Interaction between the Retinal Cyclic GMP Phosphodiesterase Inhibitor and Transducin. Kinetics and Affinity Studies†

Annie Otto-Bruc, Bruno Antonny, T. Minh Vuong, Pierre Chardin, and Marc Chabre*

CNRS, Institut de Pharmacologie Moléculaire et Cellulaire, 660 route des Lucioles-Sophia Antipolis, F-06560 Valbonne, France

Received January 28, 1993; Revised Manuscript Received May 13, 1993

ABSTRACT: In the retinal cyclic GMP phosphodiesterase (PDE), catalysis by the $\alpha\beta$-heterodimer is inhibited in the dark by two identical γ-subunits and stimulated in the light by the GTP-bearing α-subunit of the heterotrimeric G-protein transducin (T$\beta\gamma$–TαGDP). Two TαGTP molecules, dissociated from T$\beta\gamma$, bind to and displace the PDEγ subunits from their inhibitory sites on PDE$\alpha\beta$. With GTPγS in lieu of GTP, this association becomes persistent. Under physiological conditions, the PDE$\alpha\beta(\gamma T\alpha)_2$ active complex stays on the membrane. But in low-salt buffers, it becomes soluble and dissociates into a partially active PDE$\alpha\beta$ catalytic moiety and two PDEγ–TαGTPγS complexes. This indicates that Tα binds preferentially to PDEγ. We have studied the interaction of recombinant bovine PDEγ with purified Tα in solution or with retinal rod outer segments (ROS) containing both T$\beta\gamma$–TαGDP and PDE$\alpha\beta\gamma_2$. When added to dark ROS, recombinant PDEγ did not bind to inactive PDE$\alpha\beta\gamma_2$ but extracted TαGDP from membrane-bound holo-transducin to form a soluble PDEγ–TαGDP complex. PDEγ also bound to purified TαGDP in solution. The kinetics and affinity of the interaction between PDEγ and TαGDP or TαGTPγS were determined by monitoring changes in the proteins' tryptophan fluorescence. The K_d's for the binding of recombinant PDEγ to soluble TαGTPγS and TαGDP are $\leq$0.1 and 3 nM, respectively. PDEγ–TαGDP falls apart in 3 s. This slow dissociation means that, *in situ*, Tα–PDEγ cannot physically leave the active PDE$\alpha\beta$, since after GTP hydrolysis, an isolated Tα–PDEγ complex would dissociate too slowly to allow a fast PDE reinhibition by the liberated PDEγ. When recombinant PDEγ was added to PDE that had been persistently activated by TαGTPγS, reinhibition occurred and TαGTPγS, complexed to the native PDEγ, was released, indicating that both had hitherto stayed bound to PDE$\alpha\beta$. The mutation W70F does not prevent recombinant PDEγ from inhibiting PDE$\alpha\beta$ but diminishes its affinity for TαGTP and TαGDP 100-fold. Thus, W70 of PDEγ must take part in its binding to TαGTP and TαGDP. When reconstituted with [W70F]PDEγ, PDE was fully inhibited but could no longer be activated by TαGTP or TαGTPγS.

Vertebrate visual excitation is mediated by a light-triggered G-protein cascade. Its effector, the cyclic GMP phosphodiesterase (PDE), causes closure of the cationic channels on the plasma membrane by lowering the level of free cGMP in the rod outer segment (ROS). PDE is a peripheral membrane protein constituted of a large, undissociable heterodimer catalytic complex, PDE$\alpha\beta$ (99.2 + 98.3 kDa), whose cGMP hydrolytic activity is blocked, in the basal state, by two tightly bound, low molecular weight (9.7 kDa) PDEγ inhibitory subunits (Baehr et al., 1979; Hurley & Stryer, 1982; Ovchinnikov et al., 1986, 1987; Deterre et al., 1988; Lipkin et al., 1990). Upon illumination, photoexcited rhodopsin (R*) catalyzes a rapid GDP/GTP exchange on the α-subunit of the hitherto inactive heterotrimeric G-protein transducin (T$\beta\gamma$–TαGDP). The active TαGTP subunit dissociates from T$\beta\gamma$, binds to the PDE, and unleashes the cGMP hydrolytic activity by displacing a PDEγ subunit from its inhibitory site on PDE$\alpha\beta$. PDEγ is thus the control element which, by interacting with both PDE$\alpha\beta$ and TαGTP, regulates the phosphodiesterase activity. Being in large part inhibited in dark-kept ROS, PDE is transiently activated by TαGTP upon flash illumination in the presence of GTP and can be persistently activated by TαGTPγS if GTPγS is used instead of GTP. The complex PDEγ–TαGTPγS can be extracted from illuminated membranes with a low ionic strength buffer and chromatographically separated from active PDE$\alpha\beta$ (Deterre et al., 1986). Coimmunoprecipitation experiments confirmed a one-to-one stoichiometry for the interaction between TαGTPγS and PDEγ (Fung & Griswold-Prenner, 1989). Addition of PDEγ reverses the activation of PDE by TαGTPγS (Wensel & Stryer, 1986, 1990). These observations led to the suggestion that the activation event is the physical removal of the inhibitory subunits from the catalytic complex. However, given the very high affinity of PDEγ for PDE$\alpha\beta$, a high activation rate means that TαGTP must, at least initially, interact with PDEγ on the holo-PDE complex and alter its high-affinity conformation to cause the rapid release of its inhibitory lock on PDE$\alpha\beta$. Recent work (Erikson & Cerione, 1991; Clerc & Bennett, 1992; Catty et al., 1992) suggests that, under physiological ionic conditions and at high concentrations of ROS membranes, both TαGTP and PDEγ remain associated with PDE$\alpha\beta$. Thus, TαGTP would latch on to PDEγ and displace it from its inhibitory site on PDE$\alpha\beta$, but the tight Tα–PDEγ complex would remain weakly bound to the activated PDE$\alpha\beta$. This mechanistic clarification aside, it remains clear that TαGTP and PDEγ are tightly associated during the active phase of the physiological response. Hence, the *in vitro* extractable, soluble Tα–PDEγ complex is a valid minimalist model that can shed some light on the molecular details of the interaction between transducin and PDE. We have so far referred to the mammalian (cattle) ROS. In frog ROS, Yamazaki et al. (1983, 1990) reported the release of a soluble Tα–PDEγ complex, while the work of Gray-Keller

† This work was supported by grants from the Human Frontier Science Program and INSERM (Grant No. 910502).

* Address correspondence to this author. Telephone (33)93-95-77-75. Fax (33)93-95-77-10.

0006-2960/93/0432-8636$04.00/0

et al. (1990) suggests that the transducin fraction activating the PDE remains membrane bound.

Locating the domains through which PDEγ interacts with TαGTP and PDEαβ was initiated by the expression of the 87 amino acid PDEγ polypeptide in *Escherichia coli* (Brown & Stryer, 1989; Griswold-Prenner et al., 1989). The last five C-terminal residues seem necessary for the inhibitory interaction with PDEαβ, while a positively charged domain in segment 24–45 is critical for the activation by transducin (Cunnick et al., 1990; Brown, 1992; Artemyev & Hamm, 1992; Artemyev et al., 1992). Using the synthetic gene provided by Brown and Stryer (1989), but with a different expression vector, we have produced in *Escherichia coli* a recombinant PDEγ that is not a fusion protein, but has an isoleucine added in position 2 after the initial methionine. This recombinant PDEγ effectively inhibits trypsin-activated PDE. When mixed with purified TαGTPγS in solution, it formed, as expected, a stable PDEγ–TαGTPγS complex. When added to nonilluminated ROS membranes, PDEγ does not bind to the already saturated, quiescent PDEαβγ$_2$, but quite unexpectedly interacts with inactive membrane-bound holo-transducin from which it extracts the TαGDP subunit. The resulting PDEγ–TαGDP complex is soluble and dissociated from Tβγ.

Interactions of PDEγ with TαGTP or TαGDP perturb the intrinsic fluorescence of tryptophan residues in the interacting proteins: fluorescence emission spectra of the complexes differ significantly from the summed spectra of the two individual components. Thus, the binding affinities can be estimated by monitoring the tryptophan fluorescence changes upon stepwise addition of PDEγ to solutions of purified Tα. Isolated PDEγ binds with a very high affinity (K_d < 0.1 nM) to TαGTPγS. Its weaker affinity for TαGDP is still in the nanomolar range and confirms our finding that PDEγ can interact with transducin in dark ROS.

The tryptophan at position 70 of PDEγ was conservatively replaced by a phenylalanine. Compared to the unmodified PDEγ, this [W70F]PDEγ mutant inhibits PDEαβ with equal efficiency, but its affinities for TαGTP and TαGDP are both weaker by about a 100-fold. W70 does not lie within the positively charged domain 24–45 which is usually considered the interaction domain with transducin. Yet, our results suggest that this tryptophan contributes significantly to the binding of PDEγ to TαGTP and to TαGDP as well.

