Partial G Protein Activation by Fluorescent Guanine Nucleotide Analogs

EVIDENCE FOR A TRIPHOSPHATE-BOUND BUT INACTIVE STATE*

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N-methyl-3'-O-anthranoyl (MANT) guanine nucleotide analogs are useful environmentally sensitive fluorescent probes for studying G protein mechanisms. Previously, we showed that MANT fluorescence intensity when bound to G protein was related to the degree of G protein activation where MANT-guanosine-5'-O-(3-thiotriphosphate) (mGTP*S) had the highest fluorescence followed by mGTP and mGDP, respectively (Remmers, A. E., Posner, R., and Neubig, R. R. (1994) J. Biol. Chem. 269, 13771–13778). To directly examine G protein conformations with nucleotide triphosphates bound, we synthesized several nonhydrolyzable MANT-labeled guanine nucleotides. The relative maximal fluorescence levels observed upon binding to recombinant myristoylated Gαon (myrGαon) and myrGαoff were: mGTP*S > MANT-5'-guanylyl-2-(thio)diphosphate > MANT-guanylyl-(β,γ-methylene)-diphosphonate > MANT-guanosine 5'-O-2-(thio)diphosphate. Using protection against tryptic digestion as a measure of the activated conformation, the ability of the MANT guanine nucleotides to maximally activate myrGαon, correlated with maximal fluorescence. Biphasic dissociation kinetics were observed for all of the MANT guanine nucleotides. The data were consistent with the following model,

\[ \frac{k_{on}}{k_{act}} G + GXP \Leftrightarrow G - GXP \Leftrightarrow G^* - GXP \]

where G protein activation (G*-GXP) is determined by a conformational equilibrium between two triphosphate bound states as well as by the balance between binding and hydrolysis of the nucleotide triphosphate. Compared with myrGαon, maximal mGTP fluorescence was only 2-fold higher for the myrGαoff Q204L mutant, a mutant with greatly reduced GTPase activity, and only 24% that of mGTP*S, indicating that partial activation by mGTP was not just due to hydrolysis of mGTP. These results extend our previous conclusion that GTP analogs do not fully activate G protein.

Receptor-mediated activation of heterotrimeric GTP binding proteins is a common mechanism for signal transduction in biological systems. Receptors on the cell surface regulate G protein function by catalyzing the release of GDP from the α subunit of the G protein, allowing GTP to bind and activate the G protein. G protein activation is thought to be concomitant with α subunit dissociation from βγ subunits. Activated G protein α and βγ subunits interact with effector proteins to modulate intracellular second messenger metabolism (for review, see Refs. 1–3). G protein deactivation is mediated by α subunit GTPase activity. The deactivation rate, which may be limited by the rate of phosphate release from α-GDP-Pi (4), is catalyzed by membrane-bound GTPase accelerating proteins (5, 6). Upon deactivation, α and βγ subunits reassociate. Perhaps the best understood G protein mechanism is the rhodopsin-mediated activation of transducin in the retinal rod outer segment. The rhodopsin conformations can be easily studied because of the retinal chromophore attached to the protein. In addition, changes in G protein intrinsic fluorescence (due largely to changes in tryptophan 207 (7)) can be used to monitor transducin activation and deactivation (8, 9) and have been used to model the kinetic mechanism of the rhodopsin-stimulated activation/deactivation cycle of transducin (10).

Kinetic mechanisms of the predominant G proteins purified from brain (Gα and Gγ) have also been studied extensively in solution or when reconstituted into phospholipid vesicles (for review, see Ref. 11). Gα is a G protein abundant in brain that contains two tryptophans in the α subunit. Upon activation by GTP and magnesium, an increase in the intrinsic fluorescence of G protein tryptophan is observed (12). The effects of βγ subunits, ions, and activating ligands on Gα intrinsic fluorescence have been well characterized in detergent solutions (13, 14). Recent structural data of transducin bound to GDP and GTPγS indicate differences in structure in the α2 helix containing Trp207. In the GTPγS-bound state the α2 helix rotates such that the Trp is in a more hydrophobic environment, which is presumably the cause of the increase in its quantum yield. However, the receptor-stimulated G protein activation/deactivation cycle has not been studied, most likely due to the high background and relatively small signals available from intrinsic tryptophan fluorescence. One of our goals has been to study G protein activation and deactivation kinetics in real time. To that end, we have synthesized fluorescently labeled G protein α and βγ subunits (15) and studied the kinetics of their association (16). In addition, we have found that N-methyl-3'-O-anthranoyl (MANT) guanine nucleotide analogs are useful

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The abbreviations used are: GTPγS, guanosine-5'-O-(3-thiotriphosphate); MANT, N-methyl-3'-O-anthranoyl; mGTP*S, MANT-GTPγS; mGDPβS, guanosine-5'-O-2-(thiodiphasphate); mGDPγS, MANT-GDPβS; Gpp(CH2)2p, guanylyl-(β,γ-methylene)di-phosphonate; MANT-mGpp(CH2)p, MANT-mGpp(CH2)p; Gpp(NH)p, 5'-guanylyl-2-(thio)diphosphate; mGpp(NH)p, MANT-mGpp(NH)p; GXP, guanine nucleotide; mGXP, MANT-GXP; Gαon and G*, activated form of the G protein; r-myrGαon and r-myrGαoff, recombinant myristoylated G protein α subunit.

