Proc. Natl. Acad. Sci. USA
Vol. 74, No. 12, pp. 5372–5376, December 1977
Biochemistry

Guanosinetriphosphatase activity of tubulin associated with microtubule assembly

(microtubule-associated proteins/brain/colchicine/vinblastine)

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Communicated by Martin D. Kamen, September 15, 1977

ABSTRACT **Tubulin, purified by cycles of assembly followed by phosphocellulose chromatography, exhibits a characteristic GTPase activity that is polymerization dependent and can be attributed to the tubulin itself. This activity has been observed, in a standard reassembly buffer containing low Mg^{2+}, under three conditions that induce microtubule assembly: in the presence of microtubule-associated proteins, in the presence of DEAE-dextran, or after addition of high Mg^{2+} and glycerol. The phosphocellulose-purified tubulin showed no GTPase activity under the following nonpolymerizing conditions: in buffer with low Mg^{2+} in the absence of microtubule-associated proteins or DEAE-dextran, in buffer with high Mg^{2+} and glycerol at tubulin concentrations below the critical concentration, or when microtubule assembly was inhibited by vinblastine. Colchicine, on the other hand, while blocking microtubule assembly, induced a significant GTPase activity in the phosphocellulose-purified tubulin. During the process of assembly, GTP appears to be hydrolyzed as a free tubulin dimer polymerizes into a microtubule. A constant GTPase activity when polymerization equilibrium is reached apparently reflects the cyclic polymerization-depolymerization of tubulin dimers at the ends of the microtubules.**

Microtubules can be reversibly assembled *in vitro* from brain homogenates. Polymerization occurs at 37° in the presence of GTP, Mg^{2+}, and an appropriate buffer, and the microtubules are rapidly depolymerized at 4° (1, 2). Microtubules purified from the brain homogenates carry with them a number of microtubule-associated proteins (MAPs), which can be separated from the tubulin by ion-exchange chromatography (3, 4). After separation, isolated tubulin is unable to reassemble into microtubules unless MAPs are added back (3, 4). However, the self-assembly property of highly purified tubulin can be restored by at least two other procedures: addition of 5–15 mM of Mg^{2+} and 3.4 M glycerol to the assembly buffer (5) or addition of a polycation such as DEAE-dextran (6).

The stable subunit of microtubules, the 110,000 molecular weight tubulin dimer, possesses two high-affinity binding sites for the guanine nucleotides (7). On the "E" site, the nucleotide is readily exchangeable, while on the "N" site it exchanges very slowly (8), if at all. Previous reports indicate that GTP binding to the "E" site is necessary for the polymerization reaction to occur while, after polymerization, the "E" site on microtubules contains essentially GDP (8–10). Therefore, hydrolysis of "E" site GTP apparently takes place during polymerization.

The hydrolysis of GTP during polymerization has been studied by assaying directly the release of inorganic phosphate (11). In this study a polymerization-linked GTP hydrolysis has been claimed to be detected by subtracting a high-background GTPase activity observed in the presence of colchicine, which inhibits microtubule formation. A serious objection to this study arises from the observation reported here that colchicine induces a significant GTPase activity in tubulin itself. Thus, the subtraction of the background activity obtained in the presence of colchicine is not a reliable basis for determination of polymerization-linked GTPase activity.

To obviate this difficulty, we have studied the GTPase activity of purified tubulin separated from the MAPs by ion-exchange chromatography. Using the DEAE-dextran and the Mg^{2+}/glycerol procedures to promote microtubule polymerization, we have been able to determine the GTPase activity intrinsic to tubulin in the absence of assembly and during the assembly process.

MATERIALS AND METHODS

Tubulin was isolated from pig brain homogenates by three cycles of polymerization and depolymerization as described by Shelanski *et al.* (2). The reassembly buffer used for purification and assembly experiments contained 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) adjusted to pH 6.6 with NaOH/1 mM ethylene bis(oxyethylene nitrilo)tetraacetic acid (EGTA)/0.5 mM $MgCl_2$. Reassembly experiments were done either in reassembly buffer or in half-strength buffer.

