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†

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ABSTRACT: In this work, we have used resonance energy transfer to determine the relative positions of a reactive cysteine residue on the γ subunit of the retinal cyclic GMP phosphodiesterase (γ_{PDE}) and a reactive lysine residue on the α subunit of transducin (α_T). The single cysteine residue on γ_{PDE} (residue 68) is located at a site that is sensitive to the binding of both the inactive and active forms of α_T. This is demonstrated by the finding that the addition of an α_TGDP complex to a γ_{PDE} subunit labeled with the environmentally-sensitive probe 2-(4-maleimidoanilino)naphthalene-6-sulfonate (MIANS) results in an enhancement in the MIANS fluorescence. The α_TGDP-induced fluorescence enhancement is dose-dependent and yields an apparent K_d value of ~3 μM. Activation of α_TGDP by aluminum fluoride, when bound to the MIANS-labeled γ_{PDE} (M-γ_{PDE}), then results in a quenching of the MIANS fluorescence. The aluminum fluoride-induced change in M-γ_{PDE} fluorescence occurs on a time scale identical to that observed for changes in the intrinsic α_T fluorescence that correspond to activating conformational changes in the α_T "switch II" region. These results suggest that the induction of the activated state of the α_T subunit results in a change in conformation close to cysteine 68 in γ_{PDE}. The reactive lysine residue on the α_T subunit also appears to be located in a conformationally-sensitive region, since previous studies have shown that the modification of this residue within an α_TGTPγS complex prevents the stimulation (by α_T) of the cyclic GMP phosphodiesterase. However, the lysine-modified α_TGTPγS complex still binds to γ_{PDE} with high affinity, suggesting that an activated α_T subunit must make at least two contacts with the effector enzyme, with one contact being responsible for high-affinity binding and the other (which is sensitive to lysine modification) being responsible for stimulation of enzyme activity. Here we present evidence that the reactive lysine on the α_T subunit is lysine 267 located within the guanine ring binding domain of α_T. The distance between cysteine 68 on γ_{PDE} and lysine 267 on α_T was measured through resonance energy transfer, using (iodoacetamido)fluorescein (IAF) attached to cysteine 68 as the energy donor and eosin isothiocyanate (EITC) attached to lysine 267 as the energy acceptor. On the basis of the observed efficiency of energy transfer at saturation, the distance between these residues is estimated to be 45 Å. This measured distance is larger than that separating a previously proposed stimulatory contact site for the γ_{PDE} and the guanine ring binding domain, based on the crystal structure of α_TGTPγS, and will be considered within the context of different models for the high-affinity binding of the α_T subunit to its effector.

Receptor-coupled, GTP binding protein-mediated signaling has been a focus of fundamental research for the past several years. One of the best understood examples of such a biochemical pathway is that responsible for vertebrate vision. In this model system, [reviewed by Pugh (1987)], the photoreceptor rhodopsin converts light into a chemical and, ultimately, an electrical signal by interacting with the heterotrimeric GTP binding protein (G protein) transducin. This interaction results in an exchange of GDP on the α subunit of transducin (α_T) for GTP. In its active, GTP-bound state, the α_T subunit binds to its effector target, the retinal cGMP-dependent phosphodiesterase enzyme (PDE).[1] Upon interaction with the activated α_T subunit, the cGMP hydrolysis activity of PDE is greatly increased, resulting in the closure of the cGMP-gated channel located in the plasma membrane of the rod outer segments. The closing of this channel initiates the action potential that leads to the perception of visual stimuli by the central nervous system.

Various studies have implicated the inhibitory γ subunit of the PDE (γ_{PDE}) as a primary site of interaction between the α_T subunit and the PDE molecule (e.g., Wensel & Stryer, 1990). Two regions on the γ_{PDE} subunit have been suggested to be involved in the binding of the α_T subunit: a polybasic stretch within the center of the γ_{PDE} subunit (Brown, 1990), and a region near the carboxyl terminus in the vicinity of the tryptophan at position 70 (Faurobert et al., 1993). In the case of the α_T subunit, studies using synthetic peptides have shown that the amino acid sequence corresponding to

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[1] Abbreviations: PDE, phosphodiesterase enzyme; EITC-α_TGTPγS, eosin isothiocyanate-modified form of the GTPγS-bound α subunit of transducin; IAF-γ_{PDE}, 5-(iodoacetamido)fluorescein conjugate of the retinal phosphodiesterase inhibitory γ subunit; M-γ_{PDE}, 2-(4-maleimidoanilino)naphthalene-6-sulfonate-labeled γ subunit.

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residues 293−314 of α_T is capable of binding to PDE and stimulating cGMP hydrolysis (Rarick et al., 1992). In addition, site-directed mutagenesis studies of the α subunit of the stimulatory G protein of the adenylyl cyclase system (Berlot & Bourne, 1992), coupled with a consideration of the tertiary structure of the GTPγS-bound form of the α_T subunit (Noel et al., 1993), argue that multiple regions of the α_T subunit primary structure join to form a surface that contacts the effector PDE molecule. Moreover, modification of a reactive lysine residue on the α_TGTPγS complex prevents the stimulation of the cyclic GMP phosphodiesterase but does not prevent the high-affinity binding of the α_TGTPγS complex to the effector enzyme (Hingorani & Ho, 1989; Erickson & Cerione, 1991). These results suggest that at least two points of contact are present on α_T for the PDE: one that provides a major contribution to the observed affinity of α_T for the effector and the other conditionally mediating the stimulation of effector enzyme activity. It is the latter interaction that presumably is prevented as a consequence of the modification of the reactive lysine on the α_T subunit. Interestingly, various lines of evidence also suggest that the GDP-bound form of α_T binds to the PDE (e.g., Faurobert et al., 1993) and at high concentrations stimulates cGMP hydrolysis (Kutuzov & Pfister, 1994; Leibman & Devenny, 1994). However, at micromolar concentrations, the α_TGDP complex does not stimulate effector enzyme activity, and in some cases actually inhibits activity (Kroll et al., 1989), and it would therefore seem that the α_TGDP complex is incapable of making extensive secondary stimulatory contacts with the PDE molecule necessary for cGMP hydrolysis under these conditions.

