Real Time Conformational Changes in the Retinal Phosphodiesterase γ Subunit Monitored by Resonance Energy Transfer*

(Received for publication, February 6, 1996, and in revised form, August 19, 1996)

Allan L. Berger, Richard A. Cerione‡, and Jon W. Erickson§

From the Department of Pharmacology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

The γ subunit of the retinal cGMP phosphodiesterase (γPDE) acts as an inhibitor of phosphodiesterase (PDE) catalytic activity and mediates enzyme regulation by the α subunit of the GTP-binding protein transducin (αT). In order to characterize conformational changes in the 87-amino acid γPDE subunit that may accompany the activation of the holoenzyme, γPDE was labeled with the fluorescent probes 5-iodoacetamidofluorescein and eosin-5-isothiocyanate for use in resonance energy transfer measurements. 5-Iodoacetamidofluorescein specifically labeled a cysteine residue at position 68 and served as a resonance energy transfer donor. The site of modification of eosin-5-isothiocyanate, which served as the resonance energy transfer acceptor, was determined to be within the first seven residues of the amino terminus of γPDE. Energy transfer between the labeled sites on free, unbound γPDE indicated that they were separated by a distance of 63 Å, consistent with a random conformation. Upon binding the catalytic αγ subunits of the PDE, the distance between the two probes on γPDE increased to 77 Å. Binding of the labeled γPDE by αγ-guanosine 5′-3-O-(thio)triphosphate did not affect the distance between the probes under conditions where the PDE was activated. These data are consistent with the view that the binding of activated αγ to γPDE, which is essential for the stimulation of PDE activity, does not impart significant alterations in the tertiary structure of the γPDE molecule. They also support a model for PDE activation that places active αγ in a complex with the holoenzyme.

The vertebrate phototransduction system has served as a paradigm for understanding how receptors containing seven transmembrane helices couple to heterotrimeric GTP-binding proteins (G proteins) and how activated G proteins regulate the activities of their biological effectors. The receptor in this system, rhodopsin (made up of the protein backbone opsin and the chromophore retinal), initiates the signaling pathway following the absorption of light. This leads to the formation of a complex between rhodopsin and the G protein transducin (which consists of a 39-kDa α subunit, designated αT, a 35-kDa β subunit, and an ~8-kDa γ subunit, designated γPDE). Within this complex, rhodopsin stimulates the exchange of GDP for GTP, which in turn causes the dissociation of transducin into an αγ-GTP species and intact βγ complex. The αγ-GTP species then stimulates the biological effector, the cyclic GMP phosphodiesterase (PDE), a tetrameric enzyme consisting of two larger subunits (designated αPDE and βPDE, molecular mass ~85 kDa) and two identical smaller subunits designated γPDE (~14 kDa). The γPDE subunits serve as the binding sites for the GTP-bound αγ subunit, although the specific mechanism by which αγ binding to γPDE results in the stimulation of cyclic GMP hydrolysis by the catalytic core of the enzyme (i.e. the αPDE and βPDE subunits) is still not understood. The stimulation of enzyme activity continues until the bound GTP is hydrolyzed to GDP; thus, the GTPase activity returns the signaling system to its starting point.

Recently, a significant amount of information has been reported regarding the tertiary structural features of G protein α subunits, including x-ray crystallographic structures for GDP- and GTPγS-bound forms of αγ (Noel et al., 1993; Lambright et al., 1994) and αγ (Coleman et al., 1994). This structural information has raised a number of possibilities regarding the identity of the regions and amino acid residues on the G protein α subunits that are involved in the regulation of effector activity. However, thus far, no tertiary structural information is available for a G protein-effector complex, and consequently, very little is known regarding the specific mechanisms by which G protein binding is translated into effector regulation. The phototransduction system would seem to be especially amenable to such structure-function characterization, given that the target sites on the effector molecule for the G protein (i.e. the γPDE subunits) are relatively small. However, based on NMR structure studies performed in this laboratory, all indications are that the γPDE subunits do not possess significant secondary structure, at least when these subunits are free in solution (i.e. when dissociated both from the larger PDE subunits and the αγ subunit).

In studying the protein-protein interactions important in visual signal transduction, our aim has been to develop fluorescence spectroscopic approaches to examine different aspects of the GTP-binding/GTPase cycle of transducin (Phillips and Cerione, 1986; Guy et al., 1990; Mittal et al., 1994) and to probe the mechanisms underlying the activation of the cyclic GMP PDE (Erickson and Cerione, 1989, 1991; Erickson et al., 1995). In the present study, we have used resonance energy transfer approaches to determine whether the γPDE subunit adopts a unique tertiary structure when it is bound to the αPDE and βPDE subunits (versus when it is free in solution) and when it binds GTP-bound αγ. To do this, we developed procedures for generating doubly labeled γPDE subunits, with one label serving as an energy donor and the other as an energy acceptor.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Supported by National Institutes of Health Grant EY06429.
§ Supported by National Eye Institute Fellowship 06204.

