ELSEVIER

International Journal of Biological Macromolecules
27 (2000) 263–268

INTERNATIONAL JOURNAL OF
Biological
Macromolecules
STRUCTURE, FUNCTION AND INTERACTIONS

www.elsevier.com/locate/ijbiomac

The fluorescent characterization of the polymerized microtubule-associated protein Tau

Jianying Luo [a], Rongqiao He [a,*], Wei Li [b]

[a] *Molecular Neurobiology & Developmental Biology Group, Laboratory of Visual Information Processing, Institute of Biophysics, The Chinese Academy of Sciences, Beijing, PR China*
[b] *Department of Neurology and Neurosurgery, McGill University, Canada*

Received 1 June 1999; received in revised form 26 January 2000; accepted 5 April 2000

Abstract

A new fluorescence formed while microtubule-associated protein tau was incubated at 25 and 37°C for hours, with its maximum excitation at 230 and 280 nm, respectively. The fluorescence completely formed after tau was incubated in phosphate buffer and Tris–HCl buffer for approximately 20 h, with a relaxation phase about 2–4 h. The light scattering of the sample solution improved during formation of the fluorescence when tau was incubated. Both the fluorescence and tau oligomers did not form when tau was incubated in the buffers containing DTT. On the other hand, heparin improved both tau aggregation and the fluorescence formation. It suggests that the fluorescence comes from tau polymerization, which may follow the mechanism of tyrosine-tyrosinate emission for a protein not containing any tryptophan residues. This new fluorescence could be used as a probe to tau polymers.

Keywords: Tau; Human neuronal tau; Fluorescence; Alzheimer's disease

1. Introduction

Filaments with a straight or paired helical morphology are the major components of the neurofibrillary tangles 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 protein 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]. Tau protein has a flexibile molecular structure under natural conditions. Recently, some authors reported that tau had only a minimal content of the secondary structures at room temperature, which was called a worm-like structure [4]. That is to say, the polypeptide backbone of this protein is folded at random states in solutions. In fact, the conformation of tau molecule in solution has not been clear and it needs further investigation.

So far, Goedert et al. demonstrated that tau consists of six isomers with apparent molecular masses of 48–67 KD [5,6]. The most striking feature of the primary structure of tau as predicted from molecular cloning is a stretch of 31 or 32 amino acids that is repeated three or four times in the carboxy-terminal half of the molecule [6]. The human neuronal tau-40 is found in adult brain and it does not contain any tryptophan residues [5,6], but it contains five Tyr residues. Hence, the intrinsic fluorescence of this molecule comes from these Tyr residues [7]. As we know, the intrinsic fluorescence of a protein is an important characterization as probe for research of the molecular conformation [8]. Furthermore, it was reported that tau polymerizes itself while incubated in solutions in a few hours at 37°C [9]. In this case, changes in conformations of the molecule were involved in the polymerization procedure. It may be related to PHF of Alzheimer's disease if tau polymerization occurs in vivo. In addition, some small molecules were found to improve tau's polymerization, such as heparin [1], alcohol [10] and poly-L-glutamic

Abbreviations: GuHCl, guanidine hydrochloride.

* Correspondinng author. Tel.: +86-10-64888531; fax: +86-10-64877837.
E-mail address: herq@sun5.ibp.ac.cn (R. He)

0141-8130/99/$ - see front matter
PII: S0141-8130(00)00126-4

acid [11]. During the course of tau polymerizing, we observed that a specific fluorescence was forming while the protein was incubated in solutions. This paper is concerned with some properties of this fluorescence.

2. Materials and methods

2.1. Materials

The clone of the recombinant human tau-40 came from Prof. 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 use [12]. The absorbance of 5 M GuHCl at 250 nm was less than 0.05. DTT and Pipe buffer were also purchased from Sigma Company. Other reagents used were of analytic grade without further purification. The fluorescence was measured on Hitachi F-4500 fluorescence spectrophotometer and the absorbance was measured on Perkin Elmer Lamda-12 VIS/UV spectrophotometer.

2.2. Expression and purification of tau

Tau protein was purified from lysates of *E. coli*,which overexpressed htau-40, as described by Paudel et al. [3].

