Subtype-Specific Binding of Azidoanilido-GTP by Purified G Protein α Subunits†

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ABSTRACT: Azidoanilido-GTP (AA-GTP), a hydrolysis-resistant, photoreactive GTP analog, is becoming an increasingly popular tool for identifying activation of specific G proteins by receptors within native plasma membranes. Despite the use of AA-GTP as an affinity probe, surprisingly little is known regarding the ability of various G protein α subunits to bind this analog. To directly address this issue, we compared the ability of four purified G protein α subunits (Go, Gi_2, Gs, and Gz) to bind AA-GTP with their ability to bind GTPγS, a GTP analog commonly used to characterize the GTP-binding properties of G proteins. All four Gα subunits tested bound AA-GTP in a manner distinct from their binding of GTPγS. One of these proteins, Gsα, required millimolar levels of free Mg^{2+} for significant binding of AA-GTP, while Goα and $Gi\alpha_2$ displayed peak AA-GTP binding at approximately 100 μM free Mg^{2+}. The fourth Gα subunit, Gz, bound AA-GTP very poorly relative to GTPγS regardless of the magnesium concentration. These results indicate that individual G protein α subunits differ markedly in their ability to bind AA-GTP. Use of AA-GTP to identify specific G protein–receptor interactions must therefore take into account the varied abilities of Gα subunits to bind this analog.

Guanine nucleotide-binding regulatory proteins (G proteins)[1] are key players in the transduction of many extracellular signals to intracellular second messages (Gilman, 1987; Birnbaumer, 1990; Hepler & Gilman, 1992). Signal transducing G proteins are heterotrimeric molecules consisting of subunits designated α, β, and γ; they are classified according to the identity of their unique α subunit. These G proteins reside predominantly at the intracellular face of the plasma membrane and function by means of their ability to couple receptors for extracellular ligands to effector molecules. An agonist-bound receptor interacts with an appropriate G protein, driving the exchange of GTP for GDP on the α subunit of the G protein. GTP binding leads to dissociation of the oligomer into α and $\beta\gamma$ components, and the α–GTP complex can then interact with specific effector molecule(s) to modulate their activity. Recent evidence indicates that the free $\beta\gamma$ complex can also directly regulate effectors, in addition to its other roles (Federman et al., 1992; Gao & Gilman, 1991; Blank et al., 1992; Tang & Gilman, 1991).

Biochemical, pharmacological, and molecular characterization of G protein signaling pathways has revealed considerable complexity. There are multiple forms of G protein α, β, and γ subunits; the number of Gα subunits identified in mammalian cells has reached about 20 (Hepler & Gilman, 1992). More than 150 G protein-coupled receptors have been identified. Thus, a major focus of research in this field is to characterize receptor–G protein interactions and thereby assign specific G proteins to particular signal transduction pathways.

Nucleotide analogs have proven to be valuable tools in identifying the participation of G proteins in signaling. A particularly useful analog introduced by Pfeuffer in 1977 is P^3-(4-azidoanilido)-P^1-5′-guanosine triphosphate (AA-GTP) (Pfeuffer, 1977). AA-GTP is a nonhydrolyzable GTP analog containing a photoactivatable cross-linking group covalently attached to the γ phosphate. AA-GTP has been used as an affinity probe to identify receptor-activated G proteins (Devary et al., 1987; Im & Graham, 1990; Offermanns et al., 1990; Schafer et al., 1988; Wange et al., 1991). In this procedure, agonists are identified which lead to increased photolabeling of specific G protein α subunits in membranes incubated with [^{32}P]AA-GTP. Studies have also been performed examining the binding properties of total membrane G proteins in different tissues for AA-GTP (Pfeuffer & Eckstein, 1976; Gordon & Rasenick, 1988). On the basis of these studies, it has been suggested that, in addition to their characteristic nucleotide-exchange and hydrolysis properties for guanine nucleotides in general, individual Gα proteins may differ in their ability to bind AA-GTP in particular (Wong & Martin, 1985; Hatta et al., 1986; Gordon & Rasenick, 1988). In attempting to define distinct receptor-activated G proteins, condition-dependent variation of the ability of individual G proteins to bind AA-GTP could be particularly troublesome. Employing unfavorable experimental conditions (or the possible inability of a given G protein to bind AA-GTP at all) could result in one missing or misinterpreting a putative receptor–G protein interaction. Thus, information is needed on the interactions of AA-GTP with G protein α subunits.

