The Colchicine-Induced GTPase Activity of Tubulin: State of the Product. Activation by Microtubule-Promoting Cosolvents†,‡

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ABSTRACT: Colchicine induces a weak assembly-independent GTPase activity in calf brain tubulin [David-Pfeuty, T., Erickson, H. P., & Pantaloni, D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5372–5376; Andreu, J. M., & Timasheff, S. N. (1981) *Arch. Biochem. Biophys. 211*, 151–157]. Kinetic analysis shows a turnover number of 2×10^{-4} s^{-1} in 0.01 M sodium phosphate and 4 mM $MgCl_2$, pH 7.0, with an apparent K_m for GTP of 10 μM. This activity, which requires Mg^{2+} ions and attains a plateau at 4 mM $MgCl_2$, is independent of pH over the pH range of 6.6–7.4. This GTPase activity was induced by all colchicine analogues that contain rings A and C (or C'), the strength varying in a manner parallel to the free energy of binding of the ligand. The specific GTPase activity was found to be independent of the tubulin–colchicine complex concentration over the range of 2–20 μM. Sedimentation velocity examination of the product of the reaction showed that GDP–tubulin–colchicine generated by hydrolysis of the E-site GTP was indistinguishable from that produced by nucleotide exchange at the site, the protein assuming the "curved" conformation in both cases. Steady-state kinetic analysis in the presence of high concentrations of microtubule-inducing cosolvent additives revealed an increase in k_{cat}/K_m of up to 1 order of magnitude that followed the order poly(ethylene glycol) 6000 (PEG-6000 > PEG-1000 = 2-methyl-2,4-pentanediol > sucrose > L-glutamate > glycerol = PEG-200 > betaine, with no apparent change in K_m. This enhancement of the GTPase activity was shown to be due neither to cosolvent-induced protein self-association nor to an effect of the additives on the solution viscosity that would affect substrate diffusion. Initiation of the GTPase reaction (in the presence of PEG-6000) by the addition of the slowly binding colchicine analogue allocolchicine or the rapidly binding 2,3,4-trimethoxy-4'-(carbomethoxy)-1,1'-biphenyl (TCB) resulted in similar lags in inorganic phosphate release. Since the rate of binding of allocolchicine and TCB to tubulin was found not to be affected by the cosolvent, this lag has been attributed to a slow step in the activation of the GTPase activity and the presence of an intermediate species in the GTPase pathway, not detected by spectroscopic techniques, whose concentration is modulated by the presence of cosolvent.

Tubulin is a dimeric protein with a molecular weight of 110 000 (Lee et al., 1973), composed of two similar, but not identical, chains designated α and β.[1] There are two guanine nucleotide binding sites on the tubulin molecule: one, the E-site, located in the β chain (Geahlen & Haley, 1977), is exchangeable with free nucleotides; the other, the N-site, is not (Weisenberg et al., 1968). When the E-site is occupied by GTP,[2] tubulin can assemble into microtubules, while tubulin–GDP cannot (Carlier & Pantaloni, 1978). Following incorporation into a microtubule, the GTP at the E-site is hydrolyzed to GDP and inorganic phosphate and the GDP remains tightly bound (Weisenberg, 1976; Zeeberg & Caplow, 1981; Carlier, 1982).

A number of drugs are known to interfere with microtubule function in cells by inhibiting microtubule polymerization. The alkaloid colchicine (COL), which arrests mitosis, binds slowly to its principal cellular receptor, tubulin, with a high apparent affinity ($\Delta G^\circ \simeq -10$ kcal mol^{-1}). Tubulin has one high-affinity binding site for colchicine (Taylor, 1965; Olmsted & Borisy, 1973; Garland, 1978; Andreu & Timasheff, 1982a; Diaz & Andreu, 1991). The kinetic pathway of the binding of colchicine to tubulin is complex. It follows a two-step mechanism that consists of a fast and reversible bimolecular binding reaction followed by a slow monomolecular reaction (Garland, 1978; Lambeir & Engelborghs, 1981). Probing of the mechanism of colchicine binding to tubulin with the single-ring analogues of the trimethoxyphenyl and tropolone methyl ether moieties of the colchicine molecule (Andreu & Timasheff, 1982a; Andreu et al., 1984, 1991; Medrano et al., 1991) (see Chart 1 for the structures of the studied COL analogues) have shown that these bind to tubulin reversibly, specifically, and noncooperatively, with small apparent standard free energy changes of –3 to –4 kcal mol^{-1} that account fully for the affinity of the whole drug for tubulin (Andreu & Timasheff, 1982a; Andreu et al., 1991; Timasheff et al., 1991). These observations have led to the description

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[1] The molecular weight of $\alpha\beta$ tubulin was taken as 110 000, which is in accordance with the value of 55 000 ± 1000 for the molecular weight of each tubulin subunit measured by five totally independent physicochemical methods (Lee et al., 1973). It is unclear why the value calculated from sequence data (Postingl et al., 1981; Valenzuela et al., 1981) is lower by 5000 M_r units.

[2] Abbreviations: ALLO, allocolchicine; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; GTP, guanosine triphosphate; GTPase, guanosine triphosphatase; GDP, guanosine diphosphate; HPLC, high-performance liquid chromatography; MPD, 2-methyl-2,4-pentanediol; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; PEG, poly(ethylene glycol); PMG buffer, 0.01 M sodium phosphate, 0.1 mM GTP, and 4 mM $MgCl_2$, pH 7.0; TCB, 2,3,4-trimethoxy-4'-(carbomethoxy)-1,1'-biphenyl; TKB 2,3,4-trimethoxy-4'-acetyl-1,1'-biphenyl; TMB, 2,3,4,4'-tetramethoxy-1,1'-biphenyl.

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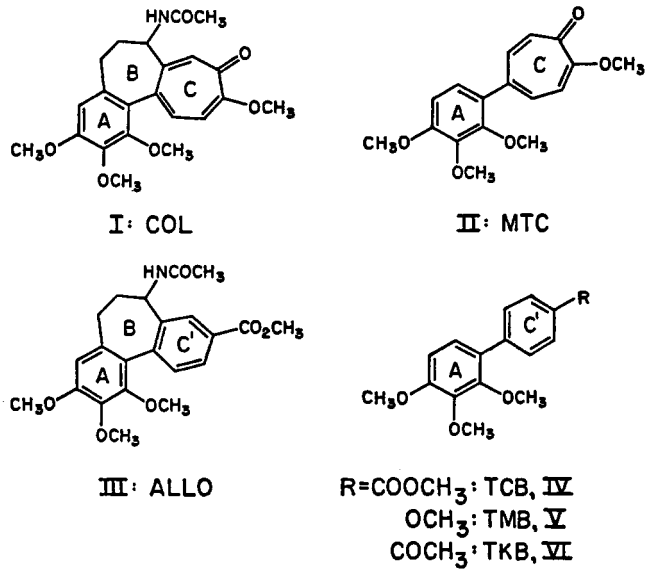
Chart 1



