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G_z, a Guanine Nucleotide-binding Protein with Unique Biochemical Properties*

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Cloning of a complementary DNA (cDNA) for $G_{z\alpha}$, 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 G_z from bovine brain is tedious, and only small quantities of protein have been obtained. The protein copurifies with the $\beta\gamma$ 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 $G_{z\alpha}$ ($rG_{z\alpha}$) from *E. coli* is simple, and quantities of homogeneous protein sufficient for biochemical analysis are obtained. Purified $rG_{z\alpha}$ has several properties that distinguish it from other G protein α subunit polypeptides. These include a very slow rate of guanine nucleotide exchange (k = 0.02 min^{-1}), which is reduced >20-fold in the presence of mM concentrations of Mg^{2+}. In addition, the rate of the intrinsic GTPase activity of $G_{z\alpha}$ is extremely slow. The hydrolysis rate (k_{cat}) for $rG_{z\alpha}$ at 30 °C is 0.05 min^{-1}, or 200-fold slower than that determined for other G protein α subunits. $rG_{z\alpha}$ can interact with bovine brain $\beta\gamma$ but does not serve as a substrate for ADP-ribosylation catalyzed by either pertussis toxin or cholera toxin. These studies suggest that G_z 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 proteins)[1] control an array of cellular processes by modulating the concentrations of crucial second messengers within the cell (1, 2). Members of this family of proteins function by means of their ability to control the activity of effectors such as adenylyl cyclase, cGMP phosphodiesterase, phospholipases, and ion channels in response to activation by specific membrane receptors. G proteins share a heterotrimeric structure, with subunits designated α, β, and γ. G protein oligomers are classified by the identity of their distinct α subunit, although multiple forms of the β (3, 4) and γ (5, 6) subunits exist as well. These proteins function via a cycle of subunit ($\alpha\cdot\beta\gamma$) dissociation, superimposed upon a cycle of receptor-stimulated guanine nucleotide exchange. Thus, a specific agonist-receptor complex catalyzes the exchange of GDP bound to the α subunit for GTP; the $\alpha\cdot$GTP complex dissociates from $\beta\gamma$ and interacts with (regulates) its respective effectors. Deactivation results from hydrolysis of protein-bound GTP and association of $\alpha\cdot$GDP with $\beta\gamma$. The $\beta\gamma$ complex may also regulate effectors directly (7), although this issue is still controversial.

The G protein family accommodates an increasing number (>9) of highly homologous but distinct α subunits (2, 8). A major focus is to identify the specific functions of these proteins, some of which are known only as cDNAs. The availability of *Escherichia coli* expression systems capable of producing active protein from these cDNAs has provided a means to evaluate and compare the characteristics of the individual members of this family (9, 10).[2]

The G protein α subunits characterized to date contain sites for NAD-dependent ADP-ribosylation that is catalyzed by bacterial toxins. $G_{s\alpha}$ can be ADP-ribosylated by cholera toxin; $G_{o\alpha}$ and the α subunits of the G_is can be modified by pertussis toxin; and $G_{t\alpha}$ can serve as a substrate for both toxins (11, 12). Modification of G proteins by these toxins results in characteristic alterations of their function (13, 14). Thus, these toxins have been extremely useful for delineating signaling pathways that are under control of G proteins. However, several such pathways respond to guanine nucleotides but are insensitive to treatment of cells with cholera or pertussis toxin. A great deal of interest has been focused on GTP-dependent pertussis toxin-insensitive events; these include hormonal regulation of phosphoinositide-specific phos-

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[1] The abbreviations used are: G proteins, guanine nucleotide-binding regulatory proteins; G_s and G_i, G proteins that mediate stimulation and inhibition of adenylyl cyclase, respectively; G_o, a G protein abundant in brain; G_t, the predominant G protein in retina; G_z, a G protein of unknown function; $G_{z\alpha}$, example of nomenclature to designate the α subunit of G_z; $rG_{z\alpha}$ and $rG_{s\alpha}$, recombinant $G_{z\alpha}$ and $G_{s\alpha}$; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol.

[2] M. Linder and A. G. Gilman, unpublished observations.

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 Matsuoka and associates (19) recently isolated a cDNA that encodes a unique G protein α subunit (termed G_z or G_x). 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 $G_{i\alpha}$ 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 GTP-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 GTP-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 $G_{s\alpha}$((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 G proteins (25) and their resolved α and $\beta\gamma$ subunits (27, 28) were purified by established procedures. The 52-kDa form of recombinant $G_{s\alpha}$ ($rG_{s\alpha}$), purified after expression in *E. coli* (10), was a gift of Dr. Michael Freissmuth (University of Vienna). ADP-ribosylation factor was a gift of Dr. Richard Kahn (National Cancer Institute).

Production of Antisera—Peptides to be utilized as antigens (see Table I) were synthesized according to amino acid sequences deduced from the human $G_{z\alpha}$ 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 $G_{z\alpha}$ 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 6MB (1 ml, Pharmacia LKB Biotechnology Inc.) as described (26). Immunoblots were processed as described previously (30) except that ^{125}I-labeled goat anti-rabbit IgG F(ab′)$_2$ fragments (Du Pont-New England Nuclear) were used as the secondary antibody. Antisera A-569 and U-49 were a gift of Dr. Susanne Mumby (University of Texas Southwestern Medical Center) (30).

