

## Structural evaluation of conformational transition state responsible for self-assembly of tau microtubule-binding domain

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Received 30 November 2004

Available online 31 December 2004

### Abstract

In the brains of Alzheimer's disease patients, the tau protein abnormally aggregates to form an insoluble paired helical filament (PHF). Since the third repeat structure (R3) of the tau microtubule-binding domain plays an essential role in PHF formation and self-aggregates most significantly in an aqueous solution of 20–40% trifluoroethanol (TFE), its possible conformation was estimated from the combination of (i) the TFE-dependent deviations of NH and C $\alpha$ H proton chemical shifts from those of the random structure in water and (ii) the TFE-dependent NOE effect connectivity diagrams between the neighboring protons. Consequently, it was indicated that the extended structure of the N-terminal VQIVYK moiety and the  $\alpha$ -helical-like structure of the LSKVTSKC region provide a structural scaffold for initiating the self-assembled filament formation of the R3 structure. To the best of our knowledge, this is the first study that demonstrated the initial structural moiety and its structural feature necessary for starting the tau PHF formation.

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**Keywords:** Tau protein; Microtubule-binding domain; Assembly; Conformational transition

Alzheimer's disease (AD) is the most common cause of dementia in the elderly population. AD is accompanied by a number of structural and metabolic alterations in the brain and is characterized by two histopathological hallmarks, the extracellular deposits of  $\beta$ -amyloid in neuritic plaques and intracellular neurofibrillary tangles. The latter is composed of bundles of paired helical filaments (PHFs), which result from the abnormal aggregation of the tau protein [1]. The tau protein is one of the neuronal microtubule-associated proteins and contributes to the stabilization of an axonal microtubule (MT). Although the tau protein is highly soluble and shows hardly any tendency to

aggregate under physiological conditions, it dissociates from MT and aggregates to form insoluble PHF fibers in the brains of AD patients [2–4]. Since these aggregates are toxic to neurons because they damage the cell interior, it is important to find a means of preventing this pathological aggregation. Therefore, many in vivo and in vitro studies have been performed, and several models of the mechanism of aggregation of the tau protein into PHFs have been proposed to date [5–8]. However, a definite conclusion has not yet been drawn because of the lack of PHF structural information at the atomic level.

On the other hand, it is important upon considering the method of dementia prevention to clarify which part and what structural change of the tau protein are most responsible for the PHF formation. Since it is now

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presumed that the repeat structure of the MT-binding domain (MBD) (Fig. 1) constitutes the core moiety of the PHF structure [9,10], the analysis on the self-assembly mechanism of each repeat peptide could provide important information on the step-by-step process of MBD-filament formation, even though the filament itself does not directly reveal the true feature of the tau-PHF structure. Therefore, we have analyzed the conformational feature and the initial *in vitro* aggregation behavior of each repeat peptide [11–13].

Recently, we have reported [14] that the self-assembly of four-repeat MBD is most significantly promoted in the Tris–HCl buffer containing 10–30% trifluoroethanol (TFE), the concentration of which corresponds to an intermediate transition state of MBD from a random structure to an  $\alpha$ -helical structure. Since such an assembly does not occur in the buffer containing <10% or >40% TFE, it could be considered that the intermediate conformation between the random and  $\alpha$ -helical structures is most responsible for the assembly of MBD into PHFs. On the other hand, we examined the TFE-dependent filament formation behaviors of four repeat peptides in MBD (Fig. 1) and found that the R2 and R3 peptides have the optimal TFE contents required for the filament formation, which almost correspond to that of four-repeat MBD [14].

The structural evaluation of such an intermediate conformation is definitely important for identifying the initial structural requisite to starting the filament formation. Since the R3 peptide shows the most significant filament formation profile among the four repeat peptides [13,14], the possible conformational feature of the R3 peptide in the optimal TFE aqueous solution was estimated on the basis of the TFE-dependent NH and C $\alpha$ H proton chemical shift changes and the NOE connectivity diagrams between the neighboring protons. We report here these results. To the best of our knowledge, this is the first paper to report the initial structural moiety and its structural feature necessary for PHF formation.

## Materials and methods

**Peptide.** The R3 peptide, which corresponds to the third (306–336) repeat moiety of the full-length human tau protein, was synthesized using a solid-phase peptide synthesizer. The peptide was characterized by mass spectrometry and had a purity of >95.0% as assessed by reverse-phase high-pressure liquid chromatography. The sample (including trifluoroacetic acid as counter ion) was obtained in the form of a lyophilized powder.

