Resonance Energy Transfer as a Direct Monitor of GTP-Binding Protein–Effector Interactions: Activated α-Transducin Binding to the cGMP Phosphodiesterase in the Bovine Phototransduction Cascade†

Jon W. Erickson‡ and Richard A. Cerione*

Department of Pharmacology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853-6401

Received April 3, 1990; Revised Manuscript Received April 22, 1991

ABSTRACT: Resonance energy-transfer approaches have been used to directly monitor the interactions of the GTPγS-bound α subunit of transducin (α_TGTPγS) with the retinal cyclic GMP phosphodiesterase (PDE). The PDE was labeled with 5-(iodoacetamido)fluorescein (IAF–PDE) and served as the fluorescence donor in these experiments while the α_TGTPγS was labeled with eosin-5-isothiocyanate (EITC–α_TGTPγS) and served as the energy acceptor. The EITC–α_TGTPγS species was able to quench a significant percentage of the IAF–PDE fluorescence (typically ≥30%) due to resonance energy transfer between the IAF and EITC moieties. The quenching by the EITC–α_TGTPγS species was dose-dependent, saturable (K_d = 21 nM), and specific for the GTPγS-bound form of the α_T subunit. Limited trypsin treatment of the IAF–PDE, which selectively removes a fluorescein-labeled γ subunit (γ_{PDE}), completely eliminates the quenching of the IAF fluorescence by the EITC–α_TGTPγS complex. Although the EITC–α_TGTPγS complex competes with the unlabeled α_TGTPγS for a binding site on the IAF–PDE, as well as for a site on the native PDE, it is not able to stimulate PDE activity. Thus, the modification of a single EITC-reactive residue on the α_TGTPγS complex prevents this subunit from eliciting a key activation event within the retinal effector enzyme.

Much recent research in cell biology has focused on delineating the molecular mechanisms of signal transduction across biological membranes. It is now clear that one of the recurring biochemical designs found in nature employs a family of structurally and genetically related receptors that regulate second-messenger levels through their interactions with guanine nucleotide binding proteins (G-proteins). These G-proteins, when in their active state, interact with a host of different effector proteins and in this way deliver the biochemical stimulus initiated by ligand-binding or photon-initiated receptor events (Gilman, 1987). In this work, we focus on the G-protein-coupled phototransduction system and, in particular, the interaction of the G-protein transducin with its effector molecule, the retinal cyclic GMP phosphodiesterase.

In the signaling pathway responsible for vertebrate vision, the coupling of the G-protein transducin to photolyzed rhodopsin has been shown to facilitate the exchange of GDP for GTP by increasing the rate of dissociation of GDP from the α subunit (α_T) of the G-protein (Chabre & Deterre, 1989). The binding of GTP induces a conformational change in α_T which corresponds to the conversion to its active state (Phillips & Cerione, 1988). During the lifetime of the active state, the GTP-bound form of α_T is apparently capable of interactions with the regulatory subunit (γ_{PDE})[1] of the cyclic GMP (cGMP) phosphodiesterase (PDE), the effector enzyme in the phototransduction system (Miki et al., 1975; Hurley & Stryer, 1982). In the intact outer segment of the rod cell, this interaction initiates the hydrolysis of cGMP by the PDE which in turn reduces the Na^+ conductance and results in the hyperpolarization of the retinal rod plasma membrane (Pugh, 1987). The hydrolysis of the GTP bound to the α_T subunit deactivates the transduction cascade by returning α_T to its inactive, GDP-bound state, thereby precluding further interactions between α_T and the PDE (Fung & Stryer, 1980).

Due to the fact that the proteins comprising the phototransduction cascade are obtainable in milligram quantities, and given the recent progress in the cloning and expression of native and mutant opsin (Oprian et al., 1987), α_T (Lochrie et al., 1985), and the γ_{PDE} (Brown & Stryer, 1989; Griswold-Prenner et al., 1989), this signaling system is particularly attractive for biophysical studies. A primary aim of our current work is to develop real-time spectroscopic assays for examining the kinetics of the visual cascade within well-defined reconstituted systems. Toward this end, we are examining the feasibility and usefulness of different fluorescent modifications of the purified components of the bovine visual system and are exploring the use of resonance energy transfer as a monitor of the interactions between these proteins. In the present study, we have used covalently modified derivatives of α_T and PDE for the purpose of directly monitoring the interactions between the activated retinal G-protein and its effector enzyme. Following the covalent attachment of appropriate donor and acceptor probes to the PDE and the α_TGTPγS subunit, we find that the fluorescence of a donor-labeled PDE molecule is quenched in a saturable fashion when titrated with an acceptor-labeled α_TGTPγS species. The functional consequence of a single-residue modification of α_TGTPγS is a loss of

† This research was supported by National Institutes of Health Grants EY06429 and GM40654 and by grants from the PEW Biomedical Research Scholars Program and the Cornell Biotechnology Institute, which is supported by the New York State Science Foundation, the United States Army, and a consortium of industries.

