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Stereoselectivity of the Guanyl-Exchangeable Nucleotide-Binding Site of Tubulin Probed by Guanosine 5′-*O*-(2-Thiotriphosphate) Diastereoisomers†

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ABSTRACT: The active site of the exchangeable nucleotide-binding site of tubulin was studied by using diastereoisomers A (S_P) and B (R_P) of guanosine 5′-*O*-(2-thiotriphosphate) (GTPβS) where the phosphorus atom to which sulfur is attached is chiral. Turbidimetric measurements were used to follow kinetics, and electron microscopy was used to evaluate polymeric forms. Both isomers at 0.5 mM promoted the assembly of tubulin in buffer containing 0.1 M 2-(*N*-morpholino)ethanesulfonic acid, 30% glycerol, 3 mM $MgCl_2$, and 1 mM EGTA, pH 6.6, 23–37 °C. GTPβS(A) promoted assembly into microtubules, although a few bundles were also found by electron microscopy. However, GTPβS(B) induced assembly of tubulin into bundles of sheets and microtubules. As expected, 0.5 mM GTP induced tubulin to assemble into microtubules, thin sheets, and a few bundles. Both GTP and GTPβS(A) were hydrolyzed in the tubulin polymers. However, more than 95% of the bound GTPβS(B) was not hydrolyzed. Higher concentrations of GTPβS(B), i.e., 1 mM, also induced bundles of sheets and microtubules, with 86% of the thionucleotide bound as the triphosphate. The GTPβS(B)-induced polymers were considerably more cold stable than the GTBβS-(A)-induced microtubules, which were more cold stable than GTP-induced polymers. Mg(II) (2–5 mM) had minimal effects on the structures induced by GTPβS(A) or -(B) isomers in the tubulin assembly system. However, at 1 mM Mg(II), no assembly was found with GTPβS(A) and tubulin. The assembly studies on GTPβS(A) and -(B) with microtubule protein (tubulin plus microtubule-associated proteins) showed similar kinetics at 0.5 mM Mg(II) in buffer without glycerol, and microtubules were the major structures. Most of the bound nucleotide was hydrolyzed. With varying Mg(II) and GTP or GTPβS(B) concentrations, only microtubules were found. However, different Mg(II)/GTPβS(A) ratios resulted in different polymeric forms with rings and cross-linked rings being the predominant polymers at high Mg(II) or GTPβ(A) concentrations. These studies demonstrate stereoselectivity at the β phosphorus of the exchangeable nucleotide-binding site on tubulin. The GTPβS(A) isomer is a better GTP analogue than GTPβS(B) in the tubulin assembly system, whereas GTPβS(B) is a better GTP analogue in the microtubule protein assembly system.

Tubulin is a unique guanine-binding protein with one exchangeable and one nonexchangeable binding site (Weisenberg et al., 1968; Dustin, 1984). The nonexchangeable binding site contains tightly bound GTP, which is released only when the protein is denatured. GTP at the exchangeable site is needed for optimal assembly into microtubules and is hydrolyzed. There is still some question as to whether hydrolysis takes place simultaneously or after subunit addition (Carlier & Pantaloni,

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1981; Farrell et al., 1983; Caplow et al., 1985; Hamel et al., 1986; O'Brian et al., 1987). Both nucleotide-binding sites are nonexchangeable after assembly into microtubules. The guanine nucleotide at the exchangeable site is able to exchange GTP or GDP with several guanine triphosphate analogues. Nonhydrolyzable analogues of GTP have also been reported to support microtubule assembly from microtubule proteins (tubulin plus microtubule-associated proteins), although at a slower rate. Chromium guanosine triphosphate (CrGTP)[1] also promotes assembly and is only partially hydrolyzed (MacNeal & Purich, 1978; Gaskin, 1981). Guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) induces microtubule protein or purified tubulin to assemble into microtubules or bundles of sheets and microtubules, and GTPγS is not hydrolyzed in the polymers (Kirsch & Yarbrough, 1981; Roychowdhury & Gaskin, 1986a). It has been reported that microtubules induced by nonhydrolyzable analogues of GTP have greater stability than GTP-induced microtubules (Weisenberg et al., 1976; Arai & Kaziro, 1976; Weisenberg & Deery, 1976), although others contradict this (Penningroth & Kirschner, 1977; Hamel et al., 1984). The diastereoisomers of guanosine 5′-*O*-(2-thiotriphosphate) GTPβS(A) or (S_P) and GTPβS(B) or (R_P) have also been reported to promote assembly of tubulin in buffer containing glycerol or microtubule protein in buffer (Hamel & Lin, 1984), and the B-isomer-induced microtubule protein polymer was more cold resistant. We reexamined GTPβS(A) and -(B) isomers for their induction of tubulin and microtubule protein assembly with an emphasis on the morphology and stability of the polymers and GTPBS hydrolysis. Since Mg(II) is required for optimal assembly with GTP or CrGTP (Gaskin, 1981) and the GTPβS isomers have different binding affinities for metals (Jaffe & Cohn, 1978), we also looked at the effect of Mg(II) and the Mg/nucleotide ratio on assembly parameters.