Purification of G_z from Bovine Brain—Bovine brain membranes (12 g of protein) were washed and extracted with 1% sodium cholate (25). The 100,000 × *g* supernatant (extract) was supplemented with $AlCl_3$ (20 μM), $MgCl_2$ (6 mM), and NaF (10 mM) (AMF). This solution was applied to a 1.0-liter column of DEAE-Sephacel which had been equilibrated with 3 liters of 20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1 mM DTT, 1% sodium cholate, and AMF (buffer 1). The column was washed with 250 ml of buffer 1 and eluted with a 2-liter linear gradient of NaCl (0–500 mM) in the same buffer. Fractions of 24 ml were collected, beginning with the start of the gradient. The column was assayed for $G_{z\alpha}$ by immunoblot analysis of 12-μl aliquots of the fractions with antiserum $G_{z\text{-}111}$. G_z eluted from this column in a broad peak centered at 200 mM NaCl. The peak fractions from the DEAE column were pooled and concentrated to 30 ml by pressure filtration through an Amicon PM-30 membrane. This material was applied to a 1.2-liter column of Ultrogel AcA 34 (LKB), equilibrated in buffer 1 containing 100 mM NaCl, and the column was eluted with the same buffer. Fractions of 14 ml were collected. $G_{z\alpha}$ immunoreactivity eluted in a symmetrical peak centered at 670 ml. The fractions were pooled, concentrated to 30 ml as above, and diluted to 200 ml with buffer 1 (with 0.1% sodium cholate) containing 400 mM NaCl. This sample was applied to a 100-ml column of heptylamine-Sepharose, equilibrated in the same buffer. The column was washed with 50 ml of buffer 1 (with 0.3% sodium cholate) containing 400 mM NaCl and was eluted with a 600-ml linear gradient (NaCl decreasing to 0 and sodium cholate increasing to 1.2% in buffer 1); fractions of 8 ml were collected. $G_{z\alpha}$ immunoreactivity eluted in a broad peak centered at 425 ml into the gradient. The peak fractions were pooled and concentrated to 2 ml, and the buffer was changed to buffer 1 (without AMF) by three successive dilutions to 10 ml and concentrations to 2 ml. The pool was then diluted to 10 ml with 10 mM Tris-Cl, 10 mM potassium phosphate (pH 8.0), 1 mM DTT, 1.0% sodium cholate, and 10 μM GDP (buffer 2). This sample was applied to a Bio-Rad high performance hydroxylapatite column; the column fittings were adapted to allow use with the Pharmacia fast protein liquid chromatography system. The column was washed with 5 ml of buffer 2 and eluted with a 22-ml linear gradient to 100 mM potassium phosphate, pH 8.0, in the same buffer; fractions of 0.75 ml were collected. $G_{z\alpha}$ immunoreactivity eluted at 80 mM potassium phosphate. ($G_{z\alpha}$ can also be visualized as a 40-kDa protein on silver-stained gels at this point.) The peak fractions were pooled and concentrated to 1 ml, and the buffer was changed to buffer 1 (0.4% sodium cholate) containing 1.0 M ammonium sulfate and 20 μM GDP (buffer 3) by successive dilution and concentration as described above. The diluted sample (10 ml) was applied to a phenyl-Superose fast protein liquid chromatography column (0.5 × 5.0 cm) (Pharmacia). The column was washed with 5 ml of buffer 3 and eluted with a 20-ml linear gradient of ammonium sulfate decreasing to 0 in the same buffer. $G_{z\alpha}$ eluted in a broad peak at 6–10 ml into the gradient as a 40-kDa immunoreactive protein accompanied by a 35/36-kDa doublet identified immunologically as the β subunits of G proteins (see Fig. 1). A protein of 26 kDa was also apparent if more protein was applied to the gel, and the gel was stained with Coomassie Blue (see "Results"). The fractions containing G_z were pooled and concentrated to 0.2 ml using a Centricon 30 (Amicon), and the buffer was changed to buffer 1 containing 20% glycerol by three successive dilutions to 2 ml and

TABLE I

$G_{z\alpha}$ 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_z	$\alpha_{i\text{-}o}$	α_s	α_t
CRQSSEEKEAARRSRR	$G_{z\text{-}3}$	++	—	—	NT
CRQSSEEKEAARRSRR	P-961	++++	—	—	NT
CGTSNSGKSTIVKQMK	P-960	++	++++	++	++
CTGPAESKGEITPELL	$G_{z\text{-}111}$	+++	—	—	NT