2.3. Preparation of 333 nm fluorescence derivative

The purified tau protein (30 μg/ml) was resuspended in phosphate buffer (25 mmol/l, pH 7.2). The fluorescence at 333 nm by excitation at 280 nm was measured at 25°C after the protein had been incubated at 25 or 37°C for about 36 h. To identify the effect of phosphate on tau's intrinsic fluorescence, we used 100 mM Tris–HCl buffer (pH 7.5) as a control to repeat this experiment. According to Geodert, heparin improved aggregation of tau molecules in vivo and in vitro [1]. Therefore, the effect of heparin on formation of the new fluorescence was detected under the same conditions. The concentrations of heparin were employed from 200 through 400 μg/ml.

2.4. Kinetics of formation of the fluorescence at 333nm

Tau (final concentration, 30 μg/ml) was added to the phosphate buffer at 37°C, followed by measurement of the fluorescence by excitation at 230 and 280 nm, until the intensity of the emission at 333 nm did not have a further increase. The procedure of fluorescent formation with heparin was also monitored during the incubation. Data were analyzed according to Tsou et al. [13,14].

2.5. DTT test

It was observed by Wille et al. that formation of Cys–Cys cross-links between tau molecules followed the polymerization of tau-40, and such cross-linking bonds may stabilize the oligomers [9]. Hence, we added DTT (3.0 mM) to tau solution and incubated for 36 h before measurement of the fluorescence at 333 nm, conditions as mentioned above.

2.6. SDS-PAGE of the polymerized tau

While the sample was incubated in the phosphate buffer (pH 7.2), aliquots were taken at different time intervals, then added with 0.001 μM glutaraldehyde at room temperature for 30 min before it was loaded onto SDS-PAGE [15]. The sample buffer (50 mM Tris–HCl, pH 6.8, 1% SDS, 10% glycerol, 0.005% Bromphenol blue) was used to stop the polymerization and reaction with glutaraldehyde, as described by Paudel et al. [3]. It was necessary to check if the samples of tau contaminated with some proteases. We incubated tau with DTT (conditions as mentioned above) for 18 h before running SDS-PAGE.

2.7. Measurement of light scattering

On the basis of the method reported by Zhou et al. [17], the maximum light scattering of tau solution was at 460 nm. Therefore, both light scattering at 460 nm and the fluorescence at 333 nm were used to monitor tau's aggregation in solutions during the incubation. The final concentration of tau was 250 μg/ml. The intensities were measured at different time intervals during the incubation.

2.8. Determination of the fluorescent quantum yield

Quantum yield was determined at 25°C compared with that of Trp (the quantum yield of Trp in the solution is 0.2 and not changing with excitation wavelength). The absorbance of the sample and Trp solutions at corresponding wavelength is no more than 0.03. We calculated the quantum yield according to Scott et al. [16].

2.9. Measurements of changes in the fluorescence at 333 nm in GuHCl solutions of different concentrations

Tau (final concentration, 30 μg/ml), which had been incubated with and without heparin in the phosphate buffer (pH 7.2) for more than 42 h, was dissolved and incubated in GuHCl solutions of different concentrations at room temperature for 24 h, respectively. GuHCl was at desired concentrations. No further changes in the fluorescence were detected when tau was

incubated with GuHCl over 24 h. The intensity of the emission at 333nm was measured after the incubation in GuHCl solutions.

2.10. Quenching the fluorescence by KI

KI of different concentrations was added to tau possessing the fluorescence at 333 nm in the phosphate buffer (pH 7.2), followed by measurements of the intensity of the fluorescence after incubated at 25°C for 10 min. Data were plotted according to stern Volmer [15].

3. Results and discussion

3.1. The intrinsic fluorescence of tau

As we know, the primary structure of tau protein does not contain any Trp residues although it has six isomers [5,6] with apparent molecular masses of 48–67 KD. The recombinant tau (htau-40) we used contains five Tyr residues in its peptide chain. The results showed that tau fluoresced at 305 nm in 25 mM phosphate buffer (pH 7.2) and in Tris–HCl buffer (pH 7.5) by excitation at 230 and 275 nm, respectively. As usual, the intrinsic fluorescence could be used as a probe to the conformational changes of proteins [18]. We observed that intensities of the intrinsic fluorescence changed after tau had been incubated with GuHCl of different concentrations at 25°C. The relative emission intensity increased (20%) distinguishably when GuHCl concentrations were from 1.0 up to 1.5 M (data not shown). This is to say, tau's conformation unfolded extensively in the denaturant solutions at these concentrations. It suggested that tau may have a relatively stable conformation as a whole in solution.

