EFFECT OF GUANINE NUCLEOTIDES ON THE ASSEMBLY OF BRAIN MICROTUBULES: ABILITY OF 5'-GUANYLYL IMIDODIPHOSPHATE TO REPLACE GTP IN PROMOTING THE POLYMERIZATION OF MICROTUBULES *in vitro*

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Summary: Polymerization of microtubule requires the presence of GTP, and the tubulin-bound GTP is hydrolyzed during microtubule formation. However, it was found that an unhydrolyzable analog of GTP, 5'-guanylyl imidodiphosphate (Gpp(NH)p), was able to replace GTP. The hydrolysis of the terminal phosphate group of GTP, therefore, does not seem to be a prerequisite to *in vitro* assembly of microtubules. The microtubules formed in the presence of Gpp(NH)p were indistinguishable from those formed in the presence of GTP under electron microscopy, but a remarkable decrease was noted in their sensitivity to depolymerization by calcium ions.

Tubulin, a major constituent of microtubules, is a dimeric protein with a molecular weight of approximately 110,000, consisting of two nonidentical, but closely related subunits. It binds two moles of guanine nucleotides per mole of protein; one is readily exchangeable with the nucleotide in the medium, whereas the other is not (1). We have studied the properties of binding of guanine nucleotide to the exchangeable site of bovine brain tubulin using a nitrocellulose membrane filtration technique (2). The binding required the presence of Mg^{2+} and free sulfhydryl groups, and the dissociation constants for GTP and GDP were estimated as 0.5×10^{-6} M and 1.9×10^{-6} M, respectively. Among other nucleotides tested, only Gpp(NH)p showed an appreciable interaction with tubulin, while another unhydrolyzable

analog of GTP, Gpp(CH_2)p, was found to be almost completely inert.

Weisenberg (3) was first to demonstrate the polymerization of microtubules *in vitro* in the presence of EGTA, Mg^{2+}, and GTP or ATP. Later, it was found that the hydrolysis of the GTP bound at the exchangeable site takes place during assembly of microtubules (4,5). More recently, Olmsted and Borisy (6) and Gaskin *et al*. (7) have reported that the polymerization of microtubules is dependent on the addition of nucleoside triphosphates, and analogs in which the terminal phosphate group was nonhydrolyzable are all inactive. From these results, they concluded that the hydrolysis of the terminal phosphate group is essential for polymerization of microtubules.

We have studied the *in vitro* assembly of microtubules in the hope of clarifying the molecular mechanism by which GTP promotes the polymerization. With a partially purified preparation from rat brain free of ATPase activity, it was shown that stoichiometric amounts of GTP are sufficient for maximal polymerization, and neither ATP nor GDP had any effect (T. Arai and Y. Kaziro, manuscript in preparation). Furthermore, it was found that Gpp(NH)p could substitute for GTP in promoting the polymerization of microtubules.

Experimental Procedure

Preparation of microtubules free of ATPase activity —— Microtubule proteins were partially purified by two cycles of assembly-disassembly according to Shelanski (8) with some modifications. After depolymerization, the insoluble materials were removed by centrifugation for 45 min at 30,000 x g and 0°. The supernatant was then fractionated with ammonium sulfate between 33 to 50% saturation. The precipitate of 50% saturation was dissolved in 0.1 M 2-(N-morpholino)ethanesulfonate (MES) buffer (pH 6.5), 0.5 mM magnesium acetate, and 1 mM EGTA (Buffer A) containing 25% glycerol and 10 μM GTP. After dialysis against the same buffer solution, sufficient GTP was added to obtain the final concentration of 100 μM and the solution was incubated for 30 min at 37°. The microtubules formed were collected by centrifugation for 45 min at 30,000 x g and 25°. The preparation did not contain any detectable ATPase activity.

Measurement of microtubule polymerization —— The polymerization reaction was carried out in a reaction mixture containing, in a final volume of 20 µl, 0.1 M MES buffer (pH 6.5), 0.5 mM magnesium acetate, 1 mM EGTA, 20 µM GTP and depolymerized microtubule proteins as indicated. After incubation for 30 min at 37°, the polymerized products were harvested by centrifugation for 45 min at 30,000 x g and 25°. The pellet was dissolved in 20 µl of 20 mM Tris-HCl buffer (pH 7.5) and 5 mM magnesium acetate, and the amount of tubulin present in microtubules was determined as described previously (2).

