

## Low Affinity Interactions of GDP $\beta$ S and Ribose- or Phosphoryl-substituted GTP Analogues with the Heterotrimeric G Protein, Transducin\*

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Evelyn M. Zera†, David P. Molloy§, Joseph K. Angleson¶, Jagannath B. Lamture, Theodore G. Wensel||, and Justine A. Malinski

From the Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030

We have examined the effects of three commonly used classes of guanine nucleotide analogues on the retinal G protein, transducin ( $G_t$ ), and found them to be quite different from those that might be expected from results with other GTP-binding proteins. The most surprising results were with guanosine 5'-*O*-(2-thiodiphosphate) (GDP $\beta$ S); rather than inhibiting activation of  $G_t$ , GDP $\beta$ S addition activated  $G_t$  as a result of a trace contaminant. Even when the contaminant levels were reduced 5-fold by chromatography, its effects dominated those of GDP $\beta$ S, which binds  $G_t$  at least 1500-fold more weakly than guanosine 5'-*O*-(3-thiotriphosphate) (GTP $\gamma$ S). The affinity of  $G_t$  for GDP was found to be at least 300-fold weaker than for GTP $\gamma$ S, while the affinities of GTP and GTP $\gamma$ S were similar. Ribose-modified GTP analogues, including 2'(3')-*O*-(*N*-methylantraniloyl) GTP (mant-GTP), 2'(3')-*O*-[(2-aminoethyl)carbonyl] GTP (edGTP), and adducts of fluorescein 5-isothiocyanate and rhodamine B-isothiocyanate with edGTP, interacted extremely weakly, if at all, with the GTP binding site of the  $\alpha$  subunit of  $G_t$ . They were neither effective activators of  $G_t$  nor effective inhibitors of activation by GTP or GTP $\gamma$ S. A  $\gamma$ -phosphoryl-modified analogue, an adduct of GTP $\gamma$ S and (5-(2(iodoacetyl)aminoethyl)amino)naphthalene-1-sulfonic acid (dnsGTP), also activated  $G_t$  weakly, if at all, and did not inhibit its activation. The exclusion of these analogues points to the highly restrictive and specific nature of the GTP binding site of  $G_t$ , in contrast to those of numerous other GTP-binding proteins which are potentially activated or inhibited by these analogues.

Structural analogues of nucleotides have played an important role in developing our understanding of protein-nucleotide interactions that govern the functions of nucleotide-binding proteins. The large class of proteins containing GTP binding sites homologous to those of signal-transducing G proteins has been extensively explored by the use of hydrolysis-resistant

analogues of GTP and GDP, as well as by use of analogues modified at various positions to probe the structural and chemical characteristics of the binding sites. The G protein of retinal rod outer segments,  $G_t$ <sup>1</sup> has been probed by several series of such analogues (1–3).

For heterotrimeric G proteins, including  $G_t$ , a particularly large number of studies have been carried out using GDP $\beta$ S as a GDP analogue resistant to conversion to an activating nucleotide and to other metabolic reactions (4). In the case of  $G_t$ , this analogue has been used in studies of  $G_t$  binding to  $R^*$  (metarhodopsin II, the light-activated active form of rhodopsin) by light scattering and biochemical techniques (5) and in electrophysiological experiments where it was introduced into functional rod outer segments (6, 7). There have been very few studies aimed at directly determining whether GDP $\beta$ S actually blocks GTP activation of heterotrimeric G proteins or not. In one study of  $G_t$  (2), essentially no effect of GDP $\beta$ S on GTP binding was observed, although GDP $\beta$ S was found to inhibit weakly binding of GDP. The often cited study that first demonstrated inhibitory activity of GDP $\beta$ S in some cells (4) also reported hormone-dependent stimulation of adenylyl cyclase by GDP $\beta$ S in liver and parotid membranes. Paris and Pouyssegur (8) found that GTP $\gamma$ S effects on G protein pathways in fibroblast cells were mimicked, rather than antagonized, by addition of GDP $\beta$ S.

When we attempted to use GDP $\beta$ S to inhibit activation of  $G_t$  in experiments with bleached rod outer segments, we were surprised to observe activation by this analogue rather than inhibition of activation. This unexpected result, together with the finding of very high affinity interactions between  $G_t$  and GTP $\gamma$ S (see our companion study, Ref. 9) raised the possibility that trace contaminants may have contributed to some of the results previously observed with GDP $\beta$ S and led us to reinvestigate its interactions with  $G_t$ .

Another potentially useful class of GTP and GDP analogues are those modified with fluorescent groups. While this class of analogues, particularly those modified at the 2' or 3' positions, have been very successfully used for studying small GTP binding proteins such as p21<sup>ras</sup> (e.g. see Refs. 10–13) and elongation factor EF-Tu (e.g. see Refs. 14 and 15), it is not clear whether

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§ Present address: Dept. of Cancer Studies, University of Birmingham, Vincent Drive, Edgbaston, Birmingham, B15 2TJ UK.

¶ Supported by National Institutes of Health Training Grant EY07001. Present address: Dept. of Physiology, University of Colorado Health Sciences Center, Denver, CO 80262.

|| To whom correspondence should be addressed: Dept. of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-6994; Fax: 713-796-9438.

<sup>1</sup> The abbreviations used are:  $G_t$ , transducin, the rod cell-specific G protein;  $G_{t\alpha}$ , the  $\alpha$  subunit of transducin; GTP $\gamma$ S, guanosine 5'-*O*-(3-thiotriphosphate); GDP $\beta$ S, guanosine 5'-*O*-(2-thiodiphosphate); mant-GTP, 2'(3')-*O*-(*N*-methylantraniloyl) GTP; edGTP, 2'(3')-*O*-[(2-aminoethyl)carbonyl] GTP; dnsGTP, adduct of GTP $\gamma$ S and (5-(2(iodoacetyl)aminoethyl)amino)naphthalene-1-sulfonic acid; PDE, cGMP phosphodiesterase holoenzyme; PDE $\gamma$ , the inhibitory  $\gamma$  subunit of PDE; PEI, polyethyleneimine; ROS, rod outer segments;  $R^*$ , the photoactivated form of rhodopsin; R, total rhodopsin regardless of its form; HPLC, high performance liquid chromatography; AA-GTP, *P*<sup>β</sup>-(4-azidoanilido)-*P*<sup>α</sup>-5'-guanosine triphosphate.

these or any other fluorescent GTP analogues have sufficient affinity for the GTP binding sites of heterotrimeric G proteins in general, or of  $G_t$  in particular, to be useful for functional studies. Because of the structural conservation of GTP binding sites between  $G_t$  and proteins known to bind ribose-modified analogues, and because of preliminary accounts indicating that these analogues may interact with  $G_o$  and  $G_t$  (16–18), we have prepared several such analogues and characterized their interactions with  $G_t$ . Because it had been previously reported that a bulky aromatic group attached to the  $\gamma$ -phosphoryl group of GTP did not prevent interactions with  $G_t$  (3) or with  $G_s$  (19, 20), we examined a fluorescent analogue of this class as well.