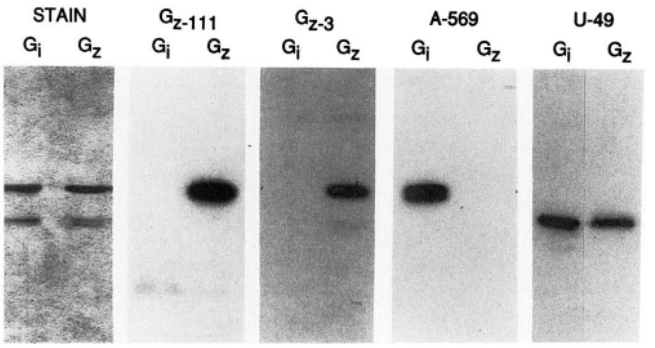

FIG. 1. **SDS-polyacrylamide gel electrophoresis and immunoblot analysis of purified bovine brain G_z.** Each *panel* consists of two *lanes* in which 150 ng each of either G_i or G_z purified from bovine brain was resolved on 11% polyacrylamide gels. The *left panel* is a silver stain of the proteins; the γ subunit ran with the dye front on this gel and is not visualized. The remaining *panels* are immunoblots using the indicated antisera. Antisera $G_{z\text{-}111}$ and $G_{z\text{-}3}$ were raised to peptide sequences unique to G_z (see Table I); A-569 is a "common" α antiserum (30) that recognizes all G protein α subunits except that of G_z, and U-49 is an anti-β antiserum (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_z Expression Vector—Plasmid pBS-G_z, which contains the complete coding sequence of human $G_{z\alpha}$ (18), was simultaneously cleaved with *Nco*I and *Pst*I, and the insert was purified by agarose gel electrophoresis. The *Nco*I-*Pst*I fragment was ligated with plasmid NpT7-5 (10) after digestion of that plasmid with the same two restriction enzymes. Transformation of *E. coli* strain BL-21/DE3 (31) with the resulting plasmid (referred to as NpT7-5/G_z) resulted in the isopropyl β-D-thiogalactopyranoside-inducible expression of immunoreactive recombinant $G_{z\alpha}$ ($rG_{z\alpha}$) in these cells. *E. coli* strain BL-21/DE3 is a T7 lysogen in which the gene for the T7 polymerase is driven by the *lac* UV-5 promoter.

Purification of $rG_{z\alpha}$—One hundred liters of LB medium was inoculated with BL-21/DE3 harboring plasmid NpT7-5/G_z and incubated in a fermenter at 30 °C to an OD of 1.5. Expression of $rG_{z\alpha}$ 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 N_2 and stored at −70 °C.

$rG_{z\alpha}$ 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 DTT (buffer 4) containing 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, and 0.5 mM Nα-*p*-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 30,000 × *g* for 90 min. The supernatant was decanted, supplemented with GDP (50 μM), and loaded onto a 5.5 × 5.0-cm column of S-Sepharose Fast-Flo (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 $MgCl_2$. $rG_{z\alpha}$ eluted from this column at 300 mM NaCl as judged by immunoblots using antiserum P-961 (a distinct peak of GTPγS-binding activity was also observed). The peak fractions were pooled, concentrated to 25 ml, and dialyzed against 15 mM Tris-Cl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, and 3 mM $MgCl_2$ (buffer 5) for 15 h (three changes, 2 liters each). A considerable amount of precipitate formed during the dialysis; this was removed by centrifugation at 30,000 × *g* for 30 min. The supernatant was loaded onto a fast protein liquid chromatography Mono Q HR10/10 column (Pharmacia), which was then washed with 50 ml of buffer 5 and eluted with a 100-ml gradient of NaCl (0–250 mM) in the same buffer. The main peaks of both P-961 immunoreactivity and GTPγS-binding activity coeluted from this column at 50 mM NaCl, SDS-PAGE analysis of column fractions revealed that the predominant protein in this peak migrated at 40 kDa (see Fig. 3). The peak fractions were pooled, supplemented with 1 M Hepes, pH 7.5, to a final concentration of 50 mM, and injected onto a Mono S HR5/5 column (Pharmacia).

TABLE II

Purification of G_z from bovine brain membranes

Step	Volume	Protein	GTPγS binding[a]	
	ml	*mg*	*nmol*	*nmol·mg protein^{-1}*
Cholate extract	1600	4500	1600	0.35
DEAE	30	300	70	0.23
AcA 34	8	70	7.5	0.11
C-7	10	6	1.5	0.25
HPHT[b]	10	2	1.0	0.50
Phenyl-Superose	0.38	0.06	0.25	4.20

[a] Other guanine nucleotide-binding proteins are present in the initial extract.

[b] HPHT, high performance hydroxylapatite.

TABLE III

Purification of recombinant $G_{z\alpha}$

Step	Volume	Protein	GTPγS binding[a]	
	ml	*mg*	*nmol*	*nmol·mg protein^{-1}*
Soluble lysate	1400	3500	170	0.05
S-Sepharose	53	43	15	0.35
Mono Q	3.0	0.56	5.1	9.1
Mono S	5.0	0.34	4.8	14.1

[a] Other guanine nucleotide-binding proteins are present in the soluble lysate.

This column was then washed with 5 ml of buffer 4 (with 5 mM $MgCl_2$) and eluted with a gradient of NaCl (0–500 mM) in the same buffer. Fig. 2 shows the protein, GTPγS-binding profile, and gradient conditions of this chromatographic step. The main protein peak and the sole peak of GTPγS-binding activity comigrated at 300 mM NaCl. SDS-PAGE analysis of the fractions across this peak revealed a single 40-kDa polypeptide. The peak fractions were pooled and supplemented with 1 mg of bovine serum albumin, and the buffer was changed to buffer 4 (with 5 mM $MgCl_2$) by concentration and dilution. 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 supplementation of the extract with 5 mM $MgCl_2$ prior to chromatography on S-Sepharose. The column elution profiles of $G_{z\alpha}$ during this procedure were essentially identical to those described above, except for the final step. Here, $G_{z\alpha}$ eluted as a doublet of both protein and GTPγS-