Materials and Methods

Preparation of Microtubule Protein and Tubulin. Microtubule protein was prepared from porcine brain by two cycles of assembly–disassembly as described previously (Shelanski et al., 1973). Tubulin was purified from microtubule protein by Mono Q anion-exchange and Mono S cation-exchange column chromatography using the Pharmacia fast performance liquid chromatography system (FPLC) as previously described (Roychowdhury & Gaskin, 1986b). This tubulin is 99% pure on the basis of PAGE and is free of nucleoside diphosphate kinase (NDPK), adenylate kinase, and ATPase activities. Both protein preparations were stored in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.6, in liquid nitrogen. On the day of the experiment 1–2 mL of protein (5–10 mg/mL) was dialyzed for 3 h against 3 × 500 mL of 0.1 M MES with 0.5 mM $MgCl_2$ and 1 mM EGTA, pH 6.6 (buffer changed hourly) in a Crowe-Englander-type thin-film microdialyzer at 4 °C. The dialyzed sample was centrifuged at 100000*g* for 30 min at 4 °C and the supernatant diluted to the desired protein concentration. The protein was warmed to 37 °C for 30 min and cooled to 4 °C for 10 min before use. These protein preparations will not assemble into microtubules at 37 °C unless appropriate cofactors are added. Several purifications were done by use of radiolabeled GTP in all the GTP additions. The purified tubulin contained 0.02 mol of radiolabeled GTP analyzed as described previously (Roychowdhury & Gaskin, 1986b; Kirsch & Yarbrough, 1981). This result suggests that the exchangeable nucleotide-binding site in purified tubulin contains 0.02 mol of GTP. No labeled GTP was detected in microtubule protein.

Characterization of Assembly. A Cary 118 C recording spectrophotometer with a thermostatable sample changer was used for turbidimetric measurements of A_{350} as a function of time at 37 °C as described previously (Gaskin et al., 1974a). Such measurements are proportional to the weight concentration of microtubules (Gaskin et al., 1974a). However, they are not quantitative for sheets and other microtubule protein aggregates (Gaskin & Kress, 1977). Thus, to quantitate assembled protein, 1-mL samples were centrifuged through 1 mL of 40% glycerol in buffer in an ultracentrifuge at 100000*g* for 45 min at room temperature, and the protein concentrations of the resuspended pellets were determined by the method of Lowry et al. (1951), using crystalline bovine serum albumin as a standard. Electron microscopy by negative staining and thin sectioning of pellets was done with a JEOL 1200 electron microscope as previously described (Gaskin & Kress, 1977; Gethner & Gaskin, 1978). Two procedures were used for negative staining. In procedure 1, one drop of the sample was applied to a Formvar grid (Ernest F. Fullam, Inc., Schenectady, NY), rinsed with six drops of 2% uranyl acetate, and blotted dry with filter paper. In procedure 2, assembled structures were fixed by adding an equal volume of 2% glutaraldehyde in MES buffer. After 15 min at room temperature, the grids were prepared as described above with the inclusion of a washing step with 10 drops of MES buffer. For length determinations, at least 100 assembled structures were measured in a field from two different grids. Both methods gave similar results, although the mean lengths were 5–10% longer in the glutaraldehyde-fixed grids. The mean lengths in the paper are for the glutaraldehyde-fixed structures. The error is 1 SD, assuming the distribution to be Gaussian. For thin sectioning, pellets were fixed overnight with 2% glutaraldehyde with or without 0.5% tannic acid in 0.1 M MES buffer containing 0.5 mM $MgCl_2$ and 1 mM EGTA. They were post-fixed with 1% osmium tetraoxide in 0.1 M phosphate buffer, pH 7.2, for 10 min, dehydrated in the usual alcohol solutions, embedded in Araldite-Epon, sectioned, and stained with uranyl acetate followed by lead citrate. Four different areas of the pellets were embedded. One-micrometer sections were cut, and every 10th section (four to five sections per area) was examined.

Synthesis of GTPβS Diastereoisomers. GTPβS(A) and GTPβS(B) isomers were synthesized enzymatically by the procedure of Connolly et al. (1982). Guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) (Boehringer Mannheim Biochemicals) was the precursor for both GDPβS(A) and -(B) isomers. The A isomer was obtained by phosphorylating GDPβS with pyruvate kinase and purification of GTPβS(A) from the mixture of A and B isomers by degradation of the B isomer with glycerol kinase. Phosphoryl transfer with acetate kinase onto GDPβS was used to prepare GTPβS(B). Trace quantities of GTP introduced from GDP contamination of GDPβS would come off the DEAE-Sephadex A-25 column before the GTPβS(B) peak, with fractions 102–108 containing GTP and fractions 120–134 containing GTPβS. No GTP was detected by A_{254}, and the GTPβS fractions had OD readings >1.0 after a 200-fold dilution. Furthermore, no GTP was detected by

[1] Abbreviations: CrGTP, chromium guanosine triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast-performance liquid chromatography; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPβS, guanosine 5′-*O*-(2-thiotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); MES, 2-(*N*-morpholino)-ethanesulfonic acid; microtubule protein, tubulin plus microtubule-associated proteins; NDPK, nucleoside diphosphate kinase; PAGE, polyacrylamide gel electrophoresis.

