Effect of human neuronal tau on denaturation and reactivation of rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase

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Human neuronal tau-40 (htau-40) has been used to study denaturation and renaturation of rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12). Inactivation of GAPDH incubated with tau was more distinguishably detected than that of control GAPDH during thermal and guanidine hydrochloride (GdnHCl) denaturation. However, tau did not influence the activity of GAPDH at room temperature or in solution without GdnHCl. A marked change in both the emission intensity and emission maximum of the intrinsic fluorescence at 335 nm of GAPDH with tau was observed when GdnHCl concentration was 0.8 M, but that of the control without tau occurred in 1.2 M GdnHCl. The first-order rate of the decrease in the fluorescence intensity of the enzyme with tau was approximately twice as great as that of GAPDH without tau. Kinetics of inactivation of GAPDH with tau in 0.2 M GdnHCl was a monophasic procedure, instead of the biphasic procedure followed by the control, as described before [He, Zhao, Yan and Li (1993) Biochim. Biophys. Acta **1163**, 315–320]. Similar results were obtained when the enzyme was thermally denatured at 45 °C. It revealed that tau bound to the denatured GAPDH but not the native molecule. On the other hand, tau suppressed refolding and reactivation of GAPDH when this enzyme was reactivated by dilution of GdnHCl solution. Furthermore, tau improved the aggregation of the non-native GAPDH in solutions. It suggested that tau acted in an anti-chaperone-like manner towards GAPDH *in vitro*. However, tau lost that function when it was aggregated or phosphorylated by neuronal cdc2-like protein kinase. It showed that tau's anti-chaperone-like function depended on its native conformation.

Key words: activity, Alzheimer's disease, anti-chaperone, chaperone, tau.

INTRODUCTION

Neuronal tau, which is a member of the microtubule-associated proteins, functions in promoting the assembly and stabilization of microtubules, and plays important roles in neuronal morphogenesis, maintenance of axonal shape and axonal transportation, through its ability to bind and regulate microtubule structure and dynamics [1–3]. In recent years, some authors reported that 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 [4,5]. Solubilization of these filaments reveals that they are composed of the microtubule-associated protein tau [6,7]. In this laboratory, a new fluorescence at 333 nm was detected when tau molecules aggregated [8,9]. Effects of ethanol and acetaldehyde on tau aggregation were studied, with an observation that the two compounds could accelerate tau aggregation [10,11]. Tau may have a flexible conformation, which contributed to its resistance to denaturation by heating and denaturants [12]. Henriquez et al. [13] observed that tau combined with actin filaments in various cell types. It suggests that tau may be involved in regulating structures of some proteins besides microtubules.

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is a key enzyme in the glycolytic conversion of glucose into pyruvic acid, which is an important pathway of carbohydrate metabolism in most organisms [14,15]. GAPDH has also been used as a substrate to research the effects of some chaperones, for example protein disulphide-isomerase (PDI), on its refolding and reactivation [16]. It was observed that PDI acted as a chaperone to GAPDH *in vitro*, although no evidence indicates that PDI is acting on GAPDH folding *in vivo*. After that, PDI was found to bind effectively to the immobilized GAPDH monomer [17]. Furthermore, in order to make an artificial chaperone, Dainiak et al. [18] used conjugates of monoclonal antibodies coupled covalently to poly(methacrylic acid) to reactivate GAPDH. The antibodies were able to bind specifically inactive dimers of GAPDH but not the native tetramers, which resulted in a significant increase in the specific activity of the enzyme. Recently, participation of chaperone GroEL in the folding of GAPDH was researched, based on the use of different oligomeric forms of the enzyme immobilized on Sepharose [19]. Last year, we observed that tau was able to raise DNA melting temperature [20], suggesting that tau was a DNA binding protein to prevent DNA from thermal denaturation. Furthermore, we used GAPDH to detect whether tau may have a chaperone-like function. Here, we report that tau can influence denaturation and reactivation of GAPDH *in vitro*.

MATERIALS AND METHODS

Materials

The clone of the recombinant human neuronal tau-40 (htau-40) came from Dr M. Goedert (University of Cambridge, Cambridge, U.K.). Sephadex G-50, Q-Sepharose and sulphopropyl (SP)-Sepharose were purchased from Pharmacia. Guani-

Abbreviations used: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; GdnHCl, guanidine hydrochloride; NCLK, neuronal cdc2-like protein kinase; htau-40, human neuronal tau-40; Ptau, phosphorylated tau; PDI, protein disulphide-isomerase; DTT, dithiothreitol.

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dine hydrochloride (GdnHCl) and dithiothreitol (DTT) came from Sigma. GdnHCl was purified according to Nozaki [21] before use. The absorbance of 5 M GdnHCl at 250 nm was less than 0.05.

