Cosolvent Modulation of the Tubulin–Colchicine GTPase-Activating Conformational Change: Strength of the Enzymatic Activity†

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ABSTRACT: The locus of action of cosolvent additives in the activation of the tubulin–colchicine GTPase was investigated. The GDP off rates were slowed down by the cosolvents in a manner that parallels their specific viscosities, indicating that diffusion-controlled release of GDP may be rate-limiting under the conditions of these studies. Yet, the net effect of cosolvents was to increase the overall rate of GTP hydrolysis. Pre-steady-state kinetics of liganded tubulin in the presence of 1%, w/v, poly(ethylene glycol) 6000 (PEG-6000) exhibited a burst of inorganic phosphate release indicating that the cosolvents act at an early step in the process. A similar conclusion was drawn from measurements of the activation energy (E_a) of the reaction, which showed that 3.4 M glycerol decreased the value of E_a to 10.6 kcal mol^{-1} from 17.3 kcal mol^{-1} in its absence. The observed difference in apparent binding free energies of the colchicine analogues allocolchicine (ALLO) and 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC, or des-ring B colchicine), when measured by fluorescence and enzyme activity titrations, identified the presence of a GTPase-activating protein conformational transition subsequent to the physicochemical binding of the ligands. The decrease of the apparent binding constant measured by enzyme activity in dilute buffer relative to that measured by fluorescence [for ALLO, K_b(fluor) = 1.46 × 10^6 M^{-1}; K_b(enz act) = 1.1 × 10^5 M^{-1}] yielded the value of the enzyme-activating conformational transition constant, K_3 = 0.08. While neither 3.4 M glycerol nor 1%, w/v, PEG-6000 affected the apparent binding constant of ALLO measured by fluorescence, both increased that measured by enzyme titration. From the differences in the binding isotherms measured by enzymatic and fluorescence titrations, the fraction of active liganded tubulin was calculated to be 7.1, 26, and 79% in dilute buffer, 3.4 M glycerol, and 1%, w/v, PEG-6000, respectively. This led to strikingly similar values of the turnover number of the active form of the tubulin–drug complex, $k_{cat}^{intrinsic}$ = (2.1 ± 0.5) × 10^{-3} s^{-1}, deduced from the kinetic measurements under the different solution conditions. This value is 15 times greater than the $k_{cat}^{apparent}$ measured in the absence of cosolvents [Perez-Ramirez, B., Shearwin, K. E., & Timasheff, S. N. (1994) *Biochemistry 33*]. Thus, the intrinsic GTPase activity of the tubulin–colchicine complex is, in fact, much stronger than that measured. The shifting of the equilibrium by the preferentially excluded cosolvents from the inactive to the active species indicates the generation of a thermodynamically favorable environment for the active state of the protein by the cosolvents. In the case of 1%, w/v, PEG-6000, the favorable linkage free energy is −2.2 kcal mol^{-1}.

In the preceding paper (Perez-Ramirez et al., 1994), a detailed characterization of the ligand-induced GTPase[1] activity of calf brain tubulin (David-Pfeuty et al., 1977, 1979; Andreu & Timasheff, 1981; Andreu et al., 1991) showed that high concentrations of preferentially excluded cosolvent additives increased the apparent turnover number of the reaction without affecting the K_m for GTP. This increase in $k_{cat}^{apparent}$ correlated neither with the solvent viscosity nor with any effect on the state of the tubulin self-association.

The observation of lags in the release of inorganic phosphate in the presence of PEG-6000 when the reaction was initiated by the addition of the slowly binding colchicine analogue ALLO [for structures, see Perez-Ramirez et al., (1994)] or the rapidly binding analogue, TCB, together with the lack of an effect on the rates of their binding to tubulin, has led to the conclusion that an additional step, which is linked to the ligand binding process but is not detected by the usual physical methods, must exist in the activation of the GTPase function. What is this slow step, and where do the cosolvents exert their effect? It can be product dissociation, the chemical process of cleavage, or a precleavage equilibrium, i.e., a conformational change of the protein. A detailed examination of this question has been carried out, and the results are the subject of this paper.

EXPERIMENTAL PROCEDURES

The purification of tubulin, the preparation of samples, and the GTPase activity measurements were performed as described in the preceding paper (Perez-Ramirez et al., 1994). ^{3}H-Labeled GDP (12.8 Ci/mmol, 1 mCi/mL) was purchased from New England Nuclear. A sample of ras p21 protein was kindly provided by Dr. A. Redfield.