of the high-affinity binding of colchicine to tubulin in terms of a simple thermodynamic model, that of the non-cooperative binding of a bifunctional ligand to two independent subsites on the protein binding site (Andreu & Timasheff, 1982b). According to this model, the colchicine tropolone methyl ether ring binds by means of ring stacking and possibly hydrogen bonding (Lambeir & Engelborghs, 1981; Andreu & Timasheff, 1982a,b; Hastie, 1989; Medrano et al., 1991), while the trimethoxyphenyl ring interacts hydrophobically with its subsite (Andreu & Timasheff, 1982a,b). The tubulin–colchicine complex differs from the unliganded protein in several properties: (i) it inhibits the formation of microtubules at substoichiometric concentrations of the drug (Margolis & Wilson, 1977; Sternlicht & Ringel, 1979; Margolis et al., 1980; Lambeir & Engelborghs, 1980; Skoufias & Wilson, 1992); (ii) it induces in tubulin an assembly-independent GTPase activity directed at the E-site-bound nucleotide (David-Pfeuty et al., 1977, 1979; Heusele & Carlier, 1981); (iii) it directs tubulin self-assembly into structures other than microtubules, but with the thermodynamic characteristics of microtubule growth (Saltarelli & Pantaloni, 1982; Andreu et al., 1983); (iv) it displays a small perturbation of the tubulin far-UV circular dichroism spectrum (Andreu & Timasheff, 1982a); (v) it exhibits a stronger interaction between the α and β subunits of the noncovalent tubulin dimer (Detrich et al., 1982; Shearwin & Timasheff, 1994). All of this suggests that the drug–protein complex exists in a different conformational state from that of the unliganded protein (Andreu & Timasheff, 1982a,b; Andreu et al., 1983, 1984).

The colchicine-induced GTPase activity was first reported by David-Pfeuty et al. (1977). In its further characterization using highly purified tubulin (Andreu & Timasheff, 1981; Monasterio & Timasheff, 1987) it was found that this activity is also induced by several simple biphenyl colchicine analogues (for structures, see Chart 1) (Andreu et al., 1984, 1991). The GTPase activity requires binding of the analogues to both the ring A and ring C subsites (Chart 1), since podophyllotoxin, which binds to the ring A locus, does not induce this activity (Andreu et al., 1991). The observation by Andreu et al. (1983) that 3.4 M glycerol did not enhance the polymerization of tubulin–colchicine, while it did the formation of microtubules, along with the observation that glycerol increased 4-fold the tubulin–colchicine GTPase activity, led to the conclusion that the rate of transformation of GTP–tubulin–colchicine into the nonassembling GDP species overhelmed the assembly-enhancing activity of glycerol. We decided, therefore, to examine the mechanism of the glycerol enhancement of the GTPase activity of tubulin–colchicine. Glycerol is known to stabilize tubulin (Shelanski et al., 1973; Na & Timasheff, 1981), as well as a number of other proteins, against thermal denaturation (Gekko & Timasheff, 1981a,b) and to induce the self-assembly of tubulin (Lee & Timasheff, 1975) and actin (Kasai et al., 1965). As a consequence, a number of other cosolvents having similar properties were also examined. Specifically, the cosolvents selected for this study were those that are known to stabilize tubulin or to promote microtubule assembly *in vitro*. These include sucrose (Frigon & Lee, 1972; Lee et al., 1975), glycerol (Shelanski et al., 1973; Lee & Timasheff, 1975), DMSO (Himes et al., 1976, 1977), PEG (Herzog & Weber, 1978; Lee & Lee, 1979), MPD (Seybold et al., 1975), and sodium glutamate (Hamel & Lin, 1981). It should be noted that some of these compounds have been shown to increase the GTPase activity of the translation initiation factor IF2 (Severini et al., 1991) and the elongation factor Tu (Harmark et al., 1992). A detailed study of the effect of these cosolvents on the tubulin–colchicine GTPase activity was carried out, and the results are reported here and in the following paper (Perez-Ramirez and Timasheff, 1994).

EXPERIMENTAL PROCEDURES

Materials. GTP and GDP were purchased from Sigma; γ-^{32}P-labeled GTP (25 Ci/mmol, 10 mCi/mL) was obtained from ICN Radiochemicals (Costa Mesa, CA); glycerol and MPD were from Aldrich Chemical Co.; and poly(ethylene glycol) was from Baker. Ultrapure ammonium sulfate and sucrose were obtained from ICN/Schwarz-Mann; guanidine hydrochloride, from United States Biochemicals (USB); and colchicine, from Aldrich. Allocolchicine (ALLO) and TCB were synthesized as described elsewhere (Medrano et al., 1989). TKB and TMB were synthesized by the procedure of Gorbunoff (Medrano et al., 1991). MTC was kindly provided by Dr. T. J. Fitzgerald (Florida State Univ., Tallahassee, FL). Other chemicals were reagent grade.

Preparation of Tubulin. Tubulin was prepared from fresh calf brains (1 h maximum after slaughter) by a modified Weisenberg procedure, as described previously (Weisenberg et al., 1968; Na & Timasheff, 1980). Protein aliquots (20 mg, 300–400 μM) were stored in liquid nitrogen in a buffer consisting of 0.01 M sodium phosphate, 0.1 mM GTP, 0.5 mM $MgCl_2$, and 1 M sucrose, pH 7.0. Prior to each experiment, samples of tubulin were thawed at 20 °C and the bulk of the sucrose was removed from the tubulin solution by a Sephadex G-25 dry column procedure (Na & Timasheff, 1980). The resulting protein solution was cleared of aggregates by centrifugation at 35000*g* for 30 min. The final equilibrium of the protein with the desired buffer was by gel filtration on a Sephadex G-25 column (Na & Timasheff, 1982). Tubulin concentrations were determined spectrophotometrically at 275 nm after dilution in 6 M guanidine hydrochloride, using extinction coefficients of 1.03 and 1.16 mg^{-1} cm^{-1} for tubulin and the tubulin–colchicine complex, respectively (Andreu & Timasheff, 1982a).

Ligand-Induced GTPase Activity. The ligand-induced GTPase activity of calf brain tubulin was assayed by a modification of the procedure described elsewhere (Andreu & Timasheff, 1981; Andreu et al., 1991). In brief, tubulin samples were subjected to Sephacryl S-300 chromatography (0.9 × 25 cm column) in 0.01 M sodium phosphate buffer, pH 7.0, in order to eliminate a ligand-independent GTPase activity (Andreu & Timasheff, 1981). This activity without purification was 13–15 × 10^{-3} mol of GTP hydrolyzed (mol

of tubulin)$^{-1}$ min^{-1}. After purification, in the absence of colchicine, the residual activity was 4×10^{-4} mol of GTP hydrolyzed (mol of tubulin)$^{-1}$ min^{-1}. Protein samples (1–10 μM) and blanks were preincubated with a 20-fold molar excess of the appropriate colchicine family ligand[3] (20 min at 20 °C). Then 2.22×10^6 dpm [γ-^{32}P]GTP and GTP were added to a fixed final substrate concentration of 0.1 mM. After addition of the cosolvent, the reaction was started by addition of $MgCl_2$ to a final concentration of 4 mM in a total reaction volume of 100 μL. The tubes were placed in a 37 °C water bath, and the reactions were stopped at selected times using the activated charcoal procedure (Nieto et al., 1975). Two hundred microliters of a charcoal suspension (100 mg/mL in 0.2 M KCl, pH 1.8) was added to 100 μL of assay mix, and the tube was kept on ice for 10 min. Charcoal was eliminated by a 2-min centrifugation in a Fisher tabletop centrifuge at 4 °C. Fifty microliters of the supernatant was taken to determine radioactivity, using 5 mL of scintillation cocktail (BCS, Amersham). The charcoal procedure was chosen for the determination of inorganic phosphate hydrolysis because some of the cosolvents interfered with the phosphomolybdate extraction procedure (Nishizuka, 1968).

