DEPENDENCE ON THE ORDER OF ADDITION OF MAPs AND GTP FOR MICROTUBULE ASSEMBLY

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1. Introduction

Microtubule assembly in vitro has been shown to depend on the presence of GTP and to be enhanced by the addition of certain high M_r proteins, known as microtubule-associated proteins (MAPs) (review [1–3]). Since the assembly is inhibited by low temperatures the process is often initiated by warming a cold solution of tubulin, MAPs and GTP to 37°C [4–6]. An alternative method of initiating the microtubule assembly involves the addition of GTP to a pre-warmed solution containing tubulin and MAPs [7]. Here, we have observed that both the rate and the extent of the microtubule assembly process are dependent on the order of addition of GTP and MAPs to the 2 min pre-warmed tubulin solution. The results suggest that the 2 agents undergo a time-dependent interaction in promoting tubulin polymerization.

2. Materials and methods

Microtubule protein was prepared from porcine brain by 3 cycles of polymerization and depolymerization method in [8] as modified [9]. Fresh porcine brains were homogenized for 1 min in (1 ml/g tissue) buffer A (0.1 M piperazine-*N*,*N*^{1}-bis[2-ethane sulfonic acid] (Pipes)/1 mM ethylene glycol-bis-[β-aminoethyl-ether)*N*,*N*^{1}-tetraacetic acid (EGTA)/1.0 mM $MgCl_2$ (pH 6.4) at room temperature. The homogenate was centrifuged at 21 000 rev./min for 120 min in a T21 Beckman rotor at 4°C. Polymerization was initiated by the addition of 1 mM GTP to a 2 min warmed solution of supernatant in 4 M glycerol, with incubation at 37°C for 30 min. This was again centrifuged at 21 000 rev./min for 120 min at 37°C and the pellet was resuspended in cold buffer A to a final volume of 1/5th of the supernatant, and incubated at 4°C for 30 min. After incubation, it was centrifuged at 37 000 rev./min for 60 min in a T50.1 Beckman rotor at 4°C. The 2 subsequent polymerization–depolymerization steps were carried out in the absence of glycerol.

Tubulin was separated from MAPs by buffer A-equilibrated phosphocellulose chromatography as in [9]. Phosphocellulose-eluted MAPs were desalted by gel filtration on buffer A-equilibrated Sephadex G-25 column. Tubulin and MAPs were either used immediately or frozen by liquid nitrogen and stored in −70°C freezer for no more than 2 weeks.

SDS-gel electrophoretic analysis indicated that tubulin samples were of homogeneity and that the MAPs contained mainly high M_r proteins with low M_r contamination.

Polymerization of tubulin was initiated by the 2 methods described. Turbidity was measured as an indication on the amount of polymerization at an absorbance of 350 nm in a 37°C water-jacketted Perkin-Elmer Spectrophotometer for ~15–20 min.

Negative staining of microtubules was performed as in [10]. Polymerization of samples was carried out for 20 min. One drop of polymerized sample was mixed with 1 drop of 0.5% phosphotungistic acid (PTA) and the mixture was directly applied to Formvar-coated copper grids. The excess solution was soaked with filter paper and the grids were examined on a Philips EM201 electron microscope.

Protein concentration was determined by Lowry reagents using bovine serum albumin as a standard.

3. Results and discussion

In most studies, the concentrations of MAPs used to promote significant assembly of microtubules were

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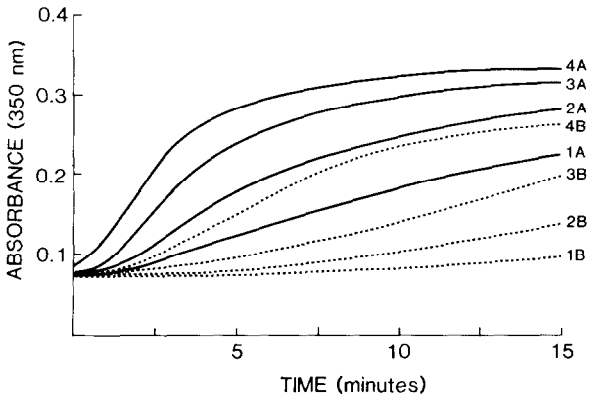