Nucleotide Exchange Reactions. [^{3}H]GDP–tubulin–colchicine was prepared after thawing of the sample (20 mg protein) and removal of sucrose and free nucleotides by passage

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

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over a drained column of Sephadex G-25 fine (Na & Timasheff, 1982). The protein was then centrifuged at 35000g for 30 min to eliminate large aggregates. GDP, containing 2×10^7 cpm/μmol [^{3}H]GDP, was added to a final concentration of 5 mM. A 20-fold molar excess of colchicine was also added at this stage. The samples were incubated for 30 min at 20 °C before passage over a second drained column and then a wet column (0.9 × 12 cm) of Sephadex G-25 fine equilibrated with the desired buffer (0.01 M sodium phosphate, pH 7.0, in the absence of $MgCl_2$). Protein concentration and radioactivity were determined in the eluent. The nucleotide contents of [^{3}H]GDP–tubulin were measured by reverse-phase ion-pair HPLC as described by Seckler et al. (1990).

Fluorescence Titration. Fluorescence titrations were performed on a Hitachi Perkin-Elmer 650-40 spectrofluorometer, operating in the ratio mode. Excitation and emission wavelengths for the different colchicine analogues employed were the same as in the preceding paper (Perez-Ramirez et al., 1994). The fluorescence intensities of ligand solutions in the absence of protein were measured, and the values, practically negligible, were subtracted from those measured in the presence of protein. To obtain a value for maximum fluorescence, the titration results were plotted as fluorescence/(free ligand concentration) versus fluorescence, using total ligand concentration as a first estimate of free ligand concentration. The abscissa intercept was taken as the first estimate of F_{max}, and the calculation was repeated until there was no change in the value of the ligand concentration (Shanley et al., 1985). This procedure eliminates the uncertainty involved in titrating a fixed amount of drug with increasing amounts of protein and extrapolating to infinite protein concentration in a 1/fluorescence versus 1/(protein concentration) graph. The experimental data were computer fitted to the binding equation with the assumption of a single binding site per $\alpha\beta$ tubulin dimer and the application of a commercial graphics/curve-fitting program (Sigmaplot 5.0, Jandel Scientific, Corte Madera, CA).

Other Determinations. The concentrations of ALLO and TCB were determined by ultraviolet absorption spectroscopy with the use of the following extinction coefficients: ALLO, 11 860 M^{-1} cm^{-1} at 288 nm and 4680 M^{-1} cm^{-1} at 315 nm; TCB, 12 100 M^{-1} cm^{-1} at 284 nm.

RESULTS

Having established that the high concentrations of cosolvents (~1 M) did not affect the K_m for GTP of the tubulin-colchicine GTPase (Perez-Ramirez et al., 1994), we thought it of interest to ask what their effects are on the individual steps of the reaction. First, the effect of the cosolvents on the rate constant (k_{off}) for dissociation of the product, GDP, from the tubulin–colchicine complex was examined by measuring their effect on the exchange of [^{3}H]GDP with GTP on the tubulin–colchicine complex. As shown in Figure 1, the rate of replacement of E-site GDP by GTP was slowed down by the cosolvents. Fitting of the experimental data to a single-exponential decay resulted in approximate rates of exchange of 4.5×10^{-2} min^{-1} in 3.4 M glycerol and 1.6×10^{-1} min^{-1} in dilute buffer. These results are qualitatively similar to the ones reported for tubulin–GDP in the absence of colchicine (Seckler et al., 1990) and for MAP-containing tubulin (Arai et al., 1975). The exchange rates in the presence of 1 M MPD and 1%, w/v, PEG-6000 were similar ($\approx 7.4 \times 10^{-2}$ min^{-1}), and that in the presence of 10%, w/v, DMSO (1×10^{-1} min^{-1}) was also slower than that in buffer alone. This order of effectiveness, glycerol > MPD ~ PEG-6000 > DMSO > buffer, parallels the specific viscosities of their solutions. This suggests an effect on the diffusion off rate of the product.

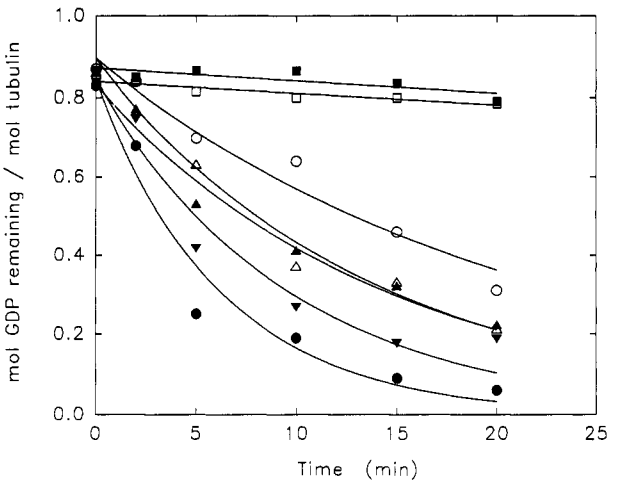


FIGURE 1: Effect of cosolvents on nucleotide exchange rates. [^{3}H]-GDP–tubulin–colchicine (4 μM), prepared as described under Experimental Procedures, was incubated at 20 °C in 0.01 M sodium phosphate buffer, pH 7.0. After addition of an excess of GTP (3 mM final concentration), 60-μL aliquots were taken at the times indicated and centrifuged (1000g, 3 min) through 1-mL columns of Sephadex G-25 which had been equilibrated with the experimental buffer and drained by centrifugation. The remaining radioactivity and protein concentration were determined in the eluent. Cosolvent additives were (●) buffer only; (○) 3.4 M glycerol; (▲) 1 M MPD; (△) 1%, w/v, PEG-6000; and (▼) 10%, w/v, DMSO. The lines represent the trends of the data. Cosolvent additives in the control experiments, in which an excess of GTP was not added, were (□) buffer and (■) 3.4 M glycerol.

