Changes in Conformation of Human Neuronal Tau During Denaturation in Formaldehyde Solution

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Abstract: Human neuronal tau was incubated in formaldehyde solution at low concentrations and the intensity of light scattering of tau-40 solution at 480 nm increased markedly. Then potassium iodide was used to quench the intrinsic fluorescence of tau. The fluorescent quenching constants decreased as formaldehyde concentrations increased. 8-anilino-1-naphthalenesulfonic acid (ANS) binding assay showed that a putative hydrophobic core formed in tau polymers during incubation with formaldehyde. Native tau was hydrolyzed by immobilized earthworm fibrinolytic enzyme-II (EFE-II), producing a digested fragment (36-37 kDa). However, formaldehyde-treated tau could not be digested under the same conditions, suggesting that aggregated protein was relatively rigidly deposited.

Keywords: human neuronal tau, 8-anilino-1-naphthalenesulfonic acid (ANS), tau aggregation, denaturation, formaldehyde.

INTRODUCTION

Tau protein plays an important role in neuronal morphogenesis, axonal shape maintenance and axonal transport though it is able to bind and regulate microtubule structure and dynamics [1, 2]. Tau has a flexible molecular structure under natural conditions. Recently, experiments using circular dichroism have shown that tau only has a minimal content of the secondary structures at room temperature, and this "denatured" state is called the worm-like conformation [3], suggesting the whole polypeptidyl backbone of tau is very flexible and changeable in solutions.

Interest in the misfolding of tau protein has increased in recent years, since tau aggregation is regarded as a key model to study pathological processes of protein deposition in some neurodegeneration diseases, for instance, Alzheimer's disease (AD). Patients with AD suffer from neurofibrillary tangles (NFTs) that are histopathological lesions and characteristic of Alzheimer's disease brain [4, 5]. Abnormally hyperphosphorylated tau and the resultant paired helical filaments PHFs are the major component of NFT [6]. The polymerization and deposition process of tau may facilitate understanding of both PHF and amyloid-like-deposit formation [7, 8].

As a widely used compound, methanol is employed as an important substance in dope, gasoline and antifreeze, especially as an organic solvent in biomedical fields. Furthermore, formaldehyde is also widely used in our daily life, such as oil painting, additive and chemical fixing reagent. Formaldehyde is the intermediate product from methanol to formic acid in the pathway in our liver cells [9], in the case of methanol toxicosis or minimal amount absorbed into our blood. It is formaldehyde that plays the most harmful role in methanol toxicosis and lesions to central nerves, especially in the optic nerve system [10]. In this lab, we have found that formaldehyde at low concentration is able to induce tau aggregation [7, 11, 12]. Here we suppose that formaldehyde is able to induce polymerization and disposition of some functionally important proteins in nerve systems, for instance neuronal tau, during formaldehyde toxicosis. To investigate how formaldehyde affects tau conformation is of importance to detect some significant pathological roles of methanol toxicosis, and gives us a clue to understand the mechanism of tau aggregation.

1. MATERIALS AND METHODS

1.1. Materials

Sephadex G50, Q-Sepharose and SP-Sepharose were from Pharmacia. 8-anilino-1-naphthalene-sulfonic acid (ANS) and potassium iodide came from Sigma (St. Louis). Rabbit antibody R134d against tau was kindly provided by Dr. Iqbal (New York State Institute of Disability, USA). I^{125}-labeled secondary goat anti-rabbit IgG were from NEM Life Science Products, Inc USA. pET-15b plasmid came from Promega. Earthworm fibrinolytic enzyme-II (EFE-II) was prepared as described previously [13, 14]. Other reagents used were of analytic grade without further purification. The fluorescence and light-scattering were measured on a fluorescence spectrophotometer [Hitachi F-4500], and absorbance was measured on a spectrophotometer [Hitachi U-2010].

1.2. Expression, Purification and Aggregation of Protein

Tau was obtained by *in vitro* expression of a recombinant human neuronal tau_{441} clone that contained the full length of

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tau_{441} gene and was kindly provided by Dr. Goedert, University of Cambridge, UK. Tau was purified in queues with Q-Sepharose, SP-Sepharose and Sephadex-G75 columns, as described [15, 16]. The expressed protein was full-length tau_{441} without any fusion peptide. The purified protein was checked by SDS-PAGE, showing a single protein band. The protein concentration was determined spectrophotometrically, by using $E^{280}_{mg/ml} = 0.27$ [17].

