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Affinity Labeling of Tubulin's Exchangeable Guanosine 5′-Triphosphate Binding Site†

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ABSTRACT: Tubulin requires GTP for maximal rate and extent of polymerization into microtubules. The localization of the guanine nucleotide in the microtubule was examined by preparing affinity probes that would permit tubulin polymerization prior to their covalent coupling to amino acids in tubulin's exchangeable GTP binding site. Two different hydrolyzable GTP analogues with modified ribose moieties, 3′-*p*-azidobenzoyl)-GTP and the periodate oxidation product of GTP, 2-(guanylformylmethoxy)-3-(triphospho)propanal, were isolated by thin-layer chromatography and high-voltage electrophoresis and identified by ultraviolet and infrared spectroscopy. The analogues bind to the tubulin molecule and promote polymerization. After tubulin polymerization and isolation of microtubules, the [^{3}H]GTP analogues were covalently coupled to tubulin by $NaBH_4$ reduction or UV irradiation. The microtubules possessed about 1 mol of acid-precipitable ^{3}H-labeled nucleotide/mol of tubulin dimer. Separation of the subunits showed that the nucleotide analogues were associated with both α and β subunits of tubulin in nearly equal amounts. The binding of analogues to both α and β subunits was saturable and competitive with GTP. Cyanogen bromide cleavage of both α and β subunits showed that the ^{3}H-labeled nucleotide was associated with a single molecular weight species of similar size (~10000) from each subunit. Two-dimensional electrophoresis of chymotryptic peptides from both (α and β) cyanogen bromide fragments showed that the ^{3}H-labeled nucleotide was associated with a peptide of nearly identical migration properties from both subunits. These results suggest that a similar peptide segment of both α- and β-tubulin has the ability to bind GTP. Furthermore, this peptide was localized to the amino-terminal one-third of the tubulin molecule.

Microtubules are involved in a variety of cellular functions (Roberts & Hyams, 1979). Assembly of microtubules from the subunit protein tubulin, a M_r 110000 heterodimer of α and β monomers, requires guanosine triphosphate (GTP)[1] (Borisy & Olmsted, 1972; Maccioni & Seeds, 1977a). The tubulin dimer has two guanine nucleotide binding sites of

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; GTPox, the periodate oxidation product of GTP; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PEI–cellulose, poly(ethylenimine)–cellulose; PMSF, phenylmethanesulfonyl flouride.

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different affinities: nucleotide bound to one site (E site) readily exchanges with exogenous nucleotide, while that bound to the other site (N site) is nonexchangeable (Weisenberg et al., 1968). Tubulin possessing bound GTP, GDP, or both has been described (Maccioni & Seeds, 1977a; Penningroth & Kirschner, 1977; Jacobs, 1979), and conformational differences between GTP–tubulin and GDP–tubulin have been reported (Purich & Karr, 1978). Although GTP is hydrolyzed during microtubule assembly, there is not general agreement as to the stoichiometry of GTP hydrolysis per tubulin dimer (Maccioni & Seeds, 1977a; David-Pfeuty et al., 1977) nor whether hydrolysis is a necessary event for assembly or for a subsequent functional event such as disassembly (Weisenberg & Deery, 1976). Thus, the specific role GTP plays in microtubule structure is still unclear.

With the hope of gaining a better understanding of GTP and its interaction with tubulin subunits in microtubules, an investigation to localize and characterize these binding sites has been undertaken. Several radiolabeled GTP analogues that can be covalently coupled to proteins have been synthesized and examined for their ability to replace GTP in the assembly step. A preliminary report of these data has been presented previously (Maccioni & Seeds, 1977b).

Materials and Methods

Materials. GTP and GDP (Sigma, St. Louis, MO, and Boehringer-Mannheim, Indianapolis, IN) were checked for purity by PEI–cellulose thin-layer chromatography (Penningroth & Kirschner, 1977). [8,5′-$^3H_2$]GTP (>35 Ci/mmol) was obtained from New England Nuclear (Boston, MA). The *p*-azidobenzoic acid was from Frinton Laboratories (Vineland, NJ), and the cyanogen bromide from Eastman Chemical Co. (Rochester, NY). Sodium borohydride and carbonyldiimidazole were purchased from Sigma (St. Louis, MO). Lamb brains were purchased locally (Wilson & Co., Denver, CO).

Tubulin Isolation. The method of Shelanski et al. (1973) was used to prepare twice recycled microtubules from lamb brain. The microtubule pellets were stored at −75 °C and subjected to an additional cycle of assembly–disassembly prior to use. Occasionally, tubulin purified by affinity deacetylcolchicine chromatography (Morgan & Seeds, 1975) or phosphocellulose chromatography (Weingarten et al., 1975) was used. The protein concentration of the tubulin solutions was determined by the method of Lowry et al. (1951) or by using a value of 1.15 A_{280}/mg of tubulin (Appu Rao et al., 1978).

Nucleotide Removal. Microtubule protein preparations were freed of unbound and exchangeable nucleotide before each experiment by two successive extractions with 0.1 M imidazole hydrochloride (pH 6.8) or 0.1 M Mes (pH 6.8) buffer containing 1 mM EDTA and 5 mg of charcoal (prewashed in a 2% albumin solution) per mL of tubulin solution (Maccioni & Seeds, 1977a).

Microtubule Polymerization. Tubulin assembly was monitored by the turbidity method, following the change in absorbance at 410 nm compared to that of a reference cuvette of the same microtubule protein solution plus 10^{-4} M colchicine or without nucleotide by using a Beckman Model 25 recording spectrophotometer with a temperature-regulated cuvette.