#### EXPERIMENTAL PROCEDURES

**Analytical Methods**—The courses of nucleotide modification reactions and nucleotide purity were routinely monitored using thin layer chromatography on either PEI cellulose or Silica Gel F254 plates (from Merck or Sigma). The PEI plates were developed in either isopropanol/HCl, system B of Hiratsuka (21), or aqueous  $\text{KH}_2\text{PO}_4$ , at various concentrations in the range 0.75–1.6 M, adjusted to pH 3.6 or 3.7 with HCl (22). The isopropanol/HCl system gave good separation of *N*-methylsaccharic anhydride from reaction products, while the phosphate systems gave good resolution of mantGTP from GTP and *N*-methylanthranilic acid. Formation of edGTP from the (2'-3') cyclic carbonate of GTP was followed using silica plates developed with 1-butanol:acetic acid: $\text{H}_2\text{O}$ , 5:2:5. Quantitative analysis of nucleotides was carried out by ion-pairing HPLC with an Altex  $\text{C}_{18}$  Ultrasphere I.P. column (25  $\times$  4.5 mm) developed with linear gradients of acetonitrile versus a buffer containing 4% (v/v)  $\text{CH}_3\text{CN}$ , 5 mM tetrabutylammonium phosphate, 30 mM  $\text{KH}_2\text{PO}_4$ , pH 6.0, with detection at 254 nm.

UV/visible absorbance was measured, using a Hewlett-Packard 8452A spectrophotometer, and fluorescence measurements were carried out using an instrument described previously (23). For detecting the fluorescence of mantGTP, 350 nm was used as the excitation wavelength and 440 nm as the emission wavelength.

**Preparation of Analogues**—GTP, GDP,  $\text{GTP}\gamma\text{S}$ , and  $\text{GDP}\beta\text{S}$  were obtained from Boehringer Mannheim or from Sigma and checked for purity by thin layer chromatography and by HPLC; purity of nucleotides from the two suppliers did not differ greatly. GDP and  $\text{GDP}\beta\text{S}$  were purified by ion exchange chromatography on DEAE-cellulose DE-52 (Whatman) using a linear gradient of 0.1 to 0.3 M triethylammonium bicarbonate, pH 7.4.

Ribose-modified analogues were purified from synthesis reaction mixtures by anion exchange chromatography on DE-52 (bicarbonate form) and lyophilized. Typically, a linear gradient from 0 to 0.8 M triethylammonium bicarbonate (pH 7.6) was used. MantGTP and edGTP were further purified by preparative PEI cellulose TLC, with development in 1.5 M  $\text{KH}_2\text{PO}_4$ , and extraction with 0.8 M triethylammonium bicarbonate, followed by lyophilization.

Synthesis of mantGTP was by the method of Hiratsuka (21). Synthesis of edGTP was by the method of Cremona *et al.* (24) as modified for GTP and ethylenediamine (11). The triethylammonium salt of GTP was prepared from the tetralithium salt by chromatography on DE-52, as described below, and converted to the tri-*n*-butylammonium salt by addition of freshly vacuum-distilled tri-*n*-butylamine in dry *N,N*-dimethylformamide (freshly distilled over  $\text{CaH}_2$ ), followed by repeated evaporation under high vacuum, redissolving first in methanol and then dioxane (freshly distilled over LiAlH<sub>4</sub>), until a dry white powder was obtained. This salt, 180 mg (0.22 mmol), was added to 243 mg (6 mmol) of carbonyldiimidazole in 5 ml of dry *N,N*-dimethylformamide, the mixture was stirred for 2 h at 25 °C, and 12 h at 4 °C, and then methanol was added to react with the excess carbonyldiimidazole. To this mixture 170  $\mu\text{l}$  (10 mmol) of freshly distilled ethylenediamine were added in a final reaction volume of 5.3 ml. As judged by the formation of a white precipitate, the reaction went to completion in 1 h at 4 °C, after which time the precipitate was removed by centrifugation, and redissolved in 2 ml of  $\text{H}_2\text{O}$ . It was then treated with aqueous acid (HCl, final pH 2) for 20 h at 25 °C to hydrolyze the aminoethylphosphoramidate of edGTP, prior to DE-52 chromatography. Fluorescein (11) and rhodamine adducts of edGTP were prepared by reaction of edGTP (16 mM) with fluorescein 5-isothiocyanate (16 mM) or rhodamine B isothiocyanate (28 mM) in a buffer containing 0.1 M sodium bicarbonate, pH 8.6.

DnsGTP was synthesized as described previously (25). A sample of dnsGTP purified by HPLC (25) was kindly provided by Dr. J. Wade Harper.

For mantGTP, the absorbance ratio (254 nm/358 nm) was used to

verify a 1:1 ratio of *N*-methylanthraniloyl chromophore to guanine base, and mild alkaline hydrolysis (0.1 N NaOH, 23 °C, 1 h) was verified to produce *N*-methylanthranilic acid and GTP (identified chromatographically and by activation of transducin). For edGTP, spectrophotometry after modification with trinitrobenzene sulfonic acid (26) was used to verify a 1:1 ratio of primary amino groups to guanine base. Conjugates of edGTP with fluorescent dyes were also analyzed by absorbance spectroscopy to verify a 1:1 stoichiometry of dye/guanine moiety. Dns-GTP was similarly analyzed, and the sample used in the experiments described here was verified to be fully active in initiation of transcription by T7 RNA polymerase (25) as evidenced by ~50% of RNA containing the dansyl moiety when dnsGTP and GTP were equimolar in the polymerase reaction (250  $\mu\text{M}$  each).

**Membranes and Protein Preparation and Assays**—Rod outer segments were prepared from frozen bovine retinas using a sucrose gradient procedure (27). Preparations of urea-stripped ROS membranes and purified transducin and PDE, as well as blue Sepharose chromatography of  $G_{ta}$  were carried out as described previously (23, 28) with an additional step for PDE of anion exchange HPLC chromatography using a Waters Protein Pak DEAE 5 PW column. PDE assays were carried out as described elsewhere (28).