1 The abbreviations used are: PDE, phosphodiesterase; GTPγS, guanosine 5′-3-O-(thio)triphosphate; DTT, dithiothreitol; IAP, 5-iodoacetamidofluorescein; EITC, eosin-5-isothiocyanate; HPLC, high pressure liquid chromatography; MALDI, matrix-assisted laser desorption-ionization; TOF, time of flight.
Using these labeled γ_PDE subunits, we are able to show that the γ_PDE subunit does change its tertiary configuration upon binding to the α_PDE and β_PDE subunits; this change extends the distance between the amino terminus and cysteine 68 of the γ_PDE molecule. However, the binding of the α_PDE subunit does not appear to perturb the relative juxtaposition of these two sites on γ_PDE. Thus, these results suggest that the changes in the γ_PDE subunit that accompany the binding of the GTP-bound α_PDE subunit and are responsible for the stimulation of cyclic GMP hydrolysis by the α_PDE and β_PDE subunits do not occur between residue 68 and the amino terminus of the γ_PDE molecule. Furthermore, measurements of PDE enzyme activation and inhibition, along with the characterization of corresponding spectroscopic states of the doubly labeled γ_PDE subunit, support a model in which the activated α_PDEγ_PDE complexes remain associated with the αβ_PDE core of the effector enzyme during the stimulation of cyclic GMP hydrolysis.

EXPERIMENTAL PROCEDURES

Materials—SP-Sepharose, phenyl-Sepharose, and blue Sepharose were obtained from Pharmacia Biotech Inc. Factor Xa was purchased from New England Biolabs (Beverly, MA). 5-Iodoacetamidofluorescein and eosin-5-isothiocyanate were purchased from Molecular Probes, Inc. (Eugene, OR). Dark-adapted bovine retina were purchased from Hornel Meat Packers (Austin, MN). All other chemicals and enzymes were purchased from Sigma. The pLCIFXSFG plasmid was a gift from Dr. Heidi Hamm (University of Illinois College of Medicine, Chicago, IL).

γ_PDE Expression and Purification—Recombinant γ_PDE was expressed in Escherichia coli, and cells were lysed as described by Brown and Stryer (1989). Briefly, E. coli strain AR68 containing the pLCIFXSFG plasmid was grown at 30°C in a Labline fermentor and induced by temperature jump to 42°C at an A600 of 0.5. After the temperature jump, the cells were grown at 37°C for an additional 2 h. Cells were harvested by centrifugation at 4000 × g and lysed with lysis buffer containing 2 mM iodoacetamide, 250 mM NaCl, 50 mM sodium phosphate (pH 8.0), and 0.5% Triton X-100. Purified protein was recovered from the lysate by centrifugation in a Beckman Airfuge (10 min, 30 p.s.i.). Supernatants were pooled and dialyzed against 20 mM NaCl, 50 mM sodium phosphate (pH 8.0), and 0.5% Triton X-100. The concentration of EITC in the doubly labeled protein was determined to be 1:1.05 ± 0.04 (S.E., n = 3) by absorbance spectroscopy using a Hewlett-Packard 8545A spectrophotometer. The fluorescein absorbance was measured at 485 nm and corrected for any contribution from EITC by deconvolution (this represented 20% of the total absorbance). The concentration of EITC in the doubly labeled protein was determined by eosin absorbance at 522 nm, using a molar extinction coefficient of 83,000 M⁻¹ cm⁻¹ for EITC (Cherry et al., 1976). There was no detectable contribution of fluorescein to this absorbance.

Purification of Component Proteins—Purification of transducin and holo-PDE from bovine retina were performed as described previously (Kroll et al., 1989). Rod outer segment were purified as described by Gierschik et al. (1984) and washed several times with isotonic buffer (10 mM HEPES, 5 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 100 mM NaCl, 0.3 mM phenylmethylsulfonyl fluoride, pH 7.5). The rod outer cell membranes were then resuspended in hypotonic buffer (10 mM HEPES, 1 mM DTT, 0.1 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, pH 7.5) to release PDE from the membranes and centrifuged at 39,000 × g. The supernatant from the hypotonic wash (containing the holo-PDE) was concentrated using an Amicon 30,000-Da molecular mass cut-off membrane.

Transducin was recovered from the hypotonically washed pellet by resuspending the membranes in hypotonic buffer supplemented with 100 mM GTP or GTPγS (for inactive GDP-bound αs or active GTP-S-bound αs, respectively). The membranes were washed several times with nucleotide-containing buffer, and the supernatants were pooled. The pooled extract containing crude transducin was purified by blue Sepharose chromatography as described by Pines et al. (1985).

Assay of PDE-inhibitory Activity—Trypsin-activated PDE (tPDE) was prepared by limited trypptic digest of purified PDE (Kroll et al., 1989). Trypsin at 65 μg/ml was added to 1 μM PDE and incubated at room temperature for 1 min. The reaction was quenched by the addition of 280 μg/ml soybean trypsin inhibitor. PDE activity was determined using a pH microelectrode as described by Yee and Liebman, 1978. Activity was measured in 5 mM HEPES, 100 mM NaCl, 2 mM MgCl₂, 5 mM cGMP, pH 7.5, at room temperature. Proton release from cGMP hydrolysis due to PDE activity was recorded in nM at one determination per second.

Activity of PDE Stimulation by Activated α-Transducin—Phospholipid vesicles were prepared by sonication of 17 mg of lecithin in 1.0 ml of deionized and distilled water. PDE purified from ROS was incubated with the phospholipid vesicles to allow binding, and the vesicles were pelleted by centrifugation in a Beckman Airfuge (10 min, 30 p.s.i.). Membrane-bound PDE was activated as described by Brown (1992). Briefly, PDE was digested with ArgC protease at a concentration of 0.1 unit per 1.1 mg of PDE at 4°C for 4 h (at pH 8.0, with no detergent). Ammonium sulfate was added to the γ_PDE to a final concentration of 0.5 M, and the clipped γ_PDE was then loaded onto a phenyl-Sepharose column. The γ_PDE eluted from the phenyl-Sepharose column when there was no (NH₄)₂SO₄ in the column buffer. This eluted protein was >99% pure as judged by Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis. Of a sample was clipped with trypsin to a final concentration of 2000 Da molecular mass cut-off tubing versus 20 mM HEPES, 150 mM NaCl, 5 mM MgCl₂, 0.2 mM DTT, pH 7.4, and stored in aliquots at −80°C until use.