Although significant breakthroughs have been recently achieved in the determination of the tertiary structures of GDP-bound and GTPγS-bound α_T (Noel et al., 1993; Lambright et al., 1994) and α_{i1} (Coleman et al., 1994), at the present time there is little known regarding the structural features of G protein−α subunit/effector complexes. In the present study, we have used resonance energy transfer to determine the proximity of two specific sites within an α_T-GTPγS/γ_{PDE} complex. Specifically, we have labeled the single cysteine residue of γ_{PDE} (at position 68) with a fluorescence donor [(iodoacetamido)fluorescein] and the reactive lysine on α_TGTPγS with an energy acceptor (eosin isothiocyanate). We show that cysteine 68 is in a region on γ_{PDE} that is sensitive to the binding of both the GDP- and GTPγS-bound forms of α_T and that the reactive lysine on α_T is residue 267 which lies within the consensus NKXD region (residues 265−268 where X is the reactive lysine) that binds the guanine ring. The results of the energy transfer measurements thus provide information regarding the location of the guanine nucleotide binding site on α_T relative to a specific site on the target molecule, γ_{PDE}, that is sensitive to the binding of α_T.

MATERIALS AND METHODS

Reagents. Quick-frozen, dark-adapted bovine retinas were purchased from Hormel Meat Packers, Austin, MN. 5-(Iodoacetamido)fluorescein, eosin isothiocyanate, and 2-(4-maleimidoanilino)naphthalene-6-sulfonate were purchased from Molecular Probes, Inc., Eugene, OR. All chemicals were of the standard grade from Sigma unless otherwise noted.

Fluorescent Labeling of Component Proteins. Isolation of the rod outer segments and the subsequent purification of transducin α subunit and the cGMP phosphodiesterase γ inhibitory subunit have been described in detail elsewhere (Erickson & Cerione, 1993). Briefly, rod outer segments (ROS) were purified in the dark under dim red light from whole bovine retinas as described by Gierschik et al. (1984). Following their isolation, ROS membranes were bleached in white light for 30 min, pelleted, and resuspended in hypotonic buffer (10 mM HEPES, 1 mM DTT, 0.1 mM EDTA, and 0.3 mM phenylmethanesulfonyl fluoride, pH 7.5). Typically, 6−8 cycles of centrifugation and resuspension in hypotonic buffer depleted the membranes of detectable PDE as determined by Coomassie blue staining. The supernatants from these washes were then pooled and concentrated. Transducin was recovered from the PDE-depleted membranes by resuspending the pellet in hypotonic buffer supplemented with 100 μM GTP (or GTPγS) and incubating the membranes on ice for 30 min in room light. The membranes were pelleted and resuspended 3 times in GTP-supplemented hypotonic buffer, and the supernatants from these washes were pooled. This pooled extract containing the crude transducin was concentrated 10-fold in an Amicon ultrafiltration cell (YM 10 membrane). The α_T subunit and the $\beta\gamma_T$ subunit complex of transducin were resolved by Blue Sepharose chromatography (Pines et al., 1985). Typically, the concentrated holotransducin extract (6−10 mg of protein in 5−10 mL of hypotonic buffer) was applied to a 50 mL Blue Sepharose column preequilibrated with 10 mM HEPES (pH 7.5), 6 mM $MgCl_2$, 1 mM DTT, and 25% glycerol (column buffer). The column was washed with 200 mL of column buffer in order to elute the unbound $\beta\gamma_T$ subunit before beginning the elution of the α_T subunit with a continuous KCl gradient (0−500 mM KCl in column buffer; 200 mL total). The α_T eluted as a single peak at a KCl concentration of ~200 mM, and fractions were pooled and concentrated to 1−2 mg/mL. This α_T stock solution was dialyzed versus 120 mM NaCl, 30 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 0.02% NaN_3 (w/w), and 40% glycerol (v/v) ("storage buffer") in no. 2 Spectrapore dialysis tubing (MW cutoff 12−14 kDa) before it was aliquoted and stored at −20 °C. The EITC-labeled α_T subunits were prepared as previously described (Erickson & Cerione, 1991).

Fluorescent modification of the crude PDE was performed with the pooled and concentrated hypotonic washes of isolated rod outer segments. Just prior to the chemical modifications, an aliquot of this concentrate was dialyzed vs phosphate saline buffer (PBS: 50 mM K_2HPO_4/KH_2PO_4, pH 7.5, 100 mM NaCl). For the reaction of PDE with IAF or MIANS, 10 μL of a 25 mM stock solution of the probe in *N*,*N*-dimethylformamide was added to 0.5 mL of this partially purified PDE preparation (~0.8 mg/mL total protein following dialysis). After the reaction was allowed to proceed for 6−8 h at room temperature in darkness, the IAF/PDE mixture was then applied in 40 μL aliquots and run on an SDS−PAGE polyacrylamide gradient gel (6−18% acrylamide). The unfixed gel slab was viewed with a UV transilluminator immediately following electrophoresis in order to identify the IAF-modified γ_{PDE} subunit. Small slices of the acrylamide slab possessing fluorescence emission under UV light and corresponding to the known mobility of the modified γ_{PDE} subunits were excised and placed in

separate 50 μL aliquots of deionized and distilled water at 4 °C overnight in order to elute the modified subunits. As previously reported, the thiol-specific modification of γ_{PDE} resulted in a probe to protein ratio of 1:1 (Erickson & Cerione, 1993) that did not diminish its ability to inhibit the cGMP hydrolysis activity of the trypsinized form of the retinal phosphodiesterase (Wensel & Stryer 1986). Preparations of fluorescently modified subunits were routinely assayed for their ability to inhibit the activity of PDE using the proton release assay described previously (Erickson & Cerione, 1993).

