Interaction of Tubulin with Guanosine 5′-*O*-(1-Thiotriphosphate) Diastereoisomers: Specificity of the α-Phosphate Binding Region†

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ABSTRACT: The exchangeable nucleotide-binding site of tubulin has been studied using diastereoisomers A (S_p) and B (R_p) of guanosine 5′-*O*-(1-thiotriphosphate) (GTPαS) in which the phosphorus atom to which sulfur is attached is chiral. GTPαS(A) (10 μM) nucleated assembly of purified tubulin (20 μM) into microtubules in buffer containing 0.1 M 2-(*N*-morpholino)ethanesulfonic acid with 3 mM Mg^{2+} and 1 mM EGTA, pH 6.6 at 37 °C. With 0.2 mM GTPαS(A), the critical concentration (Cc; minimum protein concentration required for assembly) was 8 μM tubulin. Neither 0.2 mM GTP nor GTPαS(B) promoted microtubule assembly in buffer with 0.5–6.75 mM Mg^{2+} and 20–70 μM tubulin. The Cc values for GTPαS-(A)-induced assembly of tubulin in buffer with 30% glycerol and of microtubule protein (tubulin and microtubule-associated proteins) in buffer were lower than for GTP. GTPαS(A)-induced microtubules were more stable to the cold and to Ca^{2+}. GTPαS(A) and GTP but not GTPαS(B) bound tightly to tubulin at 4 °C. Although GTPαS(B) did not nucleate assembly, it did bind to tubulin since it was incorporated into the growing microtubule. Both isomers were hydrolyzed in the microtubules. These studies show that GTPαS(A) promotes tubulin assembly better than GTP and GTPαS(B) and that there is stereoselectivity at the α-phosphate binding region of tubulin. The stereoselectivity may be due to different MgGTPαS(A) and -(B) interactions with tubulin.

Tubulin is a unique GTP binding protein and has two GTP binding sites, one nonexchangeable and one exchangeable (Weisenberg et al., 1968; Jacobs et al., 1974). In the microtubule, the exchangeable GTP is hydrolyzed and both nucleotides are nonexchangeable. Since GTP at the exchangeable binding site may have several roles [reviewed by Bayley et al. (1993)], i.e., to promote nucleation and/or propagation of the growing microtubule, to effect dynamic instability of microtubules [also reviewed by Caplow (1992), Erickson and O'Brien (1992); Mandelkow and Mandelow (1992)], and to transfer GTP to a G-binding protein (Roychowdhury et al., 1993), it is important to understand the active site of GTP. Mg^{2+} is required for GTP to bind to tubulin (Correia et al., 1987). Mg^{2+} and other metals have dramatic effects on microtubule assembly, and some of these are due to the nucleotide–metal bound at the active site on tubulin (Gaskin, 1981; O'Brien et al., 1990; Shearwin & Timasheff, 1992; Hamel et al., 1992). Whether P_i from GTP is released slowly or rapidly after incorporation has not been resolved. Melki et al. (1990) report a slow release of P_i with a $t_{1/2}$ of 50 s. Stewart et al. (1990) found no evidence for transiently bound P_i either as GTP or GDP-P_i and attribute their results to be consistent with a slow conformational change in tubulin subunits after GTP hydrolysis and P_i release.

Studies with diastereoisomers should be helpful in understanding the role of GTP and metals at the active site of tubulin. Diastereoisomers A (S_p) and B (R_p) of GTPαS[1] (Figure 1) and of GTPβS are GTP analogues which contain sulfur in place of a nonbridging oxygen atom in one of the phosphoryl units of GTP. Many kinases and nucleoside triphosphatases

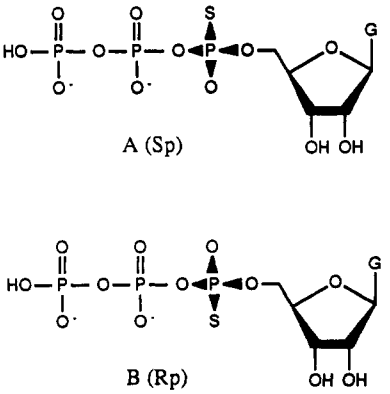

FIGURE 1: Diastereoisomers of GTPαS. G is guanine.

bind their substrates as β,γ-bidentate or as α,β,γ-tridentate chelates with Mg^{2+} (Eckstein, 1985; Leyh et al., 1985). Since it has been reported that Mg^{2+} chelates oxygen 31 000 times more strongly than sulfur (Pecoraro et al., 1984), screw sense specificity for binding may be due in part to the Mg–nucleotide complex.

Previous studies showed that most of the GTPβS(B) was not hydrolyzed in polymers (bundles of sheets and microtubules) assembled from tubulin in buffer with 30% glycerol whereas GTPβS(A) promoted assembly into microtubules and

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[1] Abbreviations: Cc, critical concentration; CrGTP, chromium guanosine 5′-triphosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EM, electron microscopy; FPLC, fast performance liquid chromatography; GDPαS, guanosine 5′-*O*-(1-thiodiphosphate); GMPS, guanosine 5′-*O*-(thiophosphate); GTPαS, guanosine 5′-*O*-(1-thiotriphosphate); GTPβS, guanosine 5′-*O*-(2-thiotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HPLC, high-performance liquid chromatography; MAPs, microtubule-associated proteins; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; MTP, two-cycle microtubule protein (tubulin + microtubule-associated proteins); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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was hydrolyzed (Roychowdhury & Gaskin, 1988). The GTPβS(B)-induced polymers were more cold stable than the GTPβS(A)-induced microtubules, which were slightly more stable than the GTP-induced polymers. Mg^{2+} (2–5 mM) had minimal effects on the tubulin polymers induced by GTPβS isomers. With microtubule protein (MTP) in buffer, both isomers promoted assembly into microtubules and 75% of the bound GTPβS(B) isomer and all of the GTPβS(A) were hydrolyzed. Studies with MTP and Mg^{2+} showed that GTPβS(A) required lower concentrations of Mg^{2+} to induce rings and cross-linked rings which were inactive in assembly. Thus, there is stereoselectivity at the β-phosphorus of the exchangeable nucleotide-binding site on tubulin.

