The GTPase activity of the stimulatory guanine nucleotide-binding regulatory protein (G,) of hormone-sensitive adenylate cyclase was investigated using purified rabbit hepatic G, and either [a-32P]- or [y-32P] GTP as substrate. The binding of [35S]guanosine 5'-O-(thiotriphosphate) (GTPyS) was used to quantitate the total concentration of G,.

1) GTPase activity was a saturable function of the concentration of GTP, with K_m = 0.3 μM. MgCl_2 monotonically increased the activity. The maximum observed turnover number was about 1.5 min^{-1}.

2) The rate of steady-state hydrolysis of GTPyS to G,GDP, GTP, and GDP is now known to be a distinct GTPase reaction associated with the control of adenylate cyclase (16). We recently demonstrated that G, can catalyze the GTPase reaction associated with the control of adenylate cyclase (16). If both G, and β-adrenergic receptors are co-reconstituted into phospholipid vesicles, the GTPase activity can be stimulated up to 15-fold by β-adrenergic agonists. A prior report (8) that pure Lubrol-solubilized G, was not a GTPase reflected the inhibition of its GTPase activity by that detergent (16). The isolated α subunit, when bound to GTPyS and thereby activated, can stimulate the activity of adenylate cyclase in the absence of the other subunits (see Refs. 15 and 10 for review).

3) The rate of formation of G,GDP and the initial GTPase rate varied in parallel as functions of the concentrations of either GTP or MgCl_2 (above 3 mM EDTA in the absence of added Mg_2^+). During steady-state hydrolysis, GDP can be trapped as a G,GDP complex and inhibition of product release may account for the inhibition of steady-state hydrolysis.

4) The rate of dissociation of assayable G,GTP was biphasic. The initial phase accounted for 20-40% of total assayable G,GTP and was characterized by a t_{1/2} of about 1 min. Lubrol 12A9 potently inhibited the GTPase reaction and the dissociation of G,GDP in parallel, and inhibition of product release may account for the inhibition of steady-state hydrolysis.

5) The β and γ subunits of G, markedly inhibited the dissociation of GDP from G, in contrast to their ability to stimulate the dissociation of GTPyS.

6) GDp, GTPγS, and guanyl-5'-yl imidodiphosphate (Gpp(NH)p) competitively inhibited the accumulation of G,GDP. GTPγS and Gpp(NH)p inhibited the GTPase reaction noncompetitively, GDP displayed mixed inhibition, and Pi did not inhibit. These data are interpretable in terms of the coexistence of two specific mechanistic pathways for the overall GTPase reaction.

Hormonal regulation of adenylate cyclase displays an absolute requirement for GTP or other closely related guanine nucleotides (1). In addition, nonhydrolyzable analogues of GTP, such as GTPγS or Gpp(NH)p, cause the persistent activation of adenylate cyclase. These and other observations led to the hypothesis that the hydrolysis of GTP is involved in the hormonal regulation of the enzyme (see Ref. 2 for review). This hypothesis was supported by the discovery by Cassel and Selinger (3) of a β-adrenergic catecholamine-stimulated GTPase activity having many properties consistent with its involvement in the control of adenylate cyclase. These authors presented data suggesting that the binding of GTP to the enzyme stimulates adenylate cyclase activity and that hydrolysis of GTP to GDP leads to deactivation. Agonist was proposed to stimulate GTPase and adenylate cyclase by increasing the rate of release of GDP, thereby allowing a new molecule of GTP to bind (4-6). While the primary importance of the release of GDP has been questioned (7-9), the importance of the GTPase cycle in the control of adenylate cyclase is now well established (see Ref. 10 for review).

The site of stimulatory regulation of adenylate cyclase by guanine nucleotides is now known to be a distinct GTP-binding regulatory protein, G,, that acts as a coupling factor between hormone receptors and the catalytic unit of the cyclase (2, 10, 11). G, has been purified from several tissues (see Ref. 10). It is a heterotrimer that consists of a GTP-binding α subunit (M_r = 45,000 or 52,000) (8), a β subunit (M_r = 35,000) that regulates the binding of nucleotides to α (12), and a γ subunit (M_r = 8,000) of unknown function (13, 14). The isolated α subunit, when bound to GTPyS and thereby activated, can stimulate the activity of adenylate cyclase in the absence of the other subunits (see Refs. 15 and 10 for review).