In the present study, we compare the ability of four G protein α subunits to bind AA-GTP. We use purified recombinant proteins and focus on the role of Mg^{2+}, as this ion is known to be important for nucleotide–G protein

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[1] Abbreviations: G proteins, guanine nucleotide-binding regulatory proteins; Goα, $Gi\alpha_2$, Gsα, and Gzα, the individual α subunits of four different heterotrimeric G proteins; AA-GTP, P^3-(4-azidoanilido)-P^1-5′-guanosine triphosphate; GTPγS, guanosine-5′-3-*O*-thiotriphosphate; GppNHp, guanosine-5′-β,γ-iminotriphosphate; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide HCl; MES, 2-(4-morpholino)-ethanesulfonic acid; PEI, poly(ethyleneimine); TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DEAE, (diethylamino)ethyl; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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interactions and also for membrane labeling by AA-GTP (Higashijima et al., 1987; Gordon & Rasenick, 1988; Offermanns et al., 1989, 1990). AA-GTP binding is contrasted to binding to another GTP analog, guanosine-5′-3-*O*-thiotriphosphate (GTPγS). This nucleotide is thought to closely mimic the structure of the physiological nucleotide GTP, since their binding properties are essentially identical (Frey & Sammons, 1985; Yamanaka et al., 1985, 1986). We find that the interaction of G protein α subunits with AA-GTP is quite distinct from that of GTPγS and discuss the relevance of this finding to previous studies conducted with AA-GTP.

MATERIALS AND METHODS

Materials. [α-^{32}P]GTP (3000 Ci/mmol) was purchased from New England Nuclear. 4-Azidoaniline was from Fluka. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide HCl (EDAC) was from BioRad. Triethylamine was from Kodak, and 2-(4-morpholino)ethanesulfonic acid (MES) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from Fisher. Tris(hydroxymethyl)aminomethane (Tris) was from Boehringer Mannheim. Lubrol was from ICN. PEI-cellulose plates were purchased from Bodman Biochemicals, and DEAE-Sephacel was from Pharmacia. BA-85 nitrocellulose filters were from Schleicher & Schuell.

Synthesis and Purification of 4-Azidoanilido-GTP (AA-GTP). [α-^{32}P]AA-GTP was synthesized and purified according to a published procedure (Schafer et al., 1988; Pfeuffer, 1977) with minor modifications. Briefly, 1 mCi of [α-^{32}P]-GTP was lyophilized to dryness. The residue was dissolved in 100 μL of 100 mM MES (pH 5.6) containing 3 mg of EDAC and 4 mg of 4-azidoaniline. The reaction was left to proceed overnight in the dark at room temperature. Unreacted azidoaniline was then extracted with three 0.5-mL portions of diethyl ether. The AA-GTP was purified by thin-layer chromatography (TLC) on a glass-backed PEI-cellulose plate which was developed with 0.8 M triethylammonium bicarbonate (pH 7.5). The band containing AA-GTP was visualized by autoradiography, and this region was scraped into a small column. AA-GTP was eluted from the resin with 0.25 M triethylammonium bicarbonate (pH 7.5) containing 10% (v/v) ethanol. The eluate was frozen and lyophilized, and the residue was dissolved in 100 μL of H_2O; this product was tested by TLC. Its mobility during TLC on PEI-cellulose developed with 0.8 M triethylammonium bicarbonate (pH 7.5) was similar to that of GDP as reported (Thomas & Pfeuffer, 1991). Also, after loading the analog on PEI-cellulose plates and irradiating with UV light, the compound formed a spot which did not migrate on subsequent chromatography (data not shown). The purified [α-^{32}P]AA-GTP was frozen in aliquots and stored at −80 °C.

Nonradioactive AA-GTP was synthesized and purified as described above, except that unlabeled GTP (2 mg) was used and the product obtained from the TLC step was further purified by chromatography on DEAE-Sephacel. Briefly, the TLC product was diluted >15-fold with 10 mM Tris (pH 7.5) and loaded onto a 1-mL DEAE column preequilibrated in that buffer. The column was washed with four column volumes of 10 mM Tris (pH 7.5) and then eluted with a 5-mL linear gradient of NaCl (0–1 M) in that same buffer. AA-GTP eluted in a sharp peak centered at 600 mM NaCl. Yield was determined colorimetrically by quantitating the ribose content of the product using a published method (McRary & Slattery, 1945; Brown, 1946). This assay is based on the fact that pentoses react with orcinol in the presence of ferric chloride and concentrated HCl to produce a green color satisfactory for colorimetry. Using GDP as a standard, an extinction coefficient for purified AA-GTP was determined to be 28 000 cm^{-1} M^{-1}. This value is in close agreement with the published extinction coefficient (Pfeuffer, 1977).

Preparation of Membranes and Purification of Recombinant Gα Subunits. Bovine brain membranes were prepared as described (Sternweis & Robishaw, 1984). Recombinant G protein α subunits were expressed in *Escherichia coli* and purified as described (Graziano et al., 1989; Linder et al., 1990; Casey et al., 1990). Preparations of both membranes and purified G proteins were flash-frozen in aliquots and stored at −80 °C until use.

Photolabeling and Immunoprecipitation. Membrane-bound G proteins were photolabeled with [^{32}P]AA-GTP as described previously (Offermanns et al., 1991). Bovine brain membranes containing 50 μg of protein were incubated with [^{32}P]AA-GTP (50 nM) for 5 min at 30 °C in 50 μL of 50 mM HEPES (pH 7.6), 1 mM EDTA, 50 mM NaCl, 1.6 mg/mL BSA, and 1.5 mM $MgCl_2$. Additions or changes to this incubation mixture for specific experiments are described in the appropriate figure legends. Reactions were stopped by placing the tubes on ice, and membranes were harvested by centrifugation for 10 min at 14000*g* and 4 °C. The pellets were resuspended in buffer containing 50 mM HEPES (pH 7.5), 1 mM EDTA, 2 mM $MgCl_2$, 50 mM NaCl, and 2 mM DTT. The suspensions were then irradiated with a UV lamp (4 W, 254 nm) at a distance of 2 cm for 5 min at 4 °C. Membranes were harvested by centrifugation as above, solubilized in Laemmli sample buffer, and processed by SDS–PAGE (Laemmli, 1970) and autoradiography.