MATERIALS AND METHODS

Construction of the Expression Vector. The expression construct developed by Brown and Stryer (1989), pLcIIFXSG, was cleaved using the *Bam*HI and *Hind*III restriction sites. The *Bam*HI–*Hin*dIII fragment was cloned in an M13mp18 vector, and the single-stranded template (M13∼PDEγ) was mutagenized to introduce a *Bcl*I site just at the beginning of the PDEγ coding sequence. This adds an isoleucine between the first two residues (Met-Asn) of PDEγ:

5′...GGGTATGATCAACCT...3′

The mutated *Bam*HI–*Hin*dIII restriction fragment was cloned in a pUC18 vector and amplified in the dam I methylase deficient GM2163 strain. Then, the *Bcl*I–*Hin*dIII fragment containing the entire PDEγ coding sequence was introduced into a pT7 vector under control of the T7 phage promoter. This vector is derived from pET-3c of Rosenberg et al. (1987) by including a *Bcl*I restriction site. The plasmid pUC18∼PDEγ carrying the modified PDEγ insert was cut with *Hin*dIII, filled in with Klenow polymerase, cut with *Bcl*I, and subcloned in the pT7 vector, which had been cut with *Bam*HI, filled in with Klenow polymerase, and cut with *Bcl*I.

Addition of the isoleucine residue at position 2 means that the PDEγ expressed with this pT7 vector does not rigorously qualify as wild type. This minor structural addition is apparently of little consequence, as the expressed protein behaves indistinguishably from the wild type. We will therefore refer to this form of PDEγ as wild type.

Expression and Purification of Recombinant PDEγ. The expression construct pT7∼PDEγ was transformed into the *Escherichia coli* BL21(DE3)+pLys S strain, which contains the T7 RNA polymerase gene under control of the ptac promoter. A 5-L culture was grown at 37 °C to an OD of 0.7, induced by addition of 0.5 mM IPTG, and immediately heat shocked by adding 0.1 volume of boiling L-broth to raise the temperature to 42–43 °C. Cells were kept at 42 °C for 10 min and then grown for 2 h at 37 °C. Cells were washed once with 10 mM Tris, pH 8, and 1 mM EDTA. Lysis and purification of the aggregated PDEγ were essentially as described by Brown and Stryer (1989) but with slightly modified buffers. Lysis buffer: 50 mM Tris, pH 8, 30% sucrose, and 1 mM EDTA. Detergent buffer added after incubation with DNAse I: 10 mM Tris, pH 8, 0.4% deoxycholate, and 0.4% Tween 20. Detergent buffer used for washing the pellet twice: 20 mM Tris, pH 8, 0.4% Tween 20, and 0.1 M NaCl. Urea buffer: 7 M urea, 20 mM HEPES, pH 7.5, and 1 mM DTT. Dialysis buffer: 20 mM Tris, pH 7.5, 0.1 M NaCl, and 1 mM DTT. From each 5-L culture we routinely obtained about 20 mg of PDEγ at 50–60 μM. Purity was >90%, as judged by Coomassie-stained SDS–poylacrylamide gels. Wild-type PDEγ was sequenced to verify the identity of the first ten amino acids. The purified proteins were divided into 300-μL aliquots, quickly frozen in liquid N_2, and stored at −70 °C until use. No apparent aggregation was detected either before freezing or after thawing. Each thawed PDEγ aliquot was used for 1–2 days and then discarded.

Site-Directed Mutagenesis. Site-directed mutagenesis of the tryptophan (TGG) at position 70 to phenylalanine (TTT) was performed with single-stranded M13∼PDEγ preparations, following the method of Frech et al. (1990). The mutagenesis oligonucleotide was

5′-pATCTGCCCATTTGAGGCTTCA-3′

The mutant M13 plaques were identified by hybridization with the ^{32}P-labeled mutagenesis oligonucleotide. The sequence was verified, and the double-stranded replicative form of M13 was prepared. The mutated *Xho*I–*Sac*I restriction fragment of PDEγ was put in place of the corresponding fragment in the pT7∼PDEγ expression construct.

Extraction of Proteins from Rod Outer Segments. TαGTPγS and PDE were extracted from illuminated ROS membranes according to their sensitivity to light and ionic strength (Kühn, 1980). Illuminated ROS were washed sequentially with medium ionic strength buffer M (20 mM Tris, 120 mM KCl, and 100 μM $MgCl_2$), low ionic strength buffer L (5 mM Tris and 100 μM $MgCl_2$), and buffer M supplemented with 100 μM GTP or GTPγS. At each step some proteins were eluted preferentially: isotonic supernatants contained soluble proteins, hypotonic supernatants contained PDE, and isotonic supernatants with GTP or GTPγS contained Tα in the GDP- or GTPγS-bound form, respectively. Using this procedure, extraction of ROS membranes at 100 μM rhodopsin yielded about 1 μM PDE and 5 μM Tα with >80% purity. Tα was then purified on a polyanion SI column

(Pharmacia-LKB, Uppsala, Sweden) with a 0.66 M Na_2SO_4 linear gradient as previously described (Deterre et al., 1986).

Protein Assay. Protein concentrations were determined with a standard Bradford assay (Pierce, Rockord, IL). This assay depends somewhat on the amino acid composition, which may lead here to an overestimate of the concentration of PDEγ over that of PDE$\alpha\beta$ or Tα. On the basis of the exact compositions of these proteins, we calculated that PDEγ concentrations are overestimated by a factor of 1.2 with respect to those of PDE$\alpha\beta$ and a factor of 1.3 with respect to those of Tα. This latter correction factor agrees with the results of the fitting performed on the PDEγ–Tα binding data (Figure 5A,B), where stoichiometric binding is observed for an uncorrected ratio of 0.75 PDEγ per Tα. This suggests an overestimation for the PDEγ concentration by a factor of 1.33 over that of Tα in the uncorrected Bradford assay.

Polyacrylamide Gel Electrophoresis. SDS–Polyacrylamide gel electrophoresis followed the method of Laemmli (1970). The resolving gel contained 15 or 17% polyacrylamide. The gels were stained with Coomassie blue or silver. When needed, densitometry of the Coomassie-stained bands was performed with a gel scanner (Model GS300, Hoeffer Scientific, San Fransisco, CA). Formation of soluble PDEγ–TαGDP upon addition of PDEγ to unilluminated ROS was assayed biochemically by sedimenting the membranes in buffer M and titrating the eluted Tα by densitometry of Coomassie-stained SDS gels.

Gel Filtration. Gel filtration of holo-transducin, wild type PDEγ, or a mixture of both was performed on a Superose 12HR 16/30 column (Pharmacia-LKB, Uppsala, Sweden). Typically, 0.2-mL samples containing 240 pmol of T$\beta\gamma$–TαGDP and/or 1.92 nmol of wild-type PDEγ were loaded onto the column, and elution was performed at 0.4 mL/min at room temperature. The elution buffer contained 20 mM Tris, pH 7.5, 120 mM NaCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 100 μM PMSF, and 0.1% Lubrol PX. One-half-milliliter fractions were collected for analysis on SDS gels.

Reconstitution of Holo-PDE with Recombinant PDEγ. Wensel and Stryer (1990) showed that in illuminated ROS membranes the replacement of endogenous PDEγ in the native holo-PDE by fluorescein-labeled PDEγ was accelerated in the presence of GTP, indicating that activated transducin enhanced the dissociation of endogenous PDEγ from PDE$\alpha\beta$. We used this mass-action technique to substitute endogenous PDEγ with recombinant PDEγ (wild type or W70F mutant). Illuminated ROS membranes (25 μM rhodopsin) were washed twice with buffer M and incubated for 10 min at room temperature with 1.5 μM recombinant PDEγ in buffer M supplemented with 200 μM GTP. After centrifugation, the pellet was washed twice with buffer M to remove the excess recombinant PDEγ and the displaced native PDEγ. The thus substituted holo-PDE was extracted from the ROS membranes by washing with low ionic strength buffer L.

Activation of PDE by Trypsin Digestion, Incorporation of Holo-PDE into Phospholipid Vesicles, and Assay of cGMP Hydrolysis Activity. Extracts of holo-PDE ($\sim$1 μM) were proteolyzed with 50 μg/mL TPCK-treated trypsin (Sigma, St. Louis, MO) for 5 min at 25 °C. The reaction was stopped with 500 μg/mL lima bean trypsin inhibitor (Sigma). The PDE$\alpha\beta$ thus activated was purified as described by Deterre et al. (1988).

Large unilamellar vesicles were prepared according to Szoda and Papahadjopoulos (1978). Ten milligrams of azolectin was dissolved in 3 mL of diethyl ether; 500 μL of buffer A (10 mM HEPES, pH 7.5, 120 mM KCl, and 2 mM $MgCl_2$) was added, and the mixture was sonicated 1–2 min at 0 °C in a bath type sonicator. The solvent was removed with a rotatory evaporator, and the resulting aqueous suspension of vesicles was centrifuged and resuspended at 1 mg/mL in buffer A. PDE was incorporated by a 15-min incubation at 25 °C in this vesicle suspension.

