The Free Energy for Hydrolysis of a Microtubule-Bound Nucleotide Triphosphate Is Near Zero: All of the Free Energy for Hydrolysis Is Stored in the Microtubule Lattice

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Abstract. The standard free energy for hydrolysis of the GTP analogue guanylyl-(a,b)-methylene-diphosphonate (GMPCPP), which is −5.18 kcal in solution, was found to be −3.79 kcal in tubulin dimers, and only −0.90 kcal in tubulin subunits in microtubules. The near-zero change in standard free energy for GMPCPP hydrolysis in the microtubule indicates that the majority of the free energy potentially available from this reaction is stored in the microtubule lattice; this energy is available to do work, as in chromosome movement. The equilibrium constants described here were obtained from video microscopy measurements of the kinetics of assembly and disassembly of GMPCPP-microtubules and GMPCP-microtubules. It was possible to study GMPCPP-microtubules since GMPCPP is not hydrolyzed during assembly. Microtubules containing GMPCP were obtained by assembly of high concentrations of tubulin-GMPCP subunits, as well as by treating tubulin-GMPCPP-microtubules in sodium (but not potassium) Pipes buffer with glycerol, which reduced the half-time for GMPCPP hydrolysis from >10 h to ~10 min. The rate for tubulin-GMPCPP and tubulin-GMPCP subunit dissociation from microtubule ends were found to be about 0.65 and 128 s^{-1}, respectively. The much faster rate for tubulin-GMPCP subunit dissociation provides direct evidence that microtubule dynamics can be regulated by nucleotide triphosphate hydrolysis.

THE hallmark for energy-transducing systems such as myosin (Bagshaw and Trentham, 1973), ion-transporting ATPases (Taniguchi, and Post, 1975; Pickart and Jencks, 1984), the mitochondrial ATPase (Grubmeyer et al., 1982) and chloroplast coupling factor (Feldman and Sigman, 1982) is a near-zero free energy for hydrolysis of bound nucleotide triphosphate (NTP)[1]. Negligible free energy is released during NTP hydrolysis since this is stored in the protein conformation until useful work can be done. We have used the hydrolyzable GTP analogue GMPCPP, which contains a methylene linkage between the alpha and beta phosphates, to determine the free energy for hydrolysis of microtubule-bound NTP. We found that the majority of this free energy is stored in the microtubule lattice, so that the standard free energy for hydrolysis is near zero. This stored energy can do work, as in the NTP-independent movement of chromosomes in a reaction coupled to microtubule disassembly (Koshland et al., 1988; Coue et al., 1991). The retention of the energy of NTP hydrolysis by microtubules parallels the behavior of several energy-transducing systems.

In addition to these equilibrium studies, we have characterized the dynamics of microtubules formed with GMPCPP. The recent observation that this substance is not hydrolyzed in microtubules (Hyman et al., 1992) suggested its use in determining the role of the gamma phosphate moiety in microtubule dynamics. Our rationale was that if conditions could be found for forming microtubules containing GMPCP, it would be possible to determine the kinetic behavior of microtubules with a cognate pair of bound nucleotide tri- and diphosphates. Such a comparison was not possible with GTP analogues containing a nonhydrolyzable linkage between the beta and gamma phosphates (Weisenberg and Deery, 1976; Penningroth and Kirschner, 1978; Arai and Kaziro, 1976; Karr and Purich, 1978).

We found two routes for forming GMPCP-microtubules: by treating GMPCPP-microtubules with glycerol in sodium (but not potassium) Pipes buffer, which induces rapid hydrolysis of bound GMPCPP and stabilizes the resulting polymer; by assembling high concentrations of tubulin-GMPCP subunits. Our observation that subunits containing GMPCP dissociate much faster than subunits containing GMPCPP provides direct evidence that microtubule dynamics can be regulated by NTP hydrolysis. This conclusion was previously inferred from the requirement for GTP for dynamic instability behavior.

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1. *Abbreviation used in this paper*: NTP, nucleotide triphosphate.

Materials and Methods

Materials

Pig and beef brain tubulin was purified by two cycles of thermal-induced assembly and disassembly, followed by chromatography on phosphocellulose (Weingarten et al., 1975). For some experiments the protein was further purified by a glutamate-induced polymerization step (Voter and Erickson, 1984). The small amount of glutamate that contaminated the resultant protein was removed by chromatography on Sephadex G-25. Some studies with calf brain tubulin were with protein provided by T. J. Mitchison (University of California, San Francisco, CA). We found no significant difference in the properties of beef and pig tubulin.

The amount of bound and free guanine nucleotide contained in purified tubulin was determined by an isotope dilution procedure. Microtubules were assembled with 0.5 mM ATP, a trace amount of [^{3}H]GDP, 0.17 U/ml of recombinant nucleoside diphosphate kinase (2500 U/mg, Nm23-1 expressed in *Escherichia coli* and purified essentially as described by Randazzo et al., 1991) and varying amounts of added GDP; polymer was isolated with an Airfuge (see below). Since the amount of label that is incorporated from radioactive nucleotide into polymer is proportional to the specific activity of the nucleotide, and the specific activity is inversely proportional to the nucleotide concentration, the endogenous guanine nucleotide contained in purified tubulin (E) is described by the relationship

$$\frac{\text{CPM in pellet (GDP added = 0)}}{\text{CPM in pellet (GDP added = N)}} = \frac{E + N}{E} \quad (1)$$

N in this equation corresponds to the concentration of added GDP. We found that the amount of radiolabel incorporated into polymer was reduced by two when N was equal to the tubulin concentration. This result indicates that the tubulin used here contained ~1.0 mol exchangeable guanine nucleotide/mol tubulin; the nucleotide was 30% GTP and 70% GDP.

[^{3}H]Guanylyl-(a,b)-methylene-diphosphonate ([^{3}H]GMPCPP) was obtained in 1984 from ICN Biomedicals, Inc. (Costa Mesa, CA); GMPCPP was also obtained in 1984 from ICN, or was synthesized as described (Hyman et al., 1984). The material obtained from ICN was repurified by chromatography on a 0.7 × 10 cm column containing Waters Protein-Pak DEAE 40HR, developing with a 50 ml 0.05–0.225 M triethylammonium bicarbonate gradient. Gamma-[^{32}P]GMPCPP was synthesized from GMPCP (provided by T. J. Mitchison or synthesized in this laboratory), using ATP-32 and nucleoside diphosphate kinase, as described previously (Hyman et al., 1992).