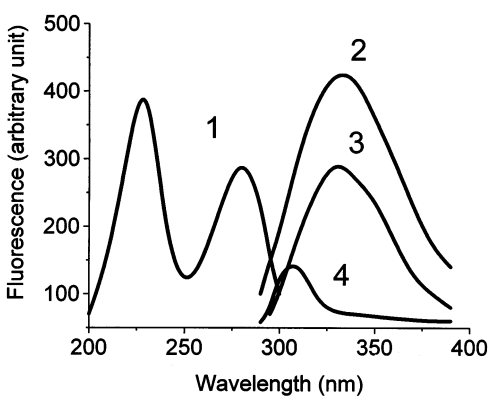

Fig. 1. Spectra of the new fluorescence formed. The clone of htau-40 was from Dr Geodert and isolated with Q- and SP-Sepharose columns according to Paudel et al. [3]. Tau (final concentration, 60 μg/ml) was incubated in 25 mM phosphate buffer (pH 7.2) at 25 or 37°C for 20 h before measurements of the fluorescence with slits of (Ex/Em), 5.0 nm/10 nm. Curve 1: the excitation spectrum. Curve 2 and 3: the emission spectra by excitation at 230 and 280 nm, respectively. Curve 4: the emission of native tau under the same conditions.

3.2. Formation of a new fluorescence at 333 nm of tau

A new fluorescence at 333 nm with a quantum yield of 0.03 was detected after tau was incubated in the phosphate and Tris–HCl buffers at both 25 and 37°C for 24 h. The excitation wavelengths were at both 230 and 280 nm (Fig. 1). The emission intensity excited at 230 nm was higher than that excited at 280 nm at room temperature. It was suggested that the human neuronal tau gave rise to a new fluorescent characterization after the incubation.

Now, we should clarify the possibilities that were correlated to this fluorescence. Firstly, we checked tau solution before the incubation, and we could not detect any fluorescence around 333 nm except the intrinsic emission (305 nm) under the same conditions, as mentioned above. This was because tau does not contain any tryptophan residues in its peptide chain [5,6]. Secondly, the buffers we used did not have any fluorescence. Thirdly, It was necessary to clarify if the sample had been contaminated with tyrosine, tryptophan and other proteins [19]. We had used tyrosine and tryptophan as controls, and they fluoresced at 303 and 355 nm in the buffers under the same conditions. This is to say, the fluorescence at 333 nm could not be contributed by the contamination of tyrosine, tryptophan and other proteins.

The new fluorescence was completely formed after tau incubated in the phosphate and Tris–HCl buffers for approximately 20 h. The kinetics in formation of this fluorescence went through a relaxation phase about 2–4 h (Fig. 2). Goedert also observed a relaxation time (5 h) for tau to polymerize itself in Mops buffer (pH 7.4) when it was incubated at 30°C [1]. The relaxation phase of formation emission at 333 nm was coincident to that reported by Goedert. At the same time, the absorbance at 230 and 280 nm increased with the procedure of the formation. It increased from 0.22 to 0.55 at 230 nm and from 0.01 to 0.27 at 280 nm, respectively, that is, the formed fluorophore absorbed energy specifically at these wavelengths for its emission.