Purified recombinant Gα proteins were photolabeled in a similar manner. The proteins were incubated with [^{32}P]AA-GTP for 10 min at 30 °C in 50 mM HEPES (pH 7.6), 50 mM NaCl, 0.3 mg/mL BSA, 10 μM ATP, 0.05% Lubrol, and EDTA and $MgCl_2$ so that the free Mg^{2+} concentration was as indicated in the figure legend. The reactions were stopped by placing the tubes on ice. DTT was then added to a final concentration of 2 mM, and the samples were irradiated as above. Laemmli sample buffer was added, and the samples were processed by SDS–PAGE. For quantitation of photolabeling, Gα proteins were processed as above (except that ATP and BSA were excluded in the incubations so that conditions matched those used in binding experiments) and visualized by Coomassie Blue staining. The appropriate bands were excised, and incorporated radioactivity was determined by scintillation counting.

Labeled-membrane G proteins were immunoprecipitated using a published method (Buss et al., 1987). Briefly, membranes containing approximately 50 μg of protein were solubilized in 25 μL of buffer containing 50 mM sodium phosphate (pH 7.4), 1 mM DTT, and 0.5% SDS. After the solution was heated for 5 min at 60 °C, 100 μL of a second buffer was added such that the final concentrations of components were 50 mM sodium phosphate (pH 7.4), 1 mM DTT, 0.5% SDS, 1% NP-40, 1% Na deoxycholate, and 150 mM NaCl. After preclearing with Pansorbin (Calbiochem), antiserum was added and the solution was incubated overnight at 4 °C. Fifty microliters of 10% Pansorbin (Calbiochem) was added, and the sample was incubated for 30 min on ice. The resultant protein–antibody–Pansorbin complex was collected by centrifugation and solubilized in Laemmli buffer for SDS–PAGE and autoradiography.

Nucleotide-Binding Assays. Nucleotide binding by purified Gα proteins was quantitated by filtration through nitrocellulose as described previously (Northup et al., 1982). The standard

reaction contained the following in a volume of 50 μL: recombinant G protein α subunit (~2 pmol as determined by [^{35}S]GTPγS binding under optimal conditions), 50 mM Hepes (pH 7.6), 0.05% Lubrol, EDTA and $MgCl_2$ at concentrations such that the free-Mg^{2+} concentration was as indicated in the figure legend, and 1 μM guanine nucleotide ([^{35}S]GTPγS or [^{32}P]AA-GTP). To calculate specific binding, the quantity of radioactivity retained on nitrocellulose after filtration of reaction mix alone (no protein) was subtracted from each point. For both GTPγS and AA-GTP, these no-protein controls were typically <10% of the maximum binding in a given experiment. Free magnesium was calculated using a K_d of EDTA for Mg^{2+} at pH 7.6 of 1 μM (Higashijima et al., 1987). Incubation times and temperature conditions were determined separately for each Gα subunit (see figure legends) because each protein has a unique intrinsic rate of nucleotide exchange. The $t_{1/2}$ of nucleotide exchange for each Gα subunit tested has been published (Graziano et al., 1989; Linder et al., 1990; Casey et al., 1990).

Experiments examining the dissociation of AA-GTP from purified Giα$_2$ and Gsα were performed by a variation of the assay described above. Either Gα subunit (~10–15 pmol) was added to standard reaction mixtures containing 1 mM free Mg^{2+} and 1 μM guanine nucleotide (either [^{32}P]AA-GTP or [^{35}S]GTPγS) and incubated for either 55 min at 30 °C (Giα$_2$) or 22 min at 20 °C (Gsα). The mixture was then diluted with an equal volume of reaction buffer that contained either 1 mM GDP or 1 mM GDP plus additional EDTA sufficient to lower free Mg^{2+} to 100 nM. At the time points indicated in the figures, aliquots were withdrawn and nucleotide remaining on the Gα protein was quantitated by filtration through nitrocellulose and scintillation counting as above.