**NMR measurements.** The R3 peptide was dissolved in water/TFE- $d_2$  mixtures with 0%, 10%, 20%, 30%, 40%, 60%, 80%, and 100% TFE contents.  $^1\text{H}$  nuclear magnetic resonance (NMR) spectra were recorded on a Varian unity INOVA 500 spectrometer using a variable temperature-control unit.  $^1\text{H}$  chemical shifts were referenced to 0 ppm 3-(trimethylsilyl)-propionic acid (TSP). From the comparison of respective NMR spectra under different pHs and temperatures, the conditions for conventional NMR measurements were finally determined as follows: concentration 1 mM, temperature 298 K, and pH 4.2 (pH > 5.0 will decrease the solubility of the peptide in solution). The pH was adjusted by adding HCl or NaOH. Two-dimensional total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) were acquired in a phase-sensitive mode using standard pulse programs available in the Varian software library. The proton peak assignments in the mixtures of different water/TFE ratios were performed by combining connectivity information via the scalar couplings in phase-sensitive TOCSY experiments and the sequential NOE networks along peptide backbone protons. Assuming the same correlation time for all the protons, the offset dependence of NOESY cross-peaks was also used for the estimation of the secondary structure of the peptide, where the NOE intensities were classified into three groups (strong, medium, and weak).

## Results

### Amide proton chemical shifts at different water/TFE ratios

Since the chemical shifts of amide protons are sensitively influenced by environmental changes and the peptide is induced to a helical conformation by alcohol solvents such as TFE, the TFE-dependent chemical shift changes of amide protons were measured to determine which structural part is most sensitive to the conformational change of the R3 peptide. As the

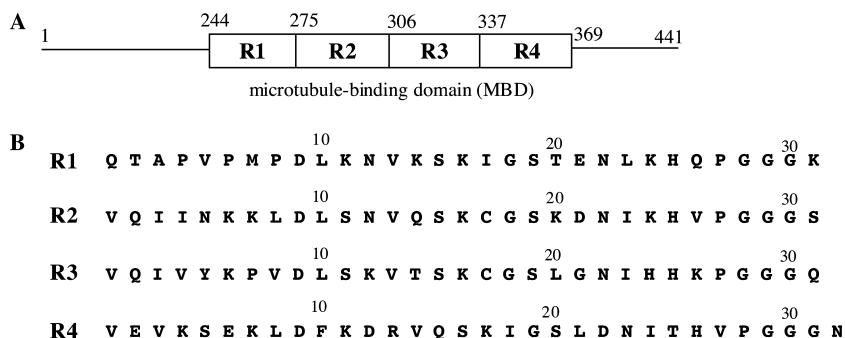


Fig. 1. Schematic of human full-length tau protein (A) and amino acid sequences of four-repeat MBD moiety (B). The regions from the first repeat structure to the fourth repeat structure in MBD are named R1 to R4, respectively. The number of the amino acid residues in (A) refers to the longest isoform of the human tau protein (441 residues).

standard value for the estimation of TFE-dependent conformational variation, the deviation from the chemical shift in water was used, because the R3 peptide in water takes a random conformation [12]. The deviation profile of each residue in different TFE contents is shown in Fig. 2A. It is characteristic that the amide proton chemical shifts of the Ile3-Leu10 and His24-Gln31 sequences showed substantial upfield shifts in proportion to TFE content. We previously reported that these sequences take the extended and random structures in both water and TFE solvents, respectively [12]. Therefore, it could be considered that these upfield shifts result from a decreased polarity of solvents with an increase of TFE content, because each residue of an extended or random structure is equally influenced by the solvent. On the other hand, the amide proton chemical shifts of the Lys12-Ile23 sequence showed the upfield shifts in 0–20% TFE and then the downfield shifts in >40% TFE. On the basis of the solution conformation in 100% TFE [11], we presume that these downfield shifts result from the electrostatic interactions (including some hydrogen bonds) of the NH protons with the neighboring carbonyl oxygen atoms, caused by the formation of the  $\alpha$ -helical structure.

#### $\alpha$ proton chemical shifts at different water/TFE ratios

It is known that the positive deviations of C $\alpha$  protons from the chemical shifts in water correlate with the structural propensity towards the  $\beta$ -structure, whereas the negative deviations correlate with the  $\alpha$ -helical structure [15,16]. Therefore, we estimated the residual structure of the R3 peptide from the deviation change of the C $\alpha$ H proton chemical shift (Fig. 2B). Each residue

of the Gln2-Lys6 sequence exhibited a small and nearly the same positive deviation in >20% TFE, indicating the preference of this region for a  $\beta$ -structure. This deviation profile is almost independent of TFE content and is dissimilar to that of the C-terminal flexible His25-Gln31 region. On the other hand, the Leu10-Cys17 sequence, except Lys16, showed the negative deviation in >20% TFE. Although the deviation is not necessarily proportional to TFE content, this profile indicates a preference for an  $\alpha$ -helical structure. In particular, the relationship of the TFE content—chemical shift deviation showed a large slope for the Lys12, Val13, Thr14, and Cys17 residues, suggesting the easy  $\alpha$ -helical formation of this region at an early stage of the TFE-induced conformational transition from a random structure in water.

