Gz, a Guanine Nucleotide-binding Protein with Unique Biochemical Properties

(Received for publication, August 9, 1980)

Patrick J. Casey†, Henry K. W. Fong§, Melvin I. Simon¶, and Alfred G. Gilman**

From the †Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235, the §Departments of Ophthalmology and Microbiology, University of Southern California School of Medicine, Los Angeles, California 90033, and the ¶Department of Biology, California Institute of Technology, Pasadena, California 91125

Cloning of a complementary DNA (cDNA) for Gz, a newly appreciated member of the family of guanine nucleotide-binding regulatory proteins (G proteins), has allowed preparation of specific antisera to identify the protein in tissues and to assay it during purification from bovine brain. Additionally, expression of the cDNA in Escherichia coli has resulted in the production and purification of the recombinant protein.

Purification of Gz from bovine brain is tedious, and only small quantities of protein have been obtained. The protein copurifies with the βγ subunit complex common to other G proteins; another 26-kDa GTP-binding protein is also present in these preparations. The purified protein could not serve as a substrate for NAD-dependent ADP-ribosylation catalyzed by either pertussis toxin or cholera toxin.

Purification of recombinant Gz, (rGz) from E. coli is simple, and quantities of homogeneous protein sufficient for biochemical analysis are obtained. Purified rGz, has several properties that distinguish it from other G protein α subunit polypeptides. These include a very slow rate of guanine nucleotide exchange (kcat = 0.02 min⁻¹), which is reduced 20-fold in the presence of mM concentrations of Mg²⁺. In addition, the rate of the intrinsic GTPase activity of Gz, is extremely slow. The hydrolysis rate (kcat) for rGz, at 30 °C is 0.05 min⁻¹, or 200-fold slower than that determined for other G protein α subunits. rGz, can interact with bovine brain βγ but does not serve as a substrate for ADP-ribosylation catalyzed by either pertussis toxin or cholera toxin. These studies suggest that Gz may play a role in signal transduction pathways that are mechanistically distinct from those controlled by the other members of the G protein family.

Guanine nucleotide-binding regulatory proteins (G pro-

† Supported by the Jules and Doris Stein Research Fund to Prevent Blindness.
‡ Supported by the Raymond and Ellen Willie Chair of Molecular Neuropharmacology, the Perot Family Foundation, and The Lucille P. Markey Charitable Trust.

1 The abbreviations used are: G proteins, guanine nucleotide-binding regulatory proteins; Gz, and Gx, G proteins that mediate stimulation and inhibition of adenyl cyclase, respectively; Gz, a G protein abundant in brain; Gx, the predominant G protein in retina; Gα, a G protein of unknown function; Gα, example of nomenclature to designate the α subunit of Gz; rGz, recombinant Gz; GTPyS, guanosine 5’-3-O-(thio)triphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid; DTT, dithiothreitol.
pholipase C in several tissues (15, 16) and regulation of certain ion channels (17). These observations have prompted speculation on the existence of G proteins that are refractory to modification by these bacterial toxins.

Fong and colleagues (18) and Matsuno and associates (19) recently isolated a cDNA that encodes a unique G protein α subunit (termed G or G). RNA blot hybridization analysis showed that the gene for this α subunit is transcribed predominantly in neural tissues. The deduced amino acid sequence of the protein resembles that of the Gα subunits, but it has differences in two particularly notable regions. First, the cysteine residue near the carboxyl terminus that serves as the site for pertussis toxin-catalyzed ADP-ribosylation is not present. Thus, this protein is a candidate for involvement in the GDP-dependent pertussis toxin-insensitive events noted above. Second, 3 amino acid residues in the first portion of the guanine nucleotide-binding domain differ from those that are strictly conserved in all other known α subunits. Structural studies on the GDP-binding proteins elongation factor Tu (20) and ras (21, 22) suggest that this region is involved in the catalytic step of GTP hydrolysis, and mutations in this region of ras (23) and Gα (24) cause significant changes in the rate of nucleotide hydrolysis. We undertook purification of this protein in order to determine its properties and to search for its functions.