This question was probed further by examining the effect of cosolvent on the GTP hydrolysis under pre-steady-state conditions where the catalytic activity of the protein is less than a single turnover. The cosolvent used was 1%, w/v, PEG-6000 because it was found to be the strongest activator (Perez-Ramirez et al., 1994). The colchicine analogue used was TCB because of its rapid binding to tubulin (Medrano et al., 1989). The results presented in Figure 2 showed an initial burst of substrate utilization in the presence of the cosolvent. This burst, with a lifetime of the order of 1 min, had an approximate[2] rate of $50–65 \times 10^{-2}$ mol of GTP hydrolyzed (mol of tubulin)$^{-1}$ min^{-1} as evaluated from the slopes of the kinetic curves. This was followed by a slower rate of GTP hydrolysis, approximately 8×10^{-2} mol of GTP hydrolyzed (mol of tubulin)$^{-1}$ min^{-1}. Hence, GTP hydrolysis proceeds initially as pre-steady state with the release of inorganic phosphate, while the subsequent turnover of the substrate is limited by the relatively slow dissociation of the product, GDP, from the tubulin–TCB complex in the exchange reaction. This result indicates that the cosolvent exerts its action either at the cleavage step or earlier in the reaction sequence. This conclusion was confirmed by measurement of the activation energy (E_a) of the reaction in the presence and absence of 3.4 M glycerol. The results presented in Figure 3, reveal linear Arrhenius plots with apparent E_a values of 10.7 and 17.3 kcal mol^{-1} in the presence and absence of glycerol, respectively. The effect of the cosolvent can then be attributed to a reduction of the energy barrier that the tubulin–colchicine system must overcome in carrying out its GTPase function.

Whether this takes place in a late stage of the drug binding process or in the formation of the activated complex was probed

[2] Precise evaluation of the initial burst would require rapid kinetic techniques not available in our laboratory.

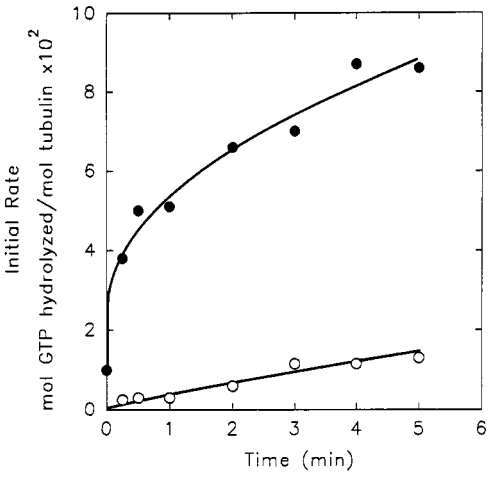


FIGURE 2: Pre-steady-state kinetics of the TCB-induced GTPase activity of tubulin. Five hundred microliters of tubulin (4 μM) was preincubated with 200 μM TCB for 20 min at 20 °C in PMG buffer containing 10 μCi of [γ-^{32}P] GTP. After the preincubation, 500 μL of PMG buffer (○) or PMG buffer containing 2%, w/v, PEG-6000 (●), both heated at 65 °C, was added to the tubes to yield a final temperature of 37 °C. This temperature was maintained during the duration of the experiments by a thermostated water bath. At selected times, 100-μL aliquots were taken, placed in 200 μL of charcoal suspension, and kept on ice for 5 min. Inorganic phosphate was determined as described before (Perez-Ramirez et al., 1994).

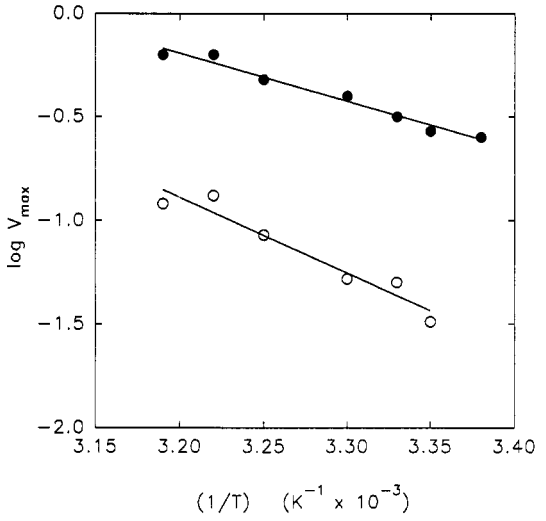


FIGURE 3: Arrhenius plots showing the activation energy (E_a) for the colchicine-induced GTPase activity of tubulin. Enzymatic reactions were carried out in a 100-μL final assay volume in PMG buffer using 6 μM tubulin and 30 μM colchicine in the absence (○) and presence (●) of 3.4 M glycerol, respectively. The lines were obtained by least-squares fitting of the data. The activation energies were calculated from the slopes.