3.3. The fluorescence at 333 nm involving tau's polymerization

What contributes to this new fluorescence of tau? To answer this question, we did the experiments as follows. First of all, let us suppose that the formation of the new fluorescence comes from tau's polymerization itself in solutions because this molecule aggregates and polymerizes during incubation at room temperature. As we know, protein polymerization leads to an increase of light scattering of the solution. It was observed that the

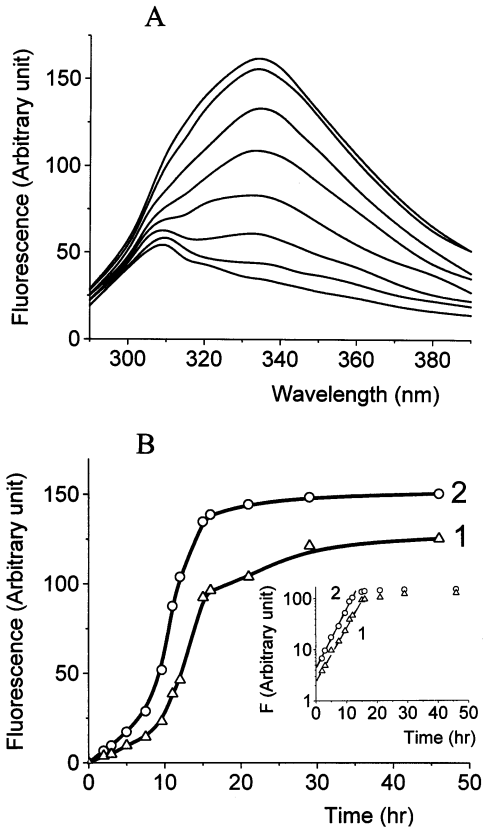

Fig. 2. Formation of the fluorescence at 333 nm. Conditions were as for Fig. 1, except time course of formation of the emission at 333 nm was monitored. (A) The emission spectra of tau incubated with heparin (400 μg/ml) measured at different time intervals. Curves from bottom to top were spectra measured at 0, 4, 9,12, 14, 17, 29 and 47 h. (B) Kinetics of the fluorescence formation. Tau was incubated with heparin. (curve 2). Curve 1 was control. The inset was the same data plotted in semilogarithm.

intensity of light scattering of this solution was improving during formation of the fluorescence (Fig. 3). Consequently, kinetics in the light scattering at 460 nm was coincident with the procedure of formation of the fluorescence. This suggested that tau was polymerizing in solution while the fluorescence formed.

Wille et al. and Wilson et al. have proved that tau polymerized itself into Alzheimer-like filaments under the similar conditions as we employed [2–9]. Some author reported that assembly of microtubule-associated protein tau into Alzheimer-like filaments could be induced by sulphated glycosaminoglycans, especially by heparin [1–11]. We compared the kinetics in the fluorescence formation and in light scattering while tau was incubated with and without heparin (Table 1). The first order kinetic rate of light scattering of tau with heparin was greater than that of tau without heparin under the same conditions. Furthermore, the rate of formation of the fluorescence with heparin was also greater than that of tau without heparin. The rate of light scattering was as one order magnitude as that of formation of the fluorescence. These results revealed that heparin improved both tau aggregation and the fluorescence formation. Here, we say the fluorescence is related to tau polymerization, however, we do not have any direct evidence to prove that the fluorescence comes from paired helical filaments (PHF), which need further studying.

Consequently, we should check whether the fluorescence formed or not, when tau molecules did not aggregated and polymerized. According to Wille et al. [9], both Cys-291 and Cys-322 would involve in cross-