Modification of γ_PDE by 5-Iodoacetamidofluorescein (IAF) and Eosin-5-isothiocyanate (EITC)—γ_PDE was reacted with 1 mM IAF at pH 7.4 for 1 h. The reaction was quenched with the addition of 30 mM DTT, and the labeled protein was separated from free probe by SDS-polyacrylamide gel electrophoresis. Labeled protein was visualized in the gel using UV trans-illumination and excised (Wensel and Stryer, 1990; Erickson and Cerione, 1991). Gel purification of the labeled γ_PDE subunit provides a means to obtain only γ_PDE that contains the IAF (fluorescence donor) molecule. This is due to the fact that the γ_PDE subunit possesses a single reactive residue at cysteine 68 and that the labeled γ_PDE subunit undergoes an apparent purification step, measured to −15 kDa after IAF labeling. Thus, pure IAF-γ_PDE subunit can be isolated using a preparative gel; amino acid analysis together with fluorescein absorption measurements at 495 nm (εmax = 75,000 M⁻¹ cm⁻¹) (Carraway et al. 1989) indicate a stoichiometry of IAF incorporation of 1:0.1 mol of IAF/mol of γ_PDE. The gel slice containing the labeled γ_PDE was incubated in 4 volumes of distilled water at 4°C overnight, and the gel eluate was dialyzed against a 100 × volume of 20 mM HEPES, 150 mM NaCl, 5 mM MgCl₂, 0.2 mM DTT, pH 7.4. The fluorescein-labeled γ_PDE was then reacted in this buffer with 1 mM EITC for 3 h. The reaction was quenched with 15 mM Tris, pH 6.8, and the protein was run on SDS-polyacrylamide gel electrophoresis and again eluted from a gel slice.

The ratio of eosin to fluorescein in the doubly labeled γ_PDE was determined to be 1:1.08 ± 0.04 (S.E., n = 3) by absorbance spectroscopy using a Hewlett-Packard 8541A spectrophotometer. The fluorescein absorbance was measured at 485 nm and corrected for any contribution from EITC by deconvolution (this represented 20% of the total absorbance). The concentration of EITC in the doubly labeled protein was determined by eosin absorbance at 522 nm, using a molar extinction coefficient of 83,000 M⁻¹ cm⁻¹ for EITC (Cherry et al., 1976). There was no detectable contribution of fluorescein to this absorbance.
When making resonance energy transfer measurements for the doubly labeled γ_PDE, we measured the change in IAF (donor) fluorescence that occurred after trypsin treatment (since this treatment effectively separates the donor (IAF) and acceptor (EITC) probes and eliminates energy transfer) and subtracted any changes caused by trypsin treatment of singly labeled IAF-γ_PDE. The efficiency of energy transfer (E) was calculated as follows,

\[ E = 1 - \frac{F_{PE}}{F_{R}} \]  
(Eq. 1)

where \( F_{PE} \) is the relative fluorescent fluorescence of the intact doubly labeled γ_PDE, and \( F_{R} \) is the relative fluorescent fluorescence of the γ_PDE control following complete trypsinization of the singly labeled flavescence species. Using the efficiency of energy transfer, the distance between the two probes (r) can be estimated using the following equation (Lakowicz, 1983),

\[ R = R_0 \left( E^{-1} - 1 \right)^{1/6} \]  
(Eq. 2)

and the \( R_0 \) for fluorescent eosin and eosin was calculated to be 56.8 Å, using the integral overlap method (Carraway et al., 1989; Epe et al., 1983). In calculating \( R_0 \), the orientation factor \( \kappa^2 \) was assumed to be 0.5, indicating equal probability for all dipole-dipole orientations. Polarization values of the free, labeled γ_PDE were determined to be \( \kappa^2 = 0.1 \), which indicates errors of <10% if \( \kappa^2 \) is set to 0.5 (data not shown; see Tables II and III in Hass et al. (1978)).

Fluorescence emission anisotropy was measured on an SLM 8000C fluorometer. Emission in the horizontal and vertical orientations was measured simultaneously using a monochrometer set at 520 nm and a band pass filter of 520 nm. The G factor was set to 1.0 by adjusting the photomultiplier gain prior to beginning acquisition. All measurements were made at 20 °C.

Data Analysis for γ_PDE Binding to the αPDE and βPDE Subunits—Activity data in Fig. 2 were fit to the binding equation,

\[ A = A_0 - (A_r - A_0) \left[ \frac{(K_D + L_r + R_D) - \sqrt{(K_D + L_r + R_D)^2 - 4R_D L_r}}{2R_r} \right] \]  
(Eq. 3)

where A is the activity, \( A_0 \) is the initial activity value, \( A_r \) is the final (inhibited) activity, \( L_r \) is the total γ_PDE concentration, \( R_r \) is the total αPDE concentration, and \( K_D \) is the inhibition constant.

Anisotropy data in Fig. 2 were fit to the binding equation,

\[ r = r_0 + r_1 \left[ \frac{(K_o + L_o + R_o) - \sqrt{(K_o + L_o + R_o)^2 - 4R_o L_o}}{2R_r} \right] \]  
(Eq. 4)

where r is the anisotropy value, \( r_0 \) is the initial anisotropy value, \( r_1 \) is the maximum anisotropy, \( L_o \) is the total γ_PDE concentration, \( R_o \) is the total αPDE concentration, and \( K_o \) is the dissociation constant. All fits were generated using IgorPro software using a least squares fit criterion (WaveMetrics, Inc., Lake Oswego, OR).

