The Effects of Various GTP Analogues on Microtubule Assembly

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ABSTRACT. We synthesized 27 GTP analogues with modification or substitution at positions C2, C6, C8 and ribose moiety to investigate their effect on microtubule (Mt) assembly. It was found that C2 and C6 are both functional for the analogues supporting Mt assembly. It was surprising to find that 2-amino-ATP (n2ATP) substantially supports assembly, and that the appearance of the assembled Mts was indistinguishable from those assembled in the standard GTP assembly buffer solution. Furthermore, 2-amino dATP and dGTP are even more potent than GTP in supporting assembly. The substitution of oxo group at C6 with reactive thiol largely reduced the activity of the analogue to support assembly. When free rotation of the glycosidic linkage of GTP was blocked by the introduction of sulfur atom between C8 and C2' of ribose moiety, it resulted in total suppression of assembly. Purine nucleoside triphosphate was found to support assembly better than GTP, and even more efficient was 2-amino purine nucleoside triphosphate. Interestingly, their deoxy-type analogues were totally inhibitory. Although 2-amino 8-hydroxy ATP and other analogues supported assembly much better than did GTP, their diphosphate analogues were totally incapable of supporting assembly. Finally, bulky fluorescent probes were introduced at C3' of ribose moiety (Mant-8-Br-GTP or Mant-GTP) to visualize the fluorescent signal in assembled Mts. Even in this case, the number of most protofilaments was found to be 14, consistent with that found in Mts assembled in GTP standard buffer solution.

Key words: tubulin/microtubules/GTP analogues/microtubule assembly

Two binding sites for GTP on the $\alpha\beta$ tubulin heterodimer have well been known (5, 14, 33), one non-exchangeable (N-site) and the other exchangeable (E-site) with exogenous GTP (33).

GTP is required for tubulin to polymerize and the E-site has been analyzed to be localized on β -tubulin (4, 24). The role of guanine nucleotides on microtubule (Mt) assembly has long been discussed on the assumption that the conformation of tubulin heterodimer will be maintained by GTP for tubulin polymerization. Non-hydrolyzable GTP analogues have been reported to support tubulin polymerization (1, 24, 27, 34), and some analogues of GTP was shown to react with tubu-

lin heterodimer (3, 35). The significance of the micro-environment of the GTP binding sites, however, has not been clarified, in part because of the difficulty of analyses of the tubulin conformation.

Recent electron crystallographic data on zinc-induced tubulin sheets suggest the positioning of GTP in the $\alpha\beta$ tubulin hetero-dimer and interactions of the phosphate, ribose and base moieties with surrounding amino acid residues (25). The position of the nucleotide agreed with models based on comparison with other nucleotide-containing proteins.

Various analogues of GTP would further help our understanding on the tubulin heterodimers in terms of the role of guanine nucleotide in polymerization. A few reports have been published describing the effect of GTP analogues modifying ribose moieties of the guanine nucleotide on Mt assembly (5–7), and some modification of the purine base supported Mt assembly although it was under non-physiological conditions (3). However, a systematic modification of the base moiety of guanine nucleotide has not been investigated, possi-

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Abbreviations: EGTA, ethyleneglycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; MAPs, microtubule-associated proteins; Mts, microtubules; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid). For abbreviations of the GTP analogues, see Table I.

bly because of time-consuming work for synthesizing various analogues of GTP.

We have been working on the synthesis of various analogues of GTP or ATP during the past ten years. This helped us to analyze the effect of various analogues of GTP, in which three positions of the base (carbon 2, 6, and, 8) and the ribose moiety were modified on Mt assembly in the presence of microtubule-associated proteins (MAPs). The present paper demonstrates that the modification of the purine base as well as the ribose moiety profoundly affects Mt assembly under physiological assembly conditions.

Materials and Methods

Syntheses of GTP analogues

The following 27 analogues (all sodium salts) with abbreviations shown in the 3rd column in Table I were synthesized and purified according to the procedures described in the references listed in the Table. dGTP, ITP and XTP were purchased from Sigma.

Preparation of Mt proteins

Mt protein was purified from fresh porcine brain essentially by the method of Shelanski and Canter (30) with the following modification. Temperature-dependent two cycled Mt protein fraction prepared in polymerization buffer which consisted of 0.1 M PIPES, 1 mM EGTA, 1 mM MgCl₂ (standard buffer) and 1 mM GTP at pH 6.8 was stored at -80°C.