Periodate-Oxidized GTP. A modification of the procedure described by Easterbrook-Smith et al. (1976) was used. One micromole of GTP was mixed with 350 μL of [^{3}H]GTP in ethanol–water, giving a final specific activity of 0.16 mCi/μmol, and adjusted to neutral pH with NaOH. The excess ethanol from the [^{3}H]GTP was evaporated under N_2 to a final volume of 160 μL. One micromole of $NaIO_4$ as 20 μL of an aqueous solution (pH 5.5–6) was added to the solution and allowed to react in the dark at 4 °C for 30 min; then an additional 0.5 μmol of $NaIO_4$ (10 μL) was added and allowed to react for an additional 30 min. The excess $NaIO_4$ was reduced by the addition of 0.5 μmol of glycerol. Under these conditions oxidation is complete and 100% yield can be obtained. The product was isolated by high-voltage paper electrophoresis at 1 kV in 50 mM citrate buffer (pH 5.0). The dialdehyde product (GTPox) was verified by hydrolysis in 0.1 N NaOH at 37 °C for 12 h followed by chromatography on Whatman No. 1 paper in isobutyric acid–NH_4OH–H_2O (60:1:33) with 0.2 mM EDTA (pH 3.7). The presence of the new carbonyl groups was verified by IR spectra at 1635 and 1675 cm^{-1}. Ultraviolet spectra showed a minimum at 223 nm compared to GTP's minimum at 232 nm at neutral pH. The GTPox migrated ahead of GTP of PEI–cellulose plates in 1.2 M LiCl. The product was also an active substrate for 3-phosphoglycerate kinase (EC 2.7.2.3).

3′-p-Azidobenzoyl-GTP. The procedure of Jeng & Guillory (1975) was used. Carbonyldiimidazole (18 mg) was mixed with *p*-azidobenzoic acid (18 mg) in 50 μL of dimethylformamide and stirred for 10 min at room temperature. An aliquot (40 μL) of [^{3}H]GTP (0.5 μmol) with a specific activity of 0.08 mCi/μmol was mixed with 6 μL of the *p*-azidobenzoic acid–carbonyldiimidazole solution and kept at room temperature for 3 h. The mixture was extracted twice with ethanol (1 mL) and the nonextracted product was lyophilized and washed twice with ether to remove imidazole and *p*-azidobenzoic acid. The residue after ether extraction was dissolved in 40 μL of H_2O and chromatographed on cellulose thin-layer plates in butanol–acetic acid–H_2O (5:3:2). There were several UV-absorbing spots: the one nearest the origin was unreacted GTP and that with an R_f = 0.15 was 3′-(*p*-azidobenzoyl)-GTP. The product has an ultraviolet absorption maximum at 273 nm and a 273/255 absorption ratio of 1.28 compared to 0.86 for GTP. Infrared spectra showed absorption bands in the region of 2120 and 1180 cm^{-1}, characteristic of the azido groups. The product's identity was also verified by thin-layer chromatography in 90% EtOH (R_f = 0.15), while GTP remains at the origin and *p*-azidobenzoic acid migrates with the solvent front. The (4-azidobenzoyl)hydrazone of GTPox was prepared by the procedure of Girshovich et al. (1976).

Affinity Labeling of Tubulin. Microtubule protein free of exchangeable nucleotide was suspended in an assembly buffer of either 0.1 M imidazole hydrochloride (pH 6.8), 0.1 M Mes (pH 6.8), or 0.1 M Pipes (pH 7.4) containing 1 mM EGTA, 1 mM $MgCl_2$, and usually 1 mM [^{3}H]GTPox or 3′-(*p*-azidobenzoyl)-[^{3}H]GTP and incubated at 37 °C for 20 min. The polymerized tubulin was collected by centrifugation at 28000*g* for 25 min. When [^{3}H]GTPox was used, the Schiff's base was reduced by resuspending the microtubule pellet in warm Tris-HCl (pH 6.8) buffer containing 0.02 M $NaBH_4$ and placing it at 4 °C for 30 min; then the sample was dialyzed against three changes of 0.01 M Tris-HCl (pH 6.8).

When the azido analogues were used, the microtubule pellet was resuspended, placed in a quartz cuvette, and then irradiated at a distance of 6 cm with a wide-spectrum UV source for wavelengths less than 320 nm at room temperature for 2.5 h.

[^{3}H]GTPox Incorporation. [^{3}H]GTPox bound to microtubule samples was assessed following a 5-min reduction with $NaBH_4$ at 4 °C. The reduced samples were precipitated with cold 8% trichloroacetic acid, collected on Millipore filters, and washed with 15 mL of acid. The filters were dried prior to suspending in 10 mL of toluene–Permafluor scintillation

cocktail and counting in a Searle Mark III.

Tubulin Subunit Isolation. The α and β-tubulin subunits were isolated by preparative polyacrylamide gel electrophoresis in 5% acrylamide gels according to the procedure of Bryan (1974) with Tris–glycine–$NaDodSO_4$ buffer. The tubulin subunits had been dansylated prior to electrophoresis (Talbot & Yphantis, 1971).

Some qualitative studies used a 0.1% $NaDodSO_4$–7.5% acrylamide discontinuous electrophoretic system of Laemmli (1970). The gels were stained, sliced, and digested with 30% H_2O_2 containing 1% NH_3 at 50 °C for 30 min. Triton–toluene–Permafluor (1:1:0.135) scintillation fluid was added and the samples were counted in a Searle Mark III.

Cyanogen Bromide Cleavage. Tubulin subunits were digested at room temperature for 24 h in 0.2 mL of a 70% formic acid solution containing cyanogen bromide at a 300:1 molar ratio to protein subunits. The reaction was terminated by evaporation to dryness under N_2, and the protein was diluted with H_2O and lyophilized twice. The cleavage fragments were dissolved in 1% $NaDodSO_4$–8 M urea–1% 2-mercaptoethanol–0.01 M H_3PO_4 and adjusted to pH 6.8 with Tris, followed by incubation at 60 °C for 10 min. The peptides were resolved by using the continuous $NaDodSO_4$–urea–polyacrylamide electrophoresis system of Swank & Munkres (1971). The gels were stained, sliced, and processed as described above for liquid scintillation counting.