by comparing the effects of cosolvents on the binding isotherms of the drugs to tubulin generated from physicochemical measurements, namely, fluorescence, and from titration of the GTPase activity. This was prompted by the observation that the apparent binding constant of the colchicine analogue MTC deduced from GTPase activity titration was 2.5 times smaller than that measured by fluorescence (Andreu et al., 1984), which had led to the proposal of a step in the binding process that is not detected by physical techniques (Timasheff et al., 1991). Therefore, the binding of ALLO to tubulin in the presence and absence of 1%, w/v, PEG-6000 was measured by the two methods, the first physical, i.e., fluorescence of the ligand, and the other biochemical, i.e., titration of the GTPase activity. ALLO was chosen for these experiments because of its high quantum yield compared to the other available

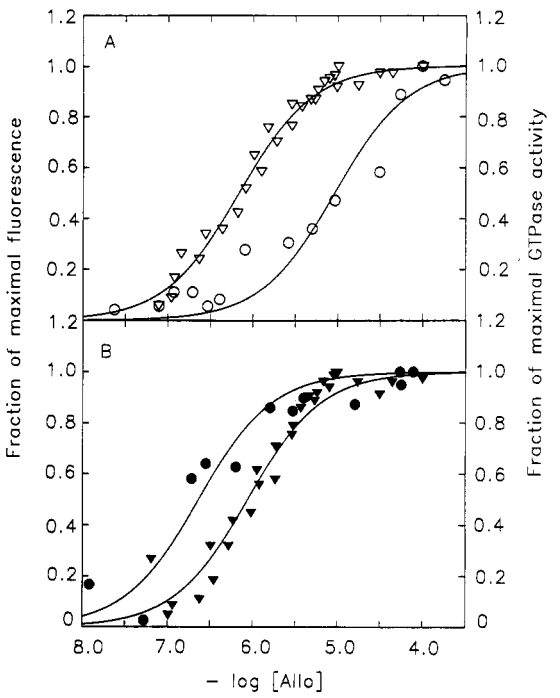


FIGURE 4: Binding isotherms of ALLO to tubulin in 0.01 M sodium phosphate, 4 mM $MgCl_2$, and 0.1 mM GTP, pH 7.0, at 37 °C, measured by ligand fluorescence (triangles) and titration of GTPase activity (circles): (A) no cosolvent; (B) in the presence of 1%, w/v, PEG-6000. The ligand concentrations plotted are free concentrations calculated from total ligand concentrations, protein concentrations and fractional fluorescence or enzyme activation, based on a binding stoichiometry of 1. Protein concentrations were 1.8 and 3.8 μM for the fluorescence titration experiments and 2 μM for the enzymatic titration. The solid lines are the theoretical curves based on the binding constants given in the text.

colchicine analogues. As shown in Figure 4A, in the absence of cosolvent, the two binding isotherms are significantly different: the midpoint measured by enzymatic activity is at a free drug concentration of 7.1×10^{-6} M, i.e., 1 order of magnitude greater than that measured by fluorescence. The results are summarized in Table 1. Fluorescence measurements gave a value of the binding constant, $K_b = (1.47 \pm 0.07) \times 10^6$ M^{-1}, at 37 °C, which is in agreement with Medrano et al. (1989). Titration of the GTPase activity with ALLO, however, resulted in $K_b = (1.1 \pm 0.2) \times 10^5$ M^{-1}, i.e., 1 order of magnitude lower than the physicochemical value,[3] which corresponds to a binding interaction weaker by 1.6 kcal mol^{-1} of standard free energy, $\Delta G°$. In the presence of 1%, w/v, PEG-6000, the results were just the opposite. As seen in Figure 4B, the binding isotherm generated by fluorescence measurements was the same within experimental error as that determined in the absence of cosolvent, while that generated by GTPase titration was displaced to a lower drug concentration, with a midpoint half an order of magnitude below that of the physical measurements. The corresponding binding constants, K_b, are listed in Table 1. That measured by fluorescence, $(1.14 \pm 0.1) \times 10^6$ M^{-1}, shows that the cosolvent had no effect on the binding of the drug, while that measured by titration of the GTPase activity with the drug in the presence of PEG had a value of $(4.3 \pm 0.8) \times 10^6$ M^{-1} [based on a maximal activity of 8×10^{-2} mol of GTP hydrolyzed (mol of tubulin)$^{-1}$ min^{-1} for the fully liganded protein], i.e., 4 times

