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Interactions of a photoaffinity analog of GTP with the proteins of microtubules

(GTP analog/tubulin/GTPase)

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ABSTRACT **Tubulin dimers isolated from brain contain two GTP binding sites, a nonexchangeable site and an exchangeable site. To localize the exchangeable site, we used a photoaffinity analog of GTP, 8-azidoguanosine triphosphate (8-N_3GTP), which supports tubulin polymerization in the absence of activating light. Photolysis of tubulin polymerized in the presence of 0.01 to 0.1 mM [β,γ-^{32}P]8-N_3GTP resulted in covalent incorporation of radioactivity only onto the β monomer. Photolysis with 8-N_3GTP also prevented any further repolymerization of the tubulin whereas like treatment in the presence of GTP had no effect. Preincubation of tubulin with GTP prevented photoincorporation of [β,γ-^{32}P]8-N_3GTP whereas preincubation with ATP did not.**

The subunit protein of microtubules, tubulin, consists of a 110,000 dalton heterodimer of α and β monomers (1). Each dimer will bind 2 mol of guanine nucleotide, one at a readily exchangeable site and a second at a nonexchangeable site (2). Isolated microbubules may contain GDP or GTP in either binding site. Tubulin with GTP present in both sites is capable of polymerization. The exchangeable site nucleotide is hydrolyzed during polymerization (3) and some reports suggest that both nucleotides are hydrolyzed (4, 5). ATP has been shown to support microtubule formation by a transphosphorylation activity associated with a nontubulin protein that copurifies with microtubules (3, 4). There is still a great deal of uncertainty as to the actual roles played by the guanine nucleotides in tubulin polymerization. Binding of GTP to tubulin may result in conformational changes that allow the dimers and rings to associate to form microtubules (3). The distribution of the two sites on the tubulin dimer is uncertain, and their locations may have important mechanistic implications.

In order to localize the tubulin binding sites for GTP, a GTP analog, 8-azidoguanosine triphosphate (8-N_3GTP), has been synthesized for use as a photoaffinity probe (Fig. 1). Previous publications have reported the synthesis of photoactive 8-azido analogs of the adenine nucleotides (8-N_3cAMP, 8-N_3AMP, 8-N_3ADP, and 8-N_3ATP) and have examined their utility as membrane structural probes (6–9). By analogy, results reported herein indicate that 8-azido analogs of the guanine nucleotides should also be useful for the study of nucleotide–protein interactions.

MATERIALS AND METHODS

[β,γ-^{32}P]8-N_3GTP was synthesized as follows. 8-N_3GMP was prepared from GMP (Sigma Chemical Co.) by a modification of the method for preparing 8-N_3AMP (6, 9). GMP (0.5 mmol) was dissolved in 10 ml of 1 M sodium acetate (pH 3.9) and brominated by the sequential addition of 3.5 ml of Br_2 in H_2O (10 μl/ml). Separation was achieved on a 3 × 50 cm DEAE-cellulose-HCO_3 column with a linear gradient of 2 liters of 0–0.3 M triethylammonium bicarbonate (60% yield). The resulting 8-Br-GMP was incubated for 48 hr at 50° in anhydrous dimethylformamide/isobutyric acid, 5:1 (vol/vol), with a large excess of triethylammonium azide (10 mmol). Separation of 8-N_3GMP (30% yield) was the same as for 8-Br-GMP. [^{32}P]-Pyrophosphate (ICN) was coupled to 8-N_3GMP to form [β,γ-^{32}P]8-N_3GTP by the Michelson method (10). N_3GTP was identified by: (*i*) the incorporation of ^{32}P from labeled pyrophosphate, (*ii*) its ultraviolet spectrum, (*iii*) its photolability, (*iv*) its R_F value, and (*v*) its biological activity. Chromatographic separations were performed on Whatman DE81 DEAE-cellulose paper with 0.1 M citric acid/sodium acetate, pH 3.75, 1:1 (vol/vol), as the solvent system.