Purification of GAPDH from rabbit muscle, recrystallized six times, was as described before [22]. The enzyme showed a single band on SDS/PAGE after purification. The specific activity was approx. 110 units [23] (1 unit is defined as the specific activity required to convert 1 μM substrate/min per mg of enzyme). The absorption coefficient at 280 nm of 149 $mM^{-1} \cdot cm^{-1}$ was used for concentration determination of the enzyme. Assay of GAPDH was carried out in a mixture of 0.1 M phosphate buffer (pH 8.9) containing 0.001 M NAD^+, 0.001 M D,L-glyceraldehyde 3-phosphate and 0.001 M EDTA at 25 °C. Tau was purified from lysates of *Escherichia coli* that overexpressed htau-40, as described by Paudel [24]. The isolated tau was purified with a Sephadex G-50 column to remove small molecules before use. This microtubule-associated tau showed a single band on SDS/PAGE after purification. The concentration was estimated by the absorption (280 nm) ratio 0.27/mg of tau [24]. Other reagents used were of analytical grade without further purification. Absorbance was measured on a Perkin–Elmer λ-12 spectrophotometer.

GdnHCl denaturation

GAPDH (final concentration, 0.2 μM) with tau (0.8 μM) was incubated in 0.05 M phosphate buffer (pH 7.2) containing GdnHCl of different concentrations at 25 °C overnight, until the denaturation of the enzyme reached completion. The concentration of GAPDH used was the same in the following text except where stated otherwise. The intensity of the intrinsic fluorescence at 335 nm was measured by excitation at 295 nm to prevent disturbance of Tyr residues at room temperature. Fluorescence was measured on a Hitachi F-4500 fluorescence spectrophotometer. The residual activity of GAPDH was assayed as described before [23]. No further changes in activity and fluorescence were detected when the samples were incubated for more than 12 h. GAPDH without tau, under the same conditions, was used as a control.

Thermal denaturation

The enzyme with tau was incubated at the desired temperatures (25, 37, 45, 55 and 65 °C etc.) for 30 min, and aliquots (10 μl) were taken for assays. The substrate mixture (1 ml) [25] was incubated at each desired temperature for 10 min for thermal equilibrium before addition of the enzyme. Disturbance of the mixture's temperature on adding the enzyme was less than 0.2 °C. GAPDH without tau was used as a control.

Kinetic measurements of fluorescence and activity during thermal and GdnHCl denaturation

GAPDH with tau (20 μl; molar ratio, tau/GAPDH, 4:1) was added to 1.0 ml of 0.05 M phosphate buffer (pH 7.2) containing 0.2 M GdnHCl and 0.001 M EDTA at 25 °C, followed by measurement of the intrinsic fluorescence intensity at 335 nm until the intensity did not change. Under the same conditions, aliquots were taken for assays at different time intervals.

For thermal denaturation, the assay mixture had been incubated at 45 °C for 10 min before 10 μl of GAPDH with tau was added. All the data were analysed according to Tsou's method [26].

Denaturation and reactivation of GAPDH

The enzyme (final concentration, 5 mg/ml) was incubated in 6 M GdnHCl with 1 mM DTT at 4 °C overnight, to reach a complete denaturation and to ensure a sufficiently low concentration of the residual GdnHCl after 100-fold dilution of the denatured enzyme. It was reported that GAPDH was completely unfolded in 3 M GdnHCl, as the intrinsic fluorescence decreased to a minimal value at 2 M GdnHCl without further changes up to 4 M [27]. Reactivation of the enzyme at final concentrations ranging from 0.28 to 1.4 μM was not affected by the initial GdnHCl concentrations from 3 to 6 M for denaturation [16,17]. Following the dilution, tau at different molar ratios (from 0.4 to 4) to GAPDH was added to the solutions, which contained the denatured GAPDH. GAPDH without tau was used as a control.

Reactivation of the denatured GAPDH was performed by dilution of the denatured enzyme in phosphate buffer (pH 7.5), containing 1 mM DTT and different concentrations of GdnHCl to ensure that all the solutions were in the same concentration of denaturant after dilution by different amounts. The reactivation mixture was first kept at 4 °C for 30 min and then at 25 °C for 3 h before an aliquot containing 0.4 μg of GAPDH was taken for assay at 25 °C. It is necessary to explain that this enzyme is completely stable during the same treatment without GdnHCl.

Measurement of light scattering on GAPDH aggregation

On the basis of the method reported by Cai et al. [16], light scattering at 488 nm was used to monitor GAPDH aggregation during incubation with tau of different concentrations. The final concentration of GAPDH was 0.3 μM. Similarly, light scattering of GAPDH with tau was also detected at different temperatures. The light scattering was measured on a Hitachi F-4500 fluorescence spectrophotometer.

Phosphorylation of tau

Purification of neuronal cdc2-like protein kinase (NCLK) and phosphorylation of tau were carried out according to Paudel [24]. Phosphorylation was carried out for 12 h. As described previously [28], SDS/PAGE was used to monitor the phosphorylation, as on SDS/PAGE phosphorylated tau (Ptau) migrates more slowly than native tau. No further band shift could be detected when phosphorylation was prolonged over 12 h. To test the effect of the phosphorylation, the Ptau was prepared freshly and added to GAPDH during denaturation and renaturation.