**Assays of Analogue Binding by Competition with  $\text{GTP}\gamma\text{S}$** —For GDP and  $\text{GDP}\beta\text{S}$ , equilibrium binding experiments were carried out using bleached ROS as described (9), with sufficient GDP or  $\text{GDP}\beta\text{S}$  added to give the indicated final concentrations, just before addition of  $\text{GTP}\gamma^{35}\text{S}$ .

For the 2'(3')-GTP analogues and for GTP itself, transient experiments rather than equilibrium ones were carried out to minimize hydrolysis of the  $\beta$ - $\gamma$ -phosphoanhydride bonds, which occurs with a rate constant of  $\sim 1/(30 \text{ s})$  for GTP bound to  $G_{ta}$  (29).  $\text{GTP}\gamma\text{S}$  uptake assays were carried out by timed vacuum filtration through nitrocellulose as described (23). Inhibition of  $\text{GTP}\gamma\text{S}$  binding was measured by incubating purified transducin with urea-stripped ROS membranes,  $\text{GTP}\gamma^{35}\text{S}$ , and varying concentrations of competing analogues for one minute before nitrocellulose filtration.  $\text{GTP}\gamma^{35}\text{S}$  and the competing nucleotides were premixed before addition to the sample containing protein and membranes. Apparent  $K_i$  values were estimated by nonlinear least squares fitting of the data to the expression  $B = B_0 + (B_{\text{max}} - B_0)G/[K_d(1 + I/K_i) + G]$ , where  $B$  is the bound  $\text{GTP}\gamma\text{S}$ ,  $B_{\text{max}}$  is the amount bound in the absence of inhibitor,  $I$  is the concentration of inhibitor (GTP, mantGTP, or edGTP),  $G$  is the concentration of  $\text{GTP}\gamma\text{S}$ ,  $K_d$  is the dissociation constant for  $\text{GTP}\gamma\text{S}$ , determined independently to be 50  $\mu\text{M}$  (9), and  $K_i$  is the inhibition constant characteristic of each inhibitor. The parameter  $B_0$ , corresponding to the amount of  $\text{GTP}\gamma\text{S}$  bound at infinite inhibitor, was determined to be 11% of  $B_{\text{max}}$  by nonlinear least squares fitting to the GTP data with both  $B_0$  and  $K_i$  allowed to vary, while  $K_i$  values for edGTP and mantGTP were determined by fitting with  $B_0$  held constant at 11% and only  $K_i$  allowed to vary. It is possible that this 11% residual binding may represent the covalent radiolabeling by  $\text{GTP}\gamma\text{S}$  previously reported to occur at high  $[\text{GTP}\gamma\text{S}]$  (30, 31), because, in contrast to conditions of the equilibrium studies (9), the concentrations of protein and  $\text{GTP}\gamma\text{S}$  in these kinetic experiments are sufficiently high for some thiophosphorylation to occur.

#### RESULTS

**$G_t$  Interactions with  $\text{GDP}\beta\text{S}$  and GDP**—Our experiments with  $\text{GDP}\beta\text{S}$  reported here were motivated by the outcome of experiments in which we attempted to inhibit  $G_t$  activation by GTP in bleached ROS. The key observations made in these initial experiments, for which data are not shown, were as follows. 1) No inhibition was observed at any concentration tested. 2) As we increased the  $\text{GDP}\beta\text{S}$  concentration in an attempt to force inhibition, we found that  $\text{GDP}\beta\text{S}$  induced activation of PDE (and therefore, presumably of  $G_t$ ) although such activation was not observed at much higher concentrations of 5'-GMP, the principal contaminant of commercial  $\text{GDP}\beta\text{S}$ . 3) No effect of  $\text{GDP}\beta\text{S}$  was observed when it was tested with purified PDE, implying its PDE effects were mediated by  $G_{ta}$ . 4) The activation turned off much more slowly ( $k_{\text{inact}} < 1/500 \text{ s}$ ) than activation by low concentrations of GTP ( $k_{\text{inact}} \sim 1/30 \text{ s}$ ) (29), suggesting a nonhydrolyzable or slowly hydrolyzed contaminant was responsible. 5) The activation of PDE was a small fraction (<10%) of that observed with trypsin activation (*i.e.* complete activation by removal of PDE $\gamma$ ), but did not increase after the first several seconds, indicating that activa-

tion was not due to degradation of the inhibitory PDE $\gamma$  subunit by contaminating proteases. 6) Prolonged incubation of GDP $\beta$ S with ROS did not give rise to increased activation; rather, activation was decreased slightly by such incubation. Including ATP prevented the decrease, possibly by replacing a phosphate group slowly released by hydrolysis.

Because of these unexpected results, we carried out equilibrium competition experiments using GTP $\gamma$ <sup>35</sup>S as shown in Fig. 1A. These indicated that  $G_{\text{tc}}$ 's apparent  $K_d$  for GDP $\beta$ S (assuming the inhibition was due to GDP $\beta$ S and not a contaminant) was approximately 350-fold higher than that for GTP $\gamma$ S. However, at ratios of GDP $\beta$ S to activating nucleotide sufficient to give measurable inhibition of GTP $\gamma$ S binding in the equilibrium studies (e.g. 100  $\mu$ M GDP $\beta$ S and 76 nM GTP), no inhibition of GTP-stimulated PDE activation was observed, but 100  $\mu$ M GDP $\beta$ S did give rise to easily measurable activation of PDE, about 40% of the level stimulated by 1  $\mu$ M GTP (data not shown).

