Nucleoside Triphosphate Specificity of Tubulin†

Gopal Chakrabarti,‡ Marisan R. Mejillano,‡,§ Young-Hee Park,‡ David G. Vander Velde,|| and Richard H. Himes*,‡

Department of Molecular Biosciences and The NMR Laboratory, University of Kansas, Lawrence, Kansas 66045-2106

Received April 27, 2000; Revised Manuscript Received June 20, 2000

ABSTRACT: We have determined the binding affinity for binding of the four purine nucleoside triphosphates GTP, ITP, XTP, and ATP to E-site nucleotide- and nucleoside diphosphate kinase-depleted tubulin. The relative binding affinities are 3000 for GTP, 10 for ITP, 2 for XTP, and 1 for ATP. Thus, the 2-exocyclic amino group in GTP is important in determining the nucleotide specificity of tubulin and may interact with a hydrogen bond acceptor group in the protein. The 6-oxo group also makes a contribution to the high affinty for GTP. NMR ROESY experiments indicate that the four nucleotides have different average conformations in solution. ATP and XTP are characterized by a high anti conformation, ITP by a medium anti conformation, and GTP by a low anti conformation. Possibly, the preferred solution conformation contributes to the differences in affinities. When the tubulin E-site is saturated with nucleotide, there appears to be little difference in the ability of the four nucleotides to stimulate assembly. The critical protein concentration is essentially identical in reactions using the four nucleotides. All four of the nucleotides were hydrolyzed during the assembly reaction, and the NDPs were incorporated into the microtubule. We also examined the binding of two γ-phosphoryl-modified GTP photoaffinity analogues, p^3-1,4-azidoanilido-GTP and p^3-1,3-acetylanilido-GTP. These analogues are inhibitors of the assembly reaction and bind to tubulin with affinities that are 15- and 50-fold lower, respectively, than the affinty for GTP. The affinity of GTP is less sensitive to substitutions at the γ-phosphoryl position that to changes in the purine ring.

The subunit of microtubules, tubulin, is a heterodimeric protein consisting of two closely related polypeptide chains, α and β. Both chains bind a molecule of GTP. GTP bound to the α-subunit does not exchange with GTP in solution, but the nucleotide bound to the β-subunit is exchangeable. An electron crystallographic structure of tubulin offers an explanation for the different properties of the two sites; the nucleotide in α-tubulin is situated at the interface between the two subunits and is not accessible to the solvent, while the site in β-tubulin is exposed to the solvent (*1*). Although exchangeable, the guanine nucleotide in the β-subunit binds with high affinity, and when isolated, tubulin contains GTP or GDP at the exchangeable site (E-site).[1]

When tubulin assembles into microtubules, the GTP at the E-site undergoes hydrolysis, although hydrolysis of GTP is not necessarily coupled to the additon of a tubulin dimer to the end of a microtubule. As a result, microtubules may contain tubulin dimers with either GDP, GTP, or GDP and P_i at the E-site (*2*−*4*). The use of nonhydrolyzable or slowly hydrolyzable GTP analogues has demonstrated that hydrolysis is not a requirement for tubulin assembly (*5*−*9*), but rather, the conversion of GTP to GDP at the E-site functions to produce a microtubule lattice structure that is unstable and can readily be disassembled. Growth and shortening of individual microtubules occur at steady state, a condition at which there is no net change in polymer mass. This property of microtubules is known as dynamic instability (*10*) and is essential for their function and positioning in the cell. Microtubules are thought to be prevented from disassembly by a "cap" of tubulin−GTP_E or tubulin−$(GDP+P_i)_E$ dimers at the end of the microtubule. Loss of this cap by hydrolysis or breakage would expose a tubulin−GDP_E end, resulting in rapid disassembly.

We have been interested in the interactions that determine the specificity tubulin shows for the guanine nucleotides. Photoaffinity labeling studies have identified regions in the β-subunit that are in proximity to the E-site nucleotide (*11*−*15*), and the electron crystallographic structure (*1*) has confirmed that these peptide sequences do indeed constitute a nucleotide binding pocket. Structural studies and comparisons with the related prokaryotic protein FtsZ (*16*, *17*) are beginning to provide insight into the nature of the interactions between amino acid residues and the nucleotide. As a complement to these studies, it is useful to know the contributions to the binding affinty of GTP made by the

† This research was supported in part by NIH Grants GM 36952 and CA 55141 to R.H.H. G.C. was the recipient of a Kansas Health Foundation (Wichita, KS) Postdoctoral Cancer Scholar Grant. M.R.M. was the recipient of a Kansas Health Foundation Predoctoral Cancer Scholar Grant.

* To whom correspondence should be addressed. Telephone: (785) 864-3813. Fax: (785) 864-5294. E-mail: himes@ukans.edu.