**Experimental Procedures**

Preparation of Membranes, G Proteins, and Subunits—Bovine brain membranes were prepared as described by Sternweis and Robishaw (25) and stored at a protein concentration of 20 mg/ml at -70°C until use. Membranes from other tissues were prepared as described (26). Bovine brain Gα proteins (25) and their resolved α and β subunits (27, 28) were purified by established procedures. The 52-kDa form of recombinant Gα (Gαγ) purified after expression in E. coli (10), was a gift of Dr. Michael Freissmuth (University of Vienna). The 52-kDa form of recombinant Gα (Gαγ), purified after expression in E. coli (10), was a gift of Dr. Michael Freissmuth (University of Vienna).

Production of Antisera—Peptides to be utilized as antigens (see Table I) were synthesized according to amino acid sequences deduced from the human Gαα cDNA (18). The peptides were cross-linked to keyhole limpet hemocyanin (Sigma) with m-maleimidobenzoyl N-hydroxysuccinimide ester (29). Rabbits were immunized with the peptide-protein conjugates as described previously (30). Rabbits developed antibody titers to peptide and Gα protein as assayed by enzyme-linked immunoassay and immunoblotting, respectively. Antibodies to peptide P-961 were purified from the antisera by affinity chromatography using a column of peptide (3 mg) coupled to CNBr-activated Sepharose 4MB (1 ml, Pharmacia LKB Biotechnology Inc.) as described (26). Immunobots were processed as described previously (30) except that 125I-labeled goat anti-rabbit IgG Fab(‘) fragments (Du Pont-New England Nuclear) were used as the secondary antibody. Antisera A-569 and U-49 were a gift of Dr. Susanne Mumba (University of Texas Southwestern Medical Center) (30).

Purification of Gα from Bovine Brain—Bovine brain membranes (12 g of protein) were washed and extracted with 1% sodium cholate to facilitate coupling to keyhole limpet hemocyanin. Specificity of reactivity was determined by immunoblotting using the indicated purified proteins. NT, not tested. Number of + denotes intensity of response; — denotes no reaction.

<table>
<thead>
<tr>
<th>Peptide</th>
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<th>α, αα, ααα, αααα</th>
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<td>CRRSSSEEEKARRSRR</td>
<td>Gα3</td>
<td>++</td>
<td>CRSSSEEEKARRSRR</td>
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<td>P-961</td>
<td>++</td>
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<td>CTPAESKEITPEL</td>
<td>Gα3</td>
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**Table 1**

Gαα sequences used for generation of antipeptide antisera

Peptides were synthesized with a cysteine at the amino terminus to facilitate coupling to keyhole limpet hemocyanin. Specificity of reactivity was determined by immunoblotting using the indicated purified proteins. NT, not tested. Number of + denotes intensity of response; — denotes no reaction.

**Properties of Gα**

Gαα sequences used for generation of antipeptide antisera

Peptides were synthesized with a cysteine at the amino terminus to facilitate coupling to keyhole limpet hemocyanin. Specificity of reactivity was determined by immunoblotting using the indicated purified proteins. NT, not tested. Number of + denotes intensity of response; — denotes no reaction.

Sequence Designation α, αα, ααα, αααα | CRSSSEEEKARRSRR | CRSSSEEEKARRSRR | P-961 | GTSNKSITVQMK | CTPAESKEITPEL |
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**Figure 1.** SDS-polyacrylamide gel electrophoresis and immunoblot analysis of purified bovine brain Gα. Each panel consists of two lanes in which 150 ng each of either Gα or Gβ α purified from bovine brain was resolved on 15% polyacrylamide gels. The left panel is a silver stain of the proteins; the γ subunit ran with the dye front on this gel and is not visualized. The remaining panels are immunoblots of the indicated antisera. Antisera A-569 and Gαααα were raised to peptide sequences unique to Gα (see Table 1); A-569 is a "common" α antisemur (30) that recognizes all G protein α subunits except that of Gαα and U-49 is an anti-β antisemur (30).
concentrations to 0.2 ml. The final preparation was stored at -70°C.