Tubulin was purified further and MAPs were isolated on a phosphocellulose column equilibrated with half-strength reassembly buffer as described (4). No more than 1 mg of protein per ml of phosphocellulose was applied, and the flow rate was less than one column volume per hr. Tubulin was eluted in the void volume of the column and the MAPs, which were adsorbed on the phosphocellulose column, were eluted in a single step with 0.8 M NaCl in half-strength reassembly buffer and dialyzed against reassembly buffer for several hours.

Turbidity measurements were made at 350 nm on a Beckman Acta V spectrophotometer in cells of 0.5-cm light path. The polymerization reaction was started by a temperature jump from 4° to 37°, which required a half-time of 4 sec.

The liberation of ^{32}P-labeled inorganic phosphate was measured according to the procedure of Nishizuka *et al.* (12). Before experimentation, free nucleotides were removed by adsorption on activated charcoal and the samples were preincubated at 4° in the presence of a given concentration of γ-^{32}P-labeled GTP for at least 15 min.

For our routine assay of $^{32}P_i$ released, aliquots of the sample were removed at different times and put into perchloric acid solution in order to stop the reaction by protein precipitation. We considered the possibility that tubulin precipitation by perchloric acid might provoke inorganic phosphate release from

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; MAPs, microtubule-associated proteins.

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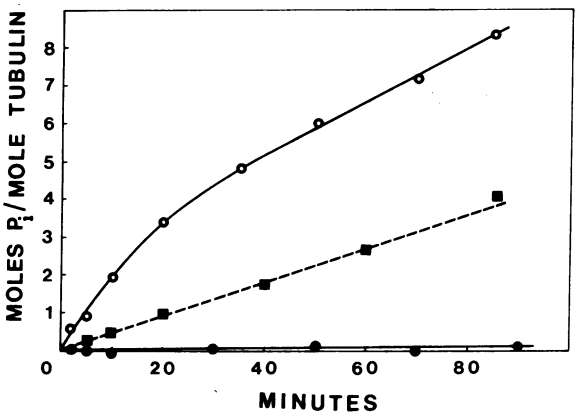


FIG. 1. Kinetics of GTP hydrolysis. Tubulin (8.5 μM), purified by the assembly procedure, was induced to polymerize by a temperature jump from 4° to 37° in reassembly buffer with 0.2 mM GTP. One aliquot was assayed for assembly by turbidity measurements at 350 nm while another was assayed for GTPase activity (○). Under these same conditions, the GTPase activity phosphocellulose-purified tubulin at 37° was measured (●). The GTPase activity of MAPs at 37° was measured in reassembly buffer in the presence of 0.5 mM GTP (■). The results have been normalized per μmol of protein, assuming a molecular weight of 110,000 for tubulin and, for convenience, for the MAPs as well.

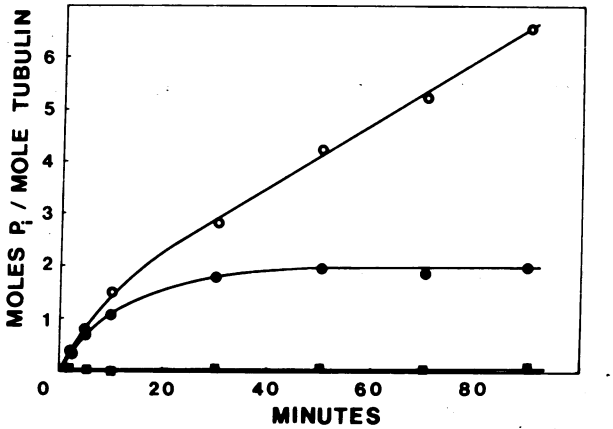


FIG. 2. Kinetics of GTP hydrolysis during polymerization of phosphocellulose-purified tubulin in the presence of DEAE-dextran at 37°. The conditions were: 10 μM tubulin, 0.1 M Mes, 30 μM Mg^{2+}, 0.3 mM GTP, and 0 (■), 0.15 (●), and 2 (○) mg of DEAE-dextran per ml.