We compared these results to those obtained with GDP. As reported previously (32) GDP also activated PDE in ROS, even when highly purified (data not shown). In equilibrium binding experiments (Fig. 1A) GDP behaved similarly to GDP $\beta$ S. Assuming, probably incorrectly, that GDP itself was entirely responsible for the inhibition of GTP $\gamma$ S binding, its  $K_d$  for  $G_{\text{tc}}$  appeared to be about 300-fold higher than  $K_d$  for GTP $\gamma$ S. Two successive rounds of purification of GDP by DE-52 anion exchange chromatography reduced contaminating GTP below levels detectable by our HPLC assay, and by calibration with radiolabeled GTP it was estimated to remove ~85% of any contaminating GTP each round. However, these procedures did not greatly reduce the ability of GDP to activate PDE in ROS (data not shown). The levels of PDE activity observed were much too high, given the concentration of  $G_t$  present in the ROS, to be accounted for by the weak activation of PDE by  $G_{\text{tc}}$ -GDP reported by Kutuzov and Pfister (33). Because results with GDP are complicated by a GDP phosphotransferase activity in ROS that is difficult to eliminate (32)<sup>2</sup> and leads to GTP production from added GDP, we did not pursue further the reason for its ability to stimulate PDE activity. From assays of [<sup>32</sup>P]GMP production from [ $\alpha$ -<sup>32</sup>P]GDP, we estimate that, under the conditions of Fig. 1A, GDP conversion to GTP should occur at a rate of approximately 0.03% min<sup>-1</sup>. Conversion of as much as 5% of the GDP to GTP could have occurred over the 3 h time course of Fig. 1A, but the effect of this conversion would be greatly reduced by hydrolysis of GTP bound to  $G_{\text{tc}}$ .

GDP $\beta$ S is presumably resistant to the same reaction (4) and even if phosphorylated yields a product, GTP $\beta$ S, that is not an efficient activator of  $G_t$  (2). Therefore we focused on GDP $\beta$ S and attempted to purify away the activating contaminant(s) present in commercial stocks by ion exchange chromatography (Fig. 1B). It is clear, from comparing the profiles of GDP $\beta$ S elution and PDE activation, that it is a contaminant, rather than GDP $\beta$ S itself, that is responsible for PDE activation. It can also be seen from Fig. 1B that a distinct peak of 254-nm absorbance can be observed for GMP, the major contaminant of GDP $\beta$ S, but that the activating contaminant is either present in much lower amounts than GMP, or absorbs at 254 nm much more weakly, as there is no identifiable peak of absorbance corresponding to it. Because the leading edge of the GDP $\beta$ S peak had the lowest PDE activation potency, only these early fractions from the chromatogram shown, or from a similar one (i.e. those corresponding to pool i, Fig. 1B), were used for further studies. GDP $\beta$ S prepared in this way showed a ~5-fold lower apparent potency in competition studies with GTP $\gamma$ S

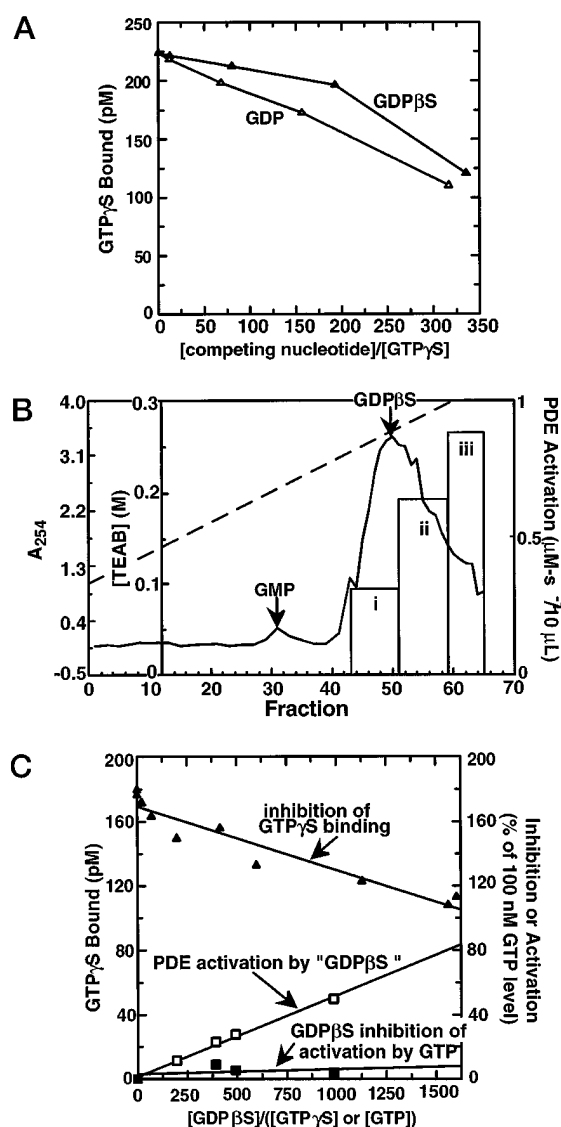


FIG. 1. Apparent effects on GTP $\gamma$ S binding and PDE activation of GDP and GDP $\beta$ S. A, equilibrium GTP $\gamma$ S (300 pM total) binding to  $G_{\text{tc}}$  in ROS (30 nM R) was measured at different concentrations of competing nucleotide (GDP, open triangles, or GDP $\beta$ S, filled triangles) from commercial stocks. B, chromatographic profiles of GDP $\beta$ S and transducin activating activity in commercial GDP $\beta$ S. Commercial GDP $\beta$ S was loaded onto a DEAE-cellulose column and eluted with a gradient of triethylammonium bicarbonate (TEAB) as shown (dashed line). Absorbance at 254 nm of each fraction is represented by the solid curve, and the arrows indicate the peaks of GMP or GDP $\beta$ S as determined by thin layer chromatography. Other species that may have been present were not present at sufficient levels to be observed on TLC. Bars indicate the transducin activating activity of pools (i-iii) containing the fractions they span, and their heights indicate the relative PDE activity induced in bleached ROS by equal volumes of the pools. C, effect of rechromatographed "GDP $\beta$ S" (a pool equivalent to pool i, panel B) on interactions of GTP and GTP $\gamma$ S with  $G_{\text{tc}}$ . The upper curve (filled triangles) shows inhibition of GTP $\gamma$ S binding as in A, and the lower curves represent either GDP $\beta$ S inhibition of activation by GTP (filled squares) or activation of PDE by GDP $\beta$ S (open squares) with all lines representing linear least squares fits to the data.