We recently demonstrated that G, can catalyze the GTPase reaction associated with the control of adenylate cyclase (16). If both G, and β-adrenergic receptors are co-reconstituted into phospholipid vesicles, the GTPase activity can be stimulated up to 15-fold by β-adrenergic agonists. A prior report (8) that pure Lubrol-solubilized G, was not a GTPase reflected the inhibition of its GTPase activity by that detergent (16).

The maximum hormone-stimulated rate of G,-catalyzed GTPase is quite low, with a turnover number of <2 min^{-1} either in the vesicles (16) or in plasma membranes (3). Because the binding of GTPγS by G, is also slow and is similarly regulated (8, 16-18), we suggested that the binding of GTP by G, might be the rate-limiting step in its hydrolysis. Identification of the rate-limiting step (or steps) in the GTPase cycle is important because the rate of the individual steps in the cycle is critical for the overall regulation of adenylate cyclase (10, 16). A key step in the regulation of adenylate cyclase is the binding of hormone to the hormone receptor, a step that is essential for activation of adenylate cyclase (4-6). The maximum hormone-stimulated rate of G,-catalyzed activation of adenylate cyclase in the absence of the other subunits (see Refs. 15 and 10 for review).

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the cycle will regulate the steady-state concentration of the activated species, G-GTP. Furthermore, while numerous studies have investigated the activation of GTPase activity, the effect of nucleotide analogs of GTP on the activation of Gs has not yet been characterized. In the present study, we have used Gs alone to initiate the characterization of its GTPase activity and to investigate the turnover of Gs-nucleotide intermediates. We have also examined the inhibition of hydrolytic activity by Lubrol. When the reaction was quenched in 0.0 ml of an ice-cold suspension of Norit A (5%, w/v) in 50 mM NaH2PO4, the mixture was centrifuged (1500 rpm, 10 min), and an additional 0.5 ml aliquot of the Norit A suspension was added. After a second centrifugation, the 3P in 0.6 ml of the supernatant was determined by Cerenkov counting. For some experiments, GTPase activities are expressed as turnover numbers, which normalize the activity to the total amount of [32P]GTP-S binding present in the assay.

The stability of Gs and, hence, the linearity of the time course of the GTPase reaction varied with the concentrations of Mg2+ and guanine nucleotides. Stability was not altered by the addition of Lubrol up to 0.1%. For all experiments shown, preliminary assays were performed to ensure the measurement of the initial rate. We also monitored the possible denaturation of Gs by assaying its ability to activate the catalytic unit of adenylate cyclase in plasma membranes from cyc-S49 lymphoma cells after its activation by GTPyS (19). In the GTPase reaction mixture containing 10 mM MgCl2 and in the absence of guanine nucleotides, at least 70% of the total Gs remained active for 20 min.

RESULTS

Steady-state GTPase Activity—Gs displays specific GTPase activity if it is separated from excess detergent or reincorporated into phospholipid vesicles (16). When the GTPase reaction was initiated in detergent-free medium by the addition of Gs, there was no perceptible lag, and the rate of hydrolysis was constant for over 20 min (Fig. 1). The specific GTPase activity was constant at concentrations of Gs between 5 and 50 nM. Higher concentrations of Gs displayed a reduced specific activity of GTPase because of the presence of Lubrol 12A9, the detergent in which Gs is stored and which inhibits hydrolysis of GTP by Gs.

FIG. 1. Time courses for the binding of [32P]GTPyS and [32P]GTP to Gs and for the hydrolysis of [32P]GTP. Gs (750 fmol) was incubated at 30 °C for the times shown in the assay medium described under "Experimental Procedures" and containing 10 mM MgCl2 and GTP (1 μM each). Binding of [32P]GTPyS (C) and [32P]GTP (D) represents means of duplicate samples. The binding of [32P]GTPyS did not reach a plateau until 45 min. GTPase activity (E) represents means of triplicate samples. The standard deviations of the GTPase data were less than 5% of the means.
its GTPase activity (see below).

