Stabilization of an Intermediate Activation State for Transducin by a Fluorescent GTP Analogue†

Sekar Ramachandran and Richard A. Cerione*

Department of Molecular Medicine, Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853

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ABSTRACT: The GTP-binding protein (G protein), transducin, serves as a key molecular switch in vertebrate vision through the tight regulation of its GTP-binding (activation)/GTP hydrolytic (deactivation) cycle by the photoreceptor rhodopsin. To better understand the structure−function characteristics of transducin activation, we have set out to identify spectroscopic probes that bind to the guanine nucleotide-binding site of this G protein and maintain its ability to interact with its specific cellular target/effector, the cyclic GMP phosphodiesterase (PDE). In this study, we describe the characterization of a fluorescently labeled GTP analogue, BODIPY−FL GTPγS (BOD-GTPγS), that binds to the alpha subunit of transducin (α_T) in a rhodopsin- and G$\beta\gamma$-dependent manner, similar to the binding of GTP or GTPγS, with an apparent dissociation constant of 100 nM. The rhodopsin-dependent binding of BOD-GTPγS to α_T is slow, relative to the rate of binding of GTPγS, particularly under conditions where rhodopsin must act catalytically to stimulate the exchange of BOD-GTPγS for GDP on multiple α_T subunits. This reflects a slower rate of dissociation of rhodopsin and G$\beta\gamma$ from α_T−BOD-GTPγS complexes, relative to their rates of dissociation from α_T−GTPγS. The binding of BOD-GTPγS occurs without a change in the intrinsic tryptophan fluorescence of α_T, indicating that only a subtle movement of the Switch 2 domain on α_T accompanies the binding of this GTPγS analogue. Nevertheless, the BOD-GTPγS-bound α_T subunit is able to bind with high affinity to the recombinant, purified γ subunit of PDE (γ_{PDE}) labeled with 5-((((2-iodoacetyl)-amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS ($K_d \approx 13$ nM)), as well as bind to and stimulate the activity of PDE, albeit less efficiently compared to α_T−GTPγS. Taken together, these findings suggest that the binding of BOD-GTPγS to transducin causes it to adopt a distinct conformation that appears to be intermediate between the inactive and fully active states of α_T, and this fluorescent nucleotide analogue can be used as a reporter group to characterize the interactions of α_T in this conformational state with its biological target/effector.

The vertebrate visual system provides an excellent model for understanding how heptahelical receptors within the plasma membranes of cells interact with heterotrimeric G-proteins to initiate signaling cascades (*1*). In this phototransduction signaling pathway, light causes the isomerization of the rhodopsin-containing chromophore, 11-cis-retinal, to all trans-retinal. This leads to a change in the conformation of rhodopsin, resulting in the photoisomerization state known as metarhodopsin II (meta II).[1] The meta II species binds to the heterotrimeric G protein, transducin, which contains a GDP-bound α subunit (α_T−GDP), as well as β and γ subunits, which are noncovalently complexed to each other and can be dissociated only by denaturation (often referred to as G$\beta\gamma$ or simply $\beta\gamma$). Binding of light-activated rhodopsin to the heterotrimeric holotransducin (α_T−GDP/$\beta\gamma$) complex results in a weakening of its affinity for GDP, yielding a complex of rhodopsin and nucleotide-free transducin. GTP then binds to α_T, leading to its dissociation from both rhodopsin and $\beta\gamma$.

Structural studies have indicated that, like other Gα subunits, α_T is composed of two distinct domains: one that highly resembles the GTPase domain of the small G-protein Ras and a second domain which is mainly α-helical in content and thus referred to as the helical domain (*2*, *3*). The guanine nucleotide is trapped within the interface of the Ras-like and helical domains. The binding of GTP to α_T induces structural changes within three regions of the Ras-like domain, designated as Switches 1, 2, and 3. The GTP-bound form of α_T then activates the downstream effector, the cGMP phosphodiesterase (PDE). The PDE is a tetrameric enzyme composed of α (α_{PDE}), β (β_{PDE}), and two γ subunits (γ_{PDE}), in the ratio 1:1:2. The α_{PDE} and β_{PDE} subunits are responsible for catalyzing cGMP hydrolysis, whereas the two γ_{PDE} subunits bind to, and block, cGMP hydrolysis by the catalytic subunits (*4*). Upon binding to the γ_{PDE} subunits, the GTP-bound α_T is thought to relieve the inhibition of PDE activity. The hydrolysis of cGMP then leads to a closure of

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* To whom correspondence should be addressed. E-mail: rac1@cornell.edu.

[1] Abbreviations: GTP, guanosine-5′-triphosphate; BOD-GTPγS, bodipy-FL modified form of GTPγS; GTPγS, guanosine 50-*O*-(3-thiotriphosphate); IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)-naphthalene-1-sulfonic acid; α_T−GTPγS, GTPγS-bound α subunit of transducin; GDP, guanosine 5′-diphosphate; PDE, CGMP phosphodiesterase; MANT, methyl-3′-*O*-anthranolyl group; RGS, regulators of G-protein signaling

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cGMP-gated channels on the rod cell membrane. This hyperpolarization results in an inhibition of neurotransmitter release, which represents the signal that is conveyed to the optic nerve.

Our laboratory has been interested in developing assays utilizing fluorescence spectroscopy to characterize the various interactions that underlie phototransduction. The fluorescence emission from the tryptophan at position 207 (W207) in α_T increases upon exchange of either GTP or non-hydrolyzable GTP analogues for GDP (*5*, *6*). The $\beta\gamma$ subunit complex has been labeled on its cysteine residues with the environmentally sensitive fluorescent reagent, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (*7*) and used as a reporter group to study rhodopsin−$\beta\gamma$ and α_T−$\beta\gamma$ interactions (*7*, *8*). Also, the reactive lysine on α_T was labeled with fluorescent reagents (*9*) and used to assay α_T−$\beta\gamma$ interactions (*10*) as well as α_T−PDE interactions (*11*, *12*) using fluorescence resonance energy transfer.

However, while several probes are available to study the interactions between individual components in this system, it has been difficult to label α_T in a manner that allows it to be activated and stimulate its target/effector (PDE) activity. For example, when α_T−GDP is labeled at lysine 267 with a fluorescent probe, it is unable to bind rhodopsin and exchange GTP for GDP (*11*), whereas, a preactivated α_T−GTPγS can be labeled at this lysine residue and bind γ_{PDE}, but it cannot stimulate cGMP hydrolysis by PDE (*12*). Thus, we have set out to introduce a fluorescent probe into the guanine nucleotide-binding site of α_T that can be used to directly monitor its interactions with its other binding partners in the phototransduction signaling system.