Preparation of aggregated tau

We prepared tau polymers by incubating the protein in 25 mM phosphate buffer (pH 7.2) at 37 °C overnight, as described by Wilson and Binder [29]. Luo et al. [8,9] observed that a new fluorescence would form during tau polymerization. So, formation of the fluorescence at 333 nm was monitored when tau (0.5 mg/ml) was incubated in 0.05 M phosphate buffer (pH 7.2) at 37 °C overnight, until the emission intensity did not change. The effect of the aggregated tau was tested while GAPDH was denatured and renatured under the same conditions as mentioned above.

RESULTS

Changes in activity and conformation of GAPDH with tau in GdnHCl solutions

During measurement of conformational changes of GAPDH with tau, we detected the intrinsic fluorescence of the enzyme at

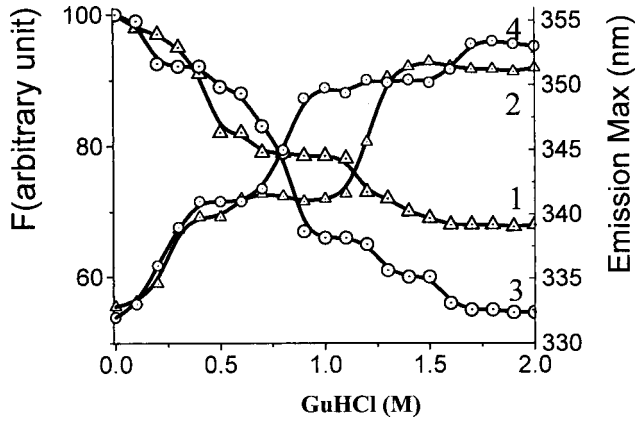

Figure 1 Changes in the intrinsic fluorescence of GAPDH with tau in GdnHCl solutions of different concentrations

GAPDH (final concentration, 0.2 μM) with htau-40 (0.8 μM) was dissolved and incubated in 50 mM phosphate buffer (pH 7.2) containing GdnHCl (GuHCl) of different concentrations at room temperature overnight. The intensity of the intrinsic fluorescence was measured by excitation at 295 nm. Curves 3 and 4 represent the emission intensity and emission maxima of GAPDH with tau, respectively. Curves 1 and 2 represent those of control GAPDH. The error of emission intensity was $\pm$2.4% and that of the emission maximum was $\pm$1.8 nm from four experiments under the same conditions.

335 nm. Consequently, we are concerned about the fluorescence background from tau, which might interfere with the fluorescence data of GAPDH. In fact, tau does not contain any Trp residues [30] and its intrinsic fluorescence comes from Tyr residues [9], whose fluorescence intensity is much weaker than that of Trp residues. Furthermore, Tyr residues fluoresce at 304 nm, instead of 335 nm in the case of Trp. The ultraviolet absorbance (240–300 nm) of 2 μM tau solution is less than 0.05 at room temperature. The intensity of the intrinsic fluorescence of tau at 335 nm by excitation at 295 nm is 1% of that of GAPDH under the experimental conditions used. So the background from tau should not interfere with the fluorescence data of GAPDH. It is believable that GAPDH, not tau protein, contributes most of the intrinsic fluorescence at 335 nm described here.

Similar to the results described previously [25,31], the relative emission intensity of GAPDH decreases with red shift at the emission maximum, following an increase in GdnHCl concentration (Figure 1). A plateau of emission intensity was detected when GAPDH was incubated in GdnHCl solutions of 0.4–1.2 M. An observable red shift at the emission maximum of GAPDH occurred when GdnHCl was around 1.2 M; however, different results were obtained when we incubated GAPDH with tau under the same denaturation conditions. First, we did not detect the plateau (0.4–1.2 M) of the sample with tau, but a marked decrease in the emission intensity when GdnHCl was around 0.7 M, accompanied by a distinguishable red shift at the emission maximum. This is to say, the conformation of GAPDH with tau is more susceptible to disturbance by the denaturant than control GAPDH. For the background, little change in the intrinsic fluorescence (335 nm) of the neuronal tau was detected during GdnHCl denaturation. The changed emission intensity of tau does not exceed 1% under the same conditions. GAPDH contains three Trp residues whose positions are 83, 193 and 310 [31], and they contribute to the intrinsic fluorescence. It suggests that tau improves unfolding of conformation of GAPDH as a whole molecule.