(Fig. 1C; cf. Fig. 1A), implying that at least 80% of the inhibitory activity observed in experiments with commercial GDP $\beta$ S was due to contaminants that had been at least partially removed by chromatography. However, when PDE assays were carried out, this purified GDP $\beta$ S again showed no inhibition of GTP-stimulated PDE activity, but did show activation of PDE at concentrations that correlated very well with those capable of inhibiting GTP $\gamma$ S binding (Fig. 1C). From these results we

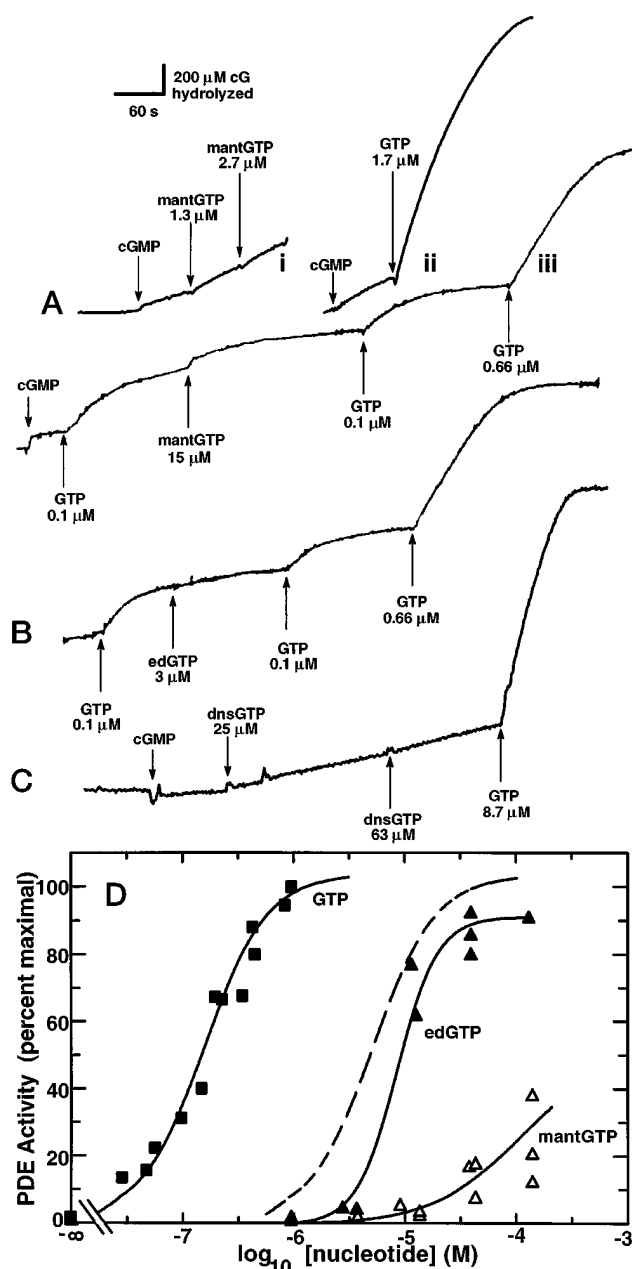
<sup>2</sup> J. K. Angleson, unpublished results.

conclude that any inhibitory interactions of GDP $\beta$ S with G<sub>t</sub> are too weak to be detected by our methods (*i.e.* more than 1500-fold weaker than those of GTP or GTP $\gamma$ S), and that even highly purified GDP $\beta$ S contains a persistent activating contaminant whose activity dominates the apparent effects of GDP $\beta$ S on G<sub>t</sub>. Our results are not conclusive with respect to the identity of the contaminant, nor with respect to the question of whether it is the contaminant itself or its metabolic product that directly interacts with G<sub>t</sub>.

**G<sub>t</sub> Interactions with 2' (3') Analogues**—Because PDE activation in ROS is an easy, sensitive, and robust assay for G<sub>t</sub> activation by nucleotides, we initially used this assay to assess the effects of the modified GTP analogues. Fig. 2 shows results typical of numerous attempts to detect either activation of G<sub>t</sub> or inhibition of its activation by these analogues. When 1.3  $\mu$ M to 15  $\mu$ M mantGTP (Fig. 2A) was added to bleached ROS, very little activation of PDE was observed. In the experiments depicted in *trace i* of Fig. 2A, addition of 1.3  $\mu$ M mantGTP appeared to stimulate a less than 60% increase over basal activity, to a final activity that was somewhat less than the basal activity in the subsequent control trace (*ii*), and that represented an activity increase only 6% of that stimulated by GTP in the control trace (*ii*). Tripling the concentration, to 4  $\mu$ M total mantGTP, did not measurably increase the PDE activity. The lower mantGTP trace (Fig. 2A, *iii*) shows that the increase in PDE activity when 15  $\mu$ M mantGTP was added was less than 5% of the increase induced by only 100 nM GTP. At 15  $\mu$ M, mantGTP was also a poor inhibitor of transducin activation; in its presence, 100 nM GTP elicited an increase in PDE activity only 13% lower than that elicited by the same concentration of GTP in the absence of this 150-fold excess of mantGTP (Fig. 2A, *trace iii*). The high affinity of GTP for G<sub>t</sub>,  $K_d \sim 50$  pM (9), implies that in this experiment, with 5  $\mu$ M R and  $\sim 200$  nM G<sub>t</sub>, free GTP must be only about 13 nM, the amount prevented from binding by mantGTP. Therefore, this result implies that mantGTP must be competing for the GTP binding site at least 1000-fold less potently than GTP.

Similar results were obtained for edGTP (Fig. 2B). Essentially no increase in PDE activity was observed when 3  $\mu$ M edGTP was added, and its presence gave rise to at most a 25% inhibition of activity induced by addition of a 30-fold lower concentration of GTP (120-fold lower free concentration). The fluorescein and rhodamine conjugates of edGTP gave results similar to those observed with edGTP (data not shown). At concentrations comparable to those at which GTP potentially activates transducin, no activation by these fluorescent analogues was observed in PDE assays (data not shown). At much higher concentrations, some activation by the rhodamine conjugate was observed, but this could be attributed to trace contamination by GTP. Likewise dnsGTP gave rise to weak if any stimulation of PDE activity (Fig. 2C); at 88  $\mu$ M dnsGTP the activity stimulated by the fluorescent nucleotide was less than 4% of that induced by GTP; no inhibition of GTP stimulation was observed at this concentration.

Fig. 2D shows that at very high concentrations of the 2' (3') modified GTP analogues, significant PDE activation could be observed. In the case of edGTP, however, the 3% contamination with GTP would be expected to give rise to even greater PDE activation than observed (Fig. 2D, *dashed line*), consistent with *no* activation by edGTP, but a slight (<20%) inhibition of GTP-dependent activation by edGTP in >30-fold molar excess. In the case of mantGTP, the weak activation observed could be explained by as little as 0.1% contamination with GTP, an amount of contamination that could not be ruled out because it was at the level of noise in the chromatogram. Thus it remains unclear whether mantGTP can activate transducin at all.