The phosphodiesterase activity was assayed with the pH-metric technique (Liebman & Evanczuk, 1982) using a pH microelectrode (Model U-402-M3-S7, Ingold Messtechnik GmbH, Steinbach/Ts, Germany). The initial concentration of cGMP added was 1 mM. The 500-μL sample was thermostated at 25 °C and vigorously stirred.

Fluorescence Measurements. Intrinsic fluorescence of proteins was measured with a scanning fluorimeter (Model RF-5000, Shimadzu Corp., Kyoto, Japan) using a 1 × 1-cm quartz cuvette. The sample was vigorously stirred and thermostated at 25 °C. The buffer contained 20 mM Tris, pH 7.5, 120 mM KCl, 2 mM $MgCl_2$, and 1 mM DTT. For emission spectra, the excitation was at 292 nm and the emission scan speed was 0.8 nm/s; the bandwidth was 5 nm for both excitation and emission. Fluorescence contribution from the buffer was substracted. Time-scan recordings were performed with excitation at 292 nm (5-nm bandwidth); the emission wavelength was either 320 or 357 nm, with a bandwidth of 30 nm.

Fluorescence Determination of the Tα–PDEγ Dissociation Constant. Stepwise additions of PDEγ (25 nM per step) were made to solutions containing 100 nM purified Tα while the fluorescence was continuously recorded. As a control, the same experiments were performed in the absence of transducin. Assuming a simple association model between the two proteins, we have the equilibrium

$$T\alpha + PDE\gamma \Leftrightarrow T\alpha\text{–}PDE\gamma$$

Tα refers to TαGTPγS or TαGDP, and PDEγ refers to the wild type or the W70F mutant. We want to find the dissociation constant, K_d, for this equilibrium: $K_d = [T\alpha][PDE\gamma]/[T\alpha\text{–}PDE\gamma]$. The concentration [T$\alpha$–PDE$\gamma$] as a function of x, the total amount of PDEγ added, is

$$[T\alpha PDE\gamma] = \frac{(x + [T\alpha]_t + K_d) - [(x + [T\alpha]_t + K_d)^2 - 4[T\alpha]_t x]^{1/2}}{2} \quad (1)$$

where $[T\alpha]_t$ refers to the total concentration of Tα. If f_T, f_P, and f_{TP} are the molar fluorescences of Tα, PDEγ, and Tα–PDEγ, respectively, and if the fluorescence level of Tα before any PDEγ addition is taken as 0, the signal F after the nth addition of PDEγ is

$$F = \Delta f[T\alpha\text{–}PDE\gamma] + nf_P\Delta x$$

where $\Delta f = f_{TP} - (f_T + f_P)$, and Δx is the increment of PDEγ added (i.e., 25 nM in our case). $nf_P\Delta x$ is simply the fluorescence due to PDEγ if it was alone by itself and is subtracted using recordings from the control experiment. This gives the corrected fluorescence, F_c, as

$$F_c = \Delta f\frac{(x + [T\alpha]_t + K_d) - [(x + [T\alpha]_t + K_d)^2 - 4[T\alpha]_t x]^{1/2}}{2} \quad (2)$$

A nonlinear least-squares fit of eq 2 (Bevington, 1969) yields values for the proportionality constant, Δf, and, more im-

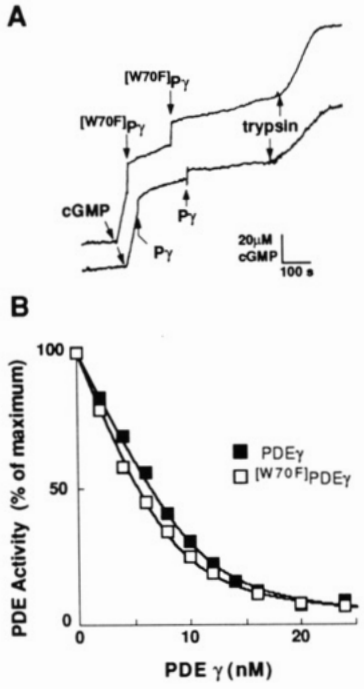

FIGURE 1: Inhibition of trypsin-activated PDE by wild-type or W70F recombinant PDEγ subunits. (A) pH-metric recording of PDE activity from illuminated ROS (1 μM rhodopsin) in the presence of 0.8 μM GTPγS. The activity became detectable upon addition of 0.12 mM cGMP and was inhibited upon injections of 80 nM wild-type PDEγ (lower trace) or [W70F]PDEγ (upper trace). Trypsin (4 μg/mL) was added at the end of the experiments to reactivate the PDE. (B) Inhibition of trypsin-activated PDE (~4 nM) in solution by increasing concentrations of wild-type PDEγ (■) or [W70F]PDEγ (□). The protein concentrations were estimated from Bradford assays. The fits use the following parameters: concentrations of PDE catalytic sites of 11 and 8.2 nM for the inhibition by wild-type PDEγ and [W70F]PDEγ, respectively; K_i = 1 nM.

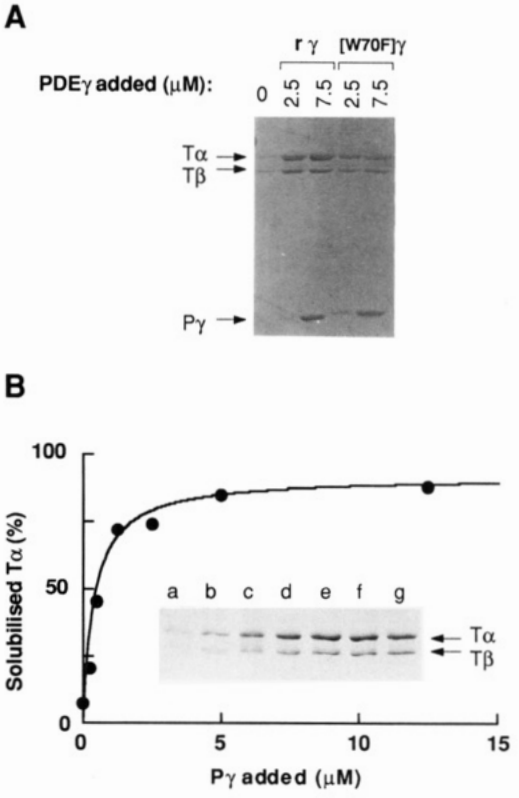

FIGURE 2: Solubilization of TαGDP by addition of PDEγ to dark ROS membranes. (A) SDS gels of supernatants from dark-adapted ROS membrane suspensions (25 μM rhodopsin, ~2.5 μM transducin) that had been centrifuged after a 10-min incubation in buffer M with wild-type PDEγ or [W70F]PDE, as indicated. The pellets were illuminated and resuspended in buffer M with 50 μM GTPγS to solubilize the remaining transducin and centrifuged, and the supernatants were analyzed by using SDS gels (not shown). Addition of 2.5 μM wild-type PDEγ solubilizes nearly all the TαGDP pool, while the same addition of [W70F]PDEγ solubilizes only a small fraction. (B) Fraction of Tα solubilized, measured by scanning the stained gels shown in the inset, as a function of the concentration of added PDEγ. Dark ROS membranes (25 μM rhodopsin) were incubated with 0 (a), 0.25 (b), 0.5 (c), 1.25 (d), 2.5 (e), 5 (f), or 12.5 μM wild type PDEγ (g).

portantly, for the dissociation constant, K_d which is a measure of the affinity between PDEγ and Tα.

RESULTS

Association between PDEγ and TαGTPγS or TαGDP. Biochemical Studies. Unlike the recombinant protein first produced by Brown and Stryer (1989), both forms of our expressed PDEγ have no fusion components but carry an additional isoleucine inserted at position 2 after the initial methionine. The tryptophan-less mutant will be called (W70F)PDEγ, while the unmodified form will be called "wild type" despite the extra isoleucine. With the conservative W70F mutation we only aimed to suppress the tryptophan fluorescence from PDEγ in order to facilitate the study of the interaction between Tα and PDEγ and did not expect to greatly alter the functional aspects of the latter. The functions of these expressed PDEγ's were first explored biochemically. Their ability to inhibit transducin-activated or trypsin-activated PDE was checked (Figure 1). Limited trypsin treatment of holo-PDE is known to selectively digest the γ-subunits without affecting the αβ dimer's catalytic function (Wensel & Stryer, 1986; Catty & Deterre, 1991). Both recombinant PDEγ's exhibit nanomolar efficiency in inhibiting cGMP hydrolysis by trypsinized holo-PDE. This value is comparable to that reported by Brown and Stryer (1989) for the inhibition of trypsin-activated PDE by native PDEγ extracted from bovine retina or by their fusion recombinant PDEγ. Given the limited precision afforded by the range of protein concentrations used here, this experiment suggests, but does not prove, that the wild type and the W70F mutant display similar affinities for PDEαβ. It is in its interaction with Tα that [W70F]PDEγ differed markedly from the wild type (see below).