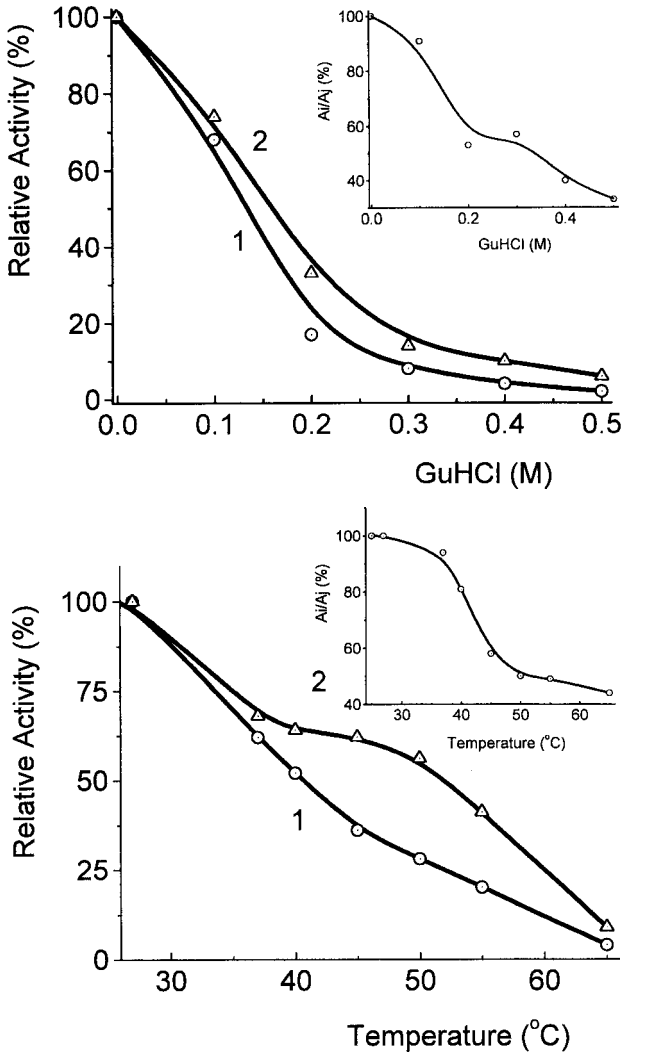

Figure 2 The residual activity of GAPDH with tau during GdnHCl (GuHCl) and thermal denaturation

Ai represents the activity with tau at different concentrations or temperatures and Aj represents that of the control. (Top panel) The residual activity in GdnHCl of different concentrations. Conditions were the same as for Figure 1, except that enzyme activity was measured. Curves 1 and 2 represent the activity of GAPDH with tau and that of GAPDH as control, respectively. The error was $\pm$3.2%. (Bottom panel) The residual activity of GAPDH with tau (molar ratio, tau/GAPDH = 4: 1) was measured at different temperatures. Curves 1 and 2 represent the residual activity of GAPDH with tau and that of GAPDH as control, respectively. The error was $\pm$4.6%.

Now let us see the effect of tau on inactivation of GAPDH during GdnHCl denaturation. Figure 2 (top panel) shows that inactivation of GAPDH with tau was not detected when the denaturant concentration was less than 0.05 M. But it became more marked while GdnHCl concentration increased. The residual activity of GAPDH with tau was approx. 18%, but that of the control was 35% in 0.2 M GdnHCl solution. The relative activities (Ai/Aj; where Ai represents the activity with tau at different concentrations or temperatures and Aj represents that of control) of GAPDH with tau were 50 and 33% of the control when the denaturant concentrations were 0.2 and 0.5 M, respectively (Figure 2, top panel, inset). Similar results were obtained when GAPDH was thermally denatured (Figure 2, bottom panel). The activities of GAPDH both with and without

