High Affinity Interactions of GTPγS with the Heterotrimeric G Protein, Transducin

EVIDENCE AT HIGH AND LOW PROTEIN CONCENTRATIONS*

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A well known difference in nucleotide binding characteristics between heterotrimeric G proteins and small GTP binding proteins of the Ras superfamily is that the former bind GTP or guanosine 5′-O-(3-thiotriphosphate) (GTPγS) with a much lower affinity (Kd = 10^{-6}–10^{-7} M) than the latter (Kd = 10^{-11}–10^{-10} M). We report here that the α subunit of the heterotrimeric G protein transducin (Gα) binds GTPγS with an affinity comparable to that of Ras. High affinity binding was suggested by GTPγS titrations of rod outer segment samples with Gα concentrations in the range of 7 nM to 300 nM; the results were more consistent with a dissociation constant for GTPγS in the subnanomolar range, than with one in the 10^{-7}–10^{-8} M range typically reported for heterotrimeric G proteins. Equilibrium binding experiments with G protein concentrations in the subnanomolar to nanomolar range confirmed this conclusion and revealed a dissociation constant of 50 nm. Thus, transducin’s affinity for GTPγS, and by inference, for GTP, appears to be approximately three orders of magnitude higher than previously reported. These results raise the possibility that some results obtained with high concentrations of nucleotide analogues may be due to minute traces of contaminants such as GTP, GTPγS, or GTPβS, that have high affinities for Gα.

Transducin (Gα),1 the heterotrimeric GTP-binding protein of the vertebrate visual system, couples light activation of rhodopsin to activation of its effector enzyme, cGMP phosphodiesterase (PDE). Photoisomerized rhodopsin (R*) catalyzes the exchange of GTP for GDP on the α subunit of transducin, and Gα,GTP activates PDE (see Refs. 1 and 2 for reviews of phototransduction). Because of its abundance in rod outer segments (ROS) and the relative ease of its purification, Gα has served as a paradigm for many studies of G protein function. Obviously, one of the most important properties of G proteins is their ability to bind GTP with sufficiently favorable energetics to allow rapid and dramatic changes in their functional properties, so they can switch from inactive to active states when stimulated to release GDP by activated receptors. In the case of Gα, the differences between the GDP and GTP forms and the speed with which the switch occurs are remarkable. The GTP form shows a dramatic decrease in affinity for R* and Gα,GTP, as compared to the GDP form (3, 4). Activation by GTP can occur on a millisecond time scale at physiological levels of GTP (5–7). Most importantly, perhaps, while the GDP form of Gα, appears to be capable of activating the effector PDE only at concentrations on the order of 50 μM (8), the GTP or GTPγS form interacts with it much more efficiently, with an apparent affinity for membrane-bound holo-PDE of <0.2 nm determined by activation (9), or of 2.5 nm determined by light scattering (7). The Kd of Gα,GTP for the inhibitory PDE, subunit of PDE has been reported to be 3 nm, while the GTPγS form binds with a Kd of \( \leq 0.1 \) nm (10), corresponding to a \( \geq 30 \) fold higher affinity.

The observation of Kd values for Gα,GTPγS binding PDE or PDEγ in the low nanomolar or subnanomolar range seems hard to reconcile with earlier reports of Kd values for formation of the Gα,GTPγS complex itself (i.e. Gα,GTPγS) on the order of 50–100 nm (4, 11). This apparent paradox raised the question of whether the Gα,GTPγS complex itself could be capable of activating its effector at a concentration so dilute that the activator itself should fall apart. Furthermore, titrations measuring PDE activity in rod outer segments as a function of added GTPγS yielded curves whose shapes were more consistent with stoichiometric binding of a very high affinity ligand than with reversible association of a ligand with a Kd value similar to those reported for Gα and other G proteins (9).