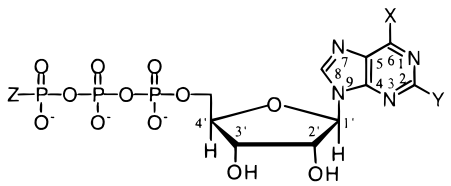
‡ Department of Molecular Biosciences.

§ Present address: Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX 75235-9040.

|| The NMR Laboratory.

[1] Abbreviations: Pipes, 1,4-piperazinediethanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; buffer A, 0.1 M Pipes (pH 6.9), 1 mM $MgSO_4$, and 1 mM EGTA; AzAGTP, p^3-1,4-azidoanilido-GTP; AcAGTP, p^3-1,3-acetylanilido-GTP; E-site, exchangeable nucleotide site in tubulin; tubulin−GTP_E, tubulin with GTP in the E-site; NTP, purine nucleoside triphosphate; ROESY, rotating-frame Overhauser enhancement spectroscopy.

10.1021/bi000966n CCC: $19.00
Published on Web 07/26/2000



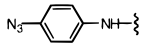
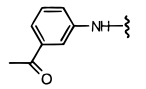
Nucleotide	X	Y	Z
GTP	O	NH_2	OH
ITP	O	H	OH
XTP	O	O	OH
ATP	NH_2	H	OH
AzAGTP	O	NH_2	
AcAGTP	O	NH_2	

FIGURE 1: Structures of purine nucleoside triphosphates.

various functional groups in the nucleotide molecule. In this study, we have studied the interaction of four purine nucleoside triphosphates and two γ-phosphoryl-modified GTP analogues with tubulin (Figure 1). The results provide information about the interactions of the base and γ-phosphoryl group with tubulin.

EXPERIMENTAL PROCEDURES

Materials. ITP, XTP, and ATP were from Sigma Chemical Co. GTP and alkaline phosphatase were purchased from Boehringer Mannheim. [^{3}H]GTP was obtained from Amersham. p^3-1,4-Azidoanilido-GTP (AzAGTP) and p^3-1,3-acetylanilido-GTP (AcAGTP) were synthesized as described by Rasenick et al. (*18*) and Zor et al. (*19*), respectively.

Purification of Tubulin. Microtubule protein (tubulin containing microtubule-associated proteins) was purified from bovine brain by two cycles of temperature-dependent assembly and disassembly (*20*). Tubulin was purified from microtubule protein by chromatography on phosphocellulose (*21*) and was stored at −70 °C in 0.1 M Pipes (Na^+ form) (pH 6.9) containing 1 mM $MgSO_4$ and 1 mM EGTA (buffer A) as frozen pellets. The purity of tubulin was checked by SDS−PAGE. Phosphocellulose-purified tubulin contains nucleoside diphosphate kinase (NDPK) activity (*22*). To obtain tubulin lacking NDPK activity, microtubule protein was taken through three cycles of temperature-dependent assembly and disassembly in 0.4 M Pipes and 8% DMSO and stored at −70 °C in buffer A as frozen pellets. To verify that NDPK activity was absent, 30 μM tubulin containing GDP in the E-site was incubated in the presence of buffer A, 8% DMSO, and 3 mM ATP at 37 °C, and assembly was monitored spectrophotometrically at 350 nm. In the presence of NDPK, ATP supports assembly under these conditions by transferring a phosphate to the GDP in the E-site. However, ATP did not support the assembly of tubulin purified through cycles of assembly and disassembly in 0.4 M Pipes and 8% DMSO.

Assembly Reaction and Critical Concentration Determination. Assembly reactions were performed in buffer A containing 8% DMSO at 37 °C. The extent of assembly was monitored either from the increase in turbidity at 350 nm or by the amount of pelletable protein formed in the incubation.

To determine the critical tubulin concentraion for assembly in the presence of each of the four purine nucleoside triphosphates, the E-site had to be depleted of GDP and GTP. This was accomplished in the following way. First, any residual GTP in the E-site was replaced with GDP by incubating tubulin with a 50-fold molar excess of GDP on ice for 30 min followed by centrifugation through a Sephadex-25 spin column in buffer A. The resulting tubulin−GDP was incubated with alkaline phosphatase (40 units/mL) in the presence of GMPPNP (*6*, *7*, *23*) on ice for 2 h. The sample was centrifuged through a Sephadex-25 spin column in buffer A. A portion of the eluent was used for nucleotide analysis after centrifugation through another spin column. The remaining portion was incubated in the presence of a 100-fold molar excess of NTP on ice for 15 min and was centrifuged through a spin column to remove excess NTP. A portion of the sample was used for nucleotide analysis after passage through another spin column, and the remainder was concentrated in the presence of 3 mM NTP to obtain a protein concentration of >30 μM and was used for assembly experiments.