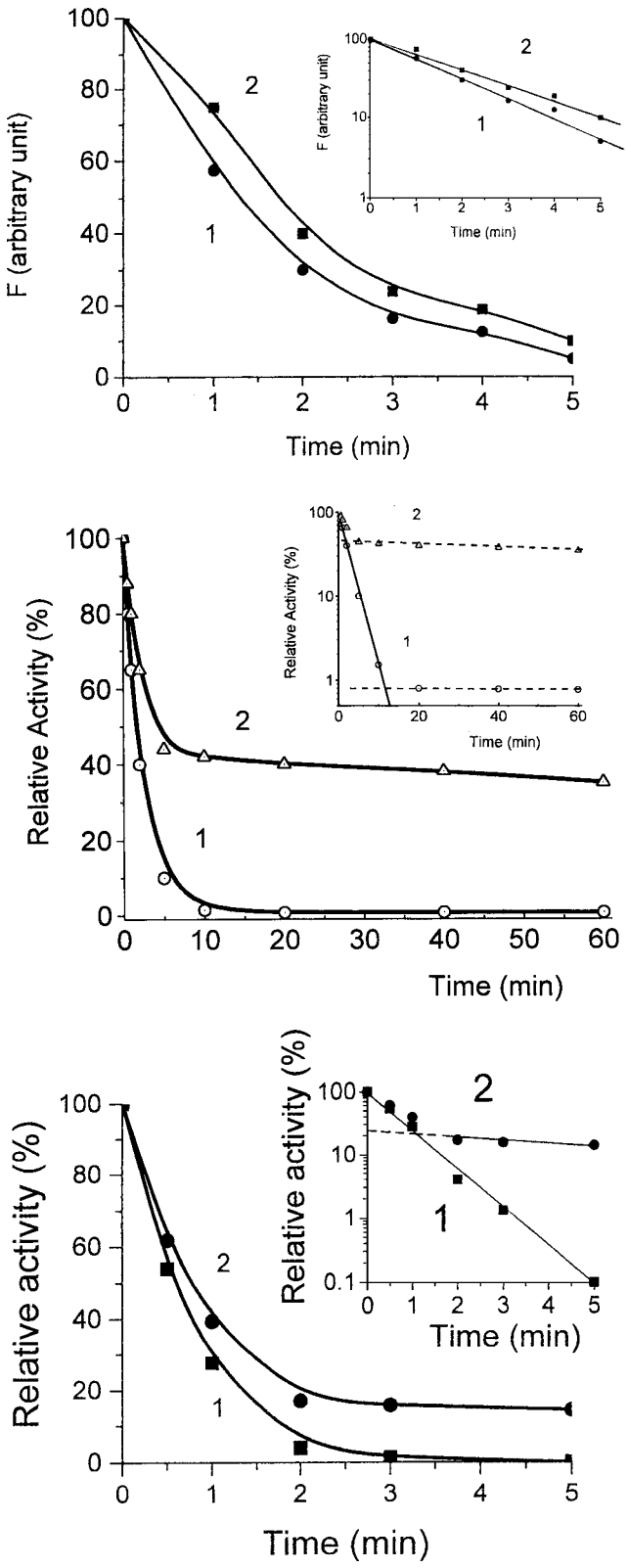

Figure 3 Kinetics of the intrinsic fluorescence and inactivation of GAPDH during GdnHCl and thermal denaturation

Curves 1 show the data from GAPDH with tau and curves 2 are those from the control. The insets are the same data plotted semi-logarithmically, according to Tsou [26]. (Top panel) GdnHCl denaturation. GAPDH with tau (tau/GAPDH = 4: 1) was added to 0.2 M GdnHCl solution, followed by measurement of the intensity of the intrinsic emission at room temperature at different time intervals. (Middle panel) The kinetics of inactivation of GAPDH with tau under the same conditions. (Bottom panel) Thermal denaturation: 10 μl of GAPDH with tau was added to 3 ml of phosphate buffer (pH 7.2) at 45 °C, followed by measurement of the activity.

tau were similar when kept at 25 and 27 °C. However, inactivation of GAPDH with tau was observed distinguishably when the temperature was 45–55 °C. The relative activities (Ai/Aj) of the enzyme with tau were 60 and 48 % of control at 45 and 55 °C, respectively (Figure 2, bottom panel, inset). The results indicate that tau does not affect the activity of the native GAPDH but influences the non-native enzyme during both GdnHCl and thermal denaturation.

We are concerned about whether tau's improvement of GAPDH denaturation is related to the conformation of tau or not. Thus the phosphorylation of tau by NCLK and the aggregated tau was performed. The results showed that neither of them influenced the inactivation and conformational changes of GAPDH during denaturation (results not shown). This suggests that the effect of tau on denaturation of GAPDH depends on its native spatial structure, which is changed after phosphorylation and aggregation.

Kinetics of inactivation and unfolding during GdnHCl and thermal denaturation

As described previously [32], unfolding of GAPDH in GdnHCl solution is a monophasic procedure. Kinetics of the conformational unfolding of the enzyme with tau is also monophasic (Figure 3, top panel). The first-order rate of GAPDH unfolding with tau was approximately twice as great as that of the control (Table 1). This is to say, tau is able to accelerate GAPDH unfolding during GdnHCl denaturation. Under the same conditions, however, inactivation of control GAPDH was a biphasic procedure, with a fast and slow phase (Figure 3, middle panel). The rate of the slow phase was two orders of magnitude lower than that of the fast phase. Nevertheless, inactivation of GAPDH with tau at room temperature was monophasic, whose first-order rate was as great as that of the fast phase of control GAPDH in 0.2 M GdnHCl solution. It suggests that tau improves the slow phase of the inactivation.

Similar results were observed during thermal denaturation, when GAPDH with tau was inactivated at 45 °C (Figure 3, bottom panel). The first-order rate of GAPDH inactivation with tau was similar to that of the fast phase of the control. Here we need to explain that tau does not inactivate GAPDH with GdnHCl at low concentrations (< 0.05 M), nor at room temperature. On the basis of the results reported previously [32], GdnHCl at such a low concentration did not influence the activity. The effects were not observed with the aggregated tau or the Ptau on the kinetics of denaturation. It showed that tau influenced inactivation of the slow phase. This suggests again that tau is able to combine with the denatured GAPDH and to accelerate the inactivation.

The effect of tau on reactivation of GAPDH

GAPDH could reactivate partially (30 %) by itself in the presence of NAD^+ and DTT after GdnHCl denaturation. A lower level of reactivation was observed in the absence of NAD^+, similar to the results of other authors [33,34]. In order to prevent NAD^+ interfering with the results, we did not add any NAD^+ to the further samples, and DTT was used to study the effect of tau on reactivation of the enzyme. GAPDH at a final concentration of 0.15 μM was employed, and the activity after reactivation by

Table 1 The first-order rates of inactivation and conformational changes of GAPDH during denaturation

Rates are given in 10^4 s^{-1}. Data are means ± S.D. from four experiments.