‡ Supported by NIH Postdoctoral Fellowship EY06204.

[1] Abbreviations: EITC–α_TGTPγS, purified eosin-5-isothiocyanate conjugate of a GTPγS-bound form of the α subunit of transducin; IAF–PDE, 5-(iodoacetamido)fluorescein-labeled retinal phosphodiesterase; γ_{PDE}, regulatory γ subunit of PDE.

0006-2960/91/0430-7112$02.50/0

α_TGTPγS-stimulated cGMP hydrolysis by PDE, in agreement with the previously published result of Hingorani and Ho (1987). Here we demonstrate that the loss of activity is not due to the inability of the labeled α_T subunit to bind to the inhibitory γ_{PDE} subunit but rather must reflect the inability of the bound α_T subunit to trigger an activation event within the effector enzyme.

Materials and Methods

Materials and Chemicals. Dark-adapted bovine retinas were purchased from Hormel Meat Packers, Austin, MN. 5-(Iodoacetamido)fluorescein and eosin-5-isothiocyanate were purchased from Molecular Probes, Inc., Eugene, OR. Trypsin, soybean trypsin inhibitor, GTP, and cyclic GMP were from Sigma. GTPγS and dithiothreitol were obtained from Boehringer Mannheim. Hydroxylapatite was purchased from Bio-Rad.

Purification of Transducin and the cGMP Phosphodiesterase from Rod Outer Segments. Isolation of the components of the phototransduction cascade from rod outer segments has been described in detail elsewhere (Phillips et al., 1989). Briefly, the rod outer segments (ROS) were purified in the dark under dim red light from whole bovine retinas essentially as described by Gierschik et al. (1984). Following several washes in isotonic buffer (10 mM HEPES, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 100 mM NaCl, and 0.3 mM phenylmethanesulfonyl fluoride, pH 7.5), the ROS membranes were exposed to room light for 30 min, pelleted, and resuspended in a hypotonic buffer (10 mM HEPES, 1 mM DTT, 0.1 mM EDTA, and 0.3 mM phenylmethanesulfonyl fluoride, pH 7.5). Several (>5) cycles of centrifugation and resuspension were performed, and the supernatants were then pooled and concentrated. This concentrate contained the PDE that was used in the initial step of the IAF modifications (see below). The α_T subunit and the $\beta\gamma_T$ subunit complex were was eluted from the illuminated ROS membranes by resuspending the pellet in the hypotonic buffer supplemented with 100 μM GTP (or GTPγS) and incubating the membranes on ice for 30 min in room light. The membranes were pelleted and resuspended 3 times as above, and the supernatants were pooled. The transducin-containing pool was concentrated 10-fold in an Amicon ultrafiltration cell (YM 10 membrane).

The α_T subunit and the $\beta\gamma_T$ subunit complex were resolved by Blue Sepharose chromatography (Pines et al., 1985). The concentrated transducin (6–10 mg of protein in 5–10 mL of hypotonic buffer) was applied to a 50-mL Blue Sepharose column preequilibrated with 10 mM HEPES (pH 7.5), 6 mM $MgCl_2$, 1 mM DTT, and 25% glycerol. The column was washed with 200 mL of the above buffer, and the peak fractions of the unbound $\beta\gamma_T$ were pooled and concentrated. The bound α_T was eluted from the column with the above buffer supplemented with 0.5 M KCl.