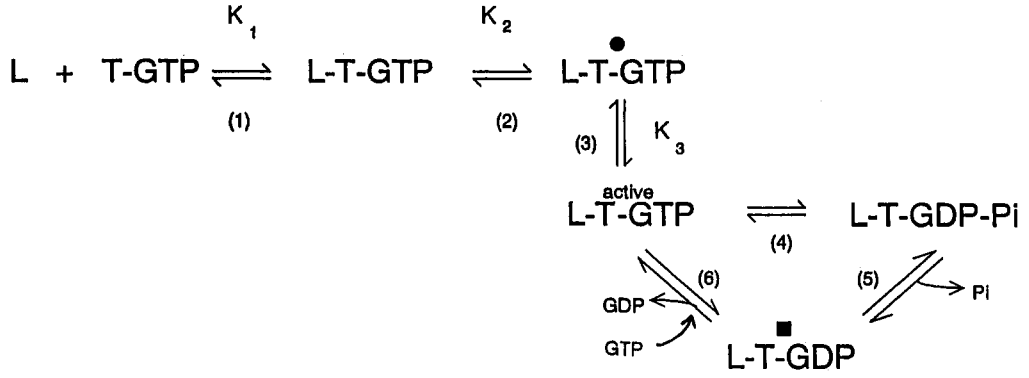
[3] The enzymatically determined binding constant was calculated with the assumption of an inactive unliganded protein, a binding stoichiometry of 1, and a maximal activity for the fully liganded protein of 7.8×10^{-3} mol of GTP hydrolyzed (mol of tubulin)$^{-1}$ min^{-1}.

Table 1: Comparison of the Binding Constants of ALLO to Tubulin at 37 °C Determined by Ligand Fluorescence and GTPase Activity

	K_b (M^{-1})					
method	buffer[a]		1%, w/v, PEG-6000		3.4 M glycerol	
ligand fluorescence[b]	$(1.47 \pm 0.07) \times 10^6$	(−8.74)[c]	$(1.14 \pm 0.1) \times 10^6$	(−8.59)	$(1.21 \pm 0.15) \times 10^6$	(−8.62)
GTPase activity[d]	$(1.1 \pm 0.2) \times 10^5$	(−7.15)	$(4.3 \pm 0.8) \times 10^6$	(−9.40)	$(4.1 \pm 0.1) \times 10^5$	(−7.96)

[a] Buffer consisted of 0.01 M sodium phosphate, pH 7.0, 4 mM $MgCl_2$, and 0.1 mM GTP. [b] Fluorescence titration was carried out using 1.8 and 3.8 μM tubulin for all experiments. [c] The numbers in parentheses are the $\Delta G°$ values in kcal mol^{-1} obtained from the equilibrium binding constants given. [d] Enzymatic titration of ligand binding was carried out at 2.0 μM tubulin.

Scheme 1



higher than the physicochemical value. This means that addition of the PEG had increased the apparent binding constant determined by titration of enzyme activity 40-fold. This corresponds to a strengthening of the process by $\Delta G° = -2.25$ kcal mol^{-1}. Similar results were obtained with 3.4 M glycerol as cosolvent additive. The binding measured by fluorescence was again unaffected by the cosolvent, while the apparent binding constant obtained by enzyme activity was increased approximately 4-fold; i.e., the free energy contribution of the cosolvent to the activation was −0.8 kcal mol^{-1}.

DISCUSSION

Tubulin Undergoes a Slow Post-Drug-Binding Conformational Change. In unraveling the mechanism of the cosolvent activation of the tubulin–colchicine GTPase activity, let us examine the effects of the cosolvents on the individual steps of the reaction. First, the cosolvents slowed down the diffusion off rates of the product, GDP (Figure 1). If this were their principal effect, it would slow down the reaction. The observation is that all of the cosolvents, except DMSO, accelerated it. This is clearly seen from the pre-steady-state kinetics. The observed burst of inorganic phosphate release in the presence of PEG-6000 (Figure 2) indicates that the cleavage, or hydrolysis, of GTP is faster in the presence of cosolvents than the dissociation of the product, GDP, from the tubulin–drug complex. GDP release could be rate-limiting under these conditions, similarly to what had been found with G_o GTPase (Higashijima et al., 1987). The effect of the cosolvents must be localized, then, in a slow step of the hydrolysis processes. This is indicated by the presence of lags of similar magnitude in the release of inorganic phosphate in the presence of cosolvent (PEG-6000) whether the reaction was initiated by the addition of a slowly binding drug, such as ALLO, or a rapidly binding one, such as TCB (Perez-Ramirez et al., 1994). The presence of such a step is further consistent with the difference between the enthalpy changes of binding ($\Delta H°$) of MTC and ALLO, measured calorimetrically and by Van't Hoff analysis of ligand fluorescence: $\Delta H°$(Van't Hoff) − $\Delta H°$(calorimetry) = 3.0 ± 0.7 and 2.6 ± 0.4 kcal mol^{-1} for MTC and ALLO, respectively (Andreu et al., 1984; Medrano et al., 1989; Menendez et al., 1989). Since both measurements of $\Delta H°$ were for the physical binding process, the slow step must occur prior to the hydrolysis step. It can be identified, therefore, as a conformational change that generates the enzymatically active form of the protein, but not the activated complex. This conformational change, which is detected calorimetrically, makes no significant contribution to the overall standard free energy change measured by several physical techniques. Therefore, it does not affect the binding process, but it is a consequence of binding. It should be noted that it does not perturb any spectroscopic properties either of the ligand or of the protein.