For the comparison of the kinetics of relative fluorescence and fluorescence anisotropy changes (e.g. Fig. 5B), relative fluorescence was determined using the mathematical relationship between fluorescence intensity and anisotropy (Lakowicz, 1983),

\[ I = \frac{3I_o}{1 + 2r} \]  
(Eq. 5)

or

\[ I = \frac{3I_o}{1 - r} \]  
(Eq. 6)

where r is the anisotropy, I is the calculated emission intensity, \( I_o \) is the intensity of emission polarized in parallel with the excitation beam, and \( I_1 \) is the intensity of emission polarized perpendicular to the excitation beam. This calculated intensity was corrected for changes in the lamp intensity to give relative fluorescence. Calculated relative fluorescence was identical when using either \( I_1 \) or \( I_0 \) and agreed very closely with data obtained when using the conventional measurement of relative fluorescence described above. In order to compare the rate of reversal by (unlabeled) γ_PDE of the αPDE/βPDE-induced enhancement in the fluorescence fluorescence of the doubly labeled γ_PDE and the rate of reversal of the increase in anisotropy (Fig. 3B), the fluorescence intensity differences for doubly labeled γ_PDE that is bound to TPDE and for doubly labeled (unbound) γ_PDE that is unbound must be mathematically corrected in the apparent anisotropy plot. This was performed using the equation,

\[ B = \frac{A_n - A}{0.34A_n - 1.34A_B + A_0} \]  
(Eq. 7)

where B is the fraction of γ_PDE that is bound to the trypsin-treated PDE, \( A_n \) is the anisotropy of unbound (free) γ_PDE (0.176 for the data in Fig. 3B), \( A_B \) is the anisotropy of γ_PDE bound to trypsin-treated PDE (0.232 in Fig. 3B), and A is the measured (uncorrected) anisotropy. The 0.34 and 1.34 factors are derived from the 34% higher fluorescence of the bound, doubly labeled γ_PDE (to αPDE and βPDE) relative to the unbound doubly labeled γ_PDE species.

RESULTS

The primary aim of these studies was to generate a γ_PDE subunit that was labeled at two distinct sites so that resonance energy transfer approaches could be used to monitor changes in the juxtaposition of these sites caused by the binding of other signaling molecules to the γ_PDE. In particular, we were interested in determining if the structure of γ_PDE changes upon its binding to the core of the PDE molecule and/or upon its binding to an activated αP subunit.

Characterization of the Doubly Labeled γ_PDE Subunit—We used the following strategy to generate a doubly labeled γ_PDE subunit. One site of labeling (with IAF) was the single cysteine residue at position 68, both because this site is located near one end of the γ_PDE molecule and because the conditions for its selective and stoichiometric modification have been well established (e.g. Erickson et al. (1995)). We then set out to label the primary amino group of the amino-terminal methionine residue, because previous studies have demonstrated that the N-terminal primary amino group of a protein can be selectively labeled when performing the modification at pH values below 7.5 (Carraway et al., 1990).

However, given that the γ_PDE subunit contains a number of lysine residues, we first examined whether the labeling with EITC was in fact occurring at the amino-terminal residue rather than at one or more of the lysine residues on γ_PDE. When mass spectrometry was performed on the full-length recombinant γ_PDE labeled with both EITC and IAF, a single predominant peak was determined with a size of 10,880 (Fig. 1A). The position of this peak is within the experimental error of the calculated size for a full-length γ_PDE molecule containing just one IAF moiety and one EITC moiety (10,762 Da).

We performed an additional experiment where the full-length γ_PDE, labeled with IAF and EITC, was trypsin-treated and then subjected to reverse-phase HPLC in order to resolve the resulting peptides. This was followed by mass spectrometry of the eosin-labeled species. The trypsin treatment resulted in the generation of only three peaks showing (EITC) absorbance at 525 nm. Two of these peaks showed lower relative levels of 525 nm absorbance and were subsequently found not to contain peptides as revealed by mass spectral analysis. It seems most likely that these peaks represented fluorescent dye aggregates, and we therefore concentrated on the third peak of 525-nm absorbance for further analysis. When this peak was subjected to mass spectrometry, two observable mass spectrum peaks were obtained (Fig. 1B). One peak of 1555 Da corresponds to the first seven amino acids from the amino terminus (ending with lysine residue 7) plus a single eosin moiety. The second peak of 2025 Da represents the first 11 amino acids (ending with arginine residue 11). Comparison of these experimentally determined labeled peptide masses revealed no other close matches when compared with hypothetical tryptic fragments in the γ_PDE sequence (not shown). These results indicate that the EITC label was attached either at the amino terminus as originally intended or at the lysine located seven residues from

Changes in γ_PDE Monitored by Resonance Energy Transfer
FIG. 1. Mass spectroscopy of the doubly labeled γPDE. Panel A shows the results of a MALDI-TOF mass spectroscopy experiment performed using approximately 100 pmol of γPDE in 25% acetonitrile and 0.1% trifluoroacetic acid. The molecular ion mass of 10,880 is within the experimental error for the expected molecular weight of 10,762, corresponding to 1 γPDE : 1 IAF : 1 EITC. Note that the predicted molecular mass for unlabeled γPDE (9660 Da) does not appear to be present. There also do not appear to be sufficient amounts of heterogeneously labeled γPDE to produce other predicted molecular weight peaks in the spectrum. Panel B shows a MALDI TOF mass spectroscopy trace for γPDE peptide that was produced from trypsin digestion of the doubly labeled γPDE. 150 pmol of protein was trypsinized, and an eosin-containing peptide species was purified by reverse-phase HPLC based upon optical absorbance at 523 nm. Mass spectroscopy of this peptide shows two components with molecular weights of 1554 and 2025. One representative spectrum is shown. The experiment was repeated one additional time with MALDI and twice with ESI mass spectroscopy techniques.

