Kinetic Control of Guanine Nucleotide Binding to Soluble Gaq

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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 Gaq 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 Gaq 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 Gaq can bind stoichiometric GTPγS, but that binding is controlled kinetically by a combination of factors. GDP (or IDP) dissociated slowly from Gaq, but the dissociation rate increased linearly with the concentration of (NH4)2SO4 up to 0.75 M (~20-fold acceleration). The resulting GDP-free Gaq was labile to rapid and irreversible denaturation, however (rate constant ≥ 1 min⁻¹ at 20°C). 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 Gaq 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 Gaq by a simple GTPγS binding assay in the presence of (NH4)2SO4, and similarly can prevent underestimation of the concentrations of other G proteins.

KEY WORDS. GTP-binding protein; guanosine 5'-O-thiotriphosphate; Gaq; G protein

Members of the Gq family of heterotrimeric G proteins (Gq, G11, G14, and G15/16) convey signals from cell surface receptors to phospholipase C-β and, perhaps, other intracellular effectors. As with other G proteins, Gq is activated by GTP binding and is deactivated when it hydrolyzes bound GTP to GDP. Receptors initiate Gq signaling by promoting dissociation of GDP from the Gq α subunit and subsequent binding of GTP.

In contrast to most other G proteins, the ability of Gq and G11 to exchange guanine nucleotides is diminished drastically by their solubilization from membranes. The rate of activation of soluble Gq by GTPγS is slow [1, 2], and significant activation of purified Gaq by GTPγS typically requires more than 10 μM nucleotide, over 10-fold more than is needed in membranes [1]. Direct measurement of [35S]GTPγS binding to solubilized Gq has yielded similar results. Blank et al. [1] detected little or no binding of [35S]GTPγS to Gaq 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 [35S]GTPγS. Using purified recombinant Gaq produced in Sf9 cells, Hepler et al. [2] established conditions under which Gq could be shown to bind about 0.6 mol of [35S]GTPγS/mol of total protein, although this stoichiometry was back-calculated to correct for 80–90% loss of Gq in the assay. The binding reaction required incubation for 90 min at 30°C with 200 μM [35S]GTPγS to reach completion. Gaq-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 Gq do not indicate its denaturation during solubilization. Soluble Gq can be activated by A13/F under conditions where binding of [35S]GTPγS is undetectable [4], and nucleotide binding is returned to normal after Gq is...
co-reconstituted into phospholipid vesicles with receptor and Gβγ [5, 6]. Such behavior is also not unique to Goq; similar difficulties were reported for Goα3 [7], and it has been impossible to demonstrate the binding of reasonable quantities of GTPγS or other nucleotides to soluble Goq [8–10].

The anomalous guanine nucleotide binding behavior of soluble Goq results, in part, from the slow rate of dissociation of Goq-bound GDP, as is the case for other G proteins [11]. In this study, we used (NH4)2SO4 to accelerate dissociation of bound GDP from Goq [11–13]. (NH4)2SO4 promotes nucleotide release from Goq 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 Goq as a combination of rate-limiting GDP release followed by the competing reactions of GTPγS binding to unliganded Goq or Goq 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 Goq.

MATERIALS AND METHODS

Mouse Goq subunit was expressed in Sf9 cells and purified as described previously [6]. The total amount of purified Goq 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 Goq, referred to throughout, although it does not distinguish active and denatured protein. The concentration of active Goq was estimated originally according to the amount of Goq-bound GDP [5] using previously described modifications of the competitive GDP binding assay of Ferguson et al. [11]. Assay of active Goq by direct GTPγS binding is described in the text.

[35S]GTPγS and [α-32P]GTP were purchased from NEN. [α-32P]ITP was synthesized by incubating [α-32P]GTP with 0.8 M NaNO2 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-32P]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].

[35S]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 MgSO4 (10 μM calculated free Mg2+), 0.05% Genapol]. The concentration of (NH4)2SO4 in the assay was varied as indicated using a pH-buffered stock solution. The concentration of Goq was 0.1 to 0.2 μM, calculated according to total protein. The concentration of [35S]GTPγS in each experiment is specified in the legend or text. Both the pH and the concentration of Mg2+ were optimized in preliminary experiments. The binding kinetics described here and the effects of (NH4)2SO4 on GTPγS binding were similar over a broad optimal range of pH (6.5 to 8.0) and Mg2+ 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 Goq, 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 MgCl2, 1 mM GTP, 0.1 mM dithiothreitol, and 0.1% Genapol, followed by adsorption of Goq to BA85 nitrocellulose membranes as described previously [17].