When mixed in solution, wild-type PDEγ and TαGTPγS came together to form one-to-one complexes that eluted from gel-filtration columns as a single peak of apparent molecular mass ~50 kDa (data not shown). This concurs with our earlier work using native transducin and PDE (Deterre et al., 1986, 1988). Addition of wild-type PDEγ to unilluminated ROS membranes induced the elution of TαGDP, probably in association with the added PDEγ. This unexpected result suggests that free PDEγ also interacts strongly with inactive transducin. Dark-adapted ROS membranes, which contain both Tβγ–TαGDP and fully inhibited PDEαβγ$_2$, were incubated in buffer M with increasing amounts of PDEγ and sedimented. Increasing amounts of solubilized Tα were detected in the supernatants by SDS gel electrophoresis (Figure 2). The release of Tα varied linearly with the amount of PDEγ added until saturation was reached. Thus the added PDEγ did not bind to the fully quenched endogenous PDEαβγ$_2$, but interacted with the TαGDP subunit of the membrane-bound Tβγ–TαGDP and brought it into solution, most likely as PDEγ–TαGDP. In this treatment, most (~70%) of the Tβγ remained membrane bound. In our hands, PDEγ is as efficient in detaching TαGDP from dark ROS as

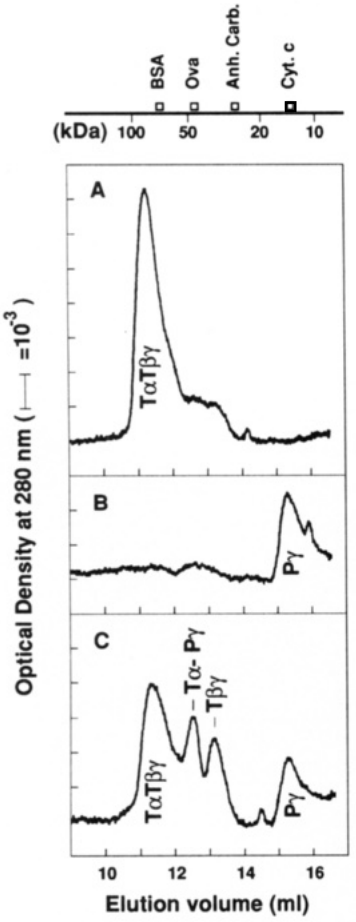

FIGURE 3: Gel-filtration assay of the binding of PDEγ to TαGDP from holotransducin. Elution profiles from a gel-filtration column of (A) 240 pmol of Tβγ–TαGDP extracted from ROS (see Materials and Methods) and (B) 1.92 nmol of wild-type PDEγ. (C) Aliquots of the two preceding samples mixed together before loading. Protein composition of the peaks was checked by using SDS gels. In (C), peaks corresponding to PDEγ–TαGDP and to Tβγ were formed at the expense of the two original components, Tβγ–TαGDP and PDEγ.

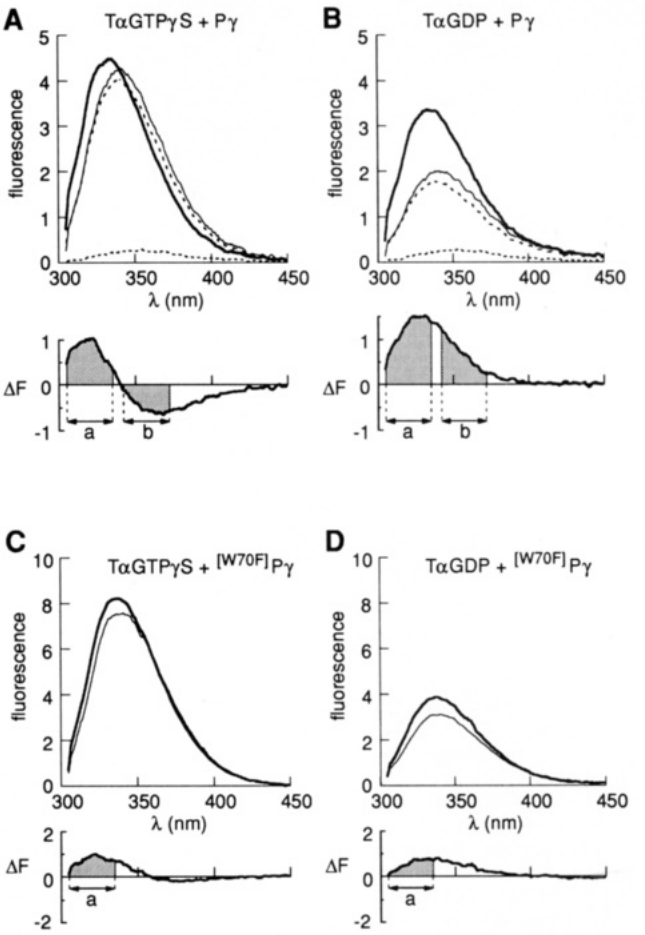

FIGURE 4: Fluorescence emission spectra of various Tα–PDEγ complexes. (A) TαGTPγS + wild-type PDEγ. (B) TαGDP + wild-type PDEγ. (C) TαGTPγS + [W70F]PDEγ. (D) TαGDP + [W70F]PDEγ. In each case, the fluorescence spectrum of a solution containing equimolar amounts of Tα and PDEγ (thick trace) is compared to the summed fluorescence spectra (thin trace) of the corresponding PDEγ and Tα solutions measured separately (dotted traces). The lower curve in each panel corresponds to the difference between the spectrum of the mixture and the sum of the individual spectra. The vertical dotted lines and the stippled areas delimit the emission bandwidths chosen for time-scan measurements (a: 320 ± 15 nm; b: 357 ± 15 nm). Tα and PDEγ concentrations were 100 nM in (A) and (B) and 200 nM in (C) and (D). The fluorescence of [W70F]PDEγ was undectectable in (C) and (D).

is GTPγS in solubilizing Tα from illuminated ROS (data not shown). We have also produced a fusion form of PDEγ that is identical to that of Brown and Stryer (1989); this fusion PDEγ extracted TαGDP from holo-transducin as efficiently as the wild-type, non-fusion form (data not shown).

Formation of the PDEγ–TαGDP complex from wild-type PDEγ and holo-transducin was revealed by gel filtration. When Tβγ–TαGDP and wild-type PDEγ were run separately on a Superose column, they eluted as single peaks at volumes corresponding to molecular masses of 85 and 12 kDa, respectively (Figure 3). When aliquots of the same protein solutions were mixed together before being loaded onto the same column, the 85- and 12-kDa peaks decreased, and two additional peaks appeared at elution volumes corresponding to molecular masses of 50 and 40 kDa. The 50-kDa peak was shown on SDS gels to contain both Tα and PDEγ, while the 40-kDa peak contained only Tβγ. Complexes of PDEγ–TαGDP that were stable enough to survive elution through the gel-filtration column had indeed formed.

Interaction between Tα and Wild-Type PDEγ. Determination of Affinities and Kinetics by Fluorescence Measurements. Tα contains two tryptophan residues, one of which (W207) is near the nucleotide site. From mutational studies (E. Faurobert et al., personal communication) W207 appears to be solely responsible for the drastic fluorescence change observed when soluble Tα switches from the GDP to the GTP conformation. The single tryptophan (W70) of PDEγ is only weakly fluorescent when the protein is all by itself. The fluorescence emission spectra of the mixtures PDEγ + TαGDP and PDEγ + TαGTPγS differ in amplitude and shape from the summed emission spectra of the two individual components (Figure 4). This confirms the existence of PDEγ–Tα complexes and provides a convenient means to determine the kinetics and affinities of the interaction between PDEγ and TαGDP or TαGTPγS. Association of PDEγ with TαGDP induces nearly a doubling of the fluorescence yield without much spectral shift. In contrast, the association with TαGTPγS induces mostly a blue shift of the emission spectrum, without much amplitude increase. Fluorescence changes resulting from interactions between [W70F]PDEγ and Tα, though less pronounced, are still detectable. We have chosen two emission windows to study the interactions between PDEγ and Tα: 320 ± 15 and 357 ± 15 nm. When TαGTPγS binds to wild-type PDEγ (Figure 4A), the former window results in a fluorescence increase, while the latter gives a decrease.