Fluorescence Measurements. All fluorescence measurements were performed using an SLM 8000c spectrofluorometer. For a typical experiment, 2 mL of buffer (120 mM NaCl, 30 mM KCl, 5 mM $MgCl_2$, and 20 mM HEPES, pH 7.5; "assay buffer") was mixed with 5−20 μL of a stock solution of the labeled subunit just prior to the addition of aliquots of a stock solution of α_T. For the experiments employing MIANS, the excitation was fixed at 322 nm and the emission scanned from 350 to 520 nm or fixed at 445 nm for time-based measurements of MIANS emission. In each case, excitation and emission slits were set at 8 nm FWHM. For energy transfer experiments using IAF-γ_{PDE} and EITC-α_T, the quenching of donor (IAF) fluorescence was monitored by fixing the excitation at 460 nm (16 nm FWHM) and scanning the emission from 480 to 610 nm (16 nm FWHM). In order to correct for acceptor (EITC) emission contributions to IAF emission, mock titrations were performed in the absence of IAF-γ_{PDE} and the resulting spectra subtracted from those corresponding to the same concentration of EITC-α_T.

The calculation of the distance between the fluorescein moiety attached to the γ_{PDE} subunit and the eosin-labeled residue on α_T was performed by applying the relationship:

$$E = 1 - Q_{DA}/Q_D$$

where Q_{DA} and Q_D are the quantum yields of the donor species in the presence and absence, respectively, of the acceptor species and E is the efficiency of donor quenching at saturation of binding. The calculated efficiency can be used to estimate a distance R between a single donor−acceptor pair using the equation:

$$R = R_0(E^{-1} - 1)^{1/6}$$

where R_0 is the distance between chromophores resulting in an efficiency of 50%. The calculation of the distance between the fluorescein in the γ_{PDE} subunit and the eosin in α_T was performed employing a value of 53 Å for R_0 (Carraway et al., 1989; Epe et al., 1983) which was calculated using the equation:

$$R_0 = (9.79 \times 10^3)(\int \kappa^2 Q_D n^{-4})^{1/6}$$

where f is an integral defining the spectral overlap of donor emission and acceptor excitation, κ^2 defines the relative orientations of the donor and acceptor dipoles, Q_D is the quantum yield of the donor, and n is the refractive index of the medium. The value of κ^2 was assumed to be $^2/_3$, reflecting random orientations of the donor and acceptor dipoles. The value used for n was 1.4, and the overlap integral was calculated as previously described (Cantley & Hammes, 1975).

Steady-state anisotropy measurements were performed on an SLM fluorometer in the T configuration with the emission monochromator set at 520 or 550 nm for fluorescein- or eosin-containing samples, respectively. Calculations of single point anisotropy values for conjugated and unconjugated fluorophore were obtained using the manufacturer's software.

Purification and Analysis of EITC-Labeled Peptides. (A) Reduction and Alkylation. Approximately 200 μg of EITC-labeled α_T destined for chymotryptic cleavage was reduced and S-carboxamidomethylated by the method of Stone et al. (1989). This sample was dissolved in 50 μL of 8 M urea/0.4 M NH_4HCO_3 and reduced with 5 μL of 45 mM dithiothreitol at 50 °C for 15 min. Cysteine residues were alkylated by reaction with 5 μL of 100 mM iodoacetamide at room temperature for 15 min. Subsequent enzymatic cleavage was carried out without further desalting for transfers described below.

(B) Proteolytic Cleavage. The above alkylation mixture containing S-carboxamidomethylated EITC-labeled α_T was diluted 4-fold without further processing to a final buffer concentration of 2 M urea/0.1 M NH_4HCO_3. Chymotrypsin was added to this solution to maintain a substrate to enzyme ratio of 25:1 (w/w), and the mixture was allowed to incubate at 37 °C for 16−20 h. The resultant peptide mixture was frozen at −20 °C until separation by reverse phase HPLC.

(C) Reverse Phase HPLC Separation of Peptides. Peptides were separated by narrow-bore reverse phase HPLC on a Hewlett-Packard 1090 HPLC equipped with a 1040 diode array detector, using a Vydac 2.1 mm × 150 mm C18 column. The gradient employed was a modification of that described by Stone et al. (1989), briefly, where buffer A was 0.06% trifluoroacetic acid/H_2O, buffer B was 0.055% trifluoroacetic acid/acetonitrile. A gradient of 5% B at 0 min, 33% B at 63 min, 60% B at 95 min, and 80% B at 105 min with a flow rate of 150 μL/min was used. Chromatographic data at 210, 277, 292, and 525 nm and UV spectra for each peak were obtained. While monitoring the absorbance at 210 nm, fractions were manually collected by peak into 1.5 mL microfuge tubes and immediately stored without drying at −20 °C in preparation for peptide sequence analysis. Details of strategies for the selection of peptide fractions and their microsequencing have been previously described (Lane et al., 1991).

(D) Amino-Terminal Peptide Sequence Analysis. Fractions were applied directly to a polybrene precycled glass fiber filter placed in a reduced volume reaction microcartridge. The sample was subjected to automated Edman degradation on an ABI Model 477A protein sequencer using the manufacturer's recommendations for faster cycle time (30 min), and the sensitivity enhancements described in Tempst and Riviere (1989). The resultant phenylthiohydantoin amino acid fractions were manually identified using an on-line ABI Model 120A HPLC and Shimadzu CR4A integrator.

Peptides that displayed absorbances at 214 and 523 nm were chosen. Many of these peaks were subsequently found to have no peptide content when subjected to microsequencing. Of those that were found to be eosin-modified α transducin-derived peptides, only one was found to contain an eosin absorbance commensurate with the estimated picomoles of peptide. Subsequent microsequencing of this peptide revealed an anomalous retention time for the lysine predicted from the known α transducin sequence at position

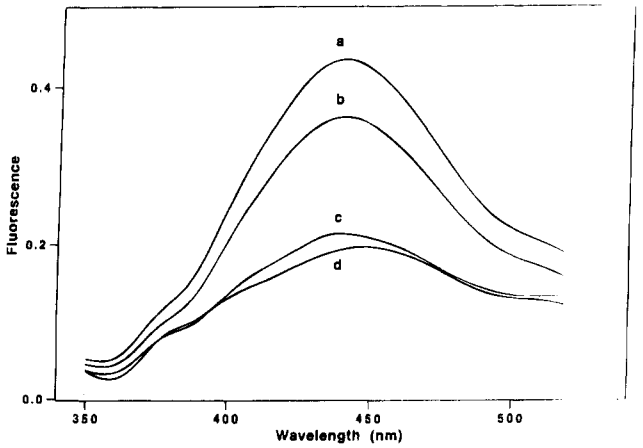

FIGURE 1: Fluorescence emission spectra of MIANS-modified γ_{PDE}. (a) 52 nM M-γ_{PDE} in the presence of 3 μM α_TGDP. (b) Same sample following the equilibration with 150 μM $AlCl_3$, 5 mM NaF. (c) Same sample as in spectrum b following the addition of 1 mM EDTA (pH 7.5). (d) Emission spectrum of 52 nM M-γ_{PDE} prior to any additions. Spectra are corrected for dilution. Excitation was set at 322 nm, and the emission was scanned as shown.