1.3. Aggregation in Formaldehyde Solution

Tau (1 μM as final concentration) was incubated with formaldehyde at different concentrations (from 0.01 to 0.5%) in 25 mM phosphate buffer (pH 7.0) at 25°C (or 37°C) overnight before use. Aliquots were taken to check the resultant aggregation in SDS-PAGE. Then an Amicon Microcon-10 column was employed to remove the excess formaldehyde before use.

1.4. Light Scattering

Light scattering was used to detect (at 480 nm) tau aggregation when it was treated with formaldehyde at different concentration at 25°C (or 37°C). BSA was used as control [12].

1.5. KI Quenching

The intrinsic fluorescence of tau that was incubated with formaldehyde at different concentrations was quenched by KI at 25°C, and the emission intensity was measured at 305 nm by excitation at 230 nm. Data were analyzed as described by Laemmli [18].

1.6. ANS binding Assay

Tau (1 μM as final concentration) was incubated with ANS (molar ratio: tau/ANS = 1/40) in 25 mM phosphate buffer (pH 7.0) containing formaldehyde at different concentrations at 25°C overnight. Aliquots were taken for measurement of fluorescence spectra (400-600 nm) by excitation at 350 nm as described previously by Fink *et al.* [19].

1.7. Digestion by Immobilized EFE-II

Immobilized EFE-II was prepared as described by Wu *et al.* [20, 21]. Both native tau (1.5 mg/ml as final concentration) and formaldehyde treated-tau were incubated with the immobilized enzyme at 37°C followed by aliquots were taken for SDS-PAGE at different time intervals.

2. RESULTS AND DISCUSSION

2.1. Formaldehyde at Low Concentration Induces Tau to Aggregate

When a soluble protein aggregates, its solution will become colloidal, enhancing the intensity of light-scattering. As shown in Figure **1**, the intensity of light-scattering of neuronal tau solution noticeably increases when formaldehyde concentrations are from 0.01-0.2 %. On the other hand, that of the BSA solution (as control) can not be detected under the same conditions. This is to say, formaldehyde at low concentration induces tau to aggregate. Similar results have been obtained with gel electrophoresis (unpublished data in this lab, [7]). It suggests that formaldehyde is an important agent involved in tau aggregation.

Neuronal tau-40 has eight aromatic amino acid residues contributed to intrinsic fluorescence, five Tyr and three Phe [22]. The fluorescence intensity of Phe is much weaker than that of Tyr. Thus, Tyr is the significant contributor to intrinsic fluorescence (Ex 230, 278 nm; Em 305 nm [23]) of tau molecule. As described by Laemmli [18], solvent molecules may have the maximum probability to collide with a fluorophore of a protein when the fluorophore is completely exposed in solution, followed by the maximum quenching of the fluorescence. On the basis of this hypothesis, we monitored intrinsic fluorescence quenching in potassium iodide solution after tau had been aggregated in formaldehyde at different concentrations (Figure **2**). The

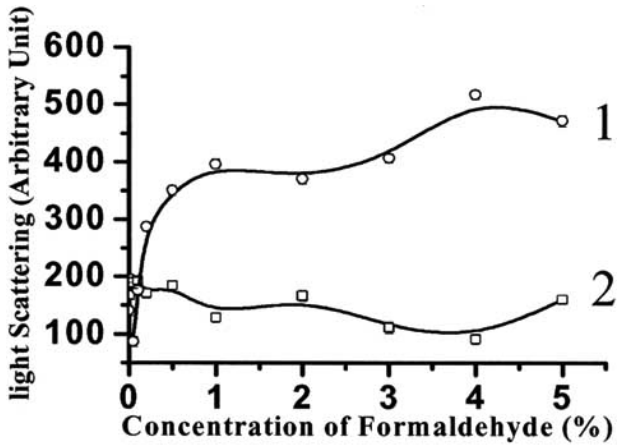

Figure 1. Light scattering of tau-40 incubated with formaldehyde at different concentrations. Tau-40 (1 μM as final concentration) was incubated with formaldehyde at different concentrations (%) in 25 mM phosphate buffer (pH 7.0) at 25°C for 24 h, before the intensity of light scattering at 480 nm was measured. BSA was used as control. Curves 1 and 2 represented light scattering intensities of tau and those of BSA, respectively.

