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CONFORMATIONAL CHANGES OF HUMAN NEURONAL TAU DURING THERMAL AND GUANIDINE-HCL DENATURATION

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Unfolding of human neuronal tau was studied during incubation with guanidine hydrochloride (GuHCl) and under thermal denaturation conditions. The intensity of the intrinsic fluorescence at 305 nm increased markedly in GuHCl solutions. Fluorescence quenching by potassium iodide showed that Tyr residues approached to the interior of the molecule during GuHCl denaturation. The difference ultraviolet absorbance at 215 nm showed a marked increase above 45°C. Light scattering of tau solution intensity decreased when temperature increased. Kinetics of the increase in the absorbance was a biphasic procedure. The first order rate of the fast phase was 1.84 × $10^{-1}s^{-1}$ and that of the slow phase was 2.68 × $10^{-2}s^{-1}$. The difference absorbance of tau was reversible. Kinetics of a decrease in the difference absorbance was a fast procedure during tau refolding. The first order rate of the fast phase was much greater than that of the unfolding procedure. This suggests that tau is not completely at a random conformation at room temperature.

Key Words: Tau; Conformation; Denaturation; Fluorescence
*Corresponding author. *Abbreviations*: GuHCl (guanidine hydrochloride); AD (Alzheimer's disease)

1. Introduction

Filaments with a straight or paired helical morphology are the major components of the neurofibrillary tangles (NFTs) that form a defining neuropathological characteristic of Alzheimer's disease [1-2]. Solubilization of these filaments reveals that they are composed of the microtubule-associated protein tau [2]. Tau plays an important role in neuronal morphogenesis, the maintenance of axonal shape, and axonal transport through its ability to bind and regulate microtubule structure and dynamics [3]. NFTs are not unique to Alzheimer's disease, they are also abundantly present in Guam-Parkinsonism dementis complex, dementia pugilistica, Postencephalitic parkinsonism and Down's syndrome, among others [4].

Tau is resistant to heat and acid treatment without losing its function to promote microtubule assembly after renaturation [5-6] and behaves in flexibility of its molecular structure under natural conditions. Some authors observed tau has only a minimal content of secondary structure at room temperature and behaves as if it was in a naturally "denatured" states [7], by having detected that tau contains little secondary structure. Therefore, tau is thought as a molecule with "a worm-like structure", the polypeptide backbone of this protein exhibits random states in solutions. However, attempts have been made to investigate the stability of tau in solution, even though it has little secondary structure. Here we report some characterizations about tau conformation when it denatures or renatures.

2. Materials and Methods

2.1. Materials The clone of the recombinant human tau-40 came from Dr. Goedert (University of Cambridge, UK). Sephadex G-50, Q-Sepharose and SP-Sepharose were purchased from Pharmacia Company. Ultra pure GuHCl came from Sigma Company, which was purified according to Nozaki before used [8]. The absorbance of 5 M GuHCl at 250 nm was less than 0.05. Tyr and oligopeptide Gly-Tyr-Ala was used as control for fluorescence and absorbance experiments, were purchased from Sigma. KI was a guaranteed reagent from a local chemical. Distilled water was purified with LabConco Water'PS before use in fluorescence measurement. Other reagents used were of analytic grade without further purification.

2.2. Expression and purification of tau Tau protein was purified from lysates of *E. coli*, which over expressed htau-40, as described by Paudel *et al* [3]. SDS-PAGE was used to check the purification of tau. Tau showed a single band in gel with an apparent molecular weight around 64 KD after purification. The protein was lyophilized and stored at -20°C before use.

2.3. Intrinsic fluorescence detection and tau denaturation in GuHCl Tau (final concentration, 1 μM) was dissolved in 0.05 M phosphate buffer (pH 7.2) before measurement of the intrinsic fluorescence at 305 nm by excitation at both 235 and 280 nm (slits: Em 5.0 nm and Ex 5.0 nm) at room temperature. A Hitachi F-4500 fluorescence spectrophotometer was used for measurement of the fluorescence. For denaturation of tau, the protein was incubated in GuHCl solutions of different concentrations at 25°C, for 24 hrs to reach complete unfolding. GuHCl was at desired concentrations. No further changes in the fluorescence were detected when tau was incubated with GuHCl over 24 hrs. The intensity of the emission maximum was measured after the incubation in GuHCl solutions. Tyr (1 μM) was dissolved in the same phosphate buffer, to compare with the fluorescence of tau under the same conditions.