HPLC using the partisil 10-SAX column as described in the next section.

We found that reverse-phase HPLC using a C_{18} column (Ultrasphere ODS, Beckman) with counterion buffer 0.3 M KH_2PO_4/5 mM tetrabutylammonium hydroxide, pH 6.0, resolved the GTPβS(A) and -(B) isomers. In a typical run, the retention times of the GTPβS(A) and -(B) isomers were 42.0 and 39.3 min, respectively. Purity of the diastereoisomers was >99%.

Analysis of Nucleotide Bound to Tubulin, Microtubule Protein, Microtubules, or Other Polymeric Forms. Nucleotide/tubulin ratios of purified tubulin and microtubule protein and the pelleted polymeric forms (100000*g* for 45 min through 1 mL of 40% glycerol in buffer cushion) were determined by precipitating protein with 50% ethanol at pH 5.6 and measuring the nucleotide concentration at A_{254} as described previously (Gaskin & Kress, 1977). The protein concentration was determined by the method of Lowry et al. (1951). A molecular weight of 100 000 was used for tubulin dimer. The microtubule protein preparation contained 80% tubulin on the basis of PAGE (Gaskin et al., 1974b).

Guanine nucleotides (GDPβS, GTPβS, GTP, GDP, and GMP) were separated by HPLC using a partisil 10-SAX anion-exchange column (Whatman) with a linear gradient from 0.007 M KH_2PO_4 and 0.007 M KCl at pH 4.0 to 0.25 M KH_2PO_4 and 1 M KCl at pH 5.5. These conditions are similar to those described by McKeag and Brown (1978) except that 1 M KCl and pH 5.5 were used instead of 0.5 M KCl and pH 5.0. The retention times of GMP, GDP, GDPβS, GTP, and GTPβS were 11.6, 24.1, 35.7, 37.4 and 42.6 min, respectively, in a typical experiment.

Results

Comparison of GTPβS(A)-, GTPβS(B)-, and GTP-Induced Assembly of Tubulin. Kinetics of tubulin assembly was followed turbidimetrically at 350 nm at 37 °C in buffer containing 30% glycerol, 0.1 M MES, 3 mM Mg(II), and 1 mM EGTA, pH 6.6, in the presence of 0.5 mM GTP, GTPβS(A), or GTPβS(B) (Figure 1A). All three nucleotides promoted tubulin assembly, and the kinetic profiles were similar. However, the lag time was greater for GTPβS(A) than for GTP, which in turn was greater than for GTPβS(B). Although the turbidity equilibrium values were different, approximately 75% of the initial protein was found in pelleted structures. Also, the A_{350} value of the GTPβS(B) structures was sensitive to shearing; i.e., transfer of 1 mL by a Pasteur pipet to another cuvette resulted in a 20–30% loss in A_{350}. No assembly was detected by turbidity or electron microscopy when no nucleotide or GDP were added.

Negative staining electron microscopy of assembled samples at 30 min showed that GTP induced primarily microtubules and thin sheets (Figure 2A), GTPβS(A) induced primarily microtubules (Figure 2B), and GTPβS(B) induced bundles of sheets and microtubules and isolated sheets and microtubules (Figure 2C,D). With GTPβS(A), sheets were rarely found, while with GTP the percentage of sheets varied from 10 to 30%. Thin sections of pellets from the GTP- and GTPβS(A)-induced tubulin aggregates showed microtubules and some bundles induced by GTP (Figure 3A) and GTPβS(A) (Figure 3B). Structures consistent with sheets and bundles of sheets were found in the GTPβS(B)-induced tubulin aggregates (Figure 3C).

Length measurements on microtubules and sheets induced by GTP and microtubules induced by GTPβS(A) were similar—6.0 ± 2.0 and 7.5 ± 2.0 μm, respectively. The lengths of polymers in bundles induced by GTPβS(B) were 5.7 ± 2.5 μm, although the isolated sheets and microtubules were considerably shorter (2.8 ± 0.6 μm).