Assembly was performed for 20 min in 400 μL at protein concentrations ranging from 0.8 to 3 mg/mL (8 to 30 μM) in the presence of buffer A, 3 mM NTP, and 8% DMSO. The samples were centrifuged for 4 min at 37 °C and 45000*g* in a Beckman TL-100 ultracentrifuge. The supernatant protein concentration was determined by the Bradford method (*24*) and was used to calculate the concentration of polymerized tubulin. Pellets from the assembly reactions were dissolved in cold buffer and recentrifuged. Protein determinations in the supernatants showed that no cold-insoluble aggregates had formed.

Nucleotide Analysis. Nucleotides were analyzed by HPLC on a 4.6 mm × 250 mm Synchropak anion exchange column (Phenomenex) using isocratic elution with a 0.4 M NaH_2PO_4/0.2 M NaCl mixture (pH 5.0) as the solvent. For XTP and XDP, the pH of the buffer was 4.3 (*25*). Detection was at 254 nm.

To determine the nucleotide content of assembled microtubules, 250 μL of the assembly solution was centrifuged through a sucrose cushion (2.5 mL of buffer A containing 40% sucrose and 8% DMSO) at 37 °C and 120000*g* for 30 min in a Beckman TL ultracentrifuge using the TLA 100.3 rotor. At the end of the centrifugation, 2 mL of warm buffer A containing 40% sucrose and 8% DMSO was carefully pipetted to the bottom of the tube, and 1 mL was removed from the top. Then 1 mL of warm buffer A containing 60% sucrose and 8% DMSO was pipetted to the bottom of the tube, and the upper layer was removed. This step was repeated, and finally the pellet was covered twice with warm buffer A containing 40% sucrose and 8% DMSO and once with 125 μL of warm H_2O. The pellet was dissolved in 125 μL of cold H_2O, and the protein was precipitated by the addition of 5% perchloric acid. After removal of the precipitate by centrifugation, the supernatant was titrated with a solution of 4 M potassium acetate and 10 M KOH to pH 5.5−6. The resulting precipitate was removed by centrifugation, and the supernatant was used for analysis by HPLC. The perchloric acid precipitate was dissolved in 250 μL of 0.1 M NaOH for protein concentration determination.

Determination of Dissociation Constants. Dissociation constants were determined by using a competitive binding assay (*26*). The tubulin−[^{3}H]GTP dimer was prepared by incubating tubulin with a 10-fold molar excess of [^{3}H]GTP (specific activity of 20 μCi/nmol) in buffer A on ice for 30 min followed by centriguation through a gel-filtration column. The tubulin−[^{3}H]GTP dimer (2 μM) was then incubated in a 400 μL volume with six different concentrations of the NTP from 100 to 5000 μM on ice for 30 min. The samples were then centrifuged through an Amicon MPS apparatus using a YMT membrane for 3 min at 1500*g*, or until about 200 μL of the solution had passed through the membrane. The concentration of the displaced [^{3}H]GTP in the lower reservoir (filtrate) was determined from the radioactivity. This value was used to calculate the concentration of the tubulin−[^{3}H]GTP dimer, the tubulin−NTP dimer, and free NTP in the upper reservoir. The K_d values were calculated using the equation (*26*)

$$K_{d(GTP)}/K_{d(NTP)} = ([NTP]_{bound}/[GTP]_{bound})([GTP]_{free}/[NTP]_{free})$$

The values reported are the mean of the calculated values obtained at each nucleotide concentration. To determine the K_d for GTP, bound [^{3}H]GTP was displaced by GDP and a K_d of 6.1 × 10^{-8} M for the tubulin−GDP dimer was used (*26*). The dissociation constants are reported for the Mg^{2+}−NTP complexes. The concentrations of the Mg^{2+} complexes were calculated using an affinty constant of 2.83 × 10^3 M^{-1} determined for the Mg^{2+}−GTP complex under similar solution conditions (*27*).

ROESY Experiments. To determine the average solution conformation of the nucleotides, ROESY experiments were performed on a Bruker Avance DRX 400 spectrometer operating at 400.13 MHz for ^{1}H. The nucleotide samples were 10 mM in 50 mM sodium phosphate prepared in D_2O (pH meter reading of 7.0). Experiments were carried out at 25 °C, and the mixing time was 300 ms.

RESULTS

Relative Affinities of the Four Nucleoside Triphosphates for Tubulin. Previous studies have shown that the E-site GDP or GTP could be replaced with ITP, XTP, or ATP (*6*, *28*), but in few cases have the binding affinities of the non-guanine nucleotides for purified tubulin been reported. We determined the dissociation constants using the competitive binding assay by incubating the tubulin−[^{3}H]GTP_E dimer with different concentrations of the nucleotides and measuring the amount of [^{3}H]GTP released from tubulin by centrifuging the samples through an Amicon YMT membrane. The K_d values for the Mg^{2+}−NTP complexes were determined because it is known that Mg^{2+} is required for efficient binding of the NTPs (*27*, *29*). A value was also obtained for the Mg^{2+}−GTP complex by using unlabeled GDP to displace the [^{3}H]GTP and was equivalent to that reported by Zeeberg and Caplow (*26*) (Table 1). The affinity of tubulin for the Mg^{2+}−GTP complex was 300-fold higher than for the Mg^{2+}−ITP complex. The differences among the non-guanine nucleotides were less striking. The Mg^{2+}−ITP complex bound with an affinity that was 4 times as high as that for the Mg^{2+}−XTP complex and about 10-fold higher than that for the Mg^{2+}−ATP complex.