A summary of the purifications is shown in Tables II and III.

Construction of G, Expression Vector—Plasmid PBS-Gm, which contains the complete coding sequence of human Gm (18), was si-
multaneously cleaved with NcoI and PstI, and the insert was purified by agarose gel electrophoresis. The NcoI-PstI fragment was ligated with plasmid pNT7-5 (18) after digestion of that plasmid with the same two restriction enzymes. Transformation of E. coli strain BL-
21/DE3 (31) resulted in the isopropyl-β-D-thiogalactopyranoside-inducible expres-
sion of immunoreactive recombinant Gm (rGm) in these cells. E. coli strain BL-21/DE3 is a T7 system in which the gene for the T7 polymerase is driven by the lac UV-5 promoter.

Purification of rGm—One hundred liters of LB medium was inoc-
ulated with BL-21/DE3 harboring plasmid pNT7-5/Gm and incubated in a fermenter at 30°C to an OD of 1.5. Expression of rGm was induced by the addition of isopropyl-β-D-thiogalactopyranoside (0.4
mM), and the incubation was continued for another 3 h. The cells were harvested with a continuous flow centrifuge, and the resulting cell paste (350 g) was frozen in liquid N2 and stored at -70°C.

rGm was purified from 50 g of cell paste. The paste was diluted
with 80 ml of 50 mM Hepes (pH 7.5), 1 mM EDTA, and 1 mM DTI
(buffer 4) containing 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM
N-tosyl-L-phenylalanine chloromethyl ketone, and 0.5 mM N-ac-
tosyl-L-lysine chloromethyl ketone, and the slurry was processed through a French pressure cell at 20,000 p.s.i. to lyse the cells. The mixture was diluted to 1.4 liters with buffer 4 containing 0.2 mM
phenylmethylsulfonyl fluoride and centrifuged at 15,000 x g for 40 min. The supernatant was decanted, supplemented with GDP (50
μM), and loaded onto a 5.5 X 5.0-cm column of S-Sepharose Fast-F10
(Pharmacia). The column was washed with 50 ml of buffer 4 and eluted with a 600-ml linear gradient of NaCl (0-1,000 mM) in buffer 4 containing 5 mM MgCl2. rGm eluted from this column at 300 mM
NaCl as judged by immunoblots using antiserum P-961 (a distinct
peak of GTPyS-binding activity was also observed). The peak frac-
tions were pooled, concentrated to 25 ml, and dialyzed against 15 mM
NaCl, 100-ml gradient of NaCl (0-250 mM) in the same buffer. The
final sample (400 μl) was stored at -70°C. A summary of the purifications is shown in Tables II and III, and SDS-PAGE analysis of the pools obtained from each chromatographic step is shown in Fig. 3.

This purification scheme was carried out once after suplementation
of the extract with 5 mM MgCl2 prior to chromatography on S-
Sepharose. The column elution profiles of Gm during this procedure were essentially identical to those described above, except for the
final step. Here, Gm eluted as a doublet of both protein and GTPyS-

![FIG. 2. Mono S chromatography of rGm. Details are under "Experimental Procedures." The mono Q pool obtained in the puri-
fication of rGm was chromatographed on the mono S column as the
last step in the purification. The full plot is the protein profile and
gradient conditions for the elution. The inset shows the GTPyS-
binding activity (conducted at 500 nM free Mg2+) of selected fractions.