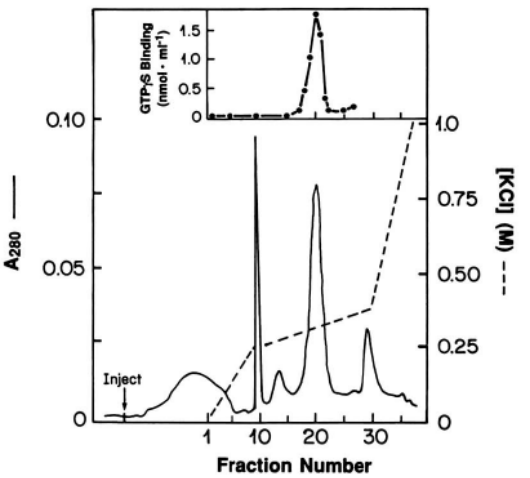

FIG. 2. **Mono S chromatography of $rG_{z\alpha}$.** Details are under "Experimental Procedures." The mono Q pool obtained in the purification of $rG_{z\alpha}$ was chromatographed on the mono S column as the last step in the purification. The full plot is the protein profile and gradient conditions for the elution. The *inset* shows the GTPγS-binding activity (conducted at 500 nM free Mg^{2+}) of selected fractions.

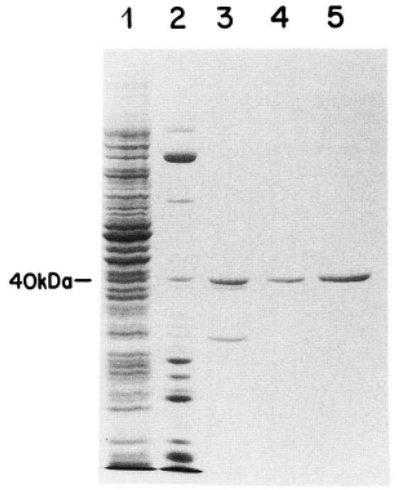

FIG. 3. **SDS-polyacrylamide gel electrophoresis analysis of $rG_{z\alpha}$ purification.** Aliquots of fractions from the various stages in the purification of $rG_{z\alpha}$ were resolved by SDS-PAGE on an 11% polyacrylamide gel, and proteins were visualized by staining with Coomassie Blue. *Lane 1*, 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.

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-S:1), whereas the second (eluting at 300 mM NaCl) was a 40-kDA species (mono-S:2). 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 $G_{z\alpha}$-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 (32). 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 ([^{35}S]GTPγS, [α-^{32}P]GTP, or [γ-^{32}P]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 $MgCl_2$, and 50 μM GTP, and the tubes were held on ice until filtration. Mg^{2+} concentrations in the individual experiments are noted in the figure legends; free Mg^{2+} was calculated using a K_d of EDTA for Mg^{2+} 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 analyzed 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 $rG_{z\alpha}$ were obtained by fitting data to the following model (34)

$$[P_i](t) = \frac{G \cdot k_{cat} \cdot k_{off}}{k_{cat} + k_{off}} \left(t - \frac{1}{k_{cat} + k_{off}} + \frac{e^{-(k_{cat} + k_{off})t}}{k_{cat} + k_{off}} \right)$$

where G is the total concentration of G protein, k_{off} is the rate constant for GDP dissociation from (or GTPγS 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 $MgCl_2$, 10 μM GDP, 2 mM DTT, 2.5 μM NAD, [^{32}P]NAD (15,000 cpm/pmol), 0.5 mM dimyristoylphosphatidyl choline, 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 40 μg/ml, and the samples were incubated for 2 h at 20 °C. Details are provided 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).

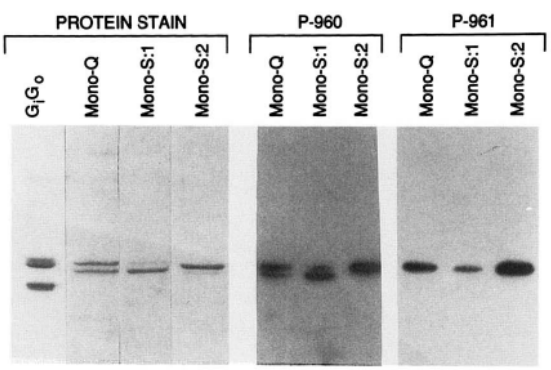

FIG. 4. **SDS-polyacrylamide gel electrophoresis and immunoblot analysis of $rG_{z\alpha}$ fractions from purification scheme that included Mg^{2+} in the extract.** Samples were resolved by SDS-PAGE on 11% polyacrylamide gels. *Left panel*, Coomassie Blue stain of a mixture of G_i and G_o (G_iG_o) in which the α (39–41 kDa) and β (35–36 kDa) subunits are visualized. In the next *three lanes*, aliquots of 250 ng each of the mono Q pool, mono S pool 1 (clipped $rG_{z\alpha}$), and mono S pool 2 (intact $rG_{z\alpha}$) were processed. The *center* and *right panels* are immunoblot analyses of the mono Q and two mono S pools using P-960 (antiserum raised to internal peptide) and P-961 (antiserum raised to amino-terminal peptide), respectively.

RESULTS

Bovine Brain G_z—An initial screening of bovine tissues by immunoblot analysis using antiserum $G_{z\text{-}111}$ 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 $G_{z\alpha}$, P-961 (Fig. 5). In contrast to recent reports (38, 39), we have not been able to detect $G_{z\alpha}$ 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_z.