RESULTS

Photolabeling of Membranes. To confirm that G proteins present in the membrane can be readily photolabeled by AA-GTP and that activation of G proteins leads to an increase in this photolabeling, we incubated bovine brain membranes with [^{32}P]AA-GTP and in the presence or absence of mastoparan. Mastoparans are small amphiphilic peptides derived from wasp venom which activate G proteins in a manner similar to that of agonist-occupied receptors (Higashijima et al., 1988). Incubation of membranes with [^{32}P]AA-GTP followed by UV irradiation results in very clear labeling of a characteristic band at approximately 40 kDa (Figure 1, left panel). This band is likely comprised largely of Goα and perhaps other Gα subunits in the 40-kDa range (e.g., Giα). Confirmation that the photolabeled 40-kDa band consisted of one or more G protein α subunits was made by immunoprecipitating the photolabeled species with an antiserum, P960, which recognizes a common region of G protein α subunits (Mumby & Gilman, 1991) (data not shown). Addition of mastoparan to the labeling mixture resulted in a slight (~20%) stimulation of this labeling when compared to incubation in the absence of mastoparan (Figure 1, left panel, lanes 1 and 2). Consistent with the results of others, inclusion of GDP in the incubation greatly increases the ratio of mastoparan-stimulated labeling to basal labeling of this same band, although total labeling is substantially reduced (Figure 1, left panel, lanes 3 and 4). This is presumably due to the ability of GDP to inhibit labeling of basal-state G proteins more than those which are stimulated by mastoparan (Offermanns et al., 1990). The specificity of AA-GTP binding was demonstrated by competition experiments with unlabeled nucleotides. Inclusion of excess ATP in the reaction mixture had little effect on labeling, while

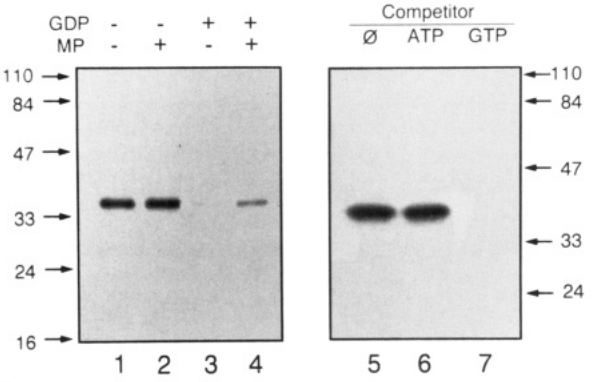

FIGURE 1: Photolabeling of G proteins in membranes with [^{32}P]-AA-GTP. Left panel: mastoparan stimulation of AA-GTP binding. Bovine brain membranes (50 μg of protein) were incubated with [^{32}P]AA-GTP (50 nM) for 5 min at 30 °C in the absence (lanes 1 and 3) or the presence (lanes 2 and 4) of 100 μM mastoparan (MP). GDP at 500 nM was included in the samples shown in lanes 3 and 4. After incubation, the reaction mixtures were photolyzed as described in Materials and Methods, processed by SDS–PAGE, and visualized by autoradiography (30-min exposure). Molecular weight standards in kilodaltons are indicated by the arrows on the *left*. For quantitation of radioactivity in each band, a phosphorimager was used. Right panel: specificity of AA-GTP binding. Bovine brain membranes (50 μg of protein) were labeled as in lane 1 of the left panel, except that either 100 μM ATP (lane 6) or 100 μM GTP (lane 7) was included in the reaction. After incubation, the samples were photolyzed, processed by SDS–PAGE, and visualized by autoradiography (15-min exposure). Molecular weight standards in kilodaltons are indicated by the arrows on the *right*.

inclusion of excess GTP abolished labeling as expected (Figure 1, right panel).

Photolabeling of Purified G Protein α Subunits. To assess the ability of [^{32}P]AA-GTP to label specific G protein α subunits, we incubated equivalent amounts of four purified Gα subunits with the analog. Four Gα subunits were expressed in *E. coli* and purified, Gsα, Goα, Giα$_2$, and Gzα. The photolabeling assays were performed in the presence of either low (150 nM) or high (3.5 mM) free Mg^{2+} to provide an initial assessment of the requirements for this metal. A short incubation time (10 min) was chosen to be consistent with labeling times commonly used in the literature for identifying specific agonist-stimulated G proteins (Offermanns et al., 1991; Schafer et al., 1988; Wange et al., 1991); these times reportedly yield the maximal ratio of agonist-stimulated to basal labeling for at least some G proteins. The results of this photolabeling are shown in Figure 2. The four proteins varied markedly in their ability to incorporate the analog. These differences are not likely due to a variability in the degree of photoincorporation among the Gα proteins, as photolabeling efficiencies were similar for each protein (Table 1). Although the intrinsic rate of nucleotide exchange is distinct for each protein, this fact can only partially explain the differences in labeling observed. Gsα, which exchanges nucleotide at a rate approximately one-half as fast as Goα, incorporated far less label than would be expected if the intrinsic nucleotide-exchange rate were the only factor influencing the quantity of label incorporated (compare lanes 4 and 6). Gzα incorporated very little AA-GTP, regardless of the Mg^{2+} concentration (lanes 7 and 8); increased time of incubation did not enhance photolabeling of this Gα subunit (data not shown). Failure of Gzα to incorporate AA-GTP was not due to an inability to bind guanine nucleotides because GTPγS binding by this preparation could be readily demonstrated (data not shown). Another surprising result from this analysis was that the three Gα subunits which incorporate AA-GTP (Gsα, Goα, and Giα$_2$) required millimolar concentrations of Mg^{2+} for

