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MICROTUBULE ASSEMBLY AFFECTED BY THE PRESENCE OF DENATURED TUBULIN

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The effects of denatured tubulin on microtubule assembly from active phosphocellulose-tubulin have been studied. The presence of denatured tubulin resulted in an inhibition of the assembly and in the increase of the critical concentration to trigger the assembly. Inhibition of both the rate and extent of microtubule assembly was dependent on denatured tubulin concentration. This perturbation of microtubule assembly by denatured tubulin is likely to be specific as non-microtubule proteins did not significantly affect the assembly.

Microtubules are cellular organelles found in almost all eukaryotic cells and are known to be involved in a variety of functions including mitosis, axoplasmic transport, secretion, receptor activity and cell motility (1,2). These organelles are formed by the assembly of tubulin, an heterodimer of 110,000 mol. wt. (3). The assembly requires GTP (4,5). Microtubule associated proteins, MAPs, have also been implicated in microtubule assembly. These MAPs include both high molecular weight proteins, MAP 1 and 2 (6,7) and low molecular weight components, the tau factor that contains two major peptides of 58,000 and 66,000 daltons (8).

The involvement of microtubules in a variety of cellular phenomena requires precise molecular regulation at both the temporal and spatial levels of the assembly process. Further understanding of the intracellular recognition of tubulin subunits and of the interactions of tubulin with other macromolecular factors involved in microtubule assembly and/or regulation is both interesting and important. Various studies have considered the role of MAPs (8,9) and other protein factors (10-12) in microtubule assembly. The present report describes the effects of denatured tubulin on the assembly of active tubulin subunits and its implications for the intermolecular recognition.

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MATERIALS AND METHODS

Microtubule preparation: Microtubule protein was purified from porcine brains by three cycles of temperature dependent assembly-disassembly according to the procedure of Shelanski et al. (13). Microtubule pellets were stored at -70°C after the second step of assembly. Prior to the experiments the pellets were resuspended in 0.1M 2-[N-morpholino]ethane sulfonic acid buffer (MES), pH 6.8, containing 1.5 mM $MgCl_2$ and 0.5 mM EGTA (Assembly buffer), centrifuged at 45,000 g at 4°C to eliminate aggregates and the supernatant subjected to the third cycle of assembly-disassembly. Tubulin and MAPs were separated by phosphocellulose chromatography according to the method of Weingarten et al. (8). Protein concentration was determined by using an A_{280} of 1.15 per mg/ml (14).

Microtubule assembly assay: Microtubule assembly was monitored by the kinetics of the increase of the absorbance at 350 nm (15) of tubulin solutions in the Assembly buffer. The assembly reaction was initiated by addition of 1 mM GTP to cuvettes containing tubulin and raising the temperature from 4° to 32°C.

Tubulin denaturation: Tubulin purified by phosphocellulose chromatography (PC-tubulin) in a buffer 0.1 M MES (pH 6.8), 1.5 mM Mg^{2+} was denatured by boiling the solution for 2 min. [^{14}C]-carboxymethylated denatured tubulin was prepared by reaction with [^{14}C]-Iodoacetate (16). The small fraction of aggregates of denatured tubulin were removed prior to the assays by centrifugation at 45,000 g. The recently prepared and non-boiled tubulin preparation will be termed "active tubulin".

Electron microscopy: Samples were collected after the assembly assays, processed for electron microscopy as described previously (17), and examined in a Phillips EM 300 electron microscope.

RESULTS

The first experiments showed that the addition of increasing amounts of denatured tubulin to a fresh and active tubulin solution (2.2 mg/ml) resulted in a decrease in microtubule assembly activity. The denatured tubulin preparation itself did not show assembly or GTP binding activities. The assembly kinetics of these samples are shown in Fig. 1a. The relationship between the assembly extent and the concentration of denatured tubulin is represented in Fig. 1b. The increase of denatured tubulin concentration resulted in a decrease of the extent of assembly, and at denatured tubulin concentrations higher than 4 mg/ml the assembly remained at a constant low value. Furthermore, a substantial decrease in the rate of assembly was also observed even after the addition of the low concentrations of denatured tubulin (Fig. 2). The presence of denatured tubulin (2 mg/ml) resulted in a decrease of the assembly rate to 12% of the control value, and in the extent of assembly to 32% of control.