Studies in this paper using GTPαS diastereoisomers demonstrate that there is also stereoselectivity at the α-phosphorus group. In particular, GTPαS(A) binds tighter than GTPαS(B) and promotes assembly much better than GTP and GTPαS(B). Both A and B isomers of GTPαS can be incorporated into the growing microtubule and are hydrolyzed.

EXPERIMENTAL PROCEDURES

Preparation of Microtubule Protein and Tubulin. Microtubule protein (MTP) was prepared from porcine brain by two cycles of assembly–disassembly as described previously (Shelanski et al., 1973). Tubulin was purified from MTP by Mono Q anion-exchange column chromatography using fast-performance liquid chromatography (FPLC) (Roychowdhury & Gaskin, 1986b). This tubulin is 99% pure on the basis of SDS/PAGE and contains little nucleoside diphosphate kinase, adenylate kinase, and ATPase activities. Aliquots of protein in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes) with 0.5 mM $MgCl_2$ and 1 mM EGTA, pH 6.6 (buffer A), were stored in liquid nitrogen until use. On the day of the experiment, 1–2 mL of protein was dialyzed for 2 h against 2 × 500 mL of buffer A (buffer change hourly) in a Crow-Englander-type thin-film microdialyzer at 4 °C. The dialyzed sample was warmed to 37 °C for 30 min, cooled to 4 °C for 30 min, and centrifuged at 260 000*g* for 6 min at 4 °C in a Beckman TL-100 centrifuge. The supernatant was diluted with buffer A or buffer containing 30% (v/v) glycerol to protein concentrations of approximately 2 mg/mL for tubulin and 1.8 mg/mL for MTP. Protein concentrations were determined by the method of Lowry (Lowry et al., 1951) using a BSA standard curve.

Assembly Studies. Tubulin or MTP in buffer A with or without 30% glycerol and with the addition of $MgCl_2$ or $MgSO_4$ (to 3 mM, unless indicated otherwise for tubulin) was incubated with different nucleotides at 4 °C for 25 min. The incubated protein was transferred to cold cuvettes and placed in a thermostatable sample changer at 37 °C. Microtubule assembly was monitored by turbidity at 350 nm by using the Varian DMS 300 recording spectrophotometer as previously described (Gaskin et al., 1974). Microtubule disassembly and reassembly was done by transferring the cuvettes with protein to 4 °C and placing them back to 37 °C. The assembly of tubulin was also monitored by centrifugation (Gaskin et al., 1974) and by negative staining electron microscopy. The JEOL 100CX was used to analyze the grids. Length determinations were done at time 10 and 30 min as described by Roychowdhury and Gaskin (1988). The error is 1 SD assuming a Gaussian distribution. The critical concentrations (Cc) for assembly were determined by using turbidimetric and sedimentation studies as previously described (Gaskin et al., 1974). Taxol (supplied by Dr. M. Suffness, Natural Products Branch, NCI) was used in taxol-induced assembly of tubulin in buffer at a final concentration of 30 μM.

Nucleotides. GTPαS(A) and GTPαS(B) were synthesized as described for ATPαS (Eckstein & Good, 1976). Guanosine 5′-*O*-(thiophosphate) (GMPS) was the starting material and was chemically synthesized from guanosine (Fisher) as previously described (Connolly et al., 1982). Tributylammonium pyrophosphate was purchased from Sigma. The isomers were separated by DEAE-Sephadex A-25 column chromatography (100 × 2.5 cm). HPLC on an anion-exchange Nucleosil 10 SB column from Mackry-Nagel was used to determine purity as previously described (Connolly et al., 1982). B isomer was not detected in the A isomer preparation which was 98% pure. However, the B isomer preparation with 95–99% purity contained up to 2% A isomer (depending on the preparation). Two methods were used to further purify GTPαS(B). The first was rechromatography through the DEAE-Sephadex A-25 column. No GTPαS(A) was detected by HPLC which was capable of detecting 0.5% GTPαS(A). Since we found tubulin binds GTPαS(A) tightly but GTPαS(B) weakly at 4 °C, a mixture of 0.4 mM nucleotide GTPαS(B) (98%) and GTPαS(A) (2%) was incubated with tubulin (7.4 mg/mL) in buffer A (3 mM Mg^{2+}) at 4 °C for 30 min. Free nucleotide was separated from tubulin by Sephadex G-25 chromatography. HPLC showed no GTPαS-(A) (<0.5%) in this GTPαS(B) preparation. GTPαS(A) was also purchased from New England Nuclear and was 96–99% pure, depending on the lot number. Both our synthesized GTPαS(A) and New England Nuclear GTPαS(A) were similar in promoting tubulin assembly. [α-^{35}S]GTPαS(A), [8-^{3}H]GTP, and GTP (Type IIS) were from New England Nuclear, ICN, and Sigma, respectively.