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environmentally sensitive fluorescent probes to study heterotrimeric G proteins (17). MANT-GTPyS (mGTPyS) displays a marked increase in fluorescence upon binding to Gα. The fluorescence increase is greater when the MANT moiety is excited via energy transfer from tryptophan (excitation, 280 nm). The fluorescence increase observed when mGTP is bound is lower. Previous studies of the mGTP fluorescence kinetics indicated that the low peak fluorescence with mGTP was not just due to slow binding and rapid hydrolysis. We concluded that mGTP does not fully activate G protein even when bound as triphosphate. It is possible that the natural nucleotide, GTP, also does not fully activate the G protein and is a partial agonist regarding G protein activation. However, the intrinsic GTPase activity of G protein makes it difficult to discern whether a conformational change resulting in partial activation limits GTP effects in addition to the well known role of GTP hydrolysis. For example, GTPγS and Gpp(NH)p inhibited cyctc S49 cell adenyl cyclase activity more than GTP (18). However, GTPγS and GTP activated cGMP phosphodiesterase to the same extent, suggesting that GTP can fully activate transducin in rod outer segment membranes (19).

To test the hypothesis that partial activation of Gα by MANT-GTP was not due to the balance between binding and hydrolysis and to explore the G protein activation/deactivation reactions, we synthesized several nonhydrolyzable MANT-labeled guanine nucleotides. The MANT-labeled nonhydrolyzable analogs displayed differing levels of maximal fluorescence, which correlated with their ability to activate G protein. In addition, the MANT guanine nucleotides with slower dissociation rates were able to more fully activate G protein, which is consistent with predictions of a two-step activation model. Partial activators showed biphasic dissociation kinetics, indicating that we are able to observe MANT-nucleotide fluorescence signals from both the inactive and active G protein states. We also compared mGTP fluorescence when bound to a hydrolysedeficient G protein, Gα12 Q204L, to that of wild type protein. While mGTP fluorescence was greater when bound to the Q204L mutant than when bound to wild type protein, it was still only 24% of the fluorescence of mGTPyS. Taken together, these results further support the use of MANT guanine nucleotide fluorescence as a measure of G protein activation and a probe of G protein conformational states. This also represents the first demonstration that nonhydrolyzable triphosphate nucleotides can produce partial activation of heterotrimeric G proteins. Regulation of this activating conformational change could be a mechanism of receptor or GTPase accelerating protein on G proteins.

**EXPERIMENTAL PROCEDURES**

Materials—Dithiothreitol, GTP-S, GDP-P-S, Gpp(CH)N-p, and Gp-p(NH)p were purchased from Calbiochem. [3H]GDP and [35S]GTP were obtained from DuPont NEN. N-Methylisatoic anhydride was purchased from Molecular Probes (Eugene, OR). G protein expression vectors containing the rat cDNA sequence for Gα and Gβ and pBB131, an expression vector containing yeast N-myristoyltransferase (20), were gifts from Drs. Maurine Linder and Jeffrey Gordon, respectively (Washington University, St. Louis, MO). Recombinant myristoylated Gα12 Q204L was a gift from Dr. Ronald Taussig (University of Michigan, Ann Arbor, MI).

MANT Guanine Nucleotide Synthesis and Purification—N-Methylisotoic anhydride was purchased from Calbiochem. [3H]GDP and [35S]GTP were synthesized according to the method of Cheng and Prusoff (22) using a PerkinElmer SL2-300 (PerkinElmer, Boston, MA) and a 12 μmol column (PerSeptive Biosystems, Inc.) equipped with a 20 mM diethylamine column (Pharmacia Biotech Inc.) followed by chromatography on Source 15Q (Pharmacia Biotech Inc.) (21).

Equilibrium Fluorescence of MANT Guanine Nucleotides—Recombinant myristoylated G protein (50 nM r-myrGα12 or 100 nM r-myrGα12) was incubated with 0.01–7 μM MANT guanine nucleotide at 30 °C for 10 min at 10°C. The fluorescence of MANT guanine nucleotides in the absence of G protein was subtracted from the total fluorescence to yield specific fluorescence, which is representative of 5–98% of total MANT-GTPyS and MANT-GDP. The fluorescence values were determined by fitting specific fluorescence values to a hyperbolic binding function (GraphPad Prism, GraphPad Software, Inc., San Diego, CA).

Equilibrium Nucleotide Binding—Binding constants for MANT guanine nucleotides were also determined from equilibrium [3H]GDP competition experiments. The G protein (20 nM r-myrGα12 in HEDNML) was preincubated with competing ligands for 20 min at 18°C. [3H]GDP was added. Following incubation for 30 min at 20°C, the samples were filtered, and the data were fit to a sigmoidal curve using GraphPad Prism. IC50 values were converted to Kd values by the method of Cheng and Prusoff (22) using a Kd of 120 nM for [3H]GDP binding to r-myrGα12. Although we propose a two-step model to describe G protein and guanine nucleotide interaction, the equilibrium behavior of that model is formally equivalent to binding with a single apparent affinity. Thus, Kd values determined here are apparent binding affinities, which reflect a composite of the equilibrium constants for the two volumes of equilibration buffer followed by a 0–1.5 M NaCl gradient over 20 column volumes. The Mono Q column was equilibrated with 20 mM Tris-Bis-Tris propane, pH 6.0, at 3 ml/min. The pooled peak from the Source 150 column was diluted with equilibration buffer (1:1) and loaded onto the Mono Q column. Following a 2-column volume wash with equilibration buffer, the MANT guanine nucleotide was eluted with a 0–2 M NaCl gradient. Volumes of 20 column volumes were collected for 0 to 2 M NaCl. Both absorbance (270-nm preparative and 252-nm analytical) and fluorescence (mercury lamp with excitation filter OD1 and pin hole filter and a GG 420 emission filter, Fluoro-tek filter fluorometer, St. John Associates, Beltville, MD) were monitored. The product purity was determined based on the fraction of total fluorescence that corresponded to the desired MANT guanine nucleotide product (some products contained small amounts < 6% of contaminating mGDP).