Changes in γPDE Monitored by Resonance Energy Transfer

FIG. 2. Effect of doubly labeled γPDE on trypsinized PDE. Panel A shows fluorescein fluorescence anisotropy of the doubly labeled γPDE. 12 pmol (15 nM) of doubly labeled γPDE (as determined by absorbance at 520 nm) was bound to successive additions of 2 pmol (2.5 nM) trypsinized PDE. The curve is fit to a quadratic binding equation for total PDE binding to doubly labeled γPDE. The apparent Kd for this binding is 7.3 nM. This experiment was repeated one additional time, which resulted in an apparent Kd of 6.0 nM. Panel B shows the inhibition of total PDE by doubly labeled γPDE. 1 pmol (5 nM) of tPDE was inhibited with successive additions of 0.9 pmol (4.5 nM) of the doubly labeled γPDE species. The rate of cGMP hydrolysis was monitored using the pH microelectrode assay (see “Experimental Procedures”; Yee and Liebman (1978)). These data are plotted as an inhibition curve, fit to a quadratic binding equation for total γPDE binding to tPDE. The concentration of labeled γPDE was determined by eosin absorbance at 520 nm. The apparent Kd for the inhibition is 8 nM. The experiment was repeated one additional time, which resulted in an apparent Kd of 7.8 nM.
Changes in $\gamma_{PDE}$ Monitored by Resonance Energy Transfer

Fig. 3. Interaction of $\gamma_{PDE}$ with PDE. Panel A shows the emission at 520 nm of IAF- and EITC-labeled $\gamma_{PDE}$ and IAF-labeled $\gamma_{PDE}$. The upper curve shows the emission of 12 pmol (15 nM) of doubly labeled $\gamma_{PDE}$. The addition of 10 pmol of (12.5 nM) trypsinated PDE caused an increase in fluorescence emission, and the further addition of another 10 pmol of PDE did not cause an additional increase in the emission. The increased fluorescence was reversed with 155 pmol (1.95 nM) of unlabeled $\gamma_{PDE}$. The lower curve shows the results for the corresponding control experiment with IAF-labeled $\gamma_{PDE}$, Inset, single exponential fit of the fluorescence changes that accompany the dissociation of doubly labeled $\gamma_{PDE}$ upon the addition of unlabeled $\gamma_{PDE}$. Panel B shows anisotropy data recorded during the relative fluorescence measurements of the doubly labeled $\gamma_{PDE}$ shown in panel A. Trypsinated PDE caused an increase in the anisotropy. This increase is saturable and reverses with the addition of the excess unlabeled $\gamma_{PDE}$, Inset, single exponential fit of the changes in the corrected fluorescence anisotropy ("Experimental Procedures") that accompany the dissociation of doubly labeled $\gamma_{PDE}$ upon the addition of unlabeled $\gamma_{PDE}$.

trypsinized $\alpha\beta_{PDE}$. No detectable change in either eosin emission (at 545 nm) or absorbance (at 525) was observed when eosin-$\gamma_{PDE}$ bound to the PDE enzyme core (data not shown).

The upper trace in Fig. 3A shows the results obtained when $\gamma_{PDE}$ labeled with both IAF and EITC was added to the $\alpha_{PDE}$ and $\beta_{PDE}$ subunits, generated by trypsin treatment of the PDE. There was a significant increase in the IAF fluorescence of the doubly labeled $\gamma_{PDE}$ upon binding to $\alpha_{PDE}$ and $\beta_{PDE}$. We have found that this increase, which ranged from 20 to 40% in different experiments (34 ± 5%, S.E., $n = 4$), could be completely eliminated by adding excess $\gamma_{PDE}$, consistent with the interaction between $\gamma_{PDE}$ and the $\alpha_{PDE}$ and $\beta_{PDE}$ subunits being fully reversible. Significantly, the addition of tryps in to the free, unbound doubly labeled $\gamma_{PDE}$ caused a significant enhancement in the IAF fluorescence. After correction for the small changes caused by the addition of tryps in to the control IAF-labeled $\gamma_{PDE}$ subunit (shown in the lower trace of Fig. 3A), the average value for tryps in-induced enhancement of the free IAF-$\gamma_{PDE}$ fluorescence was 36 ± 2% (S.E., $n = 8$). In control experiments, the magnitude of the enhancement was not influenced by the presence of other nonfluorescent proteins (e.g. bovine serum albumin; data not shown). This level of IAF fluorescence represents the donor fluorescence in the absence of resonance energy transfer, since tryptic digestion of the $\gamma_{PDE}$ molecule results in the IAF moiety being attached to a (tryptic) peptide that is distinct from the peptide containing the EITC moiety. Thus, when this level of IAF fluorescence is compared with the IAF fluorescence for the doubly labeled $\gamma_{PDE}$ molecule when it is free in solution, this yields an efficiency of energy transfer of 36%. When that value is used to calculate the distance separating the IAF and EITC moieties, using an $R_0$ value of 56.8 Å (see “Experimental Procedures”), an apparent distance between the IAF and EITC moieties of 62.7 ± 1.05 Å (S.E., $n = 8$) is obtained. The same measurement made for the doubly labeled $\gamma_{PDE}$ subunit when it is bound to the $\alpha_{PDE}$ and $\beta_{PDE}$ subunits, generated by treatment of the PDE with tryps in or with ArgC (see below), yielded an efficiency of energy transfer of 15 ± 3% (S.E., $n = 4$) and an effective distance of 77.3 ± 3.3 Å (S.E., $n = 4$). Thus, these results indicate that the $\gamma_{PDE}$ molecule becomes significantly more extended when it binds to the $\alpha_{PDE}$ and $\beta_{PDE}$ subunits.