Molecular weight estimations of these peptides were carried out on 15% acrylamide gels with 0.1% $NaDodSO_4$, 6 M urea, 0.1 M sodium phosphate (pH 7.2), and appropriate molecular weight standards.

Chymotryptic Digestion. The dansylated and radiolabeled cyanogen bromide peptide from both α- and β-tubulin was eluted from several gels in 0.1% $NaDodSO_4$–0.1 M H_3PO_4–Tris (pH 6.8) buffer. Following $NaDodSO_4$ removal the peptide was digested with 1% chymotrypsin (Worthington Biochemicals, Freehold, NJ) in 0.05 M NH_4HCO_3 at 37 °C for 4 h. The hydrolysate was lyophilized 4 times to remove the NH_4HCO_3 and resuspended in 20 μL of H_2O.

The peptides were "fingerprinted" by two-dimensional paper electrophoresis as described by Bray & Brownlee (1973). The hydrolysate was spotted on 20 × 20 cm Whatman 3 MM paper and electrophoresed in the first dimension in a buffer containing pyridine–acetic acid–H_2O (100:3:897) (pH 6.5) for 80 min at 3.0 kV (80 mA). The paper was turned 90° and electrophoresis carried out for 1 h in acetic acid–formic acid–H_2O (8:2:90) (pH 2.1). The dansylated peptides were visualized under ultraviolet light and the other peptides by ninhydrin staining. Both UV and ninhydrin spots were cut from the paper and their radioactivity was determined as described above.

Limited digestion of tubules was performed as previously described (Maccioni & Seeds, 1983). A phosphocellulose-purified tubulin sample (3 mg/mL) was induced to assemble with 1.4 mM [^{3}H]GTPox and 10% dimethyl sulfoxide. The polymer was collected by sedimentation at 28000g, resuspended in warm 0.1 M Mes (pH 6.8), 1 mM Mg^{2+}, and 1 mM EGTA buffer and reduced with $NaBH_4$ as indicated above and dialyzed against 0.1 M Mes buffer. The resulting [^{3}H]GTPox–tubulin derivative (6 mg/ml) was incubated with 0.05% w/w chymotrypsin at 15 °C. Aliquots of 10 μL were withdrawn after 160 min, the reaction was terminated by boiling in the presence of 1% $NaDodSO_4$, 3% 2-mercaptoethanol, 1% glycerol, and 0.5 mM PMSF, and samples were analyzed by polyacrylamide gel electrophoresis according to Laemmli (1970). The gels were stained, sliced into 2-mm pieces, and digested in H_2O_2, and radioactivity was measured as indicated above.

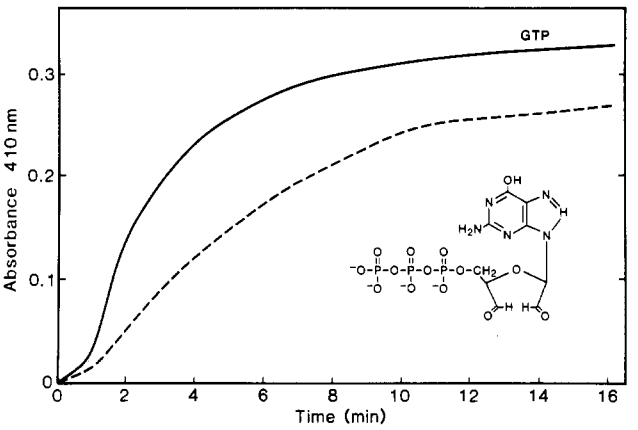


FIGURE 1: Tubulin polymerization in the presence of GTPox. A 1-mL solution of microtubule protein (3 mg/mL) free of exchangeable nucleotide was allowed to polymerize at 37 °C in the presence of either 1 mM GTP (solid line) or 1 mM GTPox (dashed line) and the turbidity increase monitored at 410 nm as compared to a sample without added nucleotide. The GTPox structure is included in the figure.

Localization of the Covalently Bound [^{3}H]GTPox in the Tubulin Molecule. A tubulin sample (4 mg/mL) was treated with 1.4 mM [^{3}H]GTPox for 30 min at 32 °C; the modified sample dialyzed against water, lyophilized, and digested in 6 N HCl at 110 °C for 22 h. The sample was evaporated to dryness and resuspended in water; small aliquots (8–10 μg of hydrolysate) were subjected to high-voltage electrophoresis on cellulose plates (Eastman No. 6064) at 1000 V (24–30 mA) for 25 min in formic acid–acetic acid–water (10:11:379), pH 1.9. [^{3}H]GTPox–lysine was used as a standard and was synthesized by reacting glycyllysine with [^{3}H]GTPox at 37 °C and pH 9.8, followed by acid hydrolysis at 110 °C for 16 h and separation of the radioactive GTPox–lysine by high-voltage electrophoresis. The amino acid hydrolysate and the standard were visualized after exposure of the plate to ninhydrin and 35 °C for 30 min. Radioactivity was determined after cutting the thin-layer cellulose strip into 0.4-mm pieces and suspending them in 1 mL of water and 10 mL of scintillation counting fluid.

Results

In general, nucleotide binding proteins have lower specificities for the ribose ring compared to the base or phosphate moieties, whose modification is more likely to affect binding (Yount, 1975). The ability of deoxy-GTP to promote microtubule assembly as well or better than GTP[2] supports this concept (Hamel & Lin, 1981). Therefore, several potential affinity labeling GTP analogues with modifications of the ribose ring were prepared. The periodate oxidation product of GTP, GTPox, promoted the polymerization of microtubule protein, as monitored by increased turbidity (Figure 1), to approximately 80% the level produced by GTP.

Thin-layer chromatography of perchloric acid extracts of the polymerized tubulin showed the radioactivity to cochromatograph with periodate-oxidized GDP. Thus, in a manner analogous to GTP, the γ-phosphate of [^{3}H]GTPox is hydrolyzed during the polymerization reaction.