G Protein Expression and Purification—Qiagen vectors containing Gα and Gβ were cotransformed into the JM109 strain of Escherichia coli with the N-myristoyl transferase vector pBB131. Protein was expressed and purified according to Mummy and Linder (21) with the following exceptions. Following sample loading, the sample was loaded on a 100-ml heptylamine-Sepharose column using the same conditions as reported for the phenyl-Sepharose column (21). Fractions containing G protein were identified by a fluorescence assay utilizing mGTPyS. An aliquot (10–20 μl) was incubated at room temperature with 400 nM mGTPyS in HEDNML (50 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 10 mM MgCl2, 20 μM deionized Lubrol) for either 10 min for recombinant myristoylated Gα12 (r-myrGα12) or 30 min for recombinant myristoylated Gα12 (r-myrGα12). Fluorescence was monitored at 440 nm (280-nm excitation) as described below. The background fluorescence of 400 nM mGTPyS in buffer alone was subtracted to determine specific fluorescence.

G proteins were further purified by chromatography on Source 150 (7.8 ml, Pharmacia), hydroxyapatite (Bio-Rad), and Mono Q HR5/5 (Pharmacia) columns using a BioCAD SPRINT System (PerSeptive Biosystems, Inc., Framingham, MA). The Source 150 column was equilibrated with 50 mM Tris-Bis-Tris propane, 1 mM dithiothreitol, pH 8.0, at 10 ml/min. Before loading onto the column, the column was filtered through a 0.45-μm filter. Following sample loading, the column was washed with 2 column volumes of equilibration buffer followed by a 0–225 mM NaCl gradient column volumes. Hydroxyapatite column chromatography was performed as described previously (21). An additional final chromatography on the Mono Q HR5/5 column removed significant amounts of inactive G protein from the preparation.

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different steps in the model. The same is true for the equilibrium fluorescence $K_f$ values.

Trypsin Digestion—One microgram of recombinant myristoylated Go, was preincubated for 20 min at 20°C with 1, 10, or 100 nM MANT guanine nucleotides in HEDNML buffer. Trypsin was added (0.02 μg), and the incubation continued for 30 min. Samples were run on an 11% SDS-polyacrylamide gel prepared according to Laemmli (23) and stained with Coomassie Blue for visualization. Maximal G protein activation was determined in the presence of 100 μM GTP·S. Background was determined with no added guanine nucleotide. Four experiments were performed, the gels were scanned (ScanJet IIIC, Hewlett Packard), the 37-kDa band was quantitated (IMAGE QUANT, Molecular Dynamics), and the results were averaged.

Guanine Nucleotide Dissociation Kinetics—Recombinant myristoylated Go, (300 nM) was incubated for 20 min at 20°C with 1 μM MANT guanine nucleotides in HEDNML buffer. Fluorescence measurements were made as described above using the PTI ALPHASCAN fluorometer, and the sample was continuously stirred. Dissociation was initiated by the addition of 10 μM GTP·S. Data were acquired every second, and the data fit with a one-phase exponential decay with floating end point. Due to the fast MANT-GDP dissociation kinetics and a fast component of the dissociation kinetics of other guanine nucleotides, dissociation was also measured using a stopped-flow spectrophotometer (SX-17MV, Applied Photophysics Ltd., Leatherhead, UK). Excitation was at 280 nm (Photomultiplier tube voltage, 750 V) with 4.65-nm slits, and emission light was measured with a photomultiplier tube behind a KV399 filter. Recombinant mygGo, (200 nM) was incubated for 20 min at 20°C with 2 μM MANT guanine nucleotide in HEDNML buffer and then loaded into one syringe. The other syringe contained 10 or 50 μM GTP·S in HEDNML. Fifty μl of each reactant was used per shot. All measurements were performed at 20°C, and samples were equilibrated at that temperature for at least 3 min before beginning measurements. For mGDP·S, data were collected every 12.5 ms for 5 s with a 0.2-ms filter. Dissociation data for mGpp(NH)p·S and mGTP·S, mGpp(NH)p·S were acquired for 100, 500, and 500 s, respectively, on a log time base. Nine or 33 traces were collected and averaged. Data were analyzed using one and two component exponential dissociation equations, and an F test was used to determine the minimal model that best fit the data (GraphPad, Prism 1.03). Dissociation experiments were performed two times with similar results.