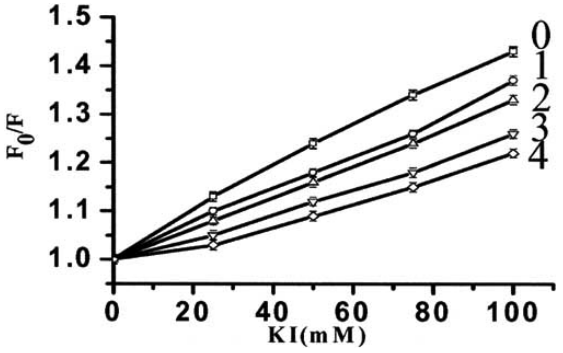

Figure 2. Quenching in the intrinsic fluorescence of neuronal tau protein. The intrinsic fluorescence of formaldehyde-incubated tau (1 μM as final concentration) was quenched by KI at 25°C. Fluorescence was measured at 305 nm by excitation at 230 nm. Curves 1 through 4 represented tau incubated with formaldehyde at 0.05, 0.1, 0.2 and 0.5 %, respectively. Tau incubated without formaldehyde was used as control (curve 0). The ordinate was F_0/F, where F_0 was the fluorescence of control and F was that tau incubated with formaldehyde.

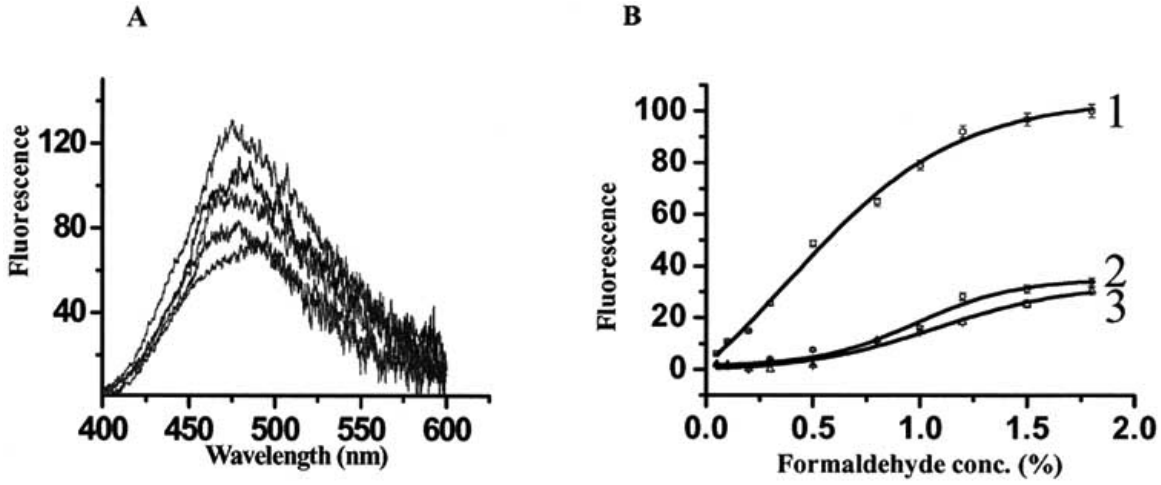

Figure 3. Changes in the fluorescence of ANS treated tau in formaldehyde solution at different concentrations. Tau (1 μM as final concentration) and BSA were incubated with ANS (molar ratio: tau/ANS = 1/40) in 25mM phosphate buffer (pH 7.0) containing formaldehyde at different concentrations at 25°C overnight. Aliquots were taken for measurement of fluorescence spectra (400-600 nm) by excitation at 350 nm. A. The fluorescence spectra of the formaldehyde treated-tau subtract those of native tau. B. Changes in ANS fluorescence of tau incubated with (curve 1) or without formaldehyde (curve 2) at different concentrations. BSA incubated with formaldehyde was used as control (curve 3).

slope of the quenching curve decreased as the formaldehyde concentration increased. That is to say, the collusion probability of KI to Tyr residues becomes smaller while tau is induced to aggregate in formaldehyde solution. At least, it indicates that the aromatic residues may shift to the interior of the molecule during aggregation. In addition, an equally likely explanation is that multiple molecules are crosslinked in the aggregated tau, which has the effect of making the Tyr residues less accessible.

2.2. ANS Binding to Formaldehyde Treated-TAU

ANS, with a strong fluorescence in a hydrophobic microenvironment, is commonly used as a fluorescent probe nonspecifically binding to hydrophobic region or core of protein [24]. As described previously [7], formaldehyde at low concentration (0.01 0.1 %) distinguishably induced tau to aggregate at room temperature at 37°C. Nevertheless, acetaldehyde could not be detected to have such an effect under the same conditions (Nie *et al.* unpublished data). Here, we are concerned about the conformational characteristic of tau when it is treated with formaldehyde. As shown in Figure **3**, the fluorescence of ANS at 480 nm is enhanced as tau was incubated with formaldehyde at different concentrations. Formaldehyde solutions, as control, do not fluoresce at the wavelength under the same conditions. It is suggested that a hydrophobic core may form when neuronal tau aggregates in formaldehyde solution.