IDP-ligated Goq was prepared by incubating purified Goq (0.9 μM) for 24–26 hr in buffer A that contained 10 mM ITP and 50 mM (NH4)2SO4. Free nucleotide and (NH4)2SO4 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 Goq-IDP. In figures that show GTPγS binding to initially IDP-bound Goq, data points represent single determinations.

The time-dependent binding of GTPγS to Goq, shown in Figs. 1 and 4 was analyzed according to the integrated rate equation derived for Scheme I (Equation 1, Appendix). Because k1 and k3 could not be independently resolved (see below), k5 was set to 10 min−1 (see text). Values for k1 and k3 were allowed to float. Values for k4, 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 (NH4)2SO4 by mixing Goq-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, [35S]GTPγS was bound by incubating Goq in buffer A, either with 10 μM [35S]GTPγS in the presence of 100 mM (NH4)2SO4 or with 100 μM [35S]GTPγS in the absence of (NH4)2SO4. [α-32P]GTP was bound to Goq under similar conditions, either with 10 μM [α-32P]GTP in the presence of 50 mM (NH4)2SO4 or with 30 μM [α-32P]GTP in the absence of (NH4)2SO4. Binding reactions were carried out at 20° for 9 hr in the presence of (NH4)2SO4 or for 17–21 hr in the absence of (NH4)2SO4. [α-32P]ITP (1 mM) was bound in the presence of 100 mM (NH4)2SO4 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 (NH4)2SO4.
RESULTS AND DISCUSSION

Effects of (NH₄)₂SO₄ on the Binding of [³⁵S]GTPγS to Gαq

Gαq 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 (NH₄)₂SO₄. Gαq bound 10 μM [³⁵S]GTPγS slowly in the absence of (NH₄)₂SO₄, but (NH₄)₂SO₄ accelerated binding over 20-fold (Fig. 1, A and B). The initial rate of [³⁵S]GTPγS binding increased linearly with (NH₄)₂SO₄ concentrations up to about 500 mM. At higher (NH₄)₂SO₄ 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.

(NH₄)₂SO₄ also increased the maximal amount of Gαq-bound GTPγS, and this effect also was biphasic (Fig. 1, C and D). In the absence of (NH₄)₂SO₄, only about 55% of active Gαq bound [³⁵S]GTPγS even at long times (Fig. 1A, and other experiments extended up to 48 hr). Active Gαq is defined according to assays of Gαq-GDP, assuming one molecule of GDP bound per active Gαq [5, 11]. Addition of 50–200 mM (NH₄)₂SO₄ increased this maximum to 80% of active Gq at 10 μM GTPγS (80 ± 24%, N = 5; Fig. 1A). Higher concentrations of (NH₄)₂SO₄ did not further increase binding at this concentration of [³⁵S]GTPγS, however. The maximum amount of bound GTPγS decreased as the (NH₄)₂SO₄ concentration was raised above 250 mM, such that < 25% of Gαq bound GTPγS above 1 M (NH₄)₂SO₄ (Fig. 1B). We have not pursued the effects of very high concentrations of (NH₄)₂SO₄ (>1 M), however, because of uncertainties about protein solubility.

The third effect of (NH₄)₂SO₄ on GTPγS binding was to shorten the period over which Gαq-bound [³⁵S]GTPγS accumulated. As the concentration of (NH₄)₂SO₄ was...
and at high (NH₄)₂SO₄ 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 Go₉**

(NH₄)₂SO₄ accelerates GTPγS binding to Go₉ 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 Go₉ and Go₁₁ by Berstein et al. [5]). Figure 2A shows that dissociation of GDP from Go₉ in solution was slow under the conditions used here (kₐₐₐ = 1.6 × 10⁻³ min⁻¹), but was accelerated by (NH₄)₂SO₄, increasing about 20-fold by 750 mM (NH₄)₂SO₄ (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 (NH₄)₂SO₄ (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 Go₉ 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 Go₉**