Electron microscopy —— Microtubule proteins purified by the assembly-disassembly procedure (8) were used for electron microscopic examinations without ammonium sulfate fractionation. The pellet from fourth assembly was fixed for 1 hour at 30° with 4% glutaraldehyde-Buffer A (1:1, v/v). It was then postfixed for 30 min with 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3), dehydrated through a graded series of ethyl alcohols and embedded in Epon 812. After standing for 48 hours, the flatembedded pellet was sectioned, and double-stained with 2% uranyl acetate in 70% ethanol and lead acetate. The sections were examined with a Hitachi HU-12A or Hitachi H-500 electron microscope.

Materials —— The sources of materials, including labeled and unlabeled nucleotides, were the same as those described previously (2).

Results

Polymerization of microtubules in the presence of Gpp(NH)p —— With the preparation free of ATPase activity, stoichiometric amounts of GTP were sufficient for polymerization and the amount of inorganic phosphate liberated from GTP during assembly was equal to that of tubulin incorporated into microtubules (to be published). Furthermore, it was found that an unhydrolyzable analog of GTP, Gpp(NH)p, could replace GTP, although higher concentrations were required to obtain maximal levels of polymerization. (Table I).

As can be seen from the table, the maximal levels were obtained by adding approximately 2 nmoles of Gpp(NH)p to the reaction mixture containing 190 pmoles of tubulin, indicating that about 10-fold excess of Gpp(NH)p was required for polymerization. This could probably be explained as follows. The tubulin dimer used for assembly experiments contained a bound GDP in its ex-

Table I

Polymerization of microtubules promoted by Gpp(NH)p

Gpp(NH)p added (nmoles)	tubulin polymerized (pmoles)
0	0
0.5	1.3
1.1	16.5
1.6	53.7
2.1	92.5
2.7	92.8
3.2	108.4

The polymerization of microtubules was carried out as described under the "Experimental Procedure", using microtubule proteins containing 189 pmoles of tubulin.

changeable site and higher concentrations of Gpp(NH)p may be required for the displacement of GDP. It was previously shown that the dissociation constant of tubulin·GDP complex was smaller than that of tubulin·Gpp(NH)p complex by almost one order of magnitude (see Fig. 8 of ref. (2)). The labeled nucleotides recovered from the microtubules polymerized in the presence of [^{3}H]Gpp(NH)p were found to consist of [^{3}H]Gpp(NH)p (data not shown). From these results, it is concluded that hydrolysis of the tubulin-bound GTP is not essential for microtubule formation.

Properties of microtubules formed with Gpp(NH)p —— As shown in Fig. 1, microtubules formed with Gpp(NH)p were indistinguishable under electron microscopy from those formed in the presence of GTP. However, a marked difference was found between them with respect to the sensitivity toward calcium ions. As shown