In preparing tubulin-GMPCP for studies of assembly it was necessary to deplete tubulin subunits of endogenous GTP and GDP. Removal of GTP was necessary since assembly might otherwise result from reaction with this nucleotide. Endogenous GDP could inhibit binding of GMPCP, which was found (see below) to have lower affinity for the E-site. Endogenous nucleotide was depleted by addition of alkaline phosphatase (P4252, 5 U/ml, 60 min, 10°; Sigma), including 1 mM GMPCP, which is not hydrolyzed by alkaline phosphatase, to stabilize the tubulin as the E-site nucleotide was hydrolyzed. To determine the extent of hydrolysis of endogenous GDP and GTP the pool of nucleotide was radiolabeled by a 10-min reaction with a trace amount of [^{3}H]GTP and nucleoside diphosphate kinase (0.7 μg/120 μl) before alkaline phosphatase addition. The nucleoside diphosphate kinase catalyzes isotopic equilibrium between GDP and GTP so that hydrolysis of these can be determined by measuring the radiolabel in GTP, GDP, GMP, and guanosine. Samples were quenched with perchloric acid and analyzed with HPLC. After a 60-min incubation only 0.1% of the initial E-site GTP and 21% of the initial GDP remained. The relatively slow digestion resulted because, as described previously for the reaction of added pyruvate kinase with E-site nucleotide (Brylawski and Caplow, 1983), the tubulin repeatedly recaptures most of the dissociated nucleotide before it can react with alkaline phosphatase. We did not extend the alkaline phosphatase digestion time to hydrolyze the 21% remaining GDP because we were concerned that this might result in loss of activity in the tubulin. Also, any observed assembly with tubulin-GMPCP subunits could not be attributed to tubulin-GDP subunits, since this material does not form microtubules under these conditions. In our calculation of the rate constant for microtubule assembly with GMPCP we have corrected for the presence of 21% tubulin-GDP.

Isolation and Characterization of GMPCPP-Microtubules

All reactions were in BRB80 buffer (80 mM Na Pipes, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8), at 37°, except for rates measured with video microscopy, where the temperature was 22–24°. Microtubules were made by two methods, in both the tubulin was incubated with GMPCPP for 20 min at 0° before a 30 min incubation at 37°. "5:1 microtubules" were generated with an ~92% yield of protein by assembly with 200 μM GMPCPP and 40 μM tubulin. The amount of GDP in this polymer was determined by including a trace amount of [^{3}H]GDP and [^{3}H]GTP in the assembly reaction; the specific activity of the added label was calculated from the amount of exchangeable GDP or GTP (see Eq. 1). "High-GMPCPP Tubes" were generated by an initial assembly at 25° with 1 mM GMPCPP and 10 μM tubulin, with a second assembly reaction using 10 μM tubulin (obtained by disassembling the initial pellet for 45 min in buffer at 0°) and 50 μM radiolabeled GMPCPP. The amount of GDP in these microtubules was determined by including a trace amount of alpha-[^{32}P]GTP and alpha-[^{32}P]GDP in the second assembly reaction.

Microtubules were isolated using an Airfuge (3–10 min, 37°, 30 psi), or by layering a 50-μl aliquot over a 2-ml cushion, made with 5% Ficoll 400 in BRB80 buffer, with centrifugation in a Beckman TLA100.3 rotor (100,000 rpm, 37°). After the supernatant was removed by suction and the pellet carefully rinsed with buffer, the portion of the tube near the pellet was wiped dry with a Kimwipe. The pellet was dissolved in 0.1 M NaOH for measurement of protein. For nucleotide analysis the pellet was treated with 1.4 M $HClO_4$ for 10 min at 0°, after which the perchloric acid was neutralized with K_2HPO_4. Precipitated salt was removed by a 30-s centrifugation in a microfuge and the nucleotide composition in the supernatant was determined using a SynChrompak AX100 column (Synchrom Inc., Lafayette, IN). GMPCP and GMPCPP eluted at 4.5 and 7.5 min (1 ml/min) when the column was developed with 0.15 M sodium phosphate buffer and 1.2 M sodium acetate, pH 7.3.

The rate of hydrolysis of GMPCPP in microtubules was determined by HPLC analysis of perchloric acid-quenched aliquots of resuspended polymer containing tritium-labeled nucleotide. Also, the rate was determined by measuring release of [^{32}P]Pi (Carlier, et al., 1987) from microtubules that had been assembled with gamma-labeled [^{32}P]GMPCPP.

Video Assay of Assembly and Disassembly at Microtubule Ends

Microtubule dynamics were analyzed using video microscopy, as described previously (Walker et al., 1988). Briefly, microtubules were assembled at the ends of sea urchin axonemes (kindly provided by E. D. Salmon) with tubulin subunits in BRB80 buffer. Disassembly was induced by displacement of subunits with buffer, using the flow cell described by Vale (1991), or, less frequently, with the cell described by Berg and Block (1984).

The rate of assembly of tubulin-GMPCPP was determined with 4 μM tubulin and 1 mM GMPCPP. It was not possible to vary significantly the tubulin subunit concentration since with 3 μM tubulin there was no nucleation at axoneme ends in 10 min and with 6 μM tubulin there was extensive nucleation of free microtubules that obscured observations of axoneme-associated tubes; formation of free microtubules also decreases the tubulin subunit concentration by an unknown amount. It is noted that the requirement for 3 μM tubulin for nucleation is not related to the critical concentration for assembly, which is much lower. Rather, as reported previously (Walker et al., 1988), there is a significant lag before axonemes are elongated with tubulin subunits and this lag was excessively long when the tubulin concentration was below 3 μM.

Tubulin-GMPCP subunits were assembled at axoneme ends by reacting 90–100 μM tubulin with 1 mM GMPCP and alkaline phosphatase. The lag for axoneme elongation was especially significant with tubulin-GMPCP, so that it was necessary to incubate the flow cell containing the reaction mixture for ~5 min at 37° (in a humidified chamber) before viewing samples at room temperature. Less frequently, axonemes were first incubated for about 5 min with 7.5 μM tubulin and 1 mM GMPCPP to generate short stubs at axoneme ends. After the tubulin-GMPCPP subunits had been washed out with buffer the stubs were elongated with the 100 μM tubulin, 1 mM GMPCP, alkaline phosphatase mixture. Electron microscopic images of elongated axonemes were obtained with samples that had been diluted 100-fold into BRB buffer containing 1% glutaraldehyde and pelleted on an electron microscope grid using a Beckman EM90 rotor.

Assays of the Rate of Disassembly of GMPCPP-Microtubules

The rate of disassembly of [^{32}P]GMPCPP-microtubules was determined by sedimenting polymer with an Airfuge (37°, 10 min, 30 psi) after a 30-fold dilution into BRB80 buffer. The radioactive nucleotide contained in the supernatant was measured. Either the microtubules were not fully sedi-

mented, or disassembly continued during a portion of the time required for centrifugation, so that an only 80% yield was obtained with microtubules that were centrifuged immediately after dilution.