an intermediate microtubule–phosphate complex and give rise to the "burst" of inorganic phosphate release during the first few minutes of polymerization. To test this hypothesis, in an experiment where tubulin polymerization was induced by DEAE-dextran, we measured the inorganic phosphate released into the medium after simply sedimenting the microtubules previously formed at different times of the reaction. In the presence of DEAE-dextran, this sedimentation was achieved in less than 5 min at 20,000 rpm. This experiment yielded the same GTP hydrolysis curve as in the control experiment, in which reaction was stopped by protein precipitation with perchloric acid.

Protein concentration was determined by the method of Lowry *et al.* (13). The color reaction for tubulin and bovine serum albumin was calibrated with solutions whose absolute concentrations were determined by refractive index increment and UV spectroscopy, respectively. The tubulin gave 11% more color (at 660 nm, per mg of protein) in the Lowry assay than did serum albumin. Routine assay of tubulin concentration was made with reference to a serum albumin standard and corrected for the color difference.

The DEAE-dextran used in these experiments was prepared as described elsewhere (14). This preparation had a charge density of 0.7 meq/g and, in limiting concentrations of the polycation, 3.4 mg of tubulin were assembled into microtubules per 1.0 mg of DEAE-dextran. The microtubules assembled with this DEAE-dextran all exhibit normal morphology (14).

RESULTS

GTPase Activity of Assembly-Purified Tubulin. We have followed in parallel the polymerization and the GTP hydrolysis under different polymerization conditions. Kinetics of polymerization measured by turbidity are characterized by a brief lag time, which is followed by an increase in turbidity. Eventually turbidity reaches a plateau as an apparent equilibrium is attained. The kinetics of GTP hydrolysis are biphasic (Fig. 1). The rate of phsophate release is maximum at the beginning of the polymerization reaction. It then slows down continuously until it reaches a constant value at about the same time, or in certain conditions some time after, the plateau of turbidity is attained. In every case, no hydrolysis occurred at 4° in the presence of tubulin nor at 37° in the absence of tubulin during the experimentation time.

The concavity of the GTP hydrolysis curves cannot be explained as an inhibition effect by the products of the reaction, i.e., by the GDP or by the phosphate produced. Indeed, at saturating GTP concentration, it is possible, after cooling and reheating the sample, to reproduce the "burst" of inorganic phosphate liberation. The kinetics of GTP hydrolysis during this second cycle of polymerization are identical to those of the first cycle in spite of the accumulated GDP and phosphate. In addition, we note that, during the depolymerization of microtubules at 4°, the amount of free inorganic phosphate remains constant. Thus, although GTP hydrolysis accompanies the polymerization reaction, GTP resynthesis does not occur in the reverse reaction of depolymerization.

GTPase Activity of Phosphocellulose-Purified Tubulin in the Absence of and during Assembly. In buffer conditions used for polymerization of the native tubulin–MAPs preparation, phosphocellulose-purified tubulin did not assemble, even at concentrations of 5–10 mg/ml. The assay for GTP hydrolysis showed that, under these buffer conditions, phosphocellulose-purified tubulin has no significant GTPase activity (less than 10^{-3} nmol of P_i/min per mg of protein).

MAPs were tested for GTPase activity under the same buffer conditions as tubulin. As illustrated in Fig. 1, MAPs catalyze GTP hydrolysis at a constant rate. The maximum GTPase activity that could be measured under different GTP and Mg^{2+} conditions was 1 nmol of P_i/min per mg of protein.