The aldehyde groups of GTPox should readily react with any available primary amine near the nucleotide binding site to form a Schiff base. The polymerized tubulin was collected by centrifugation and resuspended in buffer containing $NaBH_4$

[2] D. Fishwild and N. Seeds, unpublished results.

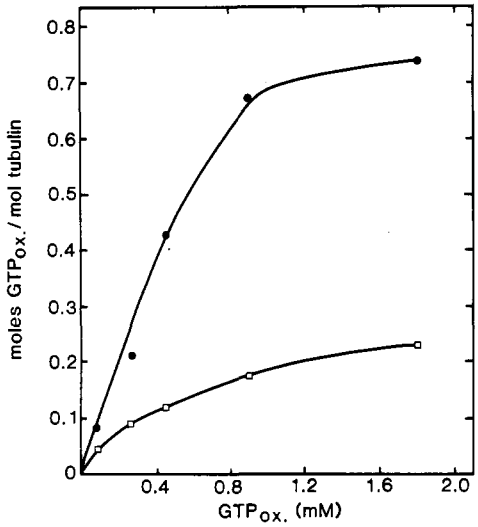


FIGURE 2: Incorporation of [^{3}H]GTPox as a function of its concentration. Microtubule protein (1.8 mg/mL) was assembled during incubation at 30 °C for 30 min in assembly buffer (0.1 M Mes, pH 6.4, 1 mM $MgCl_2$, 1 mM EGTA) with increasing concentrations of [^{3}H]GTPox as indicated (●). Analogue incorporation in the presence of 0.5 mM GTP is shown in the lower curve (□).

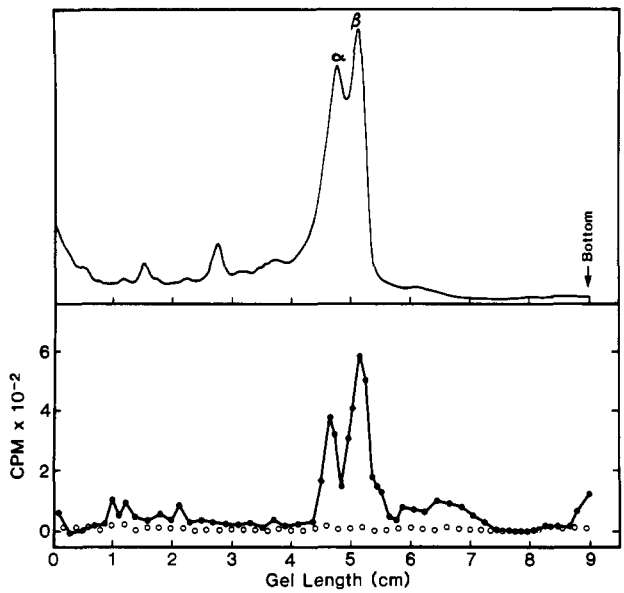


FIGURE 3: Subunit localization of bound GTPox. Microtubules formed with [^{3}H]GTPox (●) were collected by centrifugation, reacted with $NaBH_4$, and electrophoresed on acrylamide–$NaDodSO_4$ gels at pH 8.3 according to Bryan (1974) as described under Materials and Methods. Microtubules formed with GTP (○) were resuspended in [^{3}H]GTPox for 15 min, then reduced, and electrophoresed as described above. The gels were stained, sliced, and digested in H_2O_2, and the radioactivity was counted. Scanning densitometry of the stained gel is shown in the top panel.

to reduce any Schiff base formed between [^{3}H]GTPox and tubulin. Following acid precipitation the GTPox–tubulin conjugates were collected by filtration. As shown in Figure 2 the covalent binding of [^{3}H]GTPox to tubulin is saturated at GTPox concentrations above 1 mM. The binding (0.8) approaches the expected stoichiometry (1.0) for binding at the exchangeable nucleotide site. The lower curve shows that binding of GTPox is inhibited by GTP, further suggesting that the binding of GTPox is site specific.

To localize the subunit specificity of this binding, the borohydride-reduced conjugates were electrophoresed on $NaDodSO_4$–polyacrylamide gels to separate α- and β-tubulin. Surprisingly, both α- and β-tubulin possessed radiolabeled

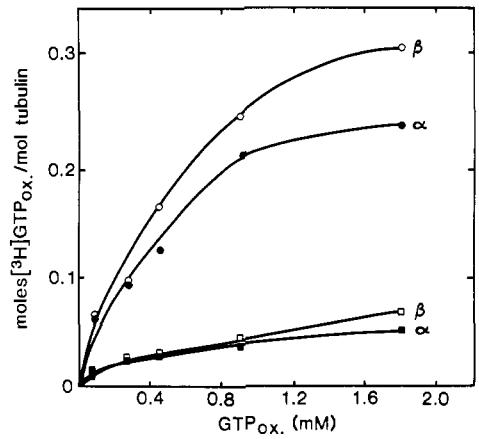


FIGURE 4: Kinetics of [^{3}H]GTPox binding to α and β subunits. Microtubule protein (1.8 mg/mL) was incubated in assembly buffer as described in Figure 2. Following $NaBH_4$ reduction the samples were electrophoresed as described in Figure 3 and stained, slices containing the α or β subunits were digested with H_2O_2, and the radioactivity was counted. Binding after 4 min (■, □) or 30 min (●, ○) at 30 °C at increasing concentrations of [^{3}H]GTPox is shown.