A noteworthy feature of the literature on affinities of GTPγS and other GTP analogues for G proteins (including several reports that are conveniently tabulated in Ref. 4) is that most of the experiments reported have been conducted at concentrations of total G protein on the same order as the apparent Kd values that were determined. Lower Kd values have tended to be reported from experiments at lower protein concentrations, while higher Kd values have been reported at higher protein concentrations. For example Gα, at concentrations of 12 nm was reported to bind GTPγS with a Kd of 5–10 nm (12), while for the same protein assayed at 400 nm (13) a Kd of 700 nm was reported. In addition, in most cases where plots of bound versus added nucleotide have been presented, no attempt has been made to distinguish between total and free (i.e. not bound to protein) ligand. It has been well established in the case of the small GTP-binding proteins of the p21 superfamily that Kd values in the picomolar range were mistaken for values in the high nanomolar range when total protein concentrations in the

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The abbreviations used are: Gα, transducin, the rod cell-specific G protein; Gαi, the inhibitory G protein; Gα, the α subunit of transducin; Gα, the β subunits of transducin; GTPγS, guanosine 5′-O-(3-thiotriphosphate); GTPγS, guanosine 5′-O-(3-thiotriphosphate); PDE, cGMP phosphodiesterase; holoenzyme; PDEγ, the inhibitory γ subunit of PDE; R*, the photoactivated form of rhodopsin; R, total rhodopsin regardless of its form; ROS, rod outer segments; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; GAP, GTPase-activating protein.
nanomolar range were assayed (reviewed in ref. 14). From a comparison of the three-dimensional structures of p21ras (15) and G\textsubscript{\alpha} (16) with nonhydrolyzable GTP analogues bound, it is far from obvious why the affinity for GTP\textsubscript{\alpha}S of the former should be 1000 times stronger than that of the latter.

High affinity interactions frequently go unrecognized because the low concentrations of binding sites (near K\textsubscript{d}) required to measure them accurately often present experimental difficulties. However, even at higher than ideal concentrations of binding sites, these interactions can be recognized by careful analysis of the shapes of binding curves, and least squares analysis of fits of the curves to either simplified or more complete binding equations (17).

We present here a reexamination of the affinity of GTP\textsubscript{\alpha}S for G\textsubscript{\alpha}, using experimental conditions that allow determination of low (picomolar) values of K\textsubscript{d}. These consist principally of the use of low G\textsubscript{\alpha} concentrations and direct measurement, rather than calculation or assumption, of the values of free [GTP\textsubscript{\alpha}]. In addition we present a comparison of the \(\chi^2\) surfaces obtained from least-squares fitting of data obtained at high and low protein concentrations. The dependence of this measure of goodness of fit, on the parameters used to calculate theoretical binding curves, reveals that important information about high affinity interactions can be obtained even at higher-than-ideal protein concentration. This analysis also reveals the limits of such information.

**EXPERIMENTAL PROCEDURES**

Materials—GTP\textsubscript{\alpha}S was obtained from Boehringer Mannheim (purity of 85% estimated by supplier). GTP\textsubscript{\alpha}S was obtained from DuPont NEN, and its radiochemical purity by TLC (18) was verified to be >95% for experiments reported here. Stocks were routinely checked for degradation, which was often found to be ~90% in stored samples. Rod outer segments (19) and purified transducin \(\beta\)G\textsubscript{\alpha} subunits (9, 20) were prepared as described; G\textsubscript{\alpha} was used to test its effects on GTP\textsubscript{\alpha} binding.