Under identical assay conditions, \[^{35}S\]GTP\(_{\gamma}S\) binds quasi-
irreversibly to G\(_p\), causing its stable activation (8, 17). At 1 
\(\mu\)M \[^{35}S\]GTP\(_{\gamma}S\) and 10 mM MgCl\(_2\), binding continued to 
increase over 20 min (Fig. 1) and did not reach a plateau until 
45 min (not shown). This concentration of GTP\(_{\gamma}S\) was well 
above its apparent \(K_d\) of 0.1 \(\mu\)M, which is 4-10-fold lower 
than the \(K_d\) originally observed in Lubrol solution (Refs. 8 
and 17; confirmed in this study). Using the total amount of 
[^{35}S]GTP\(_{\gamma}S\) bound to estimate the molar concentration of G\(_p\) 
(16, 17), the molar turnover number of the GTPase reaction 
was calculated to be 0.41 mol of GTP hydrolyzed/mol of G\(_p\) 
per min under these conditions. Turnover numbers above 1.5 
min\(^{-1}\) have been observed at higher concentrations of Mg\(^{2+}\) 
and GTP (see Fig. 4).

Hydrolysis of G\(_p\)-bound GTP to GDP—When \[^{32}P\]GTP 
was incubated with G\(_p\), under identical conditions, stable 
specific binding of \[^{32}P\] to G\(_p\) was also observed (Fig. 1). The molar 
amount of nucleotide bound was typically 20-40% of that 
observed using \[^{35}S\]GTP\(_{\gamma}S\) under the same conditions (Fig. 1) 
and 30-50% of the \[^{35}S\]GTP\(_{\gamma}S\) bound at 50 mM MgCl\(_2\) 
(see Table I). The chemical identity of the bound radioactivity 
was examined by thin-layer ion-exchange chromatography of 
the material that was trapped on the nitrocellulose filter (see 
"Experimental Procedures"). When G\(_p\) was incubated with 
[^{32}P\]GTP under a wide range of conditions that allow 
steady-state GTPase activity, the bound ligand was found to 
be essentially all GDP (Table I). Thus, we will refer below to 
the material that was trapped on the nitrocellulose filter (see 
Fig. 3). The reaction was chilled to 0 °C and 
quenched, G\(_p\)-GDP was detected, and its conversion at 
50 min (not shown). This concentration of MgCl\(_2\) 
was either zero ( ), 10 mM ( ), or 10 mM ( ) and that of GTP was 1 \(\mu\)M. 
Binding of \[^{32}P\]GTP ( ), \[^{35}S\]GTP\(_{\gamma}S\) ( ) and \[^{32}P\]GTP ( ) represents 
the average of duplicate samples.

**Table I**

Identity of nucleotide bound to G\(_p\).

G\(_p\) (4.2 pmol) was incubated for the indicated times at 30 °C in 
assay medium plus the concentrations of Mg\(^{2+}\) and Lubrol 12A9 listed 
below. The reactions were terminated by immediate filtration at 0 °C, 
and bound nucleotide was identified as described under "Experimental 
Procedures." "Other" refers to the sum of \(^{32}P\) in areas of the 
chromatography plate not associated with GTP or GDP.

<table>
<thead>
<tr>
<th>[MgCl(_2)]</th>
<th>[Lubrol]</th>
<th>Time</th>
<th>Bound nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>mg/ml</td>
<td>min</td>
<td>GTP</td>
</tr>
<tr>
<td>50</td>
<td>0.013</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
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<td>7</td>
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<td>0</td>
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<td>2</td>
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</tr>
<tr>
<td>0</td>
<td>0.013</td>
<td>10</td>
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</tr>
<tr>
<td>0</td>
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<td>2</td>
<td>33</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>10</td>
<td>28</td>
</tr>
</tbody>
</table>

**Fig. 2.** Time courses for the binding of \[^{32}P\] and \[^{35}S\]GTP 
to G\(_p\). G\(_p\) (750 fmol) was incubated in the standard assay 
mixture at 30 °C for the times shown. The concentration of MgCl\(_2\) 
was either zero ( ), 10 mM ( ), or 10 mM ( ) and that of GTP was 1 \(\mu\)M. 
Binding of \[^{32}P\]GTP ( ), \[^{35}S\]GTP\(_{\gamma}S\) ( ) and \[^{32}P\]GTP ( ) represents 
the average of duplicate samples.