Fig.1. Comparison on the effect of the order of addition of GTP and MAPs to pre-warmed tubulin with increasing MAPs concentration. For curves (1A, 2A, 3A, 4A) purified tubulin (2.56 mg/ml) was warmed to 37°C for 2 min, then pre-incubated with 1.0 mM GTP and the assembly process was started with increasing [MAPs]. In (1A) 4.87 μg/ml; (2A) 9.73 μg/ml; (3A) 19.46 μg/ml; (4A) 38.92 μg/ml. For curves 1B, 2B, 3B, 4B, purified tubulin (2.56 mg/ml) was warmed to 37°C for 2 min, pre-incubated with increasing [MAPs] and then microtubule assembly was initiated by 1.0 mM GTP. In (1B) 9.73 μg/ml; (2B) 38.92 μg/ml; (3B) 97.30 μg/ml; (4B) 194.60 μg/ml.

0.1–1 mg protein/ml [4,6,7,11]. Fig.1 shows that such high concentrations of MAPs are required if the microtubule assembly process was initiated by the addition of GTP after the tubulin and MAPs had been mixed and incubated for 2 min. If, however, the process was initiated by the addition of MAPs to a pre-warmed solution containing tubulin and GTP, much lower concentrations of MAPs were required. For example, the rate and extent of microtubule assembly achieved by the addition of 4.9 μg MAPs/ml to pre-warmed solution of tubulin and GTP was significantly greater than that induced by 97.3 μg MAPs/ml added prior to the addition of GTP (fig.1).

The differences observed in the polymerization of tubulin by using different orders of addition of MAPs and GTP were more pronounced when low concentrations of MAPs were used. At very high concentrations of MAPs, the rate and extent of the microtubule assembly became independent of the order of addition of the two agents. These results suggest that the basic mechanism of microtubule assembly is the same in the 2 methods of polymerization. However, the effectiveness of MAPs in promoting the microtubule assembly is enhanced if it is added after GTP.

In order to determine whether boiling MAPs, the so called τ proteins, are responsible for the observed difference in the extent of assembly with the order of addition, MAPs were heated for 3 min. Our results indicate that there seems to be no difference in the extent of assembly, as well as the rate of assembly. However, further studies are needed to investigate the role of τ.

Tubulin is relatively unstable in the absence of GTP [12]. Since the protein samples used did not contain GTP, one possible explanation for the lack of GTP-initiated microtubules assembly (fig.1) might be that tubulin has been inactivated during the preincubation with MAPs. An experiment was carried out to test this possibility and its results are shown in fig.2. When GTP was added to a tubulin solution which had been preincubated with a low concentration of MAPs, little turbidity developed as was expected. However, microtubules assembly could be readily induced by subsequent addition of another low dose of MAPs. Both the rate and the extent of the microtubule assembly are essentially identical to those of the control sample which was induced to assemble by the addition of MAPs to the GTP-induced tubulin. These results clearly showed that the polymerization ability of the tubulin sample was not altered during its initial incubation with MAPs.

To rule out the possibility that the observed changes in turbidity represented protein denaturation other than microtubules formation, the polymerized samples were directly negatively stained and examined by electron microscopy. Fig.3 shows that the