![FIG. 3. SDS-polyacrylamide gel electrophoresis analysis of
rGm purification. Aliquots of fractions from the various stages in
the purification of rGm were resolved by SDS-PAGE on an 11%
polyacrylamide gel, and proteins were visualized by staining with
Coomassie Blue. Lane I, 35 μg of the soluble lysate from the E. coli
expression system; lane 2, 7 μg from the S-Sepharose pool; lane 3, 0.6
μg from the mono Q pool; lane 4, 0.50 μg from the mono S pool; lane
5, 1.5 μg from the mono S pool. Apparent molecular mass was
determined from the migration of protein standards resolved on the
same gel.](https://www.jbc.org/content/1/23/2385)

<table>
<thead>
<tr>
<th>Table II</th>
<th>Purification of Gm from bovine brain membranes</th>
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<tr>
<td>Step</td>
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<td>----------</td>
<td>--------</td>
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<tr>
<td>Cholate extract</td>
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<td>AcA 34</td>
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<td>C-7</td>
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<td>Phenyl-Superose</td>
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* Other guanine nucleotide-binding proteins are present in the
initial extract.

† HPHT, high performance hydroxylapatite.

<table>
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<th>Table III</th>
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<td>Step</td>
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<td>-----------</td>
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<td>S-Sepharose</td>
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<td>Mono S</td>
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* Other guanine nucleotide-binding proteins are present in the
soluble lysate.
Properties of $G_s$

binding activity. SDS-PAGE analysis of the fractions across these peaks revealed that the first (eluting at 280 mM NaCl) was composed of a 39-kDa protein (mono-S1), whereas the second (eluting at 300 mM NaCl) was a 40-kDa species (mono-S2). SDS-PAGE and immunoblot analysis of fractions from this purification are shown in Fig. 4. These studies revealed that the 39-kDa polypeptide is almost certainly derived from the 40-kDa species by removal of the amino terminus since the $G_s$-specific antiserum raised to a peptide from an internal sequence (P-960) recognized both polypeptides, whereas the antiserum raised to the amino-terminal sequence (P-961) recognized only the 40-kDa form.

Miscellaneous Methods—Guanine nucleotide binding to $G$ proteins was quantitated as described previously (28). All measurements were made at 30°C. The standard incubation mixture contained 50 mM Hepes (pH 7.6), 1 mM EDTA, 1 mM DTT (HED), 0.05% Lubrol, and 0.5 μM guanine nucleotide ([35S]GTP[S], [α-32P]GTP, or [γ-32P]GTP). Reactions were terminated by the addition of 0.5 ml of ice-cold 20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 20 mM MgCl2, and 50 μM GTP, and the tubes were held on ice until filtration. Mg2+ concentrations in the individual experiments are noted in the figure legends; free Mg2+ was calculated using a Kd of EDTA for Mg2+ at pH 7.6 of 1 μM (33). Since all experiments were carried out with at least 1 mM EDTA present, the chelating effect of the guanine nucleotide was assumed to be negligible. Data from experiments designed to measure the rate of association of nucleotide with protein were analysed by fitting to a first-order kinetic model. GTPase activity was measured as described (34), using the same incubation mixture and conditions described for guanine nucleotide binding. Estimates of the rate of the catalytic step ($k_{cat}$) of the GTPase reaction catalyzed by $r_{G_m}$ were obtained by fitting data to the following model (34)

$$[P_i](t) = \frac{G-k_{cat} \cdot h_{off}}{k_{on} \cdot h_{off} + k_{off} \cdot h_{on}} + \frac{h_{off} \cdot k_{off}}{h_{on} \cdot k_{off}}$$

where $G$ is the total concentration of $G$ protein, $k_{on}$ is the rate constant for GDP dissociation from (or GTP/S binding to) the $G$ protein, and $t$ is time.