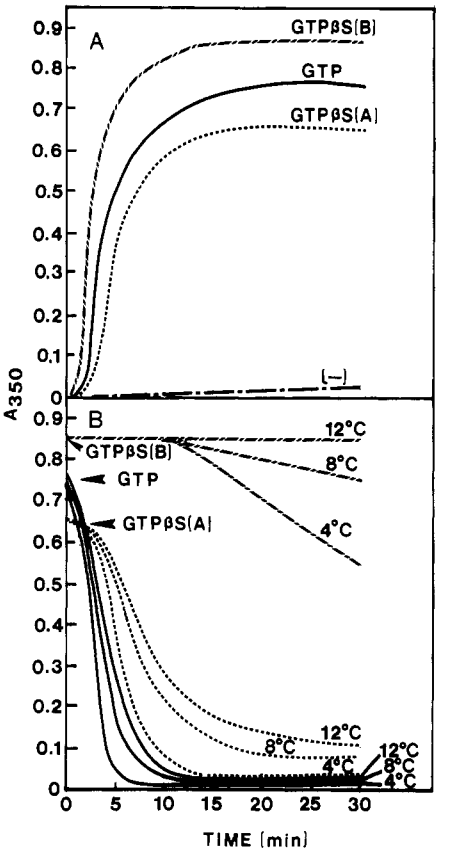

FIGURE 1: Kinetics of GTPβS(A)-, GTPβS(B)-, and GTP-induced assembly of tubulin at 37 °C and stability of the assembled tubulin at low temperatures. (A) Tubulin (1.8 mg/mL) in 30% glycerol, 0.1 M MES, 3 mM Mg(II), and 1 mM EGTA, pH 6.6, was incubated at 37 °C in the presence of 0.5 mM GTP or GTPβS diastereisomers. (B) Cold-induced disassembly of the samples in (A).

The kinetics of tubulin assembly were also followed at lower reaction temperatures (23 and 28 °C) in the presence of 0.5 mM GTPβS(A), GTPβS(B), or GTP. The assembly kinetics of nucleotide-induced tubulin at 23 and 28 °C were similar to that observed at 37 °C, although as expected the lag times and initial rates of assembly were slower (data not shown). Electron microscopy studies showed that each nucleotide induced the same polymers at 23 and 28 °C as they did at 37 °C.

Hydrolysis of GTPβS(A), GTPβS(B), and GTP during Tubulin Assembly. The binding and hydrolysis of GTPβs(A), GTPβS(B), and GTP were compared after nucleotide-induced assembly of tubulin in the presence of 3 mM $MgCl_2$. The starting purified tubulin had a nucleotide/tubulin ratio [from A_{254} N/P = 1.4] from HPLC analysis of 0.9 mol of GTP and 0.4 mol of GDP. This would suggest tubulin has 0.9 mol of GTP at the nonexchangeable site and 0.4 mol of GDP at the exchangeable site or 0.9 mol of GTP and 0.1 mol of GDP at the nonexchangeable site and 0.3 mol of GDP at the exchangeable site. Thus, purified tubulin should have 0.6–0.7 mol of free exchangeable nucleotide-binding sites. With 0.5 mM GTP, the microtubules are expected to contain 1 mol of GTP at the nonexchangeable site and 1 mol of GDP at the exchangeable site of tubulin. Table I shows 1.0 mol of GTP and 0.9 mol of GDP/tubulin dimer were bound to the pelleted microtubules and sheets. Use of radioactive GTP showed that 0.49 mol of GDP came from hydrolysis of [^{3}H]GTP at the exchangeable site. When GTPβS(A) was used to induce assembly, it was also hydrolyzed. However, with 0.5 mM

Table I: Nucleotide/Tubulin Ratio of Microtubules Assembled at 37 °C from Tubulin and GTPβS Diastereoisomers[a]

added nucleotide	A_{254} analysis	HPLC analysis				
		GTP	GDP	GDPβS	GTPβS	total
0.5 mM GTP	1.8	1.01	0.89[b]			1.90
0.5 mM GTPβS(A)	2.0	0.80	0.53	0.27	<0.01 (A)	1.60
0.5 mM GTPβS(B)	1.8	0.83	0.59		0.23 (B)	1.65
1 mM GTPβS(B)	1.7	0.79	0.45	0.06	0.36 (B)	1.66

[a] Tubulin (1.9 mg/mL) in 30% glycerol, 0.1 M MES, 3 mM $MgCl_2$, and 1 mM EGTA, pH 6.6 and GTP or GTPβS diastereoisomers were incubated at 4 °C for 30 min and then at 37 °C for 30 min. Polymer was centrifuged through 40% glycerol in buffer cushion at 34000 rpm for 45 min at 25 °C. Pellets were resuspended in buffer and analyzed for protein content. Nucleotide was released by precipitating the protein with 50% ethanol at pH 5.6 and analyzed by A_{254} and HPLC. [b] [^{3}H]GDP = 0.49, GDP = 0.40.