Kinetics Measurements. The kinetics were examined by measuring initial velocities. Phosphate release was found to be proportional to tubulin concentration and to incubation time up to 30 min, as reported earlier (Andreu & Timasheff, 1981). Determination of the kinetic constants (k_{cat}, K_m) was carried out using a 1 μM final concentration of tubulin in the assay mix. All experiments were performed at 37 °C, unless indicated otherwise. Kinetic parameters (V_{max}, K_m) were derived from nonlinear least-squares fitting of the experimental data to the Michaelis–Menten equation using either the Enzfitter (Leatherbarrow, 1987) or Sigmaplot 5.0 computer program (Jandel Scientific, Corte Madera, CA). k_{cat} was derived from the relationship $V_{max} = k_{cat}[E]$, where [E] was set equal to the final tubulin concentration. Turnover numbers were calculated assuming a single catalytic site per tubulin heterodimer, M_r 110 000 (Lee et al., 1973).

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed using a Beckman Model E analytical ultracentrifuge fitted with an RTIC temperature control and a photoelectric scanner. Runs were carried out in an An F rotor using 12-mm Epon-filled aluminum double-sector centerpieces and quartz windows. Short columns (50 μL of sample, equivalent to a 1-mm column) and overspeeding were employed in order to minimize tubulin degradation during the course of the experiments (total run time was approximately 3 h) (Sackett & Lippoldt, 1991; Shearwin et al., 1994). Three cells, each containing a different initial concentration of protein, were used in each run. The concentration gradient in the cells was determined by UV absorbance at 276 nm using the photoelectric scanner and the standard Beckman multiplexer to separate the signals. The imput voltage from the scanner was digitized and saved to a spreadsheet file using a Data Translation (Marlboro, MA) data acquisition board and custom-written software. During scanning, the photomultiplier carriage was set on the slow speed, giving approximately 4500 points across the entire cell and 250 points across the 1-mm solution column. Sedimentation data were analyzed by direct fitting of the concentration vs radial distance results to the basic sedimentation equation (Shire et al., 1991). Several association schemes were tested in order to obtain the best fit. Partial specific volumes of 0.750 and 0.736 mL/mg were used for tubulin in the presence and absence of glycerol, respectively (Na & Timasheff, 1981).

Sedimentation velocity experiments were performed at 48 000 rpm and 20 °C in 12-mm cells using an An D rotor. Tubulin–colchicine–GTP (48 μM) in 0.01 M sodium phosphate, 12 mM $MgCl_2$, and 0.5 mM GTP, pH 7.0, was incubated at a given temperature (4, 30, or 37 °C), and aliquots were removed periodically for determination of the weight average sedimentation coefficient. The schlieren patterns were recorded on Kodak OMC diagnostic film. Weight average sedimentation coefficients, reduced to standard conditions, $\bar{s}_{20,w}$, were calculated from the rate of movement of the square root of the second moment of the boundary (Goldberg, 1953) and corrected to water at 20 °C.

Fluorescence Spectroscopy. Fluorescence measurements were made on a Hitachi Perkin-Elmer 650-40 spectrofluorometer operating in the ratio mode. The excitation bandwidth was 2 nm, and the emission bandwidth was 5 nm. The kinetics of binding were measured with excitation at 315 nm and emission at 390 and 373 nm for ALLO and TCB, respectively (Medrano et al., 1989). For colchicine, the excitation and emission wavelengths were 357 and 435 nm (Andreu & Timasheff, 1982a). Narrow-path-length cells (10 × 2 mm) were used, with the shorter path length oriented in the excitation beam in order to minimize the inner filter effect.

Viscosity Measurements. Viscosity was determined at 37 °C with an Ostwald capillary viscometer (80–100 s; Fisher Scientific Instruments) immersed in a thermoregulated water bath, using a sample volume of 4 mL. The flow time was 43 s for water and 44 s for PMG buffer. The specific viscosity, defined as $\eta_{sp} = (\eta/\eta_0) - 1$, where η_0 is the viscosity of the PMG buffer and η is the viscosity of the PMG buffer that contained the cosolvent, was calculated from

$$\eta_{sp} = \frac{\text{(flow time of solvent solution)}}{\text{(flow time of PMG buffer)}} - 1 \qquad (1)$$

Other Determinations. Solution density measurements were determined using an Anton-Paar Model DMA-02 precision density meter based on the oscillating frequency of a tuning cell. The total concentrations of guanine nucleotides in the stock solutions were measured by UV absorption using molar extinction coefficients of 12 350 M^{-1} cm^{-1} at pH 1.0 and 13 700 M^{-1} cm^{-1} at neutral pH (Monasterio & Timasheff, 1987). HPLC of nucleotides was carried out as described by Seckler et al. (1990).

RESULTS

Induction of GTPase Activity by Colchicine and Analogues. The effect of solution variables on the enzymatic activity and the structural state of the tubulin–colchicine complex was examined using a variety of techniques. The Mg^{2+} ion concentration dependence of the GTPase activity, shown in Figure 1A, indicates a strong increase which reaches a plateau at 4 mM $MgCl_2$, where the initial velocity attains a rate of 9.6×10^{-3} mol of GTP hydrolyzed (mol of tubulin)$^{-1}$ min^{-1} in 0.01 M sodium phosphate and 0.1 mM GTP at 4 mM $MgCl_2$, pH 7.0 (PMG buffer). Higher concentrations of the cation, which are known to induce tubulin to associate into higher order structures (Frigon & Timasheff, 1975a,b; Lee & Timasheff, 1977), did not increase significantly the enzymatic activity. In Mg^{2+}-free buffer that contained 1 mM EDTA, the tubulin–colchicine complex showed some GTPase activity, 10–15% of the maximum. The specific activity was independent of protein concentration from 2 to 20 μM, as the

[3] As an alternative procedure, the tubulin–colchicine complex was prepared as described by Andreu and Timasheff (1982a). This procedure gave results similar to those obtained when a large excess of colchicine was present in the reaction medium.