One such group of candidates included guanine nucleotide analogues labeled with the *N*-methyl-3′-*O*-anthranoyl group (MANT), as they have proven to be very useful in studying G proteins of both the heterotrimeric and Ras families (*13*, *14*). Unfortunately, transducin appears to be unique among the class of heterotrimeric G proteins in its inability to bind MANT-labeled guanine nucleotides. However, recently Neubig and colleagues reported the binding of a fluorescent GTPγS analogue, labeled at the sulfur on the thiophosphoryl group with the BODIPY fluorophore (BOD-GTPγS), to the α subunits of the Go, Gi, and Gs proteins of the large G protein superfamily (*15*). This guanine nucleotide analogue differs from the MANT class of nucleotides in being labeled on the terminal phosphate, rather than on the ribose ring. In this study, we show that BOD-GTPγS can bind to α_T in a rhodopsin- and $\beta\gamma$-dependent manner with an almost 2.5-fold increase in the fluorescence of the BODIPY moiety. The binding of BOD-GTPγS to α_T was not accompanied by an increase in intrinsic tryptophan fluorescence, which normally occurs as an outcome of its activation by either GTPγS or AlF_4^- (*5*). Moreover, the kinetics of binding of BOD-GTPγS to α_T were slow relative to the rate for rhodopsin- and $\beta\gamma$-dependent binding of GTPγS to α_T. This was especially true under conditions where rhodopsin and/or $\beta\gamma$ needed to act in a catalytic fashion to promote the exchange of GDP for BOD-GTPγS on multiple α_T subunits, because of a reduced rate of dissociation of these proteins from each newly formed α_T−BOD-GTPγS complex. The α_T−BOD-GTPγS complex binds to isolated γ_{PDE} subunits with a high affinity but activates PDE less effectively compared to α_T−GTPγS, perhaps because the α_T−BOD-GTPγS species has a higher affinity for the $\beta\gamma$ complex, which blocks the binding of PDE. Overall, our results lead us to conclude that BOD-GTPγS has the interesting property of stabilizing α_T in what appears to represent an intermediate conformational state between the inactive and fully active forms of the G protein.

MATERIALS AND METHODS

Materials. Frozen dark-adapted bovine retina were obtained from Lawson (Lincoln, NE). IAEDANS and BOD-GTPγS were purchased from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Protein Purification. Rod outer segment membranes were isolated as described by Gierschik et al. (*16*). Holotransducin and PDE were prepared from ROS membranes essentially as described by Kroll et al. (*17*) and then were further purified on a HiPrep Superdex 200 HR 26/60 column that was equilibrated with 20 mM HEPES, pH 7.4, plus 10% glycerol. Urea-washed disk membranes that were highly enriched in rhodopsin were prepared as described in Min et al. (*18*). All proteins were flash-frozen in liquid nitrogen and stored at −80 °C. The γ_{PDE} subunit was purified from *Escherichia coli* strain BL21 (DE3) as described by Skiba et al. (*19*).

Modification of γ_{PDE} with IAEDANS. Purified γ_{PDE} (5 μM) was reacted with 1 mM IAEDANS at pH 7.4 for 1 h. The protein was separated from free probe on a PD-10 column equilibrated with 20 mM HEPES, 5 mM $MgCl_2$, and 100 mM NaCl (HMN). The concentration of labeled γ_{PDE} was measured by absorbance at 336 nm, using a molar extinction coefficient of 5700 M^{-1} cm^{-1}.

Fluorescence Measurements. Most fluorescence measurements were made using an SLM 8000c spectrofluorimeter, although in some cases, a Varian eclipse spectrofluorimeter was used. All experiments were carried out in HMN buffer containing 0.01% dodecylmaltoside (HMNDM).

When the excitation and emission spectra of BOD-GTPγS bound to α_T were measured, rhodopsin in urea-washed disk membranes (referred to from here on as simply rhodopsin) in HMNDM buffer was first mixed with transducin and incubated in room light for 5 min, prior to the addition of BOD-GTPγS. This mixture was then incubated at room temperature for 1 h. The excitation and emission slit widths were set at 4 nm.

When kinetic experiments were performed, rhodopsin was initially resuspended in HMNDM to a concentration of 500 nM, and then incubated with transducin in room light for 5 min. The BOD-GTPγS was added to the incubation mix, and the kinetics of binding were monitored continuously. When the fluorescence of the BODIPY probe was monitored, the excitation and emission wavelengths were set at 500 and 520 nm, respectively. When monitoring the intrinsic tryptophan fluorescence of the α_T subunit, excitation was at 300 nm and emission was read at 345 nm.

Measurements of cGMP PDE Activity. The analysis of cGMP hydrolysis by the retinal PDE was carried out according to Liebman and Evanczuk (*20*). Briefly, a pH microelectrode was used to measure the decrease in pH resulting from the production of a proton for each molecule of cGMP hydrolyzed by PDE. All assays were carried out

at 22 °C in a final volume of 200 μL and in buffer containing 5 mM HEPES (pH 7.4), 100 mM NaCl, and 2 mM $MgCl_2$ (assay buffer). Rhodopsin was mixed with transducin in the assay buffer and incubated in room light for 10 min. Subsequently, GTPγS or BOD-GTPγS was added to the samples to a final concentration of 10 μM and incubated at room temperature for 1 h. Purified PDE was then added and incubated at room temperature for 5 min. The reaction was initiated by addition of cGMP. At the end of each assay period, the buffering capacity (mV/nmol) was determined by adding 500 nmol of sodium hydroxide. The rate of hydrolysis of cGMP (nmol/s) was determined from the ratio of the initial slope of the pH record (mV/s) and the buffering capacity of the assay buffer (mV/nmol).

RESULTS

BOD-GTPγS Binds to Transducin in a Rhodopsin-Dependent Manner. The binding of BOD-GTPγS to transducin gives rise to a better than 2.0-fold increase in the fluorescence emission of the BODIPY moiety (Figure 1A). The increase in BODIPY fluorescence that occurs upon its binding to the α_T subunit is not accompanied by changes in either its maximal excitation (curves 1 and 3) or emission wavelengths (curves 2 and 4) and therefore is not the result of an increased hydrophobicity in the immediate environment of the fluorescent probe. Rather, the changes in BODIPY fluorescence that accompany its binding to α_T can be attributed to an extension in the structure of the nucleotide analogue such that the BODIPY moiety is no longer stacking with the guanine base and thus is less susceptible to intramolecular quenching (*15*).

The formation of the α_T−BOD-GTPγS complex can be monitored as the difference in fluorescence that occurs as a function of BOD-GTPγS concentration, in the presence and absence of transducin. As shown in Figure 1B, the binding of BOD-GTPγS to α_T is saturable and can be fit to a simple bimolecular reaction yielding an apparent dissociation constant of 110 nM (105 ± 5 nM; $n = 2$).