Protein Modifications and Characterization. The covalent modifications of α_TGDP and α_TGTPγS were carried out essentially as described by Hingorani and Ho (1987). Typically, 500 μL of a 0.5 mg/mL (~10 μM) solution of purified α_T subunit in buffer was mixed with 10 μL of a 25 mM EITC stock solution in dimethyl sulfoxide to give a final concentration of 0.5 mM EITC. When the α_TGDP species was labeled, the reaction was allowed to proceed for 2 h at ambient temperature (22 °C). In the case of the α_TGTPγS species, the reaction proceeded for an additional 2 h in order to achieve stoichiometric labeling. Following the modification of an α_T subunit, the reaction mixture was applied to a 600-μL hydroxylapatite column preequilibrated with 10 mM K_2HPO_4 (pH 7.5). The column was washed extensively with the 10 mM phosphate buffer until the eluate was free of unreacted probe. The EITC-labeled α_TGDP, or the EITC-α_TGTPγS, was eluted with 100 mM phosphate and dialyzed vs 150 mM NaCl, 5 mM $MgCl_2$, 20 mM HEPES, 1 mM dithiothreitol, 0.05% NaN_3 (w/w), and 50% glycerol (v/v), pH 7.4, overnight at 4 °C. Protein concentrations were determined by the Cu^+/bicinchoninic acid complex method (Pierce Chemicals, Inc.) using bovine serum albumin as a standard. The molecular weight of α_T was assumed to be 39K. Stoichiometries of the modification reactions were determined by measuring the optical densities of buffered aqueous solutions containing the labeled α_T subunits at the wavelength of maximal absorbance for the protein-bound eosin (523 nm; ϵ_{MAX} = 86 000 M^{-1} cm^{-1}).

Chemical modification of the cGMP PDE was performed on the pooled and concentrated hypotonic washes of isolated rod outer segments. Typically, 10 μL of a 25 mM stock solution of IAF in dimethylfuran was added to 0.5 mL of this partially purified PDE preparation (~0.8 mg/mL total protein) at pH 7.5. The reaction was carried out at room temperature for 3–12 h, depending on the extent of modification desired. The reaction mixture was then applied to a 600-μL hydroxylapatite column and washed extensively with 10 mM phosphate buffer to remove unreacted probe. In addition, a second extensive washing with 100 mM phosphate buffer was performed to elute contaminating proteins (primarily the α_T and $\beta\gamma_T$ subunits). The IAF-modified PDE was finally eluted as a single colored band after the addition of 200 mM phosphate and dialyzed vs 50% glycerol buffer as above. Electrophoretic analysis of the eluted protein demonstrated the purity of the PDE while transillumination with UV light of the same gel revealed labeling on all three subunits. The overall fluorophore to protein stoichiometry was determined by using an ϵ_{MAX} of 74 000 M^{-1} cm^{-1} for the IAF–PDE at the peak absorbance of 492 nm in 50 mM phosphate buffer (pH 8.0), and measuring the protein concentration of an equivalent sample with the Cu^+/bicinchoninic acid protein assay. The concentration of PDE was determined by using a molecular weight of 195K. Labeled protein was stored at −20 °C and generally used within 2 weeks of modification.

Fluorescence Spectroscopy. The resonance energy-transfer measurements were performed with an SLM 8000c spectrofluorometer operated in the ratio mode. The excitation wavelength was fixed at 460 nm, and the emission spectrum was scanned from 480 to 610 nm. For a typical energy-transfer experiment, 200 μL of a buffer containing 120 mM NaCl, 30 mM KCl, 2 mM $MgCl_2$, and 20 mM HEPES, pH 7.4, was made 10–100 nM in IAF–PDE by the addition of small aliquots of the stock IAF–PDE solution (1–5 μL). Titrations were performed by adding successive aliquots (1–5 μL) of the EITC-α_T stock solutions with microcapillary pipets. After being mixed thoroughly, emission spectra were taken following each addition of EITC-α_TGTPγS, and the peak fluorescence intensity was recorded following correction for dilution. Inner filter effects due to the addition of the acceptor (EITC-α_T) molecules were negligible (<0.01 absorbance unit at saturation).