267. Mass spectral analysis of this peptide confirmed the presence of a single eosin moiety.

RESULTS

Modification of Cysteine 68 on the γ_{PDE} Subunit. (A) Labeling γ_{PDE} with the Environmentally-Sensitive Reporter Group MIANS. The reactive cysteine (residue 68) on γ_{PDE} was labeled with the environmentally-sensitive reporter group MIANS to determine whether this region of γ_{PDE} is sensitive to the binding of the inactive and active forms of the α_T subunit. Spectrum a in Figure 1 shows the fluorescence emission of M-γ_{PDE} when complexed with α_TGDP following a titration of a fixed amount of M-γ_{PDE}. The peak emission of the MIANS signal represents a >2-fold increase in the intensity over that observed for the same amount of uncomplexed M-γ_{PDE} shown in spectrum d. Spectrum c in Figure 1 shows that addition of EDTA, which chelates both Al^{3+} and Mg^{2+} with high affinity, reduced the level of MIANS fluorescence to that observed for the unbound form of M-γ_{PDE} (i.e., spectrum d).

The observed increase in M-γ_{PDE} emission, upon addition of α_TGDP, provides a method for estimating the affinity of α_TGDP for M-γ_{PDE}. Figure 2 shows the results of a titration of this α_TGDP-induced fluorescence change. The solid line in Figure 2 represents the best fit to a binding function describing a single class of noninteracting sites, yielding an apparent K_d of 2.4 (±0.4; $n = 2$) μM. The relatively low affinity for the binding interaction between the GDP-bound form of α_T and the isolated γ_{PDE} [compared to the nanomolar dissociation constant measured for the binding of the GTPγS-bound α_T species; see below, and also see Artemyev et al. (1992)] is consistent with the general finding that the GTP-bound form of G protein−α subunits is the preferred species for binding effector/targets (Gilman, 1987).

The changes in fluorescence following each addition of α_TGDP were commensurate with the time of mixing (<2 s; not shown), indicating that the formation of the α_TGDP/M-γ_{PDE} complex is rapid under the conditions used. Upon the addition of a mixture of $AlCl_3$ and NaF to form an aluminum fluoride-activated α_TGDP/M-γ_{PDE} complex, a relatively slower decrease in the MIANS fluorescence was observed.

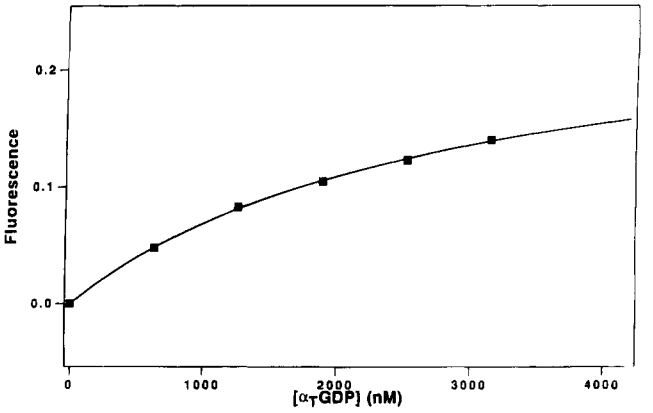

FIGURE 2: Increase of M-γ_{PDE} fluorescence emission at 445 nm with increasing concentrations of α_TGDP. The solid line represents the best fit of the equation $F_n = (F_{max}[\alpha_T GDP])/(K_d + [\alpha_T GDP])$ where the fluorescence at each point (F_n) has been corrected for dilution and the initial value of the fluorescence in the absence of any α_TGDP has been set to 0.0. The parameters used to draw the curve shown are $F_{max} = 0.264$ and $K_d = 2.83$ μM.

The fact that aluminum fluoride addition elicited a quenching of the M-γ_{PDE} fluorescence, rather than a further increase in M-γ_{PDE} emission (compare spectra a and b in Figure 1), suggested that the aluminum fluoride-induced fluorescence change is a reflection of a conformational transition occurring in the α_T subunit as it undergoes the conversion to an "activated" conformation. This interpretation is supported by kinetic analysis of the M-γ_{PDE} fluorescence decrease. In this experiment, the concentrations of the components were adjusted to ensure that all the M-γ_{PDE} was complexed to α_T-GDP (i.e., [α_TGDP] ≫ [M-γ_{PDE}]), prior to adding an excess of aluminum fluoride. The decrease in fluorescence following the addition of $AlCl_3$ and NaF is fit well by a single exponential yielding a rate constant (0.025 ± 0.007 s^{-1}, $n = 2$; data not shown) similar to that observed when monitoring the change in intrinsic α_TGDP tryptophan fluorescence induced by aluminum fluoride (Mittal et al., 1994). The change in the intrinsic fluorescence of α_T has recently been shown to be a reflection of a nucleotide (or aluminum fluoride)-dependent conformational change in the switch II region of α_T and attributable solely to changes in the local environment of tryptophan 207 (Faurobert et al., 1993). Taken together, these results suggest that conformational changes in the α_T subunit which reflect its nucleotide-bound state are sensed by the bound γ_{PDE} subunit in a region close to or at cysteine 68 in γ_{PDE}.

