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Kinetic Control of Guanine Nucleotide Binding to Soluble $G\alpha_q$

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ABSTRACT. Binding of guanine nucleotides to heterotrimeric G proteins is controlled primarily by kinetic factors, such as the release of bound GDP, rather than by affinity alone. Detergent-solubilized $G\alpha_q$ displays unusual guanine nucleotide binding properties in comparison with other G protein α subunits. Under conditions where most G proteins bind nearly stoichiometric GTPγS in 5–30 min at micromolar nucleotide concentrations, GTPγS binding to $G\alpha_q$ is slow (>1 hr to completion), markedly substoichiometric, and dependent upon high concentrations of nucleotide (0.1 to 0.2 mM). Although the latter two properties suggest low affinity, GTPγS dissociation is immeasurably slow under commonly used conditions. We found that purified $G\alpha_q$ can bind stoichiometric GTPγS, but that binding is controlled kinetically by a combination of factors. GDP (or IDP) dissociated slowly from $G\alpha_q$, but the dissociation rate increased linearly with the concentration of $(NH_4)_2SO_4$ up to 0.75 M (~20-fold acceleration). The resulting GDP-free $G\alpha_q$ was labile to rapid and irreversible denaturation, however (rate constant $\geq 1\ min^{-1}$ at 20°). Denaturation competed kinetically with relatively slow GTPγS association, such that stoichiometric binding was only attained at 100 μM GTPγS. These findings reconcile the slowly reversible binding of GTPγS to $G\alpha_q$ with the other behaviors that suggested lower affinity, and point out that events subsequent to GDP dissociation can markedly influence the rates and extents of guanine nucleotide binding to G protein α subunits. Understanding these interactions allowed the direct, accurate quantitation of active $G\alpha_q$ by a simple GTPγS binding assay in the presence of $(NH_4)_2SO_4$, and similarly can prevent underestimation of the concentrations of other G proteins. BIOCHEM PHARMACOL **58**;1: 39–48, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. GTP-binding protein; guanosine 5′-*O*-thiotriphosphate; $G\alpha_q$; G protein

Members of the G_q family of heterotrimeric G proteins (G_q, G_{11}, G_{14}, and $G_{15/16}$) convey signals from cell surface receptors to phospholipase C-β and, perhaps, other intracellular effectors. As with other G proteins, G_q is activated by GTP binding and is deactivated when it hydrolyzes bound GTP to GDP. Receptors initiate G_q signaling by promoting dissociation of GDP from the G_q α subunit and subsequent binding of GTP.

In contrast to most other G proteins, the ability of G_q and G_{11} to exchange guanine nucleotides is diminished drastically by their solubilization from membranes. The rate of activation of soluble G_q by GTPγS¶ is slow [1, 2], and significant activation of purified $G\alpha_q$ by GTPγS typically requires more than 10 μM nucleotide, over 100-fold more than is needed in membranes [1]. Direct measurement of [^{35}S]GTPγS binding to solubilized G_q has yielded similar results. Blank *et al.* [1] detected little or no binding of [^{35}S]GTPγS to $G\alpha_q$ under assay conditions considered standard for other G proteins (i.e. 100 nM GTPγS), and Pang and Sternweis [3] observed variable and substoichiometric binding (generally < 20%) at 1–3 μM [^{35}S]GTPγS. Using purified recombinant $G\alpha_q$ produced in Sf9 cells, Hepler *et al.* [2] established conditions under which G_q could be shown to bind about 0.6 mol of [^{35}S]GTPγS/mol of total protein, although this stoichiometry was back-calculated to correct for 80–90% loss of G_q in the assay. The binding reaction required incubation for 90 min at 30° with 200 μM [^{35}S]GTPγS to reach completion. $G\alpha_q$-GTPγS also had to be isolated by rapid gel filtration rather than the usual adsorption of protein to nitrocellulose. Although such determinations were reproducible, the combination of apparent low affinity (the need for 0.2 mM GTPγS), slow binding even at a high concentration of ligand, and relative stability of binding is not reconciled readily with a simple ligand binding equilibrium.

The unusual nucleotide binding characteristics of purified G_q do not indicate its denaturation during solubilization. Soluble G_q can be activated by Al^{3+}/F^- under conditions where binding of [^{35}S]GTPγS is undetectable [4], and nucleotide binding is returned to normal after G_q is

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¶ *Abbreviation*: GTPγS, guanosine 5′-*O*-thiotriphosphate.

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co-reconstituted into phospholipid vesicles with receptor and Gβγ [5, 6]. Such behavior is also not unique to $G\alpha_q$; similar difficulties were reported for $G\alpha_{13}$ [7], and it has been impossible to demonstrate the binding of reasonable quantities of GTPγS or other nucleotides to soluble $G\alpha_t$ [8–10].

The anomalous guanine nucleotide binding behavior of soluble $G\alpha_q$ results, in part, from the slow rate of dissociation of $G\alpha_q$-bound GDP, as is the case for other G proteins [11]. In this study, we used $(NH_4)_2SO_4$ to accelerate dissociation of bound GDP from $G\alpha_q$ [11–13]. $(NH_4)_2SO_4$ promotes nucleotide release from Gα subunits in much the same pattern as observed for receptor-mimetic peptides and long-chain organic amines [14], but its greater solubility makes its effects easier to control. This approach allowed us to analyze the complex GTPγS binding properties of $G\alpha_q$ as a combination of rate-limiting GDP release followed by the competing reactions of GTPγS binding to unliganded $G\alpha_q$ or $G\alpha_q$ denaturation. The data explain the unusual association kinetics and the difficulty in achieving stoichiometric binding, and their analysis allowed development of a feasible direct GTPγS binding assay to measure active soluble $G\alpha_q$.

MATERIALS AND METHODS

Mouse $G\alpha_q$ subunit was expressed in Sf9 cells and purified as described previously [6]. The total amount of purified $G\alpha_q$ protein was estimated by amido black staining using bovine serum albumin as the standard [15]. This value was used to calculate the molar concentration of total $G\alpha_q$ referred to throughout, although it does not distinguish active and denatured protein. The concentration of active $G\alpha_q$ was estimated originally according to the amount of $G\alpha_q$-bound GDP [5] using previously described modifications of the competitive GDP binding assay of Ferguson *et al.* [11]. Assay of active $G\alpha_q$ by direct GTPγS binding is described in the text.

[^{35}S]GTPγS and [α-^{32}P]GTP were purchased from NEN. [α-^{32}P]ITP was synthesized by incubating [α-^{32}P]GTP with 0.8 M $NaNO_2$ in 5 M acetic acid at 0° for 16 hr [16]. The reaction proceeded essentially to completion. After evaporation of the solvent, the product was purified from [α-^{32}P]IDP by ion exchange HPLC [6]. Genapol 24-L-75 (dodecyl, tetradecyl-polyethyleneoxide, N = 8.3) was a gift from Hoechst-Celanese. Sources of other material have been described [6].

