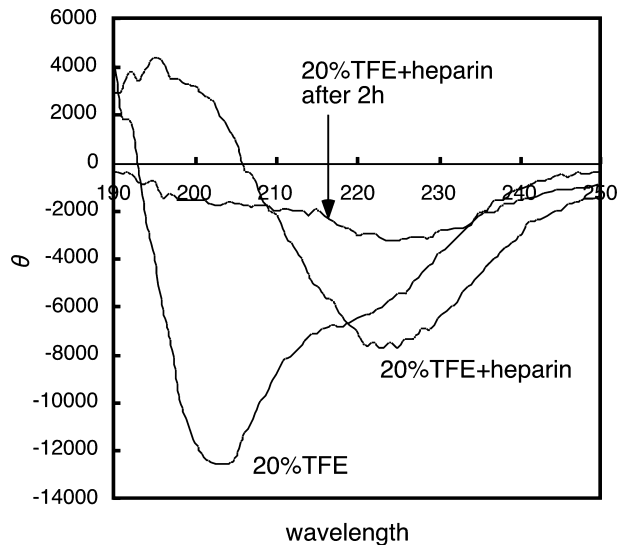


Fig. 3. Heparin-induced CD spectral change of 4RMBD in buffer A containing 20% TFE.

indicating that the aggregation form of 4RMBD is little affected by TFE.

Discussion

At present, it is not yet clarified what kind of secondary structure the aggregated form of the tau protein actually takes, whether α -helix or β -sheet. Moreover, there is no definitive conclusion concerning the conformational pathway of the tau random structure into the high-ordered PHF, although information on this pathway is very important for designing an inhibitor that can block the progression of Alzheimer's disease. The present results provide some useful information on the tau MBD conformation at the start of PHF formation. Fig. 2A shows that the reversible conformational transition of 4RMBD between the random and α -helical structures is caused by TFE. Although the transition of the random structure in buffer A occurs with the addition of TFE, the transition to the α -helical structure could be simulated by two curves, as shown in Fig. 2B, in which the slope of transition rate for buffer A with 10–30% TFE contents is considerably steep, as compared with that for buffer A with 40–100% TFE contents. Interestingly, the heparin-induced filament of 4RMBD is most significantly formed in buffer A containing 10–30% TFE; this is in contrast with that in buffer A without TFE or with 40–100% TFE, where 4RMBD undergoes filament formation at an extremely slow speed (Fig. 4A). The 10–30% TFE content corresponds just to the former curve in Fig. 2B. Although heparin ($\leq 3.8 \mu\text{M}$) in buffer A has a negative CD peak at 210 nm, its amplitude is small and is little affected by TFE. Therefore, it is clear that the intermediate conformation between the random and α -helical