(B) Labeling γ_{PDE} with a Fluorescence Donor for Resonance Energy Transfer Measurements. In order to further investigate the geometry and dynamics of the interactions of the GTPγS-bound α_T subunit with γ_{PDE}, we prepared donor and acceptor fluorescent conjugates of highly purified α_T and γ_{PDE} subunits for resonance energy transfer measurements. Introduction of a single IAF probe into the γ_{PDE} subunit was observed to result in a significant shift in electrophoretic mobility for the IAF-modified γ_{PDE}. A typical preparation of the gel-purified IAF-γ_{PDE} subunit is shown in Figure 3, lane 1. The practical advantage of gel-purifying the labeled subunit is the certainty of the probe to protein ratio. This is due to the fact that, as previously reported, the γ_{PDE} subunit possesses a single reactive residue for sulfhydryl-specific reagents corresponding to cysteine 68. Coupled with the observed mobility shift in acrylamide gels (i.e., an apparent shift in molecular mass from ~11 to ~15

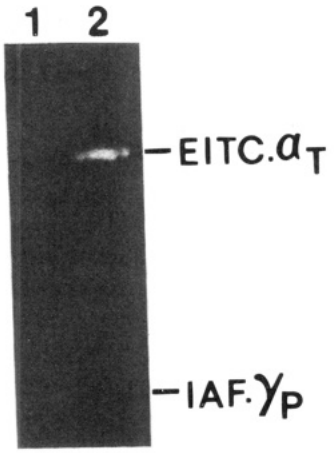

FIGURE 3: Fluorescence emission of the modified protein subunits resolved by 14% SDS−PAGE and viewed by UV transillumination. Lane 1: 10 μg of purified IAF-γ_{PDE}. Lane 2: 40 μg of EITC-α_T-GTPγS.

pep							L	N	K	*	D	V	F				
α_{T1}	T	S	I	V	L	F	L	N	K	K	D	V	F	S	E	K	I	K	K	276
α_{T2}	T	S	I	V	L	F	L	N	K	K	D	L	F	E	E	K	I	K	K	280
α_{gust}	T	S	I	V	L	F	L	N	K	K	D	L	F	Q	E	K	V	T	K	280
α_{i1}	T	S	I	I	L	F	L	N	K	K	D	L	F	E	E	K	I	K	K	280
α_s	I	S	V	I	L	F	L	N	K	Q	D	L	L	A	E	K	V	L	A	303
ras	-	M	V	L	V	-	G	N	K	C	D	L	A	A	R	T	V	E	S	127

FIGURE 4: Identification of the EITC-modified residue in α_TGTPγS. Shown is the alignment of the primary sequence in the region containing the EITC-modified residue, lysine 267 (*), with other representative GTPases. The sequences were taken from the review by Gilman (1987) except in the case of α gustducin (McLaughlin et al., 1992).

kDa after IAF labeling), this means that virtually pure IAF-γ_{PDE} subunit can be isolated using a preparative gel approach. Amino acid analysis in conjunction with fluorescein absorbance measurements performed on the γ_{PDE} preparations confirmed the presence of one fluorescein per γ_{PDE} (data not shown). The purified IAF-labeled γ_{PDE} subunits were assayed and found to fully inhibit the cGMP hydrolysis activity of trypsinized PDE as effectively as native γ_{PDE}.

Modification of a Reactive Lysine Residue on the α_TGTPγS Complex. Previously, it was reported that a single lysine residue on the α subunit of transducin was modified under nondenaturing conditions (Hingorani & Ho, 1989). In lane 2 of Figure 3 is shown the fluorescence pattern of 25 μg of a typical EITC-labeled α_TGTPγS preparation resolved on SDS−PAGE. Although the EITC-labeled α_TGTPγS species was not able to stimulate cGMP hydrolysis by the PDE, this modified α_TGTPγS subunit was shown to bind specifically to the intact holo-PDE (unlike the EITC-labeled α_TGDP complex which showed no detectable binding to holo-PDE) and to competitively inhibit the binding of unlabled α_T-GTPγS (Erickson & Cerione, 1991).

We set out to identify the precise location of the reactive lysine residue on the α_T subunit in order to properly interpret the resonance energy transfer measurements between donor-labeled γ_{PDE} and acceptor-labeled α_T (see below). Figure 4 shows the sequence obtained from a complete chymotrypsin digestion of a sample of EITC-α_TGTPγS and subsequent purification and sequencing of the eosin-containing peptide. Peptide sequence identification and knowledge of the primary sequence of bovine α_T permitted the identification of the EITC-reactive residue as lysine 267. Figure 4 shows the region of sequence that includes the labeled residue and the alignment of this region, often referred to as the G4 box, with other GTPases. As shown in Figure 4, the moderately conserved lysine at position 267 in α_T is in the third position of the highly conserved guanine nucleotide binding motif NKXD. In the recently determined crystal structures of bovine α_T (Noel et al., 1993; Lambright et al., 1994), as well as in the $p21^{ras}$ structure (Pai et al., 1990), these residues include points of contact for the guanine ring of bound nucleotide. The identification of lysine 267 as the target for isothiocyanate reagents is consistent with our observation that the modification of α_T inhibits GDP−GTP exchange activity (Mittal et al., unpublished results).

Quenching of IAF-γ_{PDE} Fluorescence by EITC-α_T Provides a Real Time Monitor for α_T/γ_{PDE} Interactions. The spectral characteristics of the fluorescent conjugates of the donor−acceptor pair used in these energy transfer experiments can provide for a significant amount of quenching. When making the energy transfer measurements, the donor-labeled IAF-γ_{PDE} was typically added to a final concentration of 5−50 nM. The acceptor-labeled EITC-α_TGTPγS was then added in small aliquots to the sample cuvette, and the extent of quenching of the IAF-γ_{PDE} fluorescence that resulted from each addition was recorded. A typical titration illustrating the quenching of IAF-γ_{PDE} with EITC-α_TGTPγS is shown in Figure 5A. A rapid quenching of the IAF-γ_{PDE} fluorescence was observed following a single addition of EITC-α_TGTPγS (not shown). That this observed quenching of the IAF-γ_{PDE} fluorescence upon EITC-α_TGTPγS addition indeed reflects specific complex formation between the two proteins is supported by several lines of evidence. This includes the observation that the quenching by EITC-α_TGTPγS was not observed when the IAF-γ_{PDE} was trypsinized prior to the titration with EITC-α_TGTPγS. In addition, the presence of an excess of unlabeled α_TGTPγS can inhibit the quenching that normally results from the addition of EITC-α_TGTPγS, indicating that the labeled and unlabeled α_TGTPγS subunits compete for the same region on IAF-γ_{PDE}. Finally, the binding of EITC-α_TGTPγS is fully reversible by the addition of an excess (>100-fold) of unlabeled α_TGTPγS. Under conditions where [α_TGTPγS] ≫ [EITC-α_TGTPγS], the recovery curve showing the increase in IAF-γ_{PDE} fluorescence emission upon the addition of excess α_TGTPγS is fit well by a single rate constant of 0.03 s^{-1} (±0.01, $n = 2$, inset, Figure 5A), that is in agreement with the k_{off} estimated by the intrinsic fluorescence for the unlabeled α_TGTPγS/γ_{PDE} subunits (Otto-Bruc et al., 1993). Although the time resolution of our methods did not allow a determination of the kinetic constants characterizing the quenching of IAF-γ_{PDE} fluorescence emission, it is possible to estimate a lower bound for a forward rate constant describing the association of the fluorescently-labeled α_T and γ_{PDE} subunits to be on the order of 10^6 M^{-1} s^{-1}. Interestingly, this lower bound for association estimated from quenching kinetics when combined with the measured rate constant for dissociation (0.03 s^{-1}) yields an estimate for K_d of 30 nM, a value in good agreement with the equilibrium constant determined by titration experiments (Figure 5B). The solid line in Figure 5B represents the best fit to a binding model describing a single class of sites for the interaction of EITC-α_TGTPγS with γ_{PDE} and yields a K_d of 32.3 (±5.2; $n = 2$) nM. The latter K_d value describing the formation of the α_T/γ_{PDE} complex is also in excellent agreement with the value measured previously for the binding of the unlabeled α_T-