Data Analysis. The data obtained from the titration of the IAF-labeled PDE with the EITC-labeled α_TGTPγS were fit according to the simple bimolecular reaction scheme:

$$\alpha_T\text{GTP}\gamma\text{S} + \text{PDE} \rightleftharpoons \alpha_T\text{GTP}\gamma\text{S–PDE}$$

without any assumptions regarding the stoichometry of the interaction. If α_{tot} is defined as the total concentration of labeled α_T following the nth addition of α_T and F_n is the corresponding fluorescence intensity, then the function de-

scribing the binding of α_T to the PDE can be written as

$$F_n(\alpha_{tot}) = F_0 - (F_0 - F_\infty)\{[(\alpha_{tot} + K_d + P_{tot}) - (\Gamma^2 + 4K_dP_{tot})^{1/2}]/2P_{tot}\} \quad (1)$$

where $\Gamma = \alpha_{tot} + K_d - P_{tot}$. K_d represents the dissociation constant characterizing this interaction, F_0 is the initial value of the IAF–PDE (donor) fluorescence, P_{tot} is the total concentration of PDE, and F_∞ is the level of donor fluorescence when all PDE-binding sites are occupied. The goodness of fit was evaluated by using a least-squares criterion. The fitting was performed by fixing the values of F_0 and P_{tot} and allowing the values of K_d and F_∞ to vary. As a final step in determining the best parameter values, F_∞ was allowed to vary along with P_{tot} while the value of the K_d was fixed following the initial convergence.

Measurement of cGMP PDE Activity. The analysis of cGMP hydrolysis by the retinal PDE was carried out according to the method described originally by Yee and Liebman (1978). Briefly, a pH microelectrode was used to measure the decrease in pH resulting from the production of one proton for each cGMP molecule hydrolyzed by PDE. All assays were carried out at 22 °C in a final volume of 200 μL and in a buffer containing 10 mM HEPES (pH 8.0), 3 mM $MgCl_2$, 60 mM KCl, and 1 mM DTT. All assays contained 5–25 pmol of either the native PDE or the IAF-labeled enzyme. Additions of other components are noted in the appropriate figure legends. The assay was always initiated by the addition of cGMP (final concentration = 5 mM), and the change in pH (as millivolts) was measured with an acquisition each second for ~200 s. At the end of each assay period, the buffering capacity (in millivolts per nanomole) was determined by adding 500 nmol of KOH to the reaction mixture. The rate of hydrolysis of cGMP (nanomoles per second) was determined from the ratio of the initial slope of the pH record (millivolts per second) and the buffering capacity of the assay buffer (millivolts per nanomole). The trypsinized activities for the native and the IAF-labeled PDE were the same and were generally found to fall in the range of 1200–2000 mol of cGMP hydrolyzed per mole of PDE per second.

Results

Preparation and Use of the EITC-Labeled α_T Subunit and the IAF-Labeled Cyclic GMP PDE for Resonance Energy-Transfer Studies. The strategy for the chemical modification of the cyclic GMP PDE was based on the availability of several reactive residues on the PDE which could be used to incorporate donor fluorophores and the presence of a single reactive amine on the α_T subunit (Hingorani & Ho, 1987) that was suitable for the introduction of an acceptor moiety. Fluorescent sulfhydryl reagents were used to label the cyclic GMP PDE since previous studies have shown that the modification of the reactive cysteine residues of this enzyme has no effect on enzyme activity (Wensel & Stryer, 1990; W. Phillips, unpublished observation). The chemical modification of an amine residue on the α_T subunit with an isothiocyanate reagent was based on the initial report of Hingorani and Ho which stated that this modification had no effect on α_T–rhodopsin or α_T–$\beta\gamma_T$ interactions, although it did decrease the intrinsic GTPase activity of α_T as well as the ability of activated α_T to stimulate PDE activity.

We find that the time course of labeling of both the GTPγS- and GDP-bound forms of α_T with EITC exhibits the same kinetics as that described for the FITC modifications (Higorani & Ho, 1987). Specifically, the activated α_TGTPγS species reacts more slowly with the isothiocyanate reagent than the α_TGDP complex, and the limited trypsin digestion patterns of both forms of the α_T subunit show that the EITC fluorescence is associated with the 32-kDa tryptic fragment. The EITC modification appears to markedly reduce the rate of generation of the 23- and 9-kDa tryptic fragments of the α_TGDP species (i.e., the appearance of these fragments requires >2 h for the EITC–α_TGDP species compared to <1 h for the native α_TGDP), again like the case for FITC. The incorporation of EITC appears to be restricted to the 23-kDa tryptic fragment, although we cannot rule out that a small percentage of label also is present on the 9-kDa fragment (data not shown). Despite the fact that Hingorani and Ho had shown that the labeling of the α_TGTPγS subunit with FITC appeared to compromise the regulation of the PDE activity by transducin, we were interested in further investigating the structure–function implications of this amine modification. In this regard, it was of interest to determine whether the native, GDP-bound form of α_T, or the preactivated GTPγS-bound form of the α_T subunit, when labeled at this reactive amine with fluorescent reporter groups, was capable of directly coupling to the retinal effector enzyme as monitored by using resonance energy-transfer approaches.