**FIG. 2. Effects of GTP and analogues on PDE activation in rod outer segments.** cGMP hydrolysis was monitored continuously using a pH electrode in suspensions of rod outer segments, while GTP or an analogue was added to the indicated total concentration. Initial concentration of cGMP was 2 mM in each case. A, activation by GTP or mantGTP at 2.9  $\mu$ M R (*i* and *ii*) or 5  $\mu$ M R (*iii*). B, activation by edGTP or by GTP, at 5  $\mu$ M R. C, activation by dnsGTP and by GTP $\gamma$ S in the presence of dnsGTP, 2.8  $\mu$ M R. Different preparations of ROS were used for each experiment shown (except the top two traces in A (*i* and *ii*) recorded from the same preparation), and each result shown is representative of several similar traces recorded with different batches of ROS and of nucleotide analogues. D, concentration dependence of PDE activation in bleached ROS (5  $\mu$ M R) by GTP and analogues. Concentration is plotted as added nucleotide, which is approximately identical to free nucleotide for the analogues. The free GTP concentration is approximately 1000–5000-fold lower than that plotted for GTP concentrations below 500 nM. Maximal activity (100%) was 23  $\mu$ M cGMP hydrolyzed per second. The *dashed line* represents the activity expected from the  $\sim 3\%$  contamination of GTP in the edGTP sample; it is the *solid line* drawn through the GTP points shifted on the abscissa by the appropriate distance.

As the effects on PDE activity caused by the analogues were so weak as to be barely detectable, we decided to use a more direct GTP $\gamma$ S binding assay to detect competition for the GTP

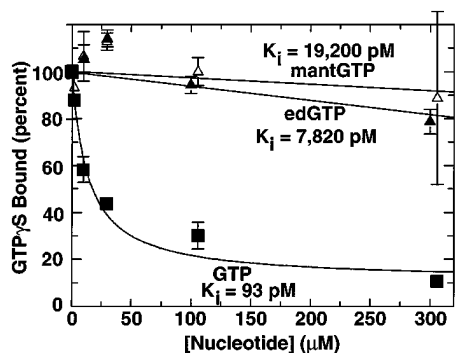


FIG. 3. **Inhibition of GTP $\gamma$ S binding by GTP and 2'(3') derivatives.** The amounts of GTP $\gamma$ <sup>35</sup>S bound to transducin (1  $\mu$ M) during 60-s incubations in the presence of R\* (urea-stripped ROS membranes) and the indicated concentrations of competing nucleotides were determined by nitrocellulose filter binding and are plotted as a percentage of the amount bound in control samples without a competing nucleotide: mantGTP (open triangles), edGTP (filled triangles), GTP (squares). The curves shown represent nonlinear least squares fits for competitive inhibition, using a  $K_d$  for GTP $\gamma$ S of 50 pM. GTP $\gamma$ S concentrations were 7.3  $\mu$ M (squares and open triangles) or 7.8  $\mu$ M (filled triangles). R concentrations were 1  $\mu$ M (open triangles and filled squares) or 0.75  $\mu$ M (filled triangles). Each point is the average ( $\pm$  standard deviation) of two to six replicates in an individual experiment, and each titration is representative of results from at least seven similar titrations.

binding site. This assay is done on a somewhat faster time scale than the PDE assays, so breakdown of the analogues is also less of a concern. Fig. 3 shows that GTP competes very effectively with GTP $\gamma$ S in this assay, yielding an apparent  $K_i$  of 93 pM, comparable to the GTP $\gamma$ S  $K_d$  of 50 pM. In contrast, mantGTP and edGTP gave rise to marginal inhibition at the highest concentrations tested. If the results are taken at face value,  $K_i$  values are obtained that are approximately 2600-fold higher (mantGTP) or 156-fold higher (edGTP) than the  $K_d$  for GTP $\gamma$ S.

Even with such low affinity, we thought it might be possible to detect mantGTP binding to  $G_{t\alpha}$  by following the distinctive fluorescence of the *N*-methylantraniloyl group using an affinity column procedure which purifies  $G_{t\alpha}$  with GTP $\gamma$ S or GDP still tightly bound. This procedure does not suffer from ambiguities due to hydrolytic release of GTP from the analogue discussed below. Fig. 4 shows the elution profiles of four such Cibacron Blue affinity columns. GTP $\gamma$ S eluted with  $G_{t\alpha}$  irrespective of the presence or absence of a 6-fold excess of mantGTP, confirming the results of Figs. 3 and 4 and verifying that the column procedure works as expected. Incubation with mantGTP in the absence of any competing nucleotide, other than a stoichiometric amount of endogenous GDP, did not result in the detection of a peak of mantGTP fluorescence corresponding to  $G_{t\alpha}$ . Subsequent experiments have demonstrated that even GDP, which binds  $G_{t\alpha}$  much more weakly than GTP or GTP $\gamma$ S (see Fig. 1A) remains tightly bound to  $G_{t\alpha}$  on this type of column.<sup>3</sup> While we cannot rule out the possibility that mantGTP fluorescence is quenched when bound to  $G_{t\alpha}$ , such quenching would be surprising given the enhancement of mantGTP (or mantGTP $\gamma$ S) fluorescence reported upon binding to  $G_{o\alpha}$  (16, 17).

The results of these different assays, taken together, make it clear that, if these analogues bind  $G_t$  at the GTP-binding site at all, they do so with very low affinities compared to GTP $\gamma$ S or GTP and do not induce a conformation of  $G_{t\alpha}$  that resembles that induced by GTP with respect to its ability to activate PDE. Ambiguity arises over whether they bind  $G_{t\alpha}$  at all, because in the assays shown in Figs. 3 and 4, production of GTP from analogues might account for some or all of the inhibition or

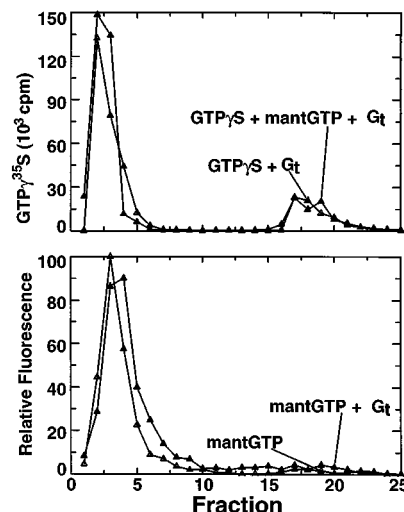


FIG. 4. **Blue Sepharose chromatography of transducin-nucleotide complexes.** Top panel, GTP $\gamma$ <sup>35</sup>S (1.6  $\mu$ M) was incubated for 1 min with urea-stripped ROS membranes (1  $\mu$ M R) and  $G_t$  (1  $\mu$ M), in the presence or absence of 10  $\mu$ M mantGTP, and chromatographed on blue Sepharose as described in the text. Lower panel, stripped ROS membranes were incubated as in top panel with 10  $\mu$ M mantGTP, in the presence or absence of  $G_t$  (1  $\mu$ M) and chromatographed on blue Sepharose. Fluorescence of mantGTP was detected as described in the text.