Pertussis toxin-catalyzed ADP-ribosylation of $G$ proteins was conducted as described (28). Briefly, proteins were suspended in 40 μl of 75 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1 mM MgCl2, 10 μM GDP, 2 mM DTT, 2.5 μM NAD, [32P]NAD (15,000 cpm/μmol), 0.5 mM diethylstilbestrol, and 5 μg/ml of pertussis toxin (List Biologicals). Incubations were for 2 h at 20°C. Cholera toxin-catalyzed ADP-ribosylation was performed as described by Kahn and Gilman (35). Proteins were mixed with bovine ADP-ribosylation factor and the other components of the reaction mixture (35); activated cholera toxin (Calbiochem) was added to a final concentration of 20 μg/ml, and the samples were incubated for 2 h at 20°C. Details included in the legend to Fig. 8. SDS-PAGE was performed by the method of Laemmli (36). Protein was quantitated by dye binding (37) (Bio-Rad).

RESULTS

Bovine Brain $G_s$—An initial screening of bovine tissues by immunoblot analysis using antiserum to $G_s$ revealed detectable levels of a 40-kDa immunoreactive protein in membranes derived from brain and adrenal medulla (results not shown). We also observed significant amounts of immunoreactive protein in membranes from human platelets. These results were confirmed with an affinity-purified preparation of an antiserum directed toward the amino terminus of $G_s$ (P-961) (Fig. 5). In contrast to recent reports (38, 39), we have not been able to detect $G_s$ in either the cytosolic (high speed supernatant) fractions of these tissues (results not shown) or in membranes derived from liver or erythrocytes (Fig. 5). Bovine brain was selected as the starting material for the purification of $G_s$.

The purification of $G_s$ from bovine brain required five chromatographic steps and yielded only small amounts of protein (Tables II and III). Recoveries at each step were judged to be >50% by immunoblot analysis of the fractions obtained. The omission of AMF from the buffers used in the purification led to a dramatic loss in recovery; under these conditions, the protein eluted in the void volume of the A2 34-column, and it adsorbed so tightly to the heptylamine-Sepharose resin that urea was required for elution. It appears that AMF stabilizes $G_s$ during purification; this is likely due to the ability of Mg2+ to stabilize the GDP-bound form of the protein (see below).

Since the interaction of a $G$ protein $α$ subunit with guanine nucleotides is a crucial facet of its function, analysis of the intrinsic ability of the protein to exchange GDP for GTP and to hydrolyze the bound triphosphate provides a starting point for its characterization. The divalent cation Mg2+ also serves an important role in this process (34, 40). Initial studies of the ability of $G_s$ to bind GTPyS and to hydrolyze GTP yielded low stoichiometries and rates, respectively, for these two processes. However, one consistent observation was that Mg2+ at concentrations in excess of 1 mM greatly inhibited both phenomena. Since this behavior is similar to that observed...
with ras proteins (41), we examined more closely the polypeptide composition of our purified samples of bovine brain Gα. By increasing the quantity of protein analyzed and by staining gels with Coomassie Blue rather than silver, we visualized a contaminant in the preparations with an apparent Mr of 26,000 (results not shown). By transferring the proteins to nitrocellulose and incubating with [α-32P]GTP (42), we detected binding of this nucleotide to the 26-kDa protein, suggesting that it was one of the many GTP-binding proteins in this size range. Binding of GTP to the 40-kDa protein was not observed with this technique; however, attempts to demonstrate binding to Gα and Gβ were also unsuccessful. Several attempts were made to resolve the two species chromatographically, but this was never completely successful. Since the presence of at least two GTP-binding proteins in the sample made analysis of protein-nucleotide interactions difficult, we attempted to express the cDNA for Gα1 in E. coli; this bacterium contains no GTP-binding proteins that are detectable with the GTP overlay technique (results not shown). We are exploring the possibility that the apparent association of G, with a small GTP-binding protein is functionally significant.

Guanine Nucleotide-binding Characteristics of Gα1—Transformation of plasmid pNPV-β/G into the BL-21(DE3) strain of E. coli and induction with isopropyl β-D-thiogalactopyranoside resulted in the accumulation of immunoreactive protein in these cells to concentrations of about 1 mg/liter of culture; approximately 20% of this protein was present in the high speed supernatant of the cell extract. The protocol summarized in Tables II and III allows the rapid purification of Gα1, from this supernatant, and immunoblot analysis is not required to follow the elution of the protein from chromatographic supports.