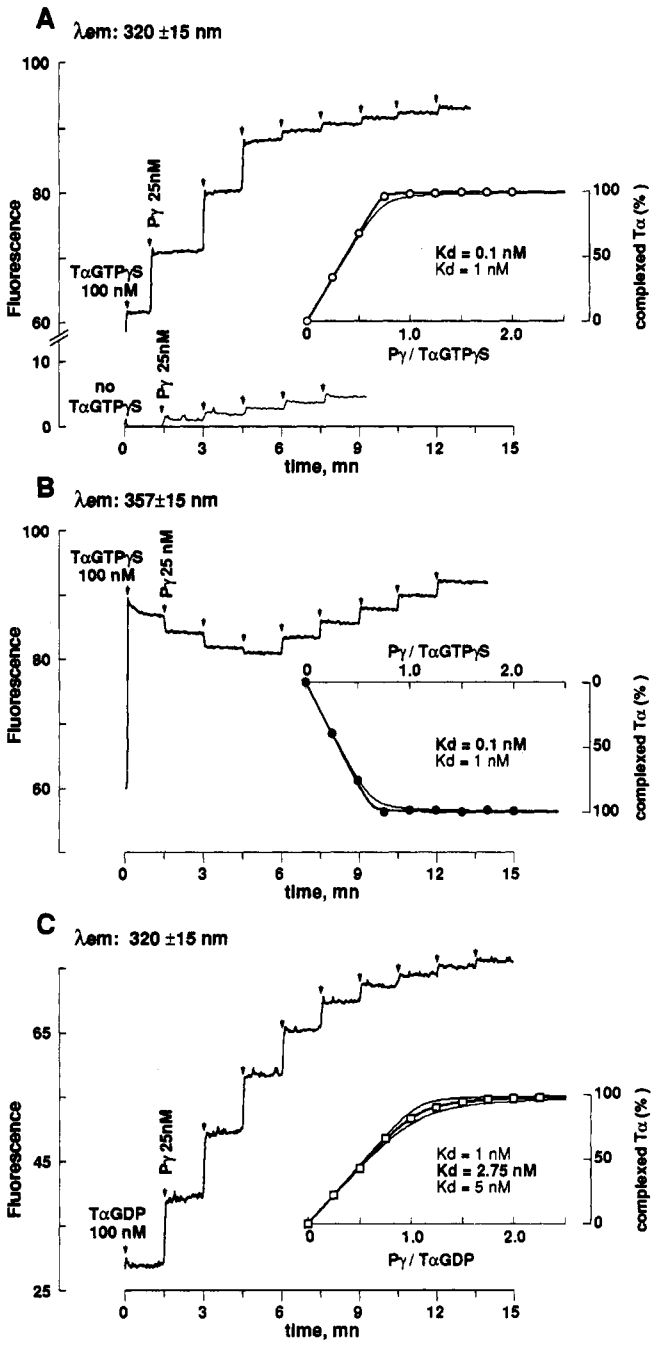

FIGURE 5: Determination of the affinity of PDEγ for TαGTPγS and TαGDP. Eight to ten 25 nM aliquots of PDEγ were sequentially injected into solutions containing 100 nM TαGTPγS (A, B) or 100 nM TαGDP (C). The increases or decreases in fluorescence emission intensity were monitored at 320 ± 15 nm (A, C) or at 357 ± 15 nm (B). The insets represent the molar fractions of Tα complexed to PDEγ as functions of the [PDEγ] / [Tα] ratios. These were computed from the fluorescence changes induced by the formation of Tα–PDEγ (see Materials and Methods). The best fits to the data are drawn in thick lines, and the corresponding K_d values are shown in bold type.

Fluorescence intensity changes were recorded as successive PDEγ aliquots (25 nM) were added to a solution containing 100 nM TαGTPγS or 100 nM TαGDP. The data were analyzed as described in Materials and Methods. The TαGTPγS–PDEγ association was monitored at two emission windows as shown in panels A and B of Figure 5. With the 320 ± 15-nm window, the fluorescence increase that occurs on the first addition of PDEγ is much larger than when the same concentration of PDEγ is injected into buffer containing no Tα. When the total concentration of PDEγ exceeds that

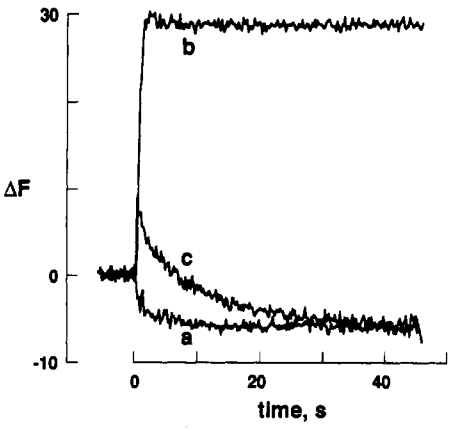

FIGURE 6: Competition between TαGDP and TαGTPγS for the binding to wild-type PDEγ. Fluorescence changes monitored at 357 ± 15 nm upon addition of 100 nM wild-type PDEγ to a solution containing 100 nM TαGTPγS (a), 100 nM TαGDP (b), or 100 nM TαGDP and 100 nM TαGTPγS (c). The amplitude of trace c's fast rise is about half of the bracket defined by traces a and b. This suggests that the added PDEγ splits about evenly between TαGDP and TαGTPγS. The slow decay of trace c toward trace a monitors the progressive substitution of TαGDP by TαGTPγS in the Tα–PDEγ complexes.

of Tα in the final injections, the fluorescence jumps become more comparable to those due to free PDEγ alone. With the 357 ± 15-nm emission window, the early injections of PDEγ into TαGTPγS induce decreases in fluorescence intensity, as predicted by the spectral changes described in Figure 4A. With this emission window, going past the equivalence point produces a more dramatic effect as the "staircase" switches from climbing down to climbing up (Figure 5B). Again, the fluorescence increments seen once [PDEγ] exceeds [TαGTPγS] are reminiscent of those due to free PDEγ alone. Although the raw recordings look very dissimilar at these two emission windows, the processed data, once corrected for the fluorescence of PDEγ alone, yield identical binding curves. This demonstrates the reliability of the technique and the analysis. The affinity of TαGTPγS for PDEγ is indeed too high to be accurately determined by this technique, but an upper limit of 0.1 nM for K_d is estimated. For the association of TαGDP with PDEγ, the lower affinity is already visible on the raw fluorescence recording, and the fit yields a best estimate of 2.75 nM for K_d.

The rapidity of the fluorescence changes observed upon addition of PDEγ means that in all cases the on rates for the Tα–PDEγ associations are too high to be measured by this simple technique. But the high affinities observed imply that the off rates should be low enough to be measurable. Thus, competition experiments were done to see if the large differences in affinities of PDEγ for the two forms of Tα arise from differences in k_{on} or in k_{off}. In the first competition experiment (Figure 6) the fluorescence was monitored at 357 ± 15 nm as PDEγ was added to an equimolar mixture of TαGDP and TαGTPγS. At this emission wavelength, binding of PDEγ to TαGDP gives a fluorescence increase, while the binding to TαGTPγS gives a decrease (Figure 4). This is illustrated by traces a and b, which were obtained by adding 100 nM PDEγ to 100 nM TαGTPγS or 100 nM TαGDP, respectively. In trace c, 100 nM PDEγ was added to an equimolar mixture of TαGDP and TαGTPγS. The fluorescence signal is biphasic: The quasi-instantaneous jump corresponds to a fast but temporary allocation of the added PDEγ to TαGDP and TαGTPγS according to the ratio of the respective on rates. Then the TαGDP that has been bound to PDEγ dissociates and is progressively replaced by the excess

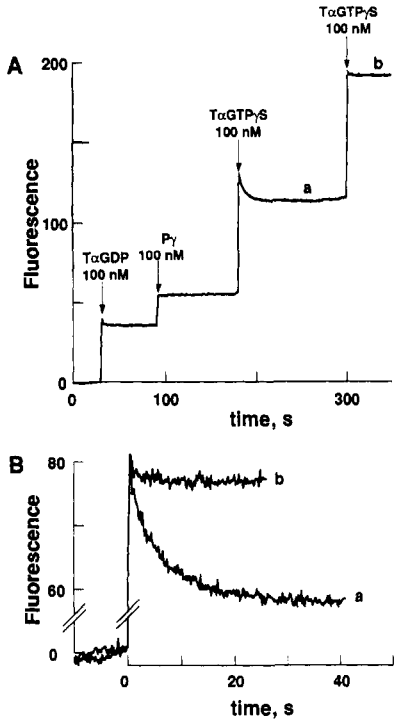

FIGURE 7: Dissociation kinetics of wild-type PDEγ from PDEγ–TαGDP. (A) A solution containing 100 nM TαGDP, whose fluorescence was monitored at 357 ± 15 nm, was supplemented successively with 100 nM PDEγ and 100 nM TαGTPγS. Upon the latter injection, a biphasic trace is observed: an initial jump whose amplitude corresponds to the fluorescence of the free TαGTPγS followed by a fluorescence decrease that monitors the dissociation of TαGDP from PDEγ and its replacement by TαGTPγS. A second addition of TαGTPγS induces a second jump of the same amplitude, but no decay follows, indicating that the exchange is complete after the first TαGTPγS injection. (B) The two parts of the trace that correspond to the first (a) and the second injection (b) of TαGTPγS are superimposed. The decay of trace a monitors the dissociation of PDEγ from TαGDP with a time constant of about 3 s.

free TαGTPγS. This replacement results in a final fluorescence level that is equal to that of pure PDEγ–TαGTPγS like in trace a. Comparison of the fluorescence level reached at the peak of the instantaneous phase with the fluorescence levels due to PDEγ–TαGDP and PDEγ–TαGTPγS alone yields an estimate for the ratio of the on rates: $k_{on(T\alpha GTP\gamma S)}/k_{on(T\alpha GDP)} \approx 1.5$. Thus the association kinetics are very similar. The slow decay of trace c toward trace a is rate limited by the dissociation of PDEγ–TαGDP. From the kinetics of this slow phase, the rate of dissociation of PDEγ–TαGDP can be estimated at 0.3 ± 0.05 s^{-1}.