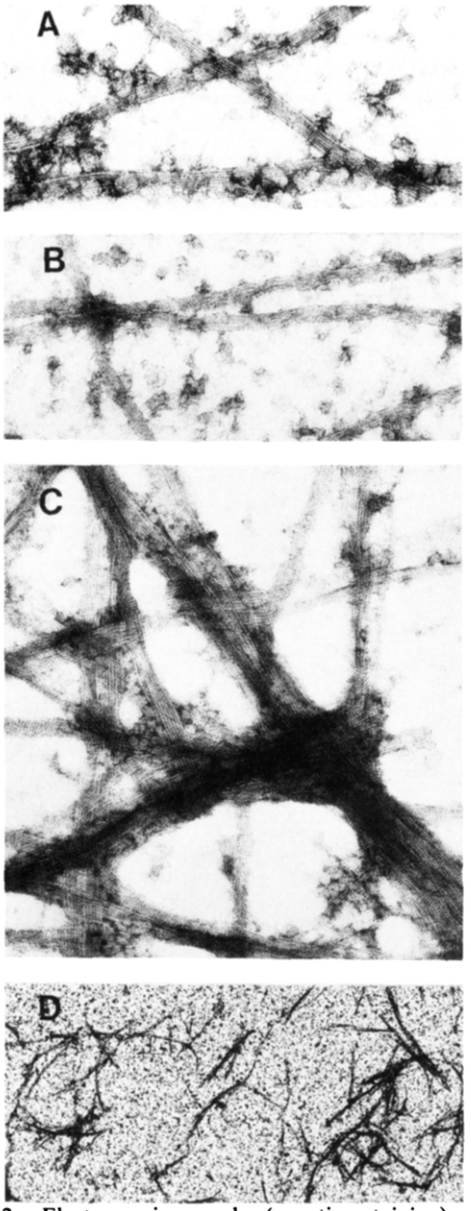

FIGURE 2: Electron micrographs (negative staining) of tubulin polymers formed with 0.5 mM GTP, GTPβS(A), or -(B) isomers and purified tubulin in buffer containing 0.1 M MES, 30% glycerol, 3 mM Mg(II), and 1 mM EGTA, pH 6.6, at 37 °C for 30 min. (A) Microtubules and thin sheets induced by GTP. Magnification 90000×. (B) Microtubules induced by GTPβS(A). Magnification 90000×. (C) Bundles of sheets and microtubules induced by GTPβS(B). Magnification 90000×. (D) Low magnification showing bundles and isolated sheets and microtubules induced by GTPβS(B). Magnification 2250×.

GTPβS(B) less than 5% of the bound thionucleotide was hydrolyzed, whereas with 1 mM GTPβS(B) 14% of the bound GTPβS was hydrolyzed.

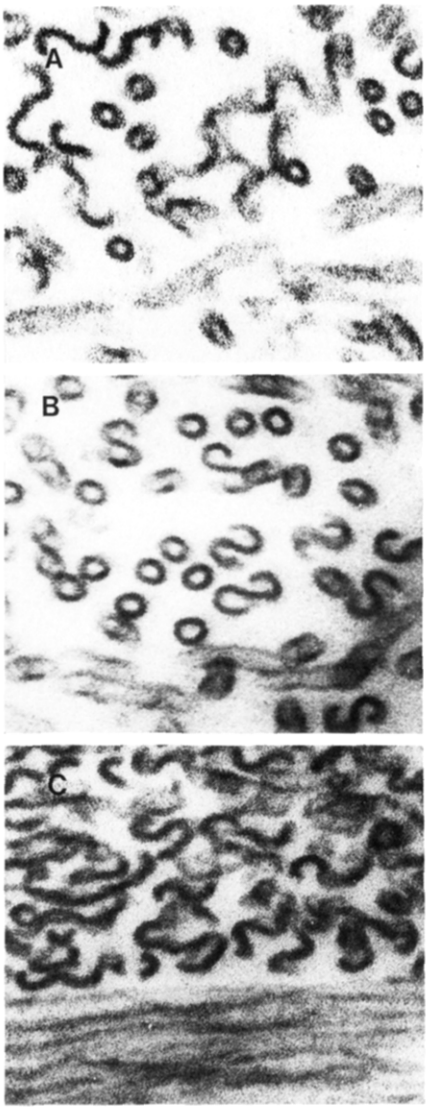

FIGURE 3: Electron micrographs of thin sections of pellets of tubulin polymers. (A) Pellet from GTP-induced polymer shows microtubules, S-shaped structures, and sheets. (B) Pellet from GTPβS(A)-induced polymer has more microtubules than the GTP-induced polymer. However, S-shaped structures similar to those in GTP-induced polymers are also found. (C) GTPβS(B) polymers show structures consistent with bundles of sheets, sheets, and occasional microtubules. Magnification 180000×.

We did several purifications using radiolabeled GTP in all steps and found the starting FPLC purified tubulin contained 0.02 mol of radiolabeled GTP. This would suggest that the exchangeable nucleotide-binding site in purified tubulin contains 0.02 mol of GTP. When the pellets containing the GTP- and isomer-induced purified tubulin aggregates were analyzed for radiolabeled nucleotide, the GTP- and GTPβS(A)-induced aggregates contained no detectable counts and the GTPβS(B)

Table II: Effect of Mg(II) on GTPβS(A)-, GTPβS(B)-, and GTP-Induced Assembly of Tubulin[a]

	$MgCl_2$			
	1 mM	2 mM	3 mM	5 mM
GTP	mts, thin sheets few bundles A_{350} = 0.5	mts, thin sheets few bundles A_{350} = 0.6	mts,[b] thin sheets few bundles A_{350} = 0.7	mts,[b] thin sheets few bundles A_{350} = 0.8
GTPβS(A)	no mts A_{350} = 0.1	mts few bundles A_{350} = 0.6	mts[b] few bundles A_{350} = 0.6	mts few bundles A_{350} = 0.6
GTPβS(B)	bundles of sheets and mts A_{350} = 0.6	bundles of sheets and mts A_{350} = 0.8	bundles of sheets and mts[b] A_{350} = 0.9	bundles of sheets and mts A_{350} = 0.9

[a] Assembly was done in buffer containing 30% glycerol and 0.5 mM nucleotide at 37 °C. mts = microtubules; A_{350} = turbidity at 30 min. [b] Optimal assembly based on lag time, kinetics, and final extent of assembly.

isomers contained 0.01 mol of radiolabeled nucleotide.