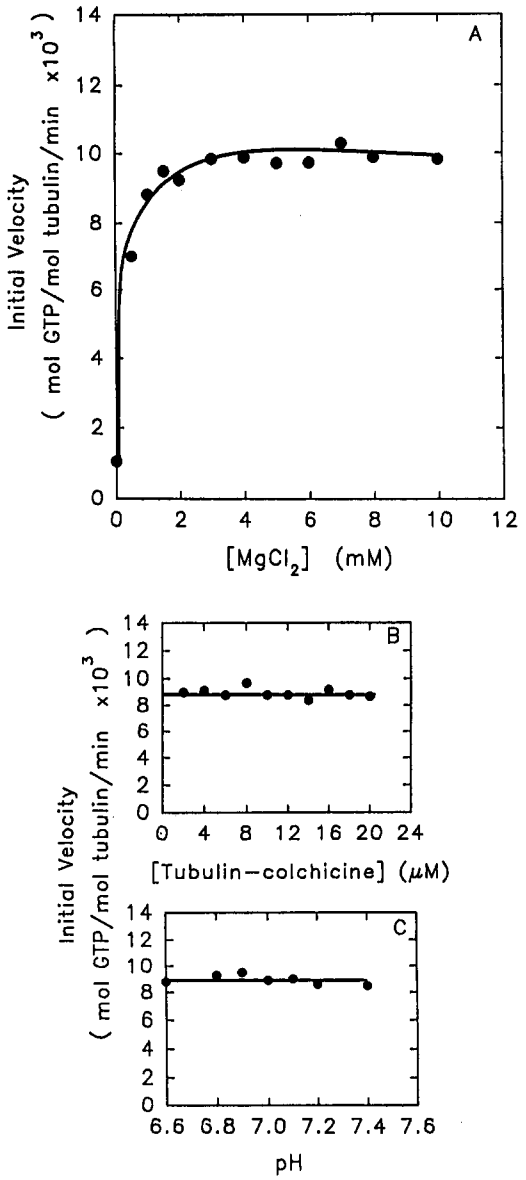


FIGURE 1: (A) Effect of $MgCl_2$ on the colchicine-induced GTPase activity of calf brain tubulin. Tubulin (4 μM) and 1 μCi of [γ-^{32}P]-GTP in PMG buffer were preincubated with a 20-fold molar excess of colchicine at 20 °C for 20 min. $MgCl_2$ was added from a stock solution to give a final volume of 100 μL. Reactions were initiated by placing the samples at 37 °C, and the incubation was allowed to proceed for 10 min. Hydrolysis was stopped by addition of 200 μL of acidified charcoal suspension, and the amount of inorganic phosphate was determined as described under Experimental Procedures. (B) Effect of the tubulin–colchicine complex concentration on the initial rate of formation of inorganic phosphate at 4 mM $MgCl_2$. The tubulin–colchicine complex was prepared as described by Andreu and Timasheff (1982a) and purified further by Sephacryl S-300 chromatography (see Experimental Procedures). (C) Effect of pH on the colchicine-induced GTPase activity. Tubulin (5 μM) was preincubated in a 20-fold molar excess of colchicine. The GTPase activity was measured in 0.01 M sodium phosphate and 4 mM $MgCl_2$ at the pH values indicated.

initial velocity remained constant at 9.6×10^{-3} mol of GTP hydrolyzed (mol of tubulin)$^{-1}$ min^{-1} (Figure 1B). The GTPase activity was also independent of pH in the zone from 6.6 to 7.4 (Figure 1C).

Enzymatic activity measurements were also performed in the presence of several structural analogues of colchicine (Chart 1), and the results are summarized in Table 1. The values of k_{cat}/K_m were smaller for all of the analogues than the one obtained in the presence of colchicine, in agreement with the initial velocity measurements of Andreu et al. (1991). As shown in Table 1, the order of GTPase activation was

Table 1: Kinetic Parameters for the Tubulin GTPase Activity Induced by Colchicine and Analogues[a]

ligand	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
colchicine	$2.0 \times 10^{-4} \pm 4.6 \times 10^{-6}$	9 ± 2.0	2.22×10^1
ALLO	$1.33 \times 10^{-4} \pm 4.5 \times 10^{-6}$	8 ± 0.6	1.66×10^1
MTC	$1.75 \times 10^{-4} \pm 1.8 \times 10^{-5}$	12 ± 3.0	1.46×10^1
TCB	$1.26 \times 10^{-4} \pm 1.6 \times 10^{-5}$	10 ± 1.7	1.26×10^1
TKB	$1.25 \times 10^{-4} \pm 1.3 \times 10^{-5}$	11 ± 2.1	1.14×10^1
TMB	$(9.5 \pm 1.0) \times 10^{-5}$	15 ± 2.4	0.63×10^1

[a] The ligand concentration in the assay was 100 μM for all the analogues. The reactions were carried out in 10 mM sodium phosphate buffer and 4 mM $MgCl_2$, pH 7.0. Tubulin, 10 μM, was used for all the determinations. The concentration of GTP was varied from $0.2K_m$ to $2.0K_m$. Further details are given under Experimental Procedures.

colchicine > ALLO > MTC > TCB > TKB > TMB. The K_m values for GTP, however, were very similar to those obtained with tubulin–colchicine (K_m = 10 μM), with the possible exception of TMB, which was slightly higher. The progression of the k_{cat}/K_m values, 75% (ALLO), 66% (MTC), 57% (TCB), 51% (TKB), and 28% (TMB) of that found for the colchicine-induced GTPase activity, follows roughly the variation of the standard free energy changes of ligand binding, which are –8.6 kcal mol^{-1} for ALLO, –7.14 kcal mol^{-1} for TCB (Medrano et al., 1989; Menendez et al., 1989), –7.8 kcal mol^{-1} for MTC (Andreu et al., 1984), –7.2 kcal mol^{-1} for TKB, –6.76 kcal mol^{-1} for TMB (Medrano et al., 1991), and –10.0 kcal mol^{-1} for colchicine (Diaz & Andreu, 1991).

Nature of the GTPase Reaction Product. To test whether the GDP–tubulin–colchicine complex formed as the product of the GTP hydrolysis was conformationally the same as that formed on replacement of the E-site GTP by GDP, the GTPase reaction was followed by sedimentation velocity. Figure 2 shows the progression of the $\bar{s}_{20,w}$ value as a function of the degree of conversion of GTP to GDP. At time zero, $\bar{s}_{20,w}$ of a 48 μM solution of the protein–drug complex was 8.1 S. This is identical with the value obtained for tubulin–GTP at the same concentration and under the same conditions (Frigon & Timasheff, 1975a). When the protein was incubated at 4 °C, the sedimentation coefficient remained unchanged over a period of 48 h, consistent with the absence of any GTPase activity at this temperature and the known structural stability of the tubulin–colchicine complex over this time period (Prakash & Timasheff, 1992). When the incubation was carried out at 37 °C, however, the sedimentation coefficient increased, reaching after 12 h a maximum value of 29.5 S which did not change further after this time. This is the value expected for tubulin–GDP under identical conditions (Howard & Timasheff 1986), and it reflects the strong propensity of tubulin–GDP to form double rings in the presence of magnesium ions. Addition of a large excess of GTP at this time lowered $\bar{s}_{20,w}$ to its initial value (8.1 S), which indicates the reversibilities both of the self-association and of the nucleotide binding. Figure 3A shows the change in the profile of the schlieren patterns as a function of the length of incubation at 37 °C. At a fixed protein concentration (48 μM), bimodality gradually developed and the area under the fast peak increased with time, while that under the slow peak diminished. Analysis of the nucleotide contents of the 37 °C samples by HPLC indicated a progressive decrease with time of the GTP contents of the samples and a corresponding increase in the GDP contents, shown in the inset of Figure 2. Incubation at 30 °C produced a lower rate of increase in the sedimentation coefficient (Figure 2) and a slower evolution of the bimodal pattern (Figure 3B), reflecting a slower rate of GTP hydrolysis. Control experiments, in which tubulin–colchicine–GTP and tubulin–colchicine–GDP prepared by nucleotide exchange were mixed in various ratios, generated