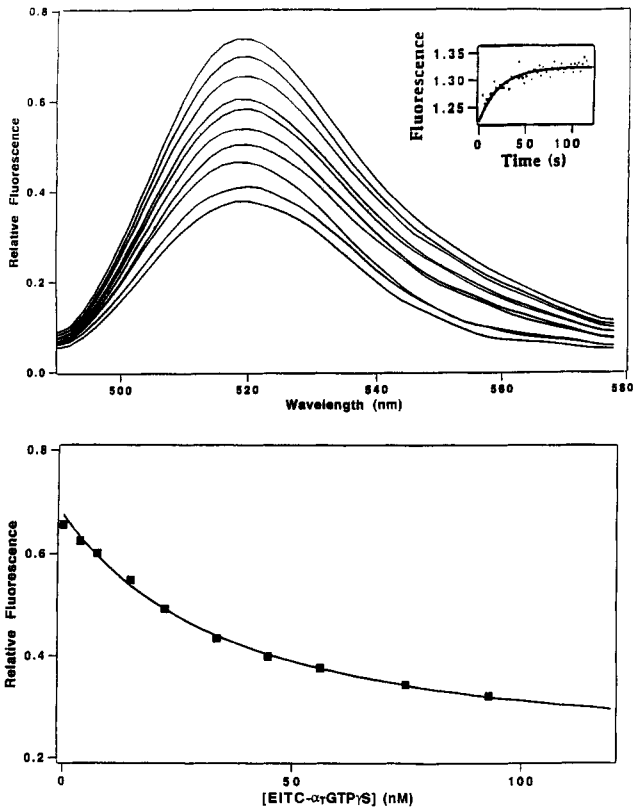

FIGURE 5: (A) Fluorescence emission for IAF-γ_{PDE} following additions of EITC-α_TGTPγS. The concentration of IAF-γ_{PDE} was 10.4 nM. The inset shows the effect of adding 1 μM unlabeled α_TGTPγS to a titrated IAF-γ_{PDE} sample. The fluorescence recovery curve was fit using a single time constant of 0.04 s^{-1}. (B) Titration data for IAF-γ_{PDE} quenching by EITC-α_TGTPγS. The solid line represents the best fit to the equation $F_n(L_{tot}) = F_0 - (F_0 - F_\infty)\cdot\{[(L_{tot} + K_d + S_{tot}) - (\Gamma^2 + 4K_dS_{tot})^{1/2}]/2S_{tot}\}$ where $\Gamma = L_{tot} + K_d - S_{tot}$ and where F_0 and F_∞ represent, respectively, the initial and final values of the binding function F_n, which describes the fluorescence observed at a given total concentration of ligand (L_{tot}), K_d is the dissociation constant defining the equilibrium S−L $\rightleftharpoons$ S + L, and S_{tot} and L_{tot} are the total ligand binding sites and total ligand, respectively. Parameters obtained from the nonlinear least-squares fitting of the data are $F_0 = 0.676$, $F_\infty = 0.192$, $K_d = 30.2$ nM, and $S_{tot} = 10.4$ nM.

GTPγS subunit to a cysteine 68-labeled γ_{PDE} subunit (K_d = 36 nM; Artemyev et al., 1992). Care should be taken in interpreting these measurements of interaction as values representing the α_T/γ_{PDE} interaction of the unmodified proteins given that conservative mutations at position 70 in γ_{PDE}, two residues away from the modified cysteine in γ_{PDE}, have been reported to result in large decreases in the observed affinity of α_TGTPγS for γ_{PDE} (Otto-Bruc et al., 1993). Interestingly, the fluorescent modification at cysteine 68 does not result in a different rate of dissociation for the labeled and unlabeled α_TGTPγS/γ_{PDE} complexes whereas mutation of tryptophan 70 in γ_{PDE} results in a more than 50-fold increase in the rate of dissociation (Otto-Bruc et al., 1993).