Table I. NAMES OF VARIOUS GTP ANALOGUES, THEIR ABBREVIATIONS AND THE NUMBER OF REFERENCES BY WHOSE PROCEDURES THE ANALOGUES WERE SYNTHESIZED EXCEPT 4, 6 AND 7 WHICH WERE PURCHASED FROM SIGMA

No.	Name of GTP analogues	Abbreviation	Ref. No.
1	2,6-diamino-9- β -D-ribofuranosylpurine 5'-triphosphate	n2ATP	20
2	6-amino-2-oxo-9- β -D-ribofuranosylpurine 5'-triphosphate	isoGTP	*
3	2,6-diamino-9-(2-deoxy- β -D-ribofuranosyl)purine 5'-triphosphate	n2dATP	21
4	6-oxo-9- β -D-ribofuranosylpurine 5'-triphosphate	ITP	From Sigma
5	2-anilino-6-amino-9- β -D-ribofuranosylpurine 5'-triphosphate	2-Anil-ATP	*
6	2,6-dioxo-9- β -D-ribofuranosylpurine 5'-triphosphate	XTP	From Sigma
7	2-amino-6-oxo-9-(2-deoxy- β -D-ribofuranosyl)purine 5'-triphosphate	dGTP	From Sigma
8	2-amino-6-anilino-9-(2-deoxy- β -D-ribofuranosyl)purine 5'-triphosphate	6-Anil-n2dPuTP	21
9	2-amino-6-morpholino-9-(2-deoxy- β -D-ribofuranosyl)purine 5'-triphosphate	6-Mor-n2dPuTP	21
10	2-amino-6-cyclohexylamino-9-(2-deoxy- β -D-ribofuranosyl)purine 5'-triphosphate	6-CHexN-n2dPuTP	21
11	6-amino-8-carboxy-9- β -D-ribofuranosylpurine 5'-triphosphate	8-COOH-ATP	*
12	6-ethoxycarbonylmethyl-9- β -D-ribofuranosylpurine 5'-triphosphate	6-EtCOOCH ₂ -PuTP	*
13	2-amino-6-mercapto-9- β -D-ribofuranosylpurine 5'-triphosphate	6-SH-GTP	2, 3, 11, 35
14	2-amino-6-methylmercapto-9- β -D-ribofuranosylpurine 5'-triphosphate	6-CH ₃ S-GTP	2, 3, 11, 35
15	2,6-diamino-8-hydroxy-9- β -D-ribofuranosylpurine 5'-triphosphate	8-OH-n2ATP	20
16	8-bromo-guanosine 5'-triphosphate	8-Br-GTP	20
17	2,6-diamino-8-bromo-9- β -D-ribofuranosylpurine 5'-triphosphate	8-Br-n2ATP	20
18	8,2'-anhydro-2,6-diamino-8-mercapto-9- β -arabinofuranosylpurine 5'-triphosphate	8,2'-S-n2ATP	20
19	8,2'-anhydro-8-mercapto-9- β -D-arabinofuranosyladenine 5'-triphosphate	8,2'-S-ATP	20
20	8,2'-anhydro-8-mercapto-9- β -D-arabinofuranosylguanine 5'-triphosphate	8,2'-S-GTP	19
21	9- β -D-ribofuranosylpurine 5'-triphosphate	PuTP	*
22	2-amino-9- β -D-ribofuranosylpurine 5'-triphosphate	n2PuTP	*
23	9-(2-deoxy- β -D-ribofuranosyl)purine 5'-triphosphate	dPuTP	*
24	9-(2,3-dideoxy- β -D-ribofuranosyl)purine 5'-triphosphate	ddPuTP	*
25	2,6-diamino-8-hydroxy-9- β -D-ribofuranosylpurine 5'-diphosphate	8-OH-n2ADP	20
26	2,6-diamino-9- β -D-ribofuranosylpurine 5'-diphosphate	n2ADP	20
27	2,6-diamino-9-(2-deoxy- β -D-ribofuranosyl)purine 5'-diphosphate	n2dADP	20
28	3'-O-methylanthoraniloyl-guanosine 5'-triphosphate	Mant-GTP	9, 10
29	8-bromo-3'-O-methylanthoraniloyl-guanosine 5'-triphosphate	Mant-8-Br-GTP	9, 10
30	2,6-diamino-8-hydroxy-9-(3-O-methylanthoraniloyl- β -D-ribofuranosyl)purine 5'-triphosphate	Mant-8-OH-n2ATP	9, 10

* represents unpublished procedure

Preparation of Mt protein fraction dissolved in a buffer solution containing GTP analogue

After thawing frozen Mt protein fraction (ca. 1.5 ml, 2–3 mg/ml), the solution was clarified by centrifugation in a ultracentrifuge (Hitachi CS100GX or Beckman TL-100) at $150,000 \times g$ for 10 min, followed by incubation at 37.5°C for 30 min to reconstitute Mts. The Mt suspension was centrifuged at $150,000 \times g$ to collect assembled Mts. The surface of the Mt pellet was rinsed once with a buffer solution free of nucleotides, followed by homogenization in a buffer solution containing GTP analogue. The homogenate was clarified by centrifugation at $150,000 \times g$ for 10 min and the supernatant was used for Mt assembly. Under this condition, the effect of the remaining trace amounts of GTP on Mt assembly was negligible, because turbidity development was not observed after the Mt pellet was depolymerized and processed for Mt assembly in the standard buffer solution free of GTP.