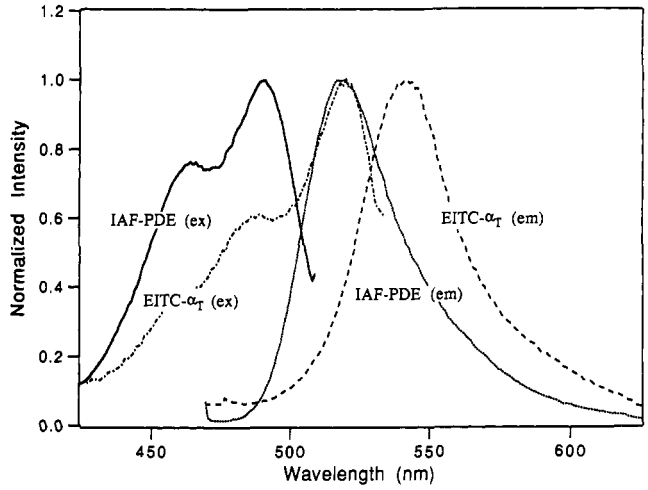

FIGURE 1: Normalized fluorescence excitation and emission spectra of labeled proteins. The emission spectrum for IAF-labeled PDE (20 nM) was obtained by exciting the sample at 460 nm (···). The excitation spectrum was recorded by monitoring the emission at 518 nm (—). The excitation spectrum for EITC–α_TGTPγS (50 nM) was obtained by recording the emission at 545 nm (–·–). The emission spectrum for EITC–α_TGTPγS was obtained at a constant excitation of 525 nM (- - -).

The energy-transfer measurements were performed by using an IAF-labeled cyclic GMP PDE as the fluorescence donor species and an EITC-labeled α_TGTPγS as the acceptor species. Figure 1 shows that this combination of chromophoric adducts represents an excellent donor–acceptor pair for resonance energy transfer, i.e., the IAF fluorescence emission strongly overlaps the excitation spectrum for the EITC adduct. The results presented in Figure 2 illustrate that the EITC-labeled α_TGTPγS complex is able to effectively interact with the IAF-labeled PDE. Specifically, dose-dependent quenching of the IAF–PDE fluorescence emission was observed. The quenching was saturable and could not be attributed to the simple absorption of the exciting light by the acceptor (EITC) chromophore, i.e., inner filter effects. The possible contribution of inner filter effects to the total quenching of the donor (FITC) fluorescence could be determined empirically by adding free eosin to a sample of IAF–PDE. We found that at the highest concentrations of EITC–α_TGTPγS used in these