The kinetics for dilution-induced disassembly were also measured with high-GMPCPP tubes containing gamma-[^{32}P]GMPCPP, from the rate of release of [^{32}P]Pi formed in the reactions in Eqs. 2 and 3:

$$[^{32}\text{P}]\text{GMPCPP-Microtubule} \xrightarrow{\text{200-fold dilution}} [^{32}\text{P}]\text{GMPCPP-tubulin subunits} \quad (2)$$

$$[^{32}\text{P}]\text{GMPCPP-tubulin subunit} \rightarrow [^{32}\text{P}]\text{GMPCPP} \xrightarrow{\text{alkaline phosphatase}} [^{32}\text{P}]\text{Pi} \quad (3)$$

With alkaline phosphatase at 1 U/ml the cleavage of GMPCPP was rapid (0.56 s^{-1}), so that the observed rate of Pi release closely tracked the rate of disassembly. The molecular rate constant for disassembly was obtained by multiplying the % microtubule disassembled/s times the mean number of tubulin subunits/microtubule. The microtubule length distribution was determined by diluting an aliquot from the reaction mixture 3,000-fold into 1% glutaraldehyde, before the microtubules were sedimented with an Airfuge EM90 rotor onto an electron microscope grid. Lengths were determined by measuring >300 microtubules.

Equilibrium Constants for Nucleotide Binding

The equilibrium constant for GMPCP and for GMPCPP binding to tubulin was determined by competition experiments in which these nucleotides displaced bound [^{3}H]GTP from the tubulin E-site. Tubulin-[^{3}H]GTP subunits were prepared by adding a trace amount of [^{3}H]GTP to the 14.2 μM tubulin that eluted in the void volume when a mixture of 40 μM tubulin and 200 μM GTP was chromatographed on a Sephadex G-25 column. It was necessary to determine the amount of GTP in this mixture, since this is required in calculating the nucleotide specific activity, used in deriving the stoichiometry for nucleotide binding. The amount of GTP was determined by adding a trace amount of [^{3}H]GTP with and without 10 μM GTP before the 14.2 μM tubulin was rechromatographed on Sephadex G-25. Since 10 μM added GTP reduced the amount of radioactive nucleotide that eluted with the protein by 42%, it was concluded that the 14.2 μM tubulin contained 13.8 μM GTP (see Eq. 1).

The K_d values for GMPCPP and GMPCP were determined by incubating these nucleotides with 14.2 μM tubulin-[^{3}H]GTP for 5 min before a 40-μl aliquot was chromatographed on a 1-ml Sephadex G-25 column. In a control reaction without added GMPCPP or GMPCP the tubulin eluted with 0.57 mol[^{3}H]GTP/mol tubulin; however, since 20% of E-site GTP dissociates from tubulin during chromatography under these conditions (see Fig. 1 in Zeeberg, 1980), the E-site stoichiometry was 0.57/0.8 = 0.71.

Results

Nucleotide Composition of Microtubules Assembled with GMPCPP

We were puzzled by the discrepancy between results showing that either GMPCPP (Hyman et al., 1992) or GMPCP (Sandoval et al., 1977) is found in microtubules assembled with GMPCPP. We reinvestigated this problem using [^{3}H]GMPCPP, rather than the gamma-[^{32}P]GMPCPP used by Hyman et al. (1992), so that both GMPCPP and GMPCP could be tracked by the tritium in the guanine moiety.

Table I. Nucleotide Composition of Isolated Microtubules

Isolation Procedure	Pelleted protein	Pelleted [^{3}H]	Pelleted [^{32}P]
	%	%	%
Cushion	85	28	2.7
No cushion	100	30	20

Microtubules were assembled with tubulin (51 μM) and gamma-[^{32}P], [^{3}H]GMPCPP (150 μM) and the resultant polymer was isolated by centrifugation in an Airfuge, or by centrifugation through a cushion containing 25% glycerol.

Microtubules assembled with [^{32}P], [^{3}H]GMPCPP and isolated through a glycerol cushion were found to contain very little radioactive phosphorus (Table I). The 10/1 ratio of [^{3}H] and [^{32}P] indicated that ~90% of the GMPCPP that had been incorporated was hydrolyzed in the pelleted microtubules. In contrast, microtubules that were isolated without using a glycerol cushion contained nearly equal amounts of [^{3}H] and [^{32}P] (Table I). The possibility that the presence of [^{32}P] in the product isolated without glycerol resulted from subunits containing [^{3}H]GMPCP and [^{32}P]Pi was ruled out by HPLC analysis of bound nucleotide: 83% of the tritium was in [^{3}H]GMPCP and 17% in [^{3}H]GMPCPP.

Rate of Hydrolysis of GMPCPP in Microtubules

To determine the basis for the dramatic difference in the extent of GMPCPP hydrolysis in microtubules isolated with or without glycerol (Table I), the rate of nucleotide hydrolysis was determined in microtubules that had been assembled with [^{3}H]GMPCPP and isolated with a Ficoll cushion. Hydrolysis was slow: the fraction of bound tritium as [^{3}H]GMPCP increased from 19.3% in the initially isolated polymer to 30.9% in 2 h and to 59% in 18 h. The rate constant calculated from results during the first 2 h of incubation is 2.1 × 10^{-5} s^{-1}; this is twice the value calculated from the longer incubation, where protein denaturation may have influenced the rate. The rate of release of [^{32}P]Pi from microtubule bound gamma-[^{32}P]GMPCPP was linear with time and an observed 2.4% release of label in 40 min corresponds to a rate constant equal to 1 × 10^{-5} s^{-1} (19 h half-life).

To confirm that glycerol affected the rate of hydrolysis of GMPCPP in microtubules parallel analyses were performed with glycerol added to microtubules in solution. The half-time of GMPCPP hydrolysis was reduced to ~10 min (Fig. 1). To test whether other commonly used chemicals affect the hydrolysis rate, we examined GMPCPP hydrolysis in the

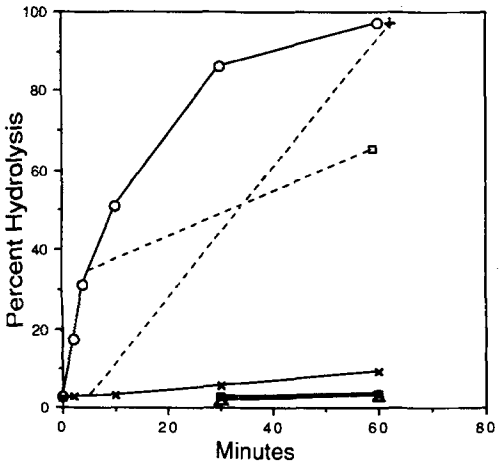


Figure 1. Effect of glycerol on the rate of hydrolysis of GMPCPP in microtubules. The rate of release of [^{32}P]Pi was determined when [^{32}P]GMPCPP-microtubules were diluted into buffer containing alkaline phosphatase and Na- (○) or K-Pipes (×) in glycerol, or in Na- (■) or K-Pipes (△) in the absence of glycerol. The dotted lines describe the reaction when GMPCPP-microtubules in either Na or K-Pipes and glycerol for 5 min were diluted 10-fold into glycerol buffer containing Na-Pipes (+) or K-Pipes (□). The rate in 1 M Na glutamate in Na-Pipes was indistinguishable from that in Na-Pipes and glycerol.