HPLC Analysis of Nucleotide Bound to Tubulin or to Microtubules. Unassembled proteins (0.6 mg/300 μL) were separated from free nucleotide by Sephadex G-25 column chromatography (5 × 0.25 cm) at 4 °C. Microtubules and other polymeric forms were pelleted through 0.6 mL of a 40% glycerol in buffer A cushion at 260 000*g* for 6 min at 35 °C in the TL 100 ultracentrifuge. Protein at pH 5.6 was precipitated with 50% ethanol, and A_{254} was used to estimate the nucleotide concentration (MacNeal & Purich, 1978; Roychowdhury & Gaskin, 1986a). Nucleotides were further analyzed by HPLC using the anion exchanger Nucleosil 10 SB column which separates GTPαS(A) and -(B) (Connolly, et al., 1982). We modified this method by using a linear gradient elution from 0.05 M KH_2PO_4 and 0.1 M KCl at pH 4.5 to 0.05 M KH_2PO_4 and 0.4 M KCl at pH 6.9 and a flow rate of 1 mL/min. In a typical experiment, the retention times of GMP, GMPS, GDP, GTP, GDPαS(A), GDPαS(B), GTPαS(A), and GTPαS(B) were 11.0, 14.0, 17.7, 21.0, 22.2, 23.5, 25.8, and 26.9 min, respectively. Concentrations of nucleotides were calculated from standard curves. Trapped nucleotide (based on radioactivity) was less than 0.5% of the starting nucleotide and was subtracted assuming no hydrolysis. A molecular mass of 100 000 kDa was used for tubulin dimer. MTP was estimated to contain 80% tubulin on the basis of SDS/PAGE (Roychowdhury & Gaskin, 1986b).

Analysis of Radiolabeled Nucleotide Bound to Microtubules. Microtubules were pelleted through a glycerol cushion as described above. The pellets were resuspended in 0.1 M Mes buffer, pH 6.6, and 5 μL samples were counted.

Analysis of Association Constants for Nucleotide Binding to Tubulin. The binding constants for GTP and GTPαS(A) to tubulin were determined using the Hummel and Dreyer (1962) method as modified by Levi et al. (1974) and Davis et al. (1993). Briefly, 100 μL aliquots of tubulin (0.7 mg/mL) in buffer with 3 mM Mg^{2+} and radiolabeled nucleotide

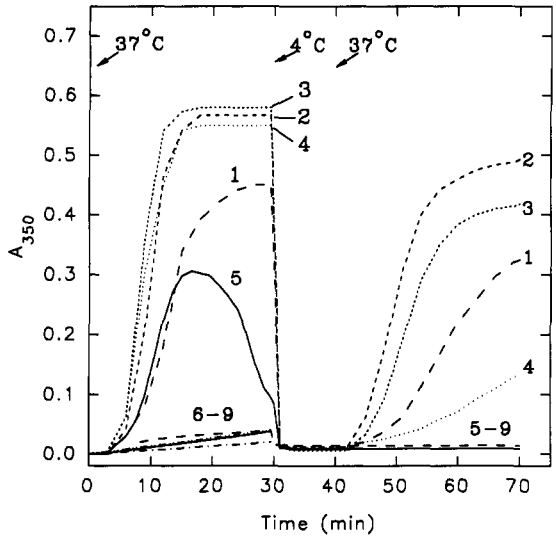

FIGURE 2: Effect of the GTPαS(A) concentration on the kinetics of assembly, disassembly, and reassembly of tubulin (20 μM) in buffer with 3 mM Mg(II). A_{350} was followed at 37 °C for 30 min and at 4 °C for 10 min and then warmed again to 37 °C for 30 min. Curves 1–6 were done at the following concentrations of GTPαS(A): (1) 0.5 mM; (2) 0.2 mM; (3) 0.1 mM; (4) 0.05 mM; (5) 0.01 mM; (6) 0.005 mM. Curves 7–9 were with 0.2 mM GTPαS(B), 0.2 mM GTP, or no added nucleotide, respectively. Microtubules (EM) were found with 1–5 for the first assembly and 1–4 for the second assembly.

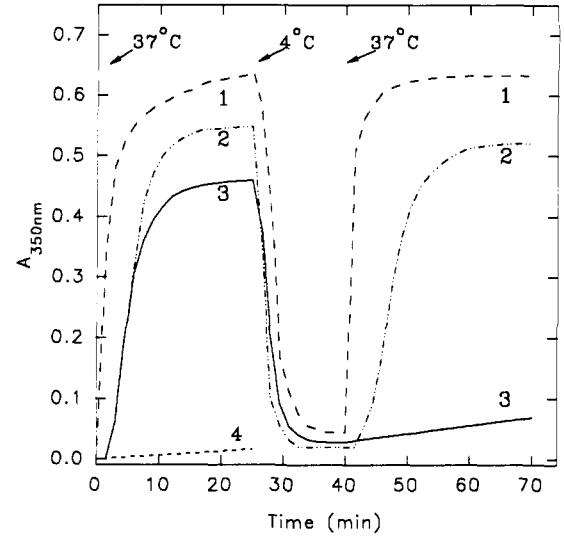

FIGURE 3: Kinetics of GTPαS(A)-, GTPαS(B)-, and GTP-induced assembly of tubulin (20 μM) in buffer with 3 mM Mg(II) and 30% glycerol. Nucleotides were at 0.2 mM (1) GTPαS(A), (2) GTP, or (3) GTPαS(B); or (4) with no added nucleotide. GTPαS(B) contains less than 0.5% GTPαS(A).