[^{35}S]GTPγS binding assays were carried out at 20° in buffer A [50 mM sodium HEPES (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 0.9 mM $MgSO_4$ (10 μM calculated free Mg^{2+}), 0.05% Genapol]. The concentration of $(NH_4)_2SO_4$ in the assay was varied as indicated using a pH-buffered stock solution. The concentration of $G\alpha_q$ was 0.1 to 0.2 μM, calculated according to total protein. The concentration of [^{35}S]GTPγS in each experiment is specified in the legend or text. Both the pH and the concentration of Mg^{2+} were optimized in preliminary experiments. The binding kinetics described here and the effects of $(NH_4)_2SO_4$ on GTPγS binding were similar over a broad optimal range of pH (6.5 to 8.0) and Mg^{2+} concentrations (1–100 μM) (data not shown). The temperature was chosen to optimize the rate of binding but minimize the rate of denaturation. The stability of $G\alpha_q$, either GDP-bound or unliganded, was not improved in the presence of several other detergents (cholate, CHAPS, or octyl glucoside), at lower concentrations of Lubrol, or in the presence of added glycerol. Binding reactions usually were carried out in a total volume of 200–600 μL, and were stopped at the times indicated by the transfer of two or more 20- or 30-μL aliquots to 100 μL of an ice-cold solution containing 20 mM Tris–Cl (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM GTP, 0.1 mM dithiothreitol, and 0.1% Genapol, followed by adsorption of $G\alpha_q$ to BA85 nitrocellulose membranes as described previously [17].

IDP-liganded $G\alpha_q$ was prepared by incubating purified $G\alpha_q$ (0.9 μM) for 24–26 hr in buffer A that contained 10 mM ITP and 50 mM $(NH_4)_2SO_4$. Free nucleotide and $(NH_4)_2SO_4$ were removed just before use by centrifugal gel filtration on Sephadex G-25 in buffer A. Control experiments (not shown) indicated that the concentration of residual free IDP was less than 5% that of $G\alpha_q$-IDP. In figures that show GTPγS binding to initially IDP-bound $G\alpha_q$, data points represent single determinations.

The time-dependent binding of GTPγS to $G\alpha_q$ shown in Figs. 1 and 4 was analyzed according to the integrated rate equation derived for Scheme I (Equation 1, Appendix). Because k_3 and k_5 could not be independently resolved (see below), k_5 was set to 10 min^{-1} (see text). Values for k_1 and k_3 were allowed to float. Values for k_4, the rate constant for GTPγS dissociation, were taken from the regression line shown in Fig. 2B. Nonlinear least-squares fits used the Marquardt-Levenberg routine in the SigmaPlot program package (Jandel Scientific).

Nucleotide dissociation was measured at 20° in the presence of various concentrations of $(NH_4)_2SO_4$ by mixing $G\alpha_q$-bound nucleotide with unlabeled nucleotide to yield a 100- to 1000-fold isotopic dilution and up to a 10-fold volume dilution. Duplicate samples were removed and quenched as described for the binding assays. For dissociation assays, [^{35}S]GTPγS was bound by incubating $G\alpha_q$ in buffer A, either with 10 μM [^{35}S]GTPγS in the presence of 100 mM $(NH_4)_2SO_4$ or with 100 μM [^{35}S]GTPγS in the absence of $(NH_4)_2SO_4$. [α-^{32}P]GTP was bound to $G\alpha_q$ under similar conditions, either with 10 μM [α-^{32}P]GTP in the presence of 50 mM $(NH_4)_2SO_4$ or with 30 μM [α-^{32}P]GTP in the absence of $(NH_4)_2SO_4$. Binding reactions were carried out at 20° for 9 hr in the presence of $(NH_4)_2SO_4$ or for 17–21 hr in the absence of $(NH_4)_2SO_4$. [α-^{32}P]ITP (1 mM) was bound in the presence of 100 mM $(NH_4)_2SO_4$ for 2–3 hr. Dissociation was first-order and complete (Fig. 2A and data not shown), and dissociation rates did not vary whether radioligand was initially bound in the presence or absence of $(NH_4)_2SO_4$.