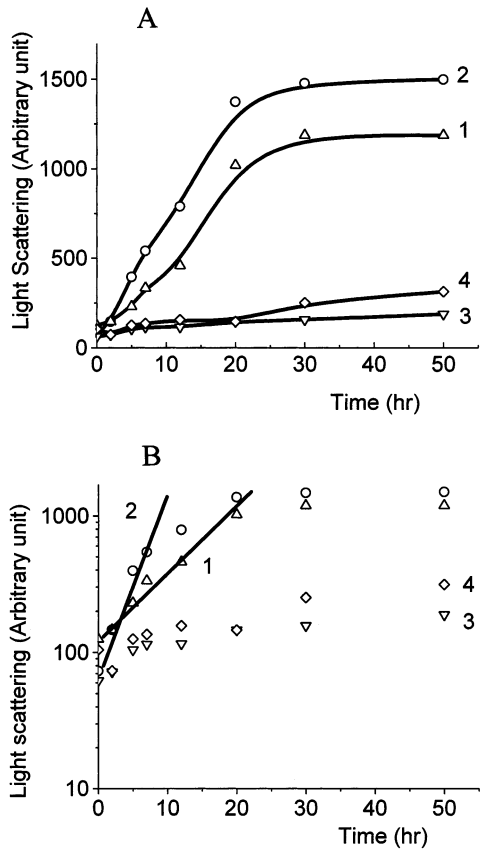

Fig. 3. Changes in the intensity of light scattering of tau solution. Tau (final concentration, 250 μg/ml) was incubated in the phosphate buffer at 37°C, and the light scattering at 460 nm was measured during the formation of the fluorescence at 333 nm. Kinetics of light scattering of tau incubated with (curve 2) heparin (200 μg/ml) was measured with Fluorospectrometer of Hitachi-F4500. 3.0 mM DTT was added to tau with (curve 4) or without (curve 3) heparin for measurement of changes in light scattering. Curve 1 was control. The inset was the same data in semilogarithmic plot.

Table 1
The first order rate constants of formation of the new fluorescence and those of light scattering[a]

	Tau	Tau with heparin
Light scattering (460 nm)	91.4	192.0
Fluorescence (333 nm)	6.7	12.0

[a] The data were the first order rates in 10^{-5} per s.

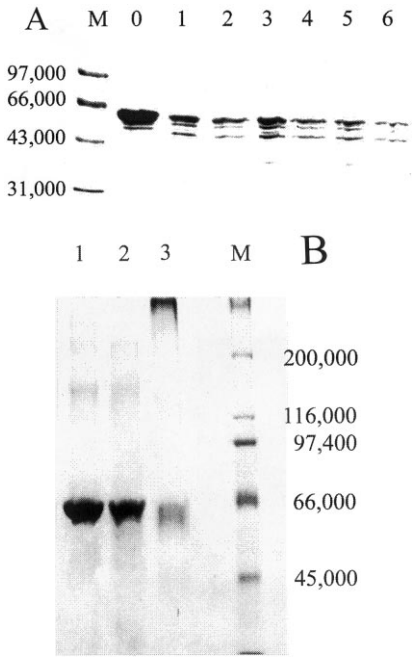

Fig. 4. SDS-PAGE of tau incubated in phosphate buffer at different time intervals. Aliquots of tau incubated at different time intervals were taken for SDS-PAGE. 0.001 μM Glutaraldehyde was added to each sample and incubated for 30 min before electrophoresis. (A) In lanes 1–6, tau was incubated with heparin at 25°C for 10, 11, 12, 13, 16 and 18 h, respectively. 0 was control. (B) In lanes 1–3, non-incubated tau was as control, tau incubated with 3.0 mM DTT, and tau incubated without DTT for 18 h. M was marker.

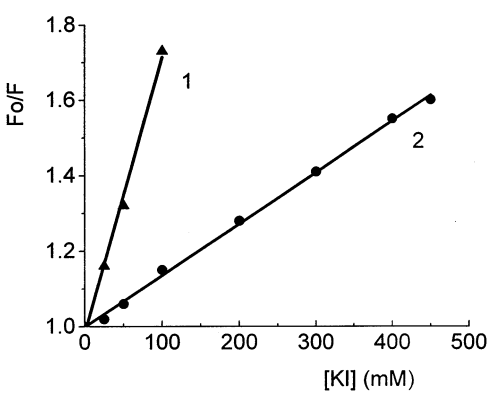

Fig. 5. KI quenching the intrinsic and 333 nm fluorescence. Tau (final concentration, 60 μg/ml) and tau oligomers had been resuspended in phosphate buffer (pH 7.2) before KI was titrated at room temperature. The intensities of the fluorescence at 305 nm for tau (curve 1) and 333 nm for tau oligomers (curve 2) were measured after KI had been added for 10 min. Data were plotted according to Stern–Volmar.

links and form disulfide bridges between tau molecules during aggregation and polymerization. They observed that tau's oligomers did not form when tau was incubated in solutions containing DTT. Hence, we added DTT to tau's solutions and incubated them at room temperature and we did not detect both the fluorescence at 333 nm and tau's oligomers by SDS-PAGE, except its intrinsic fluorescence of Tyr residues. As shown in Fig. 3, DTT could also prevent tau solution from increasing in light scattering both with heparin and without heparin. However, after tau oligomers formed, DTT at the same concentration did not quench the fluorescence at 333 nm. It suggests that the new fluorescence may be contributed by tau's oligomers as a fluorescent excimer.