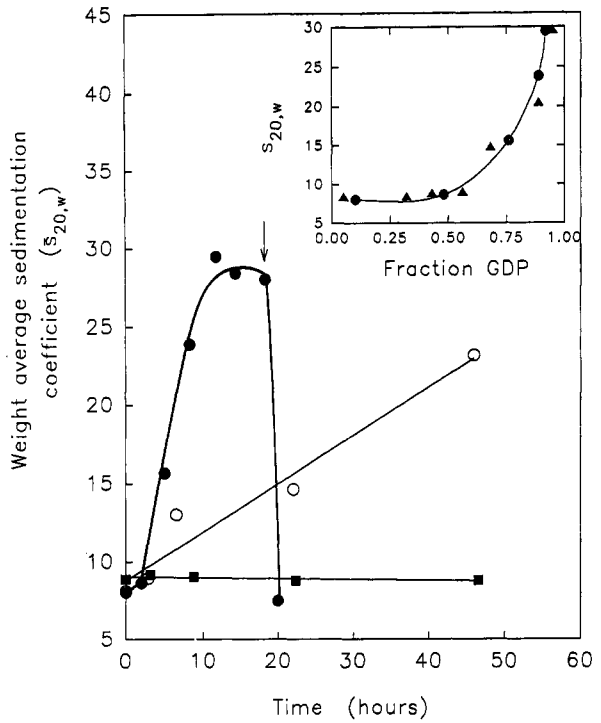


FIGURE 2: Evolution of the formation of tubulin–colchicine–GDP from tubulin–colchicine–GTP upon incubation at 4 (■), 30 (○), and 37 °C (●), as monitored by sedimentation velocity. Tubulin–colchicine–GTP (48 μM in 0.01 M sodium phosphate, 12 mM $MgCl_2$, and 0.5 mM GTP, pH 7.0) was incubated at the indicated temperatures. At given times, aliquots were removed and examined in the analytical ultracentrifuge (48 000 rpm, 20 °C) to evaluate the weight average sedimentation coefficient. At the point indicated by the arrow, a large excess of GTP was added (10 mM final concentration) to an aliquot of the 37 °C sample; this sample was incubated for 30 min (4 °C), and the sedimentation coefficient was determined. The inset shows the weight average sedimentation coefficient as a function of the fraction of GDP in solution [GDP/(GDP + GTP)] for artificial mixtures (▲) and samples incubated at 37 °C (●).

schlieren profiles indistinguishable from those produced by GTP → GDP transformation *in situ* by the GTPase activity of tubulin–colchicine. For example, in Figure 3, pattern C (b) is essentially indistinguishable from pattern A (c); both have GTP/GDP ratios of 1:4. In addition, at identical nucleotide compositions, the weight average sedimentation coefficients for the artificial mixtures were the same as those of protein–nucleotide complexes obtained via GTP hydrolysis (Figure 2, inset).

Enhancement of Activity by Cosolvent Additives. The effect of the structure-stabilizing or microtubule-enhancing cosolvents on the colchicine-induced GTPase activity of tubulin was examined using a tubulin–colchicine complex concentration below the critical concentration needed for polymerization.[4] The results are shown in Figure 4. All of the solvents analyzed were found to increase the rate of GTP hydrolysis, except DMSO, for which the initial rate remained constant at 9.6×10^{-3} mol of GTP hydrolyzed (mol of tubulin)$^{-1}$ min^{-1}; 3.4 M glycerol was found to enhance the initial rate 4-fold as reported previously (Andreu et al., 1983), as did sucrose, while PEG-6000 gave a 10-fold increase. Similarly, the GTPase activities induced by the colchicine analogues ALLO and TCB

[4] In our hands tubulin prepared according to the modified Weisenberg procedure (Weisenberg et al., 1968; Na & Timasheff, 1980) has a critical concentration for assembly of 0.9–1.0 mg/mL in 0.01 M sodium phosphate, 16 mM $MgCl_2$, 1 mM GTP, and 3.4 M glycerol. This value will be higher (2.6 mg/mL) in the present buffer, which contains a lower magnesium concentration (4 mM).

Table 2: Effect of Cosolvents on the Kinetic Parameters for the Colchicine-Induced GTPase Activity of Tubulin

solvent	concn[a]	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})	η_{sp}[b]
PM[c]		2.0×10^{-4}	9 ± 2	2.22×10^{1}	0.03
PEG-6000	1%, w/v	1.8×10^{-3}	12 ± 3	1.63×10^{2}	0.18
PEG-1000	2%, w/v	1.5×10^{-3}	11 ± 3	1.36×10^{2}	0.16
MPD	1 M	1.4×10^{-3}	11 ± 3	1.27×10^{2}	0.52
sucrose	1 M	1.1×10^{-3}	9 ± 3	1.22×10^{2}	1.63
PEG-200	6%, w/v	8.3×10^{-4}	8 ± 2	1.03×10^{2}	0.23
glycerol	3.4 M	8.5×10^{-4}	10 ± 4	8.5×10^{1}	1.20
L-glutamate	1.5 M	9.3×10^{-4}	11 ± 2	8.5×10^{1}	1.09
betaine	2 M	6.2×10^{-4}	11 ± 3	5.6×10^{1}	0.61
DMSO	10%, w/v	2.3×10^{-4}	12 ± 2	1.94×10^{1}	0.22

[a] Concentration of the cosolvent at which maximal activation was observed in the initial velocity experiments. [b] Specific viscosity. [c] PM buffer consists of 0.01 M sodium phosphate and 4 mM $MgCl_2$, pH 7.0. The concentration of GTP was varied from $0.2K_m$ to $2.0K_m$.

were also enhanced 4-fold by 3.4 M glycerol, while there was no change in activity in the presence of DMSO (not shown). For all of the cosolvents, high concentrations were necessary to produce the enhancement of the rate of hydrolysis (1 M or above for sucrose, MPD, sodium glutamate, and glycerol). The kinetic parameters (K_m, k_{cat}) of the ligand-induced GTPase activity of tubulin, determined as described under Experimental Procedures, are listed in Table 2 for the various cosolvent systems. These measurements were carried out at cosolvent concentrations at which the activation was maximal. As seen from Table 2, for all of the cosolvents analyzed, k_{cat} increased significantly (except for DMSO), while the K_m values remained unchanged within experimental error at about 10 μM. The apparent second-order rate constant (k_{cat}/K_m) increased with solvents in the order buffer ≤ DMSO < betaine < glycerol = L-glutamate < PEG-200[5] < sucrose < MPD < PEG-1000 < PEG-6000. It is interesting to note from Figure 4 that glycerol is needed at a molar concentration 4 times greater than that of sucrose to give the same enhancement. This is true also of the glycerol/sucrose concentration ratios needed to induce microtubule assembly (Lee & Timasheff, 1977). On a weight scale, however, 1 M sucrose is equivalent to 4 M glycerol. In the case of the PEGs, the effectiveness increases with an increase in the molecular weight of the polymer, even though, at identical mass concentrations, the molar concentrations of $-CH_2-CH_2-O-$ groups are identical.

These results suggest that the transformation of the tubulin–colchicine complex required to activate the hydrolytic activity is promoted by a common change in the protein environment produced by these different cosolvents. A common property of all of these cosolvents is their preferential exclusion from the domain of proteins (Timasheff, 1993). What is the factor that increases the rate in the presence of the cosolvents? It could be an effect on the self-association of the protein, on substrate diffusion modulated by the viscosity of the medium, or on the active site chemistry. These were examined in turn.