Equilibrium Binding Assays—Equilibrium binding experiments were carried out using Buffer A consisting of 20 mM MOPS, pH 8.2, 150 mM KCl, 2 mM MgCl\textsubscript{2}, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mg/ml ovalbumin (for comparison to conditions used in pH-based PDE assays), or Buffer B consisting of 10 mM MOPS, pH 7.4, 60 mM KCl, 30 mM NaCl, 2 mM MgCl\textsubscript{2}, 1 mM DTT, 0.1 mg/ml ovalbumin (for comparison to previous studies done at similar pH values). No significant differences were detected in binding results obtained with these two buffers. Samples freshly bleached rod outer segments were mixed with GTP\textsubscript{\alpha}S (100 Ci/mmol) to various final concentrations and incubated on a rotator mixer at 23°C (1 or 2 ml, final volume) for periods of time ranging from 3 to 17 h. Then they were filtered through 0.22-μm pore-size nitrocellulose filters, in a vacuum filter manifold, and washed with 1 ml of ice-cold buffer. Filters were removed with the vacuum to remove solution from the edges. The solution passing through the filters was collected using funnels and scintillation vials positioned below the filters. Radioactivity bound to the filters and in the flow-through solutions was measured by scintillation counting. Nonspecific binding was determined by adding 1 μM nonradioactive GTP\textsubscript{\alpha}S to the ROS 30 min prior to addition of GTP\textsubscript{\alpha}S, and the average radioactivity bound to filters in these samples (typically 0.95 ± 0.05% of the total radioactivity added) was subtracted from the total radioactivity bound to the other filters to calculate specific binding. The sum of the radioactivity in the flow-through samples was found to correspond to essentially all the radioactivity added.

Analysis of Covalent Incorporation of \(35\)S—Three different approaches were taken to test whether thiosphorylation of G\textsubscript{\alpha} (21, 22) might contribute to the filter-bound radioactivity. 1) In equilibrium binding experiments such as those described above, control samples were treated with 8% (final, w/v) trichloroacetic acid at 0°C just prior to filtration to denature the protein and release noncovalently bound radioactivity. 2) Identical samples to those used in binding assays (not treated with acid) were washed from nitrocellulose filters with SDS sample buffer, applied to a gel for SDS-PAGE (23), and subjected to fluorography, along with standards of known amounts of GTP\textsubscript{\alpha}S to estimate detection limits. 3) In an attempt to force the thiosphorylation reaction, ROS (5, 15, or 29 μM) R* were treated with GTP\textsubscript{\alpha}S (1000 Ci/mmol) at a concentration of 100 nM for 40 min at 23°C. The membranes were separated from soluble proteins by centrifugation, and both supernatant and pellet fractions were subjected to SDS-gel electrophoresis and fluorography to locate radiolabeled bands. Radioactivity incorporated into protein was quantitated on dried gels using a Beta Scope 603 Biol Analyzer (Amersham).
Picomolar GTPγS Binding by Transducin

Fig. 1. GTPγS activation of PDE in rod outer segment membranes, 500 nM R. GTPγS was added at the indicated concentrations to a suspension of bleached ROS (0.5 μM R) and PDE activity was monitored by pH recording. PDE activity is plotted as a percent of the maximal activity, determined by averaging the observed hydrolytic rates at the 10 highest GTPγS concentrations. The theoretical curves represent the best fit curves derived A, using Equation 1 (Kd = 33 pm, Smax = 6.95 nm) or B using Equation 2 assuming free [GTPγS] = total GTPγS, with maximal activity in the fits either fixed at the observed value (Vmax = 100%, Kd = 0.9 nm, short dashes) or allowed to vary to obtain the best fit (Vmax = 122%, Kd = 1.9 nm, long dashes). C–F, χ² surface analysis of activation by GTPγS. Data from A were fit using nonlinear least squares analysis, with either A, C, D, Equation 1, or B, E, F, Equation 2. Reduced χ² values are plotted as a function of the fit parameters, for Equation 1 without assumptions (C and D), or for Equation 2 (E and F), assuming free [GTPγS] = total GTPγS and allowing Vmax to vary. Note the different scales used for χ².

d of PDE is activated at saturating [GTPγS] in ROS suspensions, and PDE can be further activated by addition of exogenous activated Gt, it is reasonable to assume that PDE activity in this curve reports in a linear way on the fraction of Gt, activated by GTPγS.