	GAPDH with tau		GAPDH without tau	
	Fast phase	Slow phase	Fast phase	Slow phase
In 0.2 M GdnHCl (25 °C)				
Decrease of emission (335 nm)	–	3.8 ± 0.34	–	1.7 ± 0.15
Inactivation	80 ± 9.6	–	91 ± 11	3.8 ± 0.35
At 45 °C without GdnHCl				
Inactivation	25 ± 1.9	–	30 ± 3.3	7.7 ± 0.92

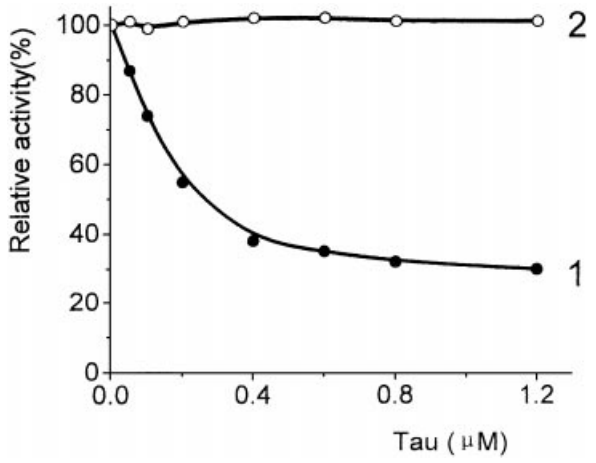

Figure 4 Effect of tau concentration on reactivation of the denatured GAPDH

Conditions were as described in the text with GAPDH at a concentration of 0.28 μM. Curve 1 represents the reactivation of GAPDH as diluted by the phosphate buffer. BSA was used as a control (curve 2).

diluting the denaturant solution was 15 % of the original activity. This reactivated activity was used as the control (100 %) for analysis of the effect of tau on GAPDH reactivation.

Dilution of the GdnHCl-denatured GAPDH (final concentration, 0.28 μM) in the presence of htau-40 at a molar ratio (tau/GAPDH) of 0.4–4 resulted in a decrease in the recovery of activity (Figure 4). With increasing concentrations of tau, the reactivation yield of GAPDH decreased from 100 to 30 %. BSA, which is used commonly as a control in the study of chaperones and anti-chaperones [35], did not have any observable effect on the reactivation of GAPDH under the same conditions. Furthermore, we did not detect any effect of tau on the activity of the native GAPDH by adding tau to the assay mixture. It suggests that tau is involved in anti-chaperone-like activity with this enzyme.

Consequently, the reactivation of GAPDH following thermal denaturation was carried out. GAPDH aggregated and precipitated markedly in solution when the temperature was higher than 45 °C. Reactivation of the enzyme was not detected after it was kept either at room temperature for 4–8 h or at 4 °C overnight. The thermal denaturation of GAPDH was irreversible under these conditions. Thus it was difficult to observe the effects of tau on reactivation of GAPDH from thermal denaturation.

The effect of Ptau on reactivation of GAPDH

NCLK catalysed phosphorylation of tau at Ser-195, Ser-202, Thr-205, Thr-231, Ser-235, Ser-396 and Ser-404 [36], which led to a decrease in tau's ability to bind and regulate microtubule structure and dynamics [37]. We prepared the Ptau with NCLK, as described by Paudel [24,28], and used Ptau to test the reactivation of GAPDH. The experiments showed that reactivation of GAPDH with Ptau (at a Ptau/GAPDH molar ratio of 3:1 or 4:1) was the same as that of the control. That is to say, tau loses its anti-chaperone-like function to GAPDH after phosphorylation by NCLK.

The effect of the aggregated tau on reactivation of GAPDH

As described by Wilson and Binder [29], tau polymers were prepared by incubation in phosphate buffer overnight. SDS/PAGE showed that tau aggregated completely when it was incubated for 72 h. The aggregated tau was added to the denatured GAPDH to test its effect on reactivation of GAPDH. Aliquots were taken at different time intervals. We observed that the ability to suppress reactivation of GAPDH decreased to 10 % as tau was incubated for 48 h, and to 0 % after 72 h, at a molar ratio of tau/GAPDH = 3:1. It revealed that tau lost its anti-chaperone-like function when it aggregated, suggesting that the native conformation of tau was involved in this function.

The effect of tau on aggregation of GAPDH

Whether a protein binds to a substrate that is in its non-native state is one of the requirements to identify if the protein is a chaperone or an anti-chaperone [38]. Thus the effect of a protein on aggregation of its substrate is an important criterion to test [39,40]. The binding of tau with the non-native GAPDH has been shown by the improvement in aggregation of the GdnHCl-denatured enzyme (final concentration, 0.28 μM), which aggregated strongly and rapidly, as monitored by light scattering (Figure 5). The intensity of the light scattering increased 5 min after dilution and approached a constant value in 10 min. The intensity increased distinguishably in the presence of htau-40. Both the rate and extent of GAPDH aggregation improved markedly with the increase in concentration of tau. In contrast, little increase in light scattering was observed in the presence of BSA at the same molar ratio, compared with GAPDH alone. Neither tau nor BSA alone changed the intensity of light scattering. Both Ptau and the aggregated tau were not observed to improve the light scattering under the same conditions. Experiments showed that tau could not bind to the non-native GAPDH when it was aggregated or phosphorylated by NCLK.