2.4. Fluorescence quench by KI After tau was denatured in GuHCl solutions of different concentrations (conditions were the same as described above), KI was titrated to tau solution at 25°C, followed by measurement of the intensity of the intrinsic fluorescence. KI (final concentration, 5.0 M) was dissolved in the phosphate buffer (pH 7.2) before titration. Data were plotted according to Stern and Volmer [9].

2.5. Measurements of the ultraviolet difference absorbance during thermal denaturation and renaturation Tau (final concentration, 60 μg/ml) was resuspended in 25mM Phosphate buffer (pH 7.2) and incubated at graded temperatures (25-65°C). Tau was incubated for 10 min at each temperature before measurement of the difference absorbance. No further changes in the absorbance were detected when tau was incubated over 10 min. For thermal renaturation, conditions were as for the denaturation, except temperature decreased by itself. We measured the spectra before tau had been incubated at each temperature for 10 min. Gly-Tyr-Ala was used as control under the same conditions. The absorbance was measured on Perkin Elmer Lamda-12 VIS/UV spectrophoto-meter.

2.6. Light scattering Tau (500 μg/ml as final concentration) was resuspended in 50 mM phosphate buffer (pH 7.2) and incubated at different temperatures for 30 min before measurement of the intensity of light scattering at 480 nm was carried out [10]. A Hitachi-F4500 fluorophotometer was employed.

2.7. Kinetic measurements 0.1 ml tau (0.2 mg/ml as final concentration) was added to 0.9 ml of 0.05 M phosphate buffer (pH 7.2) at 60°C, followed by measurement of the absorbance at 215 nm during denaturation. For renaturation, tau was incubated at 70° for 30 min before added to the phosphate buffer which kept at 25°C. Detection of the absorbance at 215 nm followed the addition of tau.

3. Results

3.1. Changes in the intrinsic fluorescence of tau during GuHCl denaturation. The intrinsic fluorescence is one important characterization of protein related to its conformation. It has been widely used for protein conformational studies [9]. Two excitation maxima (235 and 280 nm) of the intrinsic fluorescence of human neuronal tau-40 are detected by emission at 305 nm at room temperature (Fig. 1). Apparently, as Table 1 shows, Tyr residues contribute to these fluorescent spectra since these residues fluoresce around 305 nm and tau does not contain any Trp residues [11-13]. Two spectra at 305 with different intensities were exhibited by excitation at either 235 or 280 nm. Thus, the two excitations should be due to the specific electronic energy levels of Tyr residues as a fluorophore in the protein. However, the excitation and emission maxima of Tyr are slightly different from those of tau (Table 1). This may have resulted from the microenvironment at which Tyr residues are located is different from Tyr.

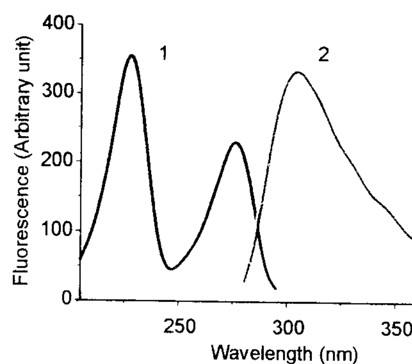

Fig. 1. Intrinsic fluorescence of human neuronal tau protein Tau (final concentration, 30 μg/ml) was dissolved in 25 mM phosphate buffer (pH 7.2) before measurement of the intrinsic fluorescence with slits of Ex 5 nm and Em 5 nm at room temperature. The abscissa is excitation wavelength and the ordinate is fluorescence intensity. Curves 1 and 2 represent excitation spectrum and emission spectrum.