The results shown in Fig. 3B show the corresponding real time fluorescence assay for doubly labeled $\gamma_{PDE}$ interactions with the $\alpha_{PDE}$ and $\beta_{PDE}$ using the changes in fluorescence anisotropy for the IAF moiety bound to $\gamma_{PDE}$. The fluorescence anisotropy change that occurs upon the addition of tryps in-treated PDE to the doubly labeled $\gamma_{PDE}$ subunit can be rapidly reversed by the addition of excess $\gamma_{PDE}$, as the change in IAF fluorescence can also be rapidly reversed; Fig. 3A). The addition of tryps in to this sample then results in an immediate decrease in the anisotropy for IAF due to the degradation of the labeled $\gamma_{PDE}$ subunit and the dissociation of labeled $\gamma_{PDE}$ peptide fragments. The raw anisotropy data shown in Fig. 4B can be further analyzed taking into account the disproportionate contribution of the $\alpha_{PDE}$-bound state of the doubly labeled $\gamma_{PDE}$ to the overall 520 nm emission (see “Experimental Procedures”). When the corrected data for the decrease in the fluorescence anisotropy, upon the addition of unlabeled $\gamma_{PDE}$, and the corresponding decrease in the fluorescence enhancement, are fit to a single exponential decay, the rate of the decay in the IAF fluorescence (0.066 s$^{-1}$) is similar to the decrease in doubly labeled $\gamma_{PDE}$ anisotropy (0.042 s$^{-1}$).

Fluorescence Read-out for the Binding of $\gamma_{PDE}$ to an Activated $\alpha_{G}$-GTP-$\gamma_{S}$ Complex—We next examined whether the position of cysteine 68 relative to the amino terminus of $\gamma_{PDE}$ changed upon binding to an activated $\alpha_{G}$ subunit. This was done employing the same fluorescence read-outs as those used to examine changes in $\gamma_{PDE}$, that accompanied its binding to the $\alpha_{PDE}$ and $\beta_{PDE}$ subunits. Fig. 4A shows the results from a control experiment where the $\alpha_{G}$-GTP-$\gamma_{S}$ complex was added to an IAF-labeled $\gamma_{PDE}$ subunit. We found that the $\alpha_{G}$-GTP-$\gamma_{S}$/IAF-$\gamma_{PDE}$ interaction resulted in an ~10% enhancement in the IAF fluorescence, presumably reflecting an $\alpha_{G}$-induced change in the microenvironment of cysteine 68, as previously reported (Erickson et al., 1995). This fluorescence change was immediately reversed upon the addition of excess (unlabeled) $\gamma_{PDE}$ due to its competition with the IAF-labeled $\gamma_{PDE}$ for the activated $\alpha_{G}$ subunit. As shown earlier (Fig. 3A), trypsin treatment of the IAF-$\gamma_{PDE}$ then results in a minor increase in the IAF fluorescence.

Fig. 4B shows the results obtained when the same type of experiment was performed using the doubly labeled $\gamma_{PDE}$. Essentially, the same results were obtained as those seen with the singly (IAF-) labeled $\gamma_{PDE}$. Specifically, there was an immediate enhancement (~10%) in the IAF fluorescence of the doubly
Changes in \( \gamma_{PDE} \) Monitored by Resonance Energy Transfer

**FIG. 4. Interaction of labeled \( \gamma_{PDE} \) with transducin.** The relative fluorescence changes due to fluorescein only and doubly labeled \( \gamma_{PDE} \) are shown. Panel A shows the fluorescence emission of 4.5 pmol of (5.7 nM) control fluorescein-only-labeled \( \gamma_{PDE} \). The addition of 25 pmol of (31.2 nM) purified \( \alpha_z\)-GTP\( \gamma \)S caused an increase in the fluorescence. Additional \( \alpha_z\)-GTP\( \gamma \)S caused no further increase. This increase was reversed with the addition of 99.2 nM of (48.4 \( \mu \)M) unlabeled \( \gamma_{PDE} \). Panel B shows the relative fluorescence of 12 pmol of (15 nM) doubly labeled \( \gamma_{PDE} \). The addition of 12.5 pmol (15.6 nM) of purified \( \alpha_z\)-GTP\( \gamma \)S caused an increase in fluorescein emission comparable with that for the fluorescein-labeled \( \gamma_{PDE} \) control in panel A. Additional \( \alpha_z\)-GTP\( \gamma \)S caused no further increase, and 99.2 nM (48.4 \( \mu \)M) of unlabeled \( \gamma_{PDE} \) fully reversed the increased relative fluorescence.

An important question was whether any change in the \( \gamma_{PDE} \) structure occurred when an activated \( \alpha_T \) subunit bound to an intact PDE molecule (i.e., an \( \alpha_{PDE}\beta_{PDE}\gamma_{PDE} \) complex). The results shown in Fig. 4 suggest that this is not the case. In this experiment, the IAF fluorescence of an IAF and EITC-labeled \( \gamma_{PDE} \) subunit was monitored after protease treatment of the holo-PDE molecule. The protease ArgC was used because it has been shown to have some selectivity in degrading the \( \gamma_{PDE} \) subunits of the holo-PDE complex, leaving intact the ability of an activated \( \alpha_T \) subunit to stimulate cyclic GMP hydrolysis following the addition of this G protein subunit together with fluorescently labeled \( \gamma_{PDE} \) subunits (Brown, 1992).