The possibility that the cosolvents induced self-association of the protein which affected the GTPase activity was examined by sedimentation equilibrium, even though in the presence of 3.4 M glycerol no increase in specific activity was found with an increase in protein concentration. The results of experiments in the presence and absence of glycerol are shown in Figure 5. These were fitted by nonlinear least squares to several association schemes. In the absence of glycerol, the best fit (as judged by the root mean square deviation of residuals) was obtained for a monomer–dimer association (monomer M_r = 110 000), with an association constant (K_a) of 8×10^3 M^{-1}. In the presence of 3.4 M glycerol, the best

[5] The numbers indicate the average molecular weight of PEG.

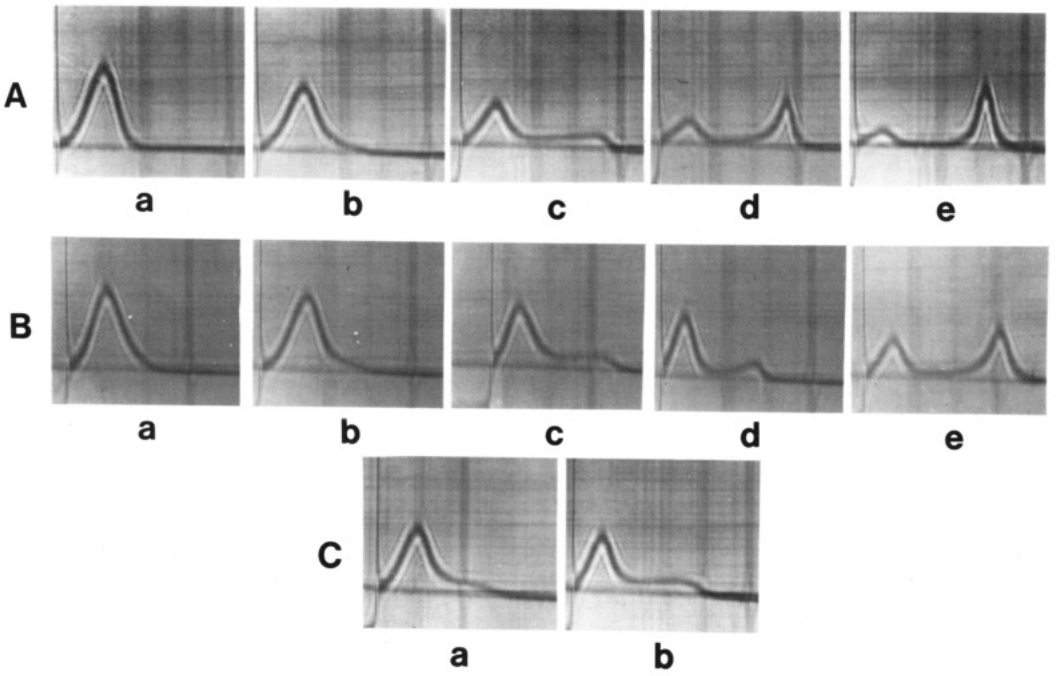


FIGURE 3: Schlieren profiles obtained on monitoring the GTPase activity of the tubulin–colchicine complex by sedimentation velocity. Protein concentrations and buffer conditions are the same as those described in the caption of Figure 2. The times at which the photographs were taken after the centrifuge came up to speed are indicated in parentheses. (A) Protein was incubated at 37 °C, and samples were run (a) 0 (5 min), (b) 2 h (5 min), (c) 5 h (5 min), (d) 8.25 h (2 min), and (e) 11.8 h (2 min) after the start of incubation. (B) Incubation was at 30 °C, and samples were run (a) 0 (5 min) (b) 2.9 h (5 min) (c) 6.5 h (2 min), (d) 22.1 h (2 min), and (e) 45.9 h (2 min) after the start of incubation. (C) Schlieren patterns obtained when tubulin–colchicine–GTP and tubulin–colchicine–GDP were prepared by nucleotide exchange and mixed in the given proportions. In panel C, profile a (5 min) has approximately the same GTP/GDP ratio as profile b in panel A, while profile b in panel C (5 min) has the same ratio as profile c in panel A, as determined by HPLC. In all cases, photographs were taken at a bar angle of 65°. Sedimentation is from left to right.

fit was to a single species of molecular weight 110 000. These results are consistent with measurements of the equilibrium association constant for the interaction of the α and β subunits of tubulin carried out in the absence of Mg^{2+}, in which 3 M glycerol decreased slightly the strength of association (unpublished observation). The inset of Figure 5 shows the variation of the molecular weight as a function of protein concentration. Within the concentration range employed in the kinetic studies (0.2–1 μM), the weight average molecular weights are very similar. For example, at 1 μM tubulin, the species distributions are 100% of 110 000 in the presence of glycerol and 98% of 110 000 and 2% of 220 000 in the absence of glycerol. When sedimentation velocity experiments were carried out with tubulin in 0.01 M sodium phosphate, 0.1 mM GTP, and 1 M MPD, pH 7.0, a high proportion of irreversible fast sedimenting species (12 S) was observed (data not shown), similar to the results obtained during the vinca alkaloid induced aging of tubulin (Prakash & Timasheff, 1992). Addition of 4 mM $MgCl_2$ to the above buffer induced a faster aggregation of the protein. This process was completely prevented by colchicine, where the protein sedimented under a single 6.9-S peak in 0.01 M sodium phosphate, 0.1 mM GTP, 4 mM $MgCl_2$, and 1 M MPD buffer, pH 7.0 (not shown). Hence, cosolvent-induced self-association of tubulin is not the cause of the enhancement of the colchicine-induced GTPase activity.

One feature common to all of the additives employed is their tendency to increase the viscosity of water. Hence, the viscosities of the cosolvent mixtures were measured at the same solvent compositions as those used in the kinetic studies. The results reported in Table 2 as specific viscosity (η_{sp}) did not show any correlation with the effect of the cosolvent additives on k_{cat}/K_m. A clear example is given by the comparison of the effects of 10%, w/v, DMSO and 6%, w/v, PEG-200, which have similar specific viscosities. Yet, DMSO had no effect on the rate of GTP hydrolysis, while PEG-200 induced a 5-fold increase in k_{cat}/K_m. Therefore, the cosolvents did not exercise their effect through a diffusional constraint of the reaction.

To probe possible effects on drug binding and active site chemistry, the enzymatic activity was examined at early times of the reaction. Tubulin (4 μM) in PMG buffer was preincubated at 37 °C in the presence or absence of 1%, w/v, PEG-6000. The reaction was started by addition of colchicine, ALLO, or TCB. As shown in Figure 6A, similar lags in the release of inorganic phosphate were observed when the reaction, carried out in the presence of PEG-6000, was started by addition of either ALLO or TCB. The magnitude of these lags could not be quantitated due to uncertainty in the measurements at these short time intervals (the time scale for the experiments was 15 s), while the slowness of the reaction precluded the drawing of any conclusions on log when the cosolvent was absent or when the drug was colchicine. Such a lag could be caused by either a slowing down of the binding of the drugs (in dilute buffer the binding of ALLO is slow, while that of TCB is fast) or a direct effect on the active site chemistry. Therefore, the effects of the cosolvent on the kinetics of COL, ALLO, and TCB binding to tubulin were monitored fluorometrically at 37 °C. As shown in Figure 6B, the rates of binding of all these drugs were not affected by the cosolvent, the curves in its presence and absence being identical within experimental error: the binding of TCB was fast, with the emission intensity reaching a maximum in less than 10 s, and that of ALLO was complete in 4 min, while that of colchicine was much slower, in agreement with the findings of Medrano et al. (1989) under different conditions. Therefore, the lag observed in the presence of ALLO and TCB must be due to causes other than the perturbation of the kinetics of drug binding by the cosolvent; i.e., the solvent must act directly on the chemical processes undergone by the protein in its activation as a GTPase enzyme.