Table 1. Some Fluorescence characterizations of Tyr and tau

	Ex	Em	
Tyr			
Wavelength (nm)	226	276	306
Intensity	196	120	415
Tau			
Wavelength (nm)	235	280	305
Intensity	350	225	330

1μM Tyr or tau in 0.05 M phosphate buffer, pH 7.2 at room temperature.

An increase of the intrinsic emission intensity has been detected after denaturation in GuHCl solutions at different concentrations (Fig. 2A), followed by a small blue shift of emission maxima (approximately 3 nm). A distinguishable change in the intensity is observed when the denaturant concentration is around 2 M. As described previously [14], changes in the intrinsic fluorescence are correlated to the conformational changes of protein molecule. Unfolding of the peptide chain of the molecule results in alterations onto the microenvironment where Tyr residues resides. In addition (Fig. 2B), changes in the fluorescence of Gly-Tyr-Ala are much smaller than those of tau. It has not been observed that an oligopeptide, for example a tripeptide, to fold into tertiary structure, even secondary structure. Therefore, it is reasonable that changes in the fluorescence of Gly-Tyr-Ala are not marked under the same conditions. These results indicate that tau may unfolds during GuHCl denaturation, especially when the denaturant concentration is around 2 M.

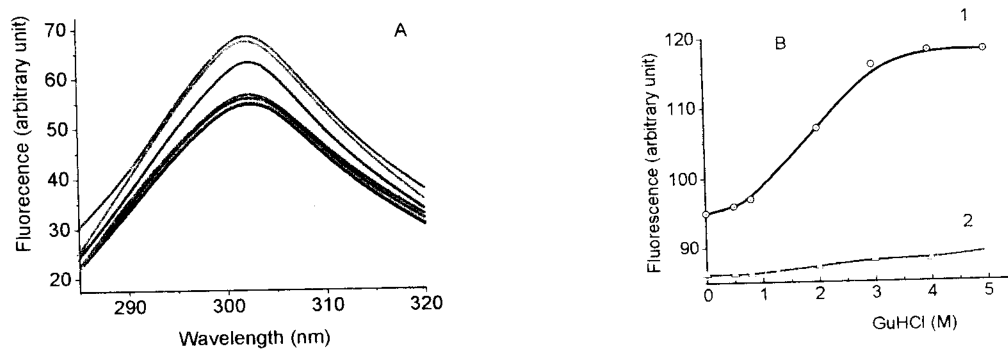

Fig. 2. Effect of GuHCl on the intrinsic fluorescence of tau. Conditions were as for Fig. 1 except GuHCl was employed to denature tau at room temperature. GuHCl was used at desired concentrations. Fluorescence at 305 nm was measured by excitation at 275 nm after tau was incubated with GuHCl of different concentrations for 24 hr. The abscissa is GuHCl concentration and ordinate is the emission intensity. (A) Spectra in GuHCl solutions at different concentrations from bottom to top (0, 0.5, 0.8, 2.0, 3.0 and 5.0 M). (B) Changes in the fluorescence intensities, curve 1and 2 represent tau and Tyr respectively.

3.2. Fluorescent quench by KI KI is used to quench the intrinsic fluorescence of tau, which denatured at different GuHCl concentrations (Fig. 3A). The mechanism of the fluorescence quench by KI is on the basis of collision theory [9]. Stern and Volmer has introduced a quenching constant which is widely used for study of the fluorescence quench of protein. The iodide collides with Tyr residues in solution, which consumes energy from the fluorophores. Thus, it leads to a decrease in the emission intensity while KI quenching. In fact, the quenching constant depends on the spatial position where the fluorescent amino acid residue resides in proteins.

When the fluorophore is located at the interior of the molecule, the quenching constant will be low. In contrast, the constant will increase when the fluorescent residue approaches to the exterior. As shown in Fig. 3B, the KI quenching constant depends on GuHCl concentrations. The constants decrease as tau unfolds following the increasing GuHCl concentration. It indicates that Tyr residues are approaching to the interior of the molecule during the denaturation.