#### NOE connectivity between neighboring protons at different water/TFE ratios

The sequential NOE networks along the peptide backbone protons at different water/TFE ratios are shown in Fig. 3. Since the NOE cross-peak patterns of  $d_{\alpha N(i,i+1)}$  in the N-terminal VQIVYK sequence did not significantly change in all solutions of different water/TFE ratios, the conformation of this sequence could be extended and rigid. In contrast, the Leu10-Val13, Ser11-Thr14, and Thr14-Cys17 residual pairs started to show the  $d_{\alpha N(i,i+3)}$  and  $d_{\alpha\beta(i,i+3)}$  NOE cross-peaks at 30% TFE and were clearly observed at 40% TFE, and these NOE patterns increased with TFE content. These NOE connectivity patterns indicate the formation of an  $\alpha$ -helical structure at the Leu10-Cys17 sequence, and this agrees with the behavior of their C $\alpha$ H proton chemical shift changes.

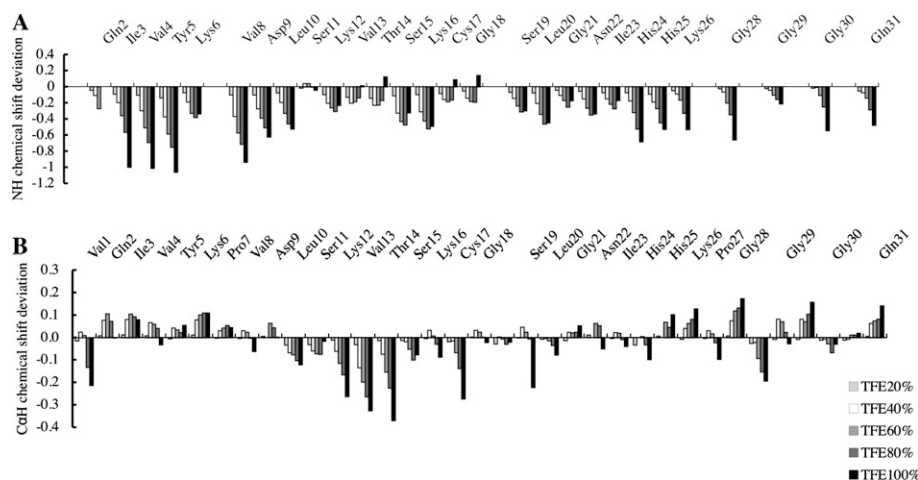


Fig. 2. TFE content-dependent deviations of the amide NH (A) and C $\alpha$ H (B) proton chemical shifts of R3 peptide compared with those in water. The bars arranged from left to right for each residue represent the differences (in ppm) in chemical shifts in 20%, 40%, 60%, 80%, and 100% TFE compared with those in water, respectively. The horizontal line corresponds to the chemical shift of each residue in water. The upper and lower sides of the baseline represent the downfield and upfield shifts of the proton, respectively.



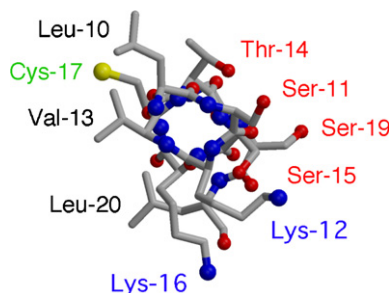


Fig. 5. Helical wheel drawing of a NMR-derived stable conformer of R3 with Leu10–Leu20 amino acid residues in 100% TFE, viewed from the N-terminal side. The residues are classified by their hydrophobic (black), hydrophilic (red), and basic (blue) behaviors, whereas Cys17 is shown in green for clarity. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

induced in 30–40% TFE, the following mechanism is proposed for the initial process of filament formation: the two neighboring R3 peptides are associated with each other by hydrophobic interactions, and an intermolecular disulfide bond formation between the neighboring Cys17 residues is most easily performed under oxidative conditions, leading to dimer formation, which would provide the effective building block for enhanced filament formation [9].

Although a unified scheme has not yet been established concerning the mechanism underlying PHF formation, the transformation of a soluble random structure  $\rightarrow$  intermediate structure via  $\alpha$ -helical structure  $\rightarrow$   $\beta$ -sheet structure  $\rightarrow$  insoluble filament is now considered as the major PHF formation process of the tau protein. The present study on the conformational behavior of the third repeat fragment at different water/TFE ratios is the first to demonstrate that the rigid extended Val1–Lys6 sequence and the TFE-sensitive helical-forming Leu10–Cys17 sequence could be most responsible for initiating MBD assembly.

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