Stability of the GTPβS(A)-, GTPβS(B)-, and GTP-Induced Tubulin Polymers at Low Temperatures. After assembly at 37 °C for 30 min, the nucleotide-induced polymers in cuvettes were transferred to 4, 8, or 12 °C and the kinetics of disassembly followed (Figure 1B). Both turbidity and electron microscopy showed that 0.5 mM GTPβS(A)- and (B)-induced polymers had different sensitivities to the cold. The bundles of sheets and microtubules induced by GTPβS(B) were stable at 12 °C for at least 30 min and lost 10% of the turbidity at 8 °C and 30% of the turbidity at 4 °C after 30 min. The GTPβS(B)-induced polymers were more cold resistant than the GTPβS(A)-induced polymers, which were more stable than the GTP-induced microtubules.

Effect of Mg(II) on GTPβS(A)- and GTPβS(B)-Induced Assembly of Tubulin. Tubulin assembly was examined at 0.5 mM GTPβS(A), GTPβS(B), or GTP at 1, 2, 3, and 5 mM Mg(II) in buffer containing 30% glycerol. GTPβS(A) did not induce assembly at 1 mM Mg(II) (Table II) . Microtubules and a few bundles were found with GTPβS(A) at 2 and 3 mM Mg(II), although optimal assembly was at 3 mM Mg(II). At 5 mM Mg(II) and GTPβS(A), there were more microtubule bundles, although isolated microtubules were still predominant structures. Bundles of sheets and microtubules were found with GTPβS(B) at all concentrations of Mg(II). Again, optimal assembly was found at 3 mM Mg(II). GTP induced primarily microtubules at all Mg(II) concentrations with optimal assembly at 3–5 mM Mg(II). With all three nucleotides, some spirals and nonspecific protein aggregates were also seen at 5 mM Mg(II).

GTPβS(A)-, GTPβS(B)-, and GTP-Induced Assembly of Microtubule Protein. GTPβS(A)-, GTPβS(B)-, and GTP-induced assembly kinetics of microtubule protein at 0.5 mM nucleotide and 0.5 mM Mg(II) in buffer without glycerol at 37 °C were similar (Figure 4A) . Only microtubules were seen in all three samples. With all three nucleotides, incorporation of nucleotide into the assembled microtubules was variable and depended on the microtubule protein preparation, which contained 1.3–1.5 mol of nucleotide/tubulin dimer after thin-film dialysis. With low GTPβS nucleotide-binding numbers (<0.3), all of the exchanged nucleotide was hydrolyzed. At higher binding numbers, i.e., 0.7–0.8, 25% of the GTPβS(B) was not hydrolyzed, while all of the GTPβS(A) and GTP were.

Disassembly kinetics at 4 °C suggest that GTP-induced microtubules (L = 7.2 ± 4.6 μm) are more labile ($t_{1/2}$ = 3.6 min) than GTPβS(A)-induced microtubules (L = 1.8 ± 0.7 μm and $t_{1/2}$ = 5.2 min), which are in turn more labile than GTPβS(B)-induced microtubules (L = 2.5 ± 1.0 μm and $t_{1/2}$ = 7.9 min) (Figure 4B). At 20 min, no microtubules were found in the GTP and GTPβS(A) samples. A few microtubules were found with GTPβS(B). Lack of complete reversibility with the diastereoisomers may be due to nonmicrotubule aggregates that are not cold sensitive or denaturation.

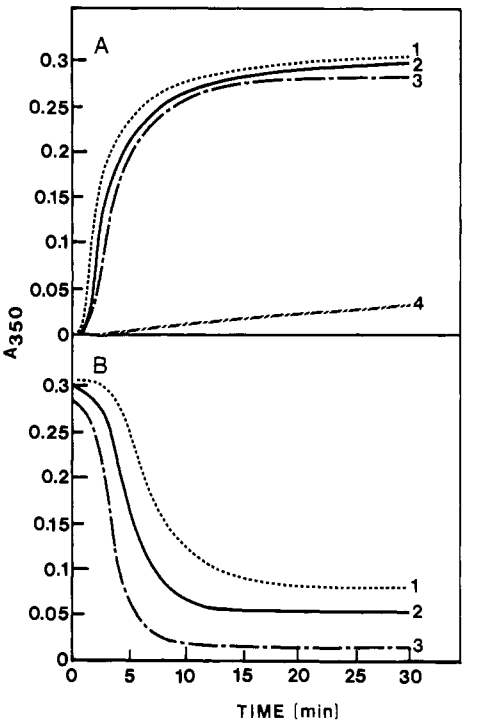

FIGURE 4: Kinetics of GTPβS(A)-, GTPβS(B)-, and GTP-induced assembly of microtubule protein (tubulin plus microtubule-associated proteins) at 37 °C and stability of the assembled microtubules at 4 °C. (A) Microtubule protein (1.8 mg/mL) in 0.1 M MES, 0.5 mM $MgCl_2$, and 1 mM EGTA, pH 6.6, was incubated at 37 °C with 1 mM GTPβS(A) (curve 2), GTPβS(B) (curve 1), GTP (curve 3), or no added nucleotide (curve 4). (B) Cold-induced (4 °C) disassembly of the samples in (A).