3.3. Changes in ultraviolet difference absorbance during thermal denaturation As described previously, tau has a tendency to self-associated into polymers (dimer, tetramer, octamer and so on) [15-16]. Under the physiological condition, tau exists mainly as monomer. As reported previously [3], a few of the monomers polymerize into dimers at room temperature. This polymerization may be reversible at an equilibrium rate *in vivo* and *in vitro*. As we know, protein conformation depends upon temperature. The protein unfolds itself at higher temperatures. Thus, we treat tau with heating and attempt to observe how polymers form when temperature increases. As we know, protein aggregation improves light scattering of its solution. Therefore, we determined if tau aggregation depends on temperature.

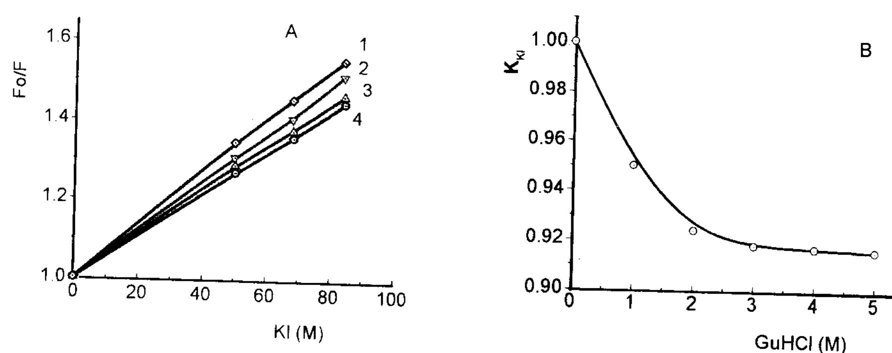

Fig.3. KI quenching of the intrinsic fluorescence of tau in GuHCl solutions of different concentrations. The denaturation conditions were the same as in Fig. 2. KI was titrated into the solutions, which contained the denatured tau by GuHCl at different concentrations, followed by detection of intensity of the intrinsic fluorescence. (A) Curves 1 through 4 represent Stern Volmer plots of tau incubated with GuHCl at 0, 1, 3 and 5 M respectively. F_0 is the emission intensity in the absence of KI and GuHCl. F is the intensity at different concentrations of KI and GuHCl. (B) The relative quenching constants (K_{KI}) plotted as a function to the concentration of GuHCl.

As Fig.4 shows, light scattering of tau solution depends on temperature. Furthermore, the light scattering decreases following temperature increase, unlike some other proteins, for example *D*-glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The light scattering of GAPDH solution increases when temperature is increased (data not shown). It is well known that the conformation of tau becomes more random following temperature increase. Formation of polymers depends upon tau conformation, which is disturbed when temperature increases. This suggests that tau molecule possesses some transition conformation which is relatively stable at room temperature, even though this molecule is flexible.

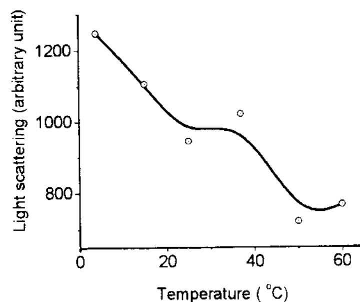

Fig. 4. Changes in Light scattering of tau incubated at different temperatures. Tau (final concentration, 500 μg/ml) was resuspended in 50 mM phosphate buffer (pH 7.2) and incubated at different temperatures for 30 min, followed by measurement of the intensity of light scattering at 480 nm.

For a detailed observation of thermal denaturation of tau, we used ultraviolet difference spectra to monitor the procedure of the conformational changes during the thermal unfolding. That tau does not contain any Trp residue leads to a unmarked difference spectrum around 280 nm, unlike the other proteins containing Trp. Although the absorbance of tau at 280 nm is too small (1mg/ml = 0.27 A_{280}) to be detected, the absorbance around 215 nm is sufficiently distinguishable. Thermal denaturation (Fig. 5A) shows that the difference absorbance around 215 nm increases when tau denatures by heating. But changes in the difference absorbance of the oligopeptide Gly-Tyr-Ala are not markedly detected (Fig. 5B). This short peptide does not contain any secondary and tertiary structures and is not able to unfold like protein [14]. As mentioned above, the difference absorbance around 215 nm is much greater than that at 280 nm. In fact, the absorbance at 215 nm is mainly contributed by peptidyl backbones of protein molecule. When conformational changes occur, it will be correlated to peptidyl backbones and lead to a variation of the absorbance at 215 nm. Therefore, we monitor changes in the absorbance at this wavelength when tau is denatured by heating. Thermal denaturation of tau involves in an increase of the difference absorbance and red shift in the absorbance maxima (Fig. 5C). Unfolding of tau is markedly observed when temperature is around 55°C. It suggests that tau conformation is not completely random when temperature is less than 40°C.