**Fig. 5. Effects of tPDE and transducin on doubly labeled \( \gamma_{PDE} \) fluorescence under catalytic conditions.** The fluorescence trace shows the emission of 12 pmol of (15 nM) doubly labeled \( \gamma_{PDE} \) at 520 nm under catalytic conditions in the presence of 250 \( \mu \)M cGMP. The addition of approximately 25 pmol of (31 nM) ArgC-treated PDE caused an increase in fluorescence. The subsequent addition of 12.5 pmol (15.6 nM) of purified \( \alpha_z\)-GTP\( \gamma \)S caused a slight further increase in the fluorescence. The addition of another the 12.5 pmol of \( \alpha_z\)-GTP\( \gamma \)S then caused no further increase. The inset shows that \( \alpha_z\)-GTP\( \gamma \)S can stimulate ArgC-treated PDE under the same conditions used to measure the fluorescence changes described above. Approximately 12.5 pmol (62 nM) of ArgC-treated PDE was inhibited with 12 pmol of (60 nM) doubly labeled \( \gamma_{PDE} \). The addition of 12.5 pmol (62.4 nM) \( \alpha_z\)-GTP\( \gamma \)S caused a 2.5-fold increase in the rate of cGMP hydrolysis. The addition of trypsin caused a further 5.8-fold increase in activity.

**DISCUSSION**

In the present study, we set out to use fluorescence spectroscopic approaches to determine whether structural changes occur within the \( \gamma_{PDE} \) molecule when it binds to the larger \( \alpha_{PDE} \) and \( \beta_{PDE} \) subunits of the effector enzyme. We also were interested in determining whether the tertiary structure of \( \gamma_{PDE} \) is
affected by the binding of an activated αPDE subunit, because such information could provide insight into the molecular mechanism by which the retinal G protein stimulates PDE activity (i.e., cyclic GMP hydrolysis). Our general strategy for examining tertiary structural changes within γPDE was to attach a fluorescence donor moiety at cysteine 68 (IAF) and an acceptor chromophore (EITC) at the amino-terminal end of the γPDE molecule and then to use resonance energy transfer to monitor the juxtaposition of these labels under different experimental conditions. By using this approach, we found that the distance between the fluorescent probes (i.e., the distance between the EITC-labeled amino group and the IAF-labeled cysteine) was increased from 63 Å when γPDE was free in solution to 77 Å when it was bound to the larger subunits of the PDE molecule. However, there was no significant change in the proximity of these two sites on the γPDE molecule upon the binding of an activated αP subunit.

Previous resonance energy transfer measurements suggested that upon the formation of an αγPDET complex, cysteine 68 of γPDE (which is thought to be close to the αγ-binding site (Faurobert et al., 1993)) is a significant distance (~40 Å) from the guanine nucleotide binding site on αγ. Various studies have implicated residues 300–310 of αγ as being involved in the stimulatory interaction with the PDE molecule (Rarick et al., 1992; Artemyev et al., 1993); however, the coordinates from the x-ray crystallographic structure of αγ indicate that this region of the αγ subunit is only 20–25 Å away from lysine 267 at the guanine nucleotide-binding site. Thus, one possible explanation for the resonance energy transfer measurements that suggest a greater distance (~20–25 Å) between the nucleotide-binding site and the γPDE-binding site on αγ is that αγ makes at least two contacts with γPDE, one involving residues 300–310 and another involving a site within the large helical domain of αγ (e.g., residues 106–116). If the contact region in the helical domain of αγ is bound to γPDE in the vicinity of cysteine 68, this would be consistent with the measured distance of ~40 Å between cysteine 68 of γPDE and lysine 267 of αγ. In this view, residues 300–310 of αγ would bind in the vicinity of lysine residues 41, 44, and 45 of γPDE, consistent with the results obtained from chemical cross-linking studies (Artemyev et al., 1993). Our present results would suggest that the distance between residues 41–45 and cysteine 68 (i.e., the two proposed αγ-binding sites on the γPDE molecule) does not change when γPDE binds to an activated αγ subunit, either when γPDE is free in solution or bound to the αγPDE and βγPDE subunits. We would expect that even subtle αγ-induced conformational changes occurring upstream from cysteine 68 would have been detected by a change in the effective distance between cysteine 68 and the amino-terminal end of the γPDE molecule. However, we (Erickson et al., 1995) and others (Faurobert et al., 1993) have found that αγ binding perturbs the microenvironment surrounding cysteine 68. Thus, in light of our present findings, we hypothesize that these αγ-induced changes are communicated to the carboxy-terminal domain of the γPDE molecule, resulting in the activation of cyclic GMP hydrolysis by the αγPDE and βγPDE subunits.