presence of glutamate or following substitution of Na-Pipes buffer with K-Pipes. The former was chosen because it is commonly used in tubulin purification; the latter was used because this had been used in a previous study (Hyman et al., 1992) which found intact GMPCPP. Like glycerol, glutamate stimulated hydrolysis, reducing the half-time to ~10 min. Most surprisingly, the switch from sodium to potassium cations strongly slowed hydrolysis, yielding a rate similar to that reported earlier (Hyman et al., 1992). The rate was also mildly stimulated by glycerol in Li-Pipes buffer: hydrolysis was increased from about 2% to 14% by glycerol, when gamma-[^{32}P]GMPCPP-microtubules that had been assembled in Li-Pipes buffer were incubated for 30 min. These cation effects are reversible, since a buffer change by a 10-fold dilution reduced hydrolysis in K-Pipes and increased it in Na-Pipes (Fig. 1).

Since both glycerol (Solomon, et al., 1973) and glutamate (Hamel and Lin, 1981) stabilize microtubules we examined whether taxol would also affect the hydrolysis rate. Isolated GMPCPP-microtubules were reacted with 25 μM taxol and the [^{3}H]GMPCPP remaining was analyzed by HPLC after a 5-, 45-, and 90-min incubation. This revealed that hydrolysis was essentially unaffected by taxol, as 82–84% of GMPCPP remained intact even at the latest time point.

To determine whether the presence of adjacent tubulin-GDP subunits could simulate the effect of glycerol on the rate of GMPCPP hydrolysis, microtubules were assembled under conditions where GMPCP and GMPCPP constituted only ~5% of the total bound nucleotide. Adjacent tubulin-GDP subunits did not simulate the effect of glycerol since there was no enhanced hydrolysis of GMPCPP in microtubules under these conditions (Table II).

Microtubules Containing GMPCPP Are Very Stable

The rate of microtubule disassembly upon dilution was sensitive to relatively small differences in the degree of substitution with analogue (Fig. 2). The sedimentation assay used here was not suited for determining the molecular rate constant for subunit dissociation, since in 30 min there was only a small increase in radiolabel that was not pelleted, over a relatively high (20%) background (lower curve in Fig. 2). To surmount this technical problem we assayed the rate of release of [^{32}P]Pi from [^{32}P]GMPCPP-microtubules after the polymer had been diluted into buffer containing alkaline phosphatase (see Eqs. 2 and 3). This revealed an initial rate

Table II. Effect of Coassembly of GMPCPP and GTP on GMPCPP Hydrolysis

GTP in assembly	μM Analogue in pellet*	% GMPCPP Hydrolyzed
50 μM	0.64 (3.9%)	25
200 μM	.54 (3.3%)	15
400 μM	.46 (2.8%)	6

Microtubules were assembled by a 30-min incubation of 40 μM tubulin, a GTP regenerating system (250 μM ATP and nucleoside diphosphate kinase) and 16 μM [^{3}H]GMPCPP and isolated by centrifugation in an Airfuge. A Ficoll cushion could not be used here since microtubules containing GDP are not stable to dilution in Ficoll. It was determined that 10.4 μM GDP was in microtubules, from measurement of the fraction of the label pelleted in an identical reaction with label in [^{3}H]GTP.

* The amount of nucleotide is expressed as concentration, after normalizing the volume of the resuspended pellet to the initial reaction volume. The value in parenthesis gives percent total [^{3}H]GMPCPP in the pellet; this exceeded the 0.5–.8% nonspecific entrapment of nucleotide under these conditions.

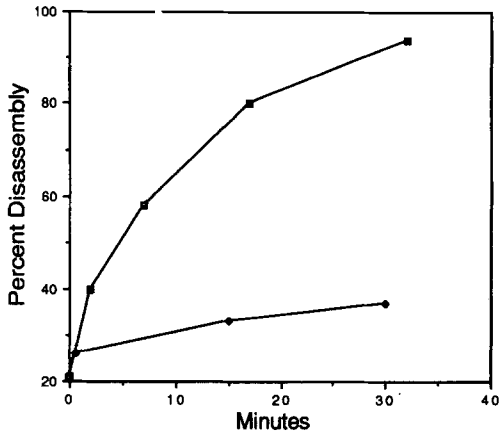


Figure 2. Rate of dilution-induced disassembly of GMPCPP-microtubules measured with a sedimentation assay. Rates with High GMPCPP Tubes (●) and 5:1 microtubules (□) are shown. High GMPCPP Tubes were generated by two cycles of assembly with GMPCPP; these contained 0.82 mol analogue/mol tubulin and <0.07 mol GDP/mol tubulin. 5:1 Tubes were assembled in a single cycle with GMPCPP/tubulin equal to 5. These contained 0.07 mol GMPCP, 0.46 mol GMPCPP, 0.05 mol GDP (from coassembly with tubulin-GDP subunits), and 0.11 mol GDP (from coassembly with tubulin-GTP subunits) per mol tubulin in polymer; this corresponds to 0.69 guanine nucleotide/tubulin, with 77% substitution with analogue.

of 1.7%/min (Fig. 3), and with a mean microtubule length equal to 2.8 μm, the molecular rate constant for subunit dissociation is 1.3 s^{-1}. Since the disassembly rates are about equal at the two ends (see below) the molecular rate constant is 0.65 s^{-1}. The rate of subunit dissociation was not significantly influenced by the identity of the monovalent cation: when microtubules assembled in Na-Pipes were diluted 100-fold the disassembly rate was 0.48%/min when dilution was in Na-Pipes and 0.33%/min in K-Pipes.