The final extent of the fluorescence change that accompanies the binding of BOD-GTPγS to α_T is essentially independent of rhodopsin concentration. This suggests that rhodopsin can act catalytically to stimulate the binding of the fluorescent analogue to multiple α_T subunits, similar to the case for the rhodopsin-stimulated binding of GTP or GTPγS to α_T. However, the rate of binding of BOD-GTPγS to α_T is highly dependent on rhodopsin concentration. In the absence of the photoreceptor, the binding of the fluorescent analogue to α_T is extremely slow, such that half-maximal binding is not achieved, even after a 1 h incubation at room temperature (Figure 2A, curve 1). With increasing concentrations of rhodopsin, the rate of binding is then markedly accelerated (see curves 2 and 3 in Figure 2A). Still, the rate of the rhodopsin-dependent binding of BOD-GTPγS to α_T is slow relative to the rhodopsin-stimulated binding of GTPγS, particularly under conditions where rhodopsin needs to act catalytically to promote nucleotide exchange on the entire pool of α_T subunits. For example, when monitoring rhodopsin-stimulated GDP-GTPγS exchange by changes in the intrinsic tryptophan fluorescence of α_T, the rate of binding of GTPγS occurs within seconds (data not shown; also see ref 6). This is also evident when comparing the rates for the exchange of BOD-GTPγS for GDP, as read-out by the enhancement in the BODIPY fluorescence (Figure 2A), versus the rates for the exchange of GTPγS for BOD-GTPγS, as monitored by the quenching of the emission of the fluorescent analogue (Figure 2B). In the latter case, the total pool of α_T is loaded with GTPγS within 2−3 min, even at substoichiometric levels of rhodopsin (Figure 2B), whereas the rhodopsin-stimulated exchange of BOD-GTPγS for GDP requires at least 30 min to 1 h under identical conditions (Figure 2A). These differences appear at least in part to reflect a slower rate of dissociation of rhodopsin from the α_T−BOD-GTPγS complex compared to the rate of dissociation of the photoreceptor from the α_T−GTPγS species, such that a significantly longer period of time is required for substoichiometric levels of rhodopsin to activate the entire α_T pool when BOD-GTPγS is used as the activating

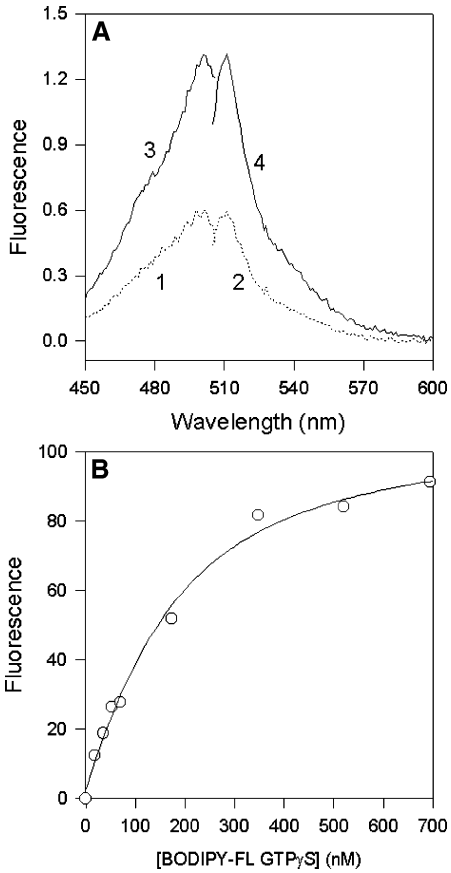

FIGURE 1: Binding of BOD-GTPγS to transducin. (A) Excitation and emission spectra of BOD-GTPγS. Transducin (440 nM) was incubated with rhodopsin (25 nM) in room light, and BOD-GTPγS was added to a final concentration of 50 nM. Curves 1 and 2 represent the excitation and emission spectra, respectively, of free BOD-GTPγS (50 nM), while curves 3 and 4 represent the excitation and emission spectra, respectively, for BOD-GTPγS in the presence of transducin. The excitation spectra were obtained with the emission wavelength fixed at 510 nm. The emission spectra were obtained with the excitation wavelength fixed at 500 nm. (B) Titration of transducin with BOD-GTPγS. Transducin (125 nM) was incubated with rhodopsin (100 nM) in room light for 5 min. BOD-GTPγS was added at varying concentrations, and the samples were incubated at room temperature for 1 h. The fluorescence of each sample was monitored with excitation at 500 nm and emission at 520 nm. The fluorescence readings at each concentration of BOD-GTPγS were corrected for emission in the absence of transducin. The corrected fluorescence readings were fitted to a simple bimolecular reaction model, and a K_d of 100 nM was calculated.

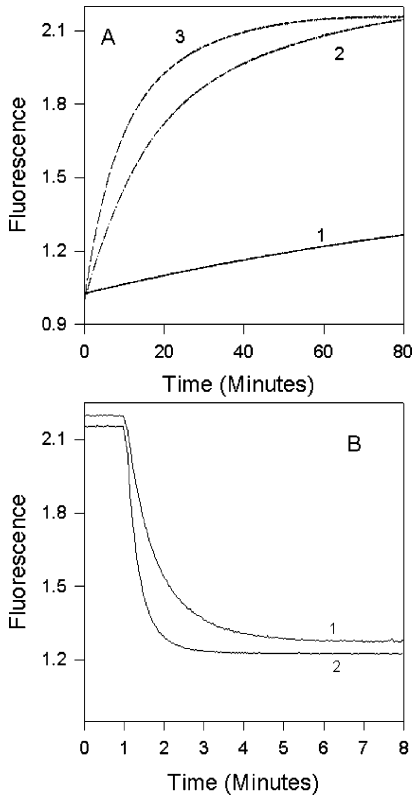

FIGURE 2: Kinetics of trandsucin−BOD-GTPγS interactions. (A) The binding of BOD-GTPγS (460 nM) to transducin (190 nM) was monitored. For curve 1, no rhodopsin was present. For curves 2 and 3, transducin was incubated with 49 and 98 nM rhodopsin, respectively, in room light for 5 min prior to the addition of BOD-GTPγS. The fluorescence was monitored continuously with excitation at 500 nm and emission at 520 nm. (B) Transducin was incubated with rhodopsin (curve 1, 49 nM; curve 2, 98 nM) in room light for 5 min. BOD-GTPγS (460 nM) was added, and the increase in fluorescence was monitored. At saturation, GTPγS (24 μM) was added, and the decrease in fluorescence was monitored.

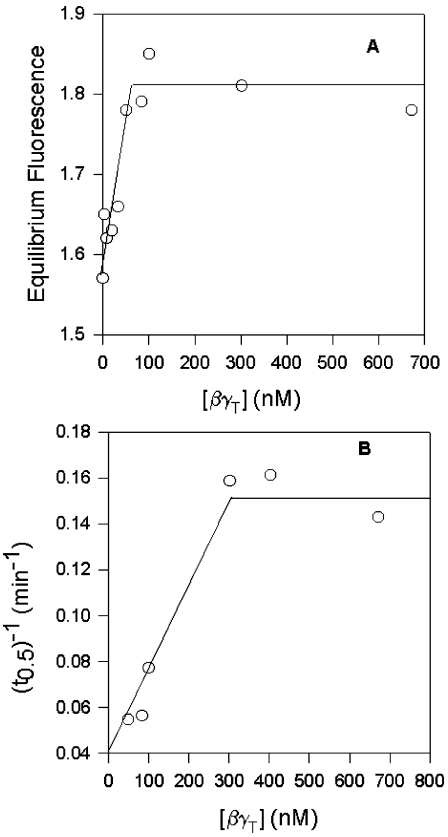

FIGURE 3: Binding of BOD-GTPγS to α_T at various $\beta\gamma_T$ concentrations. α_T (192 nM) was incubated with rhodopsin (98 nM) and varying concentrations of $\beta\gamma_T$ in room light for 10 min. Subsequently, BOD-GTPγS (460 nM) was added, and the fluorescence of the sample was monitored. (A) Fluorescence emission of the sample at equilibrium is plotted at various concentrations of $\beta\gamma_T$. (B) The time required to attain half of the total change in fluorescence was calculated at each concentration of $\beta\gamma_T$. The reciprocal of $t_{0.5}$ is plotted at various $\beta\gamma_T$ concentrations.

nucleotide (also, see below). It is in fact the relatively slow rate of dissociation of rhodopsin from the α_T−BOD-GTPγS complex that allows us to measure the exchange of GTPγS for BOD-GTPγS. Note that the reverse reaction would not be feasible to measure, as the α_T−GTPγS complex rapidly dissociates from rhodopsin and thus is essentially a non-exchangeable species.