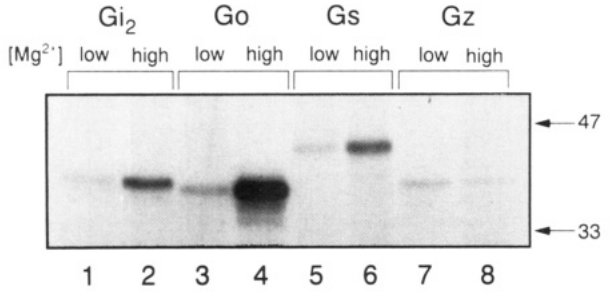

FIGURE 2: Photolabeling of purified G protein α subunits with [^{32}P]-AA-GTP. Equivalent amounts of each G protein α subunit ($\sim$4 pmol quantitated by [^{35}S]GTPγS binding) were incubated with [^{32}P]-AA-GTP (50 nM) for 10 min at 30 °C at either low (150 nM) (lanes 1, 3, 5, and 7) or high (3.5 mM) (lanes 2, 4, 6, and 8) free Mg^{2+} as described in Materials and Methods. After photolysis, proteins were resolved by SDS–PAGE and visualized by autoradiography. The film was exposed for $\sim$3.5 h. The identity of each Gα subunit assayed is indicated above the appropriate lanes. Molecular weight standards in kilodaltons are indicated by the arrows on the *right*.

Table 1: AA-GTP Photolabeling Efficiency[a]

G protein	efficiency (%)	G protein	efficiency (%)
Giα_2	4.3	Gsα	6.1
Goα	5.2	Gzα	10

[a] Efficiency is defined as the ratio of AA-GTP incorporated to AA-GTP bound by each Gα in parallel experiments. See Materials and Methods for details. The numbers represent the mean of three separate determinations.

labeling; little radioactivity was incorporated in the presence of nanomolar concentrations of Mg^{2+}.

Direct Quantitation of AA-GTP Binding to G Protein α Subunits. Since the photolabeling of G proteins by AA-GTP requires binding of the analog by protein, differences in binding of this nucleotide likely account for the differences in photolabeling observed in Figure 2. The high Mg^{2+} requirement for photolabeling of Gα subunits, then, probably reflects a requirement for high concentrations of this metal to support binding of AA-GTP. In contrast, nanomolar concentrations of Mg^{2+} are sufficient to stabilize the binding of GTP and other nonhydrolyzable analogs (GTPγS and GppNHp) by these proteins (Gilman, 1987; Higashijima et al., 1987). Thus, levels of AA-GTP binding by a particular G protein cannot be predicted by that G protein's ability to bind other commonly used guanine nucleotides. To examine this distinction directly, we compared the ability of the four Gα subunits to bind both AA-GTP and GTPγS at a variety of free Mg^{2+} concentrations. For these experiments, extended incubation times were used so that we could directly compare binding ability without regard for the distinct nucleotide-exchange rates of each Gα subunit (see Materials and Methods). The results are shown in Figure 3.

AA-GTP binding by Giα_2 and Goα is clearly dependent on free Mg^{2+} (Figure 3A,B). At high concentrations of Mg^{2+}, the stoichiometry of AA-GTP binding by each of these Gα subunits is similar to their GTPγS-binding capacity; maximal AA-GTP binding for both proteins occurs at $\sim$100 μM Mg^{2+}. In contrast, GTPγS binding for these proteins is essentially independent of Mg^{2+} within the range of concentrations tested. Giα_1 and Giα_3 are nearly identical to Giα_2 in their ability to bind AA-GTP and in the Mg^{2+} sensitivity of this binding (data not shown). Gsα requires substantially higher concentrations of free Mg^{2+} than either Giα_2 or Goα for maximal AA-GTP binding (Figure 3C); significant AA-GTP binding is not detected unless millimolar concentrations of free Mg^{2+} are present. Furthermore, when one compares maximal AA-GTP binding for the Gα subunits, Gsα does not bind AA-GTP as well as either Goα or Giα_2 within the range of free Mg^{2+} concentrations examined. As with Giα_2 and Goα, GTPγS binding by Gsα is largely independent of free Mg^{2+} at levels above 10 nM. For Gzα (Figure 3D), GTPγS binding is suppressed by free Mg^{2+} concentrations above 50 μM, as previously reported (Casey et al., 1990). However, AA-GTP binding by Gzα is detectable only between 30 and 100 μM free Mg^{2+}. Moreover, detectable AA-GTP binding is only a small fraction ($\sim$10%) of the maximal GTPγS-binding capacity for this protein; the combination of Mg^{2+}–suppressible nucleotide exchange and the requirement for high concentrations of this metal to stabilize AA-GTP binding result in this unique condition for AA-GTP binding by Gzα.