Microtubules can be polymerized from phosphocellulose-purified tubulin in reassembly buffer after addition of a polycation such as DEAE-dextran (6). Assembly can also be obtained in half-strength buffer containing 5–15 mM Mg^{2+} and 3.4 M glycerol. This is slightly different from the buffer of Lee and Timasheff (5), but the assembly reaction is apparently the same. We have demonstrated that whenever the phosphocellulose-purified tubulin is induced to polymerize, either in reassembly buffer plus DEAE-dextran or in buffer containing Mg^{2+} and glycerol, the polymerization is accompanied by GTP hydrolysis (Figs. 2 and 3). In both cases, the kinetics of GTP hydrolysis resemble those for polymerization of the unfractionated tubulin–MAPs preparation. In fact, under optimum polymerization conditions, the initial rate of hydrolysis is practically the same for polymerization induced by MAPs, DEAE-dextran,

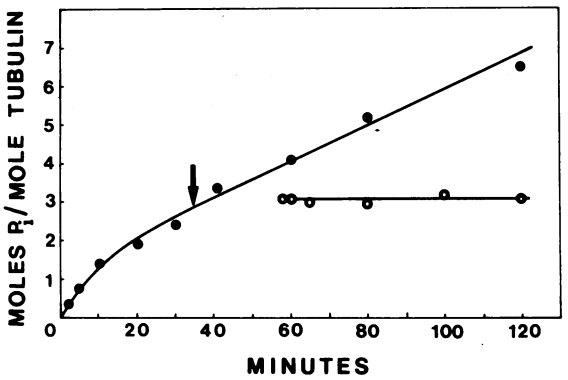


FIG. 3. Kinetics of GTP hydrolysis during polymerization of phosphocellulose-purified tubulin (14 μM) at 37° in 0.05 M Mes/12 mM Mg^{2+}/3.4 M glycerol/0.6 mM GTP. At the time indicated by the arrow, one part of the sample was centrifuged at 120,000 × *g* for 20 min at 37°. The supernatant was removed and tested for GTPase activity (○).

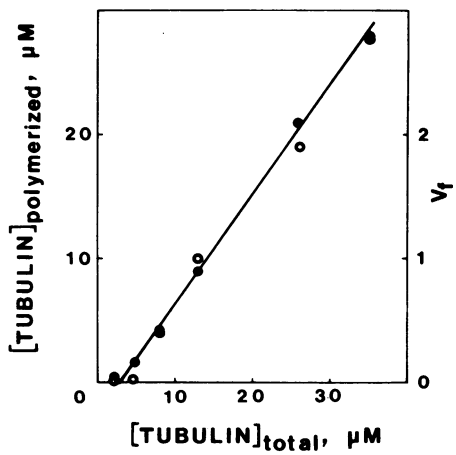


FIG. 4. Quantity of polymerized tubulin (○) pelleted after 2 hr at 37° in buffer containing Mg^{2+} and glycerol as described in the legend of Fig. 3 and steady-state values of the rate of GTP hydrolysis (V_f) (●), as a function of total tubulin concentration.

and half-strength buffer containing Mg^{2+} and glycerol (V_i = 2.4, 1.8, and 2.0 nmol of P_i/min per mg of protein, respectively). On the other hand, we have obtained large differences in the steady-state GTP splitting, in particular with DEAE-dextran-induced assembly. Indeed we observe (Fig. 2) that, at limiting DEAE-dextran concentration and low Mg^{2+} concentration, the steady-state GTP hydrolysis is essentially eliminated. Increasing the DEAE-dextran concentration from 0.15 to 2.0 mg/ml, raises the steady-state GTP splitting to 0.6 nmol/min per mg of protein. Increasing the Mg^{2+} concentration from 30 μM to 8 mM produces the same effect as excess DEAE-dextran. The length and appearance of microtubules as seen by electron microscopy is essentially the same under these different conditions, but the quantity of microtubules formed is somewhat reduced in excess Mg^{2+} and in excess DEAE-dextran.