BOD-GTPγS Binds to α_T in a $\beta\gamma$-Dependent Manner. It has been well established that the activation of heterotrimeric G proteins requires both a heptahelical receptor and the $\beta\gamma$ subunit complex. The latter has been suggested both to increase the binding affinity of the receptors for the Gα subunits as well as to participate directly in the activation event (*21*). In the case of the functional coupling between rhodopsin and transducin, it has been clearly demonstrated that the $\beta\gamma$ complex, like the photoreceptor rhodopsin, can act catalytically to promote the activation of multiple α_T subunits, as a result of the dissociation of $\beta\gamma$ from α_T that occurs upon the binding of GTP (*5*, *22*). As shown in Figure 3A, the fluorescence enhancement that accompanies the rhodopsin-dependent exchange of BOD-GTPγS for GDP on α_T is dependent on the concentration of $\beta\gamma$ (Figure 3A). At low concentrations of $\beta\gamma$ and under equilibrium conditions, a percentage of the α_T−BOD-GTPγS is associated with $\beta\gamma$, because of its slow dissociation from this α_T species. Under such conditions, substoichiometric amounts of $\beta\gamma$ are not able to activate the entire pool of α_T. However, at levels of $\beta\gamma$ approaching the concentration of α_T, complete exchange of BOD-GTPγS for GDP can occur, and maximum BODIPY fluorescence is observed.

The rate of binding of BOD-GTPγS to the α_T subunits also increases with increasing concentrations of $\beta\gamma$ (Figure 3B). Here again, this reflects the requirement that rhodopsin and $\beta\gamma$, when present in substoichiometric amounts relative to α_T, must act catalytically to bind and debind from each additional α_T subunit that is loaded with the fluorescent nucleotide as an outcome of nucleotide exchange, thus requiring a significant amount of time for rhodopsin and $\beta\gamma$ to cycle among the total pool of α_T subunits. Ultimately, as saturating levels of $\beta\gamma$ are added to the system, each α_T subunit contains a bound $\beta\gamma$ subunit complex. Under these conditions, the rate of rhodopsin-stimulated exchange of GDP for BOD-GTPγS is no longer stimulated by adding increasing amounts of $\beta\gamma$, because the rate is no longer limited by $\beta\gamma$ dissociation from the α_T−BOD-GTPγS complexes. However, at saturating levels of $\beta\gamma$, the rate of exchange of GDP for BOD-GTPγS on α_T is still slower than the rate of exchange of GDP for GTPγS. This is also the case at high levels of rhodopsin (data not shown). Thus, in addition to the slower rate of dissociation of the $\beta\gamma$ complex (and rhodopsin) from α_T−BOD-GTPγS compared to the α_T−GTPγS species, the apparent rate of association of BOD-

GTPγS with α_T (at saturating levels of rhodopsin and $\beta\gamma$) is also slow compared to the rate of association of GTPγS.

Binding of BOD-GTPγS Causes No Increase in the Fluorescence of Trp 207. The ability of α_T to dissociate from $\beta\gamma$ and subsequently from rhodopsin is thought to depend on a conformational change that occurs within the Switch 2 domain and weakens the affinity between the Gα subunits and the $\beta\gamma$ subunit complex (*2*). A sensitive real-time readout for this conformational transition is the change in the fluorescence emission of a tryptophan residue within the Switch 2 domain (Trp 207 in α_T), as this reflects the movement of Switch 2 and the burying of its tryptophan residue in a less solvent-accessible environment (*2*). Interestingly, we find that the binding of BOD-GTPγS to α_T does not result in a detectable change in the emission of Trp 207. This is clearly demonstrated by the data in Figure 4A, which shows that under conditions where the rhodopsin-stimulated exchange of GDP for BOD-GTPγS yielded an obvious enhancement in BODIPY emission (curve 1), there was absolutely no change in the α_T tryptophan emission (curve 2).

When excess GTPγS was added to the assay system (i.e., after the binding of BOD-GTPγS to α_T had reached equilibrium), a relatively rapid quenching of the BODIPY fluorescence occurred (Figure 4B, curve 1). This quenching reflected the rhodopsin-stimulated exchange of the fluorescent GTP analogue for GTPγS and was complete in 2−4 min. The time course for quenching of the BODIPY fluorescence matched exactly the time course for the enhancement in Trp 207 fluorescence (Figure 4B, curve 2), reflecting the activating conformational change in the Switch 2 domain of α_T that accompanies the binding of GTPγS.

Taken together, the results presented in Figure 4, parts A and B highlight a number of interesting differences between the rhodopsin-dependent binding of BOD-GTPγS versus GTPγS to α_T. Specifically, they further demonstrate that the rate of the rhodopsin-dependent exchange of GDP for BOD-GTPγS is slow relative to the rate for rhodopsin-stimulated exchange of BOD-GTPγS for GTPγS, under conditions where rhodopsin needs to act catalytically to stimulate this exchange reaction on multiple α_T subunits. As indicated above, the relatively slow rate of dissociation of rhodopsin and $\beta\gamma$ from α_T−BOD-GTPγS explains why rhodopsin is able to stimulate the exchange of BOD-GTPγS for GTPγS, as reflected by the ability of added GTPγS to reverse the fluorescence enhancement that accompanies the binding BOD-GTPγS to α_T (i.e., curve 1 in Figure 4B). The inability of BOD-GTPγS to induce the necessary conformational change within α_T to elicit an enhancement in the fluorescence emission of Trp 207 suggests that the binding of BOD-GTPγS to α_T, at best, induces only subtle changes in Switch 2. This may represent the underlying reason for the relatively slow rate of dissociation of rhodopsin and $\beta\gamma$ from α_T−BOD-GTPγS complexes. Given the relative ineffectiveness of BOD-GTPγS to induce the typical "activating" conformational change in Switch 2, it was then of interest to see whether α_T when bound to BOD-GTPγS was able to bind and stimulate effector (PDE) activity.