Polymerization measurements

Mt assembly in the standard buffer solution containing nucleotides at a constant concentration of 0.5 mM was monitored turbidimetrically at 350 nm by using a Hitachi 200-20 double beam recording spectrophotometer equipped with jacketed cells thermostated with a TAITEC pump unit (p-11). Turbidimetric values were expressed as $\text{OD}_{350}/\text{mg protein}$. In each set of measurements (4 samples at a time) three independent samples containing the same GTP analogue were standardized by simultaneous measurement of Mt assembly in the standard buffer containing GTP.

Observation of assembled Mts

Mts have been routinely observed by dark-field contrast microscopy equipped with mercury-arc lamp. Instead, we have recently found that an Olympus BX60 microscope with dark-field contrast condenser (Olympus U-DCW) and halogen lamp enabled us to see assembled Mts with MAPs easier than using a mercury-arc lamp.

Observation of Mts labeled with fluorescently-labeled GTP analogues (Mant GTP and Mant 8-Br GTP) was then performed using the same fluorescence microscope. This confirms the work already reported by Hiratsuka (10). The microscope was equipped with a filter [U-MWU (U-M536)] and an objective lens [U-APO40X01/340 (1-UB768)], which allowed us to observe Mts assembled from tubulin fluorescently labeled with Mant GTP, with excitation at 350 nm and emission at 442 nm in an aqueous solution containing 50 mM Tris-HCl (Hiratsuka, 1983).

Negative staining of assembled Mts was carried out by routine procedure using 2% uranyl acetate (26) and the number of protofilaments of Mts assembled in the presence of some GTP analogues was measured by staining with tannic acid (18, 22).

Others

Protein concentration was determined by the method of Lowry *et al.* (16) with bovine serum albumin as a standard. Electron microscopy was performed as described previously (23).

Results and Discussion

Modification of position 2 of purine base

It is well known that ATP does not support Mt assembly, since neither ATP nor ADP associates with Mts (15). However, it was surprising to find that the addition of amino group to position 2 of the ATP purine base (n2ATP) largely enhanced the property of the analogue to cause Mt assembly (Fig. 1). The addition caused the initial growth and steady state of assembly up to more than half the Mt assembly in the GTP standard assembly system. Electron microscopy revealed that Mts assembled in n2ATP containing buffer were indistinguishable from those assembled in the GTP standard buffer (Fig. 2A and B). It was, therefore, of interest to test the effect of isoGTP in terms of whether 2 amino group and 6 oxo group of GTP are exchanged on Mt assembly. The result, shown in Figure 3 was that isoGTP does not support Mt assembly. In addition, we synthesized n2dATP to determine the effect of the modification of ribose moiety of nucleotides on Mt assembly. Figure 1 shows that the deoxy type of the analogue supported Mt assembly better than GTP as previously shown by Hamel and Lin (5) and

Fig. 1. Effect of n2dATP, dGTP, 6-CHexN-n2dPuTP, 6-CH3S-GTP, n2ATP, 6-Mor-n2dPuTP, 6-SH-GTP and 6-Anil-n2dPuTP compared with that of GTP in Mt assembly.

Fig. 2. Negative staining of Mts assembled in buffer solutions containing GTP (A) and n2ATP (B). Many rings are shown in photograph C which is another evidence that tubulin did not polymerize in the presence of 6-SH-GTPs. Bars, 100 nm.

Fig. 3. Effect of ITP, 2-Anil-ATP, XTP and isoGTP compared with that of GTP on Mt assembly.

Hamel *et al.* (7).