Table I: Selective Labeling of α- and β-Tubulin with [^{3}H]GTPox[a]

	control[b]	+GTP (0.5 mM)	+GTP (3.5 mM)
α	0.74	0.26	0.10
β	1.00	0.34	0.12

[a] 1.4 mM [^{3}H]GTPox. [b] Binding to β subunit: 0.052 nmol of [^{3}H]GTPox. Tubulin per gel: 22 μg.

nucleotide in nearly equal amounts (43:57) as seen in Figure 3. The specificity of the GTPox interaction with tubulin was further examined. Microtubules assembled with GTP were isolated by centrifugation and resuspended in warm buffer containing [^{3}H]GTPox for 15 min prior to the addition of $NaBH_4$. The absence of radioactivity (open circles in Figure 3) indicates that the binding of GTPox to tubulin appears to be very specific for those GTP sites involved in microtubule assembly, since the GTP sites are known to be inaccessible in polymerized tubulin. Furthermore, GTPox binding to both α- and β-tubulin showed similar kinetics and was time dependent and saturable with nucleotide (Figure 4). Similar results have been obtained by using [α-^{32}P]GTPox. The binding to both α and β subunits was inhibited to the same extent by increasing concentrations of GTP, demonstrating the specificity of the GTPox binding to both subunits (Table I). Furthermore, ATP did not inhibit binding to either subunit. The slight decrease in the ratio of bound ligand per tubulin dimer (or monomer) reflects the loss of ^{3}H during the acid precipitation and electrophoresis.

Separated α- and β-tubulins possessing covalently bound nucleotide were subjected to cyanogen bromide digestion. The cyanogen bromide fragments were resolved by electrophoresis on $NaDodSO_4$–urea–polyacrylamide gel electrophoresis as described by Swank & Munkres (1971) (Figure 5). The radiolabeled nucleotide is primarily associated with a single size class of peptide in both subunits, and these peptides appear to be of similar molecular weight in both α and β. However, the $NaDodSO_4$–urea–polyacrylamide gel electrophoretic system does not show a linear response with the log of the molecular weight; when a more quantitative gel system was used, the molecular weights were estimated as 10 500 for α and 9000 for the β peptide.

A closer inspection by electron microscopy of the microtubule protein polymerized at pH 6.8 in Mes or imidazole buffer

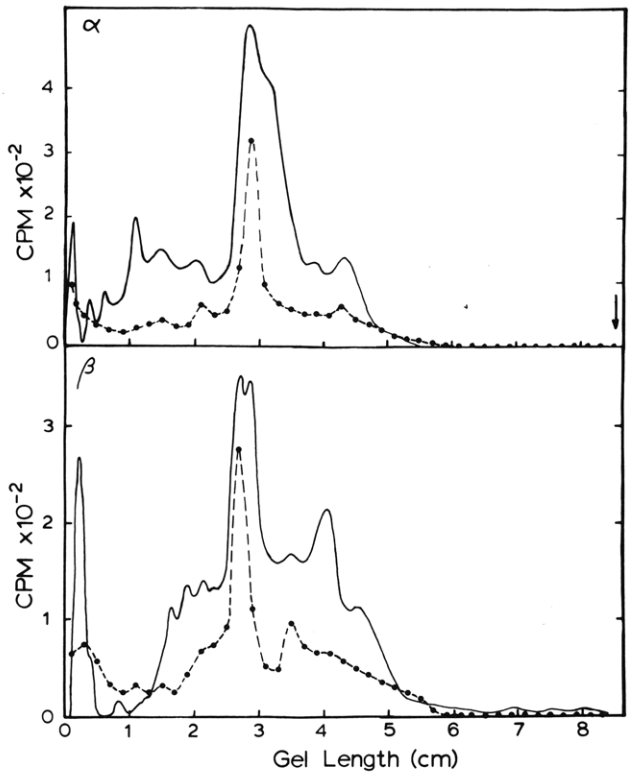


FIGURE 5: Cyanogen bromide peptides of [^{3}H]GTPox-labeled α- and β-tubulin. Tubulin subunits radiolabeled with [^{3}H]GTPox were completely separated in preparative polyacrylamide gel electrophoresis, extracted from the gels, and digested with cyanogen bromide as described under Materials and Methods. The peptides were resolved on $NaDodSO_4$–urea–polyacrylamide gel electrophoresis. The gels were stained, sliced, and digested, and the radioactivity was determined (●). The scanning densitometry of stained gels is indicated by the solid line.

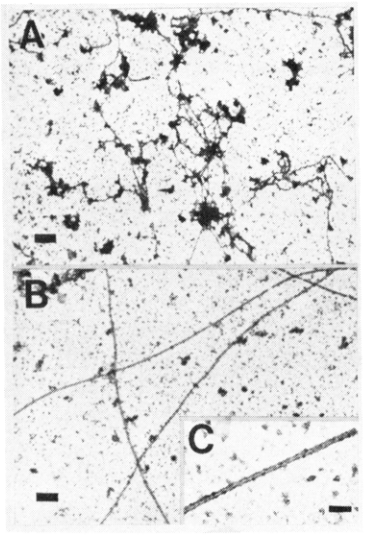


FIGURE 6: Electron microscopy of tubulin assembled with GTPox at pH 6.8 and 7.4. Tubulin polymerization was promoted by 1 mM GTPox in either 0.1 M Mes (pH 6.8) containing 1 mM EGTA, 0.1 mM EDTA, and 1 mM $MgCl_2$, shown in panel A, or 0.1 M Pipes (pH 7.4), shown in panels B and C. Samples of 10 μL were diluted 1:10 in assembly buffer containing 2% glutaraldehyde. A drop of the fixed preparation was placed on a carbon-coated grid, stained with 1% uranyl acetate, and observed in a Philips 300 EM. Magnification bars equal 2500 Å in (A) and (B) and 1135 Å in (C).

with GTPox revealed (Figure 6A) the presence of extensive filamentous structures, approximately 150 Å in diameter, and clearly distinct from normal microtubules formed by GTP. These filaments were not seen in control samples incubated