RESULTS

Maximal MANT Fluorescence Is Dependent Upon the Guanine Nucleotide Analog—We previously observed that at saturating concentrations of guanine nucleotide, peak fluorescence due to mGTP bound to Go, was 19% that of mGTP·S. Modeling the mGTP kinetics suggested that mGTP does not fully activate G protein, even when bound (17). To test the hypothesis that partial activation was not just due to the balance between binding and hydrolysis, the fluorescence and activation properties of hydrolysis-resistant MANT-labeled guanine nucleotides were examined. Specific fluorescence saturated with increasing MANT-nucleotide concentrations (Fig. 1A). The magnitude of MANT equilibrium fluorescence when bound to r-myrGo, or r-myrGo, (Fig. 1A and B) was dependent upon the type of guanine nucleotide analog (mGTP·S > mGpp(NH)p·S > mGpp(CH3)p·S > mGDP·PS). A summary of maximal fluorescence and fluorescence $K_f$ values for r-myrGo, and r-myrGo, is listed in Table 1. With the exception of mGDP·S, the apparent affinities of the MANT guanine nucleotides were greater for r-myrGo, than for r-myrGo,.

Affinities of Fluorescent Analogs for G Protein—As another method to determine affinity for G protein, the ability of the MANT guanine nucleotide analogs to compete with [3H]GDP for binding to recombinant myristoylated Go, was determined (Fig. 2). The $K_f$ values (mean ± S.E., n = 4) for mGTP·S, mGpp(NH)p·S, mGpp(CH3)p·S, and mGDP·PS were 3 ± 1 nM, 160 ± 90 nM, 1.6 ± 0.5 μM, and 250 ± 200 μM, respectively. There is a discrepancy between the $K_f$ values for some nucleotides determined by fluorescence saturation (Table I) and by competition versus [3H]GDP. Because mGTP·S binds G protein with very high affinity in both experiments, the mGTP·S is titrating the G protein. For example, when 100 nM r-myrGo, was present in the equilibrium fluorescence assay, the $K_f$ was 20 nM, and when 20 nM G protein was used in the [3H]GDP competition experiment, the calculated $K_f$ was 3 nM. Thus, both values are probably overestimates of the true $K_f$. In contrast, a shortcoming of the equilibrium fluorescence measurements for ligands with low affinity (e.g. mGDP·S) is the relatively small amount of total fluorescence that is due to specific binding. Therefore, another method to determine affinity for G protein was employed. The same order of affinity was observed for recombinant myristoylated Go, (left) and Go, (right). Maximal fluorescence and $K_f$ values are listed in Table I. Another method to determine the equilibrium fluorescence measurements for ligands with low affinity (e.g. mGDP·S) is the relatively small amount of total fluorescence that is due to specific binding. Therefore, another method to determine affinity for G protein was employed. The same order of affinity was observed for recombinant myristoylated Go, (left) and Go, (right). Maximal fluorescence and $K_f$ values are listed in Table I. Another method to determine the equilibrium fluorescence measurements for ligands with low affinity (e.g. mGDP·S) is the relatively small amount of total fluorescence that is due to specific binding. Therefore, another method to determine affinity for G protein was employed. The same order of affinity was observed for recombinant myristoylated Go, (left) and Go, (right). Maximal fluorescence and $K_f$ values are listed in Table I. Another method to determine the equilibrium fluorescence measurements for ligands with low affinity (e.g. mGDP·S) is the relatively small amount of total fluorescence that is due to specific binding. Therefore, another method to determine affinity for G protein was employed. The same order of affinity was observed for recombinant myristoylated Go, (left) and Go, (right). Maximal fluorescence and $K_f$ values are listed in Table I. Another method to determine the equilibrium fluorescence measurements for ligands with low affinity (e.g. mGDP·S) is the relatively small amount of total fluorescence that is due to specific binding. Therefore, another method to determine affinity for G protein was employed. The same order of affinity was observed for recombinant myristoylated Go, (left) and Go, (right). Maximal fluorescence and $K_f$ values are listed in Table I. Another method to determine the equilibrium fluorescence measurements for ligands with low affinity (e.g. mGDP·S) is the relatively small amount of total fluorescence that is due to specific binding. Therefore, another method to determine affinity for G protein was employed. The same order of affinity was observed for recombinant myristoylated Go, (left) and Go, (right). Maximal fluorescence and $K_f$ values are listed in Table I. Another method to determine the equilibrium fluorescence measurements for ligands with low affinity (e.g. mGDP·S) is the relatively small amount of total fluorescence that is due to specific binding. Therefore, another method to determine affinity for G protein was employed. The same order of affinity was observed for recombinant myristoylated Go, (left) and Go, (right). Maximal fluorescence and $K_f$ values are listed in Table I.
peptide of 21 amino acids from the aminoterminus of Go induces a conformation such that trypsin only released a short GTP followed by mGpp(NH)p and mGpp(CH2)p, which were both the MANT-nucleotides to activate r-myrGo.

Following by mGpp(NH)p and mGpp(CH2)p, which were both the MANT-nucleotides to activate r-myrGo.100 G protein.
much more effective than mGDP guanine nucleotides,2 there was a difference in the ability of activating r-myrGo.