Microtubular protein was isolated from sheep brain by the method of Shelanski *et al.* (11) and stored in the absence of GTP. Tubulin solutions were concentrated with a Millipore immersible molecular filter unit (10,000 nominal molecular weight limit) and passed through a 1 × 25 cm Sephadex G-25 column to remove free and loosely bound nucleotides. Microtubule formation was followed by the light scattering assay of Gaskin *et al.* (12) at 37° with a Beckman model 25 spectrophotometer equipped with a heated cuvette chamber. Solutions were monitored at 500 nm because of the ultraviolet-absorbing properties of N_3GTP. Protein concentrations were determined by the method of Lowry *et al.* (13).

Photolysis of N_3GTP with tubulin was done with a short wavelength (253.4 nm) UVS-11 Mineralight (Ultraviolet Products, Inc.) at a distance of 10 cm for 5 min. After photolysis, proteins were solubilized in an equal volume of protein-solubilizing solution (25% sucrose/2.5% sodium dodecyl sulfate/Pyronin Y, 0.25 mg/ml/25 mM Tris·HCl, pH 8.0/2.5 mM EDTA/dithiothreitol, 15.4 mg/ml). Proteins were separated by a slab gel electrophoresis system using a linear gradient (6–12%) of polyacrylamide with a 4% stacking gel as described (8). Autoradiography of the dried slab gels was performed as described (7). An Ortec model 4310 densitometer was used for scanning dried gels and autoradiographs.

RESULTS

The 8-azidoguanine moiety showed an absorbance maximum at 278 nm and had a molar extinction coefficient of 1.2×10^4 as determined by total phosphate analysis of chromatographically pure 8-N_3GMP, 8-N_3GDP, and 8-N_3GTP and assuming N_3guanine/phosphate ratios of 1:1, 1:2, and 1:3. Chromatographically pure GMP, GDP, GTP were used as standards because both the extinction coefficient and number of phosphates per base were known. Total phosphate was determined as previously reported (14).

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Abbreviations: 8-N_3GTP, 8-azidoguanosine triphosphate; MES, 4-morpholinethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

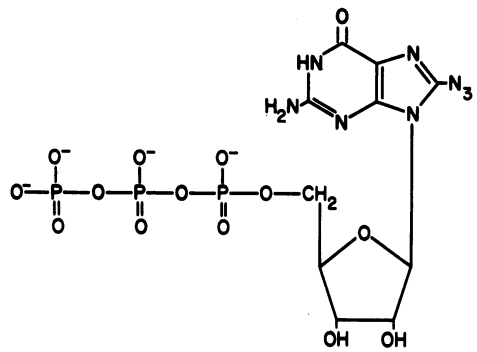

FIG. 1. Structure of 8-azidoguanosine triphosphate (8-N_3GTP).

Fig. 2 shows the effect of increasing duration of photolysis on the ultraviolet absorption spectrum of 8-N_3GMP. These nucleotide analogs are very sensitive to ultraviolet irradiation, and the spectrum of a solution of 1.95 absorbance is completely altered within 70 sec. Similar changes were seen for 8-N_3GDP, 8-N_3GTP, and 8-N_3GMP with an isosbestic point at 252 nm.

Tubulin isolated from sheep brain and stored in the absence of GTP in 0.1 M 4-morpholinethanesulfonic acid (MES), pH 6.4/1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA)/0.5 mM $MgCl_2$ will fully polymerize in the absence of additional nucleotides. Two rounds of reversible polymerization/depolymerization were sufficient to hydrolyze the endogenous GTP, and additional guanine nucleotide must then be added to restore its ability to polymerize. Alternatively, tubulin concentrated by membrane filtration with repeated changes of buffer and desalted on a Sephadex G-25 column required the addition of GTP for microtube formation. GTP-dependent tubulin prepared by either process will polymerize with the addition of either GTP or 8-N_3GTP. Fig. 3*A* shows the effect of 8-N_3GTP and GTP on microtubule formation. With two preparations the degree of polymerization obtained with 8-N_3GTP varied from 60% to 80% of that obtained with GTP. After depolymerization at 4° for 1 hr, both tubulin solutions repolymerized to the same extent as they did initially.