The purification of G_z 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 AcA 34 column, and it adsorbed so tightly to the heptylamine-Sepharose resin that urea was required for elution. It appears that AMF stabilizes G_z during purification; this is likely due to the ability of Mg^{2+} 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 Mg^{2+} also serves an important role in this process (34, 40). Initial studies of the ability of G_z to bind GTPγS and to hydrolyze GTP yielded low stoichiometries and rates, respectively, for these two processes. However, one consistent observation was that Mg^{2+} at concentrations in excess of 1 mM greatly inhibited both phenomena. Since this behavior is similar to that observed

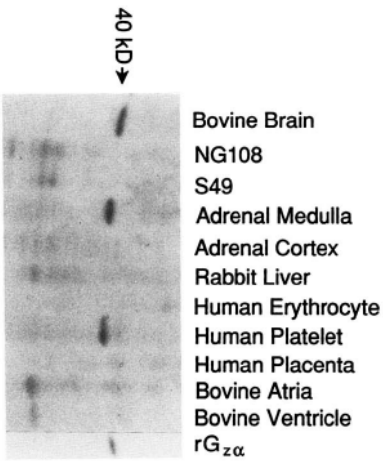

FIG. 5. **Immunoblot analysis of membrane proteins resolved by SDS-polyacrylamide gel electrophoresis.** Membrane fractions (50 μg of protein) from the tissues or cell lines indicated were processed by SDS-PAGE, and the resolved proteins were transferred to nitrocellulose. Adrenal samples were bovine. Immunoblot analysis was performed as described under "Experimental Procedures" using affinity-purified P-961 antiserum (Table I) at a 1:500 dilution. Film was exposed to blots for 12 h using an intensifying screen. The $rG_{z\alpha}$ standard contained 15 ng of the purified protein.

with *ras* proteins (41), we examined more closely the polypeptide composition of our purified samples of bovine brain G_z. 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 M_r of 26,000 (results not shown). By transferring the proteins to nitrocellulose and incubating with [α-^{32}P]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 $G_{o\alpha}$ and $G_{i\alpha}$ 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 $rG_{z\alpha}$ 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_z with a small GTP-binding protein is functionally significant.

Guanine Nucleotide-binding Characteristics of $rG_{z\alpha}$—Transformation of plasmid NpT7-5/G_z 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 $rG_{z\alpha}$ from this supernatant, and immunoblot analysis is not required to follow the elution of the protein from chromatographic supports.

Confirmation that G_z is in fact a guanine nucleotide-binding protein came from analysis of the binding of GTPγS to the two forms of $rG_{z\alpha}$ purified from the *E. coli* expression system. Stoichiometry of binding was approximately 0.5 mol/mol, based on the protein determination. Time courses for the binding of nucleotide to both the clipped (mono-S:1) and intact (mono-S:2) forms of $rG_{z\alpha}$ in the presence of 500 nM free Mg^{2+} are shown in Fig. 6. Both forms exhibit essentially identical rates of nucleotide binding—0.02 min^{-1}. However, in the presence of 5 mM free Mg^{2+}, this rate is reduced dramatically. Under these conditions, the rate of GTPγS binding to the intact form of $rG_{z\alpha}$ is less than 0.001 min^{-1}.

The effect of Mg^{2+} on the rate of nucleotide binding to the intact form of $rG_{z\alpha}$ was examined in more detail (Fig. 7). Binding is optimal in the nM to low μM range of Mg^{2+} concentrations, but the rate falls off dramatically as Mg^{2+} concentrations exceed 50 μM. This inhibition of nucleotide binding by Mg^{2+} 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 $G_{z\alpha}$ limits the rate of nucleotide exchange. Millimolar concentrations of Mn^{2+} inhibit nucleotide binding to an extent similar to that of Mg^{2+}; similar concentrations of Ca^{2+} had only a small effect (results not shown).

The ability of various purine and pyrimidine nucleotides to compete for the binding of GTPγS to $rG_{z\alpha}$ was also examined (Table IV). Of the nucleotides tested (at a 100-fold M 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).

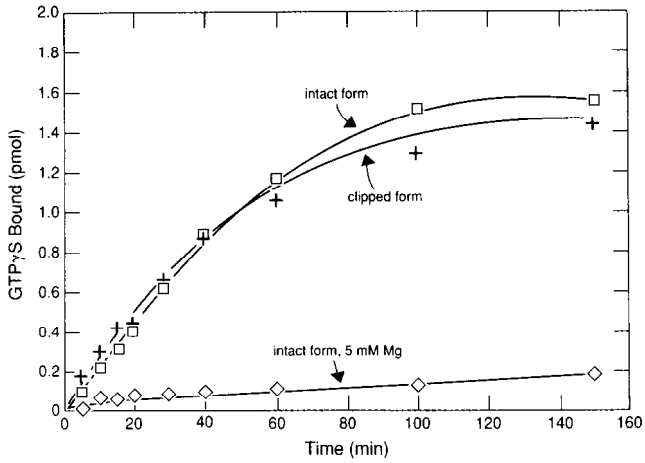

FIG. 6. **Time course of GTPγS binding to $rG_{z\alpha}$.** Binding was carried out at 30 °C in 50 mM Hepes (pH 7.6), 1 mM EDTA, 1 mM DTT, 0.5 μM GTPγS (20,000 cpm/pmol), and 0.05% Lubrol in a total volume of 60 μl. Experiments using the intact (□) and amino-terminal clipped (+) forms of $rG_{z\alpha}$ were conducted at 500 nM free Mg^{2+}. A second time course using the intact form of $rG_{z\alpha}$ (◇) was conducted at 5 mM Mg^{2+}. Incubations with the two forms of $rG_{z\alpha}$ (3 pmol of protein/point) were conducted for the indicated times, reactions were terminated, and bound nucleotide was determined as described under "Experimental Procedures." Data shown represent the mean of triplicate determinations from a single experiment, which is representative of four such experiments.