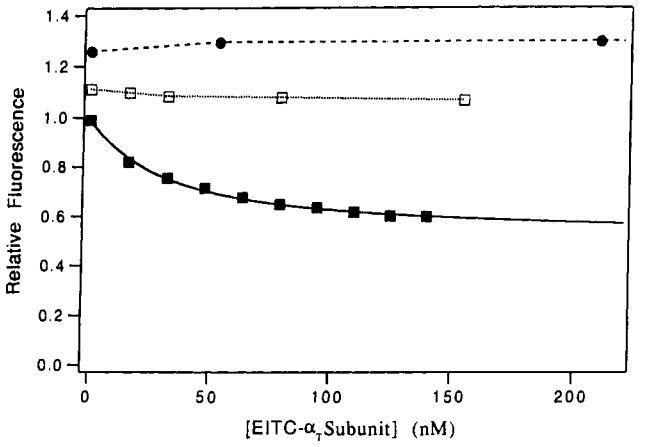

FIGURE 2: Dependence of the quenching of IAF–PDE fluorescence on [EITC–α_T]. Titrations of IAF–PDE with EITC–α_TGTPγS (■) and EITC–α_TGDP (●); the data are plotted as the relative emission intensities of IAF–PDE (at 518 nm) measured in the presence and absence of different α_T species and represent the raw fluorescence data from three different samples, each exhibiting a slightly different initial value of fluorescence prior to any additions. The ratio of EITC to α_T subunit was 1:1 for both the EITC–α_TGTPγS and EITC–α_TGDP modifications (see Materials and Methods). The solid curve drawn through the data points represents the best fit for a single class of binding sites for the interaction of EITC–α_TGTPγS and IAF–PDE (see text, eq 1). The parameters used to draw this curve are as follows: initial relative fluorescence, $F_0 = 0.991$; dissociation constant for the EITC–α_TGTPγS/IAF–PDE interaction, $K_d = 18$ nM; total IAF–PDE binding sites, $P_{tot} = 31$ nM; relative fluorescence when all IAF–PDE sites are occupied, $F_\infty = 0.545$. (□) Quenching of IAF–PDE fluorescence by EITC–α_TGTPγS following trypsin treatment. IAF–PDE samples were trypsinized as previously described (Kroll et al., 1989), resulting in a level of activation of the cGMP hydrolysis activity that was identical with the unmodified enzyme. The [IAF–PDE] in these titrations determined by a protein assay of the stock IAF–PDE solution was 22 nM.

experiments, the inner filter correction is negligible (<2%). Since under the conditions of these experiments there is no detectable eosin emission at 518 nm, the fluorescence intensity for the IAF–PDE, at any concentration of EITC–α_TGTPγS, can be determined in a straightforward manner. The maximum intensities of these emission spectra, as a function of [EITC–α_TGTPγS], are shown in Figure 2 (■). In this particular experiment, a 30% decrease in the total fluorescence emission at 518 nm was observed in the presence of a saturating amount of EITC–α_TGTPγS. For eight different modifications of the IAF-labeled PDE (derived from eight different preparations of this enzyme), the extent of quenching by the EITC-labeled α_TGTPγS ranged from ~12 to 49%. Some of this variability appeared to correlate with variations in the abilities of the different PDE preparations to hydrolyze cyclic GMP, either following trypsin activation or upon stimulation by α_TGTPγS complexes, prior to any chemical modification of either the enzyme itself or the α_T subunit. Those cyclic GMP PDE preparations which displayed relatively high levels of cyclic GMP hydrolytic activity could be quenched to a significant degree, following their labeling with IAF, by the EITC-labeled α_TGTPγS complex. The concentration of half-maximal quenching was observed to occur in the range of 15–30 nM EITC–α_TGTPγS. This range of values for the half-maximal quenching was consistently observed for all of the preparations studied and was independent of the total extent of quenching obtained with a given IAF–PDE preparation.

Characterization of the Interactions of the EITC-Labeled α_T Subunits with the IAF-Labeled Cyclic GMP PDE. If the quenching of the IAF–PDE by the EITC–α_TGTPγS reflects the direct coupling of these signaling proteins, then this quenching should be specific for the GTPγS-bound form of the α_T subunit. This was observed to be the case. The results presented in Figure 2 show that while the addition of the EITC–α_TGTPγS complex (eosin:α_T ratio = 0.98) to a solution of IAF–PDE resulted in a dose-dependent loss in the fluorescein fluorescence emission, the EITC–α_TGDP species (eosin:α_T ratio = 1.2) caused no such change even at levels as high as 500 nM.

As a further test of the specificity of the interactions between the EITC–α_TGTPγS and IAF–PDE species, we measured the extent of energy transfer following trypsin treatment of the IAF–PDE. On the basis of the widely held view that activated α_T subunits bind to the PDE via the γ_{PDE} subunit of the effector enzyme [cf. Deterre (1988)], it might be expected that the brief trypsin treatment of PDE, which results in its activation (due to the specific proteolysis of the γ_{PDE} subunits), would preclude α_TGTPγS–PDE interactions and thus eliminate the fluorescence quenching of IAF emission. Again, this prediction was confirmed as shown by the results in Figure 2. Specifically, there was no significant energy transfer observed between the EITC–α_TGTPγS and the trypsin-treated IAF–PDE samples in experiments where the trypsin treatment was performed on the identical IAF–PDE preparation that was quenched by EITC–α_TGTPγS to a final extent of about 30%. Further support for the view that this lack of observed energy transfer is due to the specific proteolysis of the γ_{PDE} subunit is the absence of the fluorescent band at ~13 kDa, corresponding to the γ_{PDE} subunit, in the SDS–PAGE analysis of trypsin-treated IAF–PDE samples (data not shown). Thus, there is a clear correlation between the presence of the γ_{PDE} subunit and the occurrence of energy transfer between the EITC–α_TGTPγS species and the IAF-labeled PDE.