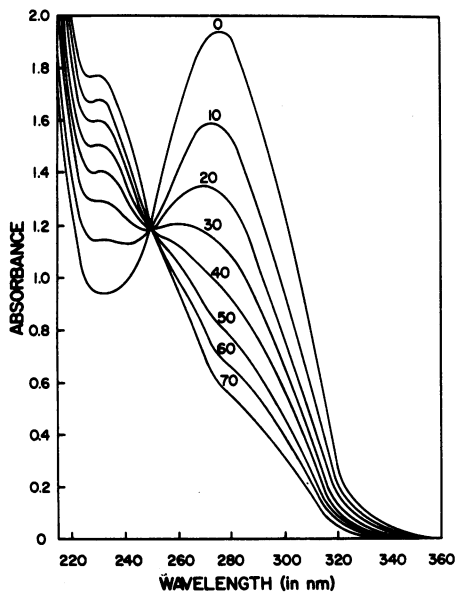

FIG. 2. Effect of photolysis on ultraviolet spectrum of 8-N_3GMP. The ultraviolet profile of 8-N_3GMP (0.16 mM, neutral pH) is extensively altered by exposure to ultraviolet irradiation for increasing lengths of time (10-sec increments). Photolysis was performed at a distance of 1 cm with a UVS-11 Mineralight.

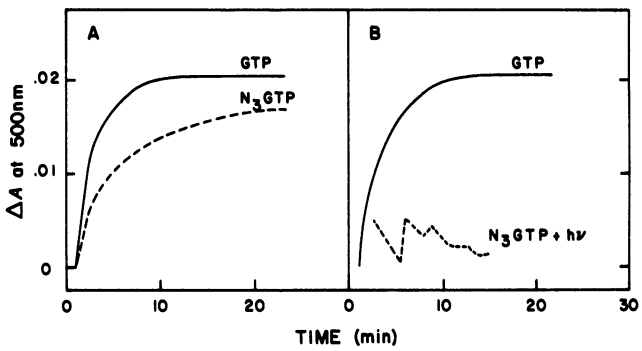

FIG. 3. Effect of photolysis of tubulin polymerization of 8-N_3GTP. GTP-dependent tubulin (0.5 mg/ml) was incubated at 0° in 0.1 mM MES, pH 6.4/0.5 mM $MgCl_2$/1 mM EGTA/1 mM GTP or 8-N_3GTP for at least 30 min. (*A*) Polymerization was initiated by transfer to a heated (37°) cuvette chamber. Each solution was depolymerized by incubation at 4° for 1 hr followed by a 2-min photolysis in the cuvette at a distance of 1 cm. (*B*) Repolymerization was initiated as before.

To rule out the possibility that a small impurity in the N_3GTP preparation (e.g., GTP) was causing polymerization, the nucleotide concentrations were doubled and the experiment was repeated. The resulting kinetics were essentially identical. Four successive rounds of polymerization and depolymerization were reversible with both GTP and N_3GTP. Also, addition of 1 mM GTP to the microtubules polymerized in the presence of 1 mM N_3GTP did not increase the final degree of polymerization to the level obtained with GTP, indicating that the polymerization was not due to contaminating GTP. At present we have no explanation for the lower maximum level of polymerization observed with 8-N_3GTP.

Tubulin that has been polymerized and depolymerized in the presence of 8-N_3GTP will not repolymerize if photolyzed for 2 min; the GTP supported system, treated identically, remains unaffected (Fig. 3*B*). Failure of the N_3GTP-supported tubulin to repolymerize may have been due to photodestruction of the nucleotide, covalent attachment of the nucleotide to the tubulin, or a combination of both.

To determine which monomer(s) would bind N_3GTP, solutions of tubulin containing 0.01 mM, 0.1 mM, and 1 mM [β,γ-^{32}P]8-N_3GTP were allowed to polymerize at 37° for 30 min to "lock" labeled 8-N_3GTP onto the exchangeable site (3). The polymerized system was then photolyzed for 5 min to effect covalent attachment of radioactively labeled nucleotide to the tubulin monomer. Separation of proteins on sodium dodecyl sulfate/gel electrophoresis revealed label incorporated only in the β-monomer band at the two lower nucleotide concentrations. Fig. 4 shows the densitometer tracings of the Coomassie brilliant blue-staining profile of tubulin photolyzed with 0.1 mM [β,γ-^{32}P]8-N_3GTP and separated on sodium dodecyl sulfate gels and the resulting autoradiograph made from the same gel. A small degree of labeling was obtained in the α-monomer band only at 1 mM nucleotide concentration (not shown). Such incorporation could be due to a low affinity binding site (or a site otherwise relatively unavailable to 8-N_3GTP) or to nonspecific photolabeling due to the high concentration of analog present during photolysis. Incubation of tubulin with 1 mM [β,γ-^{32}P]8-N_3GTP without photolysis revealed no incorporation, ruling out the possibility of labeling due to enzymatic phosphorylation.