In the second competition experiment (Figure 7), 100 nM TαGDP was first fully complexed with a stoichiometric amount of PDEγ. Then, injection of 100 nM TαGTPγS again gave a biphasic trace: an initial fluorescence jump whose amplitude corresponds to the fluorescence of 100 nM unbound TαGTPγS followed by a fluorescence decrease as TαGTPγS displaces TαGDP from the original PDEγ–TαGDP complex. This displacement is practically complete, as the fast jump induced by a second addition of TαGTPγS is of the same amplitude as before but is not followed by any decay. Thus, the affinity of PDEγ for TαGTPγS is indeed at least an order of magnitude higher than for TαGDP. From the measured values of K_d and k_{off}, one deduces that $k_{on} = 3 \times 10^8$ and 4.5×10^8 M^{-1} s^{-1} for the binding of PDEγ to TαGDP and TαGTPγS, respectively, while $k_{off} \leq 4.5 \times 10^{-2}$ s^{-1} for the dissociation of PDEγ from TαGTPγS. The on rates are close to being diffusion controlled and the off rates correspond to slow

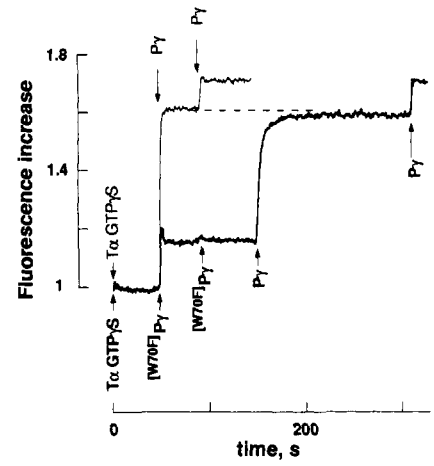

FIGURE 8: Fluorescence assay of the competition between the mutant [W70F]PDEγ and wild-type PDEγ for the binding to TαGTPγS. The two traces monitor the fluorescence at 320 ± 15 nm of samples containing 100 nM TαGTPγS and supplemented with two successive injections of 175 nM wild-type PDEγ (control sample, thin trace) or two injections of 600 nM [W70F]PDEγ, followed by two injections of 175 nM wild-type PDEγ (test sample, thick trace). For the test sample, total replacement of the mutant PDEγ bound to TαGTPγS by wild-type PDEγ is seen upon the first injection of wild-type PDEγ.

dissociations with time constants of more than 25 s for PDEγ–TαGTPγS and 3 s for PDEγ–TαGDP.

Interaction between Tα and [W70F]PDEγ. As expected, the [W70F]PDEγ mutant shows no tryptophan fluorescence of its own, but it does perturb that of Tα when the two proteins interact with each other. Parts C and D of Figure 4 show the changes in the fluorescence spectra of TαGTPγS and TαGDP induced by stoichiometric amounts of [W70F]PDEγ. These spectral changes are smaller than their counterparts of Figure 4A,B with wild-type PDEγ, but they are sufficient to provide monitors for the interactions of [W70F]PDEγ with Tα. Using the procedure described in Figure 5, the affinities of [W70F]PDEγ for TαGTPγS and TαGDP were determined to be on the order of 10 and 300 nM, respectively (data not shown). The affinity of PDEγ for Tα is thus lowered by 2 orders of magnitude as tryptophan 70 of PDEγ is conservatively replaced by phenylalanine, indicating that this residue is directly involved in the interaction. Competition experiments between wild type and [W70F]PDEγ for TαGTPγS are illustrated in Figure 8. The two fluorescence recordings at 320 ± 15 nm are from a test and a control sample, both containing 100 nM TαGTPγS. One hundred seventy-five nanomolar wild-type PDEγ and 600 nM [W70F]PDEγ are added to the control (thin trace) and test (thick trace) samples, respectively. The resulting fluorescence increases are larger than if there were only free PDEγ alone, indicating that Tα–PDEγ interaction has taken place. On the other hand, when the same additions are repeated a second time, the fluorescence jumps are either much smaller or nonexistent. This verifies that binding of both forms of PDEγ to TαGTPγS is already saturated on the first additions. One hundred seventy-five nanomolar wild-type PDEγ is then added to the test sample, which has so far received only [W70F]PDEγ (thick trace, third injection). The resulting large fluorescence increase attains the level of the control sample's first plateau, which is due only to addition of wild-type PDEγ to TαGTPγS. Since free [W70F]PDEγ is hardly fluorescent, this large increase from the test sample monitors the complete displacement of [W70F]PDEγ from TαGTPγS by wild-type PDEγ. Its rise time is limited by the dissociation of [W70F]PDEγ from TαGTPγS and provides a lower limit of 0.2 ± 0.05 s^{-1} for the

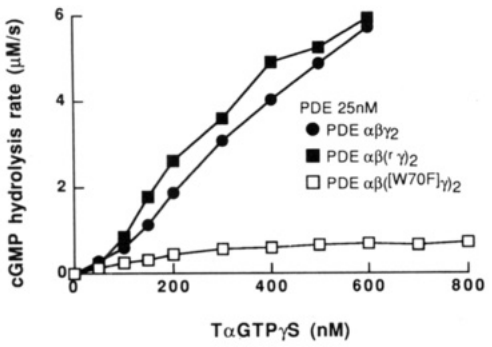

FIGURE 9: Activation by TαGTPγS of native PDEαβγ2 (●) extracted from ROS membranes and of holo-PDE's that had been reconstituted (see Materials and Methods) with wild-type PDEγ (■) or with [W70F]PDEγ (□) before their extraction from ROS membranes. All three PDE samples (25 nM per sample) were incubated with phospholipid vesicles (1 mg/mL), and their activity was assayed by the pH-metric method. When reconstiuted with the mutant [W70F]PDEγ, holo-PDE can no longer be activated by TαGTPγS.

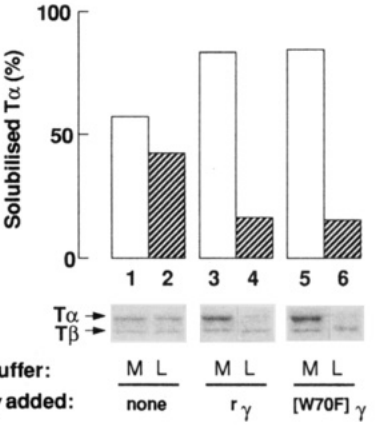

FIGURE 10: Release of PDEγ–TαGTPγS induced by addition of wild-type PDEγ or of [W70F]PDEγ to transducin-activated PDE. Native PDEαβγ2 (350 nM) and TαGTPγS (720 nM) extracted from ROS membranes were incubated with illuminated and stripped ROS membranes (25 μM R*) in buffer M. Excess PDE and Tα were removed by centrifugation. The pellet was resuspended in the same volume of buffer M, incubated with 840 nM wild-type PDEγ, [W70F]PDEγ, or nothing for control, and centrifuged. The supernatant (M) contained the Tα that had been solubilized upon addition of PDEγ. A second washing of the pellet in buffer L extracted the remaining membrane-bound tranducin (L). The supernatants were analyzed by using SDS gels, and the proportions of Tα solubilized in M and L were estimated by densitometry of the Coomassie-stained bands. Tα was released to the same extent upon addition of wild-type PDEγ or [W70F]PDEγ.

corresponding off rate. A last addition of wild-type PDEγ to both test and control samples now gives identical fluorescence increases which are characteristic of free PDEγ. That TαGTPγS binds nearly exclusively to wild-type PDEγ in the presence of a 7-fold excess of [W70F]PDEγ confirms that the mutation has lowered the affinity of PDEγ for TαGTPγS by a factor of at least 50. Similar experiments carried out with TαGDP verify that it, too, binds much less tightly to [W70F]PDEγ than to wild-type PDEγ. This also accounts for the observation that the mutant is less efficient than the wild-type PDEγ in solubilizing TαGDP from dark ROS membranes (Figure 2).