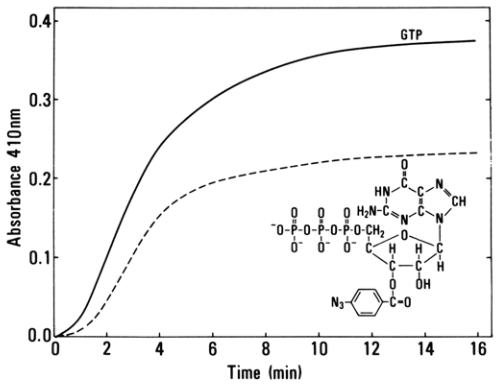


FIGURE 7: Tubulin polymerization in the presence of 3′-(*p*-azidobenzoyl)-GTP. A 1-mL solution of microtubule protein (3.5 mg/mL) freed of exchangeable nucleotide was allowed to polymerize at 37 °C in the presence of 1 mM GTP (solid line) or 1 mM 3′-(*p*-azidobenzoyl)-GTP (dashed line), and the turbidity increase was monitored at 410 nm as compared to a sample without added nucleotide. The structure of 3′-(*p*-azidobenzoyl)-GTP is inserted.

in the absence of nucleotide which failed to polymerize. However, reduction of GTPox to the dialcohol with borohydride prior to its addition to the assembly buffer also resulted in normal microtubules. This finding suggested that GTPox was possibly too reactive and prevented normal subunit interactions. Elevation of the assembly buffer's pH to 7.4 resulted in morphologically normal microtubules with GTPox (Figure 6B). Additional studies on these GTPox microtubules formed at pH 7.4 showed that the α and β binding specificities and the cyanogen bromide peptide fragment were the same as those found previously with the filaments produced at pH 6.8.

The possibility that the labeling profile seen with GTPox was artifactual or unique to this affinity reagent was examined by preparing chemically different analogues of GTP. The photoactive reagent *p*-azidobenzoic acid was used to synthesize 3′-(*p*-azidobenzoyl)-GTP and the (4-azidobenzoyl)hydrazone of GTPox. The latter compound failed to promote microtubule protein polymerization. However, the 3′-(*p*-azidobenzoyl)-GTP induced microtubule protein assembly, although at a slower rate and to a lesser extent that GTP (Figure 7). The azido compound is extremely photoreactive and will react to form covalent bonds with any amino acid within the GTP binding site when exposed to ultraviolet light. Ultraviolet irradiation of the polymerized tubulin resulted in covalent attachment of the GTP analogue.

Separation of α- and β-tubulin by $NaDodSO_4$–polyacrylamide gel electrophoresis showed that both subunits were labeled with the [^{3}H]GTP analogue (Figure 8). However, the 3′-(*p*-azidobenzoyl)-[^{3}H]GTP consistently labeled the α subunit more than the β (55:45). The radiolabeled subunits were separated and digested with cyanogen bromide and the fragments separated by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis as described above. Again the radiolabel was associated with the same size class (M_r 10 000) peptide from both α and β (data not shown). This chemically and mechanistically different affinity probe appeared to label the same cyanogen bromide fragments as found with GTPox.

The similarities of the [^{3}H]GTPox-labeled cyanogen bromide peptide from α- and β-tubulin were compared. To facilitate peptide separation and identification the tubulin preparation was dansylated prior to separation of the cyanogen bromide peptides by preparative polyacrylamide gel eletrophoresis. The radiolabeled cyanogen bromide peptide from α and β was digested by chymotrypsin and fingerprinted by two-dimensional electrophoresis at pH 6.4 and 2.1. The fingerprints were

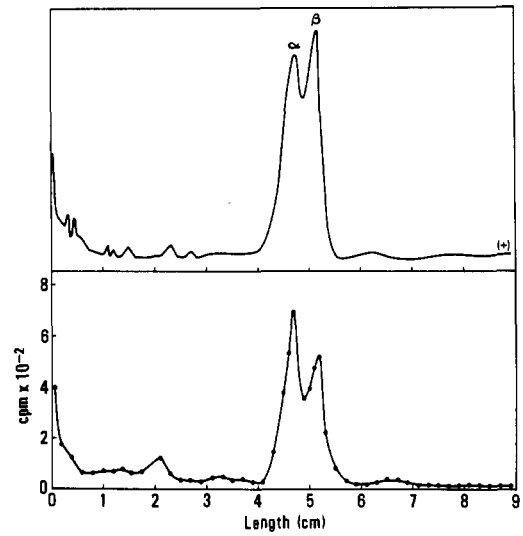
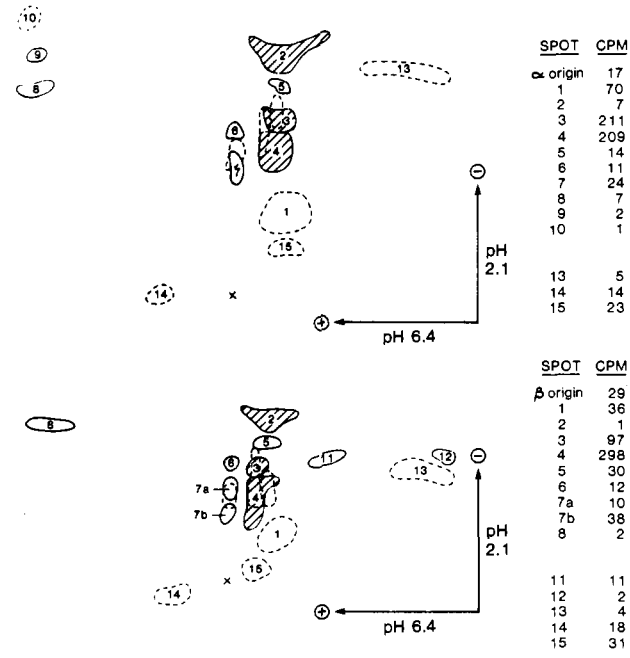


FIGURE 8: Subunit localization of bound 3′-(*p*-azidobenzoyl)-GTP. Microtubules formed with 1 mM 3′-(*p*-azidobenzoyl)-[^{3}H]GTP were collected by centrifugation and irradiated with a wide-spectrum UV source (<323 nm), and the subunits were separated on NaDodSO$_4$–polyacrylamide gel electrophoresis according to Bryan (1974). The gels were processed as described in Figure 3. The top panel is the densitometric scan and the bottom panel is the covalently bound analogue.