To evaluate the importance of GDP dissociation to the overall kinetics of GTPγS binding, we compared the binding of GTPγS to GDP-bound Go₉ with binding to IDP-bound Go₉. Inosine nucleotides bind G proteins with lower affinity than do the cognate guanine nucleotides, and IDP dissociation from Go₉ was about 15-fold faster than GDP at all (NH₄)₂SO₄ concentrations (0 to 0.75 M) (Fig. 2B). (NH₄)₂SO₄ 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 Go₉-IDP about 15-fold faster than to Go₉-GDP. Apparent initial rates of binding increased linearly with the concentration of (NH₄)₂SO₄ 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 Go₉-IDP. Although the loss of bound GTPγS at 400 and 750 mM (NH₄)₂SO₄ seems to be less in the Go₉-IDP experiments because of the difference in time scales, the calculated downward terminal slopes are about the same as when Go₉-GDP was used.

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**FIG. 2.** Dissociation of GDP, GTPγS, and IDP from Go₉. (A) [α-³²P]GTP was incubated with Go₉, as described in Materials and Methods, and dissociation of bound [α-³²P]GDP was then monitored in the presence of 0 (solid), 100 (open), 400 (hatched), or 750 mM (NH₄)₂SO₄. The lines show nonlinear least-squares fits to single exponential decay functions. To fit the data obtained at 750 mM (NH₄)₂SO₄, 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 (NH₄)₂SO₄, 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 (NH₄)₂SO₄. 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 (NH₄)₂SO₄, and the line is extrapolated to the axis.

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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 (NH₄)₂SO₄, accumulation of bound GTPγS terminated prematurely (Fig. 1, B–D).

Last, concentrations of (NH₄)₂SO₄ above 0.4 M caused the ultimate loss of Go₉-bound GTPγS after the binding reaction had reached its maximum (Fig. 1C). At long times...
Dissociation of GTPγS from Goq

The eventual loss of bound GTPγS at high (NH₄)₂SO₄ concentrations suggested that GTPγS dissociates from Goq at an appreciable rate. Although GTPγS dissociation could not be detected in the absence of (NH₄)₂SO₄ (data not shown), (NH₄)₂SO₄ accelerated dissociation over the same range of concentrations that stimulated GTPγS binding (Fig. 2B). The (NH₄)₂SO₄-promoted dissociation of GTPγS from Goq was fast enough to account for the decline in bound GTPγS that was observed at high concentrations of (NH₄)₂SO₄ (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 Goq.

Rapid Denaturation of Unliganded Goq

To evaluate the rate of binding of GTPγS to Goq after GDP dissociation, we tried to prepare unliganded Goq. However, unliganded Goq denatured essentially as soon as it was produced. Incubation of active Goq 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 Goq is at least 0.4 min⁻¹ at 20°, the fastest dissociation rate measured for IDP. We also attempted to detect unliganded Goq by adding [35S]GTPγS immediately after (NH₄)₂SO₄-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 Goq. Data from these experiments (not shown) indicated that the denaturation rate constant for unliganded Goq must be well above 1 min⁻¹. We could not stabilize free Goq significantly by manipulating detergent, pH, ionic strength, or other conditions.

Kinetic Mechanism of Guanine Nucleotide Binding to Goq

The data of Figs. 1 and 2 suggest that the rate of GTPγS binding to Goq 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 Goq and its regulation by (NH₄)₂SO₄, 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 Goq denatures rather than binding free nucleotide.

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. (Goq is shown simply as α.) Dissociation of GTPγS, which is frequently ignored, is included because it is significant in the presence of (NH₄)₂SO₄ (Fig. 2B). Dissociation rate constants for GDP (IDP) and GTPγS, k₁ and k₄, respectively, are all determined by the concentration of α-GDP α-GTPγS α-GDP α-GTPγS

\[
\begin{align*}
\text{α-GDP} & \quad \Leftrightarrow \quad \alpha \\
\alpha & \Leftrightarrow \alpha \text{-GTPγS} \\
\text{αd} & \quad \downarrow \quad k_5
\end{align*}
\]