To confirm that microtubules were stabilized by GMPCPP, video measurements were made on individual microtu-

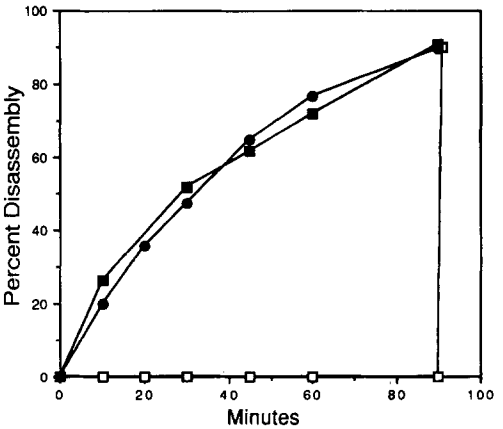


Figure 3. Rate of dilution-induced disassembly of GMPCPP-microtubules. Results from experiments run on consecutive days (●,■) and a reaction where alkaline phosphatase was not included in the diluent (□) are shown. In the latter reaction alkaline phosphatase was added at 90 min, and the quantitative release of Pi demonstrated that the microtubules had disassembled fully.

bules. High GMPCPP microtubules were very stable at both ends (Table III, Fig. 2). Subunit dissociation was extremely slow. Assuming that loss of fewer than 600 subunits (~2 mm on the video screen) would go undetected during a 30-min observation yielded a maximal rate constant for subunit loss of 0.4 s^{-1}, in agreement with the bulk measurements above (Fig. 3). About 15% of the microtubules were observed to disassemble at a rate of about 2 s^{-1}; we have no explanation for this faster rate. Microtubules assembled with a 5:1 GMPCPP/tubulin ratio so that they were 77% substituted with analogue (see legend to Fig. 2) had reduced stability. After dilution to remove tubulin subunits the majority of microtubules disassembled relatively slowly at one end (Table III), while a small fraction of these microtubules did not disassemble at a measurable rate.

To characterize further GMPCPP-microtubules, the rate of elongation from axonemes was determined for 4 μM tubulin-GMPCPP. This revealed a rate of 6.1 (n = 12, SD 1.8) s^{-1}, with no significant difference in the rate at the two microtubule ends. It was not possible to determine the elongation rate at widely varying concentrations of tubulin-GMPCPP because, as described in Materials and Methods, there is massive nucleation of microtubules that are not at axoneme ends when the tubulin concentration exceeds ~6 μM. Thus, the rate constant for elongation with tubulin-GMPCPP could not be determined from a linear plot of the concentration dependence of the rate. Instead, the observed rate with 4 μM tubulin, corrected for the rate for tubulin-GMPCPP subunit dissociation (0.65 s^{-1}, the average for the two ends from results in Fig. 3), was divided by the 4-μM subunit concentration used for assembly. The so-derived second-order rate constant was 1.36×10^6 M^{-1} s^{-1} (at 23°); the rate constant at 37° was reported to be 5×10^6 M^{-1} s^{-1} (Hyman et al., 1992). For comparison, rates equal to 1.2–5.3 $\times$ 10^6 M^{-1} s^{-1} have been reported for the reaction of tubulin-GTP subunits (Mitchison and Kirschner, 1984; Walker et al., 1988).

Table III. Kinetics for Disassembly of GMPCPP-microtubules Measured with Video Microscopy

Polymerization conditions	Glycerol treatment*	Rate end 1	Rate end 2
		s^{-1}	s^{-1}
High-GMPCPP Tubes	–	0.4‡ (41)	2.1 (11, 2)
High GMPCPP Tubes	+	146 (4, 39)	5.5 (6, 3)
5:1 Tubes‡	–	.5§ (19, .4)	10 (15, 7)

Rates were measured after microtubules assembled at axoneme ends were diluted with buffer. Values in parenthesis are number of microtubules analyzed and standard error. Results were from studies where both ends of an axoneme could be observed; however, numerous observations of microtubules on axonemes that could not be viewed at both ends confirmed the results reported here. The properties of High GMPCPP Tubes and 5:1 Tubes are described in the legend for Fig. 2.

* Microtubules that had been elongated at the end of axonemes were exposed to 60% glycerol for 40 min, after which the glycerol was displaced by excess buffer to induce disassembly.

‡ This is an upper limit for the rate since 16 of the 18 microtubules analyzed did not measurably shorten in 25 min of observation. The rate was calculated by assuming that a 2-mm shortening on the video screen would not be detected during the period of observation.

§ There was no measurable disassembly for 15 of 19 microtubules examined. An upper limit to the rate was calculated by assuming that a 1-mm change in length would not be detected in the 10 min of observation.

Stability of GMPCP-microtubules

The stability of GMPCP-microtubules was determined with microtubules that had been assembled with GMPCPP and then treated with glycerol to induce hydrolysis. On subsequent dilution these microtubules disassembled very rapidly, but only at one end (Table III). Microtubules containing GMPCP were also generated by assembling tubulin-GMPCP subunits at axoneme ends. This reaction could be induced with high concentrations of tubulin (91 μM) and GMPCP, using alkaline phosphatase to cleave excess endogenous nucleotides. The possibility that assembly resulted from reaction with residual tubulin-GTP subunits was ruled out since only 0.1% of the initial E-site GTP was found to survive the alkaline phosphatase treatment. Evidence that microtubules were formed was obtained by electron microscopy (Fig. 4). The previous failure to form microtubules in a reaction of purified tubulin with GMPCP (Sandoval, 1977) probably resulted from use of low protein concentrations.

Video microscopy was used to measure the elongation rate with GMPCP. A wide range of rates, much wider than that attributable to experimental error, was observed for elongation of a single axoneme end. For example, two microtubules at one axoneme end grew at rates of 5.7 s^{-1}, while another at the same end grew at 1.6 s^{-1}. A similarly wide range of rates of microtubule assembly (Gildersleeve et al., 1992; Toso et al., 1993) and disassembly (O'Brien et al., 1990) with GTP has also not been accounted for. Also, in agreement with previous reports (Gildersleeve et al., 1992; Toso et al., 1993), we occasionally observed periods during which microtubules temporarily stopped growing, as well as variable rates for both assembly and disassembly of a single microtubule. When results from experiments on three successive days were plotted the assembly rates clustered in two ranges, with rates equal to 16 s^{-1} (n = 16, SD 4) and 8 s^{-1} (n = 11, SD 1.7); two microtubules assembled at 1 s^{-1}. Since assembly with tubulin-GTP occurs more rapidly at the microtubule plus end we assume that the 16 s^{-1} rate is for reaction at this end.