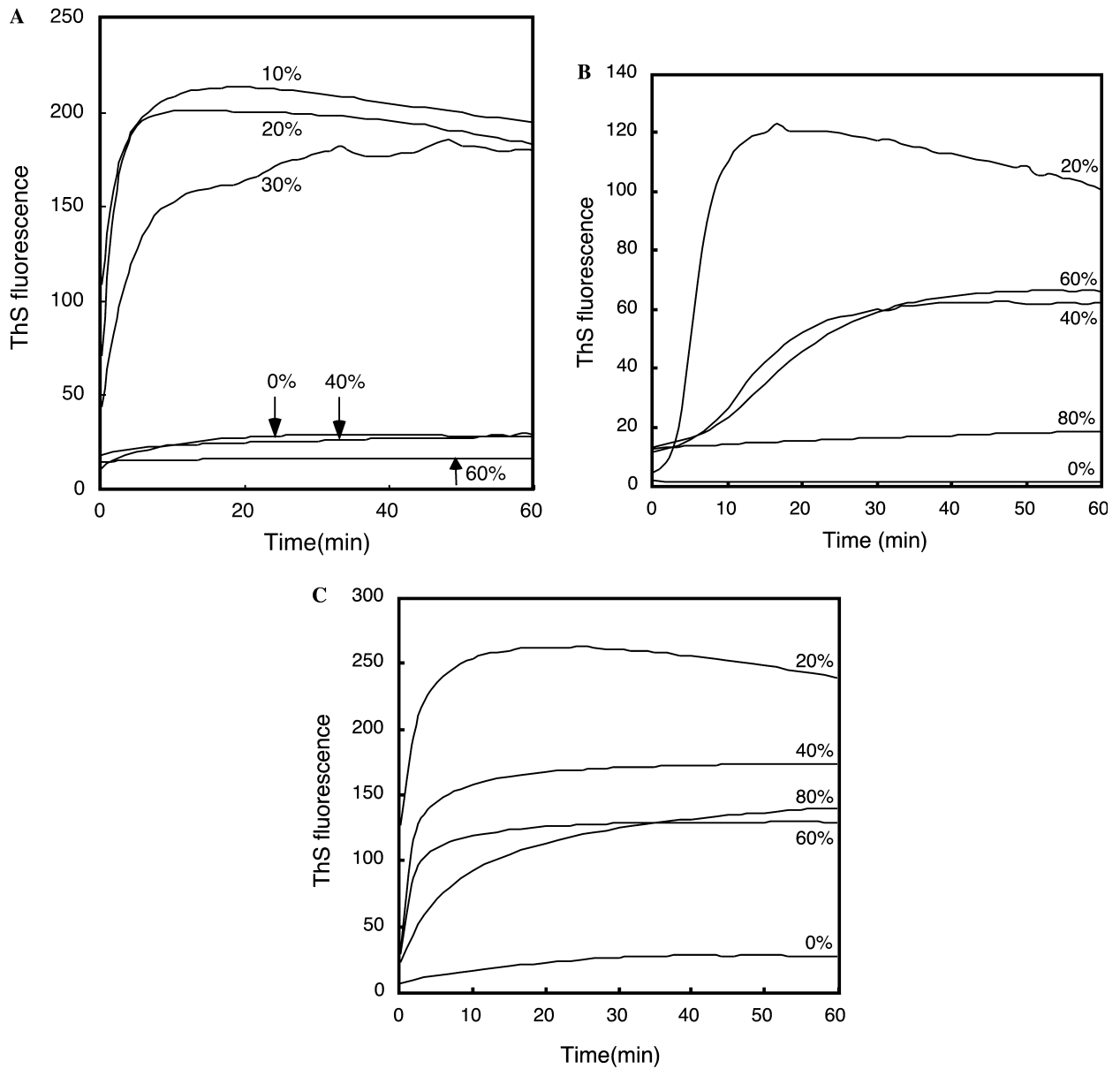


Fig. 4. Time profiles of 4RMBD (A), the R2 (B), and R3 peptide (C) aggregations in buffer A with different TFE contents.

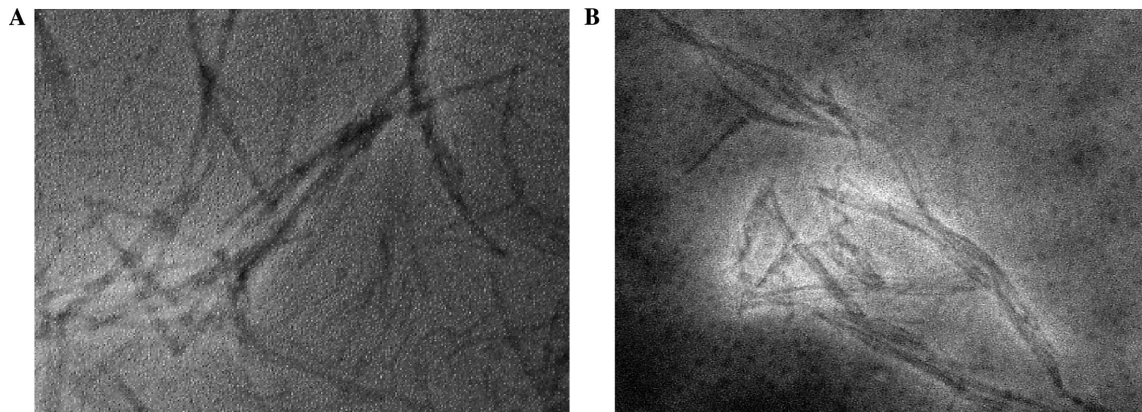


Fig. 5. Negative-staining EM image of 4RMBD filaments polymerized with heparin. The filaments assembled in buffer A without TFE (A) and with 20% TFE (B).

structures (here referred to as the ‘non-native α -helical intermediate’) is responsible for 4RMBD filament formation; that is, neither the CD-detectable random structure nor the α -helical structure itself is directly related with the filament formation in the absence of any conformational change.

It is interesting to note that the R2 and R3 peptides show a TFE-induced filament profile similar to that of 4RMBD (Fig. 4), whereas the R1 and R4 peptides do not exhibit any notable assembly during the experimental period. This suggests that 4RMBD filament formation is mainly promoted by R2 and R3 repeats of MBD, and the cysteine residues involved in R2 and R3 repeats, not in R1 and R4 repeats, of 4RMBD play the important role in the filament formation; the close relation between the disulfide bond formation and PHF formation of the tau protein has been proposed [2]. On the other hand, the faster assembly of the R3 peptide than that of the R2 peptide would be due to the different conformational behaviors between them. Recently, we have analyzed the solution conformations of R3 and R2 peptides in TFE by a combination of two-dimensional ^1H NMR measurements and molecular modeling calculations. The constructed NMR conformers of the R3 peptide showed that the N-terminal Val1–Lys6 sequence takes an extended conformation, whereas the Leu10–Leu20 moiety forms the α -helical structure with an amphipathic distribution of the respective side chains, although the C-terminal moiety is highly flexible without any secondary structure [10]. In contrast, the Val1–His25 sequence of the R2 peptide exhibited the sequential α -helical structure of similar amphipathic propensity [14]. The present results may indicate that the coexistence of different secondary structures within one repeat sequence in the R3 peptide is important for the filament formation; the importance of the R3 repeat of MBD for PHF formation has already been reported [15].

The CD spectrum of Fig. 3 indicates that 4RMBD filaments are composed of the β -like structure. Therefore, a possible pathway for MBD conformational change at the start of the assembly could be described as follows: random coil \rightarrow ‘non-native α -helical intermediate’ \rightarrow β -structure \rightarrow aggregation of the extended helical structure, where the most critical structure for the assembly is the non-native α -helical intermediate in transition from random coil conformation and thus differs from the energetically stable α -helical structure observed in the buffer containing $>40\%$ TFE. This assembly mechanism may also be applicable to the case of amyloid peptides [16]. In any event, this is the first report to clarify what conformation is responsible for the MBD assembly; it is useful for the understanding of the biological events occurring at the initial step of abnormal filament aggregation of the tau protein in Alzheimer’s disease.

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