There are two important features of this curve. One is that the concentration of GTPγS at which a half-maximal response is observed is 3.5 nm, a value more than an order of magnitude lower than the previously reported Kd for binding of GTPγS to Gt. The other is that a good fit to the data is obtained with Equation 1 by assuming that Kd is much lower than the total concentration of binding sites (i.e. total Gt ≈ Kd, Fig. 1A), while assuming that free GTPγS is equal to the GTPγS added and using Equation 2 gives a much poorer fit (Fig. 1B). Fig. 1C–F, shows the results of nonlinear least squares analysis using these two approaches; the best χ² value for the "low affinity model" (Equation 2) is an order of magnitude higher than that for the "high affinity model" (Equation 1). However, Fig. 1C shows that a reliable estimate of Kd cannot be obtained from a fit to Equation 1, exactly because Gt ≈ Kd. While the best fit value is 33 pm, any value below 150 pm fits about as well. In fact, simply drawing two straight lines through the data points in Fig. 1A, which is equivalent to assuming an infinitely tight binding (Kd = 0), gives a curve that fits the data quite well. The fit does make it possible to establish an upper limit for a Kd value consistent with these data; 200 pm would be a conservative estimate.

GTPγS Binding at Low [Gt]—The obvious solution to the problem of determining Kd when its value is apparently much lower than the concentration of Gt commonly assayed is to carry out assays at much lower concentration. While PDE assays are not as convenient at low concentrations of ROS, direct binding assays using radiolabeled GTPγS can be used instead. The kinetics in this case might be much slower (although not necessarily so for membrane-confined reactants) so we carried out long incubations to ensure that equilibrium was reached. Fig. 2 shows the results of a titration carried out at 30 nM R. It shows unequivocally that Kd is the order of 50 pm. A technical difficulty with this type of experiment is that prolonged incubations under dilute conditions tend to lead to some loss of total binding activity (20); in these earlier studies it was confirmed, however, that Gt–GTPγS formed by spontaneous nucleotide exchange or by R*-catalyzed exchange are functionally indistinguishable. In this particular experiment, about half (51%) of the GTPγS binding activity observed at higher ROS concentrations using PDE assays like the one described above, was retained after a 3 h incubation at 23 °C and 30 nM R. Fig. 2B shows that at appropriate protein concentrations, the method of analyzing the data is not nearly as critical as at protein concentrations well above Kd. Roughly equivalent estimates of Kd with similar χ² values are obtained using either Equation 1 (short dashes in Fig. 2A, inset, and in Fig. 2B), which implicitly calculates the value of free [GTPγS] from the total, or using Equation 2 (solid lines in Fig. 2, A and B) with the measured free [GTPγS] as the independent variable. Simply making the invalid but common assumption that free and total ligand are the same (long dashes in Fig. 2A, inset, and in Fig. 2C), again gives a much poorer fit, but in this case, the value of Kd obtained, 280 pm, is somewhat more realistic than...
which favor either the GDP or nucleotide-free states of Gt. However, corrections for these competing ligands are probably not very significant. As we report in a companion study (26), GDP competes extremely poorly with GTP-35S for Gt binding; it must be present in at least 200-fold excess for significant inhibition of GTP-35S binding to be observed. High affinity binding to R* requires association of Gt and Gt-GTPaS which have been estimated to bind one another with a Kd of 150–300 nM as detected by fluorescence intensity changes or resonance energy transfer (27). This estimate is consistent with the dependence on Gt-GTPaS concentration of pertussis toxin catalyzed ADP-ribosylation of Gt (28) and the dependence on Gt-GTPaS binding to illuminated ROS membranes (29). Thus, under the conditions of Figs. 2 and 3 essentially no Gt-GTP (with GDP or GTP-35S bound) or Gt-GTPaS should be present, although the latter presumably exists in small amounts as a transient intermediate in nucleotide exchange. If there were significant amounts of these complexes, then the measured Kd would be higher than the actual value, as these complexes favor the GDP form or the nucleotide-free form, respectively, of Gt. The effect of Gt-GTPaS on the equilibrium was tested directly under the conditions of Fig. 2A, by varying the added Gt-GTPaS from 0 to 238 nM at 85 pm free GTP-35S. The average value of bound 35S at 238 nM added Gt-GTPaS was within 1% of the value at 0 added Gt-GTPaS, and the maximum range of variation over the whole range of added Gt-GTPaS (total of 10 measurements) was ±5% (data not shown).