The disassembly rate was more difficult to measure with GMPCP-microtubules since these were out of focus during the flow of buffer and moved violently for some time after the flow was stopped. Also, microtubules were frequently lost by shear and since disassembly is relatively rapid, it was not possible to select a new field or otherwise optimize observations after the flow of buffer. Rates again mainly fell into two categories: 128 s^{-1} (n = 12, SD 23) and 63 s^{-1} (n = 12, SD 15); six other microtubules had a mean rate of 26 (SD 6). Since the ratio of the assembly to disassembly rate is equal to the equilibrium constant for tubulin-GMPCP subunit addition, and this ratio must be equal at the two ends for a reaction where there is no NTP hydrolysis (Wegner, 1976), the rapid and slow assembly rates were coupled with the corresponding disassembly rates to calculate the second-order rate constant for assembly and the equilibrium constant for subunit addition (Table IV). The appropriateness of this coupling of rapid and slow rates was indicated by analysis of the rates for microtubules where both assembly and disassembly could be observed. For example, 12 out of 17 microtubules were found to have assembly and disassembly rates that were both either in the rapid or slow category. Generally, microtubules at opposite microtubule ends were

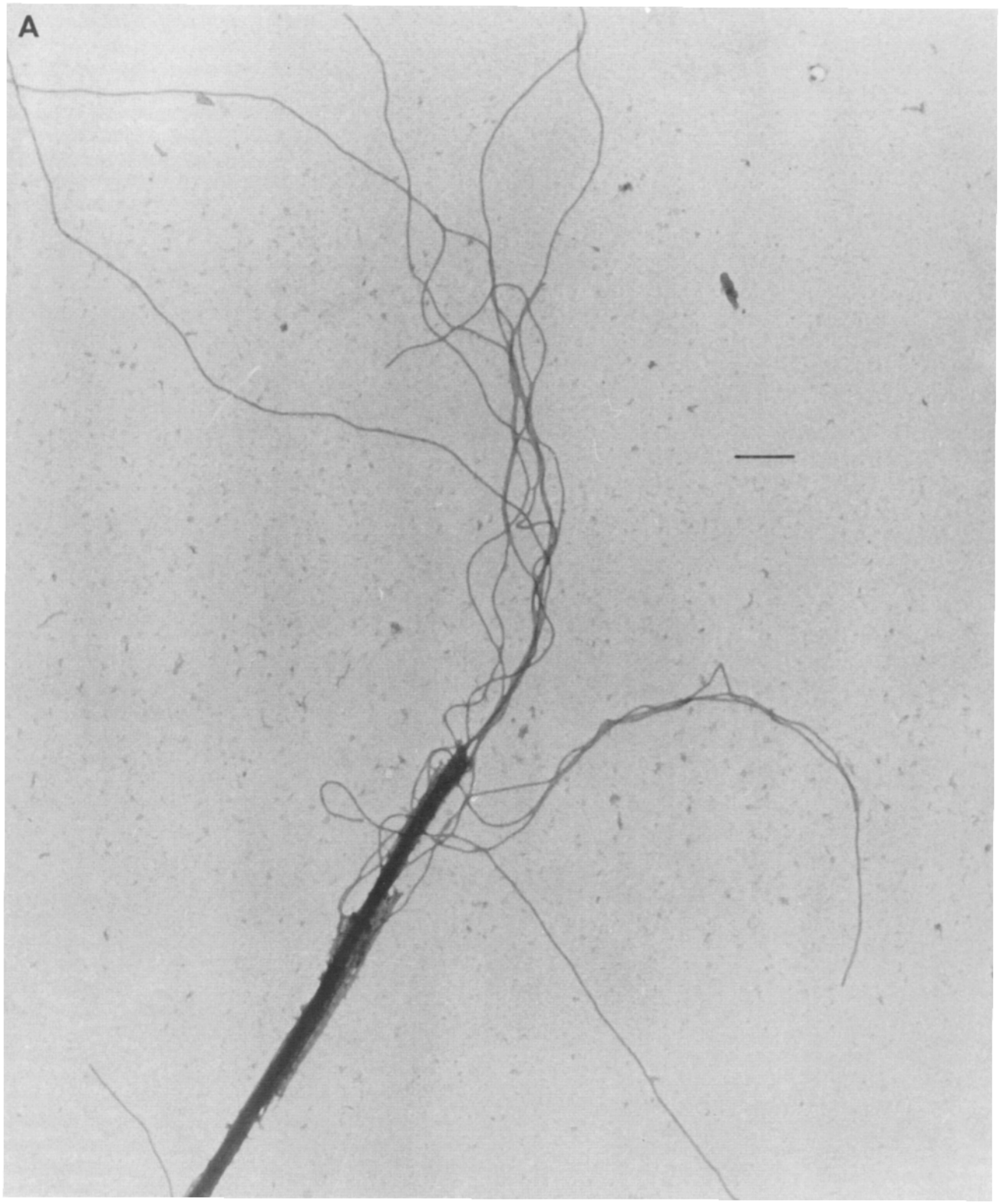


Figure 4. Structure of microtubule assembled with GMPCP. Bars, 0.5 μm.

either in the fast or slow range for assembly or disassembly. Deviation from the rule that either rapid or slow assembly and disassembly must occur at a given end may have resulted because some of the microtubules originated from axonemes with opposite polarity that were stuck together, or because microtubules grew from defects in the axoneme structure and then folded back on the axoneme so that their site of origin was mistaken. The frayed character of the axonemes is apparent in Fig. 4.