These results suggested that the introduction of the amino group into position 2 greatly promotes Mt assembly. In order to further investigate this possibility, we substituted the amino group at position 2 of GTP with hydrogen, secondary amines and oxo group. Their effects, shown in Figure 3, indicated that Mt assembly was supported in the following order: hydrogen (ITP) > primary alkyl amino group (GTP) > secondary aryl amino group (2-anil-ATP) > oxo group (XTP). The possibility was considered that some hydrogen bonding through hydrogen atoms of amino group at position C2 with nearby oxygen or nitrogen atoms in β -tubulin molecule might be involved in the maintenance of a conformation favorable for tubulin polymerization. However, this was ruled out by the fact that the substitution of amino group with hydrogen atom (ITP) was shown to be more favorable than GTP in promoting Mt assembly, although the possibility cannot be excluded that a small bulky group like the amino group is needed to form hydrogen bonds around position C2. In this connection, we tried to substitute the amino group at position C2 with carboxyl group without success.

6-substituted dGTP analogues

The results so far obtained suggested that positions 6 and 2 of the purine base seem important for the nucleotide to support Mt assembly. Therefore, we synthesized some of the 6-substituted dGTP. The reason we selected dGTPa instead of GTP was that the former was shown to be more efficient in promoting Mt assembly (Fig. 1). Figure 1 also shows that 6-Anil-n2dPuTP

and 6-Mor-n2dPuTP were inhibitory. On the other hand, the substitution of oxo group with amino group (n2dATP) or cyclohexyl group (6-CHexN-n2dPuTP) largely supported Mt assembly. The substitution of oxo group with bulky cyclohexyl group suppressed the initial velocity of polymerization but the steady state was higher than that monitored in the standard GTP buffer solution. Even in the presence of amino group at position 2, the introduction of bulky group like ethoxycarbonyl group (6-EtCOOCH₂-PuTP) at position 6 substantially suppressed assembly (Fig. 1).

When the oxo group was substituted with thiol (6-SH-GTP), it resulted in near total incapacity to promote Mt assembly (Fig. 1). Electron microscopy revealed that only ring structures without Mts were observed after 30 min incubation of C₃S solution containing only 6-SH-GTP as guanine nucleotide (Fig. 2C). This result is inconsistent with those reported by others (3, 35), probably due to the presence of glycerol at final concentration of 25% W/v (about 3.4 M glycerol) in their experiments. It was previously demonstrated that when assembly is performed in the presence of 4 M glycerol, tubulin assembles into Mts even in the absence of added GTP (30). Glycerol promotes Mt assembly even in the presence of GDP which routinely inhibits Mt assembly in a physiological medium condition (15). The inhibition was suggested to not be due to crosslinking between the introduced thiol at C6 of the nucleotide base and the cysteine residue 12 in β tubulin involved in photocrosslinking (31), because total inhibition occurred even in the presence of reducing reagent, such as DTT in excess amount. In the present study, it was intriguing to observe that when the reactivity of thiol group was sequestered by the introduction of methyl group (forming 6-CH₃S-GTP), namely, the introduction of a hydrophobic group, it largely regained the activity to promote Mt assembly (Fig. 1).

The fact that the introduction of amino group at position C2 and hydroxy group at position C8 supported assembly much more than did GTP led us to examine the effects of Mt toxins such as colchicine or podophyllotoxin on this Mt assembly system promoted by the most efficient analogue such as n2-8OH ATP. In the Mt assembly system, no turbidity development was observed in the presence of substoichiometric amount (10 μ M) of colchicine or podophyllotoxin (data not shown).

8-substituted GTP analogues

The modification of position C8 of the purine base substantially lowered the activities of the nucleotide analogues. Figure 4 shows that the substitution of hydrogen atoms at position C8 with hydroxy group (8-OH-n2ATP) or bromo group (8-Br-GTP), somehow pro-

Fig. 4. Effect of 8-OH-n2ATP, 8-Br-GTP, 8-Br-n2ATP and 8-COOH-ATP compared with that of GTP on Mt assembly.

moted assembly more than did GTP and 8-Br-n2ATP, possibly independent of modification of position C6.

8,2'-S-cyclo GTP analogues

The linkage between position N9 and ribose C1' is called a glycosidic bond which is known to rotate freely. Therefore, it was interesting to note the blocking of rotation on Mt assembly. We then synthesized 8,2'-S-cyclo GTP analogues (8,2'-S-n2ATP, 8,2'-S-ATP and 8,2'-S-GTP) which were found to be almost totally inhibitory in Mt assembly, independently of the modifica-

Fig. 5. Effect of 8,2'-S-n2ATP, 8,2'-S-ATP and 8,2'-S-GTP compared with that of GTP on Mt assembly.

tion of positions C2 and C6 so far examined (Fig. 5). This result seems to indicate that the rotation around the glycosidic linkage is inevitable for the binding of GTP to β -tubulin to sustain the heterodimer conformation favoring polymerization into Mts. However, 8,2'-anhydro-8-mercapto-purinenucleoside has a fixed high-anti conformation (8, 12, 13, 32) due to anhydro linkage between base C8 and C2' of sugar moiety, and the furanose ring takes C2'-endo type puckering. Therefore, the possibility cannot be excluded that a high-anti conformation leading to distortion of nucleotide conformation caused the suppression of assembly promoting ability of GTP.