Activation of GTPase Activity. The above described findings of the difference in the apparent binding constants of MTC (Andreu et al., 1984) and ALLO to tubulin, when measured by fluorescence and by titration of the enzyme activity (Table 1; Figure 4), are fully consistent with the presence of a postbinding conformational transition. In dilute buffer, this reaction is unfavorable and is driven by its linkage to the strong binding reaction. Thus, for ALLO, the standard free energy cost of this reaction is 1.5 kcal mol^{-1}, i.e., the difference between the binding free energies measured by enzyme titration and by a physical technique. Cosolvents reverse this situation: they favor the enzymatically active form of the protein. In the presence of 1%, w/v, PEG-6000, the binding free energy measured by enzyme activity was 0.7 kcal mol^{-1} more negative than that measured by fluorescence. Since the binding constant measured by fluorescence is unaffected by the cosolvents (Table 1), the whole cosolvent effect must be on the equilibrium step detected by GTPase activity only. This effect is large, since $\delta\Delta G° = \Delta G°$(cosolvent) − $\Delta G°$(buffer) = −2.25 kcal mol^{-1}. This analysis is summarized by Scheme 1, where L is ligand, T is tubulin, and L–T* is the end product of drug binding measured by physicochemical techniques; it can be identified with the "straight" conformation of tubulin when the E-site is occupied by GTP; $L–T^{active}–GTP$ is the GTPase-active liganded-tubulin complex detected by enzyme activity titration. The first two steps (K_1, K_2) correspond to the binding process described by Garland (1978). They are unaffected by cosolvents. The

Table 2: GTPase-Activating Equilibrium and Intrinsic Turnover Number

ligand	cosolvent	K_3	$k_{cat}^{apparent}$ (s^{-1})	[L–T^{total}–GTP]	[L–T^{active}–GTP]	$k_{cat}^{intrinsic}$ (s^{-1})
ALLO	PMG buffer	0.08	1.3×10^{-4}	1×10^{-5} [b]	7.4×10^{-7}	1.8×10^{-3}, ±19%
ALLO	3.4 M glycerol	0.34	6.7×10^{-4}	1×10^{-5}	2.5×10^{-6}	2.7×10^{-3}, ±31%
ALLO	1%, w/v, PEG-6000	3.7	1.5×10^{-3}	1×10^{-5}	7.9×10^{-6}	1.9×10^{-3}, ±26%
MTC	PMG buffer	0.4[a]	1.8×10^{-4}	1×10^{-5}	2.9×10^{-6}	6.2×10^{-4}, ±38%

[a] Calculated from the data of Timasheff et al. (1991). [b] The total amount of tubulin in the assay was 1 nmol.

action of the cosolvents is exercised on the next step (K_3), which is the formation of the enzymatically active complex. In dilute aqueous buffer, it requires the expenditure of free energy. This is overcome by the linkage free energy of interaction of the protein with the cosolvents. In the case of 1%, w/v, PEG-6000 this amounts to –2.25 kcal mol^{-1}. Hence, it is the enhancement of the formation of the L–T^{active}–GTP species by the cosolvents that is the cause of the observed increase in the rate of GTP hydrolysis.

Further steps in Scheme 1 are the simplest representation of the catalytic GTPase hydrolysis reaction. These steps are kinetic. The species L–T■–GDP is the product of the reaction. As shown in the preceding paper (Perez-Ramirez et al., 1994), it is the "curved" conformation of tubulin, and it remains as the stable product if an excess of GTP is not present to displace the GDP from the E-site and complete the cycle. In the catalytic reaction, steps 4 and 5 should be favored by a ligand with a large K_2 (step 2), i.e. formation of a stronger complex. This accounts for the order of GTPase activation observed: colchicine > ALLO > MTC > TCB > TKB > TMB (Perez-Ramirez et al., 1994). Although the cosolvent decreases the release of GDP (step 6), the increase of the active species (L–T^{active}–GTP) is sufficient to allow an overall increase in the rate of hydrolysis. In the light of this analysis, it is possible to assume that the mechanism of the enzymatic hydrolysis of GTP by the liganded tubulin is similar whether cosolvent is present or absent. The decrease of the apparent energy of activation for the GTPase reaction by 6.7 kcal mol^{-1} in the presence of glycerol (Figure 3) suggests that the action of the cosolvent is to create an energetically favorable local environment for the enzymatically active tubulin conformer, which reduces the enthalpic barrier of the transition. This is consistent with the observed difference between the van't Hoff and calorimetrically measured values of $\Delta H°$ of binding. In the present state of knowledge, however, it is not possible to evaluate quantitatively these various contributions to the experimentally measured apparent energies of activation.