The relationship between the extent of assembly and the concentration of active tubulin was examined in the presence and the absence of denatured tubulin. Results are represented in Fig. 3 and showed that addition of denatured tubulin resulted in an increase of the critical concentration necessary to trigger assembly, which

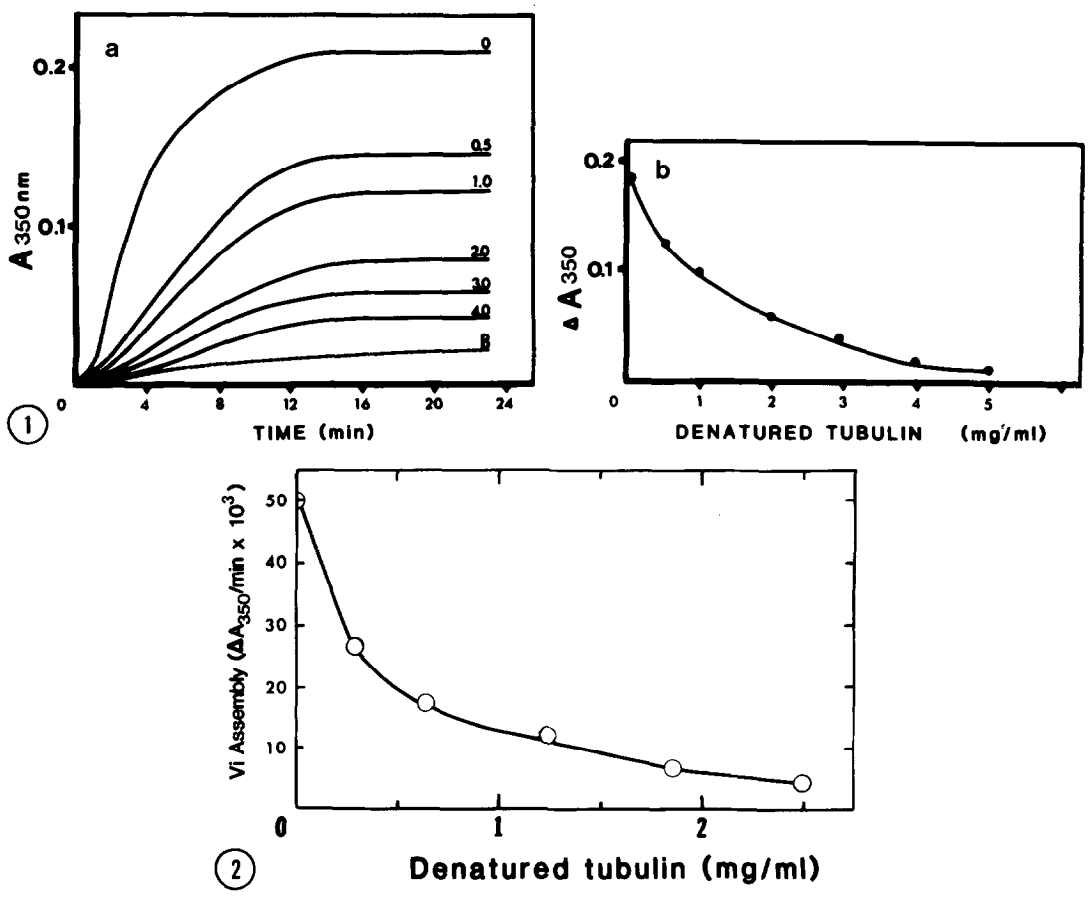

Fig. 1. Assembly kinetics of tubulin in the presence of increasing concentrations of a denatured tubulin fraction.

a. Individual 0.5 ml samples of native PC-tubulin (2.2 mg/ml) were adjusted to 0.5 mg/ml MAPs, 1 mM GTP and assembled in the presence of denatured tubulin: 0 mg/ml; 0.5 mg/ml; 1.0 mg/ml; 2.0 mg/ml; 3.0 mg/ml; 4.0 mg/ml; and sample assayed in the absence of GTP and denatured tubulin (B). The assay of samples of denatured tubulin at concentrations indicated, in the absence of active tubulin did not show assembly.

b. The maximum extent of assembly is represented as a function of the concentration of denatured tubulin in the assays.