Confirmation that Gα1 is, in fact, a guanine nucleotide-binding protein came from analysis of the binding of GTPγS to the intact (α-S:2) and clipped (α-S:1) forms of Gα1 in the presence of 500 nM free Mg2+. This inhibition of nucleotide binding is 0.02 min⁻¹. However, in the presence of 5 mM free Mg2⁺, this rate is reduced dramatically. Under these conditions, the rate of GTPγS binding to the intact form of Gα1 is less than 0.001 min⁻¹. The effect of Mg2⁺ on the rate of nucleotide binding to the intact form of Gα1 was examined in more detail (Fig. 7). Binding is optimal in the μM to low μM range of Mg2⁺ concentrations, but the rate falls off dramatically as Mg2⁺ concentrations exceed 50 μM. This inhibition of nucleotide binding by Mg2⁺ is due to a stabilization of the GDP-bound form of the protein, since the same behavior is observed if one examines the rate of dissociation of GDP rather than the association of GTPγS (results not shown). Thus, as with other members of the G protein family, the dissociation of GDP from Gα1 limits the rate of nucleotide exchange. Millimolar concentrations of Mn2⁺ inhibit nucleotide binding to an extent similar to that of Mg2⁺; similar concentrations of Ca2⁺ had only a small effect (results not shown).

The ability of various purine and pyrimidine nucleotides to compete for the binding of GTPγS to Gα1 was also examined (Table IV). Of the nucleotides tested (at 100-fold μM excess over GTPγS), only guanine nucleotides and, to a lesser extent, ITP were effective. The capacity of ITP to compete for the guanine nucleotide-binding site on other G protein α subunits is well documented (43).

GTPase Activity of Gα1—As shown in Fig. 8A, Gα1 possesses intrinsic GTPase activity. However, the protein exhibits an extremely slow steady-state rate of GTP hydrolysis (kcat < 0.005 min⁻¹), and there is a lag in the approach to this value (tₐₜ, approximately 7 min). The lag in the release of phosphate (defined as the extrapolation of the steady-state release of P, to the abscissa) is equal to (kcat + kcat⁻¹)⁻¹ (33); calculation of kcat from this relationship yields a value of 0.04 min⁻¹, given a lag of 17 min and a value of kcat (kcat for GTPγS) of 0.02 min⁻¹. A related method for determining kcat is to examine the rate of approach to steady state of GTP binding (Fig. 8B and C). The apparent kcat for [γ-32P]GTP is equal to kcat + kcat since the label is released upon hydrolysis. The data of Fig. 8B
indicate that the apparent $k_{\text{on}}$ for $[\gamma^{32}\text{P}]\text{GTP}$ is 0.09 min$^{-1}$, corresponding to a $k_{\text{on}}$ of 0.07 min$^{-1}$. The rate and extent of $[\alpha^{32}\text{P}]\text{GTP}$ binding were also measured. The $k_{\text{on}}$ for $[\alpha^{32}\text{P}]\text{GTP}$ is equal to that shown above for $[\gamma^{32}\text{P}]\text{GTP}$. Assessment of the extent of binding of $[\gamma^{32}\text{P}]\text{GTP}$ versus $[\alpha^{32}\text{P}]\text{GTP}$ permits determination of the fractional occupancy of $rG_{\alpha \gamma}$ by GTP (that is, the fraction of $G_{\alpha \gamma}$ that contains GTP, rather than GDP, in the presence of the triphosphate). This fraction is equal to $k_{\text{on}}/(k_{\text{on}} + k_{\text{off}})$. For the experiment shown in Fig. 8C, the value is 0.34; this implies a $k_{\text{on}}$ for $rG_{\alpha \gamma}$ of about 0.05 min$^{-1}$ at 30°C, in good agreement with the values estimated above (0.04 min$^{-1}$ and 0.07 min$^{-1}$). This value is relatively unaffected by Mg$^{2+}$ concentration over the range of 1 mM to 10 mM (data not shown). The $k_{\text{cat}}$ for $G_{\alpha \gamma}$ is about 200-fold slower than those determined for $G_{\alpha \gamma}$ (10) and the $G_{\alpha \gamma}$ subunits of the $G_{\alpha \gamma}$ and $G_{\alpha \beta}$ (34, 35).