If the binding site being photolabeled is specific for GTP, then N_3GTP and GTP should be competitive for the same site. Fig. 4*C* shows that preincubation of tubulin with 1 mM GTP, prior to addition of 1 mM [β,γ-^{32}P]8-N_3GTP and photolysis, completely prevented photoincorporation. Identical preincu-

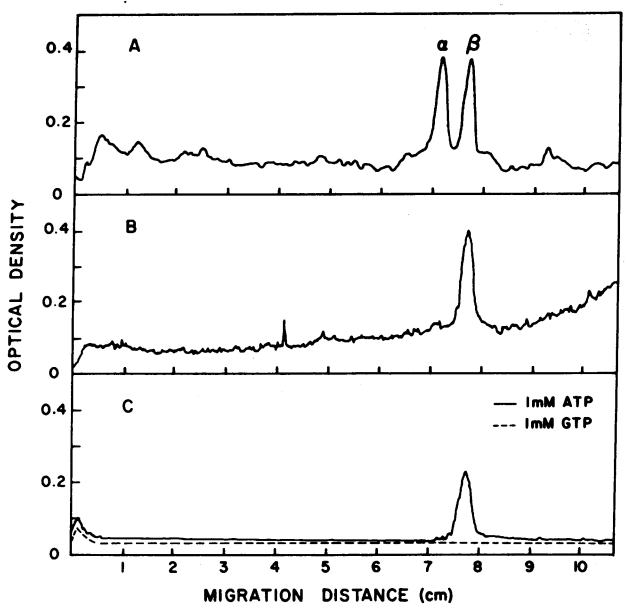

FIG. 4. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of tubulin photolabeled with [β,γ-^{32}P]8-N_3GTP. Tubulin (48 μg) in 40 mM MES/0.4 mM EGTA/0.2 mM $MgCl_2$ in a total volume of 100 μl was incubated with [β,γ-^{32}P]8-N_3GTP and photolyzed for 5 min at a distance of 10 cm. (*A*) Densitometer tracing of Coomassie blue-staining profile of tubulin. (*B*) Densitometer tracing of autoradiogram of electrophoresed tubulin after incubation at 25° for 30 min with 100 μM [β,γ-^{32}P]8-N_3GTP followed by photolysis. (*C*) Densitometer tracing of autoradiogram of electrophoresed tubulin photolyzed with 1 mM [β,γ-^{32}P]8-N_3GTP after preincubation for 15 min at 37° with 1 mM GTP (dashed line) or 1 mM ATP (solid line).

bation with 1 mM ATP did not protect the β-monomer from being photolabeled. This indicates that ATP does not compete for this GTP binding site on the β subunit. In this experiment, with 1 mM GTP or ATP present, no labeling of the α subunit appeared, even at 1 mM analog concentration.

DISCUSSION

The results in this paper show that a photoaffinity analog of GTP is able to mimic GTP biologically in the requirement for tubulin polymerization. It therefore seems likely that both nucleotides bind to identical sites on the tubulin dimer. When photoaffinity analogs that contain azide functions are exposed to activating irradiation, they form highly reactive nitrenes that are capable of forming covalent attachments to all amino acid residues of proteins. In this manner, photolysis of [β,γ-^{32}P]8-N_3GTP bound to tubulin yields a radioactive tag covalently attached to an amino acid residue(s) within the GTP binding site(s). Because the nonexchangeable site nucleotide is tightly bound, the 8-N_3GTP analog is most likely binding to the exchangeable site. Photolysis of tubulin with 0.01 or 0.1 mM [β,γ-^{32}P]8-N_3GTP yielded label only on the β-monomer, which indicates that the exchangeable GTP binding site is present only on the β subunit.