Table 1: K_d Values for the Binding of Purine Nucleoside Triphosphates to Tubulin[a]

NTP	K_d (M)[b]	NTP	K_d (M)[b]
GTP	(1.99 ± 0.37) × 10^{-8}	ATP	(5.71 ± 2.74) × 10^{-5}
ITP	(6.43 ± 1.80) × 10^{-6}	AzAGTP	(1.03 ± 0.10) × 10^{-6}
XTP	(2.78 ± 1.08) × 10^{-5}	AcAGTP	(3.16 ± 0.53) × 10^{-7}

[a] The tubulin−[^{3}H]GTP dimer (2 μM) was incubated with 100−5000 μM NTP, and the displaced [^{3}H]GTP was determined in the filtrate after centrifugation through an Amicon YMT membrane (see Experimental Procedures). [b] The values were calculated using the value of 1.99 × 10^{-8} M for GTP. This value was obtained by displacing [^{3}H]GTP with GDP and using a value for the K_d of GDP of 6.1 × 10^{-8} M reported by Zeeberg and Caplow (*26*). The K_d for GTP reported by Zeeberg and Caplow is 2.2 × 10^{-8} M. Standard deviations are given.

Table 2: Critical Concentrations for Tubulin Assembly[a]

nucleotide	critical concentration (mg/mL)
GTP	0.42 ± 0.01
ITP	0.37 ± 0.04
XTP	0.54 ± 0.02
ATP	0.54 ± 0.01

[a] Determined as described in Experimental Procedures. Average of two determinations.

Table 3: Nucleotide Content of Tubulin and Microtubules[a]

	tubulin (mol/mol of tubulin)				microtubules (mol/mol of tubulin)			
NTP	GTP	GDP	NTP	NDP	GTP	GDP	NTP	NDP
GTP	1.02	0.13	−	−	1.05	1.02	−	−
ITP	0.98	0.16	0	0	1.08	0.19	0	0.77
XTP	0.91	0.25	0	0	1.02	0.21	0	0.62
ATP	1.08	0.24	0	0	1.01	0.22	0	0.46

[a] The values are averages of two or three determinations.

Assembly of Tubulin in the Presence of Different Purine Nucleoside Triphosphates. It is known that ITP, XTP, and ATP can promote the assembly of tubulin to different degrees (*6*, *28*, *30*), but it is not clear in the previous experiments whether the E-site was saturated with the nucleotides. We compared the effectiveness of these nucleotides at a concentration that would saturate the E-site so that differences would not be due to differences in the concentration of the tubulin−$(NTP)_E$ complex that was present. To make a comparison under this condition, we used tubulin that lacked NDPK activity and had been depleted of the E-site GTP and most of the E-site GDP (see Experimental Procedures). A concentration of 3 mM NTP was used in the assembly reaction. At this concentration, the percent of tubulin in the tubulin−Mg^{2+}−NTP_E form was at least 95% for the four nucleotides. The critical protein concentration for tubulin assembly was determined by measuring the concentraion of unassembled protein at steady state. Plots of the concentration of polymerized tubulin versus total tubulin concentration were linear in the range from 0.8 to 3.0 mg/mL and yielded the critical concentrations presented in Table 2. There is little difference in the values among the four nucleotides.

We also wanted to determine the nucleotide content of the microtubules formed in these reactions. After treatment with alkaline phosphatase followed by a 100-fold molar excess of NTP, the nucleotide content of tubulin was determined (Table 3). The GTP content of tubulin after these treatments was about 1 mol/mol of tubulin, reflecting the content of the N-site. There was a residual amount of GDP,

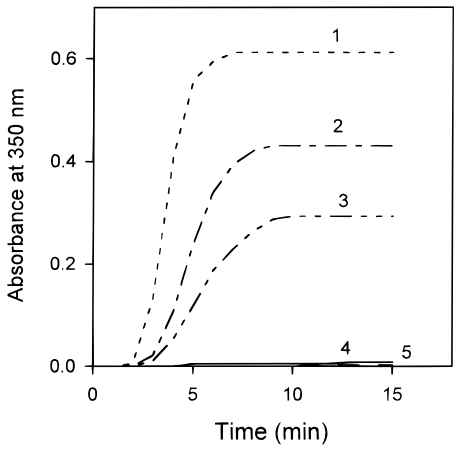


FIGURE 2: Tubulin assembly in the presence of AzAGTP or AcAGTP. Tubulin was assembled at 37 °C at a concentration of 1.5 mg/mL in PEM buffer and 8% DMSO: (1) 0.1 mM GTP, (2) 0.1 mM GTP and 1 mM AzAGTP, (3) 0.1 mM GTP and 1 mM AcAGTP, (4) 3 mM AzAGTP, and (5) 1 mM AcAGTP.

ranging from 0.15 to 0.24 mol/mol of tubulin. However, there were no other nucleotides present, indicating that their affinity is too weak to survive the two gel-filtration spin columns used in the procedure.