activation observed. We verified repeatedly that such hydrolysis does occur, as illustrated in Fig. 5 for edGTP, which is expected to be more resistant to hydrolytic GTP release than mantGTP. Fig. 5 shows the results obtained upon chromatographic analysis of a sample of edGTP that initially appeared pure and unable to activate transducin, but gradually acquired unusually high transducin activating activity upon use and several freeze/thaw cycles. Readily detectable levels of GTP had been released, presumably by spontaneous hydrolysis of the 2'(3')-carbamoyl group. From the average of hydrolysis rates observed in samples from two different preparations stored at 4  $^{\circ}$ C, the apparent first order rate constant for hydrolysis was estimated to be  $4.65 (\pm 1.1) \times 10^{-8} \text{ s}^{-1}$ . While this represents an extremely slow reaction (half-life of 173 days), consistent with the known stability of this class of compounds, it presents a serious problem for conducting comparisons with GTP, due to the extreme sensitivity to traces of contaminating GTP. Similar results have been observed with mantGTP (data not shown), and may account for some of the variability in the results observed at high [mantGTP] in Fig. 3. For the nucleotide stocks used for the experiments of Fig. 2D, a GTP contamination of  $\sim 3\%$  was found for edGTP, while for mantGTP, it was possible to establish only an upper limit of  $\sim 0.1\%$  contamination due to baseline noise at the GTP elution position. These results illustrate a fundamental problem in working with GTP analogues (such as mantGTP and edGTP) which interact very weakly (if at all) with transducin but which can generate traces of GTP (and possibly other active species) upon spontaneous hydrolysis. If these modified nucleotides even remotely resembled good analogues in their interactions with transducin's GTP binding site, such low levels of contamination would be of little concern. However, the presence of traces of GTP, which binds  $G_{t\alpha}$  with picomolar affinity, in solutions of analogues that must be used at micromolar levels for any effects to be observed, renders these analogues virtually useless for most purposes.

#### DISCUSSION

*Structural Constraints of the Nucleotide Binding Site*—The exquisite specificity of transducin's nucleotide binding site may be understood in terms of its highly restrictive geometry and

<sup>3</sup> T. Melia and T. Wensel, unpublished results.

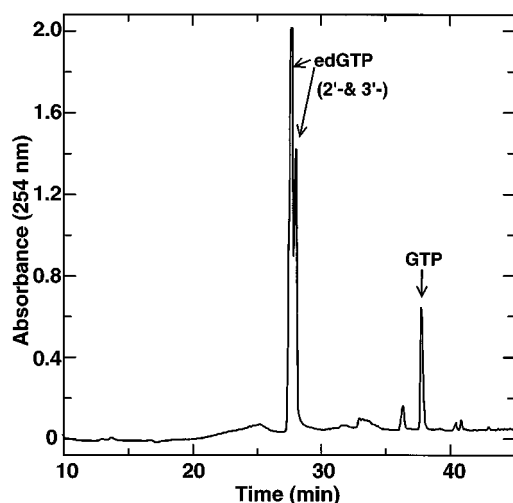


FIG. 5. HPLC analysis of hydrolysis products in edGTP. An ion-pairing solvent system and a C18 column were used as described in the text to analyze a sample of edGTP that appeared, when freshly purified, to be free of contaminating GTP, and had negligible ability to activate transducin, but developed the ability to activate transducin at high concentrations after storage at 0–4 °C. The elution positions of edGTP (verified by TLC) and of GTP (determined by injection of GTP standards) are shown. Other minor peaks were not characterized.

the large number of contacts between the protein and the nucleotide (34, 35). The much weaker interactions of  $G_{\text{tc}\alpha}$  with GDP and GDP $\beta$ S, as compared to those with GTP and GTP $\gamma$ S, can be readily explained in terms of the multiple favorable interactions contributed by the  $\gamma$ -phosphoryl itself, and those induced by substitution of GTP $\gamma$ S or GTP for GDP (35). Thus, while GDP is locked into the binding site sufficiently tightly to display extremely slow dissociation,  $<10^{-4} \text{ s}^{-1}$  (23, 36), its binding is still orders of magnitude weaker than that of GTP $\gamma$ S. In addition, the multiple interactions involving the  $\beta$ -phosphoryl position may facilitate discrimination between GDP and GDP $\beta$ S.

Failure of the 2'(3') analogues to activate  $G_{\text{tc}\alpha}$  efficiently may be understood in terms of hydrogen bonds to both the 2' and 3' hydroxyls and steric hindrance in the tightly packed GTP-binding pocket. Likewise, the multiple contacts to the  $\gamma$ -phosphoryl of GTP $\gamma$ S and tight packing of the protein around it are consistent with the failure of dnsGTP to compete efficiently for the GTP binding site or to activate  $G_{\text{tc}\alpha}$ .

**Functional Implications of the Weakness of GDP $\beta$ S Interactions**—The lack of effect of GDP $\beta$ S on  $G_{\text{tc}\alpha}$  may seem surprising in view of the widespread use of this analogue to inhibit G protein function, including that of  $G_{\text{tc}\alpha}$  (see references cited in the Introduction). However, while this result is in conflict with common *assumptions* about interactions of GDP $\beta$ S with  $G_{\alpha}$  subunits, it does not conflict with any previous measurements of which we are aware. In fact it is quite consistent with previous results with some  $G_{\alpha}$ , such as those of Yamanaka *et al.* (2) in ROS, of Paris and Pouyssegur (8) in fibroblasts, and of Eckstein *et al.* (4) in liver and parotid membranes. In all of these cases, either inhibition of  $G_{\alpha}$  by GDP $\beta$ S was so weak as to be possibly due to a trace contaminant, or  $G_{\alpha}$  activation by GDP $\beta$ S was observed.