The second-order rate constant for assembly with tubulin-

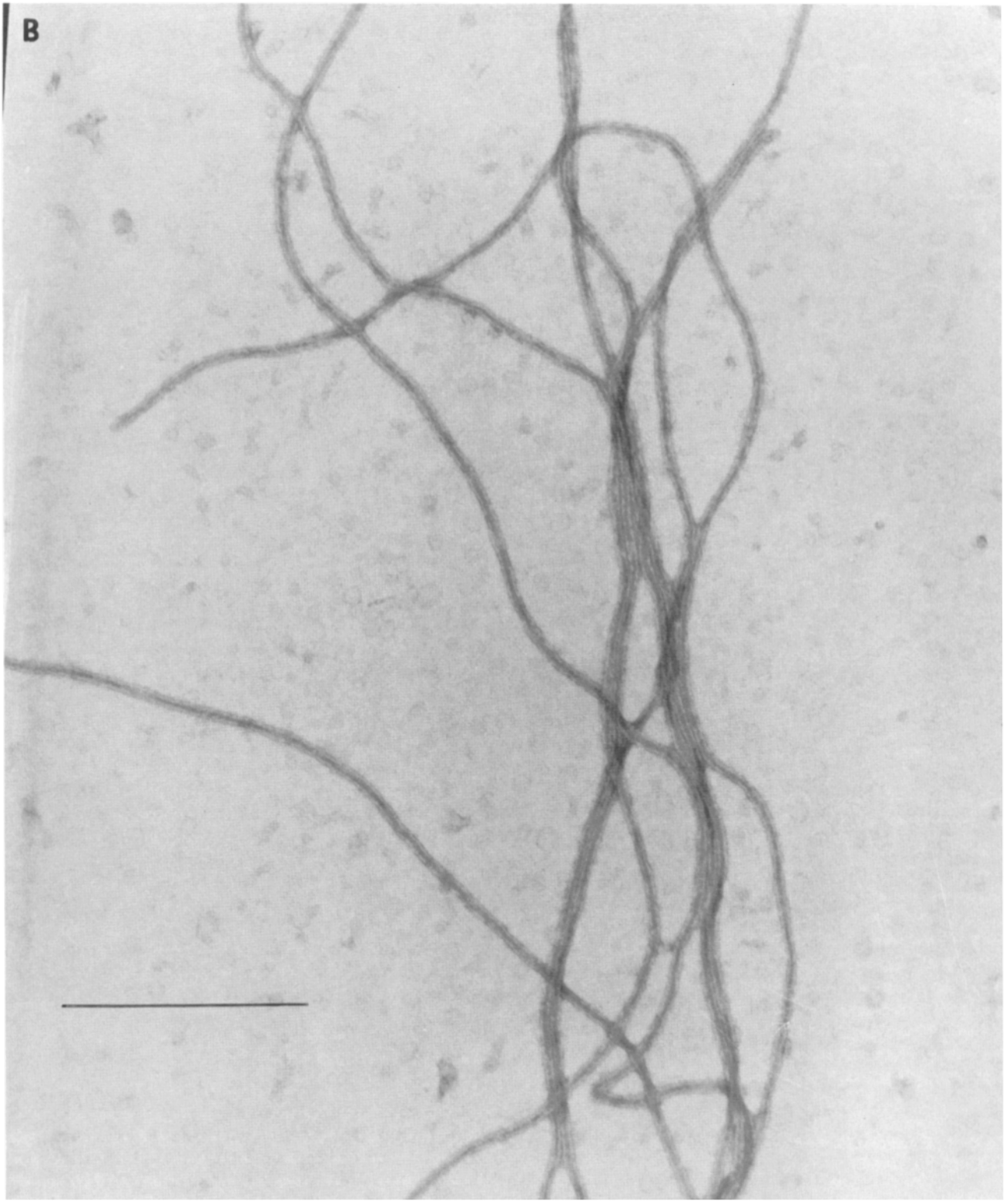


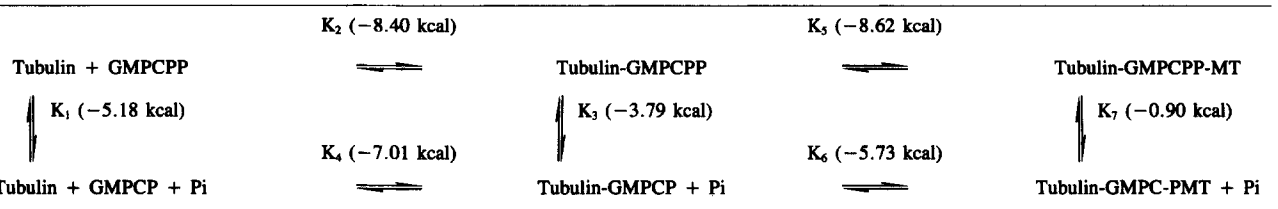
Figure 4.

GMPCP was determined by dividing the sum of the observed elongation and disassembly rates by the tubulin-GMPCP subunit concentration (73 μM, after correcting for the 21% occupancy of the E-site with GDP). The calculated second order-rate constants were 2×10^6 M^{-1} s^{-1} at one end and 1×10^6 M^{-1} s^{-1} at the other end.

Equilibrium for GMPCP and GMPCPP Binding to Tubulin Subunits

The binding affinity of GMPCPP and GMPCP to tubulin subunits was measured by their ability to displace GTP (Table V). The affinity is quite high, with K_d equal to 7.25 μM

Table IV. Equilibrium Constants for Reactions of GMPCPP and GMPCP with Tubulin



Parameter	K_{eq}	How determined
K_1	6.30×10^3	Assumed to be identical to AMPCPP*
K_2	1.45×10^6	See Table V
K_4	1.38×10^5	See Table V
K_3	3.50×10^2	$K_1 K_4 = K_2 K_3$
K_5	2.09×10^6‡	Rate constant assembly/rate constant disassembly§
K_6	1.58×10^4	See K_5‖
K_7	2.65	$K_5 K_7 = K_3 K_6$

* Milner-White and Rycroft, 1983. Since this value was determined by comparison with ATP, we use a free energy for ATP hydrolysis equal to −8.8 kcal/mol (Jencks, 1968), corrected for pH 6.8.
‡ This is equal to 5×10^7, using the rate constants derived by Hyman et al. (1992). The difference is mainly in the rate for subunit dissociation, which was found to be 0.1 s^{-1} for both ends, compared to 1.35 s^{-1} found here. A larger value for K_5 would make the free energy change for K_7 more positive, reinforcing the conclusions we have derived.
§ k(tubulin-GMPCPP subunit addition)/k (tubulin-GMPCPP subunit dissociation) = 1.36×10^6 $m^{-1}/0.65$ s^{-1}. The subunit dissociation rate was derived from the 1.3 s^{-1} measured for both ends, taking into account the fact that the rate must be equal at the two ends. This equality is required because the rate of addition of tubulin-GMPCPP subunits was the same at the two ends, and because K_5, which is equal to the ratio of the rate constants, must be identical at the two microtubule ends when there is no NTP hydrolysis in the reaction (Wegner, 1976).
‖ As with K_5, with rates equal to 2.0×10^6 M^{-1} s^{-1} and 128 s^{-1}, or 1.0×10^6 M^{-1} s^{-1} and 63 s^{-1}.

with GMPCP and 0.69 μM with GMPCPP (at 23°); this corresponds to a free energy for binding equal to −7.01 and −8.04 kcal, respectively.

Discussion

Regulation of Microtubule Dynamics by Controlled Hydrolysis of a GTP Analogue

Although the GTP-cap model for microtubule dynamic instability is generally accepted and even made its way into textbooks, there is conflicting evidence for the presence of tubulin-GTP subunits in microtubules (reviewed by Caplow, 1992). The most convincing evidence that GTP hydrolysis modulates microtubule dynamics has come from comparison of the behavior of microtubules containing nonhydrolyzable GTP analogues and GDP.

The GTP analogue GMPCPP, which is susceptible to hydrolysis because the methylene bridge links the alpha and beta phosphate residues, is unique in binding relatively tightly to the tubulin E-site and in being more effective than GTP in promoting assembly (Sandoval et al., 1977; Hyman et al., 1992).

Table V. Equilibrium Constant for GMPCPP and GMPCP Binding to Tubulin

Nucleotide	% cpm bound	K_d
		(μM)*
1 mM GMPCP	74.5-80.2	4.7, 6.5
2.5 mM GMPCP	66.7-71.0	8.0, 9.8
1 mM GMPCPP	29.6	0.67
2.5 mM GMPCPP	14.6-15.1	0.71, .69

* The equilibrium constant was determined from the relationship: eluted [Tubulin-GMPCPP/Tubulin-GTP] = K_{GTP} $(GMPCPP)_{free}/K_{GMPCPP}$ $(GTP)_{free}$, where K_{GTP} is equal to 2.2×10^{-8} (Zeeberg and Caplow, 1979).