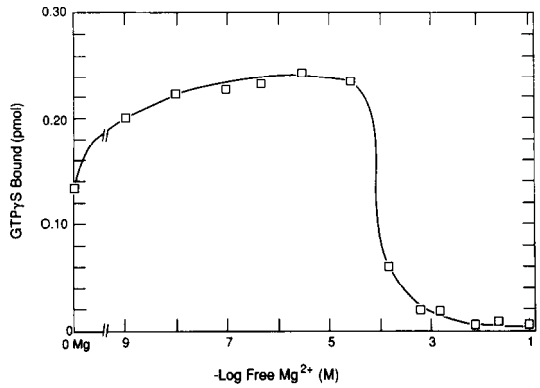

FIG. 7. **Effect of Mg^{2+} on the initial rate of GTPγS binding to $rG_{z\alpha}$.** The assay conditions were as described in the legend to Fig. 6, except that the protein was gel filtered (G-50 resin) into buffer 4 (see "Experimental Procedures") prior to the assay. Reaction mixtures were supplemented with $MgSO_4$ such that the indicated concentrations of free Mg^{2+} were achieved. The zero Mg^{2+} point was determined with 5 mM EDTA in the assay mixture. Incubations with $rG_{z\alpha}$ (0.8 pmol) were for 20 min; reactions were then terminated, and bound nucleotide was determined. Data shown represent the mean of triplicate determinations from a single experiment, which is representative of three such experiments.

GTPase Activity of $rG_{z\alpha}$—As shown in Fig. 8*A*, $rG_{z\alpha}$ possesses intrinsic GTPase activity. However, the protein exhibits an extremely slow steady-state rate of GTP hydrolysis ($k < 0.005$ min^{-1}), and there is a lag in the approach to this value ($t_{1/2}$ approximately 7 min). The lag in the release of phosphate (defined as the extrapolation of the steady-state release of P_i to the abscissa) is equal to $(k_{cat} + k_{off})^{-1}$ (33); calculation of k_{cat} from this relationship yields a value of 0.04 min^{-1}, given a lag of 17 min and a value of k_{off} (k_{on} for GTPγS) of 0.02 min^{-1}.

A related method for determining k_{cat} is to examine the rate of approach to steady state of GTP binding (Fig. 8, *B* and *C*). The apparent k_{on} for [γ-^{32}P]GTP is equal to $k_{cat} + k_{off}$ since the label is released upon hydrolysis. The data of Fig. 8*B*

TABLE IV

Ability of nucleotides to compete for GTPγS binding to $rG_{z\alpha}$

Assays were conducted as described in the legend to Fig. 5 and contained 500 nM free Mg^{2+}, 0.5 μM GTPγS, and the indicated nucleotide as competitor (50 μM). Reactions were terminated at 15 min, and bound GTPγS was determined. Results are reported as binding relative to the value with no competing nucleotide (100; 0.60 pmol).

Competing nucleotide	GTPγS bound (relative)
None	100
ATP	74
App(NH)p[a]	88
ITP	8
CTP	88
UTP	82
GTP	<5
GDP	<5
GTPγS	<5

[a] App(NH)p, adenosine 5′-(β,γ-imido)triphosphate.

indicate that the apparent k_{on} for [γ-^{32}P]GTP is 0.09 min^{-1}, corresponding to a k_{cat} of 0.07 min^{-1}. The rate and extent of [α-^{32}P]GTP binding were also measured. The k_{on} for [α-^{32}P]GTP is equal to that shown above for GTPγS—0.02 min^{-1}. Assessment of the extent of binding of [γ-^{32}P]GTP *versus* [α-^{32}P]GTP permits determination of the fractional occupancy of $rG_{z\alpha}$ by GTP (that is, the fraction of $G_{z\alpha}$ that contains GTP, rather than GDP, in the presence of the triphosphate). This fraction is equal to $k_{off}/(k_{cat} + k_{off})$. For the experiment shown in Fig. 8*C*, the value is 0.34; this implies a k_{cat} for $rG_{z\alpha}$ 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 nM to mM (data not shown). The k_{cat} for $G_{z\alpha}$ is about 200-fold slower than those determined for $G_{s\alpha}$ (10) and the α subunits of the G_is and G_o (34).[2]

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

$rG_{z\alpha}$ Interacts with βγ Subunits—The observation that G_z from bovine brain copurified with the βγ subunit complex that is associated with other G_α polypeptides (Fig. 1) suggested that $G_{z\alpha}$ was also capable of associating with βγ to form a heterotrimer. This possibility was examined by determining the ability of purified bovine brain βγ to inhibit the binding of GTPγS to purified $rG_{z\alpha}$; βγ is known to stabilize the GDP-bound form of other G protein α subunits (33). The data shown in Fig. 10 reveal that βγ does inhibit nucleotide binding to $rG_{z\alpha}$. However, the effect is a modest one and requires rather high concentrations of βγ. Several lines of evidence support the notion that the amino terminus of a G protein α