AA-GTP Dissociation from Gα Subunits. The findings described above indicate that, in contrast to GTPγS binding, high levels of Mg^{2+} are essential for significant binding of AA-GTP to G protein α subunits. Since the rate-limiting step for nucleotide exchange is GDP release from Gα, it will be the same for either AA-GTP or GTPγS binding. This suggests that the differences observed in AA-GTP binding are due to dissociation of this GTP analog after it has bound. To directly test this, we compared rates of AA-GTP and GTPγS dissociation from Giα_2 and Gsα in the presence of either high (1 mM) or low (100 nM) free Mg^{2+}. Figure 4A,B show the results at low Mg^{2+}. As expected, GTPγS binding to both Giα_2 and Gsα is very stable and consistent with previous results that low nanomolar amounts of Mg^{2+} are sufficient to prevent GTPγS dissociation from G proteins (Higashijima et al., 1987). In contrast, dissociation of AA-GTP from Giα_2 was quite rapid in the presence of 100 nM Mg^{2+} ($t_{1/2} \sim 6$ min), and dissociation of this analog from Gsα was too fast to accurately measure ($t_{1/2} < 1$ min). Consistent with the hypothesis that the high Mg^{2+} requirement for AA-GTP binding to Gα subunits is due to an effect only on dissociation, AA-GTP binding to Giα_2 is stabilized by 1 mM Mg^{2+} (Figure 4D). Quite surprisingly, though, release of AA-GTP from Gsα is still quite fast ($t_{1/2} \sim 2$ min) even at this concentration of Mg^{2+} (Figure 4D). This finding supports the observation in Figure 3 that Gsα requires higher Mg^{2+} for maximal binding of AA-GTP and helps explain the data in Figure 2, where Gsα incorporated much less AA-GTP than expected even at millimolar levels of Mg^{2+}. Thus, unlike binding of GTPγS, AA-GTP binding to Gsα and Giα_2 is readily reversible at nanomolar free Mg^{2+} concentrations. Furthermore, Gsα and Giα_2 differ in their Mg^{2+} requirement for stabilizing AA-GTP binding.

DISCUSSION

AA-GTP is regarded as an important tool for identifying specific receptor-activation of G proteins because it allows this study to be conducted within native plasma membranes. Although some photolabeling experiments have suggested that individual G proteins may vary in their ability to bind AA-GTP (Gordon & Rasenick, 1988; Wong & Martin, 1985; Hatta et al., 1986), surprisingly little has been published that directly addresses this issue. In this study, we have characterized AA-GTP binding by several purified G protein α subunits as a function of Mg^{2+} concentration. One Gα tested, Gz, does not appreciably bind AA-GTP regardless of the free Mg^{2+} concentration. This observation raises the possibility that other G protein α subunits may also be unable to stably bind AA-GTP. The other G protein α subunits tested (Gs, Gi_2, and Go) bind AA-GTP with a Mg^{2+} dependence quite distinct from that of GTPγS binding. Furthermore, the Mg^{2+}