Changes in ultraviolet difference spectra of tau were observed while it renaturates from thermal denaturation state. We kept tau at 65°C for 10 min, then let temperature go down by itself and measured the difference spectra desirably at a graded temperature. At each desired temperature, tau was incubated for 10 min before the absorbance was measured. The absorbance at 215 nm decreased with temperature going down, and finally the absorbance recovers to the original one before heated, showing a renaturation procedure similar to that of the denaturation (data not shown). Furthermore, a blue shift of the absorbance maxima was observed from 219 to

216 nm. This is to say, tau can rapidly refold by itself from the thermal denaturation states, following decrease of temperature. It suggests that the ability of tau to be resistant to heating may be due to such a characteristic, refolding readily by itself from thermal denaturation.

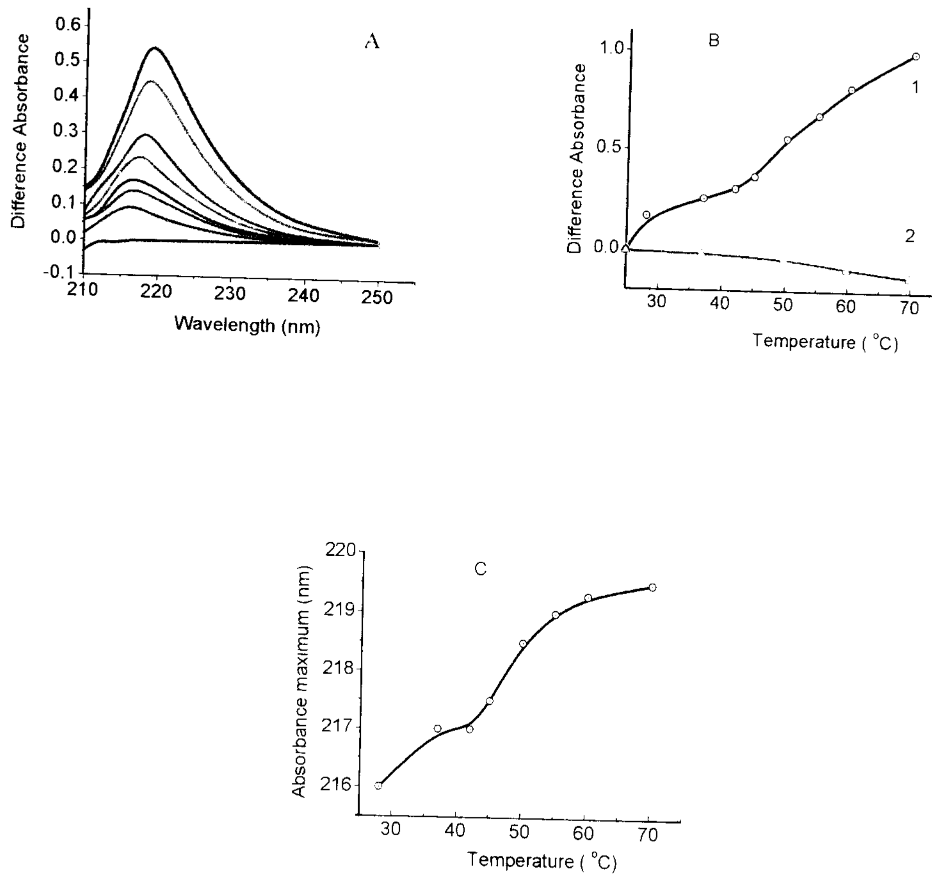

Fig. 5. Ultraviolet difference spectra of tau at different temperatures. Tau (250 μg/ml) was suspended in 25 mM Phosphate buffer (pH 7.2) and incubated at different temperatures for 10 min before measurements of the difference spectra. (A) Curves from bottom to top are difference absorbance spectra at 25, 28, 37, 42, 45, 50, 55, 60 and 70°C. (B) The same data are plotted with temperature. Curves 1 and 2 represent changes in difference intensity 215 nm and those of Gly-Tyr-Ala as control. (C) Wavelength of the maximum difference absorbance (nm).