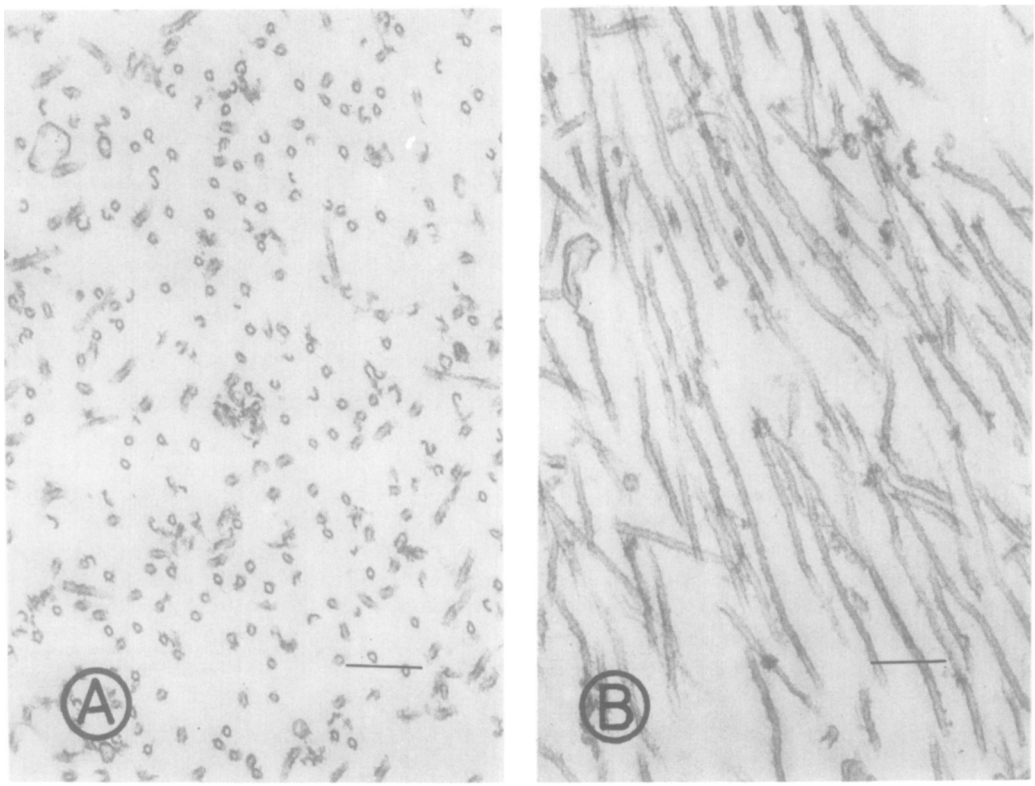

Figure 1. Electron micrographs of microtubules formed with Gpp(NH)p. Microtubule proteins were purified by three cycles of the assembly-disassembly procedure and the fourth cycle of the polymerization was carried out in the presence of 300 μM of Gpp(NH)p instead of GTP. Microtubules formed were collected, fixed and stained as described under the "Experimental Procedure". Cross section (A) and longitudinal section (B). The bars on the photographs represent 200 nm.

in Table II, under the conditions where about 80% of microtubules formed with GTP was depolymerized, those formed with Gpp(NH)p were nearly completely preserved. This indicates that the conformation of microtubules before hydrolysis of the bound GTP is different from that required for calcium binding.

Discussion

The foregoing results indicate that microtubule assembly in vitro is achieved by incubation of microtubule proteins with EGTA, Mg^{2+}, Gpp(NH)p, and glycerol at 37°, and therefore does not

Table II

Ca^{2+}-sensitivity of microtubules formed in the presence of GTP(System 1) and Gpp(NH)p(System 2)

Additions	System 1	System 2
	(pmoles of tubulin in microtubules)	
none	67.7	47.1
2.5 mM $CaCl_2$	14.3	44.0

Microtubules were formed as described under the "Experimental Procedure," using microtubule proteins containing 120 pmoles of tubulin·GDP, in the presence of either 0.4 nmoles of GTP or 6 nmoles of Gpp(NH)p. After incubation for 30 min at 37°, 1 µl of either redistilled water or 50 mM $CaCl_2$ was added to the reaction mixture, and the incubation was continued further for 30 min. Microtubules remaining were assayed as described in the text.

require the hydrolysis of the terminal phosphate bond of GTP. These results are in contrast to the previous ones of Olmsted and Borisy (6) who showed that the viscosity development during microtubule polymerization was dependent upon hydrolyzable nucleoside triphosphate; none of the nonhydrolyzable analogs like $Gpp(CH_2)p$, Gpp(NH)p, $App(CH_2)p$ and App(NH)p, being unable to replace GTP. Gaskin et al. (7) also reported that the assembly of microtubules as measured by turbidity was promoted by either GTP, ATP, or ADP, but by neither $Gpp(CH_2)p$ nor $App(CH_2)p$.

The discrepancy between their results and ours might be, in part, due to the difference in preparations and methods of measurements. However, as we have reported previously (2), $Gpp(CH_2)p$ does not interact with tubulin, and hence, would not support polymerization. Although Olmsted and Borisy (6) reported the inhibitory effect of $Gpp(CH_2)p$ on GTP-promoted polymerization, this could be explained as well by competition of two nucleotides for Mg^{2+} ions. The superiority of the β-γ-imido

analogs of nucleoside triphosphate over the corresponding β-γ-methylene diphosphonate compounds has already been indicated in other systems (9,10).

Two remarkable differences are noted between GTP- and Gpp(NH)p-promoted polymerizations of microtubules. First the additions of GDP inhibited almost completely Gpp(NH)p-promoted reaction, without affecting the GTP-promoted reaction (data not shown). Second, the microtubules formed with GTP are sensitive to Ca^{2+}, wherease the ones formed with Gpp(NH)p are not.

From these results, the role of GTP in assembly of microtubules might be interpreted as follows. The conformation of tubulin complexed with GTP, or its nonhydrolyzable analog, is favorable for polymerization. In contrast, tubulin·GDP complex probably has an unfavorable conformation for polymerization. Gpp(NH)p, when added in sufficiently higher concentrations to displace GDP from tubulin, can promote the polymerization of microtubules. The cleavage of GTP is apparently required to shift the equilibrium irreversibly toward the formation of microtubules, and also to confer the Ca^{2+} sensitivity. The elucidation of the detailed molecular mechanism of assembly and disassembly of microtubules as proposed above must await further investigation.

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