GTPase Activity of Tubulin Dimers Coexisting at Equilibrium with Microtubules. Free tubulin dimers that exist in equilibrium with microtubules at the assembly plateau can be easily separated by centrifuging the microtubules. Phosphocellulose-purified tubulin was induced for assembly at 37° in the presence of DEAE-dextran or in buffer containing Mg^{2+} and glycerol. After 35 min, when equilibrium was reached, the sample was centrifuged at 37° for 20 min at 120,000 × *g*. The supernatant was decanted and tested for GTPase activity and for reassembly ability. No microtubules were observed by electron microscopy even after 30 min at 37°, and no significant GTPase activity could be detected in this tubulin dimer supernatant. Fig. 3 illustrates the result of this experiment performed in buffer containing Mg^{2+} and glycerol. In a similar experiment, in which assembly was induced by DEAE-dextran in the presence of 2 mM Mg^{2+}, the same result was obtained.

Effect of Total Protein Concentration on Steady-State GTP Hydrolysis. The kinetics of GTP hydrolysis at 37° in buffer containing Mg^{2+} and glycerol were studied as a function of tubulin concentration over a 2-hr period. Then the solutions were centrifuged and the amount of polymerized tubulin was determined by assaying the protein in the pellet and supernatant. The amount of microtubules formed (expressed as quantity of tubulin dimers incorporated into the microtubules) and the rate of GTP splitting in the steady state were plotted on the same graph as a function of total tubulin concentration (Fig. 4). The rate of inorganic phosphate release in microtubule solutions at equilibrium was directly proportional to the total amount of polymerized protein. Moreover, below the critical concentration, there was no significant GTPase activity.

Exchange of Nucleotide Bound at the "E" Site in Microtubules. To test if GTP splitting in the steady state could be accounted for by GTP hydrolysis and exchange at the "E" site within assembled microtubules, we performed an exchange experiment on microtubule solutions. Tubulin (20 μM) was polymerized at 37° in the presence of DEAE-dextran (0.5 mg/ml), 1 mM Mg^{2+}, 0.1 mM ^{3}H- and γ-^{32}P-labeled GTP. At polymerization equilibrium, after 40 min, a 10-fold excess of unlabeled GTP was added to one sample. Another was kept at 37° without addition of GTP. Both samples were maintained at 37° for another 40 min, after which they were centrifuged at 37° for 30 min at 120,000 × *g* through a 20% sucrose phase. The pellets were resuspended and homogenized in the assay buffer and radioactivity was measured.

In parallel to this exchange experiment, we followed the liberation of inorganic phosphate during the incubation at 37°. At the polymerization plateau, the rate of GTP splitting was constant and equal to 0.5 μmol of P_i/min per mg of protein, that is, in 40 min, 20 μmol of $^{32}P_i$ were liberated. This was approximately equal to the total amount of polymerized tubulin. If the hydrolysis were occurring by exchange of nucleotide within the assembled microtubules, we would have expected a large replacement of labeled nucleotide in the sample chased with an excess of unlabeled GTP. The amount of [^{3}H]GDP found in the two pellets, however, was essentially the same. In addition, we observed no ^{32}P in the microtubule pellets, indicating that the "E" site nucleotide in microtubules is GDP. These results are consistent with other reports indicating that the GDP on the "E" site is not available for exchange in microtubules assembled in the presence of MAPs (8–10).

Effect of Microtubule Inhibitors on GTPase Activity of Tubulin and of MAPs. After 20 min of incubation at 37° of phosphocellulose-purified tubulin (10 μM) in the presence of 100 μM colchicine and elimination of excess free colchicine on activated charcoal, 80% of the tubulin dimers bound one molecule of colchicine. Under these conditions a significant GTPase activity was observed which was constant during the experimentation time and generally of the order of 0.5 nmol of P_i/min per mg of protein. Furthermore, this activity was stimulated by addition of a DEAE-dextran concentration that would induce microtubule formation in the absence of colchicine.

However, in the presence of DEAE-dextran and colchicine, no microtubules could be observed under the electron microscope. Under these conditions, the rate of GTP hydrolysis was constant over the experimentation time and always less than the initial rate but greater than the final, steady-state rate obtained without colchicine.

Addition of 2 mM Ca^{2+} or 3 μM vinblastine to the tubulin, under the conditions of limiting DEAE-dextran described in the legend of Fig. 2, inhibited at the same time the formation of microtubules and the GTPase activity. The GTPase activity of MAPs, however, under the standard assay conditions, was not affected by any of these microtubule inhibitors.