Taken together, the results presented in Figure 2 indicate that the resonance energy transfer between the IAF–PDE and the EITC–α_TGTPγS is due to a specific, high-affinity interaction between these signaling components. In order to quantify the fluorescence quenching data obtained from several different chemical modifications, the data from each titration were fit to a simple model (see eq 1) that treated the binding sites on the PDE for the α_TGTPγS species as a single class of noninteracting sites characterized by a single affinity constant. Employing this model, we obtained the best fits to titration curves with three different protein preparations which yielded an average dissociation constant of 21 ± 2.6 nM.

It should be noted that the relative intensities of the labeled subunits of the cyclic GMP PDE, as estimated by SDS–PAGE analysis, indicate that the fluorescein is predominantly attached to the larger subunits of the enzyme (i.e., the α_{PDE} and β_{PDE} subunits). Under the mild conditions used for covalent modifications (i.e., room temperature, pH 7.4), it is likely that the reaction with IAF would be selective for cysteine residues. In the case of the γ_{PDE} subunit, there is a single cysteine residue at position 68 (Ovchinnikov et al., 1986). The fact that the stoichiometries of IAF to PDE typically have been observed to be ~8:1, coupled with the observation that greater than 40% of the donor (IAF) fluorescence can be quenched by the EITC–α_TGTPγS species, suggests that the labeled α_TGTPγS complex (when coupled to the γ_{PDE}) remains bound to the core of the enzyme throughout the time period of the fluorescence measurements.

Assays of Cyclic GMP Hydrolysis Using the EITC-Labeled α_T Subunit and the IAF-Labeled PDE. As an independent approach for examining the interactions of the EITC–α_TGTPγS complex with the effector enzyme, we examined

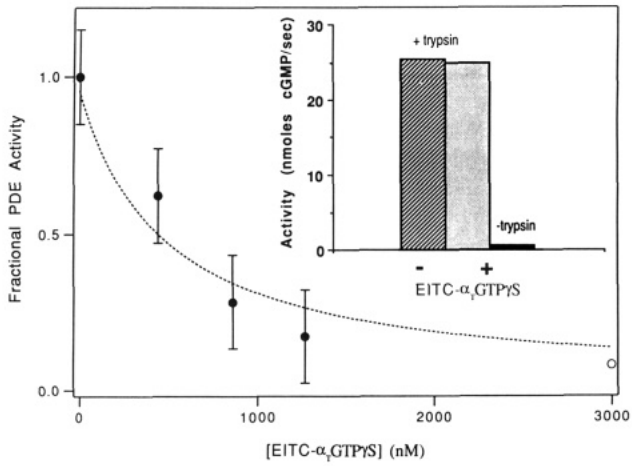

FIGURE 3: Effects of EITC–α_TGTPγS on the ability of native α_TGTPγS to stimulate the cyclic GMP PDE. Increasing amounts of EITC–α_TGTPγS were added to 200-μL test samples containing 300 nM native α_TGTPγS, 105 nM cyclic GMP PDE, and lipid vesicles (final concentration of lipid ~0.3 mg/mL) in assay buffer. The curve was drawn according to a model for α_TGTPγS-stimulated cGMP hydrolysis where both labeled and unlabeled α_TGTPγS bind to the same site on the PDE and where only α_TGTPγS can stimulate the enzyme. Each α_TGTPγS species was assigned its own dissociation constant. Assuming that only the unlabeled α_TGTPγS will stimulate PDE activity, the level of PDE activity in the presence of the competitive inhibitor EITC–α_TGTPγS can be written as $A([\text{EITC}-\alpha_T]) = \{[\alpha_T]_{tot}/(P + K_d)\}/\{[\alpha_T]_{tot}/(P + K_d) + [\text{EITC}-\alpha_T]_{tot}/(P + K_d^{EITC}) + 1\}$ where K_d and K_d^{EITC} represent the dissociation constants for the interactions of PDE with α_TGTPγS and EITC–α_TGTPγS, respectively, and P represents the concentration of free PDE at a given value of $[\text{EITC}-\alpha_T]_{tot}$. With $[\alpha_T]_{tot}$ and K_d^{EITC} held constant (the value of K_d^{EITC} being fixed at 21 nM as determined by the fluorescence titrations), the best fit of the data yielded a value of 13.5 nM for K_d. Inset: Activity of the trypsin-treated PDE (105 nM) in the presence and absence of EITC–α_TGTPγS (407 nM), compared to the level of cGMP hydrolysis observed for the nontrypsinized enzyme.