Estimation of the Proximity of Cysteine 68 in IAF-γ_{PDE} and Lysine 267 in EITC-α_T Using Resonance Energy Transfer. In experiments using protein from different ROS preparations, we observed that the highest extent of quenching of the IAF-γ_{PDE} fluorescence, upon the addition of EITC-α_TGTPγS, was reproducibly ~70%. According to the theory of Förster, for the case where two proteins are each modified by a single donor or acceptor fluorescent probe, the observed efficiency of energy transfer can be used to estimate the distance between the two labeled residues (see Materials and Methods). In the present case, using an R_0 of 53 Å (Carraway et al., 1989; Epe et al., 1983), we estimate the distance between the labeled residue on IAF-γ_{PDE} and that on EITC-α_TGTPγS to be approximately 45 Å. This value for R_0 was arrived at by assuming the orientation factor $\kappa^2 = {}^2/_3$, which implies a random sampling of all available orientations for both the donor and acceptor chromophores during their respective fluorescence lifetimes (Lakowicz, 1983). In order to place some limits on the error in R_0 that may be introduced by this assumption, we determined the steady-state polarizations (p) of the labeled proteins. Both donor and acceptor chromophores exhibited low values of p; for IAF-γ_{PDE}, the average value was 0.152 ± 0.002 (n = 5) and for EITC-α_TGTPγS, p = 0.210 ± 0.009 (n = 7). When IAF-γ_{PDE} was bound to unlabeled α_TGTPγS, p increased to 0.186 ± 0.007, an increase in anisotropy consistent with the formation of the larger complex, α_T-GTPγS/IAF-γ_{PDE}. We repeated this measurement for the complex EITC-α_TGTPγS/γ_{PDE} with essentially the same results, indicating the absence of any significant restriction of chromophore rotational mobility in the EITC-α_TGTPγS/IAF-γ_{PDE} complexes. On the basis of these values of p observed for the attached chromophores and the simulations of Haas et al. (1978), we estimate the error introduced in our calculation of R, using a value for κ^2 of ${}^2/_3$, to be less than 10% [see Table III in Haas et al. (1978)]. Given that the locations of the chromophores on γ_{PDE} (cysteine 68) and the α_T subunit (lysine 267) are known, we can propose a model for the relative positions of the effector (γ_{PDE}) binding site relative to the guanine nucleotide binding site on the α_T molecule (see below).

DISCUSSION

In this study, we have taken advantage of fluorescence spectroscopic approaches to investigate the interactions of the transducin α subunit with γ_{PDE}. This has involved labeling the γ_{PDE} subunit at a single reactive cysteine (position 68) and the α_TGTPγS complex at a single reactive lysine (position 267). Cysteine 68 on γ_{PDE} is located at, or adjacent to, a site that is sensitive to the binding of the α_T subunit, as indicated by the finding that the labeling of cysteine 68 with the environmentally-sensitive probe MIANS provides a reporter group for the binding of both the inactive and activated forms of the α_T subunit. The GDP-bound α_T subunit causes an enhancement of the MIANS fluorescence, whereas the activation of the α_TGDP/M-γ_{PDE} complex by the addition of aluminum fluoride results in a quenching of the MIANS fluorescence. The quenching occurs with a time course that mirrors the kinetics of the aluminum fluoride-induced enhancement of the α_T tryptophan fluorescence (Mittal et al., 1994), which serves as a direct readout for the activation of the α_T subunit. These results indicate that while both the inactive and active forms of the α_T subunit can bind to γ_{PDE}, they induce distinguishable changes in the micro-environment of cysteine 68 of the γ_{PDE} subunit.

The reactive lysine residue on the α_T subunit is located within a nucleotide-sensitive region based on the structural details of GTP binding proteins now available. Micro-sequencing of an eosin isothiocyanate-labeled peptide from α_T indicates that the reactive lysine is at position 267, which lies within the consensus NKXD sequence (X = lysine 267) that has been shown to be involved in the binding of the

GTP guanine ring. This finding is consistent with our observation that the modification of this lysine residue, even with a small methyl isothiocyanate moiety, results in an inhibition of the GDP−GTP exchange reaction (Mittal et al., unpublished data). However, it is interesting that the modification of lysine 267, while having no detectable effect on the binding of the α_TGTPγS complex to the PDE (Erickson et al., 1991), prevents the α_TGTPγS complex from stimulating PDE activity (Hingorani & Ho, 1989; Erickson et al., 1991). Thus, lysine 267 plays a critical role in coordinating the effector binding of α_T to the subsequent stimulation of PDE activity, such that the modification of this residue uncouples these two events.

Through the application of the Forster theory of resonance energy transfer, we calculate a distance of ~45 Å between the EITC moiety attached to lysine 267 on the α_T subunit and the IAF moiety attached to cysteine 68 on γ_{PDE}. Studies with synthetic peptides have suggested that residues 293−314 encompassing the α_4 helix and the $\alpha_4 - \beta_6$ loop can bind to γ_{PDE} (Artemyev et al., 1993) and stimulate cGMP hydrolysis (Rarick et al., 1992). Using the coordinates of the crystal structure of the α_TGTPγS complex, computer-assisted measurements indicate the distance between lysine 267 and penylalanine 303 (within the center of the putative stimulatory region) to be ~25 Å. Taking into account errors in the energy transfer measurements due to uncertainties in the orientations of the transition dipoles for the donor and acceptor chromophores (<10% based on polarization measurements), it is difficult to reconcile the differences between the calculated distance based on the observed donor quenching and the distance between the guanine nucleotide binding domain and the putative effector stimulatory domain in the known crystal structures of α_T.

Comparisons of the crystal structure for the GDP-bound and GTPγS-bound forms of α_T (Lambright et al., 1994) do not reveal detectable guanine nucleotide-dependent changes in the orientation of the α_4 helix or $\alpha_4 - \beta_6$ loop, as might have been expected if this region alone was sufficient for binding and stimulating the effector enzyme. One region on α_T that appears to undergo a guanine nucleotide-dependent change in orientation, designated "switch III" (residues 227−238), contains a cluster of acidic acids (residues 232−235) that have been proposed as a potential binding site for γ_{PDE} (Lambright et al., 1994). However, as is the case for other possible contact residues in the GTPase domain of α_T, this region is closer (~25 Å) than the distance between lysine 267 and cysteine 68 predicted from energy transfer measurements.