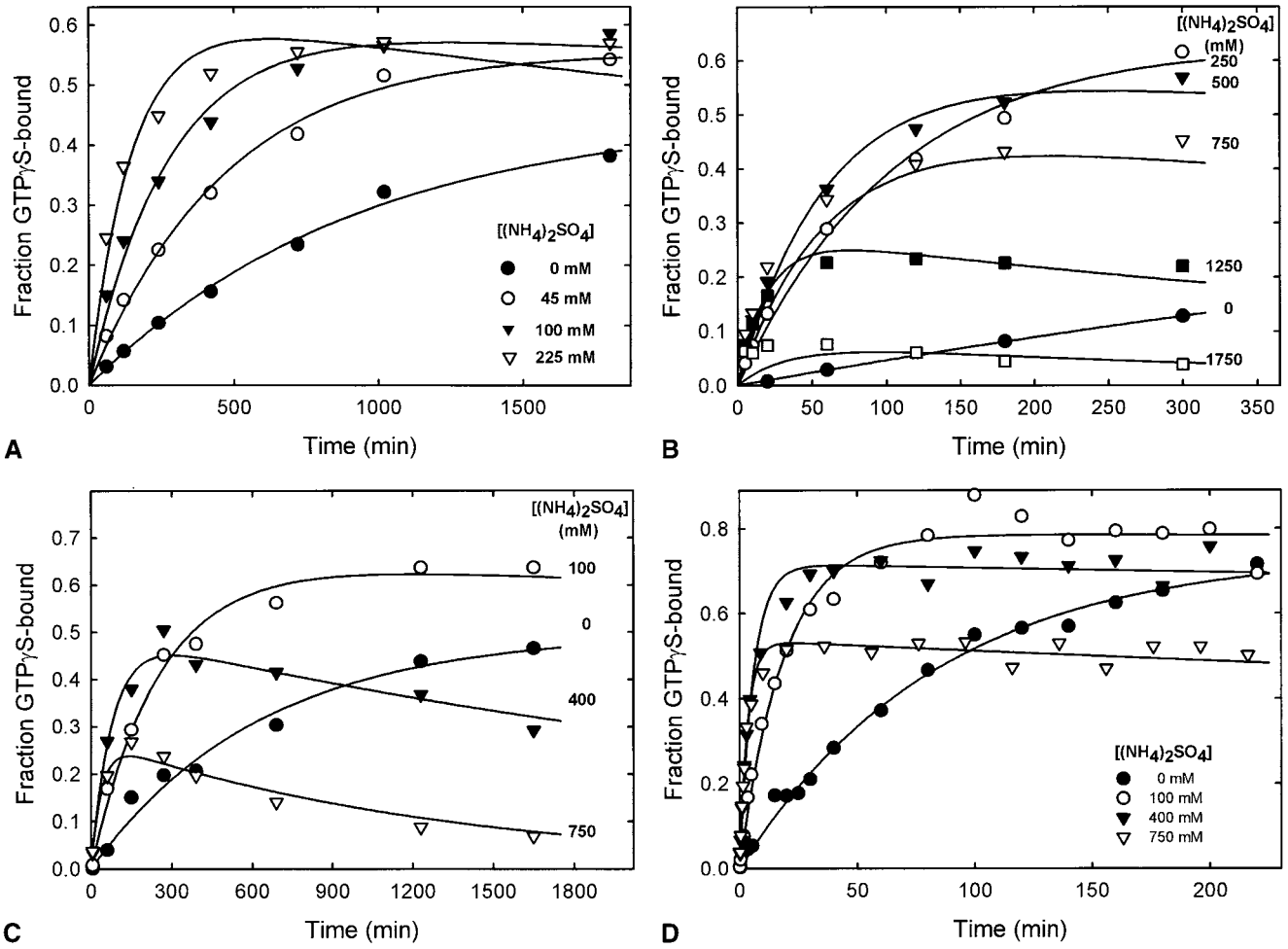


FIG. 1. Effects of $(NH_4)_2SO_4$ on the time-dependent binding of [^{35}S]GTPγS to $G\alpha_q$. Binding of 10 μM [^{35}S]GTPγS to $G\alpha_q$ was measured at 20° over several time intervals in the presence of varied concentrations of $(NH_4)_2SO_4$. In panels A–C, $G\alpha_q$ was bound initially to GDP. In panel D, $G\alpha_q$ initially was bound to IDP. The concentration of $G\alpha_q$ was 80–140 nM according to bound GDP (panels A–C) or estimated by assuming that binding was 80% complete at the maximum (panel D). Data points, expressed as the fraction of total $G\alpha_q$ bound to GTPγS, are averages of duplicate measurements representative of numerous similar experiments. The drawn lines show fits of the data to Equation 1 using values for k_4 taken from Fig. 2B and $k_5 = 10$ min^{-1}. Values for for k_1 and k_3 derived from the fits are shown in Fig. 4.

RESULTS AND DISCUSSION

Effects of $(NH_4)_2SO_4$ on the Binding of [^{35}S]GTPγS to $G\alpha_q$

$G\alpha_q$ exchanges nucleotides very slowly, it binds less than stoichiometric nucleotide after apparent completion of the binding reaction, and binding requires high concentrations of added nucleotide. Essentially all of these properties are altered by $(NH_4)_2SO_4$. $G\alpha_q$ bound 10 μM [^{35}S]GTPγS slowly in the absence of $(NH_4)_2SO_4$, but $(NH_4)_2SO_4$ accelerated binding over 20-fold (Fig. 1, A and B). The initial rate of [^{35}S]GTPγS binding increased linearly with $(NH_4)_2SO_4$ concentrations up to about 500 mM. At higher $(NH_4)_2SO_4$ concentrations, the apparent initial rate of binding reached a maximum and then declined, at least in part because accumulation of bound GTPγS terminated quickly, and true initial rates could not be observed.

$(NH_4)_2SO_4$ also increased the maximal amount of $G\alpha_q$-bound GTPγS, and this effect also was biphasic (Fig. 1, C and D). In the absence of $(NH_4)_2SO_4$, only about 55% of active $G\alpha_q$ bound [^{35}S]GTPγS even at long times (Fig. 1A, and other experiments extended up to 48 hr). Active $G\alpha_q$ is defined according to assays of $G\alpha_q$-GDP, assuming one molecule of GDP bound per active $G\alpha_q$ [5, 11]. Addition of 50–200 mM $(NH_4)_2SO_4$ increased this maximum to 80% of active G_q at 10 μM GTPγS (80 ± 24%, N = 5; Fig. 1A). Higher concentrations of $(NH_4)_2SO_4$ did not further increase binding at this concentration of [^{35}S]GTPγS, however. The maximum amount of bound GTPγS decreased as the $(NH_4)_2SO_4$ concentration was raised above 250 mM, such that < 25% of $G\alpha_q$ bound GTPγS above 1 M $(NH_4)_2SO_4$ (Fig. 1B). We have not pursued the effects of very high concentrations of $(NH_4)_2SO_4$ (>1 M), however, because of uncertainties about protein solubility.

The third effect of $(NH_4)_2SO_4$ on GTPγS binding was to shorten the period over which $G\alpha_q$-bound [^{35}S]GTPγS accumulated. As the concentration of $(NH_4)_2SO_4$ was

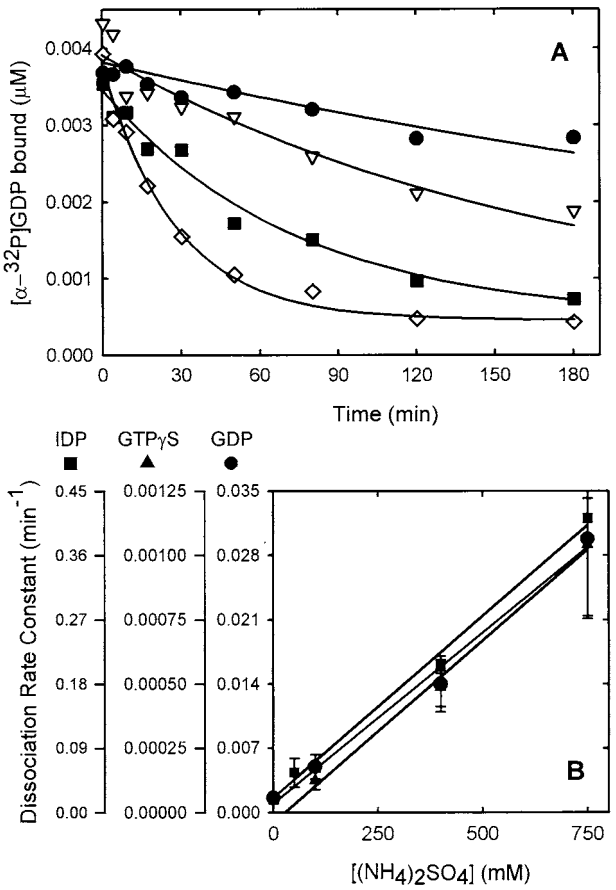


FIG. 2. Dissociation of GDP, GTPγS, and IDP from $G\alpha_q$. (A) [α-^{32}P]GTP was incubated with $G\alpha_q$ as described in Materials and Methods, and dissociation of bound [α-^{32}P]GDP was then monitored in the presence of 0 (●), 100 (▽), 400 (■) or 750 (◇) mM $(NH_4)_2SO_4$. The lines show nonlinear least-squares fits to single exponential decay functions. To fit the data obtained at 750 mM $(NH_4)_2SO_4$, maximal dissociation was allowed to float, and the value obtained from the fit was 93% of the initial amount bound. For data obtained at the other three concentrations of $(NH_4)_2SO_4$, where dissociation was incomplete at the longest time shown, the terminal value was arbitrarily constrained to 93%. Variability among the zero-time points reflects error in transfer of small volumes of the first incubation into the larger dissociation volume, such that the absolute amounts of GDP bound cannot be compared precisely among the four curves. (B) Dependence of dissociation rates on the concentration of $(NH_4)_2SO_4$. The first-order dissociation rate constants for all three nucleotides are shown, with standard deviations for each determination. Dissociation rate constants for IDP and GTPγS were determined as described above for GDP. Dissociation of GTPγS could not be observed in the absence of $(NH_4)_2SO_4$, and the line is extrapolated to the axis.

increased, the time to maximum binding decreased from about 1500 to 12 min. Thus, while initial rates of GTPγS binding were relatively high between 0.2 and 1 M $(NH_4)_2SO_4$, accumulation of bound GTPγS terminated prematurely (Fig. 1, B–D).