(12–800 nM) were gel-filtered at 4 °C at a flow rate of 0.5 mL/min through Bio-Gel P-10 columns (17 cm × 1 cm) preequilibriated with buffer containing 3 mM Mg^{2+} and the appropriate radiolabeled nucleotide at varying concentrations of 12–800 nM. [α-^{35}S]GTPαS(A)] was used at (3–44) × 10^7 cpm/mol, and [8-^{3}H]GTP was used at (3–24) × 10^7 cpm/mol. The stoichiometry of nucleotide binding to tubulin at each nucleotide concentration was determined by measuring the tubulin concentration with the Pierce (Rockford, IL) micro BCA (bicinchoninic acid) protein assay for microtiter plates and radiolabel (scintillation counting) in the protein fraction (0.5 mL) eluting from the column. The data were plotted according to Scatchard (1949), and the equilibrium association constants were determined by linear regression analysis of the data points.

RESULTS

GTPαS(A) Induced Assembly of Tubulin into Microtubules in Buffer without Glycerol and with Low Mg(II) Whereas GTPαS(B) and GTP Did Not Induce Tubulin Assembly under These Conditions. Our preliminary studies showed that 0.2 mM GTPαS(A) promoted assembly of tubulin (2 mg/mL) into microtubules in buffer A containing 3 mM Mg^{2+}. However, 0.2 mM GTP and GTPαS(B) did not promote assembly using varying Mg^{2+} concentrations of 0.5–6.75 mM and tubulin concentrations of 2–7 mg/mL. Thus, the GTPαS-(A)-induced assembly was studied in more detail.

1 mM Mg^{2+} resulted in little assembly and 2 mM resulted in 43–47% of the assembly found for 3 and 3.5 mM Mg^{2+}. Microtubules were seen by electron microscopy (EM) for 2, 3, and 3.5 mM Mg^{2+}. However, microtubules and thin sheets were found with 4 mM Mg^{2+}. In summary, optimal assembly of tubulin into microtubules was found with 3.0–3.5 mM Mg^{2+} and 0.2 mM GTPαS(A). Thus, all experiments with tubulin were done with 3 mM Mg^{2+}.

Figure 2 demonstrates the kinetics of GTPαS(A)-induced assembly of tubulin at 37 °C, disassembly at 4 °C, and reassembly at 37 °C. GTPαS(A) was used at concentrations of 0.005, 0.01, 0.05, 0.1, 0.2, and 0.5 mM. All concentrations except 0.005 mM were capable of promoting microtubule assembly (verified by EM) once. With 0.01 mM GTPαS(A) there was assembly and then disassembly at 37 °C. The rate of assembly with 0.5 mM GTPαS(A) was significantly slower than with 0.05–0.2 mM GTPαS(A) concentrations. Rapid disassembly with a $t_{1/2}$ of 1.1 min was found at 4 °C, and no microtubules were found by EM after 10 min at 4 °C. A 0.01 mM concentration of GTPαS(A) promoted assembly only once. The second assembly rate at the other concentrations was slower than the first assembly rate, and A_{350} values at 30 min were 84%, 71%, 70%, and 21% for 0.2, 0.1, 0.5, and 0.05 mM GTPαS(A), respectively, when compared to the first assembly. Again, microtubules were found by EM.

In order to learn more about the minimum concentration of tubulin required for assembly, assembly with 0.2 mM GTPαS(A) was followed with time as a function of tubulin concentration. Plots of both A_{350} and pelletable protein against initial protein concentrations resulted in straight lines with an intercept of 0.8 mg/mL (8 μM), the critical concentration (Cc) (data not shown).

GTPαS(A)-, GTPαS(B)-, and GTP-Induced Assembly of Tubulin in Buffer Containing 30% Glycerol. The assembly of tubulin (2 mg/mL) in buffer A with 3 mM Mg^{2+} and 30% glycerol was followed by A_{350} and EM at 37 °C in the presence of 0.2 mM GTPαS(A) and -(B) isomers and GTP (Figure 3). GTPαS(A) promoted assembly faster (no detectable lag) with a $t_{1/2}$ of 2 min compared to 5 min for the other nucleotides and had a slower rate of disassembly ($t_{1/2}$ = 4 min compared to 2 min). The reassembly kinetics with the A isomer were essentially the same as the first assembly kinetics, and GTP reassembly kinetics were similar. However, 0.2 mM GTPαS-(B) (<0.5% A isomer) did not promote a second assembly. If an additional 0.2 mM GTPαS(B) was added after disassembly of the GTPαS(B)-treated tubulin, there was reassembly and so the tubulin was still active (data not shown).

Although microtubules were seen in all assemblies, the A isomer of GTPαS produced more bundles than the B isomer. The GTPαS bundles were similar to GTPβS-induced bundles described by Roychowdhury & Gaskin (1988). GTP induced microtubules and thin sheets, as previously described (Roychowdhury and Gaskin, 1988). The lengths of the GTP-

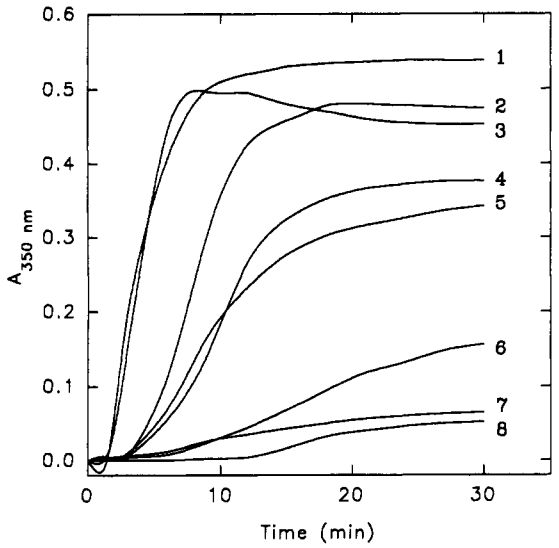

FIGURE 4: Minimum nucleotide concentration required to promote assembly of tubulin (20 μM) in buffer with 3 mM Mg^{2+} and 30% glycerol. (1) 50 μM GTPαS(B); (2) 5 μM GTP; (3) 5 μM GTPαS-(A); (4) 1 μM GTPαS(A); (5) 20 μM GTPαS(B); (6) 0.5 μM GTPαS-(A); (7) 5 μM GTPαS(B); (8) 2 μM GTP. GTPαS(B) contains less than 0.5% GTPαS(A).