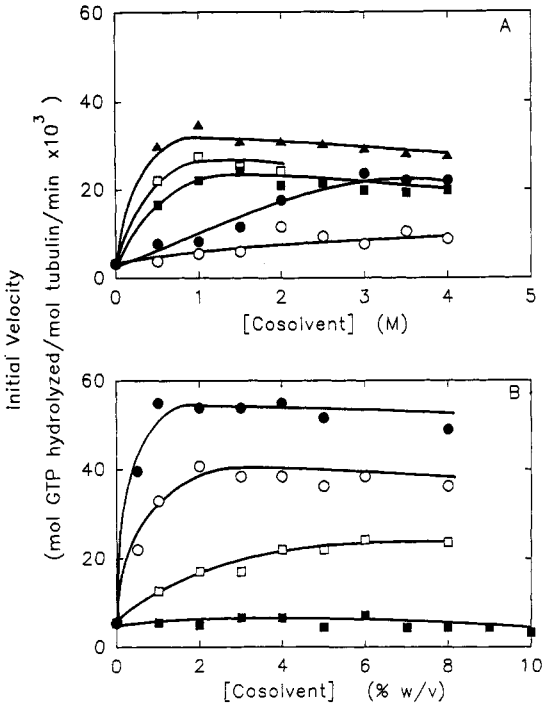


FIGURE 4: Effect of cosolvents on the initial rate of the colchicine-induced GTPase activity of tubulin. Panel A: Effect of glycerol (●), sucrose (□), L-glutamate (■), MPD (▲), and betaine (○). Panel B: Effect of DMSO (■), PEG-200 (□), PEG-1000 (○), and PEG-6000 (●). Tubulin (3 μM) in PMG buffer was preincubated at 20 °C for 20 min in the presence of a 20-fold molar excess of colchicine, 1 μCi of [γ-^{32}P]GTP, and the indicated amounts of the cosolvents. GTPase reactions were initiated by placing the tubes at 37 °C and adding $MgCl_2$ to a final concentration of 4 mM in a final volume of 100 μL. After 10 min of incubation at 37 °C, the reactions were stopped by addition of 200 μL of charcoal suspension and the radioactivity was determined as described under Experimental Procedures. The lines represent the trends of the data.

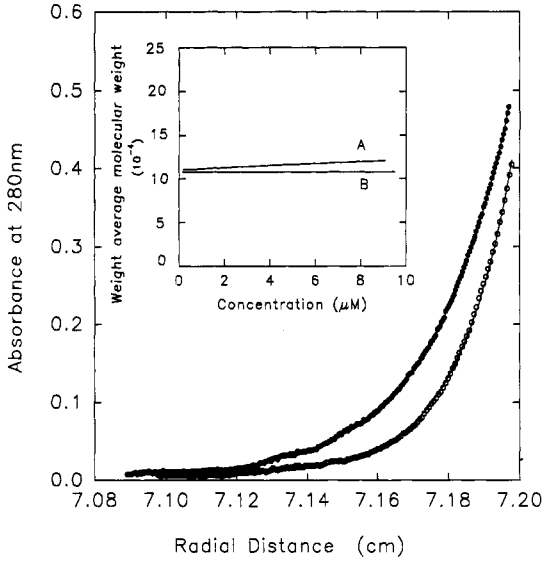


FIGURE 5: Effect of glycerol on tubulin–colchicine self-association as monitored by sedimentation equilibrium. Tubulin–colchicine complex (1.5 μM, 0.01 M sodium phosphate, 4 mM $MgCl_2$, and 10 μM GTP, pH 7.0) in the presence (●) or absence (○) of 3.4 M glycerol was sedimented to equilibrium (32 000 rpm, 30 min; 26 000 rpm, 3 h; 20 °C), and the concentration gradient in the cell was determined by ultraviolet absorption at 276 nm. The solid lines represent the best fits to the data. Inset: Concentration dependence of the weight average molecular weight calculated on the basis of the best-fit results. (A) In the absence of glycerol the best fit was a monomer–dimer association ($K_a = 8 \times 10^3$ M^{-1}), where monomer molecular weight was 110 000. (B) In the presence of 3.4 M glycerol the best fit was a single species of molecular weight 110 000.

DISCUSSION

The appearance of the GTPase activity in tubulin on the binding of colchicine or its analogues has been ascribed to conformational changes induced by the ligands (David-Pfeuty et al., 1977; Andreu & Timasheff, 1981; Monasterio & Timasheff, 1987; Medrano et al., 1991). Fluorescence energy transfer measurements have shown that the exchangeable GTP binding site and the colchicine site are more than 2.4 nm apart (Ward & Timasheff, 1988). Therefore, the activation of the GTPase function by colchicine-like ligands must be a long-range allosteric effect triggered by the binding of ring C or its phenyl analogue within the bidentate ligands. Chemically, this activation is similar to that induced in tubulin upon assembly into microtubules. In both cases, the product is a molecule with the E-site occupied by GDP. In the case of microtubule assembly, the activation is linked to the polymerization process, whereas in the drug-induced process, enzymatic activity is independent of the assembly of the protein into large microtubule-mimicking tubulin–colchicine polymers (Andreu et al., 1983). That the GTPase activity is not due to any protein self-association has been further established in the present study by the demonstration that it was independent of the concentration of the tubulin–colchicine complex up to 20 μM, a concentration at which the assembly process that leads to double rings is known to proceed under the present solvent conditions (Frigon & Timasheff, 1975a; Howard & Timasheff, 1986; Shearwin & Timasheff, 1992). Furthermore, the results reported here demonstrate that this activity is not affected by the incorporation of tubulin–colchicine into the double rings, since the enzymatic process proceeds normally when GTP–tubulin–colchicine exists in a state of equilibrium between αβ dimers and the double rings (Figures 2 and 3). Nevertheless, to avoid any possible ambiguities related to the state of tubulin association, the enzyme kinetics experiments were conducted under conditions that set the protein concentration below the critical concentration for both microtubule assembly and polymerization of the tubulin complex into the large microtubule-mimicking structures (Saltarelli & Pantaloni, 1982; Andreu et al., 1983).