Last, concentrations of $(NH_4)_2SO_4$ above 0.4 M caused the ultimate loss of $G\alpha_q$-bound GTPγS after the binding reaction had reached its maximum (Fig. 1C). At long times and at high $(NH_4)_2SO_4$ concentrations, bound GTPγS declined to background. The initial accumulation of bound GTPγS followed by its loss, all in the presence of excess free GTPγS, is inconsistent with a simple approach to equilibrium and indicates the participation of at least two distinct reaction pathways.

Dissociation of Guanine Nucleotides from $G\alpha_q$

$(NH_4)_2SO_4$ accelerates GTPγS binding to $G\alpha_q$ primarily by promoting the dissociation of bound GDP [11, 12]. G proteins bind guanine nucleotides tightly, and they are purified with 1 mol/mol of bound GDP. The binding of labeled nucleotides to purified G proteins therefore follows, and is kinetically limited by, the release of bound GDP [11] (this has been confirmed for $G\alpha_q$ and $G\alpha_{11}$ by Berstein *et al.* [5]). Figure 2A shows that dissociation of GDP from $G\alpha_q$ in solution was slow under the conditions used here (k_{diss} $\sim 1.6 \times 10^{-3}$ min^{-1}), but was accelerated by $(NH_4)_2SO_4$, increasing about 20-fold by 750 mM $(NH_4)_2SO_4$ (Fig. 2). Because the rate constant for GDP dissociation was about equal to the initial rate of GTPγS binding over the range 0–400 mM $(NH_4)_2SO_4$ (compare Figs. 1 and 2B), it seemed likely that GTPγS binding was limited simply by GDP dissociation. However, dissociation of bound GDP was complete, but GTPγS bound to ~80% of the newly available sites. Therefore, some fraction of the unliganded $G\alpha_q$ produced by GDP dissociation either was intrinsically unable to bind GTPγS or was inactivated rapidly before GTPγS binding could occur.

GTPγS Binding to Initially IDP-Liganded $G\alpha_q$

To evaluate the importance of GDP dissociation to the overall kinetics of GTPγS binding, we compared the binding of GTPγS to GDP-bound $G\alpha_q$ with binding to IDP-bound $G\alpha_q$. Inosine nucleotides bind G proteins with lower affinity than do the cognate guanine nucleotides, and IDP dissociation from $G\alpha_q$ was about 15-fold faster than GDP at all $(NH_4)_2SO_4$ concentrations (0 to 0.75 M) (Fig. 2B). $(NH_4)_2SO_4$ accelerated IDP dissociation to the same relative extent as for GDP (linear increase to about 25-fold).

As predicted by the IDP dissociation rates, GTPγS bound to $G\alpha_q$-IDP about 15-fold faster than to $G\alpha_q$-GDP. Apparent initial rates of binding increased linearly with the concentration of $(NH_4)_2SO_4$ as predicted by the increase in the IDP dissociation rate constant. The families of binding curves in panels B and D of Fig. 1 are thus strikingly similar except for the shorter reaction times for $G\alpha_q$-IDP. Although the loss of bound GTPγS at 400 and 750 mM $(NH_4)_2SO_4$ seems to be less in the $G\alpha_q$-IDP experiments because of the difference in time scales, the calculated downward terminal slopes are about the same as when $G\alpha_q$-GDP was used.

Dissociation of GTPγS from $G\alpha_q$

The eventual loss of bound GTPγS at high $(NH_4)_2SO_4$ concentrations suggested that GTPγS dissociates from $G\alpha_q$ at an appreciable rate. Although GTPγS dissociation could not be detected in the absence of $(NH_4)_2SO_4$ (data not shown), $(NH_4)_2SO_4$ accelerated dissociation over the same range of concentrations that stimulated GTPγS binding (Fig. 2B). The $(NH_4)_2SO_4$-promoted dissociation of GTPγS from $G\alpha_q$ was fast enough to account for the decline in bound GTPγS that was observed at high concentrations of $(NH_4)_2SO_4$ (compare downward slopes in Fig. 1C with dissociation rates in Fig. 2B). The similarity of the two rates suggests that the loss of bound GTPγS reflects initial dissociation of GTPγS followed by more rapid inactivation of the unliganded $G\alpha_q$.

Rapid Denaturation of Unliganded $G\alpha_q$

To evaluate the rate of binding of GTPγS to $G\alpha_q$ after GDP dissociation, we tried to prepare unliganded $G\alpha_q$. However, unliganded $G\alpha_q$ denatured essentially as soon as it was produced. Incubation of active $G\alpha_q$ in the absence of added nucleotide caused its denaturation at rates that were identical within experimental error to the previously measured rates of dissociation of GDP or IDP (compare Figs. 3B and 2B). Thus, the rate of denaturation of unliganded $G\alpha_q$ is at least 0.4 min^{-1} at 20°, the fastest dissociation rate measured for IDP. We also attempted to detect unliganded $G\alpha_q$ by adding [^{35}S]GTPγS immediately after $(NH_4)_2SO_4$-stimulated dissociation of GDP or IDP. These experiments did not produce the burst of rapid GTPγS binding that would have indicated the presence of active, unliganded $G\alpha_q$. Data from these experiments (not shown) indicated that the denaturation rate constant for unliganded $G\alpha_q$ must be well above 1 min^{-1}. We could not stabilize free $G\alpha_q$ significantly by manipulating detergent, pH, ionic strength, or other conditions.

Kinetic Mechanism of Guanine Nucleotide Binding to $G\alpha_q$

The data of Figs. 1 and 2 suggest that the rate of GTPγS binding to $G\alpha_q$ is kinetically limited by dissociation of bound GDP (or IDP), as is the case for other G proteins [11]. However, an overall mechanism must account for terminally substoichiometric binding and for the subsequent loss of bound GTPγS. The high concentration of GTPγS that is needed to drive significant binding [2] (and below), which suggests low affinity, must also be reconciled with the slow dissociation of GTPγS, which suggests very high affinity. To explain the nucleotide binding behavior of $G\alpha_q$ and its regulation by $(NH_4)_2SO_4$, we propose that the overall nucleotide exchange reaction is limited by the initial dissociation of bound GDP (or IDP), but that a significant fraction of unliganded $G\alpha_q$ denatures rather than binding free nucleotide.