Results of SDS-PAGE (Fig. 4) also showed that tau molecules were polymerizing each other while it was incubated. Tau monomer decreased distinguishably followed by an increase of oligomers, whose molecular masses were too large to run into the gel plate while electrophoresis [3]. Consequently, we should check if the samples contaminated with some proteases, which degraded tau during the incubation. As mentioned above, DTT could prevent from the formation of the fluorescence and it could not inhibit any proteases. Fig. 4B. showed tau was not degraded after incubation with 3 mM DTT. It suggests that tau polymerized during the incubation.

3.4. Tyr residues and the new fluorescence

Tau-40 contains five Tyr residues besides three Phe (Phe-8, 346 and 378) [5,6]. However, the fluorescence from Phe is much weaker than Tyr and Trp [18]. As mentioned above, the intrinsic fluorescence of tau comes from Tyr residues. Looking back at Figs. 1 and 2, we found that the wavelengths of the excitations (230 and 275 nm) of the new fluorescence are similar to those of the intrinsic fluorescence. This is to say, the two maximum excitations did not shift during the formation of the emission at 333 nm.

Some years ago, tyrosine-tyrosinate emission spectra which mimic tryptophan fluorescence were found for two cytotoxins which do not contain Trp residues [20]. These mimic spectra were formed and observed during the conformational changes in the proteins and aggregation in solutions with high salt concentration. In this case, the emission is probably due to Tyr residues which are hydrogen bonded in the ground state to glutamic or asparic acid residues in the protein [18]. As we know, formation of the fluorescence at 333 nm followed tau's aggregation and polymerization. Conformational changes in tau protein occurred during the procedure of the fluorescence formation. Furthermore, as mentioned, the wavelength of the new emission was similar to that of Trp residue at 333 nm in a protein. It appears that the new fluorescence of tau involves in the mechanism of tyrosine-tyrosinate emission.

In addition, we used the fluorescence quenching with KI to test changes in tau's conformation (Fig. 5) during formation of the new emission. The quenching data were plotted according to Stern Volmer [18]. Similar to the intrinsic fluorescence at 303 nm, the quenching curve of tau oligomers was linear. However, the quench constant

of tau oligomers (k_o) was five times lower than that of tau monomers (k_m). On the basis of the mechanism of the fluorescence quenching, the fluorophore of oligomers, which emitted at 333 nm, approached to the interior of the molecule. On the other hand, Tyr residues of the monomers were more exposed to the exterior. It suggests that Tyr residues folded and moved to the interior of tau molecule after aggregated and polymerized.

3.5. The fluorescence at 333 nm used as a conformational probe

Tsou et al. [21] found and demonstrated the NAD fluorescent derivative of *D*-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Formation of this derivative depended on the conformation of GAPDH. Similar to the NAD fluorescent derivative, formation of the fluorescence at 333 nm was also dependent upon tau's conformation. We added GuHCl (>0.1 M) to tau solutions and incubated them at room temperature for 24 h before measurement of the fluorescence at 333 nm. The result showed that both the new fluorescence and tau oligomers had formed after the incubation. As we know, GuHCl disturbed protein conformations and inactivated enzymes, when the spatial structure of the active site was slightly perturbed [22,23]. It showed that to keep tau protein in the native conformation was a requirement for formation of the new fluorescence and oligomers. However, after the fluorophore for 333 nm emission had formed, we added GuHCl to the oligomers and the effect of this denaturant on the emission was different from that of the intrinsic fluorescence of monomers. The fluorescent intensity at 333 nm of tau oligomers decreased, following the increase of GuHCl concentrations (Fig. 6). Tyr was used as control and its fluorescence did not change in the same GuHCl solutions. It was suggested that the conformation of tau oligomers disturbed by the denaturant. However, some different results were observed with tau oligomers with heparin. The fluorescence at 333 nm of the oligomers with heparin was more stable during GuHCl denaturation. However, tau oligomers without heparin were more susceptible to GuHCl than those with heparin. According to Geodert, heparin improved tau's aggregation and polymerization. This is to say, the structure of tau's oligomers formed with heparin was more stable than that of tau's without heparin.