TαGTPγS Does Not Activate Holo-PDE Reconstituted with [W70F]PDEγ. The W70F mutation drastically reduces the affinity of PDEγ for Tα but does not measurably affect either its affinity for the catalytic complex PDEαβ or its inhibitory action. The obvious and interesting question is then: Can transducin activate a membrane-bound holo-PDE in which the two endogenous γ-subunits have been replaced by two [W70F]PDEγ's? Since trypsin does not only digest the γ-subunits but also detaches PDEαβ from the membrane (Wensel & Stryer, 1986; Catty & Deterre, 1991), it cannot be used to eliminate the endogenous PDEγ. Instead, illuminated ROS membranes were incubated in the presence of GTP with an excess of wild type or [W70F]PDEγ. This treatment facilitates the replacement of the minority, native PDEγ by the excess, exogenous PDEγ probably because interaction of PDEγ with TαGTP enhances its dissociation from PDEαβ (Wensel & Stryer, 1990). After washing the membranes in medium salt buffer M to remove the excess free PDEγ, reconstituted holo-PDE was extracted from the ROS membranes by washing in low salt buffer L and reconstituted on large azolectin vesicles as described in Materials & Methods. For control, the incubation step with GTP and recombinant PDEγ was omitted. Figure 9 shows the activation by increasing amounts of TαGTPγS of holo-PDE reconstituted with either wild type or mutant PDEγ as compared to that of native PDEαβγ2. Holo-PDE reconstituted with wild type PDEγ shows the same sensitivity toward TαGTPγS as untreated PDEαβγ2. But activation by purified TαGTPγS practically disappeared when [W70F]PDEγ was used for the reconstitution. Thus TαGTPγS cannot displace the mutant PDEγ subunit from its inhibitory site on PDEαβ.

Effects of Wild-Type PDEγ and [W70F]PDEγ on Transducin-Activated PDE. Let us recall that wild-type PDEγ as well as [W70F]PDEγ efficiently inhibited the phosphodiesterase activity from illuminated ROS where the endogenous PDE had been turned on by transducin loaded with GTPγS. PDE activity was monitored by the pH-metric technique, and reinhibition by added PDEγ was essentially instantaneous on the time scale of our measurement (Figure 1). Already reported by Wensel and Stryer (1990), this reinhibition effect indicates that, upon stimulation by GTPγS-activated transducin, the inhibitory sites on PDEαβ become accessible to the added PDEγ. It was thus argued that TαGTPγS physically removes endogenous PDEγ from PDEαβ. However, recent reports (Clerc & Bennett, 1992; Catty et al., 1992) suggest that no such physical separation occurs, but that PDEγ–TαGTPγS and PDEαβ remain as a single membrane-bound complex. This model does not contradict the reinhibition experiments with exogenous PDEγ since the endogenous PDEγ does not have to leave PDEαβ in order to make the inhibitory sites on the latter available. Here we seek to verify this revised interpretation and to answer a more detailed question: Can exogenous PDEγ reinhibit the active PDE complex without displacing the resident PDEγ–TαGTPγS?

ROS membranes depleted of endogenous transducin and PDE and reconstituted with purified holo-PDE in buffer M were supplemented with an excess of TαGTPγS, whose unbound fraction was removed by centrifugation. The fraction of TαGTPγS that stayed with the ROS pellet corresponded to about twice the molar amount of membrane-bound PDE. This confirms the results of Clerc and Bennett (1992) and Catty et al. (1992). Then, addition of wild-type or mutant PDEγ induced the solubilization of this remnant TαGTPγS, probably as a complex with the native PDEγ. Thus, once the inhibitory site left vacant on PDEαβ is reoccupied by exogenous PDEγ, there is detachment of the activating transducin and concomitant reinhibition of PDEαβ. Compared to the wild type, [W70F]PDEγ has a similar affinity for PDEαβ but a much weaker one for TαGTPγS. Yet both PDEγ's are equally efficient in releasing PDEγ–TαGTPγS from ROS membranes and, presumably, from PDEαβ (Figure 10). As its affinity for transducin is much weaker than that of native PDEγ,

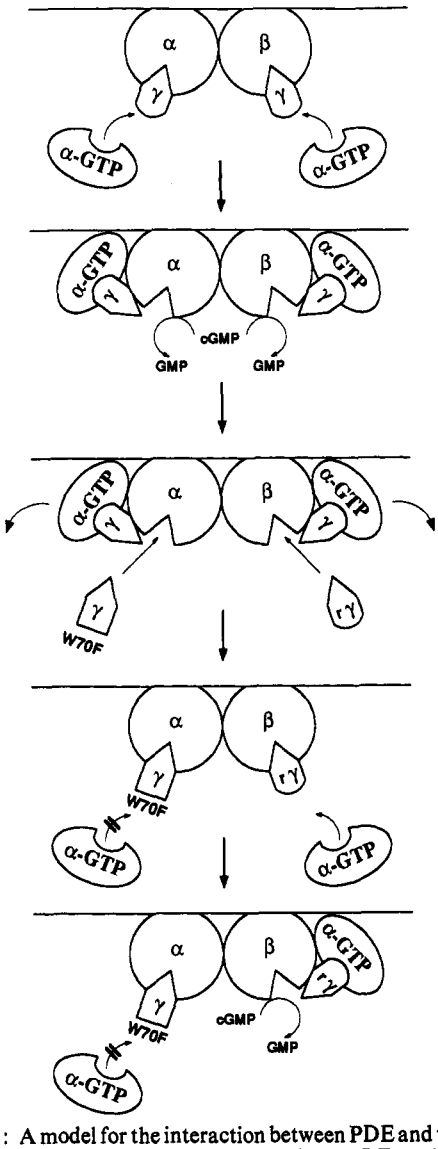

FIGURE 11: A model for the interaction between PDE and transducin. TαGTP activates PDE by displacing inhibitory PDEγ subunits from inhibitory sites on the PDEαβ subunit but does not physically carry PDEγ away. These inhibitory sites can be close to or coincident with the catalytic sites. The uncovered inhibitory sites become accessible to any added PDEγ. In this way, both wild type and mutant [W70F]PDEγ can reinhibit transducin-activated PDE, and both induce the release of the PDEγ–TαGTP complex, which has hitherto stayed bound close to the vacant inhibitory sites. When quenched by wild-type PDEγ, PDE can still be activated by TαGTP, but inhibition by [W70F]PDEγ is irreversible given the very low affinity of the mutant PDEγ for TαGTP.

[W70F]PDEγ could not have interacted directly with the already partnered TαGTPγS to effect its detachment but must have acted via PDEαβ, most likely by binding to the inhibitory site left vacant by its native counterpart. This observation further supports the notion that, *in vivo*, active PDE exists as a large PDEαβ(PDEγ–TαGTP)$_2$ complex (Figure 11).

DISCUSSION

Regulation of the cGMP phosphodiesterase activity by transducin is mediated through two sets of protein–protein contacts: the PDEγ-to-PDEαβ contact which inhibits the PDE catalytic activity and the Tα-to-PDEγ contact which enables it, provided Tα carries a GTP or an analog. Activation cannot result from a sequential occurrence of these two bimolecular interactions. Given the very high affinity involved, Tα cannot wait for PDEγ to spontaneously dissociate from PDEαβ. In order to achieve rapid activation, Tα must bind to PDEγ while the latter is still associated with an inhibitory site on PDEαβ. Once the activatory TαGTP-to-PDEγ contact is established, the inhibitory contact between PDEγ and PDEαβ is severed, and one might assume that no interaction would subsist between the PDEγ–TαGTP complex and the active PDEαβ, at least until Tα hydrolyzes its GTP. This was sugggested by the observation that, *in vitro*, PDEγ–TαGTPγS is easily dissociated from active PDEαβ when the ionic strength of the buffer is lowered. However, these *in vitro* experiments did not prove that, under more physiological conditions, such segregation also occurs on the membrane. Indeed, recent evidence indicates that the active complex is in fact a membrane-bound PDEαβ(γTα)$_2$. Regarding the exact nature of the inhibitory site, the simplest way for PDEγ to quench PDEαβ is to bind at or near the catalytic site and to cover it up. That is, inhibitory and catalytic sites might be almost the same. The work reported here does not address this issue, and we have assumed a putative, distinct inhibitory site on PDEαβ to which PDEγ binds and exerts its control of the catalytic site.

Using recombinant PDEγ, we have confirmed that it forms a very tight PDEγ–TαGTPγS complex in solution. On the other hand, TαGTPγS, when added to membrane-bound inactive PDEαβγ$_2$, binds to the endogenous PDEγ and does not dissociate it but stays with it in an active PDEαβ(γTα)$_2$ complex. Addition of excess recombinant PDEγ will induce the release in solution of TαGTPγS together with the endogenous PDEγ. This result is best accounted for by the following reaction scheme:

$$\{PDE\alpha\beta(PDE\gamma\text{–}T\alpha GTP\gamma S)_2\}_{mb} + 2(rPDE\gamma)_{sol} \rightarrow \{PDE\alpha\beta(rPDE\gamma)_2\}_{mb} + 2(PDE\gamma\text{–}T\alpha GTP\gamma S)_{sol}$$

The mb and sol indices refer to membrane-bound and soluble species, respectively, while rPDEγ denotes recombinant PDEγ. Hence, under physiological conditions, both TαGTP and PDEγ interact with PDEαβ in the active PDE complex.