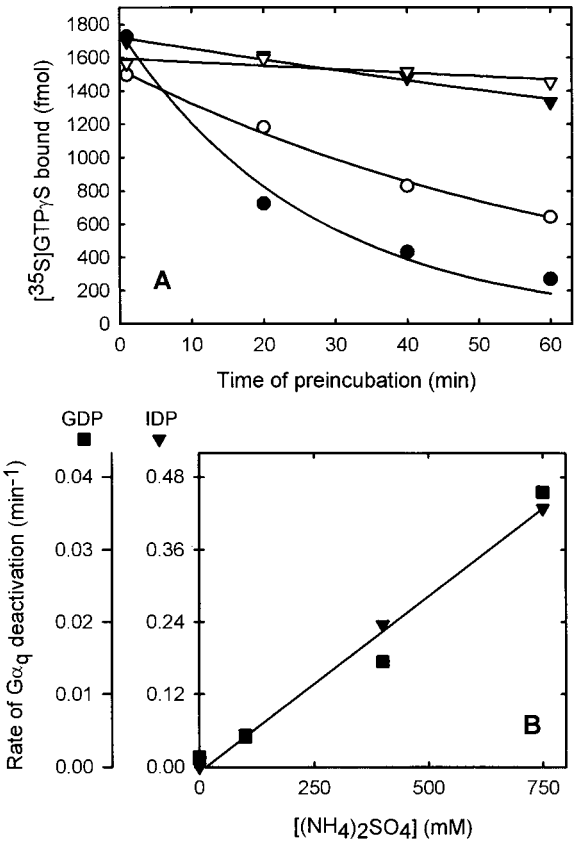
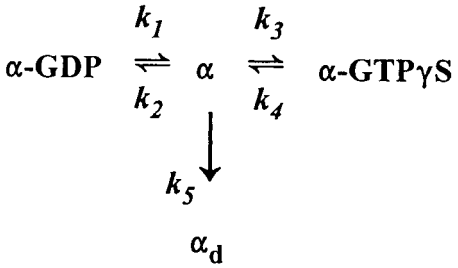


FIG. 3. Inactivation of unliganded $G\alpha_q$. (A) $G\alpha_q$-GDP (200 nM according to total protein) was incubated at 20° in buffer A, which contained 0 (▽), 0.1 (▼), 0.4 (○), or 0.75 (●) M $(NH_4)_2SO_4$. At the times indicated, remaining GTPγS binding capacity was assayed by diluting 20-μL aliquots into binding assay fluid to yield a final concentration of 10 μM [^{35}S]GTPγS and 400 mM $(NH_4)_2SO_4$. Bound GTPγS was determined after further incubation for 4 hr at 20°. Solid lines show best fits to a single exponential decay to zero. (B) Rate constants for the loss of active $G\alpha_q$ were obtained from nonlinear fits of the data in part A and of data from similar experiments performed using $G\alpha_q$-IDP. Lines in panel B show linear least-squares fits.

These reactions, shown in Scheme I, are similar to others commonly used to describe nucleotide binding to G proteins, notably the rate-limiting dissociation of bound GDP. ($G\alpha_q$ is shown simply as α.) Dissociation of GTPγS, which is frequently ignored, is included because it is significant in the presence of $(NH_4)_2SO_4$ (Fig. 2B). Dissociation rate constants for GDP (IDP) and GTPγS, k_1 and k_4, respectively, are all determined by the concentration of



SCHEME 1

$(NH_4)_2SO_4$, as shown in Fig. 2B. Although rebinding of GDP is shown (k_2), it is insignificant because the maximum concentration of free GDP is much lower than that of GTPγS and because GTPγS binds with higher affinity.

The novel feature of Scheme I that allows it to explain the multiphasic binding time courses is its explicit consideration of the irreversible inactivation of unliganded $G\alpha_q$ (k_5; α_d refers to denatured $G\alpha_q$). Unliganded $G\alpha_q$ denatures unusually rapidly for a Gα subunit (see below), and denaturation of unliganded $G\alpha_q$, therefore, competes significantly with the binding of GTPγS. This consideration allows description of both substoichiometric binding and the eventual loss of bound GTPγS. Note that the inclusion of irreversible denaturation means that accumulation of G_q-bound GTPγS is determined kinetically and is not an approach to equilibrium.

Scheme I can be used to formulate an integrated rate equation for the formation of $G\alpha_q$- GTPγS according to the experimental constraints that apply to $G\alpha_q$ (see Appendix):

$$[\alpha\text{-GTP}\gamma\text{S}]_t = \frac{Z \cdot k_1}{k_1 + (Z-1)\cdot k_4} \cdot \{e^{-k_4 \cdot (1-Z)\cdot t} - e^{-k_1 \cdot t}\} \quad (1)$$

where

$$Z = \frac{k_3 \cdot [\text{GTP}\gamma\text{S}]}{k_3 \cdot [\text{GTP}\gamma\text{S}] + k_5} \quad (2)$$

Equation 1 describes the accumulation of $G\alpha_q$-GTPγS in terms of two exponentials: an ascending limb whose rate is dependent on the dissociation of GDP (k_1) and a later descending limb whose rate is dependent on the slower dissociation of GTPγS (k_4). Both processes produce unliganded $G\alpha_q$. The ratio Z describes the fraction of unliganded $G\alpha_q$ that binds GTPγS relative to the fraction that irreversibly denatures. Fractional binding is thus determined coordinately by k_3, k_5, and the concentration of GTPγS. The initial rate of binding of GTPγS is approximately equal to $k_1 \cdot [G\alpha_q\text{-GDP}] \cdot Z$. GDP dissociation is slow and rate limiting, but not all free $G\alpha_q$ binds GTPγS. Because k_1 increases linearly with the concentration of $(NH_4)_2SO_4$ (Fig. 2B), so does the initial rate of GTPγS binding (Fig. 1A). Rates of GTPγS binding to $G\alpha_q$-GDP and to $G\alpha_q$-IDP also differ appropriately according to the dissociation rates of the two nucleotides. Values of the dissociation rate constants for GDP, IDP, and GTPγS derived from fitting [^{35}S]GTPγS binding data (Fig. 4) were all consistent with those derived directly from dissociation data (Fig. 2B). At longer times, dissociation of GTPγS from $G\alpha_q$ continued to produce unliganded $G\alpha_q$ after all of the initially bound GDP has dissociated. $(NH_4)_2SO_4$ thereby promotes the slow terminal loss of bound GTPγS at a rate equal to $[G\alpha_q\text{-GTP}\gamma\text{S}] \cdot k_4 \cdot (1 - Z)$. Behavior at intermediate times, including the maximum amount of GTPγS bound, is more complicated but is influenced similarly by Z (see Appendix). Scheme I, therefore, seemed to provide a reasonable hypothetical framework for analyzing GTPγS binding.