The presence of phospholipids may also affect the results, as there are differences in membrane binding of Gt-GTPaS and Gt-GTP-35S even to dark-adapted ROS membranes.2 Phospholipids present in the assays also probably do not influence GTP-35S binding strongly, as binding of Gt-GTP to phospholipid vesicles is half-maximal at approximately 60 µM accessible phospholipid (determined at 0.75 µM Gt-GTPaS, a value more than 50-fold higher than the accessible phospholipid in the binding experiments described here).

Degradation of GTP-35S can also lead to overestimation of the value of Kd. While this seems a likely source for at least some of the reported findings of Kd values > 10 nM for G proteins binding GTP-35S, our practice of routinely analyzing the integrity of GTP-35S stocks by thin layer chromatography and discarding those with low radiochemical purity makes this an unlikely source of error in our experiments.

Possible artifacts and errors leading to underestimation of Kd include possible binding to PDE, PDE activating protein, GAP (18, 30), the only reactions known to favor the GTP-35S or GTP form of Gt. In addition, underestimation of Kd could result from contamination with a high affinity GTP binding protein other than Gt or from covalent radiolabeling of Gt (21, 22). The low total membrane concentration means that Gt binding to PDE is unlikely to shift the equilibrium significantly because accessible ROS phospholipids are well below the concentration of 10–13 µM required for half-maximal interactions of PDE with Gt-GTP (determined at 10 nM PDE and either 0.3 µM or 2 µM Gt-GTP-35S (9). The Kd values for Gt-GTPaS binding to PDE catalytic subunits; −10 pm (31) and to Gt-GTP-35S; −0.1 nM (10) imply that Gt-GTP-35S binding to PDE is also unlikely to affect the equilibrium substantially, GAP interactions with Gt-GTPaS are also sufficiently weak as to be negligible at such low membrane concentrations, based on the failure of endogenous GAP in ROS to elicit noticeable GTPase acceleration at R concentrations of 10 µM or lower (18, 30).

Thiophosphorylation of Gt, a reaction that has been re-

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2 Z. Yang and T. Wensel, unpublished results.
3 T. Melia and T. Wensel, unpublished results.
ported to occur in the presence of R* (21, 22), is rendered unlikely by our control experiments. The ready loss of the apparent binding following denaturation by low pH or SDS-PAGE argues strongly against a significant component of the bound radioactivity being due to covalent modification. It might also be suggested that the binding is not due to Gt at all, but rather to some previously unidentified high affinity GTP binding protein in ROS. This is very unlikely the case, because the stoichiometry of binding is too high, e.g. 51% of the total activatable Gt, measured at high [ROS] bound GTP·S in the experiment depicted in Fig. 2, with the discrepancy between total GTP·S binding observed at low and high [ROS] likely due to surface-induced and other losses of active protein (20). The amount of bound GTP·S is too high to be accounted for by minor unknown proteins. Only Gt, Ras, and arrestin are abundant enough to account for such a high stoichiometry (3, 32), and of these only Gt binds GTP·S.

An additional concern might be that the long incubation times required to ensure equilibrium at low ROS concentrations could conceivably lead to overestimation of Kd through thermal denaturation and loss of native binding activity. Alternatively, prolonged incubations might lead to underestimation of Kd through some slow binding reaction distinct from the Gt activation by GTP that occurs rapidly under physiological conditions. It is reassuring in this regard that: 1) Gt, loaded with GTP·S by incubation for many hours in the absence of R* as is functional in PDE activation as Gt, activated with Ras (20) and 2) the essentially instantaneous binding reactions observed in the experiments of Fig. 1 give nearly identical Kd values to those resulting from long incubations (Figs. 2 and 3).

While appropriate caution must be exercised because of reactions with other proteins, experimental variation, and losses of activity during prolonged incubations, it is probably safe to conclude that the true Kd for Gt, binding to GTP·S is greater than 10 nM and less than 100 nM. This conclusion means that transducin binds GTP·S with an affinity 2-3 orders of magnitude stronger than has been reported previously for heterotrimeric G proteins.