Recently, based on the crystal structure for α_i (Coleman et al., 1994), a candidate effector binding site was proposed to reside in the helical domain within the αB helix and the connecting loop to the following αC, which would correspond roughly to residues 106−116 in α_T. On the basis of the strong specificity in effector interactions (α_T will not bind to adenylyl cyclase and α_i will not bind the PDE), the αB−αC helical region was originally proposed as a potential effector binding domain because of the divergence of α subunit primary sequence in this region (Masters et al., 1986). Similar to the absence of detectable conformational changes in the region of the α_4 helix and the $\alpha_4 - \beta_6$ loop, comparison of the GDP-bound and GTPγS-bound α_T structures reveals no striking differences within the helix B−C region. However, significant GTP hydrolysis-sensitive changes do occur

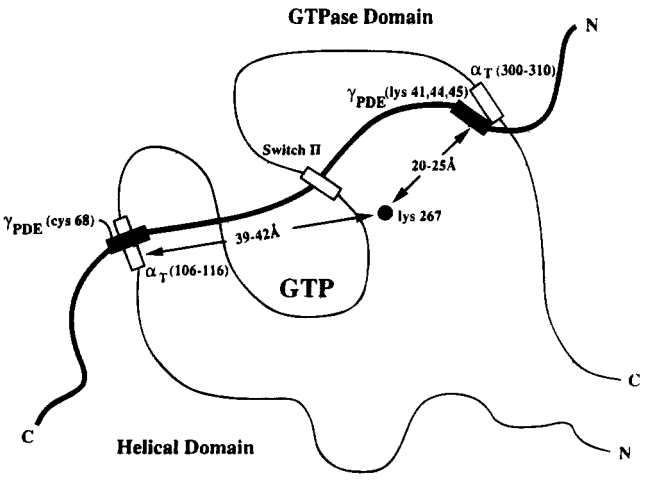

FIGURE 6: Model depicting the relative positions of lysine 267 of α_T and the α_T/γ_{PDE} contact sites. Distances between residues were measured with the program Quanta using the coordinates of the 2.2 Å α_TGTPγS structure (Noel et al., 1993). Regions of the α_T primary sequence tentatively assigned as effector contact residues are shown schematically along with their linear distance from the location of the EITC-labeled residue in α_T, lysine 267.

in α_i in this region, inviting speculation that this may be an additional effector contact site (Coleman et al., 1994). In the crystal structure for the α_TGTPγS complex, the region of helices B−C (amino acid residues 106−116) is ~41 Å away from lysine 267, similar to the distance between lysine 267 and cysteine 68 or γ_{PDE}, as determined from energy transfer measurements. Thus, in light of our results and in the context of available structural data, the B−C helical region represents a strong candidate for an additional contact site for the formation of the α_TGTPγS/γ_{PDE} complex.

Figure 6 depicts our current working model for the interaction of α_TGTPγS with γ_{PDE}. The initial binding between the activated α_T subunit and γ_{PDE} may involve establishing a contact between the helix B−C region of α_T and a region at the carboxyl-terminal domain of γ_{PDE} that is close to cysteine 68 (Otto-Bruc et al., 1993). A conditional (i.e., GTP-dependent) contact could then involve residues in the $\alpha_4-\beta_6$ loop of α_T and a polybasic region of γ_{PDE} (residues 41, 44, 45). It has previously been shown using chemical cross-linking that a site within residues 300−314 on α_TGTPγS binds to a region on γ_{PDE} between residues 24 and 45, and it was suggested that this binding interaction could involve salt bridges between lysine residues 41, 44, and 45 on γ_{PDE} and Glu 305, Asp 311, and Glu 314 on α_T (Artemyev et al., 1993) although the identification of critical contact residues is controversial (Spickofsky et al., 1994). Our results are consistent with the presence of a common locus in the region of helices B and C on α_T-GDP and α_T-GTPγS, distinct from this stimulatory domain, that is involved in binding but not stimulating γ_{PDE}. This view is supported by the finding that the crystal structures for the GDP-bound and GTPγS-bound α_T subunits do not show any significant change in the relative positions of the B−C helical region and lysine 267.

A key question concerns how the binding of an activated (GTP-bound) α_T subunit specifically stimulates effector activity, whereas the binding of a GDP-bound α_T subunit shows little or no stimulatory effect. There appears to be no detectable change in the orientation of the $\alpha_4-\beta_6$ loop amino acid residues in the GDP-bound and GTPγS-bound

α_T structures. If this region is essential for effector stimulation, the GTP-dependent regulation of effector stimulation is not manifested by a change in the local conformation of the $\alpha_4-\beta_6$ loop amino acid region on α_T. It is possible that subtle differences in the helix B−C region in the GDP-bound and GTPγS-bound states of α_T result in the observed differences exhibited by these two forms of α_T in their binding affinities for γ_{PDE} and the differences they induce in the reporter group fluorescence detected by MIANS-labeled γ_{PDE} (in the region of cysteine 68). The different conformational changes that accompany the initial binding of the inactive and active forms of α_T to γ_{PDE} may then enable a specific stimulatory interaction between a region on γ_{PDE} and the α_4 region on α_T.

However, the modification of lysine 267 must in some manner uncouple the initial binding interaction, residing in the B−C helical region in our model, from the stimulatory interaction at α_4. For the model presented in Figure 6, this is attributed to a third contact region on γ_{PDE} for the α_T switch II, a region which undergoes significant conformational changes in response to GTP hydrolysis and is reportedly important in $\alpha_T-\gamma_{PDE}$ interactions (Faurobert et al., 1993). In our model, the proposed interference in the communication between the contact in the helical domain (i.e., the B−C helical region) and the GTPase domain (the α_4 helix) induced by the modification at lysine 267 would occur through this region. Supporting the view that the modification of lysine 267 has direct effects on the conformation of switch II is the loss of tryptophan 207 fluorescence (Mittal et al., 1994) and the observed resistance to trypsin cleavage at α_T arginine 204 that accompanies the labeling of this lysine residue in α_TGDP (R. Mittal, unpublished observation). Moreover, mutational analysis of the switch II loop in α_T demonstrates a role for this region in the affinity for γ_{PDE} (Faurobert et al., 1993) and PDE stimulatory activity (Mittal et al., manuscript in preparation).

Given the suggestion of multiple contact sites between α_T and γ_{PDE}, it will be interesting to see how the proposed topology for the α_T/effector complex (Figure 6) compares with the X-ray crystallographic structure of an α_T/effector complex and ultimately for other G protein-α/effector complexes. This information also may help us to better understand the mechanisms by which α_T influences PDE activity and why the modification of lysine 267 interferes with effector stimulation without interfering with GTPγS-dependent α_T/effector interactions. Future experiments will be aimed at mutagenizing this lysine and other residues in the G4 region in order to further characterize the involvement of this domain in the propagation of guanine nucleotide-induced conformational changes throughout the α_T subunit.

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