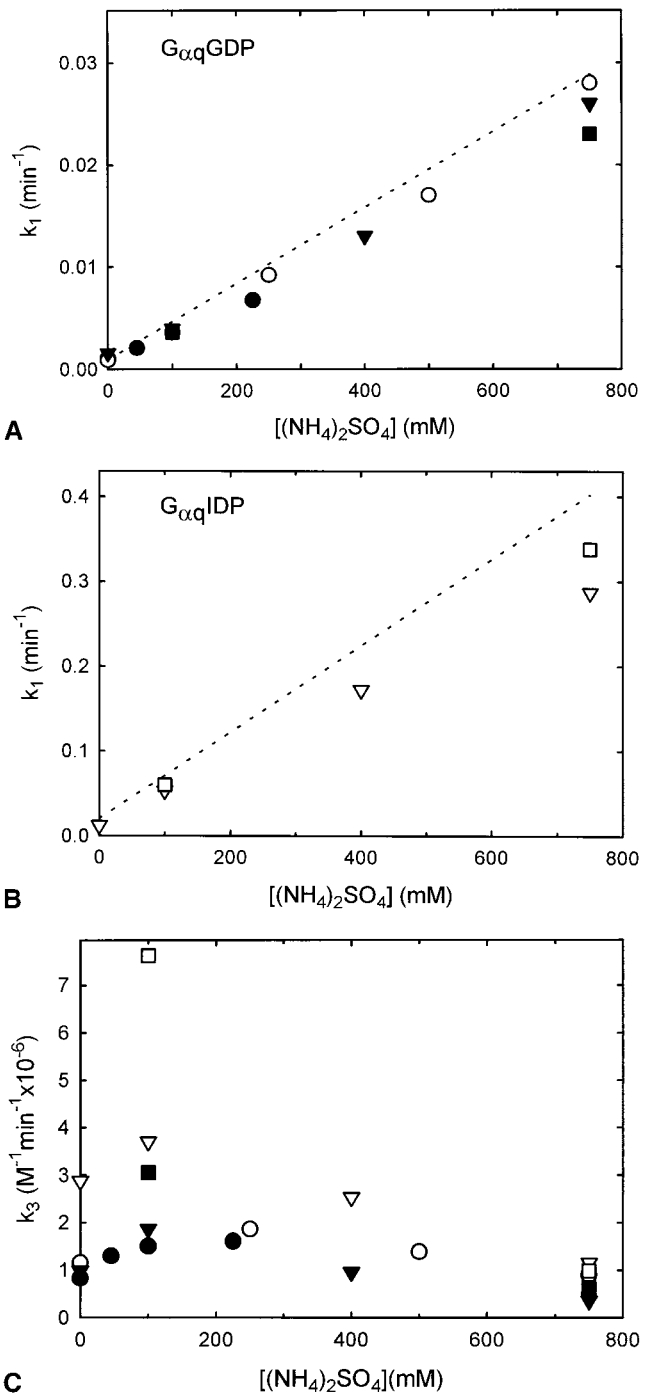


FIG. 4. Association and dissociation rate constants from GTPγS binding time courses. Values of the rate constants k_1 for GDP (A) and IDP (B) and for k_3 (C) obtained from fits of Equation 1 to the GTPγS binding data in Figs. 1 and 5. For all fits, k_5 was set to 10 min^{-1} and k_4 was set to the dissociation constants shown in Fig. 2B for the appropriate $(NH_4)_2SO_4$ concentration. For comparison, the dotted lines in panels A and B show the fits to the actual dissociation constants, and are taken directly from Fig. 2B. Parameters shown are for Figs. 1A (●), 1B (○), 1C (▼), 1D (▽), 5A, B (■), and 5C, D (□).

Quantitative Analysis of Time-Dependent GTPγS Binding

To test the applicability of Scheme I to the experimental binding data, Equation 1 was used to fit the binding time courses shown in Fig. 1. As shown, the fitted curves closely approximated the multiphasic GTPγS binding behavior of $G\alpha_q$. Initial rates, maxima, terminal loss of bound GTPγS, and the dependences of these behaviors on the concentration of $(NH_4)_2SO_4$ were all described reasonably well. In addition, values of the dissociation rate constants for GDP and IDP derived from the fits agreed well with the values determined directly (compare Fig. 2B with panels A and B of Fig. 4). In these fits, values for k_4, the rate constant for dissociation of GTPγS, were taken from Fig. 2B rather than being allowed to float because k_4 is only significant at long times and at high concentrations of $(NH_4)_2SO_4$. Without a downward limb to the curve, k_4 is not constrained by the data. However, values of k_4 taken from Fig. 2B described the loss of bound GTPγS well in those experiments where it occurred [Figs. 1 (B and C) and 4B], and allowing k_4 to float in these cases produced fitted values in good agreement with those determined directly (data not shown).

Equation 1 links the association rate constant for GTPγS (k_3) with the denaturation rate constant for unliganded $G\alpha_q$ (k_5) as the ratio Z, such that only relative values of these two rate constants can be determined from fitting GTPγS binding data to Equation 1. Neither rate could be measured independently because unliganded $G\alpha_q$ denatured too fast ($k_5 \geq 1$ min^{-1}, see above). Values of these constants derived from fits to binding data should, therefore, be considered as tests of consistency of Scheme I rather than as independent determinations. To generate initial trial fits of Equation 1 to the GTPγS binding data of Fig. 1, we allowed k_5 to float and arbitrarily set k_3 to 10^6 min^{-1} · M^{-1}. This is a very slow rate, but would still yield a pseudo-first-order rate constant of 10 min^{-1} at 10 μM GTPγS, well above k_1. Using this test value for k_3, fitted values of k_5 varied over the range 10–40 min^{-1} for 0–750 mM $(NH_4)_2SO_4$, fast enough to account for our inability to detect unliganded $G\alpha_q$. For the purposes of comparing data from separate experiments, we set k_5 equal to 10 min^{-1} and allowed k_3 to float. Fitted values of k_3 clustered in the range of 0.8 to 3.0 min^{-1} · M^{-1}, perhaps with a maximum at about 250 mM $(NH_4)_2SO_4$ (Fig. 4C). This maximum falls in the range of $(NH_4)_2SO_4$ concentrations where total binding is also maximal, but experimental variation is significant, and changes in fitted values of k_3 may reflect more complex coordinate changes in both k_3 and k_5. Regardless, the binding data place a limit on k_5 and, therefore, on k_3. If k_5 = 10 min^{-1}, then GDP binding stabilizes $G\alpha_q$ by 10^4- to 10^5-fold (see Appendix), a free energy of stabilization of 6–8 Kcal. This is reasonable, considering the depth of the GDP binding site within the Gα subunit and the number of protein-nucleotide contacts [18, 19]. Regardless, unliganded $G\alpha_q$ is strikingly unstable, much more so than is $G\alpha_i$ or $G\alpha_o$ [12]. To conform to the fast denaturation rate, k_3 must be greater than 10^5 min^{-1} · M^{-1}. Both k_3 and k_5 might be much larger, but an increase of k_3 to 10^7 min^{-1} · M^{-1}, more similar to G_i [11], would increase k_5 to at least 100 min^{-1}.