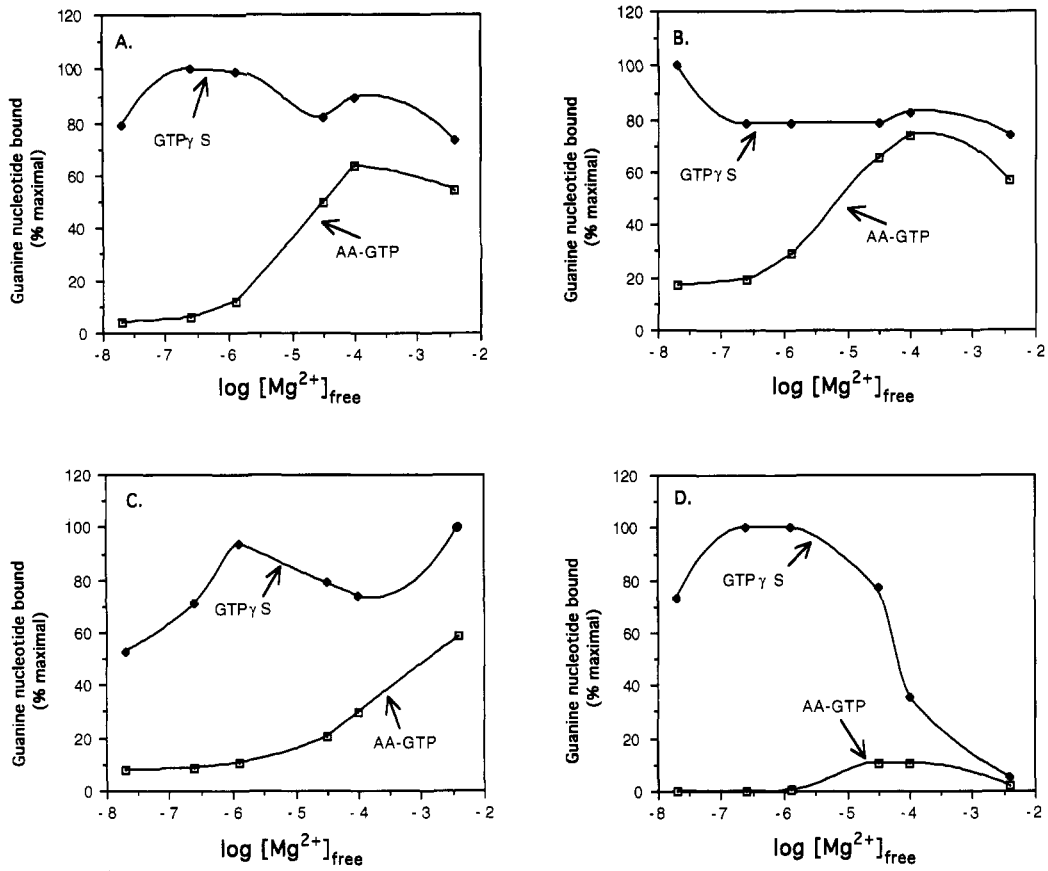

FIGURE 3: Quantitation of AA-GTP binding to purified α subunits. G protein α subunits (~2 pmol) were incubated with either [^{32}P]AA-GTP or [^{35}S]GTPγS (both at 1 μM) at the indicated free Mg^{2+} concentrations. Binding was quantitated by the filter binding assay described in Materials and Methods; 100% binding is defined as the maximum quantity of GTPγS bound (~2 pmol) in each experiment over the entire range of Mg^{2+} concentrations. The other points for both GTPγS and AA-GTP in each experiment are reported as a percentage of this maximum. Each point represents the mean of three separate determinations. Time and temperature conditions were chosen for each protein on the basis of their intrinsic rates of nucleotide exchange: (A) Gi_2, 55 min at 30 °C; (B) Go, 11 min at 20 °C; (C) Gs, 22 min at 20 °C; and (D) Gz, 150 min at 30 °C.

requirement for AA-GTP binding by Gsα is higher than for binding by $Gi\alpha_2$ or Goα; this difference is shown to be due to the inability of even millimolar levels of Mg^{2+} to stabilize bound AA-GTP on Gsα.

These data provide a direct explanation for some of the earlier photolabeling experiments with AA-GTP in native membranes (Offermanns et al., 1990; Gordon & Rasenick, 1988). The influence of Mg^{2+} on photoincorporation of AA-GTP into membranes is a reflection of the requirement of membrane G proteins for this ion to stabilize the binding of AA-GTP. Moreover, the concentration of free Mg^{2+} directly affects the pattern of G protein photolabeling observed in membranes because of the G protein subtype-specific differences in the Mg^{2+} dependence of AA-GTP binding. For example, increases in free Mg^{2+} from ~100 μM to 5 mM would not be expected to affect AA-GTP photolabeling of Giα proteins but would greatly enhance labeling of Gsα simply by stabilizing AA-GTP binding by this protein (see Figures 3 and 4).

The Mg^{2+} requirement for AA-GTP binding is presumably due to the modified structure of the analog relative to GTP's structure and the resultant alteration of interactions between protein, metal, and nucleotide. G protein structural models inferred from the crystal structures of both Ras and transducin-α complexed to GTPγS suggest that Mg^{2+} within the binding pocket of Gα subunits is coordinated to one oxygen each of the β and γ phosphates of GTP (Bourne et al., 1991; Pai et al., 1989; Noel et al., 1993). In addition, the γ phosphate makes several close contacts with the protein. It is plausible to expect then that substitutions at the γ phosphate of GTP may affect nucleotide binding. The substitution of the bulky azidoaniline for an oxygen atom on the γ phosphate of GTP to form AA-GTP could lead to the loss of stabilizing protein–nucleotide interactions or could interfere with protein–Mg^{2+} interactions by displacing the metal.

The observation that there are subtype-specific differences in the Mg^{2+} requirement for AA-GTP binding suggests that the azidoaniline moiety of this nucleotide either perturbs distinct protein–nucleotide–metal interactions in particular Gα subtypes or affects the same interactions differently in different proteins. This implies that the nucleotide-binding pockets of Gα's with different Mg^{2+} requirements for AA-GTP binding are different. For example, since Gsα has a higher Mg^{2+} requirement for AA-GTP binding than both $Gi\alpha_2$ and Goα, there are, perhaps, subtle structural differences in the nucleotide-binding pocket of Gsα relative to that of these other two proteins. However, the residues predicted by the crystal structure of transducin-α to interact with guanine nucleotide, especially the γ phosphate, and Mg^{2+} are strictly conserved among G protein α subunits (Noel et al., 1993). Thus, the differences in AA-GTP-binding behavior observed in this study for Gsα and $Gi\alpha_2$ and the possible differences in the structure of their binding pockets could not be predicted by models inferred from the crystal structure of transducin. In addition, any differences in the binding pockets do not appear to affect the degree of photoincorporation of the nucleotide, as photolabeling efficiency was similar for each protein in this study (see Table 1). While it appears that Gzα