Modification of $rG_{\alpha \gamma}$ by Bacterial Toxins—The deduced amino acid sequence of $G_{\alpha \gamma}$ reveals that an isoleucine residue replaces the cysteine near the carboxyl terminus that serves as the site of ADP-ribosylation of $G_{\alpha \gamma}$ and $G_{\alpha \beta}$. Thus, it has been assumed that $G_{\alpha \gamma}$ is refractory to this modification. Consistent with this assumption, we have been unable to ADP-ribosylate either the purified protein from bovine brain or $rG_{\alpha \gamma}$, by appropriate treatment with pertussis toxin and NAD (Fig. 9). In both cases, ADP-ribosylation of $G_{\alpha \gamma}$ conducted in parallel proceeded smoothly. Similar results were obtained when we attempted to ADP-ribosylate $G_{\alpha \gamma}$ with NAD, cholera toxin, and ADP-ribosylation factor (a protein necessary for ADP-ribosylation of $G_{\alpha \gamma}$ by cholera toxin). Cholera toxin did not modify either bovine brain $G_{\alpha \gamma}$ or $rG_{\alpha \gamma}$, but $rG_{\alpha \gamma}$ was efficiently modified in parallel experiments. Although the arginine residue that has been identified as the site of modification by cholera toxin (44) is conserved in $G_{\alpha \gamma}$, this residue is also present in other members of this family that cannot be modified by this toxin.

$rG_{\alpha \gamma}$ Interacts with $\beta\gamma$ Subunits—The observation that $G_{\alpha \gamma}$ from bovine brain copurified with the $\beta\gamma$ subunit complex that is associated with other $G_{\alpha \gamma}$ polypeptides (Fig. 1) suggested that $G_{\alpha \gamma}$ was also capable of associating with $\beta\gamma$ to form a heterotrimer. This possibility was examined by determining the ability of purified bovine brain $\beta\gamma$ to inhibit the binding of GTP$\gamma$S to purified $rG_{\alpha \gamma}$; $\beta\gamma$ is known to stabilize the GDP-bound form of other $G$ protein $\alpha$ subunits (33). The data shown in Fig. 10 reveal that $\beta\gamma$ does inhibit nucleotide binding to $rG_{\alpha \gamma}$. However, the effect is a modest one and requires rather high concentrations of $\beta\gamma$. Several lines of evidence support the notion that the amino terminus of a $G$ protein $\alpha$ subunit is important for interactions with $\beta\gamma$ and that removal of the amino terminus disrupts such interactions (45, 46). Thus, the observation that the clipped form of $rG_{\alpha \gamma}$, which is missing the amino terminus, does not show any inhibition of nucleotide exchange by $\beta\gamma$ supports the conclusion that $rG_{\alpha \gamma}$ can in fact interact with $\beta\gamma$. It is probably not surprising that the effect on nucleotide exchange is so modest, since this rate for $rG_{\alpha \gamma}$ is already more than 10-fold lower than those of other $G_{\alpha \gamma}$ subunits. This result, coupled with the copurification of $G_{\alpha \gamma}$ and $\beta\gamma$ from bovine brain, strongly implies that $G_{\alpha \gamma}$ is a heterotrimer in its basal state.