Nucleotide Concentration Dependence

The high concentrations of GTPγS needed to drive binding to $G\alpha_q$ [2] would be inconsistent with its slow rate of dissociation if binding were a simple equilibrium. Scheme I, however, predicts that the accumulation of bound GTPγS will increase with increasing concentrations of nucleotide until the rate of GTPγS binding to unliganded $G\alpha_q$ is much larger than the rate of denaturation (i.e. until Z ~ 1). As shown in Fig. 5, the effect of increasing the concentration of GTPγS over a 100-fold range conformed to the predictions of Scheme I. $G\alpha_q$ bound stoichiometric amounts of GTPγS at a nucleotide concentration of 100 μM. Fits to the model were good at either 100 or 750 mM $(NH_4)_2SO_4$ (or 400 mM, not shown), and for $G\alpha_q$ that was initially bound to either GDP or IDP. Effects of GTPγS concentration on maximum binding, initial rate, and the late declining phase were all essentially in agreement with the fits to Equation 1 in that all of the data at each concentration of $(NH_4)_2SO_4$ could be modeled with single values for k_3 and k_5. The dependence of binding on GTPγS concentration thus appeared to be a kinetic effect rather than a measure of equilibrium affinity.

Even though Scheme I is based on kinetics, it allows calculation of the equilibrium binding constant, K_d, for GTPγS and $G\alpha_q$ according to the ratio k_4/k_3. At 0.1 M $(NH_4)_2SO_4$, if k_5 = 10 min^{-1}, then $k_3 = \sim 2 \times 10^6$ M^{-1} · min^{-1} (Fig. 4C), $k_4 = \sim 1 \times 10^{-4}$ min^{-1} (Fig. 2B), and K_d = ~50 pM. At 0.75 M $(NH_4)_2SO_4$, K_d would increase to ~1 nM. If k_5 were only 0.5 min^{-1}, then K_d would be 1 nM at 0.1 M $(NH_4)_2SO_4$. Any of these values is far below the concentration of GTPγS needed to observe half-maximal binding, reinforcing the idea that accumulation of $G\alpha_q$-GTPγS is controlled kinetically, not thermodynamically.

Routine [^{35}S]GTPγS Binding Assay for G_q

The ability to accelerate GTPγS binding to $G\alpha_q$ with $(NH_4)_2SO_4$ allows the direct assay of active G_q according to the binding of [^{35}S]GTPγS. Such an assay, the standard for other G proteins, had been prohibitively difficult [2]. The experiment shown in Fig. 6 compares the measurement of active $G\alpha_q$ by direct [^{35}S]GTPγS binding assay under optimal conditions (100 μM [^{35}S]GTPγS, 100 mM $(NH_4)_2SO_4$, 1500–1800 min, 20°), and by measurement of tightly bound GDP [5, 12]. Five different preparations of purified $G\alpha_q$ were assayed over several months. All data are shown as normalized to the total molar amount of $G\alpha_q$ estimated according to the amido black dye binding assay. This assay overestimates active $G\alpha_q$ according to the amount of bound GDP present. In four of the five preparations, the two assays gave essentially the same result, that

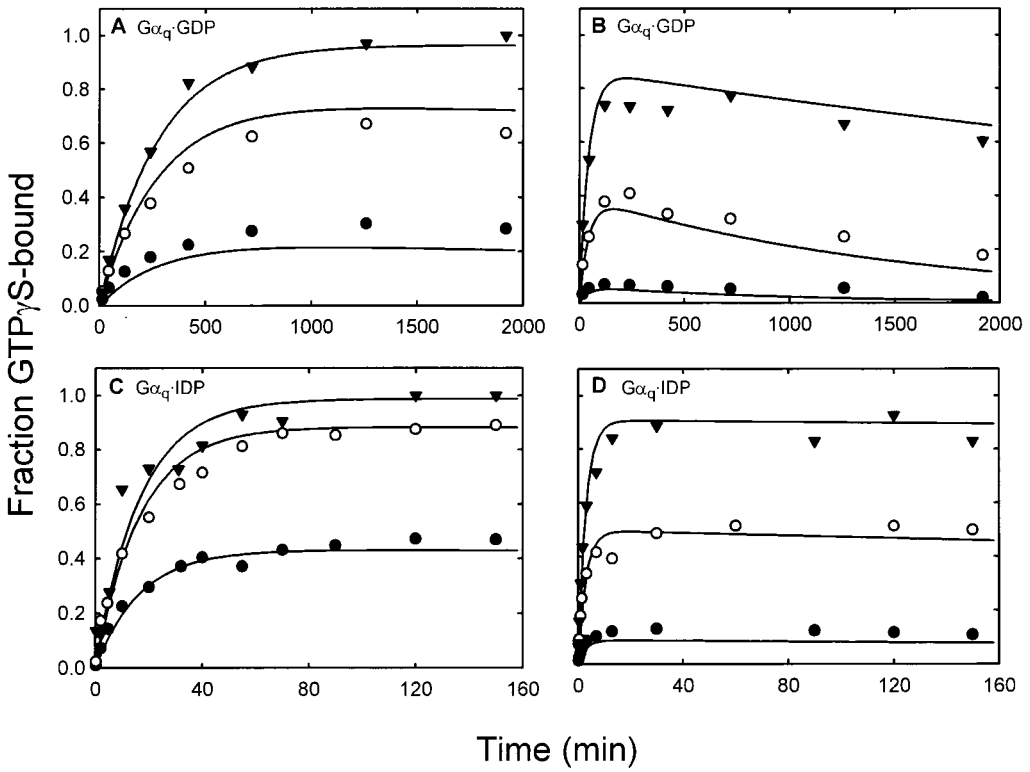


FIG. 5. Effect of the concentration of [^{35}S]GTPγS on binding to Gα$_q$ at different concentrations of $(NH_4)_2SO_4$. [^{35}S]GTPγS binding was measured at 100 mM $(NH_4)_2SO_4$ (A, C) or 750 mM $(NH_4)_2SO_4$ (B, D) using either 1 (●), 10 (○), or 100 (▼) μM [^{35}S]GTPγS. Drawn lines show fits to Equation 1 using values of k_1 and k_3 shown in Fig. 4, k_5 = 10 min^{-1}, and values of k_4 taken from Fig. 2B.

ligand-binding sites accounted for about 70% of the total protein but were in agreement with the amount of bound GDP. (Some G_q may be denatured or Gα$_q$ may bind more amido black per molecule than does the albumin standard. Numerous other direct binding assays gave similar results, and such a value is typical for other purified G proteins.) In the fourth preparation, the amount of bound GDP was unaccountably low, but the direct binding assay indicated the expected level of activity. In any event, the [^{35}S]GTPγS binding assay is a reliable and reproducible way to measure active Gα$_q$. It is markedly easier and somewhat more sensitive than the assay for bound GDP, and can be made more sensitive still by adjustment of the assay volume and the specific activity of the ligand.