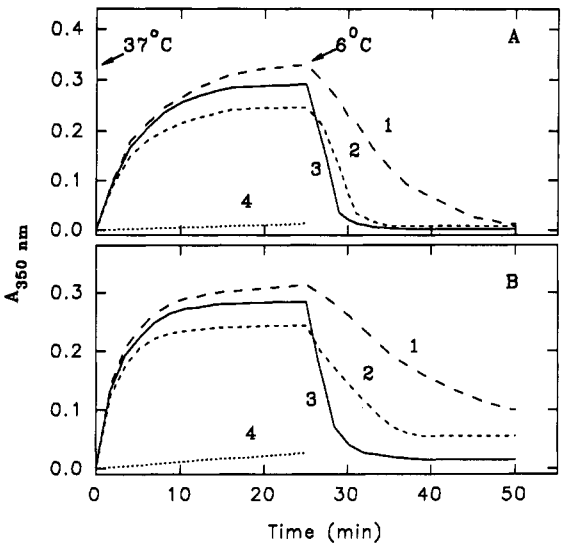

FIGURE 5: Kinetics of GTPαS(A)-, GTPαS(B), and GTP-induced assembly of microtubule protein at 37 °C and disassembly kinetics at 6 °C. (A) 0.2 mM nucleotide: (1) GTPαS(A); (2) GTPαS(B); (3) GTP. (4) No added nucleotide. (B) 0.5 mM nucleotide: (1) GTPαS(A); (2) GTPαS(B); (3) GTP. (4) No added nucleotide.

induced polymers at 10 min were approximately twice those of the GTPαS(A) polymers: 5.5 ± 1.5 μm versus 2.6 ± 0.9 μm. Comparison of these lengths with those found at 30 min showed little or no length redistribution for GTP-induced polymers (6.1 ± 2.1 μm) and GTPαS(A)-induced polymers (2.3 ± 0.7 μm), respectively. The lack of significant length redistribution for GTP-induced microtubules is in agreement with the study of Davis et al. (1993).

Cc's were determined to be 1.5 and 5.0 μM for GTPαS(A)- and GTP-induced assembly in buffer/glycerol, respectively (data not shown).

To learn more about the minimum nucleotide concentrations required to promote assembly of tubulin in buffer/glycerol, turbidity was used to monitor assembly at nucleotide concentrations of 0.5, 1, 2, 5, 20, and 50 μM. Figure 4 shows that 5 μM GTPαS(A) promotes assembly with a shorter lag (0.8 min) than 5 μM GTP (2.7 min) and that 5 μM GTPαS(B) does not promote assembly. Similar kinetics and levels of assembly are found with 5 μM GTPαS(A) and 50 μM GTPαS-(B) and with 1 μM GTPαS(A) and 20 μM GTPαS(B). These results suggest that the A isomer is more effective in promoting assembly than GTP and that 10–20-fold more B isomer than A isomer is needed for comparable rates and levels of assembly. Although the small amount of contaminating A isomer (<0.5%) in the B isomer preparation may help in nucleation, the assembly with the B isomer is still better than if the only added nucleotide was the contaminating A isomer. By EM, mainly microtubules were found with 1 μM GTPαS(A), 20 μM GTPαS(B), and 5 μM GTP. Increasing concentrations of GTPαS A and B isomers resulted in increasing numbers of bundles of microtubules similar to those described by Roychowdhury and Gaskin (1988) for GTPαS(B)-induced assembly of tubulin. Length measurements at 10 min on polymers induced by 5 μM GTP and GTPαS(A) were 6.3 ± 2.1 and 3.6 ± 1.1 μm, respectively.

GTPαS(A)-, GTPαS(B)-, and GTP-Induced Assembly of MTP in Buffer. Initially, MTP assembly into microtubules was done at 0.2 mM nucleotide. The three nucleotides promoted assembly into microtubules (EM) with a higher level with the A isomer than with GTP, which was higher than with the B isomer (Figure 5A). Disassembly at 6 °C showed $t_{1/2}$ times of 8, 2, and 4 min for the A isomer, GTP, and the B isomer respectively. The stability of the microtubules to the cold was further analyzed after assembling with 0.5 mM nucleotide (Figure 4B). The disassembly $t_{1/2}$ values at 0.5 mM nucleotide were about twice as long as with 0.2 mM nucleotide for the isomer-induced polymers, with corresponding times of 15 and 7 min for the A isomer and the B isomer, respectively. More microtubules and filaments were found after 25 min at 6 °C with the A isomer than with the B isomer. The 0.5 mM GTP-induced microtubule disassembly rate was similar to that for 0.2 mM GTP (2 min). All samples except those with 0.2 mM GTPαS(B) were able to assemble a second time.

Table 1: Comparison of Ca^{2+} Effects on MTP Assembly and Disruption of Microtubules Assembled with GTPαS(A) or GTP

sample[a]	Ca^{2+} 0.6 mM[b]	Ca^{2+} 1.5 mM
MTP + GTPαS(A)	100[c]	70
MTP + GTP	23	0
GTPαS(A)-induced MTs	100	90
GTP-induced MTs	30	0

[a] MTP (1.8 mg/mL) + nucleotide (0.5 mM) samples were assembled with or without added Ca^{2+} for 30 min. MTs: microtubules. Ca^{2+} was added to MTs after assembly for 15 min and disassembly followed until a new equilibrium was reached (15–45 min). [b] Excess concentration of Ca^{2+} assuming 1 mM EGTA binds 1 mM Ca^{2+}. [c] % A_{350} of Ca^{2+}-treated sample/A_{350} of control with no added Ca^{2+}.