**Fig. 3.** Onset of the inhibition of \[^{32}P\]Pi release from \[^{35}S\]GTP 
by added unlabeled GTP. G\(_p\) was incubated at 30 °C with 1 
\(\mu\)M \[^{32}P\]GTP\(_{\gamma}S\) and 10 mM MgCl\(_2\). At 0, 2, and 4 min, 50-\(\mu\)l aliquots (750 fmol of G\(_p\) each) were assayed for \[^{32}P\]Pi release ( ). At 5 min, 
the reaction was divided into 2 volumes. To one, 0.01 volume of 0.1 
M GTP was added at 6 min ( ). To the other, 0.01 volume of water 
was added at 6 min ( ). \[^{32}P\]Pi release was measured for each sample 
thereafter. Each time point represents triplicate determinations, with 
the standard deviation indicated by error bars.

The turnover rate of the GTPase reaction, we attempted to follow the hydrolysis of \[^{32}P\]GTP\(_{\gamma}S\) bound to G\(_p\), the presence of 10 mM Mg\(^{2+}\). 
At 30 °C, we were unable to detect a G\(_p\)-GTP complex by manual 
filtration. However, if the reaction was chilled to 0 °C and 
quenched, G\(_p\)-GTP was detected, and its conversion at 0 °C 
to G\(_p\)-GDP proceeded with a half-life of about 1 min (not shown).

Hydrolysis of G\(_p\)-GTP to G\(_p\)-GDP was dependent on the presence of Mg\(^{2+}\) or other divalent cations (Mn\(^{2+}\) for example) 
at low concentrations. If the concentration of free divalent
cation was decreased from 1 mM to below the micromolar level by the addition of EDTA; GTP hydrolysis was prevented, although the binding of $[^{32}P]GTP$ to G, was not markedly inhibited (Fig. 4). Thus, the assayable binding of $[^{32}P]GTP$ and of $[^{32}P]GTP$ to G, was similar in the absence of MgCl$_2$.

While we have not carefully measured the dependence of GTP hydrolysis on the concentration of Mg$^{2+}$, we found that hydrolysis occurs readily in the presence of 0.5 mM MgCl$_2$, 1 mM EDTA, and 1 mM GTP at pH 8. Using the equations and constants summarized by Fabiato and Fabiato (22), these concentrations yield a calculated concentration of free Mg$^{2+}$ of 0.5 μM, which is about 10 times the $K_D$ of the Mg$^{2+}$-GTP dissociation reaction under these assay conditions. It is thus plausible that the requirement for free Mg$^{2+}$ for GTP hydrolysis reflects the need to form the Me-GTP complex rather than the binding of Mg$^{2+}$ directly to G,.

In the experiment shown in Fig. 1, the calculated GTPase turnover number, 0.41 min$^{-1}$, was the same as the apparent first-order rate constant for the binding of $[^{32}P]GTP$, 0.43 min$^{-1}$, calculated according to Asano and Ross (18). The similarity of the turnover number and the association rate constant initially suggested that the low observed GTPase activity might be limited by the rate of binding of nucleotide to G, as we proposed previously (16). To test this possibility further, we investigated the stimulation of GTP binding and of GTPase activity by Mg$^{2+}$. The initial rate of both high-affinity $[^{32}P]GTP$ binding and GTPase activity were stimulated in parallel by increasing concentrations of Mg$^{2+}$ (Fig. 4), and the ratio of the initial rates of binding and of GTPase activity was constant between 1 mM and 100 mM total MgCl$_2$. Thus, nucleotide binding and GTPase activity appeared to be well correlated. However, we have consistently noted that the actual activity of the GTPase was about 3 times greater than the initial rate of high-affinity binding of $[^{32}P]GTP$, and this discrepancy has been observed using multiple G, preparations and in assays performed at varying concentrations of GTP and Mg$^{2+}$. It is discussed in more detail below.

**Dissociation of the GDP Product—** The isolation of G,-GDP after the binding of $[^{32}P]GTP$ indicated that the dissociation of the GDP product from G, was significantly slower than the hydrolysis of GTP and release of Pi. This also suggests why the binding of GTP (measured using $[^{32}P]GTP$) approaches steady state more rapidly than does the binding of GDP (measured using $[^{32}P]GTP$, Fig. 2). In Fig. 5, the dissociation of bound GDP from G, was measured directly. GDP dissociated with an initial $t_{1/2}$ of about 1.0 min; dissociation was biphasic, whether measured at 10 or 50 mM Mg$^{2+}$. The fraction of bound $[^{32}P]GDP$ that dissociated rapidly was usually about two-thirds of the total amount bound. The rate of the initial dissociation process varied up to 50% and the relative extent of the initial phase varied up to 30% among different preparations of G, but both were quite reproducible among multiple experiments using a single preparation.