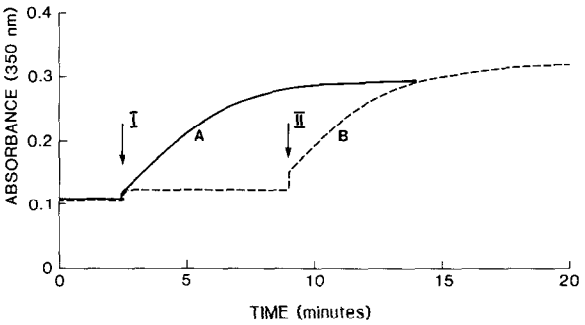


Fig.2. Effect of MAPs on the GTP-initiated microtubules assembly. Curve (A, ——): Tubulin solution (2.0 mg/ml) was incubated with 1 mM GTP for 2.5 min and 35.4 μg MAPs/ml was added to the sample to initiate assembly (arrow I). Curve (B, – – –): Tubulin solution (2.0 mg/ml) was incubated with 35.4 μg MAPs/ml for 2.5 min and 1 mM GTP was then added (arrow I). After 6.5 min 35.4 μg MAPs/ml was added to the sample to initiate assembly (arrow II).

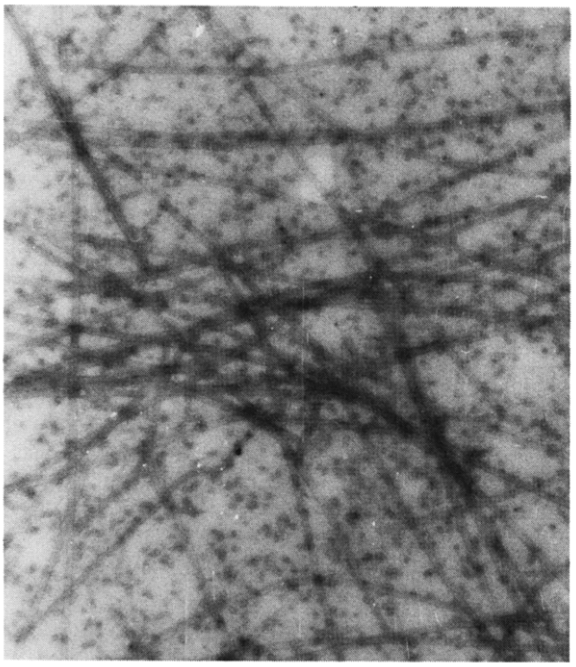


Fig.3. Electron micrograph of negatively-stained polymerized tubulin. Warmed tubulin (1.99 mg/ml) was incubated with 0.1 mM GTP for 7 min at 37°C. Microtubule assembly process was initiated by MAPs (8.9 μg/ml). After 20 min, 1 drop of polymerized sample was mixed with 1 drop of 0.5% PTA and applied to Formvar-coated copper grids: ×65 000.

microtubules were indeed formed under the conditions of low concentrations of MAPs.

The morphology of these microtubules was indistinguishable from those formed under the normal conditions, i.e., addition of high concentrations of MAPs prior to GTP addition.

The suggestions that the development of the turbidity in the tubulin solution represented the microtubules assembly was supported by the reversibility studies. When the turbid solution was incubated in an ice-water bath, the solution became clear within minutes.

The observation that MAPs added after the incubation of tubulin and GTP at 37°C showed a much higher assembly-promoting potency than if it was pre-incubated with tubulin indicated that either the protein was inactivated during its pre-incubation with tubulin, or that tubulin polymerization was potentiated upon incubation of GTP to respond to MAPs. Thus, the possibility that tubulin preparation contained proteases which destroyed MAPs during the pre-incubation period was tested. In one experiment, tubulin and 18 μg MAPs/ml were incubated at 37°C for 2 and 10 min, prior to the addition of GTP to initiate microtubule assembly. The extent of microtubule assembly appeared to be the same, but there seems to be a difference in the rate of polymerization. The 10 min incubation of MAPs with tubulin showed a greater lag period before an observable change in turbidity occurred than the 2 min incubation of MAPs with tubulin. It is, however, noted that this observation is not always pronounced with every preparation of fresh tubulin. These observations indicate that MAPs incubated with tubulin somehow become inactive but not destroyed.