Purinenucleoside 5'-triphosphate analogues

C2 and C6 were shown to be sites which are functional for the nucleotides to support Mt assembly. We then synthesized, purinenucleoside 5'-triphosphates in which amino group is deprived of C2 and C6. Figure 6 shows that the analogue (PuTP) supported Mt assembly better than did GTP. However, the substitution of hydrogen atom with amino group at C2 (n2PuTP) promoted assembly slightly better than did PuTP. Based on the fact that these analogues supported assembly better than did GTP, we tried to deoxygenize the hydroxyl group of ribose moiety. This modification largely affected the activity of the analogues to support assembly. Deoxy- and dideoxy-types of analogues (dPuTP and ddPuTP) were shown to lose their Mt assembly promoting activity almost completely (Fig. 6). These results differ markedly from those assembled in solutions containing deoxy and dideoxy GTP, which reinforce

Fig. 6. Effect of n2PuTP, PuTP, ddPuTP and dPuTP compared with that of GTP on Mt assembly.

Fig. 7. Effect of n2ADP, 8-OH-n2ADP and n2dADP compared with that of GTP on Mt assembly.

the idea that position C2 of GTP is important for supporting assembly.

GDP analogues

Next, we examined diphosphate-type analogues (8-OH-n2ADP, n2ADP, and n2dADP). Figure 7 demonstrated that irrespective of the high ability to promote Mt assembly of triphosphate-type analogues (8-OH-n2ATP, n2ATP and n2dATP), their diphosphates (8-OH-n2ADP, n2ADP and n2dADP) almost completely failed to support assembly. Since Mt assembly is accompanied by dephosphorylation of the E-site GTP on β -tubulin, the E-site needs to be in the triphosphate form before assembly (25). The above results clearly indicate that it is a triphosphate-type of guanine nucleotide that promotes assembly, and are consistent with those reported by Hamel *et al.* (6) demonstrating that guanosine tetraphosphate failed to support assembly.

Mant GTP

Finally, we synthesized Mant GTP analogues (Mant GTP, Mant-8-Br-GTP, Mant-8-OH-n2ATP) according to the procedure originally reported by Hiratsuka (9) and observed their effects on Mt assembly (Fig. 8). Mant-GTP and Mant-8-Br-GTP substantially supported assembly, but Mant-8-OH-n2ATP did not, which was surprising in view of the fact that 8-OH-n2ATP remarkably supported assembly (Fig. 4). This may show that the introduction of a bulky moiety at position 3' as well as amino group at position C6 in this

Fig. 8. Effect of Mant-GTP, Mant-8-Br-GTP and Mant-8-OH-n2ATP compared with that of GTP on Mt assembly.

case largely affects the activity of the analogues supporting Mt assembly. Although the turbidity of C_3S in the presence of Mant-8-OH-n2ATP did not increase, a few Mts could be observed by fluorescence microscopy. However, many labelled Mts were observed in the case of Mant-GTP or Mant-8-Br-GTP (Fig. 9). This corroborates the work done by Hiratsuka (10).

Fig. 9. Dark field-contrast (A) and fluorescence (B) micrographs of Mts assembled in the presence of Mant-8-Br-GTP. Similar images of Mt bundles were observed in the case of Mant-GTP (photograph not shown). Bar, 10 μ m.

Fig. 10. Cross section of Mt assembled in a buffer solution containing Mant-8-Br-GTP, fixed with glutaraldehyde and stained with tannic acid. Bar, 10 nm.

We measured the number of protofilaments composed of Mts assembled from tubulin saturated with Mant-8-Br-GTP after Mts were treated with tannic acid, postfixed with OsO_4 and thin-sectioned. Semi-quantitative counting of about 30 Mts showed most of them have 14 protofilaments. Figure 10 shows a representative image of Mt having 14 protofilaments. This was consistent with the results reported by Pierson *et al.* (24) in which the number of most protofilaments was shown to be 14 when assembled in a buffer solution containing PIPES and GTP. Therefore, it was rather surprising to learn that the introduction of a bulky group like methylantraniloyl (Mant) group into ribose moiety of GTP did not largely alter the arrangement of the microtubule lattice. This kind of Mant-derivative of GTP may provide a useful tool in determining the affinity of other non-labeled nucleotide analogues for some proteins as described by Mills and Richter (14).

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