We reported previously that Lubrol 12A9 inhibits the GTPase activity of G, (16). This inhibition is probably why the GTPase activity of G, was not observed in earlier studies (8) and is the reason why the above experiments were performed at very-low concentrations of detergent. To determine at which point in the catalytic cycle Lubrol impinges on GTPase activity, we investigated the influence of increasing concentrations of Lubrol on various segments of the GTPase reaction. The results are shown in Fig. 6. Inhibition of GTPase activity was significant at or below the critical micelle concentration of the detergent (23). In parallel, Lubrol lowered the rate of dissociation of bound GDP from G, suggesting that Lubrol inhibited steady-state GTPase activity primarily by inhibiting the rate of release of GDP. In contrast, Lubrol increased the initial rate of binding of $[^{32}P]GTP$ to G, by 30–50% (Fig. 6) and decreased, by 20–30%, the extent of total assayable $[^{32}P]GTP$ binding (i.e. bound GDP, not shown). There was no significant influence of Lubrol on the fractions of bound nucleotide that were present as GTP or GDP (Table I). Relatively low concentrations of Lubrol clearly inhibited GTPase activity and the dissociation of GDP at either 10 or

![Fig. 4. Influence of Mg$^{2+}$ on the initial rate of $[^{32}P]GTP$ binding to G, and GTPase activity.](image)

**Fig. 4.** Influence of Mg$^{2+}$ on the initial rate of $[^{32}P]GTP$ binding to G, and GTPase activity. G, (750 fmol) was incubated at 30 °C with 1 mM GTP labeled with $^{32}P$ at the $\alpha$ or $\gamma$ position and the free concentration of Mg$^{2+}$ indicated. The initial rate of binding of $[^{32}P]GTP$ (•) was determined in duplicate, and GTPase activity (○) was determined in triplicate from the initial linear regions of their reaction time courses (see Fig. 1). The inset shows the ratio of the initial rate of binding to the GTPase activity plotted against the concentration of free Mg$^{2+}$, with error bars representing the standard deviation. The value of this ratio in the absence of Mg$^{2+}$ was calculated to be 10, but its precision is unreliable due to the very low GTPase activity.

![Fig. 5. Dissociation of bound $[^{32}P]GDP$ from G,](image)

**Fig. 5.** Dissociation of bound $[^{32}P]GDP$ from G,. G, was incubated at 30 °C with 1 μM $[^{32}P]GTP$ and 10 mM MgCl$_2$. At 0, 1, and 2 min, 50-μl aliquots (750 fmol of G,) were assayed for bound $^{32}P$ (•) as described under "Experimental Procedures." At 2.5 min, the reaction was divided into 2 volumes. To one, 0.01 volume of 0.1 M GTP was added at 3 min (○). To the other, 0.01 volume of water was added at 3 min (●). Each time point represents the average of duplicate determinations.
of G, are more tightly associated. Complete inhibition at 10 mM MgCl₂, where the α and βγ subunits contributed additional Lubrol 1,2A9, a control was included which contained Gb plus an equivalent volume of Hepes (pH 8.0), and 1 mM EDTA was added to each aliquot. The buffer included either a 10-fold molar excess of heat-denatured (100 °C, 20 min) βγ subunits (O), a 10-fold molar excess of active βγ subunits (O), or no other addition (A). The final concentration of Lubrol when excess βγ subunits were added was 100 μg/ml; when GTP buffer alone was added, the final concentration was 9 μg/ml. Aliquots assayed at 3 min (50 μl) contained 750 fmol of total G, and 210 fmol of [α-32P]GTP binding activity. Subsequent to the 3-min time point, aliquot volumes were increased by 10% to maintain a constant amount of G, Each time point represents the average of duplicate determinations.

50 mM MgCl₂. However, more Lubrol was required for complete inhibition at 10 mM MgCl₂, where the α and βγ subunits of G, are more tightly associated.