Involve two steps (12, 17, 25, 26). Since the MANT nucleotides tide triphosphate binding and G protein activation most likely on intrinsic fluorescence detect the same activation state, however, 100 [3H]GDP as described under "Experimental Procedures." The 20°C with MANT guanine nucleotides followed by incubation with [3H]GDP as described under "Experimental Procedures." In the presences of GTP or the MANT guanine nucleotide analogs, the amount of 37-kDa r-myrGo was quantitated for mGTP-S, mGpp(NH)p, mGpp(CH2)p, and mGDPβS followed by incubation with trypsin and 11% SDS-polyacrylamide electrophoresis as described under "Experimental Procedures." In the lanes marked with a and *, recombinant myristoylated Go was preincubated with no additional guanine nucleotide or 100 μM GTP-γS, respectively. Shown is a representative experiment that was performed 4 times. B, following trypsin digestion in the presence of GTP-γS or the MANT guanine nucleotide analogs, the amount of 37-kDa r-myrGo was quantitated for mGTP-S (■), mGpp(NH)p ( ), mGpp(CH2)p ( ), and mGDPβS ( ) as described under "Experimental Procedures," and the results from four experiments were averaged (± S.E.). Background and maximal protection from trypsin digestion was defined using no added guanine nucleotide and 100 μM GTP-γS, respectively.

TABLE I

| Guanine nucleotide | Kd value | Maximal Fluorescence (relative to mGTP-γS) |
|--------------------|----------|------------------------------------------|
| mGTP-γS            | 0.020 ± 0.007 | 1                                           |
| mGpp(NH)p          | 0.16 ± 0.04   | 0.66 ± 0.03                                |
| mGpp(CH2)p         | 1.2 ± 0.1     | 0.54 ± 0.02                                |
| mGTP               | NDa         | ND                                         |
| mGDPβS             | 4 ± 1        | 0.34 ± 0.09                                |

a ND, not determined.

b Maximal mGTP fluorescence was determined from the peak fluorescence in a time course with 5–7 μM guanine nucleotide added to 100 nM r-myrGo, and compared with maximal fluorescence observed with the same concentration of mGTP-γS.

FIG. 2. [3H]GDP equilibrium binding in the presence of MANT guanine nucleotides. Recombinant myrGo was preincubated at 20°C with MANT guanine nucleotides followed by incubation with [3H]GDP as described under "Experimental Procedures." The K values for mGTP-γS (■), mGpp(NH)p ( ), mGpp(CH2)p ( ), and mGDPβS ( ) are (mean ± S.E., n = 4) 3 ± 1 nM, 160 ± 90 nM, 1.6 ± 0.5 μM, and 250 ± 200 μM, respectively.

In the figure, preincubation with GTP-γS (Fig. 3A, * lanes), induces a conformation such that trypsin only released a short peptide of 21 amino acids from the amino terminus of Go. The remaining 37-kDa fragment is insensitive to further trypsin digestion (24). Even at saturating concentrations of MANT-guanine nucleotides,2 there was a difference in the ability of the MANT-nucleotides to activate r-myrGo.3 In contrast to the MANT-nucleotides, the unmodified nucleotides, GTP-γS, Gpp(NH)p, and Gpp(CH2)p at 100 μM, were all equally effective at activating r-myrGo, as monitored by intrinsic tryptophan fluorescence (A. Remmers, data not shown). However, 100 μM GTP only increased tryptophan fluorescence to 16% of that observed with the other nonhydrolyzable guanine nucleotides. Because the MANT moiety quenches tryptophan fluorescence, similar experiments could not be performed with the MANT guanine nucleotides. If the trypsin protection and intrinsic fluorescence both detect the same activation state, then it appears that the MANT moiety may hinder the ability of mGpp(NH)p and mGpp(CH2)p to fully induce the active conformation of the G protein.

FIG. 3. Activation of r-myrGo by MANT guanine nucleotides. A, one microgram of recombinant myristoylated Go was preincubated with the indicated concentrations of mGTP-γS, mGpp(NH)p, mGpp(CH2)p, and mGDPβS followed by incubation with trypsin and 11% SDS-polyacrylamide electrophoresis as described under "Experimental Procedures." In the lanes marked with a and *, recombinant myristoylated Go was preincubated with no additional guanine nucleotide or 100 μM GTP-γS, respectively.

Because the MANT moiety may hinder the ability of mGpp(NH)p and mGpp(CH2)p to fully induce the active conformation of the G protein.

2 Based on the apparent Kd and K values measured here (i.e., 1.6 μM for mGpp(CH2)p) and the nucleotide concentrations used (100 μM in trypsin digest), greater than 98% of G protein is bound to ligand.

3 In contrast to the MANT-nucleotides, the unmodified nucleotides, GTP-γS, Gpp(NH)p, and Gpp(CH2)p at 100 μM, were all equally effective at activating r-myrGo, as monitored by intrinsic tryptophan fluorescence (A. Remmers, data not shown). However, 100 μM GTP only increased tryptophan fluorescence to 16% of that observed with the other nonhydrolyzable guanine nucleotides. Because the MANT moiety quenches tryptophan fluorescence, similar experiments could not be performed with the MANT guanine nucleotides. If the trypsin protection and intrinsic fluorescence both detect the same activation state, then it appears that the MANT moiety may hinder the ability of mGpp(NH)p and mGpp(CH2)p to fully induce the active conformation of the G protein.

The different MANT nucleotides would exhibit different equilibrium constants (kact/kdeact) for the activation step. The initial binding step is governed by the forward rate constant k and dissociation by kdeact, while the activation and deactivation of the triphosphate-bound G protein is determined by kact and kdeact.