The α_T−BOD-GTPγS Complex Binds to γ_{PDE} and Stimulates PDE Activity. It has been well established that a GTPγS-bound α_T subunit will stimulate cyclic GMP hydrolysis by the PDE through the ability of the activated α_T

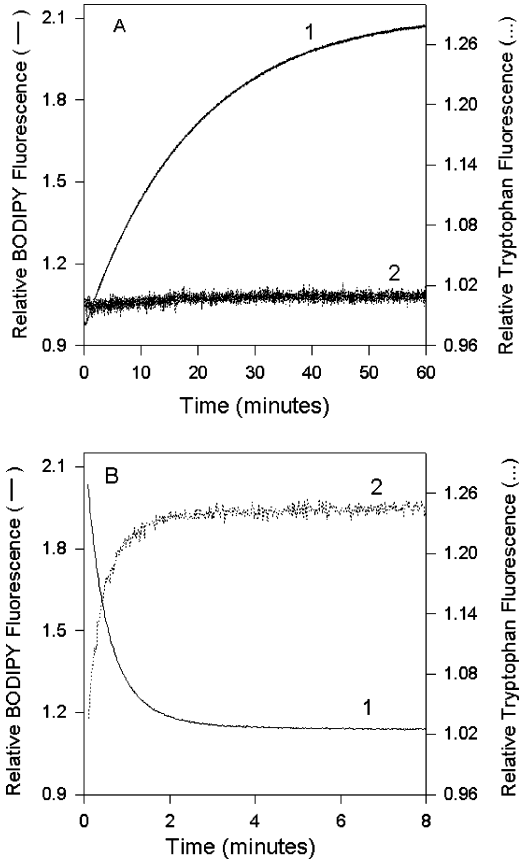

FIGURE 4: The binding of BOD-GTPγS to transducin as monitored by changes in BODIPY fluorescence or changes in the intrinsic tryptophan fluorescence. (A) Transducin (150 nM) was incubated with rhodopsin (30 nM) in room light for 10 min. BOD-GTPγS (500 nM) was added, and the fluorescence emission was monitored. For curve 1, the relative change in BODIPY fluorescence (left ordinate) was recorded for an excitation wavelength set at 500 nm and an emission wavelength set at 520 nm. For curve 2, the relative change in tryptophan fluorescence (right ordinate) was monitored with the excitation wavelength set at 300 nm, and the emission wavelength was set at 345 nm. (B) Competition between GTPγS and BOD-GTPγS for binding to transducin. BOD-GTPγS (460 nM) was incubated with transducin (192 nM) and rhodopsin (96 nM) for 90 min in room light and at room temperature. Subsequently, GTPγS (24 μM) was added, and the fluorescence emission was monitored. For curve 1, the relative change in BODIPY fluorescence (left ordinate) was monitored with the excitation wavelength set at 500 nm, and the emission wavelength was set at 520 nm. For curve 2, the relative change in tryptophan fluorescence (right ordinate) was monitored with the excitation wavelength set at 300 nm, and the emission wavelength was set at 345 nm.

subunit to bind with high affinity to the γ subunits of the effector enzyme (γ_{PDE}). We have taken advantage of the ability to generate relatively large quantities of both α_T and γ_{PDE}, and the ease of labeling the latter, to monitor the binding of activated α_T to γ_{PDE} through fluorescence energy transfer. The γ_{PDE} subunit has a single cysteine at position 68 that can be labeled with various fluorescent probes. For our studies, we have used the thiol-specific reagent, IAEDANS, to label γ_{PDE}, because the fluorescence emission of the IAEDANS probe (Figure 5A, curve 1) provides an excellent overlap with the excitation spectrum of BOD-GTPγS (Figure 5A, curve 2). Thus, IAEDANS-labeled γ_{PDE} and α_T−BOD-GTPγS represent a very good donor−acceptor pair for fluorescence resonance energy transfer. The binding of α_T−BOD-GTPγS to IAEDANS-labeled γ_{PDE} was moni-

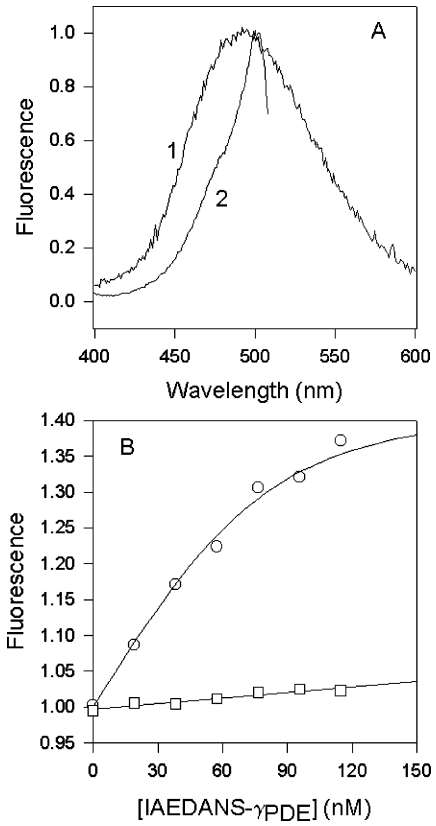

FIGURE 5: Binding of IAEDANS-γ_{PDE} to BOD-GTPγS-bound α_T. (A) The emission spectrum for IAEDANS-γ_{PDE} (150 nM) was collected by scanning the emission monochromator from 400 to 600 nm with the excitation monochromator set at 336 nm (curve 1). BOD-GTPγS (500 nM) was incubated with transducin (150 nM) and rhodopsin (30 nM) for 90 min in room light and at room temperature. Subsequently, the excitation spectrum for BOD-GTPγS was collected by scanning the excitation monochromator from 400 to 508 nm (curve 2), with the emission monochromator set at 510 nm. (B) BOD-GTPγS (500 nM) was incubated with transducin (96 nM) and rhodopsin (30 nM) for 90 min in room light and at room temperature. The binding of IAEDANS-γ_{PDE} at various concentrations was monitored by measuring the sensitized emission of BOD-GTPγS (due to energy transfer from the IAEDANS label) at 510 nm, for excitation at 335 nm. The binding curves were fitted to a simple bimolecular interaction model, yielding a K_d value of 13 nM.

tored by increases in the fluorescence emission from the BODIPY probe that occurred through the excitation of the IAEDANS label. The resulting sensitized fluorescence emission from BOD-GTPγS, as a function of increasing amounts of IAEDANS-γ_{PDE}, was saturable (Figure 5B, open circles) and could be fit to a simple model for a bimolecular reaction with an apparent K_d value of 13 nM. The increase in BODIPY fluorescence was reversed with an excess of unlabeled γ_{PDE}, as a result of its competition with IAEDANS-γ_{PDE} for the α_T−BOD-GTPγS complex (data not shown). Such competition would eliminate the possibility of resonance energy transfer between the BODIPY and IAEDANS labels. Also, pretreatment of the α_T−BOD-GTPγS complex with unlabeled γ_{PDE}, followed by the addition of IAEDANS-γ_{PDE}, abolished the sensitized emission (Figure 5B, open squares), further confirming a specific interaction between α_T−BOD-GTPγS and IAEDANS-labeled γ_{PDE}.