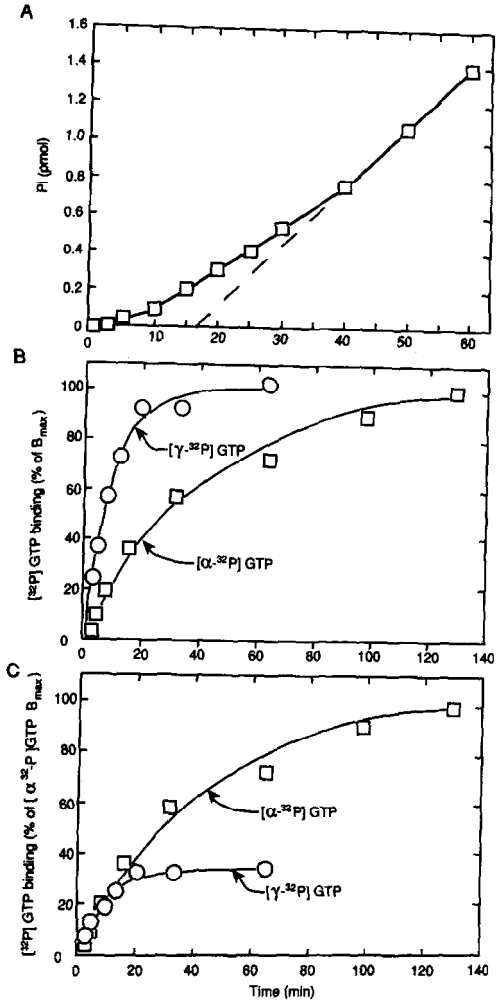

FIG. 8. **Determination of k_{cat} for $rG_{z\alpha}$.** *A*, GTPase activity of $rG_{z\alpha}$. Conditions were as described in the legend to Fig. 6, except that the GTPγS in the incubation mixture was replaced by 0.5 μM [γ-^{32}P]GTP. Reaction mixtures (50 μl) contained 5 pmol of $rG_{z\alpha}$. At the indicated times, reactions were terminated, and released $^{32}P_i$ was quantitated as described under "Experimental Procedures." Data shown represent the mean of duplicate determinations from a single experiment, which is representative of two such experiments. *B*, time course of [α-^{32}P]GTP and [γ-^{32}P]GTP binding to $rG_{z\alpha}$. Conditions were as described in the legend to Fig. 6, except that the GTPγS in the incubation mixture was replaced with either [α-^{32}P]GTP or [γ-^{32}P]GTP (1 μM). Reactions were terminated at the indicated times and bound nucleotide determined. The data are plotted as a percent of the maximum binding (B_{max}) (end point) for each assay, which was 3.55 and 1.21 pmol for [α-^{32}P]GTP and [γ-^{32}P]GTP, respectively. Data shown represent the mean of triplicate determinations from a single experiment, which is representative of three such experiments. *C*, the data obtained in the experiment described in *part B* were replotted as a percentage of the B_{max} determined for [α-^{32}P]GTP; determination of the B_{max} for [γ-^{32}P]GTP thus yields the fractional occupancy of $rG_{z\alpha}$ by GTP (see "Results").

subunit is important for interactions with βγ and that removal of the amino terminus disrupts such interactions (45, 46). Thus, the observation that the clipped form of $rG_{z\alpha}$, which is missing the amino terminus, does not show any inhibition of nucleotide exchange by βγ supports the conclusion that $rG_{z\alpha}$ can in fact interact with βγ. It is probably not surprising that the effect on nucleotide exchange is so modest, since this rate for $rG_{z\alpha}$ is already more than 10-fold lower than those of other α subunits. This result, coupled with the copurification of $G_{z\alpha}$ and βγ from bovine brain, strongly implies that G_z is a heterotrimer in its basal state.

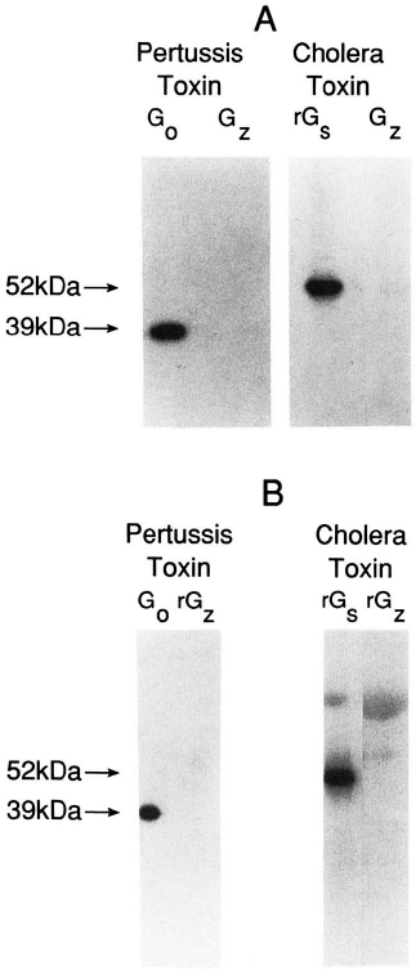

FIG. 9. **ADP-ribosylation of G_z by bacterial toxins.** *A*, purified preparations of G_o (0.7 pmol), the 52-kDa form of recombinant $G_{s\alpha}$ (rG_s, 0.5 pmol), and bovine brain G_z (5 pmol for pertussis toxin, 0.5 pmol for cholera toxin) were treated with [^{32}P]NAD and either pertussis toxin 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 $rG_{z\alpha}$ was utilized instead of bovine brain G_z. The quantities of each protein treated were: G_o, 0.2 pmol; $rG_{s\alpha}$, 1.0 pmol; $rG_{z\alpha}$ (pertussis toxin), 1.4 pmol; $rG_{z\alpha}$ (cholera toxin), 3.0 pmol. Experiments with recombinant proteins were performed in the presence of a 3–10-fold M excess of the $\beta\gamma$ subunit complex purified from bovine brain.