SPOT	CPM
α origin	17
1	70
2	7
3	211
4	209
5	14
6	11
7	24
8	7
9	2
10	1
13	5
14	14
15	23

SPOT	CPM
β origin	29
1	36
2	1
3	97
4	298
5	30
6	12
7a	10
7b	38
8	2
11	11
12	2
13	4
14	18
15	31

FIGURE 9: Chymotryptic fingerprint of [^{3}H]GTPox peptides. Radiolabeled peptide no. 7 (the peak from Figure 5) from cyanogen bromide digests was collected until 200 μg of both α- and β-derived material was obtained. The peptide was digested with chymotrypsin as described under Materials and Methods. Following lyophilization the material was spotted on Whatman 3MM (20 × 20 m) paper and electrophoresed in two dimensions as described under Materials and Methods. The dansylated peptides were visualized under UV light (dashed lines), followed by ninhydrin staining (solid lines). The hatched lines indicate the most intense ninhydrin spots. Chymotryptic peptide no. 1 was UV absorbing rather than fluorescent. The peptides were marked, cut out, and eluted, and their covalently bound radioactivity was determined (insert table). The region around peptide no. 4 possesses most of the radioactivity in both α and β subunits.

first visualized with UV light, and the position of fluorescent peptides and UV-absorbing material was marked and then stained with ninhydrin. A schematic of the ultraviolet (dashed lines) and ninhydrin visualized peptides (solid lines) is shown in Figure 9. The peptide maps from these cyanogen bromide fragments are very similar. Those peptides indicated by the dashed lines were UV fluorescent except for peptide no. 1 (Figure 9) which showed a strong UV absorbance. Both UV and ninhydrin-positive spots were cut out and the peptides eluted for liquid scintillation counting. The covalently bound radioactivity, as shown in the table to the right of Figure 9, was associated with similar chymotryptic peptides from both α and β subunits. The differences may in part reflect the choice of cutting these (no. 3 and 4) adjacent and possibly overlapping peptides from the paper. Furthermore, acid hydrolysates of [^{3}H]GTPox–tubulin showed that the radiolabel is covalently associated with a lysine residue (data not shown).

In the preceding paper (Maccioni & Seeds, 1983) we demonstrated that limited proteolytic cleavage of tubulin produced a 17-kilodalton amino-terminal fragment and a 34-kilodalton carboxyl-terminal peptide. Limited cleavage of [^{3}H]GTPox–tubulin with chymotrypsin shows that the GTPox binds to site in the 17-kilodalton amino-terminal portion of the tubulin molecule (Figure 10).

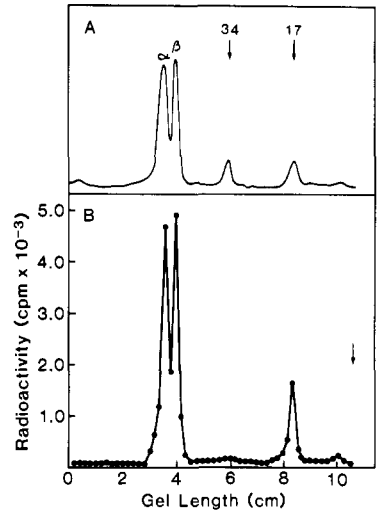


FIGURE 10: Limited chymotryptic cleavage of [^{3}H]GTPox–tubulin. (A) Densitometric profile of Coomassie brilliant blue stained cleavage products resolved by NaDodSO$_4$–polyacrylamide gel electrophoresis after incubation with 0.05% (w/w) chymotrypsin. Arrows indicate the 34- and 17-kilodalton tubulin fragments. (B) Distribution of bound radioactivity from [^{3}H]GTPox in the gel.

Discussion

Two chemically and mechanistically distinct analogues of GTP have been used to affinity label a GTP binding site(s) of tubulin. The results presented in this paper have demonstrated that the binding of the affinity ligands is saturable, competitive with the natural ligand, and apparently specific for the GTP site involved in microtubule assembly.

The formation of primarily filamentous (~150-Å diameter) structures in the presence of GTPox at the pH routinely used for tubulin assembly, while complete microtubules were formed only at a higher pH, suggests that the extent of protonation of an amino acid side chain in tubulin could markedly affect the lateral interaction between tubulin subunits containing the GTPox analogue.

Several nucleotide binding enzymes have been shown to contain a lysine residue at their nucleotide binding site (Easterbrook-Smith et al., 1976; Powell & Brew, 1976), and the dialdehydes formed by periodate oxidation of nucleotides have been shown to be specific affinity probes for these nucleotide binding sites (Kumar et al., 1979; Slebe et al., 1981). The ability to covalently attach GTPox to tubulin suggests that

a lysine residue is located at the exchangeable GTP binding site of tubulin. Furthermore, electrophoresis of tubulin hydrolysates shows that the radiolabeled material comigrates with lysyl-GTPox. Interestingly, Mellado et al. (1980) have shown that the selective carbamoylation of only one amino residue in tubulin prevents tubulin assembly and that the modification occurs equally (47:53) in both α and β subunits (Mellado et al., 1982).

The finding that both α- and β-tubulins display specific binding of the nucleotide analogues was very surprising; however, the kinetics and saturability of binding to both subunits (Figure 4; Table I), the similarity in the relative distribution of labeling between subunits (Figures 3 and 8), and the size of the labeled cyanogen bromide peptides found with both the 3'-(*p*-azidobenzoyl)-GTP and GTPox analogues (Figure 5) strongly support the site specificity of this labeling. Also, a sulfhydryl-specific analogue of GTP, 2-amino-6-(*S*-dinitrophenylthio)purine riboside triphosphate, has been shown to react in equal amounts with both subunits (Mann et al., 1978; Fasold et al., 1980).