What is the conformational nature of the product of the hydrolysis of the E-site GTP on the tubulin–colchicine complex? This was addressed by an ultracentrifugal examination of the effect of the *in situ* transformation of GTP into GDP on the ability of the protein to assemble into double rings. The findings (Figures 2 and 3) were that (i) on completion of the reaction, the weight average sedimentation coefficient of a 48 μM tubulin–colchicine solution had increased from that of tubulin–colchicine–GTP (8.1 S) to that measured for tubulin–colchicine–GDP (29.5 S), while (ii) the shape of the sedimentation reaction boundary had became transformed from that of tubulin–colchicine in the GTP state to that in the GDP state as prepared by GDP → GTP exchange, and (iii) at intermediate times, both the sedimentation pattern and the weight average sedimentation coefficient were indistinguishable between the mixtures of tubulin, GTP, and GDP present in the reaction flask and mixtures of identical composition prepared from pure tubulin–GTP and tubulin–GDP. Thus, replacement of GTP by GDP, whether by exchange or hydrolysis, leads to an identical conformational transition of tubulin from the "straight" to the "curved" state (Melki et al., 1989). That this is a rapidly reversible process is demonstrated by the fact that replacement by GTP of the hydrolysis-generated GDP in the E-site causes the sedimentation behavior to revert to that of straight tubulin, even when complexed to colchicine (Figure 2). Since tubulin–GDP generated by the disassembly of microtubules also forms

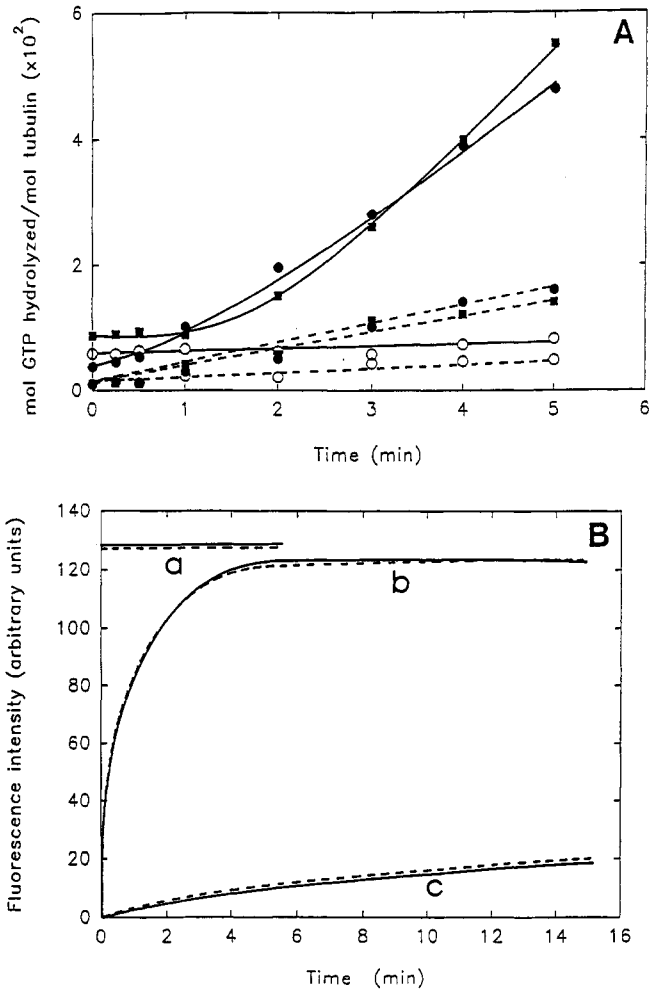


FIGURE 6: (A) Effect of PEG-6000 on the GTPase activity induced by colchicine and biphenyl analogues. Tubulin (4 μM) in PMG buffer containing 10 μCi/mL [γ-^{32}P]GTP was preincubated at 37 °C for 2 min in the presence (—) or absence (- - -) of 1%, w/v, PEG-6000. After addition of 30 μM ALLO (■), colchicine (○), or TCB (●), 100-μL aliquots were taken at the times indicated and placed over 200 μL of charcoal suspension for 5 min at 0 °C in order to stop the reaction. Following elimination of the charcoal by centrifugation at 4 °C, 50-μL aliquots were taken for determination of radioactivity in the supernatant (see Experimental Procedures). The lines represent the trends of the data. (B) Fluorescence time course of the binding of colchicine, ALLO, and TCB to tubulin at 37 °C; (—) and (- - -) indicate reactions carried out in the presence and absence of PEG-6000, respectively. The reactions were started by addition of 30 μM ligand to 4 μM tubulin solutions: (a) TCB, (b) ALLO, and (c) colchicine. Fluorometric binding measurements using colchicine were carried out with excitation a 365 nm and emission at 435 nm (Andreu & Timasheff, 1982a). Excitation was at 315 nm for both ALLO and TCB, while emission was monitored at 390 nm for ALLO and 373 nm for TCB (Medrano et al., 1989).

double rings, similarly to tubulin–GDP prepared by nucleotide exchange (Melki et al., 1989), it seems clear that the two identified basic conformational states of tubulin are essentially immutable, as they are independent of the manner by which E-site occupancy is established.

The dependence of the GTPase activity on Mg^{2+} ion concentration and the lack of its full inhibition by EDTA-containing buffers suggests that the catalytic activity requires a tightly bound cation at the E-site nucleotide. This, together with the pH independence of the tubulin GTPase over the pH range from 6.6 to 7.4, where tubulin is stable, is similar to reported observations on the Mg^{2+}-dependent GTPase of ras (Mistou et al., 1992), the α subunit of mammalian G_0 (Higashijima et al., 1987), and the EF-Tu-kirromycin GTPase (Ivell et al., 1981).

Effect of Cosolvent Additives. Following the observation that the rate of GTP hydrolysis was increased by addition of 3.4 M glycerol, the generation of the colchicine-induced GTPase activity was probed in detail by the use of a number of cosolvent additives which have been shown to stabilize tubulin or enhance microtubule formation (Frigon & Lee, 1972; Shelanski et al., 1973; Na & Timasheff, 1981; Arakawa & Timasheff, 1984). As shown in Figure 4, all of the cosolvents employed, except DMSO, increased the rate of GTP hydrolysis, but this increase required them to be present at high concentrations (>1 M). This clearly implies weak nonspecific protein–solvent interactions, rather than specific ones such as the stoichiometric binding of cosolvent molecules to particular sites on the protein (Timasheff, 1994). A kinetic examination of the parameters k_{cat} and K_m showed that none of the cosolvents employed affected the K_m for GTP, which indicates that the reaction is not diffusion controlled, while the cosolvents, except DMSO, increased k_{cat}. A possible effect of cosolvents on a weak self-association of tubulin–colchicine was eliminated by the demonstration that the weight average molecular weight was not affected by 3.4 M glycerol. Furthermore, the lack of correlation between cosolvent solution viscosity and the effect of these media on the apparent second-order rate constant (k_{cat}/K_m) (Table 2) eliminated substrate diffusion as the source of the observed effect. The influence of the cosolvents must therefore be exercised on a protein–ligand–substrate transition. This could be modulation of a conformational change and/or of dynamic properties of the protein molecule. Consistent with this, the dynamic properties of proteins may be coupled to those of the solvent (Gavish & Weber, 1979; Beece et al., 1989; Ng & Rosenber, 1991; Ansari et al., 1992). An indication of the locus of activation was given by the observation of lags (~30–60 s) in the release of inorganic phosphate (Figure 6A) when the GTPase reaction was initiated (in the presence of PEG-6000) by the addition of ALLO or TCB. The great difference in the rates of binding of these analogues in dilute buffer (TCB binds rapidly, <10 s; ALLO, at an intermediate rate, 4 min; and colchicine, slowly) and the lack of any cosolvent effect on these rates (Figure 6B) can eliminate drug binding as the locus of the cosolvent effect. These lags in the hydrolysis kinetics indicate, therefore, that another slow step must be present in the GTPase reaction. This can be either an activating conformational change in the protein or one of the steps in the actual hydrolysis reaction. In order to identify this step, further kinetic and thermodynamic probing was performed, and the results are the subject of the following paper (Perez-Ramirez et al., 1994).

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