The binding of GTPγS to the α subunit of G, is tightly coupled to the dissociation of its β and γ subunits (8, 12). Accordingly, the addition of excess βγ subunit promotes the dissociation of that nucleotide from the α subunit. The data of Fig. 7 indicate that the βγ subunits have the opposite effect on the release of GDP from G, In this experiment, [α-32P]GTP was bound to G, (αβγ), and the initial phase of dissociation of GDP was measured with and without the addition of a large excess of βγ subunits. Since the preparation of βγ subunits contributed additional Lubrol 1,2A9, a control was also included which contained Gb plus an equivalent volume of the denatured βγ subunit preparation. In this experiment, excess βγ subunit inhibited the dissociation of GDP at least 4-fold relative to the denatured βγ control. The inhibition of the dissociation of GDP shown here appeared to be maximal and, under our standard assay conditions, inhibition saturated as a 10-fold molar excess of βγ was approached (not shown).

The stabilization of the binding of GDP to G, by excess βγ, in contrast to its ability to destabilize the binding of GTPγS, suggests that βγ and GDP may both bind preferentially to the inactive conformation of α. It is certainly inconsistent with the generalization that the binding of βγ favors the release of all guanine nucleotides from G,.

Inhibition of GTPase Activity and [α-32P]GTP Binding by Guanine Nucleotides—In order to correlate the turnover of individual enzyme-nucleotide intermediates with the steady-state rate of the GTPase reaction, we investigated the inhibition of GTPase activity and of the rate of binding of [α-32P]GTP to G, by other guanine nucleotides at varying concentrations of GTP. Both GTPase activity and the initial rate of [α-32P]GTP binding saturated as functions of the concentration of GTP in the presence or absence of other nucleotides, and double-reciprocal plots of the data were linear. The data from these experiments are summarized in Table II. GDP, Gpp(NH)p, and GTPγS each inhibited both the initial rate of [α-32P]GTP binding by each nucleotide was competitive, as might be expected from the rapid onset of inhibition displayed in Fig. 3. Each nucleotide inhibited GTP binding with somewhat greater potency (lower calculated Kᵢ) than that observed when the inhibition of [35S]GTPγS binding was measured (8, 17).

In contrast to these data, Gpp(NH)p and GTPγS were non-competitive inhibitors of steady-state GTPase activity, and GDP displayed mixed inhibition. Despite the different modes of inhibition that were detected when GTPase was assayed, the inhibitory potencies of the nucleotides were similar in either assay (Table II). Some interpretation of these results is offered under “Discussion.” Inorganic orthophosphate, at concentrations up to 5 mM, did not inhibit either the steady-state hydrolysis of GTP or the rate of binding of [α-32P]GTP, as is consistent with the ordered release mechanism proposed above.

**DISCUSSION**

These studies represent an initial characterization of the GTPase cycle of G, A detailed understanding of each step in
GTPase activity and $[^{32}P]GTP$ binding were determined at 2 min (initial rates) in assay medium containing 50 mM MgCl$_2$, 0.1–10 $\mu$M GTP (6 concentrations), and either no other nucleotide or fixed concentrations of GDP, Gpp(NH)p, or GTP-S. Binding assays were performed in duplicate, and GTPase assays were performed in triplicate. Double-reciprocal plots (1/B or 1/v versus 1/[GTP]) of the data were linear and were fitted using unweighted least-squares analysis. Data obtained in the absence of inhibitors were used to determine values for $K_d$ (GTPase) and $K_i$ (binding). The slopes of the double-reciprocal plots obtained in the absence of inhibitor and in the presence of two concentrations of each inhibitor were plotted versus the inhibitor concentration to obtain values for $K_i$ (25). The numbers given in parentheses ($n$) are the number of complete experiments that were performed to obtain each constant. For $n \geq 3$, the values of each constant are means ± the standard deviation of the mean. For $n = 2$, the range of values obtained is given.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Initial rate of binding ($K_d$ or $K_i$)</th>
<th>GTPase ($K_m$ or $K_a$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>0.33 ± 0.07 ($n = 5$)</td>
<td>0.31 ± 0.07 ($n = 8$)</td>
</tr>
<tr>
<td>GTPyS</td>
<td>0.02–0.04 ($n = 2$)</td>
<td>0.04–0.06 ($n = 2$)</td>
</tr>
<tr>
<td>Gpp(NH)p</td>
<td>0.09–0.49 ($n = 2$)</td>
<td>0.21 ± 0.11 ($n = 3$)</td>
</tr>
<tr>
<td>GDP</td>
<td>0.24–0.65 ($n = 2$)</td>
<td>0.50 ± 0.13 ($n = 5$)</td>
</tr>
</tbody>
</table>

The numbers given in parentheses ($n$) are the number of complete experiments that were performed to obtain each constant. For $n \geq 3$, the values of each constant are means ± the standard deviation of the mean. For $n = 2$, the range of values obtained is given.