3. PROTEASE DIGESTION

According to Fink *et al.*, there may form a hydrophobic core in amyloid-like protein through refolding during aggregation [25, 26]. It is necessary to investigate which part of tau molecule participates to form the inner core induced by formaldehyde. To detect the buried peptide region, we incubated tau with immobilized EFE-II and digested both native tau and 0.05% formaldehyde-treated tau (Figure **4A**). SDS-PAGE shows that no digested fragments are produced, except tau polymers retard at the top of the gel. It suggests that the polymerized protein induced by formaldehyde treatment form rigid deposits that are not readily cleaved by the protease. On the other hand, native tau is readily digested, showing a multiple hydrolyzed bands in the gel at the initial stage, indicating that neuronal tau has multiple hydrolytic sites recognized by EFE-II (Figure **4B**). Consequently, all the digested bands disappear as time passes, except a transient fragment (36~37kD). However, BSA, as control, shows no polymerizations before and after incubated with formaldehyde (Figure **4C**). Formaldehyde-treated BSA can be thoroughly digested, similar to the native protein. Sequencing analysis shows the N-terminus of the fragment (M-A-E-R-Q-) is the same as native tau. It is supposed that the cleavage site may be located in the microtubule-associated region [27]. After 30 min, the transient fragment will be digested. According to Schweer *et al.* [3], tau is a worm-like protein with an unstable conformation, and thus its hydrolytic sites are readily exposed to the protease. The existence of transient fragment reveals that the buried region may be in the microtubule-associated region. When tau is aggregated in formaldehyde solution, this site buries into the molecule and constitutes a stable core of the polymers and then the protease cannot recognize and cleave it. This features are similar to amyloid-like protein [28-29], suggesting that formaldehyde-treated tau may form a amyloid-like deposits.

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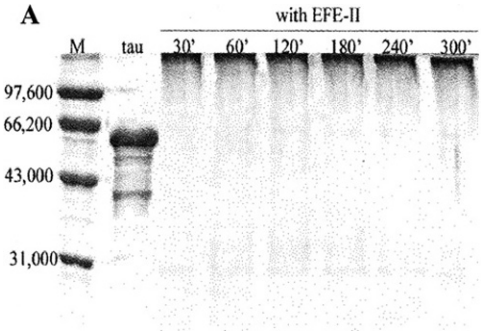
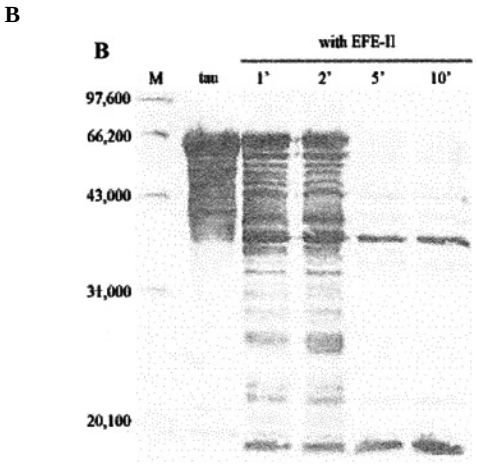
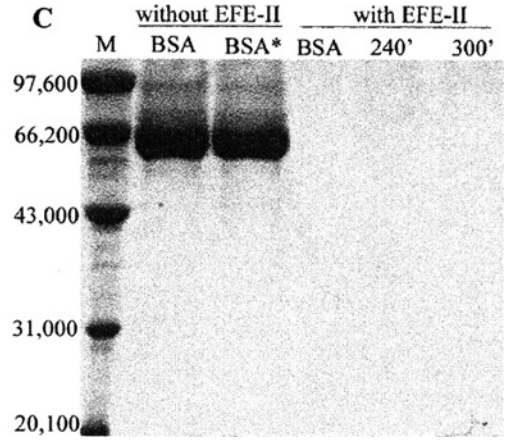


Panel A, Native tau was used.



Panel B, formaldehyde treated-tau was employed.



Panel C, both BSA and formaldehyde-treated BSA* were as controls, and incubated with EFE-II.

Figure 4. Digestion of formaldehyde treated-neuronal tau by earthworm fibrinolytic enzyme-II.
Immobilized earthworm fibrinolytic enzyme-II was prepared as described by Wu *et al.* [21]. Proteins (1.5 mg/ml as final concentration) were incubated with the immobilized enzyme at 37°C. Aliquots were taken for SDS-PAGE at different time intervals. Lane M was molecular mass markers. "tau" and number with prime represented tau as control and digestion time (min) with EFE-II, respectively.

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