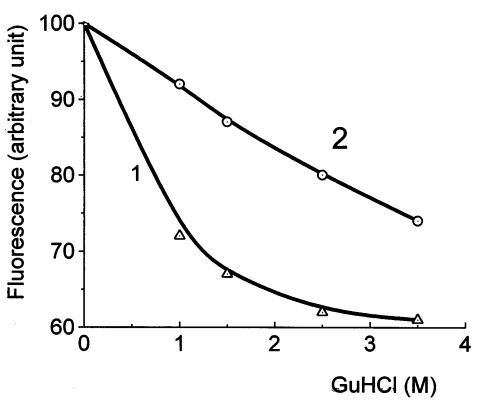

Fig. 6. Changes in the intensity of emission at 333 nm in GuHCl solutions of different concentrations. The polymerized tau was dissolved in 25 mM phosphate buffer (pH 7.2), containing GuHCl at desired concentrations for 24 h before measurements of the fluorescence. The relative intensities of the fluorescence at 333 nm of tau with (curve 2) and without (curve 1) heparin were detected, respectively.

Acknowledgements

This project is in part supported by the Director Foundation of the Chinese Academy of Sciences (C.A.S.), (STZ 98-2-07) and the Instrument Foundation of C.A.S. and in part by the Chinese National Natural Foundation (No. 39970236). We thank Dr Paudel who gives us the clone of tau, which is provided by Dr Goedert. We are grateful to Mr Yong-Hui Chen who helps us to do some work for this project.

References

[1] Goedert M, Jakes R, Spillantini MG, Hasegawa M, Smith MJ, Ctowther RA. Nature (London) 1996;383:550.
[2] Wilson DM, Binder LIJ. Biol Chem 1995;270:24306.
[3] Paudel HK. J Biol Chem 1997;272:1777.
[4] Schweers O, Schonbrum-Hanebeck E, Marx A, Mandeldow E. J Biol Chem 1994;269:24290.
[5] Goedert M, Spillantini MG, Jakes R, Rutherford D, Ctowther RA. Neuron 1989;3:519.
[6] Goedert M, Jakes R. EMBO J 1990;9:4225.
[7] Luo JY, Li W, He RQ. Sci Bull Sin 1998;44:233.
[8] He RQ, Zhao KY, Yan ZZ, Li M. Biochim Biophys Acta 1993;1163:315.
[9] Wille H, Drewes G, Biernat J, Mandelkow EM, Mandelkow J. Cell Biol 1992;118:573.
[10] He RQ, Luo JY, Li W. Protein Peptide Lett 1998;5:279.
[11] Pérez M, Valpuesta JM, Medina M, Montejo de Garcini E, Avila J. J Neurochem 1996;67:1183–90.
[12] Nozaki Y. Methods Enzymol 1972;26:43–50.
[13] Tsou CL. Acta Biochim Biophys Sin 1965;5:398–408.
[14] Tsou CL. Acta Biochim Biophys Sin 1965;5:409–17.
[15] Laemmli UK, Favre M. J Mol Chem 1973;80:575–99.
[16] Scott TG, Spencer RD, Leonard NJ. Am Chem Soc 1970;92:687–95.
[17] Zhou J, Jiang K, He RQ, Han YM, Guan JD. Acta Biophys Sin 1997;13:531–5.
[18] Lakowicz LR. Principles of Fluorescence Spectroscopy, first ed. New York: Plenum Press, 1983:258–96.
[19] Cowgill RW. Biochim Biophys Acta 1964;88:338–40.
[20] Szabo AG, Lynn KR, Krajcarski DT, Rayner DM. FEBS Lett 1978;94:249–52.
[21] Ho YS, Tsou CL. Nature (London) 1979;277:245–6.
[22] He RQ, Li YG, Wu XQ, Li L. Biochim Biophys Acta 1995;1253:47–56.
[23] He RQ, Yang MD, Zheng X, Zhou JX. Biochem J 1995;309:133–9.