Tubulin–Colchicine GTPase Is Strong: Apparent and Intrinsic Turnover Numbers. Returning to Scheme 1, the difference between the equilibrium binding constants obtained by enzymatic and fluorescence titrations leads directly to the equilibrium constant (K_3) of the GTPase-activating equilibrium, since

$$K_3 = \frac{[\text{L–T}^{active}\text{–GTP}]}{[\text{L–T}^{*}\text{–GTP}]} = \frac{K_{binding}^{GTPase}}{K_{binding}^{fluor}} = \frac{K_1K_2K_3}{K_1K_2} \quad (1)$$

Substitution in eq 1 of the values of the equilibrium binding constants of ALLO obtained by fluorescence and GTPase measurements in PMG buffer (Table 1), i.e., 1.1×10^5 and 1.47×10^6 M^{-1}, gives $K_3 = 0.076$. At ligand saturation, the total concentration of liganded tubulin, [L–T^{total}–GTP], expressed in moles of $\alpha\beta$ dimer is

$$[\text{L–T}^{total}\text{–GTP}] = [\text{L–T}^{active}\text{–GTP}] + [\text{L–T}^{*}\text{–GTP}] \quad (2)$$

Combination of eqs 1 and 2 gives

$$K_3 = \frac{[\text{L–T}^{active}\text{–GTP}]}{[\text{L–T}^{total}\text{–GTP}] - [\text{L–T}^{active}\text{–GTP}]} \quad (3)$$

Solving for the active species results in

$$[\text{L–T}^{active}\text{–GTP}] = [\text{L–T}^{total}\text{–GTP}]\frac{K_3}{1 + K_3} \quad (4)$$

Since [L–T^{total}–GTP] = 10 μM in the measurements [Table 1 in Perez-Ramirez et al. (1994)] and $K_3 = 0.076$, in dilute buffer [L–T^{active}–GTP] is found by eq 4 to have a value of 0.71. Therefore, in the absence of cosolvent only 7.1% of the liganded tubulin is in the enzymatically active conformation when the ligand is ALLO. This now permits the calculation of the intrinsic value of k_{cat} from the experimentally measured V_{max}. In the absence of cosolvents V_{max} was found to be 1.33×10^{-4} nmol of GTP hydrolyzed per second by 1 nmol of liganded tubulin [see Table 1 in Perez-Ramirez et al. (1994)]. By definition

$$k_{cat}^{apparent} = \frac{V_{max}}{[\text{L–T}^{total}\text{–GTP}]}; \quad k_{cat}^{intrinsic} = \frac{V_{max}}{[\text{L–T}^{active}\text{–GTP}]} \quad (5)$$

This gives $k_{cat}^{apparent} = 1.3 \times 10^{-4}$ s^{-1} [Table 1 in Perez-Ramirez et al. (1994)]. But the fraction in the active conformation is 0.071 nmol, which gives $k_{cat}^{intrinsic} = 1.8 \times 10^{-3}$ s^{-1}. A similar analysis was carried out for the 3.4 M glycerol and 1%, w/v PEG-6000 systems. The results are summarized in Table 2. It is noteworthy that with PEG-6000 as cosolvent the fraction of the tubulin in the active form was 79%, i.e., 10 times greater than in dilute buffer. The values of $k_{cat}^{intrinsic}$, however, are strikingly similar for all three solvent systems, with a value of $(2.1 \pm 0.5) \times 10^{-3}$ s^{-1}. It seems clear, therefore, that the action of the cosolvents is to shift the equilibrium between the inactive and active conformations of tubulin toward the active one. A similar calculation applied to the published data with MTC as ligand resulted in a somewhat lower value of $k_{cat}^{intrinsic}$. This may reflect a difference in the GTPase-activating capacities of the various colchicine analogues.