Fig. 2. Effect of the denatured tubulin on the initial velocity of assembly of active tubulin.
Samples of PC-tubulin (2.2 mg/ml) were assayed in the presence of denatured tubulin at the concentrations indicated. The initial velocities followig the lag period were calculated and plotted against the concentrations of denatured tubulin.

implies a proportional decrease of the apparent association constant (K_{app}) of tubulin to be incorporated into the growing microtubule.

Denatured [^{14}C]-carboxymethylated-tubulin was not incorporated into microtubules, since no radioactivity was found in tubules formed from active tubulin in the presence of the denatured material. However, denatured tubulin seems able to

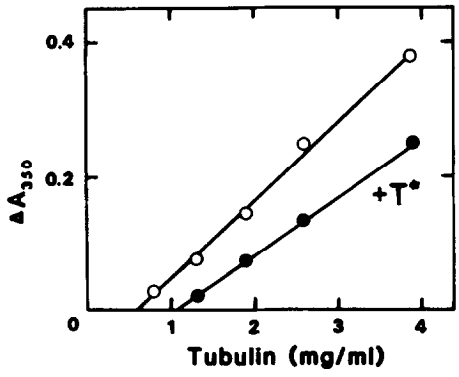

Fig. 3. Dependence of the extent of assembly (ΔA_{350}) on the concentration of active tubulin, in the absence (○) or in the presence (●) of denatured tubulin (1.6 mg/ml).

interact with the active tubulin dimer. A solution of active tubulin just below the critical concentration required for asembly was incubated with an equimolar amount of [^{14}C]-carboxymethylated, denatured tubulin. This mixture was subsequently applied to a Sephadex G-200 column and two major peaks resulted from the chromatography. The distribution coefficients (Kav) for these two peaks were 0.23 and 0.37, corresponding to molecular weights of 220,000 and 110,000 daltons respectively. The Kav = 0.23 peak showed a specific radioactivity 48% of that of the initial pool of denatured, labeled tubulin. The chromatography of the denatured, radiolabeled tubulin resulted in a single major peak with Kav = 0.37. Both preparations showed a minor peak with Kav = 0.11, which probably resulted from a small amount of aggregate formation. The results of these experiments are summarized in Table I.

The effects of the addition of non-microtubular protein were also assessed. The addition of a denatured purified neurofilament preparation (18) failed to inhibit the assembly from the active tubulin pool. In addition, neither native nor denatured bovine serum albumin significantly affected assembly, thus suggesting that the effect of denatured tubulin on the assembly is likely to be specific. Additional information was obtained from electron microscopy studies of tubules assembled either in the presence or the absence of denatured tubulin. Preparations assembled in the presence of denatured tubulin showed fewer tubules than preparations obtained in the absence of denatured protein. In addition, assembly

TABLE I

Exclusion Chromatography of [^{14}C]-carboxymethylated, denatured-tubulin in the presence and the absence of active tubulin*.

	Sample	Percentage of Total Protein		
		Protein Peak		
		Kav=0.11	Kav=0.23	Kav=0.37
A.	[^{14}C]-Denatured-Tubulin Active Tubulin	3%	26%	71%
B.	[^{14}C]-Denatured-Tubulin	4%	--	96%

*[^{14}C]-Carboxymethylated, denatured-tubulin (0.9 mg) was incubated for 30 min at 37°C in assembly buffer with an equivalent amount of active PC-tubulin in a final volume of 1 ml (A). The mixture was centrifuged at 28,000 xg and the supernatant filtered through a Sephadex G-200 column (45 x 1.2 cm). Radioactivity and protein of each fraction were determined. As a control (B) a labeled, denatured-tubulin sample was incubated under identical conditions but in the presence of boiled PC-tubulin and filtered through Sephadex G-200.

in the presence of denatured tubulin resulted in a significant amount of amorphous material and appearance of broken or incomplete microtubules (Fig. 4B), which is not a characteristic of the control sample assembled without the boiled tubulin (Fig. 4A). The formations of parallel arrays of microtubules and bent tubules in the preparation containing denatured tubulin is also noteworthy.

DISCUSSION

The experiments show that the presence of denatured tubulin affect both the initial velocity and extent of microtubule assembly from the pool of active tubulin. This effect can be interpreted as an alteration in the intermolecular recognition of active tubulin species.