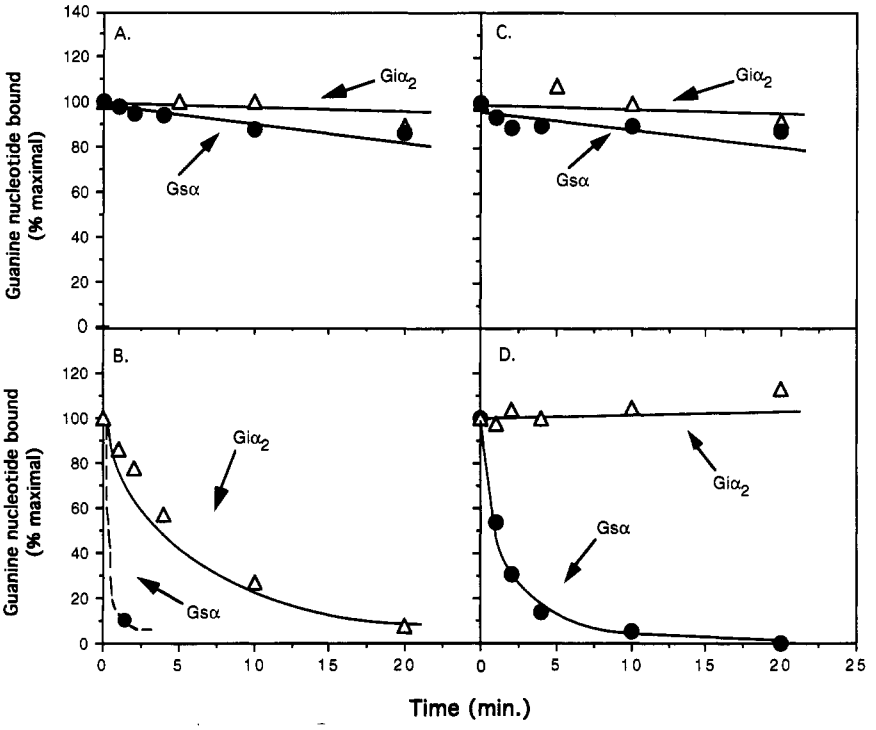

FIGURE 4: Dissociation of nucleotide from $Gi\alpha_2$ and $Gs\alpha$. Experiments were performed as described in Materials and Methods. $G\alpha$ protein (~10–15 pmol) was incubated with either [^{32}P]AA-GTP or [^{35}S]GTPγS (both at 1 μM) in a volume of 420 μL at 1 mM free Mg^{2+} for either 55 min at 30 °C ($Gi\alpha_2$) or 22 min at 20 °C ($Gs\alpha$). At the end of the incubation (designated time zero), the reaction mix was diluted with an equal volume of reaction buffer containing 1 mM GDP; for the experiments in A and B, additional EDTA sufficient to lower the free Mg^{2+} concentration to 100 nM was included in the dilution. Aliquots of 120 μL were withdrawn at various time points, and nucleotide binding was quantitated by the filter binding assay described in Materials and Methods; 100% binding was defined as the quantity of nucleotide bound at time zero (~1.5–2 pmol). Each point represents the mean of three separate determinations: (A) GTPγS dissociation at low (100 nM) free Mg^{2+}; (B) AA-GTP dissociation at low (100 nM) free Mg^{2+}; (C) GTPγS dissociation at high (1 mM) free Mg^{2+}; and (D) AA-GTP dissociation at high (1 mM) free Mg^{2+}.

may be slightly different in this regard, the higher measured efficiency for this $G\alpha$ probably reflects error introduced into the calculation by the use of AA-GTP-binding stoichiometry as the denominator; this stoichiometry is very low for $Gz\alpha$ (see Figure 3).

The Mg^{2+} requirement for AA-GTP binding has unusual consequences for $Gz\alpha$. For this protein, nucleotide exchange is suppressed by increasing Mg^{2+}. The mechanism by which Mg^{2+} suppresses nucleotide exchange on $Gz\alpha$ is not clear, but it is known that the effect is exerted at the GDP-dissociation step (Casey et al., 1990). Thus, as the Mg^{2+} concentration is raised to levels which might stabilize AA-GTP binding, nucleotide exchange on $Gz\alpha$ is inhibited with the net result that little analog binding is observed.

Another important aspect of the interaction of this analog with G proteins is that $G\alpha$ subunits can bind it reversibly. Binding of GTP and closely related analogs like GTPγS by G protein α subunits is extremely stable in the presence of even nanomolar concentrations of Mg^{2+} (Gilman, 1987; Higashijima et al., 1987) (and see Results). By contrast, dissociation of AA-GTP can be relatively rapid. In addition, dissociation of AA-GTP varies among individual $G\alpha$ proteins. AA-GTP binding to $Gi\alpha_2$ was quite stable at millimolar concentrations of Mg^{2+} but was readily reversible at nanomolar concentrations of Mg^{2+}, while AA-GTP dissociation from $Gs\alpha$ was rapid even at millimolar concentrations of Mg^{2+}. This kind of variability among $G\alpha$ subunits could be a problem when one is attempting to identify receptor-activated G proteins using AA-GTP. This study clearly demonstrates that the amount of incorporation of AA-GTP into some G protein α subunits (Gs and Gz, for example) will not necessarily reflect the level of their activation relative to that of other $G\alpha$ subunits (Gi and Go, for example). Thus, while AA-GTP is a valuable tool for identifying GTP-binding proteins and specifically receptor-activated G proteins, the variability in its interactions with G protein α subunits must be considered when designing experiments involving its use.

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