Table III: Effect of Nucleotide Concentration on Polymers Formed from Microtubule Protein (Tubulin plus Microtubule-Associated Proteins)[a]

	nucleotide		
	0.06 mM	0.2 mM	0.5 mM
GTP	mts[b] A_{350} = 0.24	mts A_{350} = 0.24	mts A_{350} = 0.23
GTPβS(A)	rings, cross-linked rings, filaments A_{350} = 0.06	mts A_{350} = 0.20	mts[b] A_{350} = 0.23
GTPβS(B)	mts[b] A_{350} = 0.24	mts A_{350} = 0.25	mts A_{350} = 0.25

[a] Assembly was done in buffer containing 0.5 mM $MgCl_2$ and 1 mM EGTA, pH 6.6 at 37 °C. mts = microtubules; A_{350} = turbidity at 30 min. [b] Optimal assembly based on lag time, kinetics, and final extent of assembly.

The nucleotide and Mg(II) were varied to study the domains of different polymeric forms. Table III shows the effect of nucleotide concentration (0.06–0.5 mM) on polymers formed from microtubule protein in the presence of 0.5 mM Mg(II). Rings, cross-linked rings, and filaments form at low concentrations of GTPβS(A), i.e., 0.06 mM, while microtubules are found with higher GTPβS(A). Only microtubules are seen with GTPβS(B) or GTP. Table IV shows that the same GTPβS(A) nonmicrotubule aggregates can be induced by increasing both the Mg(II) and GTPβS concentrations, i.e.,

Table IV: Domains of Different Polymeric Forms When Microtubule Protein Was Assembled with Variable Concentrations of Mg(II) and GTPβS(A)[a]

[$MgCl_2$] (mM)	+0.2 mM GTPβS(A)	+0.5 mM GTPβS(A)
0.5	mts A_{350} = 0.20	mts[b] A_{350} = 0.22
1.0	rings, cross-linked rings, some spirals, and filaments, few mts A_{350} = 0.16	mts A_{350} = 0.2
2.0	cross-linked rings A_{350} = 0.07	mts, rings and cross-linked rings, spirals, filaments A_{350} = 0.16
3.0	not done	spirals, filaments, rings and cross-linked rings, few mts A_{350} = 0.19

[a] Assembly was done in buffer containing 0.5 mM $MgCl_2$ and 1 mM EGTA, pH 6.6 at 37 °C. With 0.2 mM or 0.5 mM GTP or GTPβS-(B), only mts were found with 0.5–3 mM $MgCl_2$. mts = microtubules; A_{350} = turbidity at 30 min. [b] Optimal assembly based on lag time, kinetics, and final extent of assembly.

with 0.2 mM GTPβS(A) and 1 or 2 mM Mg(II) or with 0.5 mM GTPβS(A) and 2 or 3 mM Mg(II). Similar GTP/Mg-(II) or GTPβS(B)/Mg(II) ratios resulted in only microtubules.

Discussion

Our data show that there is stereoselectivity at the β phosphate of the exchangeable nucleotide-binding site of tubulin since the A and B isomers of GTPβS have different effects on tubulin assembly. GTPβS(A) closely resembles GTP in promoting assembly of tubulin into microtubules, and both nucleotides were hydrolyzed. In fact, with GTPβS(A), thin sheets are rarely found by negative staining electron microscopy, while they make up 10–30% of the structures induced by GTP. However, GTPβS(B) induces bundles of sheets and microtubules, and hydrolysis is minimal. The lack of hydrolysis of GTPβS(B) suggests that the pro (R_P) oxygen of the β phosphorus of guanosine triphosphate is involved in a critical reaction with tubulin directly or with Mg since Mg coordinates S less strongly than O. The use of metals such as Zn(II) or Cd(II) that bind O less strongly than sulfur to probe this reaction would probably lead to complications. Zn(II) has a direct interaction with tubulin (Gaskin, 1981; Eagle et al., 1983). At very low Zn(II) microtubules form with GTP (Haskins et al., 1980), but usually Zn(II) induces sheets and wrapped sheets with GTP or CrGTP (Larsson et al., 1976; Gaskin & Kress, 1977; Gaskin, 1981). Cd(II) binds to the Zn binding site (Eagle et al., 1983) and inhibits tubulin polymerization (Wallin et al., 1977). The previous finding that GTPγS is not hydrolyzed during microtubule and tubulin assembly suggests that the γ phosphate is also important in nucleotide binding to tubulin (Kirsch & Yarbrough, 1981; Roychowdhury & Gaskin, 1986a).