After assembly in the presence of a nucleotide concentration of 3 mM, the formed microtubules were examined for nucleotide content (Table 3). In all cases, no NTP was found in the microtubules. IDP, XDP, and ADP were present, indicating that all the nucleotide triphosphates had undergone hydrolysis. In a previous communication (*6*), we reported that ATP did not undergo hydrolysis during the assembly reaction. We have no explanation for this difference, but the results obtained in this work are consistent with those of Duanmu et al. (*28*). The results in Table 3 also show that GDP is present in the microtubules in the same proportion as in the unpolymerized tubulin. This result, which has been observed previously (*6*, *31*−*35*), suggests that the tubulin−E_{GDP} complex is competent to assemble onto a growing microtubule.

Interaction of Two γ-Phosphoryl-Modified Nonhydrolyzable GTP Analogues with Tubulin. Two photoaffinity analogues of GTP, p^3-1,4-azidoanilido-GTP (AzAGTP), and p^3-1,3-acetylanilido-GTP (AcAGTP) (Figure 1) were examined for their ability to support tubulin assembly and to bind to tubulin. In contrast to other nonhydrolyzable or slowly hydrolyzable GTP analogues that support tubulin assembly (*5*−*9*), AzAGTP and AcAGTP were found not to promote assembly and, in fact, to be inhibitors of assembly (Figure 2). When assembly was attempted with the tubulin−GDP_E dimer or E-site-depleted tubulin, there was no evidence of microtubule formation in the presence of the analogues, even at an analogue concentration of 3 mM. On the other hand, when the analogues were included in an assembly reaction mixture containing 0.1 mM GTP, inhibition occurred (Figure 2). An analogue concentration of 1 mM was effective in inhibiting assembly in the presence of 0.1 mM GTP. The AcAGTP analogue was somewhat more effective than AzAGTP as an inhibitor of assembly.

Both analogues were capable of displacing [^{3}H]GTP from tubulin. The K_d values for the two analogues were determined by the competitive binding assay (Table 1). The value for AcAGTP was about 15-fold higher than the K_d of GTP, while that for AzAGTP was about 50-fold higher. These analogues have higher affinities than ITP, XTP, or ATP.

Table 4: Results from the ROESY Experiments

nucleotide	normalized relative cross-peaks (H8 to H1′, H2′, and H3′)	coupling constants $J_{1'-2'}$ (Hz)
ATP	15, 52, and 33	6.1
XTP	12, 52, and 36	6.1
ITP	24, 45, and 31	5.5
GTP	34, 38, and 28	6.1

Conformation of Free Nucleotides. When the orientation of the heterocyclic ring relative to the sugar ring in purines is considered, two main conformations exist, anti and syn (*36*). In the anti orientation, the bulk of the ring points away from the sugar ring. In the syn conformation, the heterocyclic ring is over the sugar ring. The conformations are defined by the O4′−C1′−N9−C8 torsion angle χ. The value of this angle is 90° in the conformation termed high anti (*36*). In solution, the two conformations are in equilibrium, although the relative contribution of one may be preferred over the other. ROESY experiments can be used to estimate the relative contributions of the syn and anti conformations by determining the cross-peak intensities between H8 in the purine ring and H1′, H2′, and H3′ in the ribose ring. In the anti conformation, H8 is closer to H2′ than to H1′, while in the syn conformation H8 is closer to H1′ than to H2′. Studies with ATP by André et al. (*37*) led to the conclusion that, while there is substantial rotational freedom around the glycosidic bond, and a distribution of syn and anti conformations existed, on average, H8 was closer to H2′ than to H1′ and H3′, indicating a population of the high anti conformation.

We performed similar experiments on the four nucleotides to determine whether the solution conformational preferences for GTP, ITP, and XTP were different from that of ATP. The normalized cross-peak intensities and coupling constants between H1′ and H2′ are presented in Table 4. Although our experimental conditions were somewhat different (lower magnetic field and higher temperature) than those employed by André et al. for ATP (*37*), and the quantitative results were somewhat different (the normalized H8 to H1′, H2′, and H3′ cross-peak intensities were 20, 65, and 15 in their data and 15, 52, and 36 in ours, respectively), the qualitative conclusions are the same. The relative cross-peak intensities for XTP were virtually identical to those for ATP. For ITP, the H8−H1′ distance was shorter, corresponding to a lower average value of χ. For GTP, the H8−H1′ distance was even shorter. From the results, we can conclude that the average value of χ increases in the following order: GTP > ITP > XTP ∼ ATP. In contrast, the sugar pucker equilibrium (as judged from the *J* coupling constants) does not seem to be related to the differences in the affinities of the four nucleotides.