An important point raised by the energy transfer experiments is that the αγ-mediated stimulation of PDE activity occurs while the αγγPDE complex is still associated with the core of the effector enzyme (i.e., the αγPDE and βγPDE subunits). The latter suggestion has been a subject of controversy over the years, since various lines of data have argued that αγ-mediated stimulation is accompanied by the dissociation of an αγγPDE complex from the core of the enzyme (Wensel and Stryer, 1990), while other lines of evidence have suggested that γPDE dissociation is not necessary for the stimulation of PDE activity (Erickson and Cerione, 1993; Catty et al., 1992; Clerc and Bennett, 1993). However, the fact that the changes in the γPDE subunit that are specifically induced by the αγPDE and βγPDE subunits (i.e., the increase in the distance between the amino terminus and cysteine 68 of γPDE) are not reversed upon the binding of an activated αγ subunit (under conditions where αγ causes a stimulation of PDE activity) argues that the γPDEαγPDEβγPDE complex remains intact during the αγ-GTP-S-mediated stimulation of cyclic GMP hydrolysis (see Fig. 5). The activated αγ subunit does not induce similar changes within the γPDE subunit (as those induced by the αγPDE and βγPDE subunits) when γPDE is free in solution (i.e., dissociated from the αγPDE and βγPDE subunits), and αγ appears to bind to γPDE independently of the αγPDE and βγPDE subunits. Specifically, the K0 values that we have measured for the interaction of αγ-GTP-S with γPDE are similar for γPDE that is free in solution (~30 nM; Erickson et al. (1995)) or bound to αγPDE and βγPDE (21 nM; Erickson and Cerione (1989)). Thus, based on a consideration of microscopic reversibility, these data argue against the possibility that the activated αγ subunit binds to γPDE and stimulates its dissociation from the αγPDE and βγPDE subunits (during PDE activation) but still maintains the free γPDE subunit in a specific conformation that was originally induced by the larger PDE subunits.

Future work will be directed toward further examining both of the suggestions raised by the present study. In particular, we will set out to directly demonstrate that αγ binding to γPDE results in specific conformational changes within the carboxy-terminal domain of this subunit that are directly translated into the stimulation of PDE activity. We also will try to determine the types of changes that must occur in the orientation of the γPDE subunits relative to the larger subunits of the effector enzyme. This remains an important issue, because although our data argue that the γPDE subunits remain associated with αγPDE and βγPDE during αγ-mediated stimulation of enzyme activity, it has been well established that the addition of excess free γPDE molecules will inhibit the αγ-stimulated PDE activity (Wensel and Stryer, 1986). Taken together, these findings imply that a positional change in the binding interaction of the γPDE subunits with the αγPDE and βγPDE subunits occurs during PDE enzyme activation following the binding of the activated αγ subunit.

REFERENCES

Artemyev, N. O., Mills, J. S., Thornburg, K. E., Knapp, D. R., Schey, K. L., and Hamm, H. E. (1993) J. Biol. Chem. 268, 23611–23615
Brown, R. L., and Stryer, L. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4922–4926
Brown, R. L. (1992) Biochemistry 31, 5918–5925
Carraway, K. L., III, Koland, J. G., and Cerione, R. A. (1989) J. Biol. Chem. 264, 8699–8707
Carraway, K. L., Koland, J. G., and Cerione, R. A. (1990) Biochemistry 29, 8741–8747
Catty, P., Pfister, C., Bruckert, F., and Deterre, P. (1992) J. Biol. Chem. 267, 19489–19493
Cherry, R. J., Coguli, A., Opplinger, M., Schneider, G., and Semenza, G. (1976) Biochemistry 15, 3653–3656
Clerc, A., and Bennett, N. (1992) J. Biol. Chem. 267, 6620–6627
Coleman, D. E., Berghaus, A. M., Lee, E., Linder, M. A., Gilman, A. G., and Sprang, S. R. (1994) Science 265, 1405–1412
Epe, B., Steinhauser, K. G., and WOolley, P. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2579–2583
Erickson, J. W., and Cerione, R. A. (1991) Biochemistry 30, 7112–7118
Erickson, J. W., and Cerione, R. A. (1993) J. Biol. Chem. 268, 3328–3333
Erickson, J. W., Mittal, R., and Cerione, R. A. (1995) Biochemistry 34, 8693–8700
Faurobert, E., Otto-Bruc, A., Chardin, P., and Chabre, M. (1993) EMBO J. 12, 4191–4198
Gierschik, P., Simons, C., Woodard, C., Somers, R., and Spiegel, A. (1984) FEBS Lett. 172, 321–325
Guy, P. M., Koland, J. G., and Cerione, R. A. (1990) Biochemistry 29, 6954–6964
Hass, E., Katzir, E-K., and Steinberg, I. Z. (1978) Biochemistry 17, 5064–5070
Haungland, R. P. (1994) Molecular Probes Handbook of Fluorescent Probes and Research Chemicals, 5th Ed., Molecular Probes, Inc., Eugene, OR
Kroll, S., Phillips, W. J., and Cerione, R. A. (1989) J. Biol. Chem. 264, 4490–4497
Lakowicz, J. R. (1983) Principles of Fluorescence Spectroscopy, Plenum Press, NY
Lambrechts, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) Nature 369, 371–374.
Changes in $\gamma_{PDE}$ Monitored by Resonance Energy Transfer

Mittal, R., Cerione, R. A., and Erickson, J. W. (1994) Biochemistry 33, 10178–10184
Noel, J. P., Hamm, H. E., and Sigler, P. B. (1993) Nature 366, 654–663
Phillips, W. J., and Cerione, R. A. (1988) J. Biol. Chem. 263, 15498–15505
Pines, M., Gierschik, P., Milligan, G., Klee, W., and Spiegel, A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4095–4099
Rarick, H. M., Artemyev, N. O., and Hamm, H. E. (1992) Science 256, 1031–1033
Wensel, T. G., and Stryer, L. (1990) Biochemistry 29, 2155–2161
Yee, R., and Liebman, P. A. (1978) J. Biol. Chem. 253, 8902–8909