SCHEME 1
(NH₄)₂SO₄, 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 Gαₐ \( k_5 \); \( α \) refers to denatured Gαₐ. Unliganded Gαₐ denatures unusually rapidly for a Gα subunit (see below), and denaturation of unliganded Gαₐ 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 Gαₐ-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 Gαₐ-GTPγS according to the experimental constraints that apply to Gαₐ (see Appendix):

\[
\alpha \text{-GTPγS} = \frac{Z \cdot k_1}{k_1 + (Z - 1) \cdot k_4} \cdot \left\{ e^{-k_4 (1-Z) \cdot t} - e^{-k_1 t} \right\}
\]  

(1)

where

\[
Z = \frac{k_1 \cdot [\text{GTPγS}]}{k_1 \cdot [\text{GTPγS}] + k_5}
\]  

(2)

Equation 1 describes the accumulation of Gαₐ-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 Gαₐ. The ratio Z describes the fraction of unliganded Gαₐ that binds GTPγS relative to the fraction that irreversibly denatures. Fractional binding is thus determined coordinately by \( k_1 \), \( k_5 \), and the concentration of GTPγS. The initial rate of binding of GTPγS is approximately equal to \( k_1 \cdot [\text{Gαₐ-GDP}] \cdot Z \). GDP dissociation is slow and rate limiting, but not all free Gαₐ binds GTPγS. Because \( k_1 \) increases linearly with the concentration of (NH₄)₂SO₄ (Fig. 2B), so does the initial rate of GTPγS binding (Fig. 1A). Rates of GTPγS binding to Gαₐ-GDP and to Gαₐ-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 [³⁵S]GTPγS binding data (Fig. 4) were all consistent with those derived directly from dissociation data (Fig. 2B). At longer times, dissociation of GTPγS from Gαₐ continued to produce unliganded Gαₐ after all of the initially bound GDP has dissociated. (NH₄)₂SO₄ thereby promotes the slow terminal loss of bound GTPγS at a rate equal to \( [\text{Gαₐ-GTPγS}] \cdot k_4 \cdot (1-Z) \). 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.
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 Goq. Initial rates, maxima, terminal loss of bound GTPγS, and the dependences of these behaviors on the concentration of (NH₄)₂SO₄ 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 kₙ, the rate constant for dissociation of GTPγS, were taken from Fig. 2B rather than being allowed to float because kₙ is only significant at long times and at high concentrations of (NH₄)₂SO₄. Without a downward limb to the curve, kₙ is not constrained by the data. However, values of kₙ 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 kₙ 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 (kₐ) with the denaturation rate constant for unliganded Goq (kₙ) 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 Goq denatured too fast (kₙ ≥ 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 kₙ to float and arbitrarily set kₐ to 10⁶ min⁻¹ · M⁻¹. This is a very slow rate, but would still yield a pseudo-first-order rate constant of 10⁻¹ min⁻¹ at 10 μM GTPγS, well above kₙ. Using this test value for kₐ, fitted values of kₙ varied over the range 10⁻⁴ to 10⁻¹ min⁻¹ for 0–750 mM (NH₄)₂SO₄, fast enough to account for our inability to detect unliganded Goq. For the purposes of comparing data from separate experiments, we set kₙ equal to 10 min⁻¹ and allowed kₐ to float. Fitted values of kₐ clustered in the range of 0.8 to 3.0 min⁻¹ · M⁻¹, perhaps with a maximum at about 250 mM (NH₄)₂SO₄ (Fig. 4C). This maximum falls in the range of (NH₄)₂SO₄ concentrations where total binding is also maximal, but experimental variation is significant, and changes in fitted values of kₐ may reflect more complex coordinate changes in both kₐ and kₙ. Regardless, the binding data place a limit on kₐ and, therefore, on kₙ. If kₐ = 10 min⁻¹, then GDP binding stabilizes Goq by 10⁴⁻⁵ to 10⁵-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 Go subunit and the number of protein-nucleotide contacts [18, 19]. Regardless, unliganded Goq is strikingly unstable, much more so than is Go or Go, [12]. To conform to the fast denaturation rate, kₙ must be greater than 10³ min⁻¹ · M⁻¹. Both kₐ and kₙ might be much larger, but an increase of kₐ to 10⁷ min⁻¹ · M⁻¹, more similar to G₁ [11], would increase kₙ to at least 100 min⁻¹.