DISCUSSION

One purpose of this study was to determine the most important features of the guanine ring in the GTP molecule that are responsible for the strong interactions between the nucleotide and tubulin. Tubulin does not exhibit absolute specificity for the guanine nucleotide. The three purine nucleotides ITP, XTP, and ATP also bind to tubulin and

promote tubulin assembly. In fact, the critical tubulin concentration was similar for the four purine nucleoside triphosphates when they were present at concentrations that would saturate the E-site, indicating that the nucleotide hydrolysis and assembly reactions are relatively insensitive to the nature of the purine ring. This is similar to the nocodazole-stimulated GTPase reaction which shows only small differences among the four nucleotides (*25*). The K_d values we obtained for ITP and XTP are in fair agreement with those reported by Farr et al., who used a method that involved measuring the recovery of very low tubulin concentrations from a Mono Q anion exchange column (*38*). The K_d for ATP obtained by these authors was much higher than what we found. On the other hand, our value is in reasonable agreement with that reported by Zabrecky and Cole (*39*).

The major differences among the four nucleotides, however, are in their affinities for tubulin. Comparison of the K_d values in Table 1 shows that the largest decrease in affinity occurs when the 2-exocyclic amino group is removed from the purine ring, a 300-fold decrease, or replaced with an oxo function, a 1400-fold decrease. This suggests that the amino group may interact with an H-bond acceptor in the protein, either directly or through an intervening H_2O molecule. In a recent report in which the ability of 27 GTP analogues to support tubulin assembly was examined, it was found that ITP promoted assembly better than GTP, and it was concluded that there was no interaction between the 2-amino group and the protein (*40*). However, in this work, microtubule protein rather than purified tubulin was used so the contribution of NDPK activity has to be considered. Furthermore, the E-site was not depleted of nucleotide. The contribution of the 6-oxo function can be seen by comparing the affinities of ITP and ATP. There is about a 10-fold decrease in affinty when the oxygen is replaced with an amino group. Replacement of the 6-oxo function with a sulfur only changed the affinity of the nucleotide 2-fold (*41*), probably because guanine and 6-thioguanine have similar H-bonding properties (*42*). The involvement of the 6-oxo function in binding receives support from the crystal structure of FtsZ (*43*), a prokaryotic protein that is related to tubulin (*44*). The structure shows H-bonding between an asparagine residue and the 6-oxo function of GTP.

The order of specificity of tubulin for purine nucleoside triphosphates is similar to that of the G-protein H-ras; however, there are quantitative differences. The binding affinity of GTP for H-ras is about 3000-fold higher than for tubulin (*45*). And although the differences in affinity between GTP, ITP, and XTP are fairly similar for the two proteins, there is a major difference in the relative values for GTP and ATP. H-ras binds GTP with an affinity that is 10^7 times higher than for ATP (*45*), whereas in the case of tubulin, the difference is about 3×10^3. In the case of H-ras, it was concluded that, in addition to the loss of binding energy when the 6-oxo is replaced with an amino group, there is an unfavorable interaction of the 6-amino group with the protein backbone (*45*). Perhaps such an unfavorable interaction with ATP is lacking in tubulin.

From the cross-peak intensities in the ROESY experiments (Table 3), it is clear that there are differences in the average solution conformation among the four nucleotides. This is due to different values for the glycosidic torsion angle that lead to differences in the distance from H8 in the purine ring to hydrogens in the ribose ring. In ATP and XTP, H8 is closer to H2′ and H3′ than to H1′. In GTP, the average distance from H8 to H1′ is shorter than in ATP and XTP. In ITP, the distance is intermediate between that in GTP and ATP. The results are consistent with a preferred low anti conformation for GTP, a medium anti conformation for ITP, and a high anti conformation for ATP and XTP. Our results with ATP are consistent with those of André et al. (*37*), and the GTP results are consistent with those of Son et al., who concluded from studying the proton−proton Overhauser effect that the syn and anti conformations contributed equally to the conformation of GMP (*46*). Other groups have also noted the preference of guanine nucleotides for the syn conformation, and it has been suggested that this preference is due to an interaction between the 2-exocyclic amino group and the 5′-phosphate (see ref *36*). Although the preferred conformation in the bound state is not known, it is interesting that there is a correlation between the solution conformation of the nucleotides and the affinity for tubulin.[2]