The unexpected observation is that addition of excess recombinant PDEγ to dark ROS membranes induces the release of TαGDP from the inactive holo-transducin:

$$(rPDE\gamma)_{sol} + (T\beta\gamma T\alpha GDP)_{mb} + (PDE\alpha\beta\gamma_2)_{mb} \rightarrow (rPDE\gamma\text{–}T\alpha GDP)_{sol} + (T\beta\gamma)_{mb} + (PDE\alpha\beta\gamma_2)_{mb}$$

The affinity of PDEγ for TαGDP is substantial at 3 nM, as measured by our fluorescence technique. This surprisingly high affinity does not imply that excess free TαGDP might activate PDE, given the much higher affinity (10^{-11} M) of PDEγ for PDEαβ. The affinity of TαGDP for Tβγ must be weaker than 3 nM since PDEγ can efficiently cause the dissociation of Tβγ–TαGDP. The soluble PDEγ–TαGDP complex falls apart in 3 s. Such slow dissociation provides an additional argument against the *in vivo* existence of a Tα–PDEγ complex that is physically separated from PDEαβ. Under physiological conditions, PDE is quenched well within 1 s of the GTP hydrolysis by the activatory TαGTP (Vuong & Chabre, 1991). Thus, PDEγ must be let go rapidly by TαGDP in order to reinhibit PDEαβ once GTP hydrolysis has occurred. Such quick release would not be possible with a soluble PDEγ–TαGDP complex that is separated from PDEαβ. An obvious corollary of this argument is that the loose connection between Tα–PDEγ and PDEαβ would enhance the dissociaton of the former species so that reinhibition could swiftly occur after GTP hydrolysis. Further-

more, one cannot imagine that the rod cell would synthesize a vast excess of PDEγ over PDE$\alpha\beta$ because any overproduced PDEγ would be immediately taken up by the slow "buffer" that is TαGDP and would not serve any useful purpose, such as providing for a GTPase-independent quenching mechanism of transducin-activated PDE (Erickson et al., 1992).

The interaction between Tα–PDEγ and PDE$\alpha\beta$ seems to have little influence on the PDE activity: Provided it is not dissociated from its supporting membrane, PDE$\alpha\beta$ remains fully active when stripped of Tα and PDEγ. But this transducin–PDE interaction may be crucial during the deactivation phase. As mentioned above, it might be responsible for the rapid dissociation of PDEγ from TαGDP, something that does not happen when PDEγ–TαGDP is isolated. More importantly, it must be this global Tα–PDEγ–PDE$\alpha\beta$ interaction that accelerates the GTP hydrolysis by TαGTP. Indeed, the GTPase-activating protein of Tα is not PDEγ, as the soluble complex PDEγ–TαGTP hydrolyzes its bound GTP in 20 s, that is, as slowly as isolated TαGTP (Antonny et al., 1993). Yet, under conditions approximating those *in vivo*, deactivation of transducin-stimulated PDE takes less than 1 s and absolutely requires GTP hydrolysis (Vuong & Chabre, 1991).

Finally, the functional characteristics of the [W70F]PDEγ mutant suggest that W70 is only implicated in the binding of PDEγ to Tα, although it is close to the L77–I87 terminal peptide that is necessary for the inhibition of PDE$\alpha\beta$ (Brown & Stryer, 1989) but more distant from the R24–K45 positively charged peptide that has been suggested to interact with Tα (Lipkin et al., 1988; Takemoto et al., 1992). [W70F]PDEγ can fully inhibit PDE$\alpha\beta$, but its affinity for Tα is lowered by about 100-fold compared to wild-type PDEγ. This loss of affinity could correspond to the suppression of a hydrogen bond between PDEγ and Tα, since replacing a tryptophan with a phenylalanine preserves the aromatic structure and the hydrophobicity of the residue but suppresses the hydrogen bonding capacity of the nitrogen in the indole ring of tryptophan.

ACKNOWLEDGMENT

We are grateful to Nordine Belmokhtar for the design and construction of the pH-metric apparatus and to Dr. J. Bigay for help with the gel-filtration experiments.

REFERENCES

Antonny, B., Otto-Bruc, A., Chabre, M., & Vuong, T. M. (1993) *Biochemistry* (following article in this issue).

Artemyev, N. O., & Hamm, H. E. (1992) *Biochem. J. 283*, 273–279.

Artemyev, N. O., Rarick, H. M., Mills, J. S., Skiba, N. P., & Hamm, H. E. (1992) *J. Biol. Chem. 267*, 25067–25072.

Baehr, W., Devlin, M. J. & Applebury, M. L. (1979) *J. Biol. Chem. 254*, 11669–11677.

Bevington, P. R. (1969) *Data reduction and error analysis for the physical sciences,* pp 218–222, McGraw-Hill, Inc., New York.

Brown, R. L. (1992) *Biochemistry 31*, 5918–5925.

Brown, R. L., & Stryer, L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4922–4926.

Catty, P., & Deterre, P. (1991) *Eur. J. Biochem. 199*, 263–269.

Catty, P., Pfister, C., Bruckert, F., & Deterre, P. (1992) *J. Biol. Chem. 267*, 19489–19493.

Chabre, M., & Deterre, P. (1989) *Eur. J. Biochem. 179*, 255–266.

Clerc, A., & Bennett, N. (1992) *J. Biol. Chem. 267*, 6620–6627.

Cunnick, J. M., Hurt, D., Oppert, B., Sakamoto, K., & Takemoto, D. J. (1990) *Biochem. J. 271*, 721–727.

Deterre, P., Bigay, J., Pfister, C., Kuhn, H., & Chabre, M. (1986) *Proteins: Struct., Funct., and Genet. 1*, 188–193.

Deterre, P., Bigay, J., Forquet, F., Robert, M., & Chabre, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2424–2428.

Erickson, J. E., & Cerione, R. A. (1991) *Biochemistry 30*, 7112–7118.

Erickson, M. A., Robinson, P., & Lisman, J. (1992) *Science 257*, 1255–1258.

Frech, M., Schlichting, I., Wittinghofer, A., & Chardin, P. (1990) *J. Biol. Chem. 265*, 6353–6359.

Fung, B. K. K., & Griswold-Prenner, I. (1989) *Biochemistry 28*, 3133–3137.

Gray-Keller, M. P., Biernbaum, M. S., & Bownds, M. D. (1990) *J. Biol. Chem. 265*, 15323–15332.

Griswold-Prenner, I., Tuteja, N., Farber, D. B., & Fung, B. K. K. (1989) *Biochemistry 28*, 6145–6150.

Hurley, J. B. & Stryer, L. (1982) *J. Biol. Chem. 257*, 11094–11099.

Kühn, H. (1980) *Nature (London) 283*, 587–589.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Liebman, P. A., & Evanczuk, A. T. (1982) *Methods Enzymol. 81*, 532–542.

Lipkin, V. M., Dumler, I. L., Muradov, K. G., Artemyev, N. O., & Etingof, R. N. (1988) *FEBS Lett. 234*, 287–290.

Lipkin, V. M., Khramtsov, N. V., Vasilevskaya, I. A., Atabekova, N. V., Muradov, K.G., Gubanov, N. V., Li, T., Johnston, J. P., Volpp, K. J., & Applebury, M. L. (1990) *J. Biol. Chem. 265*, 12955–12959.

Ovchinnikov, Y. A., Lipkin, V. M., Kumarev, V. P., Gubanov, N. V., Khramtsov, N. V., Akhmedov, N. B., Zagranichny, V. E., & Muradov, K. G. (1986) *FEBS Lett. 204*, 288–292.

Ovchinnikov, Y. A., Gubanov, V. V., Khramtsov, N. V., Ischenko, K. A., Zagranichny, V. E., Muradov, K. G., Shuvaeva, T. M., & Lipkin, V. M. (1987) *FEBS Lett. 223*, 169–173.

Rosenberg, A. H., Lade, B. N., Chui, D., Lin, S.-W., Dunn, J. J., & Studier, F. W. (1987) *Gene 56*, 125–135.

Szoka, F., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194–4198.

Takemoto, D. J., Hurt, D., Oppert, P., & Cunnick, J. M. (1992) *Biochem. J. 281*, 637–643.

Vuong, T. M., & Chabre, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9813–9817.

Wensel, T. G., & Stryer, L. (1986) *Proteins: Struct., Funct., Genet. 1*, 90–99.

Wensel, T. G., & Stryer, L. (1990) *Biochemistry 29*, 2155–2161.

Yamazaki, A., Stein, P. J., Chernoff, N., Bitenski, M. W., & George, J. S. (1983) *J. Biol. Chem. 258*, 8188–8194.

Yamazaki, A., Hayashi, F., Tatsumi, M., Bitenski, M. W., & George, J. S. (1990) *J. Biol. Chem. 265*, 11539–11548.