Given that the α_T−BOD-GTPγS complex was able to bind to the γ_{PDE} subunit, we examined whether this α_T species was also capable of stimulating effector activity (i.e., cyclic GMP hydrolysis). We found that the α_T−BOD-GTPγS species was indeed able to stimulate cyclic GMP hydrolysis by the PDE, but with a reduced potency relative to the α_T−GTPγS complex. As shown in Figure 6A, when α_T was maintained at a constant level and PDE was varied, the ratios for the stimulation of cyclic GMP hydrolysis by α_T−BOD-GTPγS versus α_T−GTPγS progressively increased when increasing concentrations of PDE were added to the assay. When PDE was held constant and the amounts of α_T (and $\beta\gamma$) were increased in the assay incubation, we again observed that the α_T−BOD-GTPγS species formed in a rhodopsin- and $\beta\gamma$-dependent manner showed a reduced potency for stimulating PDE activity, compared to the corresponding amount of α_T−GTPγS (Figure 6B). However, under these conditions, the relative stimulation by α_T−BOD-GTPγS compared to α_T−GTPγS remained constant over the range of α_T concentrations assayed. Overall, these results suggest that the apparent higher affinity exhibited by α_T−BOD-GTPγS for $\beta\gamma$ may contribute to the reduced potency exhibited by α_T−BOD-GTPγS in stimulating PDE activity. Because $\beta\gamma$ competes with γ_{PDE} for binding to α_T, by raising the PDE concentration while keeping the level of $\beta\gamma$ fixed (i.e., Figure 6A), PDE becomes a more effective competitor. Consequently, the differences in the ability of α_T−BOD-GTPγS versus α_T−GTPγS to stimulate PDE activity were reduced as PDE levels were increased. However, when the PDE levels were held constant while the levels of α_T were varied together with $\beta\gamma$ (Figure 6B), the effective ability of PDE to compete with $\beta\gamma$ for α_T−BOD-GTPγS was not enhanced. Thus, the ratio for PDE activity stimulated by α_T−BOD-GTPγS, compared to that by α_T−GTPγS, essentially remained the same over the range of α_T concentrations assayed.

DISCUSSION

The phototransduction signaling pathway operating in vertebrate vision has served as an excellent model for studying receptor/G protein-coupled signal transduction. It is possible to isolate the primary components of this system from rod outer segments in highly purified form and in large quantities, and recombinant expression systems are available for studying the effects of different mutants of α_T. Moreover, a number of insights into how the retinal G protein acts as a molecular switch in this signaling system have been obtained from the X-ray crystal structures of the α_T subunit in both its off (GDP-bound) and on (GTPγS-bound) states (*2*, *3*), as well as from the structures of α_T in its transition state for GTP hydrolysis (i.e., the α_T−GDP-AlF_4^- complex) (*23*) and when associated with the γ_{PDE} subunit and RGS regulatory protein (*24*). Thus, it has been proposed that the movement of conformationally sensitive switch regions in α_T, which occurs as an outcome of GDP−GTP exchange, is necessary for the dissociation of activated GTP-bound α_T from the G$\beta\gamma$ complex and for the association of α_T with its binding site (the γ_{PDE} subunits) on the target/effector. Still, a number of interesting mechanistic and kinetic questions exist regarding how receptors such as rhodopsin promote GDP−GTP exchange and pertaining to the nature of the intermediate conformational states that occur during the activation event, as well as during the initial binding of an activated Gα subunit to its target/effector.

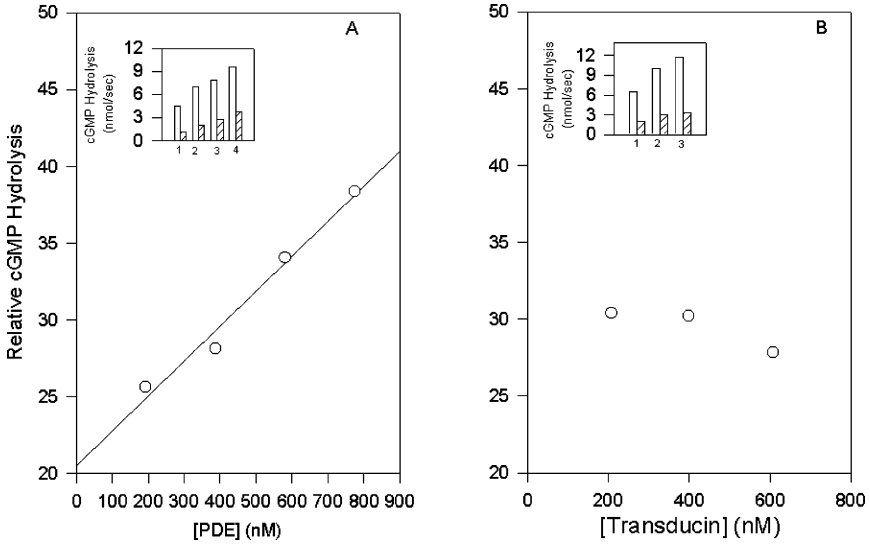

FIGURE 6: Relative stimulation of PDE activity by α_T−BOD-GTPγS versus α_T−GTPγS. (A) Transducin (256 nM) was incubated in the presence of rhodopsin (98 nM) in room light for 10 min. Subsequently, GTPγS (10 μM) or BOD-GTPγS (10 μM) was added to these samples and incubated at room temperature for 90 min. PDE was added at varying concentrations (194, 388, 581, and 775 nM), and cGMP hydrolysis was monitored as nanomoles of protons released per second. The percentage PDE activity measured in the presence of BOD-GTPγS relative to that measured in the presence of GTPγS is plotted on the ordinate. The line through the data was drawn by inspection. The inset shows cGMP hydrolysis measured as nanomoles of cGMP hydrolyzed per second for α_T−GTPγS (open bars) and α_T−BOD-GTPγS (striped bars) at different PDE concentrations (194 nM (column 1), 388 nM (column 2), 581 nM (column 3), and 775 nM (column 4)). (B) Transducin at varying concentrations (208, 400, and 608 nM) was incubated with rhodopsin (98 nM) in room light for 10 min. Subsequently, GTPγS (10 μM) or BOD-GTPγS (10 μM) was added to these samples and incubated at room temperature for 90 min. PDE was added to yield a concentration of 388 nM in the assay, and cGMP hydrolysis was monitored as nanomoles of protons released per second. The percentage PDE activity measured in the presence of BOD-GTPγS relative to that measured in the presence of GTPγS is plotted on the ordinate. The inset shows cGMP hydrolysis measured as nanomoles of cGMP hydrolyzed per second for α_T−GTPγS (open bars) and α_T−BOD-GTPγS (striped bars) at different transducin concentrations (200 nM (column 1), 400 nM (column 2), and 608 nM (column 3)).