We had hoped to use AzAGTP or AcAGTP to photolabel the E-site in polymerized as well as unpolymerized tubulin to determine whether a change occurs upon polymerization. It had been reported that AzAGTP is a nonhydrolyzable analogue of GTP that supported tubulin assembly (*48*). However, we found that both of these analogues acted as inhibitors of assembly, presumably in a competitive fashion since the inhibition could be overcome by increasing the concentration of GTP. This is consistent with the results for other γ-phosphoryl-modified GTP analogues, including GTPγF (*49*), GTPγS (*50*, *51*) [although one laboratory has reported that GTPγS supports assembly (*52*)], two fluorescent analogues of GTP (*53*), and a photoaffinity analogue with an *N*-(4-azidophenyl)-2-amino-3-(4-hydroxy-3-iodophenyl)propionamide group at the γ-position (*13*). The affinity of these analogues for tubulin varies with the complexity of the modification, but in general, it is reasonably high as seen for AzAGTP and AcAGTP (Table 1). From a strictly chemical view, the electron-withdrawing azidophenylanilido and acetylanilido groups at the γ-phosphoryl site would be expected to increase the rate of GTP hydrolysis. Moreover, other nonhydrolyzable or slowly hydrolyzable analogues promote microtubule formation. Thus, the fact that the analogues were nonhydrolyzable does not explain the lack of assembly-promoting activity. The electron crystallographic stucture of tubulin shows the E-site at one end of the dimer exposed to solvent and at the site of interaction with the α-monomer of another dimer upon polymerization (*1*, *16*). Thus, modifications at the γ-phosphoryl group could well interfere with dimer−dimer interactions during the assembly process.

GTP analogues modified in the ribose ring have also been used in tubulin assembly assays. Assembly-promoting activity has been found for a number of derivatives, including deoxyGTP nucleotides (*54*−*57*), analogues modified at the 2′- or 3′-position (*55*, *58*, *59*), and the 2′−3′ cleavage product

[2] The ROESY experiments were performed at 25 °C, while the K_d values were determined at 4 °C. Although there is some temperature dependence on the absolute syn/anti ratio of a particular nucleotide (*47*), we would not expect the relative average conformations of the four nucleotides to be temperature-dependent.

(*52*, *55*, *59*). Unfortunately, affinity constants have not been reported for these analogues, although limited competitive binding experiments demonstrated that the deoxyGTP derivatives had reduced affinty compared to that of GTP (*56*). More quantitative information would help in the elucidation of the importance of interactions of the ribose ring with tubulin.

ACKNOWLEDGMENT

We thank Dr. Chan-Ho Park for help in the synthesis of AzAGTP and AcAGTP.