The preceding results suggest that our observations are, at least in part, due to the potentiation of tubulin by GTP to respond to the effect of MAPs on microtubule assembly. To test for the time dependence of GTP effect on tubulin's response to MAPs, tubulin samples were preincubated to various times with GTP and the assembly process was initiated by the addition of 19.5 μg MAPs/ml. Fig.4 shows that if GTP and MAPs were added together to the tubulin sample, the initial rate of the microtubule assembly was not

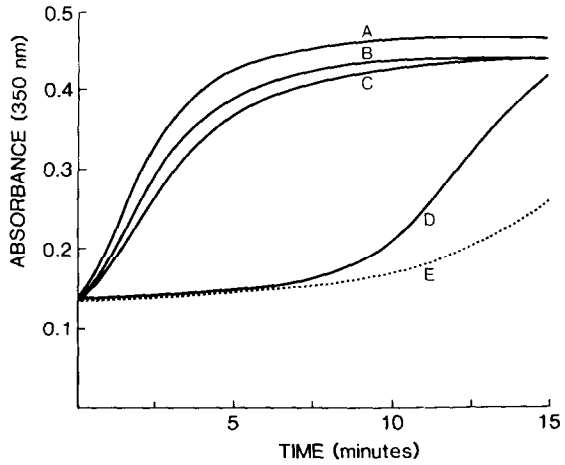
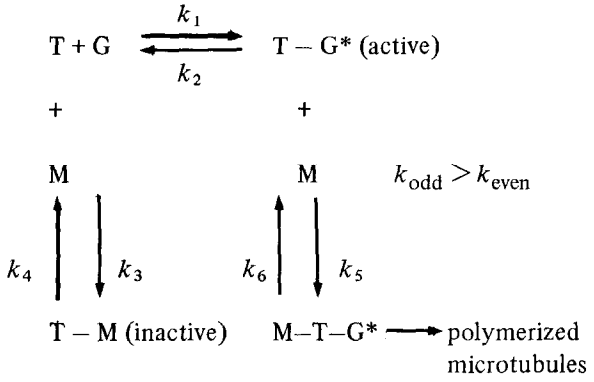


Fig.4. Effect of GTP pre-incubation with tubulin. Purified, warmed tubulin (2.56 mg/ml) was pre-incubated at 37°C with 1.0 mM GTP for: (A) 2 min; (B) 1 min; (C) 15 s; and the assembly process was initiated by MAPs (19.4 μg/ml); (D) tubulin (2.56 mg/ml) was warmed to 37°C and 1.0 mM GTP plus 19.4 μg MAPs/ml were added at the same time (i.e., 0 s incubation); (E) tubulin (2.56 mg/ml) was preincubated at 37°C with MAPs (19.4 μg/ml) and microtubule assembly process was initiated by 1.0 mM GTP (i.e., negative 2 min incubation).

significantly different from that obtained by initiating the assembly process by GTP alone. Thus pre-incubation with GTP was required for the tubulin to exhibit a good response to MAPs. The time required for the incubation however, appeared to be quite short. The sample which had been incubated with GTP for 15 s showed a time course not substantially different from that incubated for 2 min. It is also noted again, that when the assembly was initiated by GTP or by the mixture of GTP and MAPs, the time course showed pronounced lag period before the rise in turbidity.

Although, the mechanism of the dependence of microtubule assembly on the order of addition of GTP and MAPs is not known, the observarions may be explained by using the following reaction scheme:



where T, G, and M stand for tubulin, GTP and MAPs, respectively. This scheme assumes that the T–M complex is inactive and is unable to assemble into microtubules. It also assumes that the rate constant k_4 is much less than k_3, so that the regeneration of the functional MAPs is slow.

Thus, when tubulin and MAPs are mixed together prior to the addition of GTP, the formation of the T–M complex results in the inactivation of MAPs. On the other hand, a very brief period of pre-incubation of tubulin with GTP would convert tubulin into T–G complex, which is active in its response to MAPs for polymerization. This also explains that when the MAPs concentration was high, the rate of rise in turbidity became independent of the order of addition of GTP and MAPs (fig.1).

Conceivably, other mechanisms could account for most of our observations. However, the scheme postulated above explains all our findings.

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