The close similarity of the [^{3}H]GTPox peptides from cyanogen bromide and chymotryptic digests of both α- and β-tubulin (Figures 5 and 9) is very suggestive that the nucleotide binding site in α and β are identical. The primary amino acid sequences of α- and β-tubulin are $\sim$40% identical, and chick and pig brain tubulin sequences differ in only 7 of the $\sim$900 residues (Valenzuela et al., 1981; Ponstingl et al., 1981; Krauhs et al., 1981). A similar homology would be expected for lamb brain tubulin. Limited chymotryptic cleavage (Figure 10) shows that the GTP binding site is in the first 150 or so amino acids. Furthermore, our preliminary data indicate that the GTPox-containing cyanogen bromide peptides have an amino acid composition similar to the β peptide no. 73–147 and α peptide no. 37–154, both of which have a conserved lysine-containing sequence of Val-Arg-Lys and Ileu-Arg-Lys at residues no. 120-123 (Valenzuela et al., 1981; Ponstingl et al., 1981; Krauhs et al., 1981). Furthermore, Krauhs et al. (1981) has recently suggested that the glycine-rich region from residues no. 135–154 may represent part of the nucleotide binding site based on sequence homology with other nucleotide binding proteins.

During the course of this work Geahlen & Haley (1977, 1979) reported affinity labeling studies of the exchangeable GTP binding site in tubulin using an 8-azido-GTP analogue. In contrast to the results presented here, they only found specific labeling of the β subunit. The difference between our results may be related in part to the location of the active groups on the analogues; i.e., purine vs. ribose. Differences may also reflect the amount or type of residual nucleotide in the tubulin preparation. The tubulin used in the studies presented here was freed of all exchangeable nucleotide and possessed only 1 mol of nucleotide (GDP)/dimer (Maccioni & Seeds, 1982). Also, the 8-azido-GTP may bind more tightly or be more reactive toward the β subunit; this is an important consideration since the 8-azido-GTP labeling was less efficient (0.06–0.12 mol/tubulin dimer) than that found with GTPox (0.75–1.0 mol/tubulin dimer).

The near-stoichiometric binding of GTPox at saturation suggests only one site per tubulin dimer is available for binding exchangeable GTP. Therefore, the labeling of both subunits in nearly equal amounts suggests two functionally identical sites for nucleotide binding, one on α and another on β; furthermore, half of the tubulin has the α site accessible, and the other half has the β site accessible. This type of labeling may be explained by an equal opportunity for either α or β to bind GTP, but once 1 mol is bound, a conformational change occurs, masking the other site. Similarly, the nonexchangeable and exchangeable binding sites may be chemically identical but differ only in which subunit binds the first mole of nucleotide. These data can also be explained by a single site for exchangeable nucleotide that is created by both the α and β subunits, with an identical sequence region of both α and β forming the ribose binding portion. Since GTPox is a dialdehyde it could react with either subunit. The *p*-azidobenzoyl analogue may also possess enough flexibility to bind either subunit. This one-site model is more compatible with the data of Geahlen and Haley, where the purine binding portion of tubulin may be contributed only by the β subunit. In this regard, the amino acid sequences of chick and pig brain tubulin from about amino acid no. 75 to no. 150 are totally conserved and the α and β sequences are about 60% homologous through this region, which is presumed to constitute the GTP binding site.

Acknowledgments

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Registry No. GTP, 86-01-1; GTPox, 58045-02-6; 3'-*p*-azidobenzoyl-GTP, 84433-07-8.

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Mapping of Actin-Binding Sites on the Heavy Chain of Myosin Subfragment 1†

Kazuo Sutoh

ABSTRACT: When the rigor complex of actin and myosin subfragment 1 (S1) was treated with a zero-length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, covalently linked complexes of actin and S1 heavy chain with apparent molecular weights of 165 000 and 175 000 were generated. Measurements of the molar ratio of actin to S1 heavy chain in the 165K and 175K products showed that they were 1:1 complexes of actin and S1 heavy chain. Chemical cleavages of the cross-linked products followed by peptide mappings revealed that two distinct segments of S1 heavy chain spanning the 18K–20K region and the 27K–35K region from its C terminus participated in cross-linking with actin. Cross-linking of actin to the former site generated the 165K peptide while the latter site was responsible for generating the 175K peptide.

A detailed knowledge of the structure of the actin–myosin complex during a cyclic ATP hydrolysis by the complex is required to understand the molecular mechanism of muscle contraction. As a model system of the actin–myosin interaction, the structure of a stable actin–myosin subfragment 1 (S1)[1] complex formed in the absence of MgATP (the "rigor" complex) has been extensively investigated. One promising approach to reveal the structure of the actin–S1 rigor complex is to reconstitute its three-dimensional image from its electron micrographs (Moore et al., 1970; Toyoshima & Wakabayashi, 1979; Wakabayashi & Toyoshima, 1981; Taylor & Amos, 1981). Another complementary approach is to identify peptide segments participating in the actin–S1 contact. The latter approach, by employing the chemical cross-linking of the actin–S1 rigor complex with a zero-length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), has provided fruitful results (Mornet et al., 1981a,b; Sutoh, 1982a,b).

It was shown that EDC treatment of the rigor complex of actin and trypsin-treated S1 generated covalent cross-links between actin and the 20K and 50K tryptic fragments of S1 heavy chain, indicating that these two tryptic fragments of S1 heavy chain contain actin-binding sites (Mornet et al., 1981a). Detailed mapping of the actin-binding site was carried out on the 20K fragment of the heavy chain by employing the peptide mapping technique (Sutoh, 1982b). The peptide mapping showed that the N-terminal cyanogen bromide peptide of the 20K fragment spanning residues 1–20 participated in binding with actin. Similar cross-linking and peptide mapping experiments revealed that the N-terminal acidic segment of actin spanning residues 1–12 participated in binding with both of the 20K and 50K fragments of S1 heavy chain and, moreover, that a cluster of acidic residues close to the C terminus of actin was a binding site of alkaline light chain 1 of S1 (Sutoh, 1982a).

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[1] Abbreviations: S1, myosin subfragment 1; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; BNPS-skatole, 2-[(2-nitrophenyl)sulfenyl]-3-methyl-3-bromoindolenine.

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