It is of special interest that the rate of tubulin subunit dissociation from microtubules is dramatically increased when the gamma phosphate moiety of a bound NTP has been lost by hydrolysis. The more than 100-fold greater rate for dissociation of tubulin-GMPCP subunits compared to tubulin-GMPCPP subunits provides direct evidence that microtubule dynamics can be regulated by NTP hydrolysis. This conclusion was previously derived from considering the requirement for GTP for dynamic instability, as well as from comparison of the properties of microtubules assembled with nonhydrolyzable GTP analogues that were presumed to exactly mimic GTP, with the properties of GDP-microtubules.

Free Energy for Hydrolysis of Microtubule-bound GMPCPP

The equilibrium constant for hydrolysis of GMPCPP bound to the E-site of tubulin subunits and microtubules was determined from experimental measurements of five of the equilibrium constants in Scheme I (Table IV). The most striking result is the decrease in the free energy for GMPCPP hydrolysis from −5.18 kcal in solution, to −3.79 kcal in subunits, to −0.90 kcal in microtubules. The near zero free energy for hydrolysis of microtubule-bound GMPCPP is of singular interest.

It must be noted that the products for hydrolysis of microtubule-bound GMPCPP (K_7 in Table IV) are the binary complex of GMPCP and tubulin subunits in the microtubule, and free Pi at a 1 M standard state. To estimate the free energy change to form the ternary complex contain-

ing both GMPCP and Pi in the microtubule requires correcting the −0.9 kcal value for K_7 for the free energy expended for Pi dissociation at the 1 M standard state concentration; this depends on the K_d for Pi dissociation. Pi binding to GDP-subunits in microtubules is weak (Carlier, et al., 1988; Caplow et al., 1989; Melki et al., 1990; Stewart et al., 1990; Trinczek et al., 1993) and a K_d equal to 25 mM was previously estimated from the effect of Pi on the disassembly rate in glycerol buffer (Carlier, et al., 1988). Based on this value for K_d, the free energy expended for Pi dissociation at the 1 M standard state concentration is +2.18 kcal ($-RT\ln 1/K_d$). Therefore, the standard free energy for forming the GMPCP-Pi-microtubule ternary complex would be +1.28 kcal (i.e., 2.18 − 0.9), corresponding to an equilibrium constant equal to 0.114. For comparison, the free energy change is −1.3 kcal for conversion of the myosin-ATP to the myosin-ADP-Pi ternary complex (Bagshaw and Trentham, 1973).

It is emphasized that the observed free energy change for NTP hydrolysis under physiological conditions will be more negative than +2.18 kcal, since the position of the equilibrium depends on the Pi concentration. With Pi buffered in cells at 1 mM, the apparent free energy change for NTP hydrolysis in the microtubule would be 4.08 kcal more negative ($-RT\ln 0.001$) than the +1.28 kcal; ie., equal to −2.8 kcal.

The relatively small free energy change for NTP hydrolysis (even at physiological Pi concentrations) indicates that much of the free energy potentially available from this reaction is stored in the microtubule lattice, presumably as a repulsive force between subunits. This repulsive force is manifested in the enormous rate for dissociation of subunits containing GMPCP or GDP. The energy stored in the microtubule lattice can do work, by coupling disassembly to vesicle or chromosome movement (Koshland et al., 1988; Coue et al., 1991). Finally, it is noted that calculation of the free energy for hydrolysis of microtubule-bound GTP is not possible since the equilibrium constant for addition of tubulin-GDP subunits to the microtubule (K_6 in Table IV) is not available.

Glycerol and Glutamate-Induced Hydrolysis

Our observation that glycerol induces hydrolysis of microtubule bound GMPCPP in Na Pipes buffer means that glycerol binds to microtubules. Although this would appear to be a reasonable conclusion, since glycerol is used to stabilize microtubules (Solomon, et al., 1973), the effect of glycerol has primarily been taken to involve destabilization of tubulin subunits. That is, results from studies of the effect of glycerol on the apparent specific volume of tubulin (Na and Timasheff, 1981) were interpreted as proving that glycerol is preferentially excluded from the solvation sphere of tubulin dimers. This increase in the chemical potential of tubulin (and glycerol) is presumably relieved by a shift of tubulin from dimer to microtubule. According to this model, the dramatic reduction by glycerol of the rate of dilution-induced disassembly (Caplow et al., 1986; O'Brien and Erickson, 1989) results because glycerol destabilizes the transition state for disassembly, since this transition state resembles tubulin subunits. However, equally likely is the possibility that glycerol binds to and stabilizes microtubules.

As to the mechanism through which glycerol and glutamate accelerate hydrolysis of GMPCPP in microtubules, we propose that interaction of these compounds with the microtubule induces a protein conformation change. It is suggested GMPCPP is ordinarily stable because the different stereochemistry of the methylene linkage between the alpha and beta phosphate groups alters the position of the gamma phosphate, so it is remote from the residues responsible for catalysis. A protein conformation change may move the phosphate group to the active site for catalysis. The different rates of glycerol-induced hydrolysis with Na and K-Pipes is unaccounted for.

It is noted that GMPCP-microtubules formed by glycerol-induced hydrolysis in GMPCPP-microtubules did not have identical kinetic properties as microtubules formed with GMPCP. The disassembly rates at the two ends were 146 s^{-1} rate and 5 s^{-1} after glycerol treatment (Table III), compared to 128 s^{-1} and 63 s^{-1} with microtubules that had been assembled with tubulin-GMPCP. This difference may result from the ~17% of the GMPCPP that remained after treating GMPCPP-microtubules with glycerol (see discussion of results in Table I). We suggest that the slower rate at one end with glycerol-treated microtubules results because the residual sticky tubulin-GMPCPP subunits that escape hydrolysis slow down the rate. The effect of these tubulin-GMPCPP subunits may be different at the two ends because the unhydrolyzed nucleotide interfaces with the solution at the plus end and with the core of the microtubule at the minus end (Mitchison, 1993).

We are grateful to Harold Erickson for helpful comments on the manuscript and to Tim Mitchison, who used his tubulin and our reagents to repeat our study of the effect of glycerol on GMPCPP hydrolysis. These experiments allowed us to identify the role of sodium and potassium in hydrolysis of GMPCPP in microtubules. Don Cleveland's editorial assistance is greatly appreciated.

This work was supported by the National Institutes of Health (GM46773).

Received for publication 17 February 1994 and in revised form 25 July 1994.

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