### Table IV

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<th>Competing nucleotide</th>
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<td>GTP</td>
<td>45</td>
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<td>GDP</td>
<td>45</td>
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<tr>
<td>GTP$\gamma$S</td>
<td>35</td>
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*App(NH)$_2$p, adenosine 5'-($\beta\gamma$-imido)triphosphate.*
that purified rG,, was utilized instead of bovine brain G,. The quantities of each protein treated were: G,, 0.2 pmol; rG,, 1.0 pmol; rG,zn pertussis toxin or cholera toxin (in the presence of ADP-ribosylation factor), and the reaction mixtures were processed by SDS-PAGE and autoradiography. B, the identical experiment was performed except that purified rG,zn was used instead of bovine brain G,. The quantities of each protein treated were: G,, 0.2 pmol; rG,, 1.0 pmol; rG,, (pertussis toxin), 1.4 pmol; rG, (cholera toxin), 3.0 pmol. Experiments with recombinant proteins were performed in the presence of a 50-fold M excess of the @ subunit complex purified from bovine brain.

DISCUSSION

We have expressed the cDNA that encodes the @ subunit of G, in E. coli and have developed a rapid and relatively simple procedure for purification of the recombinant protein. Purified rG,, displays properties that set it apart from other G protein @ subunits. The basal rate of guanine nucleotide exchange is extremely slow (k = 0.02 min-' at 30 °C) in the absence of Mg2+, and perhaps more interesting, this rate is strongly suppressed by the metal. This effect of Mg2+ is the extremely slow rate of hydrolysis of GTP by G, (41). Quantitatively, however, the proteins differ markedly in these rates. The rate of dissociation of GDP from ras is about 10-12 min, compared with a few seconds for the other G protein @ subunits. Again, this behavior is similar to that observed with the ras proteins, which exhibit a kcat for GTP hydrolysis of 0.02 min-' at 37 °C (47).

Previous results from this laboratory have demonstrated that the interactions of guanine nucleotide with recombinant G protein @ subunits are essentially identical to those of their counterparts purified from mammalian tissues (10). Thus, we feel that the characteristics of rG,, described above accurately reflect the behavior of the mammalian protein. This conclusion is strengthened by experiments recently conducted with an altered form of rG,. As mentioned earlier, G, is the only member of the G protein family to date to exhibit a kcat for GTP hydrolysis of 0.02 min-' at 37 °C (47).

Although the fraction of rG,, which contains GTP at steady state is considerably lower than that observed with ras under similar conditions (0.3 versus 0.9), this difference is due mainly to the very slow rate of nucleotide exchange for rG,. Presumably, there exists in vivo an exchange factor (receptor?) that serves to increase the rate of exchange of guanine nucleotides on the protein. The extremely sluggish rate of GTP hydrolysis by G, would then result in a signal with a long lifetime. This slow kcat prompts speculation on the existence of a GTPase-activating protein for G, similar to those recently described for ras and related proteins (48, 49). The
GTpase-activating proteins apparently enhanced deactivation of these proteins by increasing their rate of hydrolysis of bound GTP; GTPase-activating proteins may be the actual effectors that are regulated by the ras proteins. Demonstration of such a relationship for G, might facilitate identification of the next protein in its signaling pathway.

We have been unable to ADP-ribosylate bovine brain G, or rG, with pertussis or cholera toxin. Thus, any signaling systems under the control of G, should be refractory to disruption by either of these proteins. Candidate systems include some pathways for the regulation of phosphoinositide-specific phospholipase C (16, 50) and substance P-induced inhibition of K+ channels in brain neurons (17). The availability of purified rG, will facilitate studies of its possible involvement in these and other signaling systems.

Acknowledgments—We thank Drs. Michael Graziano, Michael Freissmuth, Maurine Linder, and Susanne Mumbry for continued discussions and criticism of this work. We also thank Dr. Freissmuth for assistance with the ADP-ribosylation and Lisa Ortlepp for skillful technical assistance.

REFERENCES