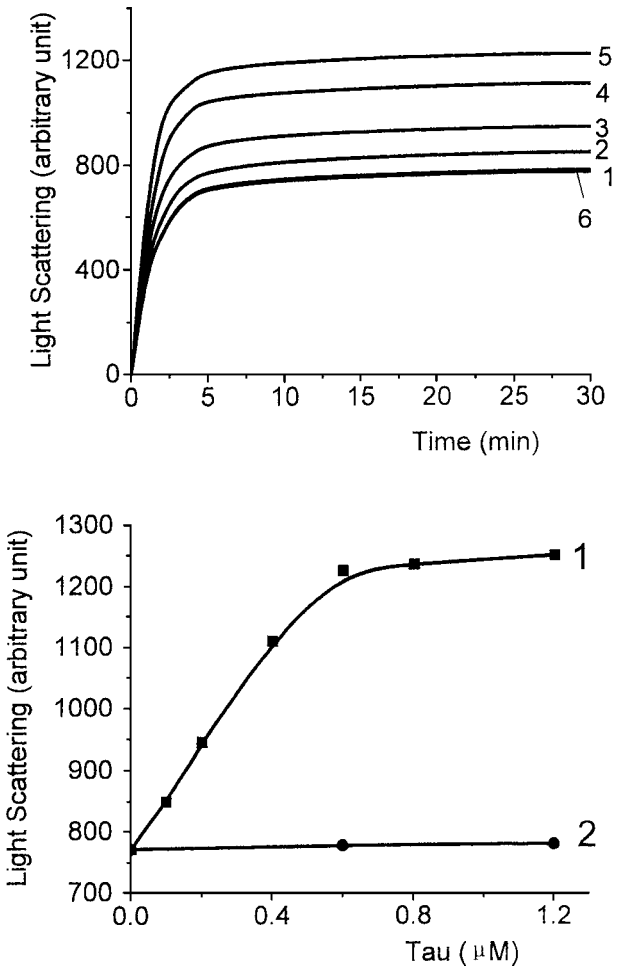

Figure 5 Effect of tau concentration on the aggregation of GdnHCl-denatured GAPDH as monitored by light scattering

On the basis of the method reported by Cai et al. [16], light scattering at 488 nm was used to monitor GAPDH (0.3 μM as final concentration) aggregation when incubated with tau at different concentrations. (Top panel) The time course of light-scattering change with tau of different concentrations (curves 1–5 represent tau concentrations of 0, 0.1, 0.2, 0.4 and 1.2 μM). Curve 6 is BSA as a control (12 μM). (Bottom panel) Effect of tau concentration on the eventual levels of aggregation of GAPDH (curve 1). Curve 2 is BSA as a control.

This supports the hypothesis that tau's anti-chaperone-like function with GAPDH depends upon its native conformation.

The effect of delayed addition of tau on GAPDH reactivation

According to Cai et al. [16] and Chen et al. [17], the effect of delayed addition of PDI on reactivation was observed in about 30 min after dilution of the denatured GAPDH (final concentration, 0.25 μM). At a molar ratio of tau/GAPDH = 4:1, the effect of delayed addition of tau on reactivation of GAPDH is shown in Figure 6. Tau suppressed reactivation of GAPDH distinguishably until 40 min after the dilution of the enzyme from the denaturation. The recovery was inhibited by 70 % before 40 min. However, tau no longer inhibited the activity after the recovery of GAPDH. These results strongly suggest that tau inhibits the refolding of GAPDH to the active state by interaction with the aggregation-prone species of GAPDH, hence improving the enzyme aggregation. We know that tau functions as an anti-chaperone to GAPDH as well as regulating the

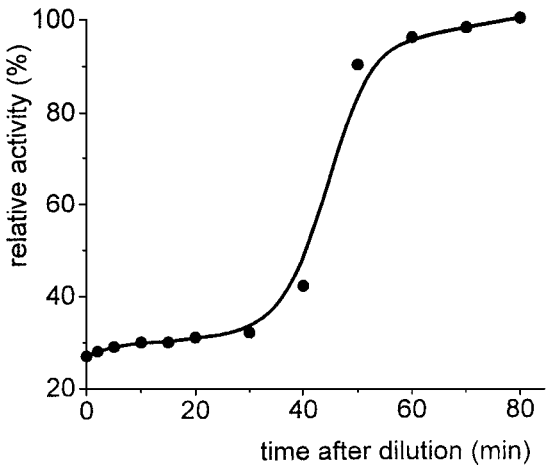

Figure 6 Effect of delayed addition of tau on reactivation of the denatured GAPDH

Tau (1 μM) was added at different times after dilution of the denatured GAPDH (0.25 μM), as decribed in the text.

conformation of tubulin to assemble and stabilize microtubules [41,42]. Furthermore, tau is demonstrably able to combine with DNA and stabilize the double strand [20]. It suggests that tau may play an important role concerning the structure of some other proteins besides its function on tubulin.