Fluorescence spectroscopy has provided a powerful real-time readout for the interactions of G proteins and their component Gα and G$\beta\gamma$ subunits with other participants in signaling cascades, as well as for the conformational changes that accompany G protein activation and target/effector stimulation (*25*). This has been particularly true for the vertebrate vision system, as the α_T subunit has been labeled at its guanine nucleotide-binding site (at lysine 267) as well as at its carboxylterminus (cysteine 351), several cysteine residues have been labeled on the β subunit of the $\beta\gamma$ complex, and both γ_{PDE} and the PDE molecule have been labeled with fluorophores. The ability to introduce fluorescent probes at these different sites has enabled a variety of resonance energy transfer studies to monitor directly the interactions between α_T and $\beta\gamma$, rhodopsin and $\beta\gamma$, α_T and γ_{PDE}, and γ_{PDE} with the larger α_{PDE} and β_{PDE} subunits (*7*, *10*, *11*). Moreover, it has been possible to take advantage of an environmentally sensitive tryptophan residue in the Switch 2 domain of α_T (Trp 207) to monitor rhodopsin-dependent GDP−GTP exchange on α_T as well as the GTP-binding/GTP hydrolytic cycle (*5*, *6*). Despite these achievements, it has not been possible to introduce a spectroscopic probe into the guanine nucleotide-binding site of α_T in a manner that enables the α_T subunit to become activated and to directly interact with and stimulate its target/effector (PDE). For example, labeling lysine 267 with fluorescein isothiocyanate, within the highly conserved NKXD motif of α_T that binds the guanine ring of GDP and GTP (where X = lysine 267 in α_T), yields an α_T subunit that cannot be activated by rhodopsin (*10*). Thus, it is not possible to use this labeled subunit to follow its dissociation from G$\beta\gamma$ following GDP−GTP exchange or the ensuing binding of the activated α_T subunit to PDE. Likewise, it has not been possible to take advantage of the widely used fluorescent guanine nucleotide analogue, MANT−GDP, or any of its related derivatives, because these fluorescent analogues do not bind to α_T. However, in the present study, we now show that the fluorescent GTPγS analogue, BOD-GTPγS, binds to the α_T subunit and enables it to stimulate PDE activity.

The binding of BOD-GTPγS to α_T requires rhodopsin and is stimulated by $\beta\gamma$, similar to the case for GTP or GTPγS (*6*). However, unlike a newly formed α_T−GTPγS complex, which immediately dissociates from $\beta\gamma$ as well as from rhodopsin, the α_T−BOD-GTPγS complex appears to dissociate more slowly from its binding partners. In fact, because of this slow rate of dissociation, it is possible to exchange BOD-GTPγS on α_T for GTPγS, whereas once an α_T−GTPγS species is formed, it is essentially nonexchangeable because of its immediate release from $\beta\gamma$ and rhodopsin. The apparent slower rate of dissociation of α_T−BOD-GTPγS from the $\beta\gamma$ complex is likely due to an altered change in Switch 2, relative to the change that accompanies GDP-GTPγS exchange. Unlike the case for α_T−GTPγS, where an enhancement in Trp 207 fluorescence occurs, there is no change in Trp 207 emission upon the formation of the α_T−BOD-GTPγS species. Nonetheless, the α_T−BOD-GTPγS complex is able to bind to γ_{PDE}, as readout by assaying the fluorescence resonance energy transfer that occurs between the BODIPY moiety and IAEDANS attached to the single cysteine (Cys 68) on γ_{PDE}. In addition, the α_T−BOD-GTPγS complex is able to stimulate cyclic GMP hydrolysis, albeit less effectively than α_T−GTPγS. This may reflect the higher

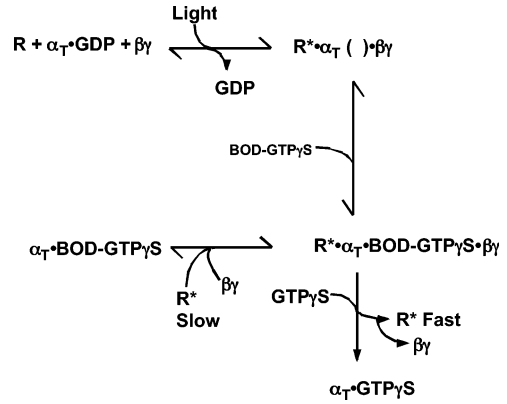

FIGURE 7: Model depicting the binding of BOD-GTPγS to the α_T subunit. This model depicts a system that contains rhodopsin (in urea-stripped rod outer segments), purified retinal α_T, and the purified retinal $\beta\gamma$ complex. Following the binding of the α_T−GDP-$\beta\gamma$ complex (i.e., transducin) to light-activated rhodopsin, there is an exchange of BOD-GTPγS for GDP. This is followed by a relatively slow dissociation of $\beta\gamma$ and rhodopsin from the α_T−BOD-GTPγS species.

affinity exhibited by the α_T−BOD-GTPγS species compared to α_T−GTPγS for the $\beta\gamma$ complex, which results in a larger portion of the total pool of α_T−BOD-GTPγS containing bound $\beta\gamma$ and therefore being unable to stimulate the target/effector. Raising the levels of PDE (at fixed levels of $\beta\gamma$) enables the PDE to compete more effectively with $\beta\gamma$ for the α_T−BOD-GTPγS species and thereby increases the α_T−BOD-GTPγS-stimulated PDE activity.

What is particularly interesting is that the ability of an α_T−BOD-GTPγS complex to stimulate PDE activity is not dependent on extensive changes in Switch 2, at least as indicated by an enhancement in tryptophan fluorescence. This is reminiscent of what we had observed with an α_T subunit, α_T(E203A), that was mutated at a highly conserved glutamic acid residue in the Switch 2 domain. The α_T(E203A) mutant was capable of undergoing rhodopsin-stimulated GDP-GTP exchange, but unexpectedly, was able to stimulate PDE activity even in the GDP-bound state and under conditions where the position of Trp 207 had not yet been altered (*26*). Thus, subtle changes in Switch 2 are apparently sufficient to convey the necessary structural changes to other regions of the α_T subunit that mediate the actual stimulation of PDE activity. The change in Trp 207 fluorescence may reflect secondary structural effects that follow those changes necessary for effector activation, as suggested by experiments showing that the rate of Trp 207 fluorescence changes lag behind the rate of dissociation of an activated α_T subunit from the $\beta\gamma$ complex (*10*).

Overall, the results that we have obtained with BOD-GTPγS suggest that this fluorescent nucleotide traps the α_T subunit in a conformational state that might represent an intermediate to those of the signaling-inactive, GDP-bound state and the fully activated state (e.g., as exemplified by an α_T−GTPγS complex) (Figure 7). The ability to spectroscopically monitor this BOD-GTPγS-induced "activated state" raises a number of interesting questions, including how the kinetics for the dissociation of both rhodopsin and the $\beta\gamma$ complex from α_T−BOD-GTPγS compare to the kinetics for the interaction of α_T−BOD-GTPγS with γ_{PDE}, and just how well the RGS proteins, which stimulate GTP hydrolysis, bind to such an intermediate state as that represented by the α_T−BOD-GTPγS complex, relative to their binding to either the α_T−GTPγS or α_T−GDP-AlF_4^- complex. There are also some interesting structural questions, as we would expect that the three-dimensional structure for the BOD-GTPγS-induced conformational state can be distinguished from the structures of both the GDP- and GTPγS-bound states of α_T (*2*, *3*). Thus, future efforts will be directed toward determining an X-ray crystal structure for the α_T−BOD-GTPγS species, as this should provide interesting insights into the minimal changes in the Switch 2 domain that are necessary to stimulate the dissociation of the $\beta\gamma$ complex and the binding and stimulation of the PDE. Finally, there are questions regarding the mechanism by which the BOD-GTPγS molecule is able to trap α_T in a distinct conformational state. While at the present time we do not have a definitive answer to this question, one rather provocative possibility has to do with Mg^{2+}. It is interesting to consider the possibility that unlike GTP or other GTP analogues (e.g., GTPγS), BOD-GTPγS is unable to bind Mg^{2+} through its γ-phosphate and that this accounts for its ability to trap α_T in an intermediate rather than fully activated state. We plan to look at this possibility more closely by examining the effects of mutations in α_T that would be expected to fail to bind properly the γ-phosphate of GTP or that should be unable to assist in Mg^{2+}-binding, to see if such mutants provide other examples of α_T subunits that assume an intermediate conformation between the inactive and fully active states.