Tubulin self-assembly occurs through series of bimolecular reactions (19) of the type:

$$(1)\quad T_{n-1} + T \rightleftharpoons T_n \text{; with dissociation constant } K_{n-1}.$$

The results of Table I indicate that denatured tubulin dimers (T*) interact with active molecules (T) of the tubulin pool, thus perturbing the effective association of the active tubulin dimers to the growing microtubule.

$$(2)\quad T + T^* \rightleftharpoons [T - T^*]$$

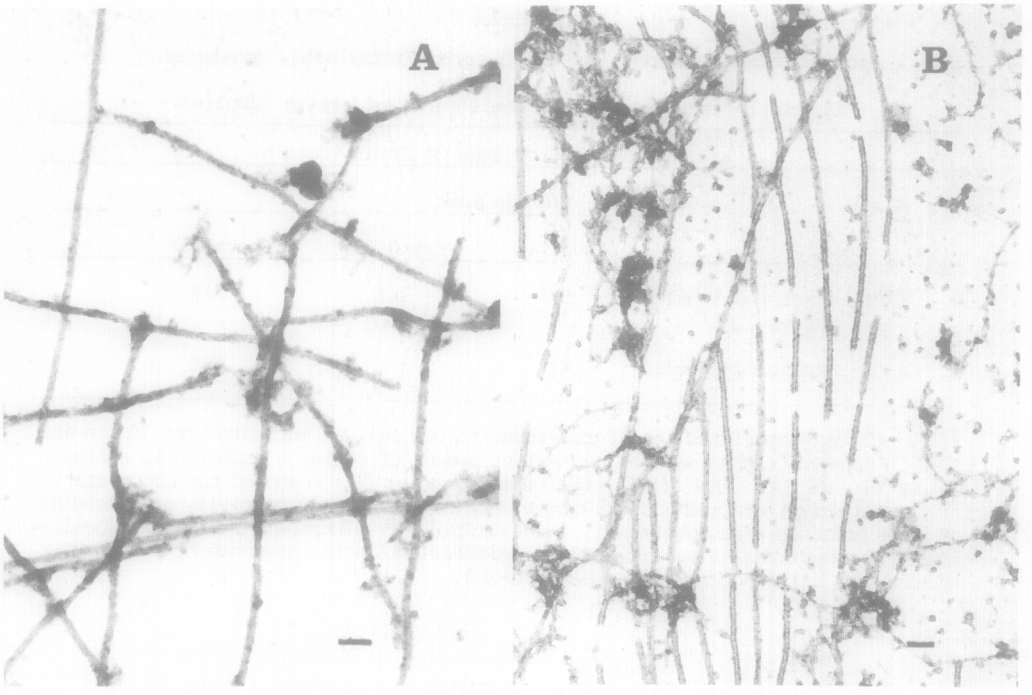

Fig. 4. Electron micrographs of PC-tubulin (2.2 mg/ml) assembled with MAPs (0.5 mg/ml) in the absence (A) or the presence (B) of denatured tubulin (1.6 mg/ml). Other conditions are indicated in Methods. The bars represent 1,000 A°.

Therefore, the non-productive interaction of active tubulin subunits with T* affects microtubule assembly. The analysis of the decrease in the distribution coefficient by exclusion chromatography has proven to be useful in the study of protein-protein interaction (20). The significant increase in the critical concentration for assembly by the addition of boiled tubulin also support the idea of the interaction between active and denatured tubulin dimers. An alternative explanation, the incorporation of denatured tubulin dimers into the growing pole of microtubules (or fragments of tubules) appears to be less likely due to the non-significant incorporation of [^{14}C]-carboxymethylated tubulin into the pelleted microtubules. Furthermore, previous studies (21) showed that the addition of carbamoylated tubulin to an unmodified tubulin sample resulted in a decrease of assembly with the failure of the modified protein to incorporate into microtubules.

The reported data show that the presence of denatured tubulin decreases the assembly ability of active untreated tubulin, suggesting that non-productive interactions between active and denatured tubullin species result in a decreased interaction between free active subunits and the growing microtubules or pieces of tubules. Several regulatory signals have been proposed on the basis of the in vitro studies of tubulin assembly. These studies suggest that the proportion of active and inactive (denatured of quasi-native structure) could play a regulatory role in the intracellular assembly-disassembly of microtubules.

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