Isolated microtubules contain equimolar amounts of α and β monomers along with several microtubule-associated proteins. Labeling of only the β-monomer argues for the specificity of this photolabeling. Only GTP, and not ATP, was effective in preventing the photoincorporation, which indicates that the analog is photolabeling specific GTP binding sites. These results also support earlier reports that ATP and GTP do not compete for binding to tubulin (4). The prevention of [β,γ-^{32}P]8-N_3GTP binding by an equimolar amount of GTP could imply that tubulin possesses a greater affinity for GTP than for the analog. However, the order of addition may be more important because polymerization during preincubation could "lock" the nucleotide added first onto the exchangeable site. Photolysis of tubulin with high concentrations (1 mM) of [β,γ-^{32}P]8-N_3GTP in the absence of any added nucleotide yielded a small amount of incorporation in the α-monomer. At such high concentrations, nonspecific labeling cannot be ruled out. Also, the possibility exists that some exchange could have occurred with the nonexchangeable site. This is not likely but requires further testing for elimination.

8-N_3GTP should prove to be a useful tool for further study of protein–nucleotide interactions involved in microtubule formation. In addition to tubulin, other proteins are known to bind GTP with a resulting activation and conformational change. Examples are adenylate cyclase and several initiation and elongation factors involved in protein synthesis. Preliminary studies have shown that 8-N_3GTP is able to completely replace GTP in an *in vitro* polyuridylate-directed polyphenylalanine synthesizing system and in an assay for AUG-dependent ribosomal binding of f[^{14}C]Met-tRNA (unpublished data). The results indicate that 8-N_3GTP will activate both elongation and initiation factors in place of GTP and may serve as an important probe for studying protein synthesis. [β,γ-^{32}P]8-N_3GTP has been shown to label specific proteins in human erythrocyte membranes and may eventually serve as a probe for studying membrane-bound adenylate cyclase (unpublished data).

In addition to 8-N_3GTP, the analogs 8-N_3GMP, 8-N_3GDP, and cyclic [^{32}P]8-N_3cGMP have been synthesized. We have utilized cyclic [^{32}P]8-N_3cGMP as a photoactivated probe for cyclic GMP sites in both membranes and whole freeze-fractured mouse spleenocytes (unpublished data). We therefore propose that the 8-azidoguanine nucleotide analogs will be useful tools for the study of nucleotide–protein interactions involved not only in tubulin polymerization but also in the numerous systems in which guanine nucleotides are involved in control and regulation.

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1. Bryan, J. & Wilson, L. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1762–1766.
2. Weisenberg, R. C., Borisy, G. G. & Taylor, E. W. (1968) *Biochemistry* **7**, 4466–4479.
3. Weisenberg, R. C., Deery, W. J. & Dickinson, P. J. (1976) *Biochemistry* **15**, 4248–4254.
4. Jacobs, M., Smith, H. & Taylor, E. W. (1974) *J. Mol. Biol.* **89**, 455–468.
5. Maccioni, R. & Seeds, N. W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 462–466.
6. Haley, B. E. & Hoffman, J. F. (1974) *Proc. Natl. Acad, Sci. USA* **71**, 3367–3371.
7. Haley, B. E. (1975) *Biochemistry* **14**, 3852–3857.
8. Owens, J. R. & Haley, B. E. (1976) *J. Supra. Structure* **5**, 91–102.
9. Haley, B. E. (1977) in *Methods in Enzymology*, eds. Jakoby, W. B. & Wilchek, M. (Academic Press, New York), Vol. 46, pp. 339–346.
10. Michelson, A. M. (1964) *Biochim. Biophys. Acta* **91**, 1–13.
11. Shelanski, M. L., Gaskin, F. & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 765–768.
12. Gaskin, F., Cantor, C. R. & Shelanski, M. L. (1974) *J. Mol. Biol.* **89**, 737–758.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
14. Haley, B. E. & Yount, R. G. (1972) *Biochemistry* **11**, 2863–2871.