DISCUSSION

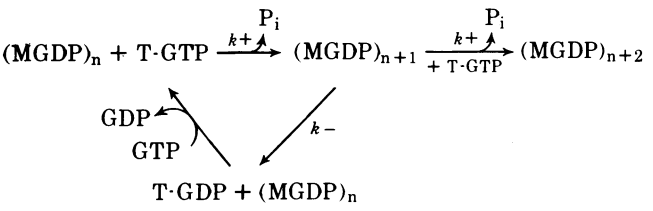
The experiments reported here demonstrate that purified tubulin freed of MAPs and unable to form microtubules does not possess a GTPase activity under the standard assay conditions. However, under self-assembly conditions, i.e., in the presence of MAPs, DEAE-dextran, or Mg^{2+} and glycerol, a GTPase activity is observed. This activity is stimulated by colchicine and inhibited by vinblastine under conditions in which these reagents are known to act specifically on tubulin. It seems unlikely then that this GTPase activity could be due to a contaminant rather than to tubulin itself. Therefore this catalytic activity may be attributed to (*a*) free tubulin subunits activated for GTPase activity under the same conditions that activate polymerization, (*b*) tubulin subunits within microtubules, (*c*) tubulin subunits located at microtubule ends, or (*d*) polymerization of free tubulin subunits at the ends of microtubules.

Hypothesis *a* could be rejected because, under conditions favorable to polymerization, no GTPase activity could be detected in tubulin dimers isolated by centrifugation from microtubule solutions at equilibrium. Above a critical concentration, at equilibrium the tubulin dimer concentration is equal to this critical concentration and is independent of total tubulin concentration (15). Under these conditions, the GTPase activity was proportional to the quantity of tubulin incorporated into microtubules. Moreover, below the critical concentration, no significant GTPase activity could be detected during more than 1 hr at 37°.

The rate of GTP hydrolysis observed in microtubule solutions at equilibrium could not be accounted for by catalytic activity due to subunits within microtubules because we found that the "E" site nucleotide did not exchange rapidly in microtubules. Sequestration of GDP on the "E" site of assembly-purified microtubules has also been reported by others (8–10). Aside from the exchange experiment, simple observation of the time evolution of GTP hydrolysis curves indicated that the rate of GTP hydrolysis could not be proportional to the quantity of tubulin incorporated into the microtubules. The quantity of polymerized tubulin continuously increased during the polymerization reaction, while the rate of GTP hydrolysis continuously decreased. By the same argument, one could reject any mechanism that could imply that the rate of GTP hydrolysis was proportional to the quantity of tubulin incorporated into microtubules, that is, hypothesis *b*.

In hypothesis *c*, the rate of GTP hydrolysis should be simply proportional to the concentration of microtubule ends. The observation that the rate of GTP hydrolysis continuously decreases during the self-assembly process would then imply that, during the polymerization reaction, there is a continual rearrangement of many short microtubules into a smaller number of long ones. This is not impossible but is contrary to our current understanding of microtubule assembly, since observations in other laboratories (16) indicate that the number of microtubule ends increases sharply at the beginning of assembly and remains constant during elongation.

The model that provides the best fit to our experimental data is *d*, in which GTP hydrolysis occurs as a tubulin subunit carrying a GTP on its "E" site polymerizes onto the end of a microtubule. In this case, the rate of GTP hydrolysis is proportional to the product of the concentrations of microtubule ends and of tubulin–GTP (T-GTP). The relationship between GTPase activity and polymerization, which follows from the model, can be illustrated thus:



where $(MGDP)_n$ stands for the microtubules. More generally, the rate of GTP hydrolysis will be determined by the frequency of tubulin–tubulin associations of the type that occur during microtubule formation or during the nucleation process.