REFERENCES

1. Arshavsky, V. Y., Lamb, T. D., and Pugh, E. N., Jr. (2002) G proteins and phototransduction, *Annu. Rev. Physiol. 64*, 153−187.
2. Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) Structural determinants for activation of the alpha-subunit of a heterotrimeric G protein, *Nature 369*, 621−628.
3. Noel, J. P., Hamm, H. E., and Sigler, P. B. (1993) The 2.2 A crystal structure of transducin-alpha complexed with GTP gamma S, *Nature 366*, 654−663.
4. Granovsky, A. E., Natochin, M., and Artemyev, N. O. (1997) The gamma subunit of rod cGMP-phosphodiesterase blocks the enzyme catalytic site, *J. Biol. Chem. 272*, 11686−11689.
5. Phillips, W. J., and Cerione, R. A. (1988) The intrinsic fluorescence of the α subunit of transducin. Measurement of receptor-dependent guanine nucleotide exchange. *J. Biol. Chem. 263*, 15498−15505.
6. Guy, P. M., Koland, J. G., and Cerione, R. A. (1990) Rhodopsin-stimulated activation−deactivation cycle of transducin: kinetics of the intrinsic fluorescence response of the α subunit, *Biochemistry 29*, 6954−6964.
7. Phillips, W. J., and Cerione, R. A. (1991) Labeling of the *β-γ* subunit complex of transducin with an environmentally sensitive cysteine reagent. Use of fluorescence spectroscopy to monitor transducin subunit interactions, *J. Biol. Chem. 266*, 11017−11024.
8. Phillips, W. J., and Cerione, R. A. (1992) Rhodopsin/transducin interactions. I. Characterization of the binding of the transducin−*β-γ* subunit complex to rhodopsin using fluorescence spectroscopy, *J. Biol. Chem. 267*, 17032−17039.
9. Hingorani, V., N. and Ho, Y. (1987) Chemical modification of bovine transducin: effect of fluorescein 5′-isothiocyanate labeling on activities of the transducin alpha subunit *Biochemistry 26*, 1633−1639.
10. Mittal, R., Cerione, R. A., and Erickson, J. W. (1994) Aluminum fluoride activation of bovine transducin induces two distinct conformational changes in the alpha subunit, *Biochemistry 33*, 10178−10184.
11. Erickson, J. W., and Cerione, R. A. (1991) Resonance energy transfer as a direct monitor of GTP-binding protein-effector interactions: activated α-transducin binding to the cGMP phosphodiesterase in the bovine phototransduction cascade, *Biochemistry 30*, 7112−7118.

12. Erickson, J. W., Mittal, R., and Cerione, R. A. (1995) Use of resonance energy transfer to determine the proximity of the guanine nucleotide binding site of transducin relative to a conformationally sensitive site on the γ subunit of the cyclic GMP phosphodiesterase, *Biochemistry 34*, 8693−8700.
13. Remmers, A. E., Posner, R., and Neubig, R. R. (1994) Fluorescent guanine nucleotide analogues and G protein activation, *J. Biol. Chem. 269*, 13771−13778.
14. Neal, S. E., Eccleston, J. F., and Webb, M. R. (1991) Hydrolysis of GTP by $p21^{NRAS}$, the NRAS Protooncogene Product, is Accompanied by a Conformational Change in the Wild-Type Protein: Use of a Single Fluorescent Probe at the Catalytic Site, *Proc. Natl. Acad. Sci. U. S. A. 87*, 3562−3565.
15. McEwen, D. P., Gee, K. R., Kang, H. C., and Neubig, R. R. (2001) Fluorescent BODIPY-GTP analogues: real-time measurement of nucleotide binding to G proteins, *Anal. Biochem. 291*, 109−117.
16. Gierschik, P., Simons, C., Woodard, C., Somers, R., and Spiegel, A. (1984) Antibodies against a retinal guanine nucleotide-binding protein cross-react with a single plasma membrane protein in nonretinal tissues, *FEBS Lett. 172*, 321−325.
17. Kroll, S., Phillips, W. J., and Cerione, R. A. (1989) The regulation of the cyclic GMP phosphodiesterase by the GDP-bound form of the alpha subunit of transducin, *J. Biol. Chem. 264*, 4490−4497.
18. Min, K. C., Gravina, S. A., and Sakmar, T. P. (2000) Reconstitution of the vertebrate visual cascade using recombinant heterotrimeric transducin purified from Sf9 cells, *Protein Expr. Purif. 20*, 514−526.
19. Skiba, N. P., Artemyev, N. O., and Hamm, H. E. (1995) The carboxyl terminus of the γ-subunit of rod cGMP phosphodiesterase contains distinct sites of interaction with the enzyme catalytic subunits and the α-subunit of transducin, *J. Biol. Chem. 270*, 13210−13215.
20. Liebman, P. A., and Evanczuk, A. T. (1982) Real time assay of rod disk membrane cGMP phosphodiesterase and its controller enzymes, *Methods Enzymol. 81*, 532−542.
21. Iiri, T., Farfel, Z., and Bourne, H. R. (1998) G-protein diseases furnish a model for the turn-on switch, *Nature 394*, 35−38.
22. Fung, B. K. (1983) Characterization of transducin from bovine retinal rod outer segments. I. Separation and reconstitution of the subunits, *J. Biol. Chem. 258*, 10495−10502.
23. Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) GTPase mechanism of Gproteins from the 1.7-A crystal structure of transducin alpha-GDP-AIF-4, *Nature 372*, 276−279.
24. Slep, K. C., Kercher, M. A., He, W., Cowan, C. W., Wensel, T. G., and Sigler, P. B. (2001) Structural determinants for regulation of phosphodiesterase by a G protein at 2.0 A, *Nature 409*, 1071−1077.
25. Cerione, R. A. (1994) Fluorescence assays for G protein interactions. In: G proteins and Signal Transduction (R. Iyengar, ed.) *Methods Enzymology 237*, 409−423.
26. Mittal, R., Erickson, J. W., and Cerione, R. A. (1996) Uncoupling of GTP binding from target stimulation by a single mutation in the transducin alpha subunit, *Science 271*, 1413−1416.

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