Of particular interest are the results of incorporation of GDP $\beta$ S into salamander rods via a patch pipette (7). Introduction of 2 mM GDP $\beta$ S resulted in a decline in the circulating current, an effect also observed with analogues known to activate  $G_{\text{tc}\alpha}$ . GDP $\beta$ S also prolonged the response to intense flashes of light, consistent with the presence of a slowly hydrolyzed activating analogue. This activator was suggested by the authors to be produced within the cell after GDP $\beta$ S introduction.

In the same series of experiments, a decrease in sensitivity and slowing of the rising phase of the light response were also observed, but these inhibitory effects were seen consistently only when the pipette contained higher [GDP $\beta$ S] (11 mM), and these effects developed much sooner after GDP $\beta$ S introduction than the prolongation of activation. Together with our results, these earlier studies suggest that there is a very weak inhibition of  $G_{\text{tc}\alpha}$  activation observable at high GDP $\beta$ S concentrations, but that it is generally accompanied by persistent (but also weak) activation due to a contaminant of GDP $\beta$ S or to a metabolic product of a contaminant.

Because radiolabeled GDP $\beta$ S was not available, and because of our failure to obtain GDP $\beta$ S completely free from contamination, we are not able to say accurately how tightly this nucleotide analogue binds  $G_{\text{tc}\alpha}$ . Results have been published demonstrating competition for GDP binding by GDP $\beta$ S (2, 5), although in spectroscopic and light scattering experiments, GDP $\beta$ S was shown not to mimic the effects of GDP on  $G_{\text{t}}$  binding to metarhodopsin II (5) and was found to inhibit the rate of GTP binding very weakly (apparent  $IC_{50}$  of 500  $\mu\text{M}$ ). What we can say unequivocally is that GDP $\beta$ S is not an efficient inhibitor of  $G_{\text{tc}\alpha}$  activation. This conclusion is in stark contrast to the well known effects of GDP $\beta$ S on  $G_{\text{s}}$ , which is half-maximally inhibited at 1000-fold lower GDP $\beta$ S concentrations, 400 nM (4). Our results also suggest rather strongly that some previous studies with GDP $\beta$ S may need to be reinterpreted in light of its failure to inhibit  $G_{\text{tc}\alpha}$  activation of PDE in direct measurements, and in light of the likely presence in most GDP $\beta$ S preparations of contaminant(s) that give rise to long lived  $G_{\text{tc}\alpha}$  activation.

**2'(3') Analogues**—It is somewhat disappointing that mant-GTP and related 2'(3')-acyl GTP analogues are not functional GTP analogues for transducin; to date no fluorescent nucleotides have been found which activate this G protein. The apparent inability of these analogues to form productive complexes with  $G_{\text{tc}\alpha}$  is not surprising, given the structural constraints discussed above. While some of these constraints are also present in p21<sup>ras</sup> and EF-Tu, for which these analogues have high affinity, both the structural data (34) and our present results indicate that there are additional constraints in  $G_{\text{tc}\alpha}$ . Because even the relatively small and flexible 2-aminoethyl group of edGTP seems to preclude productive interactions, it is likely that the hydrogen bonds to both the 2' and 3' positions of GTP play a critical role in binding. There is apparently less flexibility in this part of the nucleotide binding site in transducin than in EF-Tu which lacks analogous hydrogen bonds (37, 38) and binds mantGTP (15), as well as a rhodamine B sulfonylchloride adduct of edGTP (39), and also less flexibility than in p21<sup>ras</sup>, which forms a hydrogen bond to 2' OH (40), and binds the 2' form of mantGTP with  $\sim 6$ -fold lower affinity than the 3' form (10, 41).

$G_{\text{oc}\alpha}$  has been reported to bind mantGTP and mantGDP as well as mantGTP $\gamma$ S (16). This binding appears to be dramatically different from that of GTP or GDP; dissociation was reported to be extremely rapid for both mantGDP (0.4  $\text{s}^{-1}$ ) and mantGTP (0.03  $\text{s}^{-1}$ ) as compared to the parent nucleotides. In addition, it was reported that mantGTP does not efficiently induce activation of  $G_{\text{oc}\alpha}$ . Thus, the most important conclusions that can be drawn from the results with  $G_{\text{oc}\alpha}$ , that the mant nucleotides are poor analogues for GTP and GDP, and that the binding mode of mantGTP must differ greatly from the binding of GTP that induces the activated  $G_{\alpha}$  conformation, are consistent with our observations for  $G_{\text{t}}$ .

A previous study (3) of a GTP analogue acylated at the 2'(3') position with a 3-[N-(4-azido-2-nitrophenyl)-amino]propionyl group also provided evidence that substitutions at these posi-

tions abolish GTP's ability to activate  $G_{\text{t}\alpha}$  or bind it with high affinity. Inhibition of GTP hydrolysis was half-maximal at a 1000-fold excess of analogue over GTP, and the analytical procedures described could not rule out a 0.1% contamination with GTP. PDE activation was half-maximal at 10  $\mu\text{M}$  analogue, implying a 200,000-fold lower efficiency of activation by the analogue than by GTP $\gamma\text{S}$ , and it was not possible to induce photoreaction of  $G_{\text{t}\alpha}$  with the highly reactive 4-azido-2-nitrophenyl group.

*Substitution at the  $\gamma$ -Phosphoryl*—The failure of dnsGTP either to activate  $G_{\text{t}\alpha}$  or to block its activation by GTP is also somewhat disappointing, as the numerous studies (see references cited by Fields *et al.* (19) with AA-GTP (20)), including one demonstrating potent activation of  $G_{\text{t}\alpha}$  and PDE (3), had suggested that GTP analogues substituted at the  $\gamma$ -phosphoryl or thiophosphoryl positions with fluorescent groups might be very useful for studying  $G_{\text{t}\alpha}$ . However, the results observed for this analogue are not too surprising given the great differences observed for different  $G_{\alpha}$  binding AA-GTP as compared to GTP $\gamma\text{S}$ ;  $G_{\text{z}\alpha}$  bound AA-GTP very weakly if at all, and  $G_{\text{so}\alpha}$ ,  $G_{\text{oc}\alpha}$ , and  $G_{\text{ic}\alpha}$  required much higher concentrations of  $\text{Mg}^{2+}$  for binding AA-GTP than for binding GTP $\gamma\text{S}$  (19). Even at high  $\text{Mg}^{2+}$  concentrations, dissociation of AA-GTP from  $G_{\text{so}\alpha}$  was orders of magnitude faster than dissociation of GTP $\gamma\text{S}$ . It remains to be determined what substitutions to the  $\gamma$ -phosphoryl are compatible with high affinity binding and activation.

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