Not only was GTPβS(B) only partially hydrolyzed in bundles of sheets and microtubules assembled from tubulin but the bundles were considerably more stable to the cold than microtubules containing GDP or GDPβS that were induced by GTP or GTPβS(A). This result supports the hypothesis that hydrolysis is not necessary for assembly and that the nucleotide binding changes the conformation of tubulin so that it can assemble, while hydrolysis again changes the conformation of tubulin such that disassembly can occur at a faster rate (Weisenberg et al., 1976; Weisenberg, 1980; Kirschner & Mitchison, 1986). Differences in the length distributions of the polymers formed may give rise to apparent differences in stability since depolymerization can be treated as a first-order process and the rate is proportional to the concentration of ends. However, the GTPβS(B) bundles (L = 5.7 ± 2.5 μm) and individual sheets and microtubules (L = 2.8 ± 0.6 μm) are overall shorter than the GTP-induced microtubules and sheets (L = 6.0 ± 2.0 μm) and GTPβS(A)-induced microtubules (L = 7.5 ± 2.0 μm). In our study it is possible that the bundles stabilize lateral and/or vertical tubulin interactions and that tubulin is more slowly released from the bundles when subjected to low temperature than GDP or GDPβS tubulin from microtubules. Since the cold-induced disassembly rate of GTP-induced microtubules and sheets (L = 6.0 ± 2.0 μm) is faster than that of the GTPβS(A)-induced microtubules (L = 7.5 ± 2.0 μm), the difference in apparent stability between these two polymers may be due to differences in length distributions. Alternatively, sheets may disassemble faster than microtubules in the cold.

GTPβS(B) induces bundles of sheets and microtubules with varying Mg(II) (1–5 mM) and tubulin. Both GTP and GTPβS(A) induce tubulin to assemble into microtubules with 2–5 mM Mg(II). These studies further suggest that GTPβS(A) is a better analogue for GTP in the tubulin assembly system. Whether the GTPβS analogues promote assembly of tubulin through nucleation and/or propagation needs to be determined. The starting FPLC purified tubulin contained 0.02 mol of GTP at the exchangeable site. This amount could be sufficient to nucleate assembly. However, no assembly was detected by A_{350} or EM when GDP or no nucleotide were added. Since there was no detectable GTP contamination in the GTPβS isomers used in the study and the GTP at the exchangeable nucleotide-binding site of starting tubulin was low (0.02 mol), the results in Table I showing 0.5 mol of unlabeled GDP in the polymers support the hypothesis that tubulin with GDP at the exchangeable site will add to the microtubules or bundles using the experimental conditions described in Table I. Since GTP in the pelletable isomer-induced polymers is only 0.8, we may have 0.2 mol of GDP at the nonexchangeable site or we may have 20% inactive or denatured protein in the pellet.

Both GTPβS isomers (0.2–0.5 mM) induced microtubule protein to assemble into microtubules when 0.5 mM Mg(II) was used. In most microtubule protein preparations only low amounts of GDPβS (<0.3) were bound to the microtubules. When higher levels of nucleotide incorporation were found, i.e., 0.7–0.8, only 75% of GTPβS(B) was hydrolyzed, while all of GTPβS(A) was. The different amounts of exchanged nucleotide might be due to different levels of nucleotide-requiring enzymes, i.e., NDPK, in our microtubule protein preparations (Roychowdhury & Gaskin, 1986b). The NDPK could catalyze the reaction GTPβS + GDP–tubulin → GDPβS + GTP–tubulin, and the GTP on the exchangeable site of tubulin would be hydrolyzed during assembly.

Another interesting finding is that although with microtubule protein there are small differences in nucleotide-induced microtubule stabilities to the cold (Figure 4B), the GTP-induced microtubules are considerably longer (L = 7.2 ± 4.6 μm) but less stable than the GTPβS(A)-induced microtubules (L = 1.8 ± 0.7 μm), which are more stable but similar in length to the GTPβS(B)-induced microtubules (L = 2.5 ± 1.0 μm).

GTPβS(B) is a good GTP analogue for microtubule protein assembly since with 0.2 or 0.5 mM nucleotide only microtubules were found with 0.5–3 mM $MgCl_2$. However, with GTPβS(A), microtubules were found only with 0.2 mM nucleotide and 0.5 mM Mg(II) or 0.5 mM GTPβS(A) and 0.5–2

mM Mg(II). At the other Mg(II) concentrations and GTPβS(A), rings, cross-linked rings, or spirals were predominant structures.

In summary, over a series of Mg(II) concentrations, the GTPβS(A) isomer is a better GTP analogue than GTPβS(B) in inducing tubulin to assemble into microtubules, while with the microtubule protein assembly system, GTPβS(B) is a better GTP analogue than GTPβS(A). This suggests that the tubulin nucleotide- and/or Mg(II)-binding site(s) may be altered by microtubule-associated proteins or that one of the isomers preferentially reacts with a microtubule-associated protein or enzyme such as NDPK.

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Registry No. (S_P)-GTPβS, 81570-53-8; (R_P)-GTPβS, 81570-52-7; GTP, 86-01-1; Mg, 7439-95-4.

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