Nucleotide Concentration Dependence

The high concentrations of GTPγS needed to drive binding to Goq [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 Goq 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. Goq bound stoichiometric amounts of GTPγS at a nucleotide concentration of 100 μM. Fits to the model were good at either 100 or 750 mM (NH₄)₂SO₄ (400 mM, not shown), and for Goq that was initially bound 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 (NH₄)₂SO₄ could be modeled with single values for kₐ and kₙ. 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 Goq, according to the ratio kₐ/kₙ. At 0.1 M (NH₄)₂SO₄, if kₙ = 10 min⁻¹, then kₐ = ~2 × 10⁶ M⁻¹ · min⁻¹ (Fig. 4C), kₙ = ~1 × 10⁻⁴ min⁻¹ (Fig. 2B), and K_d = ~50 pM. At 0.75 M (NH₄)₂SO₄, K_d would increase to ~1 nM. If kₙ were only 0.5 min⁻¹, then K_d would be 1 mM at 0.1 M (NH₄)₂SO₄. Any of these values is far below the concentration of GTPγS needed to observe half-maximal binding, reinforcing the idea that accumulation of Goq-GTPγS is controlled kinetically, not thermodynamically.

Routine [³⁵S]GTPγS Binding Assay for Goq

The ability to accelerate GTPγS binding to Goq with (NH₄)₂SO₄ allows the direct assay of active Goq according to the binding of [³⁵S]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 Goq by direct [³⁵S]GTPγS binding assay under optimal conditions (100 μM [³⁵S]GTPγS, 100 mM (NH₄)₂SO₄, 1500–1800 min, 20°, and by measurement of tightly bound GDP [5, 12]. Five different preparations of purified Goq were assayed over several months. All data are shown as normalized to the total molar amount of Goq estimated according to the amido black dye binding assay. This assay overestimates active Goq according to the amount of bound GDP present. In four of the five preparations, the two assays gave essentially the same result, that
ligand-binding sites accounted for about 70% of the total protein but were in agreement with the amount of bound GDP. (Some Gq may be denatured or Gaq 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 [35S]GTPgS binding assay is a reliable and reproducible way to measure active Gaq. 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 Gaq

The present results and their interpretation in Scheme I indicate that binding of GTPgS to Gaq does not differ mechanistically from binding to other Gα subunits, but reflects its instability when unliganded and, possibly, its slow rate of association with GTPgS. GDP dissociation from Gaq is slower than from Gαi and somewhat slower than from Gαo or Gαs, but is faster than from Gαt [8–10] and about the same as from Gα13 [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 Gs by Smigel et al. [20] and Ferguson et al. [11].

FIG. 5. Effect of the concentration of [35S]GTPγS on binding to Gaq at different concentrations of (NH₄)₂SO₄. [35S]GTPγS binding was measured at 100 mM (NH₄)₂SO₄ (A, C) or 750 mM (NH₄)₂SO₄ (B, D) using either 1 ( ), 10 ( ), or 100 ( ) μM [35S]GTPγS. Drawn lines show fits to Equation 1 using values of k₁ and k₅ shown in Fig. 4, k₅ = 10 min⁻¹, and values of k₄ taken from Fig. 2B.

FIG. 6. Comparison of assays for active Gaq. Active Gaq was measured according to the binding of [35S]GTPγS or the amount of bound GDP. Five separately purified preparations of recombinant Gaq were assayed at 100 μM [35S]GTPγS and 100 mM (NH₄)₂SO₄ for their capacity to bind [35S]GTPγS (filled bars). For 24 independent determinations of [35S]GTPγS binding (duplicate samples in each assay), the value was 0.69 ± 0.2 mol/mol, assuming a molecular weight of 42,000 for Gaq. 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 Gaq protein in each assay determined by amido black binding.
The addition of (NH₄)₂SO₄ to the binding reaction mixture facilitated the investigation of nucleotide binding to Goₐₜ because it increased the rate of dissociation of GDP and thereby increased the fraction in the nucleotide-free state. The use of (NH₄)₂SO₄ 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 (NH₄)₂SO₄ increased the binding of [³²P]GTPγS to the α subunit of transducin. (NH₄)₂SO₄ also increased the assayable concentration of active Goₐₜ 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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References


APPENDIX

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

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

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

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

\[ \frac{d[\alpha_d]}{dt} = k_3 \cdot [\alpha] \] (4)

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

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

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

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

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\(_4\))\(_2\)SO\(_4\) to be less than that observed at low (NH\(_4\))\(_2\)SO\(_4\) concentrations.