We examined the rates of fluorescence decrease of prebound fluorescent guanine nucleotide, following the addition of competing ligand, GTP-γS. The rates of fluorescence decrease varied greatly among the MANT guanine nucleotide analogs (Fig. 5A). MANT-GTP-γS displayed very slow kinetics, followed by mGpp(NH)p and mGpp(CH2)p with intermediate rates and mGDPβS with the fastest kinetics. In addition, all four nucleotides showed biphasic kinetics with results of nonlinear least
FIG. 4. G protein activation by and maximal fluorescence of MANT guanine nucleotides. Plotted on the abscissa is the maximal MANT guanine nucleotide fluorescence when bound to recombinant myristoylated Go
a, relative to mGTPS (Table I). On the ordinate, the ability of 100 μM MANT guanine nucleotides to protect against trypsin digestion is plotted where protection by 100 μM GTPS is 1.00 (Fig. 3B).

FIG. 5. Kinetics of MANT guanine nucleotide fluorescence decrease. A, MANT guanine nucleotides were prebound to r-myrGo
a, and dissociation was initiated by the addition of GTPS as described under "Experimental Procedures." The data were best fit with a two-phase exponential decay with floating end point. The rate of mGTPS (data not shown). The rate of mGTPS binding was identical to wild type and Q204L Gα
a (Fig. 6) and is presumably limited by the rate of GDP release. For r-myrGo
a, the MANT guanine nucleotide signal is 10% that of mGTPS. The maximal mGTP fluorescence is doubled when bound to the hydrolysis-deficient mutant (Fig. 6, inset). Thus, hydrolysis does contribute to the low amount of activated G protein at steady state. However, it does not appear to be the sole limiting factor (see "Discussion").

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square fits summarized in Table II. We propose that the two kinetic components reflect the disappearance of the G-mGXP and G*-mGXP states. The fraction of fluorescence that decreased rapidly was dependent on the guanine nucleotide where mGpp(CH2)p (10.3 ± 0.2%) had a larger fast component than mGpp(NH)p (4.1 ± 0.2%) (Fig. 5B and Table II). The half-times for the fast and slow processes were 3.6 and 33 s for mGpp(CH2)p and 16 and 330 s for mGpp(NH)p. The fluorescence decrease for mGTPS never reached a plateau, so the rapidly decreasing fraction was estimated to be very small (~0.4%; see Fig. 5 legend for details), and GDP/S had the greatest fast component (63%). If there was an equilibrium between nucleotide-bound and activated G protein, guanine nucleotide analogs that more completely activate the G protein would have a smaller amount of fast component, which reflects direct dissociation of mGXP from the nonactivated G-mGXP state.

One puzzling aspect of the dissociation kinetics was why the fast phase of the mGpp(CH2)p fluorescence (which we attribute to dissociation of the nonactivated G-mGXP) was only 10% when it appeared from the steady state fluorescence and trypsin protection data that mGpp(CH2)p left ~50% of the G protein inactive. Similarly, the 4% fast component appeared to underestimate the 30% inactive for mGpp(NH)p. This paradox is easily understood when we take into account the relative quantum yields of the two states. If the inactive G-mGXP state has lower fluorescence than the activated G-mGXP state, then we would expect the fast phase of fluorescence decrease to be proportional to the amount of G-mGXP present at equilibrium but to be quantitatively less. Based on a comparison of the fraction of MANT guanine nucleotide fluorescence that decreases rapidly and the fraction inactive measured by trypsin digest, we estimate that the relative quantum yield of G-GXP is approximately one-fourth that of G*-GXP. The relative quantum yields for the G-mGXP state are comparable with the relative maximal fluorescence observed for mGDPβS bound to inactive mutant, indicating that the fluorescence of G-mGXP is similar to that of an inactive G protein MANT-nucleotide complex. The biphasic kinetics and the relative amount of fluorescence that decreased rapidly for each MANT guanine nucleotide suggest that these data can be described by a two-step G protein activation model (Reaction 1).

Maximal mGTP Fluorescence When Bound to r-myrGo
a, and r-myrGo
a, Q204L—We employed an additional approach to determine if mGTP also partially activates G protein by using a hydrolysis-deficient mutant of r-myrGo
a, Q204L. The steady state binding of [32P]GTP and [35S]GTPS was identical and indicative of extremely reduced GTPase catalytic activity (>100-fold lower) in this mutant (27). We found a 10-fold decrease in steady state GTPase using buffer conditions employed for our fluorescent guanine nucleotide experiments (data not shown). The rate of mGTPS binding was identical for wild type and Q204L Go
a (Fig. 6) and is presumably limited by the rate of GDP release. For r-myrGo
a, the mGTP fluorescence signal is 10% that of mGTPS. The maximal mGTP fluorescence is doubled when bound to the hydrolysis-deficient mutant (Fig. 6, inset). Thus, hydrolysis does contribute to the low amount of activated G protein at steady state, however, it does not appear to be the sole limiting factor (see "Discussion").