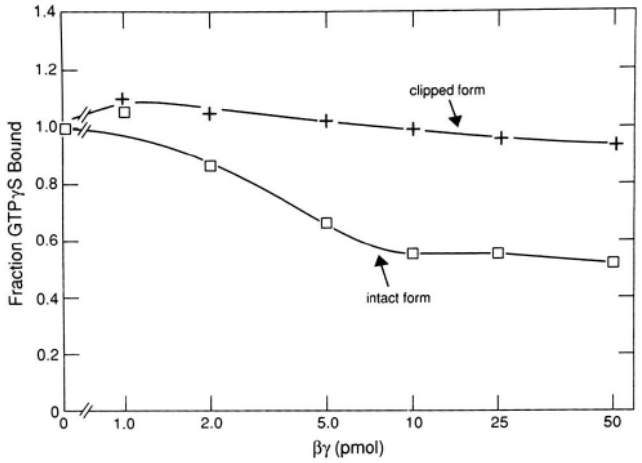

FIG. 10. **Effect of $\beta\gamma$ on the initial rate of binding of GTPγS to both the clipped and intact forms of $rG_{z\alpha}$.** The clipped form (+) and the intact form (□) of $rG_{z\alpha}$ were incubated on ice for 30 min in a solution containing 50 mM NaHepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 50 nM GDP, 0.1% Lubrol, and the indicated amount of purified $\beta\gamma$ in a total volume of 30 μl. The mixtures were then diluted 2-fold with a solution containing GTPγS such that the final concentrations of components were as described in the legend to Fig. 6. The samples were incubated for 20 min; reactions were then terminated, and bound nucleotide was determined. The results are expressed as the fraction of GTPγS bound compared with the value measured in the absence of $\beta\gamma$, which was 0.36 and 0.18 pmol for the clipped and intact forms of $rG_{z\alpha}$, respectively. Data shown represent the mean of triplicate determinations from a single experiment, which is representative of two such experiments.

DISCUSSION

We have expressed the cDNA that encodes the α subunit of G_z in *E. coli* and have developed a rapid and relatively simple procedure for purification of the recombinant protein. Purified $rG_{z\alpha}$ displays properties that set it apart from other G protein α subunits. The basal rate of guanine nucleotide exchange is extremely slow (k = 0.02 min^{-1} at 30 °C) in the absence of Mg^{2+}, and perhaps more interesting, this rate is strongly suppressed by the metal. This effect of Mg^{2+}, although not seen with other G proteins, is qualitatively similar to that observed with the *ras* proteins (41). Quantitatively, however, the proteins differ markedly in these rates. The rate of dissociation of GDP from *ras* is about 1 min^{-1} at low concentrations of Mg^{2+} and is decreased to 0.02 min^{-1} in the presence of mM concentrations of the ion (at 37 °C).

Another major difference between $rG_{z\alpha}$ and other G protein α subunits is the extremely slow rate of hydrolysis of GTP by $rG_{z\alpha}$. The k_{cat} for GTP hydrolysis by $G_{s\alpha}$, $G_{o\alpha}$, and the $G_{i\alpha}$s at 30 °C is in the range of 10 min^{-1} (10, 34),[2] or 200 times faster than that determined here for $rG_{z\alpha}$ (0.05 min^{-1}). Thus, once GTP binds to $rG_{z\alpha}$, the $t_{1/2}$ for hydrolysis (and presumably for deactivation of the protein) is 10–12 min, compared with a few seconds for the other G protein α subunits. Again, this behavior is similar to that observed with the *ras* proteins, which exhibit a k_{cat} for GTP hydrolysis of 0.02 min^{-1} at 37 °C (47).

Previous results from this laboratory have demonstrated that the interactions of guanine nucleotide with recombinant G protein α subunits are essentially identical to those of their counterparts purified from mammalian tissues[2] (10). Thus, we feel that the characteristics of $rG_{z\alpha}$ described above accurately reflect the behavior of the mammalian protein. This conclusion is strengthened by experiments recently conducted with an altered form of $rG_{s\alpha}$. As mentioned earlier, $G_{z\alpha}$ is the only member of the G protein family to date to exhibit divergence of amino acid sequence in the first portion of the guanine nucleotide-binding domain. When the sequence of this region of $G_{s\alpha}$ is changed to that present in $G_{z\alpha}$ by site-directed mutagenesis, the altered protein (mutant $rG_{s\alpha}$) shows a 50-fold reduction in k_{cat} compared with its wild-type counterpart.[3]

Although the fraction of $rG_{z\alpha}$ 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 $rG_{z\alpha}$. 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 $G_{z\alpha}$ would then result in a signal with a long lifetime. This slow k_{cat} prompts speculation on the existence of a GTPase-activating protein for G_z, similar to those recently described for *ras* and related proteins (48, 49). The

[3] G. Santoyo, P. J. Casey, and A. G. Gilman, unpublished observations.

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 a similar relationship for G_z might facilitate identification of the next protein in its signaling pathway.

We have been unable to ADP-ribosylate bovine brain G_z or $rG_{z\alpha}$ with pertussis or cholera toxin. Thus, any signaling systems under the control of G_z 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 $rG_{z\alpha}$ will facilitate studies of its possible involvement in these and other signaling systems.

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