DISCUSSION

Now denaturation and renaturation of GAPDH have been investigated, but there remains a question: what about tau under the GAPDH-denatured conditions? We considered the idea that tau is not denatured distinguishably under the denaturing conditions. This view is based on these observations: tau is resistant to heat and acid treatment without losing its ability to promote microtubule assembly [1,3] and tau flexibility is similar to that of a denatured protein [43,44]. Recently, we observed that the intrinsic fluorescence of tau at 305 nm (from Tyr residues) did not change markedly when GdnHCl concentrations were less than 2.0 M. As mentioned above, kinetics of inactivation and reactivation of GAPDH were detected in the solutions of GdnHCl that were less than 0.2 M. Tau is readily able to refold from the thermal denaturation. Furthermore, tau will not lose its activity after boiling at 100 °C for 15 min [45,46]. This characterization is beneficial to the purification of tau. After tau is expressed by *Escherichia coli*, the lysates of the bacteria are boiled and most of the contaminated proteins are precipitated, except tau. Hence, tau should not be denatured under the same conditions as GAPDH is denatured.

As mentioned above, tau will lose its effect on denaturation and renaturation of GAPDH after it is phosphorylated by NCLK *in vitro*. We prepared Ptau with the method of Paudel [28,36], both currently and previously [47]. Under those conditions, NCLK catalyses phosphorylation of tau at the amino acid residues Ser-195, -202, -235, -396 and -404, and Thr-205 and -231, according to Sengupta et al. [48] and Haque et al. [49]. So far, although we cannot indicate which phosphorylation sites are essential for tau to combine with the denatured GAPDH, it suggests that phosphorylation may influence the effect of tau on

denaturation and renaturation of GAPDH. It was reported that tau is polymerized after phosphorylation [28]. In fact, it takes more than 5 h for Ptau to start aggregating at 37 °C [29]. Kinetic studies show that Ptau does not have any effect on inactivation and conformational changes of GAPDH during denaturation. We prepared Ptau freshly and used it within 1 h. So Ptau probably had no chance to aggregate when the kinetics experiments were carried out. Similarly, the effect on GAPDH inactivation and unfolding was not detected after tau was incubated at 37 °C for 24 h. We believed that tau had been polymerized under the conditions, on the basis of the formation of the new fluorescence at 333 nm [9]. This fluorescence formed when tau was incubated in phosphate or Tris (0.1 M, pH 7.2) buffers at 37 °C for over 24 h. This is to say, tau cannot influence denaturation and renaturation of GAPDH when tau is aggregated. It appears that the effect of tau on the activity and conformation of the denatured GAPDH depends on the native conformation of tau.

According to Ellis [38], for a protein to be regarded as a chaperone it needs three characterizations: (i) the protein must be one of a functional class of unrelated families; (ii) it must assist the correct non-covalent assembly of other polypeptide-containing structures *in vivo*; and (iii) it must not be a component of the assembled structure when it is performing its normal biological functions. For an anti-chaperone, just one characterization is different from the chaperone: it functions in obstructing the correct non-covalent assembly of polypeptide. As shown here, tau does not influence the activity of the native GAPDH, but binds to the denatured enzyme, and obstructs its reactivation and aggregation. Furthermore, the delayed addition of tau does affect the reactivation of the denatured GAPDH. It indicates that tau does not interact with GAPDH as a component of the assembled structure of the enzyme. Definitely, tau is not related to the other families of chaperones and anti-chaperones reported to date. This is why we regard tau as a new anti-chaperone.

GAPDH is a key enzyme in the glycolytic conversion of glucose into pyruvic acid, which is an important pathway of carbohydrate metabolism in most organisms [14,15]. Although we observed that tau is able to obstruct renaturation of this enzyme, no evidence was obtained how tau affects the activity and conformation of GAPDH *in vivo*. Recently, some authors observed that there exists a stable cold-folding intermediate of rabbit muscle GAPDH, which is bound by chaperonin 60 (GroEL) and 8-anilino-1-naphthalenesulphonic acid [50,51]. This intermediate refolding state, which is similar to the relatively stable unfolding intermediate of the enzyme denatured in 0.5–1.0 M GdnHCl, may bind to GroEL, with suppression of both its reactivation and aggregation [52]. This suggests that tau may bind to the folding intermediate and suppress reactivation of GAPDH. Nevertheless, it is necessary to determine whether and how tau binds to the GAPDH folding intermediate. We used GAPDH as a substrate for tau, which may function as an anti-chaperone, *in vitro*. We do not have any evidence to say that tau is an essential factor for GAPDH folding and unfolding, *in vivo*. But this study gives us a suggestion that tau may act with a chaperone-like or an anti-chaperone-like function with other proteins, besides assembling tubulin and stabilizing microtubules. Whether or not tau functions as a chaperone or anti-chaperone *in vivo* still needs further study.

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