Comparative Nucleotide Binding Properties of Gα$_q$

The present results and their interpretation in Scheme I indicate that binding of GTPγS to Gα$_q$ does not differ mechanistically from binding to other Gα subunits, but reflects its instability when unliganded and, possibly, its slow rate of association with GTPγS. GDP dissociation from Gα$_q$ is slower than from Gα$_o$ and somewhat slower than from Gα$_i$ or Gα$_s$, but is faster than from Gα$_t$ [8–10] and about the same as from Gα$_{13}$ [7]. Other unliganded Gα subunits are also unstable, albeit to a lesser extent [11, 12]. A qualitatively similar pattern of competing binding and denaturation was described for G_s by Smigel *et al.* [20] and Ferguson *et al.* [11].

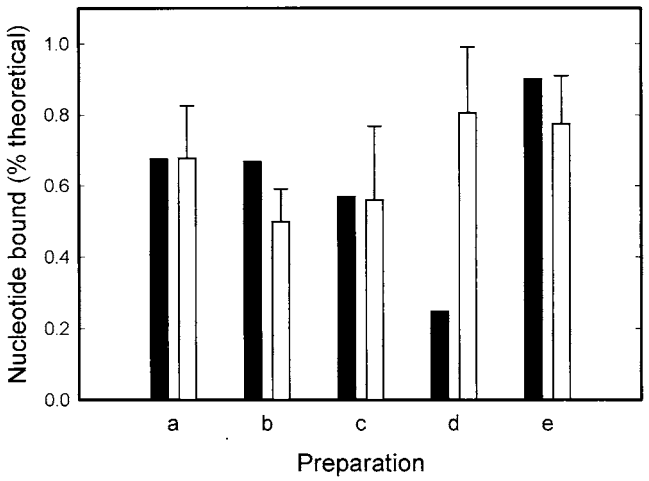


FIG. 6. Comparison of assays for active Gα$_q$. Active Gα$_q$ was measured according to the binding of [^{35}S]GTPγS or the amount of bound GDP. Five separately purified preparations of recombinant Gα$_q$ were assayed at 100 μM [^{35}S]GTPγS and 100 mM $(NH_4)_2SO_4$ for their capacity to bind [^{35}S]GTPγS (filled bars). For 24 independent determinations of [^{35}S]GTPγS binding (duplicate samples in each assay), the value was 0.69 ± 0.2 mol/mol, assuming a molecular weight of 42,000 for Gα$_q$. The same preparations were assayed for bound GDP exactly as described by Berstein *et al.* [5] (open bars). In four determinations of bound GDP ("d" excluded), the average value was 0.70 ± 0.12 mol/mol (mean ± SD, see error bars; 2–6 GDP assays in each determination). Both sets of data are normalized to the total amount of Gα$_q$ protein in each assay determined by amido black binding.

The addition of $(NH_4)_2SO_4$ to the binding reaction mixture facilitated the investigation of nucleotide binding to $G\alpha_q$ because it increased the rate of dissociation of GDP and thereby increased the fraction in the nucleotide-free state. The use of $(NH_4)_2SO_4$ in guanine nucleotide binding assays appears to be generally useful for G proteins with slow GDP dissociation rates. For example, preliminary experiments have shown that $(NH_4)_2SO_4$ increased the binding of [^{35}S]GTPγS to the α subunit of transducin. $(NH_4)_2SO_4$ also increased the assayable concentration of active $G\alpha_z$ by about 20% [21]. The techniques described here thus may be useful in quantitating levels of nucleotide binding activity in purified preparations of G proteins whose slow rates of GDP dissociation complicate or preclude assay by the usual methods.

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APPENDIX

GTPγS binding was analyzed according to the integrated rate equation for Scheme I, which was derived from the following differential rate equations. $G\alpha_q$ is shown as α.

$$d[\alpha\text{-GDP}]/dt = -k_1 \cdot [\alpha\text{-GDP}] + k_{2app} \cdot [\alpha] \quad (1)$$

$$d[\alpha]/dt = k_1 \cdot [\alpha\text{-GDP}] - (k_5 + k_{3app} + k_{2app}) \cdot [\alpha] + k_4 \cdot [\alpha\text{-GTP}\gamma\text{S}] \quad (2)$$

$$d[\alpha\text{-GTP}\gamma\text{S}]/dt = k_{3app} \cdot [\alpha] - k_4 \cdot [\alpha\text{-GTP}\gamma\text{S}] \quad (3)$$

$$d[\alpha_d]/dt = k_5 \cdot [\alpha] \quad (4)$$

with the initial conditions [α-GDP] = 1 and [α] = [α-GTPγS] = [α_d] = 0. The concentrations of unliganded $G\alpha_q$, $G\alpha_q$-GDP, and $G\alpha_q$-GTPγS are thus expressed as fractions of total $G\alpha_q$.

The pseudo-first-order association constants are defined as $k_{2app} = k_2 \cdot [GDP]$ and $k_{3app} = k_3 \cdot [GTP\gamma S]$. The derivation includes the simplifying assumptions that both k_{3app} and k_5 are much greater than k_1 and k_4, and that $k_{2app} \ll k_{3app}$. Rebinding of GDP/IDP was insignificant both because the initial concentration of $G\alpha_q$-GDP was about

0.2 μM in all experiments and the lowest concentration of GTPγS in any experiment was 1 μM (usually 10–100 μM), and because GDP binds with much lower affinity than does GTPγS.

These equations were integrated to yield

$$[\alpha\text{-GTP}\gamma\text{S}]_t = \frac{Z \cdot k_1}{k_1 + (Z-1) \cdot k_4} \cdot \{e^{-k_4 \cdot (1-Z) \cdot t} - e^{-k_1 \cdot t}\} \quad (5)$$

where

$$Z = \frac{k_3 \cdot [\text{GTP}\gamma\text{S}]}{k_3 \cdot [\text{GTP}\gamma\text{S}] + k_5} \quad (6)$$

Z is the fraction of unliganded $G\alpha_q$ that binds GTPγS at time t rather than denaturing.

According to Equation 5, binding reaches a defined maximum and then declines. Although the terms that describe the maximum and the time to maximum are complex, they simplify when $k_1 \gg k_4$ (GDP dissociates faster than GTPγS) such that

$$[\alpha\text{-GTP}\gamma\text{S}]_{max} = Z \text{ and } t_{max} = \frac{1}{k_1} \cdot \ln \frac{k_1}{k_4(1-Z)}$$

to allow an easy estimate of these parameters.

Scheme I does not include denaturation of $G\alpha_q$-GDP, which is much slower than that of unliganded $G\alpha_q$, but which does occur with a rate constant of about 10^{-3} min^{-1} over the long time courses of some experiments (data not shown). This process causes the maximum amount of GTPγS binding attained in the absence of $(NH_4)_2SO_4$ to be less than that observed at low $(NH_4)_2SO_4$ concentrations.