General Remarks. While it is not possible to draw detailed conclusions about the mechanism of GTP hydrolysis at the active site of the tubulin–colchicine complex in the absence of its three-dimensional structure, general features can be inferred from the available evidence for similar systems, such as the GTPase activity of H-ras p21 protein, where structural information is available (Schlichting et al., 1990; Pai et al., 1990). Similarly to the case of tubulin–colchicine, measurements of the initial rate of GTP hydrolysis by ras protein that we have carried out (not shown) have revealed a 7-fold increase in the GTPase activity in the presence of 1%, w/v, PEG-6000 relative to that in dilute buffer. In this system, the activation by GAP protein of the GTPase activity of H-ras p21 protein has been proposed to be due to an alteration of the ras protein

[4] T. Arakawa and S. N. Timasheff, unpublished results.

conformation that stems from the movement of side chains in the active site. This would allow contact with a water molecule which is placed perfectly to be the nucleophile attacking the γ-phosphate of GTP (Pai et al., 1990). By analogy to this mechanism, the role of the cosolvents in the activation of the tubulin GTPase activity would be to stabilize a particular configuration of side chains at the active site involved in catalysis. This is fully consistent with the lack of an effect of the cosolvent on the free energy of binding of the ligand, but a strong effect on the free energy of formation of the GTPase active complex.

How do the cosolvents exercise this effect? All are known to be preferentially excluded from the surface of proteins; i.e., in their presence the environment of the protein is enriched with water relative to the bulk solvent composition. This could stabilize a given conformation of side chains in the active site if the extents of preferential exclusion differed between the inactive and active conformations of the protein (Timasheff, 1992). The mechanisms of their preferential exclusion, however, are different (Timasheff, 1994). The PEGs are excluded by steric interference (Arakawa & Timasheff, 1985; Bhat & Timasheff, 1992), MPD is repelled by charges (Pittz & Timasheff, 1978), glycerol is excluded due to the solvophobic effect (Gekko & Timasheff, 1981a,b), and sucrose (Lee & Timasheff, 1981) and sodium glutamate (Arakawa & Timasheff, 1984) are excluded as a result of their increasing the surface tension of water. Betaine and DMSO are partially hydrophobic and, therefore, may bind to the protein.[4] This may explain their very minimal or absent effect on the reaction.

REFERENCES

Andreu, J. M., & Timasheff, S. N. (1981) *Arch. Biochem. Biophys. 211*, 151–157.

Andreu, J. M., Gorbunoff, M. J., Lee, J. C., & Timasheff, S. N. (1984) *Biochemistry 23*, 1742–1752.

Andreu, J. M., Gorbunoff, M. J., Medrano, F. J., Rossi, M., & Timasheff, S. N. (1991) *Biochemistry 30*, 3777–3786.

Arai, T., Ihara, Y., Arai, K., & Kaziro, Y. (1975) *J. Biochem. (Tokyo) 77*, 647–658.

Arakawa, T., & Timasheff, S. N. (1984) *J. Biol. Chem. 259*, 4979–4986.

Arakawa, T., & Timasheff, S. N. (1985) *Biochemistry 24*, 6756–6762.

Bhat, R., & Timasheff, S. N. (1992) *Protein Science 1*, 1133–1143.

David-Pfeuty, T., Erickson, H. P., & Pantaloni, D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5372–5376.

David-Pfeuty, T., Simon, C., & Pantaloni, D. (1979) *J. Biol. Chem. 254*, 11696–11702.

Garland, D. L. (1978) *Biochemistry 17*, 4266–4272.

Gekko, K., & Timasheff, S. N. (1981a) *Biochemistry 20*, 4667–4676.

Gekko, K., & Timasheff, S. N. (1981b) *Biochemistry 20*, 4677–4686.

Higashijima, T., Ferguson, K. M., Smigel, M. D., & Gilman, A. G. (1987) *J. Biol. Chem. 262*, 757–761.

Lee, J. C., & Timasheff, S. N. (1981) *J. Biol. Chem. 256*, 7193–7201.

Medrano, F. J., Andreu, J. M., Gorbunoff, M. J., & Timasheff, S. N. (1989) *Biochemistry 23*, 5589–5599.

Menendez, M., Laynez, J., Medrano, F. J., & Andreu, J. M. (1989) *J. Biol. Chem. 264*, 16367–16371.

Na, G. C., & Timasheff, S. N. (1982) *Methods Enzymol. 85*, 393–408.

Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabash, W., & Wittinghofer, A. (1990) *EMBO J. 9*, 2351–2359.

Perez-Ramirez, B., Shearwin, K. E., & Timasheff, S. N. (1994) *Biochemistry* (preceding paper in this issue).

Pittz, E. P., & Timasheff, S. N. (1978) *Biochemistry 17*, 615–623.

Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabash, W., Petsko, G. A., & Goody, R. S. (1990) *Nature 345*, 309–315.

Seckler, R., Wu, G.-M., & Timasheff, S. N. (1990) *J. Biol. Chem. 265*, 7655–7661.

Shanley, B. C., Clarke, K., & Winzor, D. J. (1985) *Biochem. Pharmacol. 34*, 141–142.

Timasheff, S. N. (1992) *Biochemistry 31*, 9857–9864.

Timasheff, S. N. (1994) in *Protein Solvent Interactions* (Gregory, R. H., Ed.) Marcel Dekker, New York (in press).

Timasheff, S. N., Andreu, J. M., & Na, G. C. (1991) *Pharmacol. Ther. 52*, 191–210.