DISCUSSION

In this report we demonstrate that two fluorescent nucleotide triphosphate analogs, mGpp(NH)p and mGpp(CH2)p, only partially activate the G protein as reflected in their maximal fluorescence when bound to G protein and their ability to induce a trypsin-resistant state. Rapid measurements of dissociation kinetics show nucleotide fluorescence decreases from two pools of G protein, which appear to represent triphosphate-bound but inactive as well as activated G protein, G-mGXP and G*-mGXP, respectively. Thus, the MANT guanine nucleotides reveal a novel aspect of G protein mechanisms and provide a means to dissect the detailed kinetics of the G protein activation step and study their regulation.
understand the factors (tivation properties, which must be considered separately from show that the different triphosphate analogs have distinct ac-
activate as well as the natural nucleotides (see below), they do
strate a role for the conformational activation step in regulat-
activation. With the MANT-nucleotides, we clearly demon-
theGTPhydrolysisstepstodeterminetheamountofGprotein
ply considered the balance between the GDP release step and
Consequently, most analyses of G protein activation have sim-
dependence of these two steps (12, 25, 26) is one of the strong-
triphosphate binding and differential magnesium
assumed that G protein activation is a separate step from
withthenucleotidedissociationrate(seebelow).Itisgenerally
3) themagnitude of the fluorescence signal inversely correlates
thatweareobservingnotonlyMANTguaninenucleotidebind-
g and mGTP was added to 100
S dissociated at all in the rapid mix experiments, the fraction with rapid dissociation kinetics was

![Graph](https://www.jbc.org/content/early/2019/04/26/jbc.R190001JBC.ADR)

**FIG. 6.** Maximal mGTP fluorescence when bound to r-myrGiQ204L and r-myrGiQ204L. Seven micromolar mGTP-S or mGTP was added to 100
molar r-myrGiQ204L (black line) or r-myrGiQ204L (gray line), stirring in HEDNML at 30°C and monitoring fluorescence as described under "Experimental Procedures." Data were fit to a one-component exponential rise, and the maximal fluorescence was plotted in the inset. Shown is a representative experiment that was repeated once with similar results. The relative maximal mGTP fluorescence compared with that of mGTP-S when bound to r-myrGiQ204L and r-myrGiQ204L was 10.2 ± 1.2% and 23.6 ± 0.6%, respectively (mean ± range, n = 2).

**TABLE II**

| Guanine nucleotide | Rates of fluorescence decrease | Percentage of fluorescence with rapid kinetics |
|--------------------|-------------------------------|---------------------------------------------|
| mGTP-Sb | 0.093 ± 0.007 (7.4) | 0.0012 ± 0.0002 (578) | 0.42 ± 0.01b |
| mGpp(NH)p | 0.043 ± 0.002 (16) | 0.0021 ± 0.00007 (330) | 4.1 ± 0.2 |
| mGpp(CH)p | 0.19 ± 0.02 (3.6) | 0.021 ± 0.001 (33) | 10.3 ± 0.2 |
| mGDP-Sd | 1.7 ± 0.1 (0.4) | 0.42 ± 0.08 (1.6) | 63 ± 6 |

a Data from 3 (mGDP-S) or 5 (mGTP-S) shots from a single experiment were averaged, and the mean and S.E. are shown.

b Since only a small fraction of mGTP-S dissociated at all in the rapid mix experiments, the fraction with rapid dissociation kinetics was estimated. The fluorescence amplitude with fast dissociation kinetics (0.0088 = 0.0002 V) was divided by an estimate of total mGTP-S fluorescence.

c Shown are mean and S.E. from three experiments.

in the biphasic dissociation kinetics observed for mGpp(NH)p and mGpp(CH)p, which we predicted based on their incomp-
distin-
guish among them and thus discuss the data in the context of a simple two-step equilibrium model. Based on a two-step
reversible reaction where the first (binding) step is fast and the second (activation) step is slow (Reaction 1), it is possible to assign molecular rate constants to the observed fast and slow dissociation. The slow rate of fluorescence decrease is equal to $k_{dact} \times (k_{i1}(k_{i1} + k_{in}))$, and the fast rate is equal to $k_{act} + k_{in}$ (corresponding to the disappearance of G-mGXP). The MANT derivatives with higher fluorescence dissociated more slowly, which would be expected because a small $k_{dact}$ would both increase the steady state activation and decrease the rate of dissociation. The observation that mGpp(CH)p had a larger fraction of fluorescence decrease rapidly compared with mGpp-
pp(NH)p is consistent with mGpp(CH)p being less effective in forming G*-mGXP than mGpp(NH)p.

Although only 10% of the mGpp(CH)p fluorescence decrease reduces rapidly, it appears, based on the steady state mGpp(CH)p fluorescence and the trypsin digest experiment, that approximately 50% of the G protein is activated. Thus, the small fast signal appears to account for approximately half of the G protein because G-mGXP most likely has a lower quan-
tum yield than G*-mGXP. This lower relative quantum yield (20–30% of that of G*-mGXP) is mostly dependent on the G protein conformational state. Likewise, although ~60% of mGDP-S fluorescence decreases rapidly, most of the protein is in the bound but not activated state. The ability to detect conformational transitions with a spectral probe that is more selective than intrinsic tryptophan fluorescence will enable us to examine the mechanisms of receptor, βγ, and effector pro-
teins in the cycle of G protein activation and deactivation. To evaluate potential models of G protein activation, "on kinetics" with GDP-free G protein will be measured and the molecular rate constants determined.