Comparisons with Previous Results—As discussed in the Introduction, most previous measurements of GTP·S binding to G proteins have been carried out with total G protein concentrations considerably higher than the Kd values observed here, and free [GTP·S] was not determined directly, so they are difficult to compare to the present results. However, in experiments with Gt and Gt, (33, 34) it was reported that the concentration of free GTP·S was known and was at least nine times the concentration of G protein-bound nucleotide; Gt concentrations ranged from 5–300 nM. In those studies, the reported Kd for binding of GTP·S to both Gt was around 30 nM, a value almost three orders of magnitude higher than the one we observe for Gt, and one which would lead to binding of at least 15% of the GTP·S even at the lowest protein concentration of 5 nM. This value is also intermediate between the low and high extremes of protein concentrations used. It would be interesting to determine whether there is any dependence of the apparent Kd on the concentration of Gt, used, or, alternatively, if there is actually a large difference in GTP·S affinity for Gt and Gt, as compared to Gt. In the case of Gt, previous reports indicating apparent lower affinity binding of GTP·S or GTP (nucleotide concentration for half-maximal binding or Kd $\geq 50$ nM, e.g. Refs. 4, 11, 35, 36) used high concentrations of Gt, did not directly monitor the concentration of free GTP·S or GTP, and are thus consistent with our results.

Fig. 4 shows clearly that if our results are analyzed without taking into account the difference between total nucleotide and free nucleotide (open symbols) the apparent Kd values calculated in an approximately linear way with protein concentration. Taking account of this difference explicitly (Fig. 5, closed symbols) gives nearly identical Kd values over protein concentrations spanning three orders of magnitude.

Structural Correlations—A high resolution structure of Gt, complexed to GTP·S determined by x-ray crystallography (16) reveals clearly the many contacts between the protein and GTP·S. Although there are some differences in detail even within the conserved GTPase domain, an overall comparison between the nucleotide binding sites of Gt, and p21ras (15) indicates that all the p21ras binding interactions are either found in Gt, or replaced by similar interactions, and that Gt, makes additional contacts from its insert domains unique to the Gt family. With all these interactions, the surprise is that we measure a GTP·S Kd for Gt, that is only comparable to, and not much stronger than, binding of GTP·S to p21ras.

Implications for Gt Function—Given the millimolar concentration of GTP in rod cells (37), it is reasonable to ask what functional consequences may have followed the evolution of such a high affinity binding site. Certainly the probability of occupancy would be identical even if the Kd value were micromolar. Two possibilities seem worth considering. The first is that the free energy of binding serves to drive rapidly and efficiently the conformational changes in Gt·GTP·S and its subsequent reactions: dissociation from R*, dissociation from Gt·GTP·S, and binding to PDE. Another possibility is that viselike positioning of GTP in the binding site of Gt facilitates precise control in the timing of GTP hydrolysis, making it an event that occurs only once in 20 s when Gt, is not in contact with its GAP and PDE (18, 30, 38, 39) but that happens rapidly once these interactions occur.

Experimental Implications—While the consequences of high affinity binding for Gt, function in vision require further study, some experimental ramifications are immediately apparent. One is that in kinetic experiments conducted at low concentrations of [GTP] or [GTP·S], Gt, can be expected to bind virtually all of the nucleotide present, as long as it is present at concentrations above that of the nucleotide and above ~1 nM, and dissociation of [GTP·S] for practical purposes will not occur (an observation already made in several laboratories). Another is that studies with analogues of GTP can be greatly confused by the presence of very small amounts of contaminating GTP, GTP·S, or GTP·pS (26).
detected even at concentrations far from those that allow them to be measured accurately (Fig. 1). Recognizing them is facilitated by 1) critically comparing the shapes of binding curves to low affinity and high affinity models, 2) experimentally testing for dependence on protein concentration of apparent half-maximal binding concentrations, and 3) independently measuring the free and bound ligand concentrations. It will be interesting to determine what will be revealed by application of these approaches to other heterotrimeric G proteins, and to discover whether transducin’s high GTP affinity is unique, or a common feature of this protein family.

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