Cc's were determined to be 0.6 and 2.7 μM for GTPαS(A)- and GTP-induced assemblies, respectively (data not shown).

Table 1 shows that lower concentrations of Ca^{2+} disrupt microtubules or inhibit assembly of GTP-induced microtubules when compared with GTPαS(A)-induced microtubules. A 0.6 mM concentration of Ca^{2+} has no effect on the microtubule ⇄ tubulin equilibrium with GTPαS(A) but shifts the equilibrium to more tubulin (only 23–30% of the expected mass of microtubules are found) with GTP. A 1.5 mM concentration of Ca^{2+} essentially inhibits MTP and GTP assembly and disrupts GTP-induced microtubules. However, with GTPαS(A) and 1.5 mM Ca^{2+}, 70% of the control assembly is found and addition of 1.5 mM Ca^{2+} to microtubules

Table 2: HPLC Analysis of Nucleotide Bound to Tubulin in Buffer[a]

added nucleotides	mol of GDP/mol of tubulin	mol of GTP/mol of tubulin	mol of GTPαS(A)/mol of tubulin	mol of GTPαS(B)/mol of tubulin
no additions	0.17	0.80	–[b]	–
0.2 mM GTP	0.09	1.54	–	–
0.2 mM GTPαS(A)	0.05	0.90	0.60	–
0.2 mM GTPαS(B) + 4 μM GTPαS(A)	0.06	0.81	0.21	<0.02
0.2 mM GTPαS(B) + <1 μM GTPαS(A)	0.18	1.03	<0.02	<0.02

[a] Tubulin (2 mg/mL) in buffer containing 3 mM Mg^{2+} was incubated with nucleotide for 25 min at 4 °C and chromatographed on Sephadex G-25 (5 × 0.7 cm) to separate protein from unbound nucleotide. Bound nucleotide was determined as described in the Experimental Procedures. Before incubation, the nucleotide/tubulin ratios were 0.37 mol of GDP and 1.02 mol of GTP/mol of tubulin dimer. [b] None.

results in minimal disassembly (10%) of GTPαS(A)-induced microtubules.

Nucleotide Binding to Tubulin in Buffer at 4 °C. Nucleotides bound to tubulin after incubation with 0.2 mM nucleotide at 4 °C for 25 min and after chromatography to remove unbound nucleotide (Table 2) showed that GTPαS-(A) binds as well as GTP (Table 2). The table suggests that 0.8–1.0 mol of GTP/mol of tubulin is at the nonexchangeable site and approximately 0.6 mol/mol of GTP or GTPαS(A) is bound at the exchangeable site. Even with 4 μM GTPαS-(A), 0.2 mol/mol of the isomer are bound to the exchangeable site of tubulin (20 μM). With further purification of GTPαS-(B), no GTPαS(A) isomer is detected bound to tubulin. Binding of GTPαS(B) to tubulin is not detected, suggesting it is weakly bound or does not bind in the cold. Further evidence that GTPαS(B) does not bind tightly to tubulin at 4 °C comes from an analysis of the bound nucleotides from taxol-induced polymers assembled from nucleotide-treated tubulin in buffer. GDPαS(B) and GTPαS(B) were not found in the polymer (data not shown).

The equilibrium binding constants of GTP and GTPαS(A) for tubulin in buffer containing 3 mM Mg^{2+} at 4 °C were determined using radiolabeled nucleotide and the modified Hummel and Dreyer method as described in the Experimental Procedures. The K_a values for GTP and GTPαS(A) binding to tubulin are 1.3×10^7 M^{-1} and 1.8×10^7 M^{-1}, respectively (Figure 6). Thus, GTPαS(A) binds slightly tighter than GTP. Our K_a value for GTP–Mg binding to tubulin is in agreement with published values of 0.25×10^7 to 4.5×10^7 M^{-1} (Levi et al., 1974; Jacobs & Caplow, 1976; Zeeberg & Caplow, 1979). The binding constant for GTPαS(B) to tubulin was not determined since the binding of GTPαS(B) is weak or neglible at 4 °C (Table 2) and radioactive GTPαS(B) is not available and would have to be synthesized to essentially 100% purity and free of GTPαS(A) since GTPαS(A) binds tightly to tubulin (Table 2).

Nucleotide Binding to Microtubules Assembled from Tubulin in Buffer with or without Glycerol. Table 3 shows that microtubules assembled with GTPαS(A) in buffer contain 0.8 mol of GDPαS(A)/mol of tubulin dimer and that GTPαS-(A) was not detected. Thus, GTPαS(A) is hydrolyzed during or after assembly. With GTPαS(A) and GTP, 0.8 and 0.7 mol of hydrolyzed nucleotide/mol of tubulin dimer were found, respectively, after assembly in buffer/glycerol. Only 0.4 mol of GDPαS(B)/mol of tubulin dimer was found after assembly with GTPαS(B) in buffer/glycerol. Again, only hydrolyzed isomers were found.