Table 2. The first order rates of tau during thermal unfolding and refolding

	Fast phase	Slow phase
	Denaturation (Absorbance at 215 nm)	
Tau	18.4	2.7
	Renaturation (Absorbance at 215 nm)	
Tau	> 1000	-

Rates are in $\times 10^2 s^{-1}$

3.4. Kinetics of tau during thermal denaturation and renaturation The time course of increase in the absorbance at 215 nm showed a biphasic procedure at 60 °C, which contained both fast and slow phases (Table 2). The two phases were linear in semi-logarithmic plot. The first order rate of the slow phase was much smaller than that of the fast phase. The kinetics in tau refolding from the thermal denaturation showed just a fast procedure. The absorbance at 215 nm decreased rapidly. The first order rate of the fast phase of renaturation was as least two orders magnitude greater than that of denaturation. Tau refolds itself readily from the thermal denaturation state. Now we may understand why tau is resistant to heat and acid treatment without losing its ability to promote microtubule assembly. Because the microtubule-assembly assay was carried out at 37°C, tau could easily renature itself when it was added into the assay mixture after it was heated or denatured by acid treatment [17-18].

4. Discussion

Human neuronal tau-40 is found in adult brain and it contains 5 Tyr residues, but no Trp. Although tau contains 3 Phe, its fluorescence is much weaker than those of Tyr and Trp. Hence, the intrinsic fluorescence of this molecule comes almost exclusively from Tyr residues. As we know, the intrinsic fluorescence of a protein is an important characterization for research of the molecular conformational changes during denaturation [19]. Changes in the intrinsic fluorescence during GuHCl denaturation manifest that tau unfolds from the native conformation to the denatured states. Furthermore, the difference ultraviolet absorbance changes of tau, following temperature increases, support for this view.

Methodically, the fluorescence of protein is much more sensitive than the ultraviolet absorbance at 280 nm when a protein gives rise to conformational changes. It was reported that there was no change in extinction coefficient, and the spectrum between 240-310 nm remains the same, when the concentration of urea was increased from 0 to 8 M [5]. Such high concentrations of urea (> 4 M) have noise backgrounds, which may interfere the measurement of the difference spectrum, unless tau concentration is high enough to overcome th background. GuHCl is a stronger denaturant, and at a low concentration it leads to conformational changes o protein. Thus, we employ this denaturant to test the denaturation of tau and determine the signals from th difference ultraviolet spectra with a high concentration of tau. Furthermore, we have observed changes in th

intrinsic fluorescence at 305 nm, which comes from Tyr residues, when tau denatures in GuHCl solutions. KI quenching of the intrinsic fluorescence gives an information that Tyr residues may be in situ at the exterior of the molecule. If tau conformation were at a random state, the Tyr residues would not always approach to the exterior. So, we think that tau should not be at a random state, although tau is a flexible molecule.

Recently, some authors approached to the idea that tau was regarded as a "natively denatured" protein. This view is probably suggested and based on these observations: tau is resistant to heat and acid treatment without losing its ability to promote microtubule assembly [6-7] and tau's flexibility is similar to that of denatured proteins [19]. However, as mentioned above, tau is readily able to refold from the thermal denaturation. Although tau has a little or no secondary structure [5], we can see kinetics in its unfolding and refolding. Furthermore, light scattering of tau decreases when temperature increased. It shows that tau may have a transition conformation, on which formation of tau polymers depends. We think tau is such a flexible protein that it is able to renature itself rapidly after the denatured condition is removed. It suggests that tau's resistance to heat may be resulted from the ability of renaturation.

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