REFERENCES

1. Nogales, E., Wolf, S. G., and Downing, K. (1998) *Nature 391*, 199−203.
2. Carlier, M.-F., and Pantaloni, D. (1981) *Biochemistry 20*, 1918−1924.
3. Melki, R., Fievez, S., and Carlier, M.-F. (1996) *Biochemistry 35*, 12038−12045.
4. Dougherty, C. A., Himes, R. H., Wilson, L., and Farrell, K. F. (1998) *Biochemistry 37*, 10861−10865.
5. Terry, B. J., and Purich, D. L. (1980) *J. Biol. Chem. 255*, 10532−10536.
6. Mejillano, M. R., Barton, J. S., Nath, J. P., and Himes, R. H. (1990) *Biochemistry 29*, 1208−1216.
7. Seckler, R., Wu, G.-M., and Timasheff, S. N. (1990) *J. Biol. Chem. 265*, 7655−7661.
8. Hamel, E., and Lin, C. M. (1990) *Biochemistry 29*, 2720−2729.
9. Hyman, A. A., Salser, S., Drechsel, D. N., Unwin, N., and Mitchison, T. J. (1992) *Mol. Biol. Cell 3*, 1155−1167.
10. Mitchison, T., and Kirschner, M. W. (1984) *Nature 312*, 237−242.
11. Hesse, J., Thierauf, M., and Ponstingl, H. (1987) *J. Biol. Chem. 262*, 15472−15474.
12. Linse, K., and Mandelkow, E. M. (1988) *J. Biol. Chem. 263*, 15205−15210.
13. Chavan, A. J., Kim, H., Haley, B. E., and Watt, D. E. (1990) *Bioconjugate Chem. 1*, 337−344.
14. Shivanna, B. D., Mejillano, M. R., Williams, T. D., and Himes, R. H. (1993) *J. Biol. Chem. 269*, 127−132.
15. Jayaram, B., and Haley, B. E. (1994) *J. Biol. Chem. 269*, 3233−3242.
16. Nogales, E., Downing, K. H., Amos, L. A., and Löwe, J. (1998) *Nat. Struct. Biol. 5*, 451−458.
17. Erickson, E. P. (1998) *Trends Cell Biol. 8*, 133−137.
18. Rasenick, M. M., Talluri, M., and Dunn, W. J., III (1998) *Methods Enzymol. 237*, 100−110.
19. Zor, T., Halifa, I., Kleinhaus, S., Chorev, M., and Selinger, Z. (1995) *Biochem. J. 306*, 253−258.
20. Tiwari, S. C., and Suprenant, K. A. (1993) *Anal. Biochem. 215*, 96−103.
21. Algaier, J., and Himes, R. H. (1988) *Biochim. Biophys. Acta 954*, 235−243.
22. Jacobs, M., and Huitorel, P. (1979) *Eur. J. Biochem. 99*, 613−622.
23. Purich, D. L., and MacNeal, D. L. (1978) *FEBS Lett. 96*, 83−86.
24. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−256.
25. Mejillano, M. R., Shivanna, B. D., and Himes, R. H. (1996) *Arch. Biochem. Biophys. 336*, 130−138.
26. Zeeberg, B., and Caplow, M. (1979) *Biochemistry 18*, 3880−3886.
27. Correia, J. J., Baty, L. T., and Williams, R. C., Jr. (1987) *J. Biol. Chem. 262*, 17278−17284.
28. Duanmu, C., Lin, C. M., and Hamel, E. (1986) *Biochim. Biophys. Acta 881*, 113−123.
29. Mejillano, M. R., and Himes, R. H. (1991) *Arch. Biochem. Biophys. 291*, 356−362.
30. Xu, S., and Gaskin, F. (1998) *Biochim. Biophys. Acta 1383*, 111−122.
31. Karr, T. L., Podrasky, A. E., and Purich, D. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5475−5479.
32. Bayley, P. M., and Manser, E. J. (1985) *Nature 318*, 683−685.
33. Hamel, E., Batra, J. K., and Lin, C. M. (1986) *Biochemistry 25*, 7054−7062.
34. Carlier, M.-F., Didry, D., and Pantaloni, D. (1987) *Biochemistry 26*, 4428−4437.
35. Roychowdhury, S., and Gaskin, F. (1988) *Biochemistry 27*, 7799−7805.
36. Saenger, W. (1984) *Principles of Nucleic Acid Structure* (Cantor, C. R., Ed.) Springer Advanced Texts in Chemistry, Springer-Verlag, New York.
37. André, F., Demassier, V., Bloch, G., and Neumann, J.-M. (1990) *J. Am. Chem. Soc. 112*, 6784−6789.
38. Farr, G. W., Yaffe, M. B., and Sternlicht, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5041−5045.
39. Zabrecky, J. R., and Cole, D. (1982) *Nature 296*, 775−776.
40. Muraoka, M., Fukuzawa, H., Nishida, A., Okano, K., Tsuchihara, T., Shimoda, A., Suzuki, Y., Sato, M., Osumi, M., and Sakai, H. (1999) *Cell Struct. Funct. 24*, 101−109.
41. Fishback, J. L., and Yarbrough, L. R. (1984) *J. Biol. Chem. 259*, 1968−1973.
42. Thewalt, U., and Bugg, C. E. (1972) *J. Am. Chem. Soc. 94*, 8892−8898.
43. Löwe, J., and Amos, L. A. (1998) *Nature 391*, 203−206.
44. Erickson, H. P. (1995) *Cell 80*, 367−370.
45. Rensland, H., John, J., Linke, R., Simon, I., Schlichting, I., Wittinghofer, A., and Goody, R. S. (1995) *Biochemistry 34*, 593−599.
46. Son, T.-D., Guschlbauer, W., and Guéron, M. (1972) *J. Am. Chem. Soc. 94*, 7903−7911.
47. Hemmes, P. R., Oppenheimer, L., and Jordan, F. (1974) *J. Am. Chem. Soc. 96*, 6023−6026.
48. Rasenick, M. M., and Wang, N. (1988) *J. Neurochem. 51*, 300−311.
49. Monasterio, O., and Timasheff, S. N. (1987) *Biochemistry 26*, 6091−6099.
50. Hamel, E., and Lin, C. M. (1984) *J. Biol. Chem. 259*, 11060−11069.
51. Shearwin, K. E., and Timasheff, S. N. (1992) *Biochemistry 31*, 8080−8089.
52. Kirsch, M., and Yarbrough, L. R. (1981) *J. Biol. Chem. 256*, 106−111.
53. Yarbrough, L. R., and Kirsch, M. (1981) *J. Biol. Chem. 256*, 112−117.
54. Hamel, E., del Campo, A. A., and Lin, C. M. (1984) *J. Biol. Chem. 259*, 2501−2508.
55. Hamel, E., del Campo, A. A., Lustbader, J., and Lin, C. M. (1983) *Biochemistry 22*, 1271−1279.
56. Hamel, E., Lustbader, J., and Lin, C. M. (1984) *Biochemistry 23*, 5314−5325.
57. Burns, R. G., and Symmons, M. F. (1995) *Biochemistry 34*, 2302−2308.
58. Han, Y., Malak, H., Chaudhary, A. G., Chordia, M. D., Kingston, D. G. I., and Bane, S. (1998) *Biochemistry 37*, 6636−6644.
59. Maccioni, R. B., and Seeds, N. W. (1983) *Biochemistry 22*, 1572−1579.

BI000966N