Nucleotide Binding to Microtubules Assembled from MTP in Buffer. An HPLC analysis showed that 0.9 mol of GDPαS-(A) and 0.6 mol of GDPαS(B)/mol of tubulin are found in the microtubules assembled from MTP with GTPαS(A) and -(B), respectively. Microtubules assembled with GTP contained 0.8 mol/mol of GDP. To learn more about the relative affinity of binding of GTP and GTPαS(A) to MTP that assembles into microtubules, radioactive isotopes were used in assembly. Quantification of radioactivity in the microtubule pellets showed that the GTPαS(A)/GTP relative affinity ratio is 1.5 (Table 4). This ratio is in agreement with the ratio of 1.4 that we found for the equilibrium binding constants for GTPαS(A) ($K_a = 1.8 \times 10^7$ M^{-1}) and GTP ($K_a = 1.3 \times 10^7$ M^{-1}) to tubulin in buffer at 4 °C.

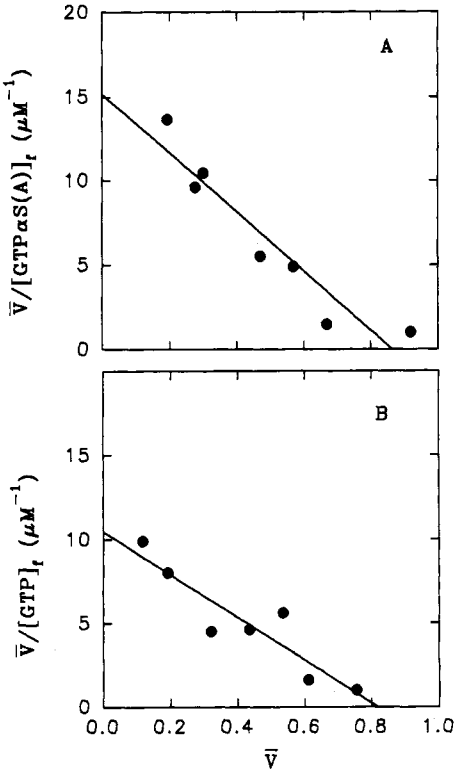

FIGURE 6: Scatchard plots for binding of GTPαS(A) and GTP to tubulin in buffer with 3 mM Mg^{2+}. Aliquots of tubulin were fractionated on 17 × 1 cm columns of Bio-Gel P-10 equilibriated with varying concentrations of radiolabeled nucleotide in buffer with 3 mM Mg^{2+}. 0.5 mL fractions were collected, and the tubulin and radiolabeled nucleotide content were determined (Experimental Procedures). $\bar{v}$ is the mol of nucleotide bound/mol of tubulin. (A) GTPαS(A) binding to tubulin. $[GTP\alpha S(A)]_f$ is the free GTPαS(A) concentration. (B) GTP binding to tubulin. $[GTP]_f$ is the free GTP concentration.

DISCUSSION

The studies described in this paper demonstrate that there is stereoselectivity at the α phosphorus of the exchangeable nucleotide-binding site of tubulin. GTPαS(A) binds tighter than GTPαS(B) and promotes nucleation. However, both nucleotides are incorporated into the growing microtubule and hydrolyzed to the corresponding GDPαS(A) or -(B) isomer. Thus, there does not appear to be stereoselectivity of

Table 3: GTPαS(A) Binding to Microtubules Assembled from Tubulin in Buffer ± Glycerol[a]

buffer	added nucleotide	A_{254}	mol of GDP/mol of tubulin	mol of GTP/mol of tubulin	mol of GDPαS(A)/mol of tubulin	mol of GDPαS(B)/mol of tubulin
MME[b]	GTPαS(A)	1.63	0.05	0.75	0.78	–[d]
MMEG[c]	GTP	1.85	0.68	0.80	–	–
MMEG	GTPαS(A)	1.73	0.08	0.72	0.79	<0.01
MMEG	GTPαS(B)	1.60	0.30	0.60	<0.01	0.40

[a] Tubulin (2.0 mg/mL) in buffer containing 3 mM Mg^{2+} was incubated with nucleotides at 4 °C for 25 min. After assembly at 37 °C for 30 min, the nucleotide bound to microtubules was determined by A_{254} and HPLC as described in the Experimental Procedures. GTPαS(B) and GTP did not promote assembly of tubulin in MME. Bound GTPαS(A) or -(B) were not detected. [b] 0.1 M Mes, 3 mM Mg^{2+}, 1 mM EGTA. 0.2 and 0.5 mM added nucleotide gave essentially the same results, and an average is given. [c] 0.1 M Mes, 3 mM Mg^2, 1 mM EGTA, 30% v/v glycerol. 0.4 mM added nucleotide. [d] None.

Table 4: Relative Affinity of Binding of GTP and GTPαS(A) to Microtubule Protein That Assembles into Microtubules[a]

sample	+GTPαS(A) (mM)	+GTP (mM)	mol of GTPαS(A)/mol of tubulin[b]	mol of GTP/mol of tubulin	relative affinity, GTPαS(A)/GTP
1	0.2		0.80 ± 0.16		
2	0.2	0.1	0.49 ± 0.09	0.16 ● 0.04	1.56
3	0.2	0.2	0.42 ± 0.07	0.27 ± 0.05	1.53
4	0.2	0.4	0.27 ± 0.01	0.39 ± 0.04	1.38
5		0.2		0.58 ± 0.07	

[a] MTP (1.8 mg/mL) in buffer was incubated at 4 °C for 25 min with either 0.2 mM [^{35}S]GTPαS(A) and various concentrations of GTP or 0.2 mM GTPαS(A) and various concentrations of [8-^{3}H]GTP. After assembly at 37 °C for 25 min, microtubules were found in all samples. The microtubules were pelleted and assayed as described in the Experimental Procedures. [b] Numbers are nucleotide binding and hydrolysis was not determined.

the α-phosphate of GTPαS in regard to the elongation step and hydrolysis.