GTPase Cycle of $G_s$

The finding that the rate of the overall GTPase reaction is 3- to 4-fold higher than the initial rate of binding of $[^{32}P]GTP$ suggests that the high-affinity binding of nucleotide may not be rate limiting for the GTPase cycle. However, the constant ratio of the binding rate to the hydrolytic rate as a function of Mg$^{2+}$ concentration, the slow rate of both these reactions, and their similar apparent $K_d$ and $K_m$ for GTP all suggest that their rates are limited by a common step (Reac-

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D. R. Brandt, unpublished data.
of an alternative pathway for the GTPase, via G_{\gamma}-GDP, that does not include a high-affinity G$_G$-GDP species.

The stabilization of the G$_G$-GDP complex by excess $\beta \gamma$ subunit (Fig. 7) is opposite to the destabilization of the active G$_G$-GTP$_\gamma$S species by $\beta \gamma$ that has been described by Gilman's group (8, 12, 15). It is plausible that $\beta \gamma$ and GDP both bind preferentially (and, thus, cooperatively) to the inactive conformation of the $\alpha$ subunit, whereas GTP$_\gamma$S binds in some other unknown way. We have no data on the effects of $\beta \gamma$ on the equilibrium binding of GDP. Given the data discussed above, however, it is tempting to propose that the binding and release of $\beta \gamma$ are the determinants of whether the GTPase reaction proceeds slowly (via Reactions 3 and 4) or quickly (via reactions 3' and 4'). When GTPase activity is measured under the conditions used in the experiment shown in Fig. 7, the addition of excess $\beta \gamma$ decreases the rate more than 50%. In this same vein, it is also plausible that Lubrol exerts its effects on the GTPase cycle by enhancing the binding of $\beta \gamma$ to favor the slower pathway, either directly or by preventing aggregation that might decrease the amount of available $\beta \gamma$.

The patterns of inhibition of the GTPase and accumulation of bound GDP are not inconsistent with the above scheme. Competitive inhibition of binding by all the guanine nucleotides tested probably reflects competition for binding at the initial stage of the GTPase cycle (Reaction 1). Noncompetitive inhibition of overall GTPase activity by GTP$_\gamma$S or Gpp(NH)p could reflect the accumulation of a stable $\alpha$-subunit-nucleotide complex, equivalent to G$_G^{\alpha \gamma}$-GTP of Fig. 8 (8, 12). The mixed-type inhibition exerted by GDP cannot be completely rationalized, but its noncompetitive component might also reflect accumulation of GDP bound to the $\alpha \beta \gamma$ trimer form of G$_G$.

We feel that our data provide a reasonable, but still incomplete, description of the intermediate steps of the G$_G$-catalyzed GTPase cycle. Eventually, detailed kinetic modeling of these steps should be possible. Preliminary calculations using our current data indicate that the rate constants for both the hydrolysis of GTP and the dissociation of GDP are accurately described by a simplified form of the reactions of Fig. 8. Several obvious questions remain. What is the relationship of the high-affinity binding of nucleotide and the onset of hydrolysis, i.e. why is the "on" rate for [$\alpha^{32}$P]GTP less than the GTPase rate? What are the detailed effects of the $\beta \gamma$ subunits of G$_G$ on each step in the cycle? This must be approached using known mixtures of resolved $\alpha$ and $\beta \gamma$ subunits in a medium where detergent or phospholipid is held constant. What effect does detergent exert on a molecular level and why does phospholipid permit the rapid hydrolysis of GTP, as we showed previously (16)? Last, what are the roles of hormone receptors and the catalytic unit of adenylate cyclase? The ability to combine G$_G$, (16-18) or G$_i$ (24) with purified $\beta\gamma$-adrenergic receptors should allow us to approach these questions quickly.

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