The rate of GTP hydrolysis is highest at the beginning of the assembly reaction, producing a "burst" of inorganic phosphate released during the assembly of microtubules. This is easily understood in terms of the model since the concentration of free tubulin is highest and a high frequency of tubulin–tubulin interactions may be expected to occur during the nucleation process. The "burst" of P_i released before the equilibrium state is reached should reflect directly the number of tubulin association steps that occur during the assembly. We expect this burst to be equal to at least 1 mol of P_i released per mol of tubulin incorporated into microtubules, and indeed the lowest stoichiometry we have found experimentally is around 1:1. In some cases, however, higher values of P_i "burst" have been observed, reaching sometimes 6 mol of P_i per mol of polymerized tubulin at low tubulin concentrations and in the presence of MAPs. In these cases we conclude that there are a number of association-disassociation steps in addition to the single polymerization step required for the incorporation of each subunit into the microtubules. This phenomenon would be observed, for example, if there were a rapid formation of small polymers, most of which dissociated and reassembled one or more times before the final incorporation into microtubules.

The observation of a steady state of GTP hydrolysis after microtubule assembly is completed is consistent with the interpretation that the assembly is an equilibrium reaction, with constant association and disassociation of subunits at the ends of the assembled microtubules. The steady-state rate should, according to the model, be proportional to the concentration of microtubule ends. It is noteworthy that, after assembly from different concentrations of tubulin, the GTPase activity at polymerization equilibrium is proportional to the weight concentration of microtubules formed (Fig. 4). This implies that the average length of the microtubules does not depend on the initial tubulin concentration. Another significant observation is that the steady state of GTP hydrolysis is often not reached until 10–50 min after the turbidity plateau. This implies that the equilibrium ratio of total polymerized tubulin to free tubulin dimers, indicated by the turbidity plateau, is established first, but that the number and size distribution of polymers continues to change as the GTPase activity decreases to its equilibrium value. Finally, the very low rate of GTP hydrolysis observed after assembly in low concentrations of DEAE-dextran

and low Mg^{2+} (Fig. 2) implies that these microtubules are extremely stable, with a low rate of disassociation.

The experiments with microtubule inhibitors are still preliminary, but a number of interesting observations have already been made. The GTPase activity of tubulin plus DEAE-dextran and the assembly of microtubules are both inhibited by very low concentrations of vinblastine. Under these conditions, electron microscopy showed more or less massive assembly into the spiral protofilaments characteristic of the vinblastine reaction (17), but the tubulin interactions leading to GTP hydrolysis and microtubule formation were apparently blocked. Colchicine, on the other hand, while blocking microtubule assembly, induces a significant GTPase activity in phosphocellulose-purified tubulin, which can be augmented by DEAE-dextran. This hydrolysis appears to be effected by the individual tubulin–colchicine complex, although we have not convincingly excluded the possibility that the colchicine stimulates a tubulin–tubulin interaction that induces GTP hydrolysis but is abortive for microtubule assembly.

Earlier reports indicated that microtubules will form in the presence of a nonhydrolyzable analogue of GTP, guanylyl imidodiphosphate (9, 18, 19), suggesting that GTP hydrolysis by tubulin as well as by MAPS is not necessary for polymerization. However, it has been reported that tubulin polymerization in the presence of guanylyl imidodiphosphate appears to be irreversible (20). It has then been proposed that GTP hydrolysis could be used to produce a more readily depolymerizable state (20). Whether this hypothesis is valid remains to be established.

The main point of this work has been to demonstrate that GTP hydrolysis accompanies microtubule polymerization under a variety of experimental conditions, an observation of obvious importance in the biology of microtubules. We note, in addition, that GTP hydrolysis provides information on the assembly reaction not previously available. According to the model we suggest, the inorganic phosphate release provides a measure of the frequency of tubulin–tubulin interactions. An important consequence of this work is then that measurement of GTP hydrolysis allows one to explore aspects of tubulin polymerization directly related to molecular events, which are not observed by turbidimetry or related techniques that measure total polymerized tubulin.

We thank Colette Simon for her technical assistance. The research was supported by grants from the Centre National de la Recherche Scientifique and the Délégation Générale à la Recherche Scientifique.

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