The Cc for GTPαS(A)-induced assembly in buffer with 3 mM Mg^{2+} was 8 μM. This number is low and in contrast to the much higher reported Cc obtained for GTP-induced assembly in buffer with 10 mM Mg^{2+}, i.e., 25 μM tubulin (Herzog & Weber, 1977). With FPLC-purified tubulin, we also find that even with high Mg^{2+} (10 mM) the Cc > 25 μM (Roychowdhury & Gaskin, 1986b). Thus, GTPαS(A) should be a useful analogue for GTP in tubulin assembly in buffer since (1) it promotes assembly at a lower Cc, (2) it is hydrolyzed (0.8 mol/mol of tubulin dimer in the microtubule), (3) it requires low concentrations of Mg^{2+}, (4) the GTPαS(A)-induced microtubules disassemble at 4 °C and the tubulin will reassemble into microtubules, 85% of the first assembly with 0.2 mM GTPαS(A) at 37 °C, and (5) the K_a values for nucleotide binding to tubulin are similar although GTPαS-(A) binds slightly tighter than GTP.

Addition of glycerol to buffer results in a lower Cc and alleviates the high Mg^{2+} requirement for tubulin assembly with GTP (Lee & Timasheff, 1975). We found that the Cc for tubulin assembly was lower (1.5 μM) with GTPαS(A) than with GTP (5 μM) with buffer/glycerol/3 mM Mg^{2+}. Studies with MTP in buffer show that the Cc was also lower for GTPαS(A) than for GTP with the corresponding Cc's of 0.6 and 2.7 μM, respectively. Since the Cc is equal to the reciprocal of the equilibrium constant for adding a monomer to polymer (Oosawa & Kasai, 1962; Oosawa & Higashi 1967), the greater the Cc, the more difficult it is to nucleate assembly. Thus, GTPαS(A) is more effective at the nucleation step than GTP. Because GTPαS(B) failed to promote reassembly of tubulin in buffer/glycerol or MTP in buffer, it may not promote nucleation. The first assembly may be due to trace GTPαS-(A) in the GTPαS(B) preparation which is used up during the first assembly. Another explanation is that GTPαS(B) is consumed in the polymerization.

In Oosawa's nucleated-condensation model, which has been used to characterize tubulin assembly (Gaskin et al., 1974), the kinetics of tubulin assembly show an initial lag which is due to the difficulty of nucleation. The longer the lag time, the more difficult the nucleation step. It follows that the shorter the lag time, the faster the nucleation step and the greater the number of nucleation centers with time, which will result in more polymers with a shorter length. Thus, the hypothesis that GTPαS(A) is more effective than GTP at the nucleation step in tubulin assembly is compatible with the shorter lag time (<$^1/_2$) and shorter polymers (approximately half the mean length) found with GTPαS(A) than with GTP.

Further support that GTPαS(A) is more effective than GTP in promoting assembly comes from the lower nucleotide requirement to promote assembly of tubulin in glycerol (Figure 4). At 5 μM nucleotide the lag time is shorter with GTPαS-(A) than with GTP. A 1 μM concentration of GTPαS promotes assembly to approximately 85% of the maximum compared to <10% for 2 μM GTP at 30 min. It is of interest to note that the assembly with the B isomer is still better than if the only added nucleotide was due to possible contaminating GTPαS(A). Thus, tubulin–GDPαS(B) stabilizes the microtubules. Only 0.4 mol of GDPαS(B) is found in the pelleted polymers compared to 0.8 mol of GDPαS(A) (Table 3). This may be explained by the stronger binding of GTPαS(A) to tubulin.

Several hydrolyzable analogues of GTP have been shown to promote microtubule assembly without hydrolysis or with a slow hydrolysis. The assembled structures are more stable to isothermal dilution or to the cold. These include (1) CrGTP–MTP in buffer (MacNeal & Purich, 1978; Gaskin, 1981); (2) GTPγS–MTP or tubulin in buffer/glycerol (Roychowdhury & Gaskin, 1986a); (3) GMPCPP–tubulin in buffer (Hyman et al., 1992); (4) GTPβS(B)–tubulin in buffer/glycerol (90% unhydrolyzed) or MTP in buffer (25% unhydrolyzed) (Roychowdhury & Gaskin, 1988). However, in this paper we show that GTPαS(A)-induced microtubules and bundles of microtubules and sheets are more stable to the cold and to Ca^{2+}, even though GTPαS(A) is hydrolyzed. In GTP-induced assembly, both β,γ-phosphate and Mg–nucleotide bonds are broken, and it has been suggested that the phosphate bond may be broken before the metal bond (Carlier

et al., 1991). Thus, increased microtubule stability with GTPαS(A) may be due to tightly bound MgGDPαS(A) instead of GDP.

The greater stability of the microtubules induced by GTPαS-(A) and containing GDPαS(A) and partial enhanced stability of microtubules containing GDPαS(B) compared to GTP suggest that the α-phosphate may also be important in conferring stability. Since Mg binds to oxygen 31 000 times stronger than to sulfur (Pecoraro et al., 1984), the diastereoisomers of GTPαS and GTPβS as well as GTPγS and GTP should have different Mg–nucleotide binding affinities for tubulin during or after hydrolysis. It would be important to determine whether these diastereoisomers bind as Mg–nucleotide complexes as GTP does and whether Mg is released after assembly as it is with GTP (Correia et al., 1987, 1988). The enhanced microtubule stability seen with other unhydrolyzed analogues which could have been hydrolyzed may be solely or partly due to the Mg^{2+}–nucleotide binding environment on tubulin.

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