MANT moiety renders mGpp(CH)p and mGpp(NH)p partial agonists—Yamanaka et al. (19) described different affinities of the guanine nucleotides in interacting with transducin in which GTP-S was most potent and Gpp(CH)p had a rather low affinity. In addition GTP-S is more potent than Gpp(NH)p to compete for the GTP binding site on G (28) and to inhibit G, GTPase (29). Differing affinities of hydrolysis-resistant GTP analogs were also observed in vivo in cardiac atrial myocytes. The GTP analogs activated Gs (one of the Gi subtypes) and opened an inwardly rectifying potassium channel with the following relative affinities GTP-S > GTP > Gpp(NH)p > Gpp(CH)p (30). However, all produced the same maximal re-
sponse. In contrast, Gpp(CH)p was only able to partially activate phosphodiesterase in rod outer segment membranes (19). We observed strikingly different abilities of MANT-labeled

G Protein Activation Model—Three lines of evidence suggest that we are observing not only MANT guanine nucleotide bind-
but also G protein activation: 1) the fluorescence increase is magnesium-dependent (17), 2) the magnitude of the fluores-
ence signal correlates with the degree of G protein activation as measured by protection from tryptic digestion (Fig. 3), and 3) the magnitude of the fluorescence signal inversely correlates with the nucleotide dissociation rate (see below). It is generally assumed that G protein activation is a separate step from nucleotide triphosphate binding and differential magnesium dependence of these two steps (12, 25, 26) is one of the strong-
est pieces of evidence in support of this model. However, there has not been a good method to study the kinetics of the activation step because GDP release is slow with purified G proteins. Consequently, most analyses of G protein activation have sim-
ply considered the balance between the GDP release step and the GTP hydrolysis steps to determine the amount of G protein activation. With the MANT-nucleotides, we clearly demonstr-
strate a role for the conformational activation step in regulat-
G protein activation. While the MANT-nucleotides may not activate as well as the natural nucleotides (see below), they do show that the different triphosphate analogs have distinct ac-
tivation properties, which must be considered separately from their affinities. Subsequent studies will be needed to better understand the factors (e.g. magnesium concentration, βγ sub-
units, and receptor activation) that control these steps in the G protein.

The strongest support for the G protein activation model lies

![Diagram](https://www.jbc.org/content/early/2019/04/26/jbc.R190001JBC.ADR)
guanine nucleotides to maximally activate r-myr Gα1, although affinities are similar to those described previously for nonfluorescent nucleotides. In the presence of saturating concentrations of mGpp(NH)p and mGpp(CH2)p, G protein was only partially activated as indicated by protection against trypsin digestion. Thus, we conclude that these fluorescent analogs only partially activate G protein. It is possible that faster dissociation rate constants for mGpp(NH)p and mGpp(CH2)p could contribute to the greater trypsin sensitivity compared with mGTPγS. However, if the nucleotides fully activated Gα1, one would expect little nonactivated Gα1 to be present for trypsin digestion. More direct methods to measure G protein activation such as use of fluorescent subunit probes (14) or adenyl cyclase inhibition by Gα (31) will be useful to definitively test this possibility.

Is partial activation of G proteins only a property of MANT-nucleotides? Additional data suggest that GTP may also function as a partial activator. Slepak et al. (32) generated r-myr Gα1Q205L, a GTP hydrolysis-deficient G protein mutant. This corresponding mutation reduced the rate of GTP hydrolysis (kcat) approximately 20-fold in Ras (33) and greater than 100-fold in Gα1 (27). They showed that GTP was only able to partially protect the Gα1Q205L mutant from trypsin digestion as compared to GTPγS. The time course of the experiment indicated that GTP hydrolysis was not a factor in the partial protection. However, Gα1Q204L was protected from trypsin digest in the presence of GTP to a greater extent than was Gα1Q205L (27).

Implications for the G Protein GTPase Cycle—Based on the classical model of steady state G protein activation by a hydrolyzable nucleotide (34) in which binding and activation are considered to occur simultaneously, the fractional activation, Gα* mGTP/Gtotal, would equal kcat/koff + kcat. In this equation koff is the GDP dissociation rate, and kcat is the rate of hydrolysis of bound GTP. If we assume the fluorescence signal represents Gα* mGTP, then when decreasing the G protein hydrolysis rate by a factor of 10 as seen with Gα1Q204L, one should see a 5-fold increase of activated G protein (Gα* mGTP) from 0.1 to 0.5. Since we observed an increase only from 0.1 to 0.2 of maximum mGTPγS fluorescence, we conclude that mGTP, like mGpp(CH2)p and mGpp(NH)p, is unable to fully activate G protein even with the reduced GTPase activity of the Q204L mutant.

We pointed out previously (17) that estimates of kcat from “single turnover” experiments (26, 27) may overestimate the true value of the rate constant of GTP hydrolysis. The actual value determined in such experiments is kcat + k1. The fact that the amount of Pi released is less than the amount of GTPγS bound indicates that the dissociation term k1 is not negligible as previously assumed. If kcat is not as large as previously estimated, then some other factor (i.e., the activation/deactivation equilibrium) is required to account for the low degree of steady state activation of G proteins by GTP.

The proposal that GTP may be a partial agonist regarding G protein activation suggests a novel role of agonist-ligated receptor. In addition to stimulating GDP release from G protein, the receptor may also shift the activation/deactivation equilibrium toward activated G protein when GTP is bound. These fluorescent GTP analogs are novel tools to discern guanine nucleotide binding from activation. In future experiments they will permit us to provide estimates of the kinetics of these processes and to assess the role of receptor, βγ, and effector in regulation of the G protein activation/deactivation conformational equilibrium.

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