the ability of the native α_TGTPγS subunit to stimulate cyclic GMP hydrolysis by the native PDE, in the presence of increasing concentrations of the EITC–α_TGTPγS species. The results of a typical experiment are shown in Figure 3. Increasing concentrations of the EITC–α_T subunit result in a marked inhibition of the cyclic GMP hydrolysis stimulated by the native α_T subunit, with the extent of the inhibition reaching 100%. This inhibition can be overcome by high levels of native α_TGTPγS, and it is specific for the α_TGTPγS-stimulated enzyme activity (i.e., the EITC–α_TGTPγS species has no effect on the trypsin-activated PDE activity; see inset), suggesting that the inhibitory effect results from a simple competition between the native and labeled α_T subunits for the PDE. If we model the inhibition data as a simple competition between the native α_TGTPγS and the EITC–α_TGTPγS for the PDE, and fix the value of the equilibrium constant for the interaction of EITC–α_TGTPγS with the enzyme at 21 nM (i.e., the value derived from the fluorescence titrations), the inhibition data in Figure 3 can be well fitted by using a K_d value of 13.5 nM for the interaction of native α_TGTPγS with the PDE (see dashed line in Figure 3).

Despite the ability of the EITC–α_TGTPγS species to tightly couple to the IAF–PDE, the labeled α_T subunit is not able to stimulate enzyme activity. We have determined that it is the modification of the reactive amine on the α_T subunit, rather than the modification of the effector enzyme, which prevents the activation (by α_T) of the cyclic GMP PDE. Specifically, trypsin activation of the IAF–PDE results in similar levels of cyclic GMP hydrolysis [~1100 mol of cyclic GMP hydrolyzed s^{-1} (mol of PDE)$^{-1}$] as those measured upon trypsin treatment of the native PDE. In addition, the α_TGTPγS complex stimulates the native PDE and the IAF–PDE to essentially identical levels [~250 mol of cGMP hydrolyzed s^{-1} (mol of PDE)$^{-1}$]. However, the EITC–α_TGTPγS complex shows no detectable stimulation of either the native PDE or the IAF–PDE activity (data not shown). Thus, taken together, these findings indicate that the reaction of the isothiocyanate-sensitive amine on the α_T subunit results in an α_T species which can bind to the effector enzyme but is incapable of triggering the stimulation of enzyme activity.

Discussion

Studies of the physiological coupling between G-proteins and effector molecules generally have relied upon indirect functional measurements of the stimulated effector activities. For example, in the case of the vertebrate visual transduction system, the coupling of the α subunit of transducin to the cyclic GMP PDE is typically monitored by observing the changes in pH that accompany the turnover of cyclic GMP by the effector enzyme. While this assay provides a convenient readout for changes in the levels of cyclic GMP, it does not provide information about the individual steps that are involved in the G-protein regulation of the effector enzyme, i.e., the initial binding of the α_T subunit to the PDE molecule and the ensuing conformational changes (and/or the dissociation of PDE subunits) that trigger the hydrolysis of cyclic GMP. In this study, we have used resonance energy-transfer approaches to focus on the first step in this process, namely, the coupling of the α_T subunit to the effector enzyme.

When the EITC–α_TGTPγS species was added to a solution containing the IAF-labeled PDE, the fluorescence of the labeled effector enzyme was quenched in a saturable fashion. There are a number of lines of evidence that indicate that this quenching is a direct outcome of the binding of α_T to the PDE molecule. One line of evidence is drawn from experiments which employ the trypsinized enzyme. The brief treatment of the IAF–PDE with trypsin, which results in the maximal stimulation of cyclic GMP hydrolysis, completely eliminates the quenching of the IAF fluorescence by the EITC–α_TGTPγS complex. The extent to which the energy transfer between the labeled α_T and PDE molecules is attenuated may be directly attributable to the selective removal of a fluorescein-labeled γ_{PDE} subunit. Since the γ_{PDE} subunit(s) has (have) been implicated as the binding site for the α_T subunit (Fung & Griswold-Prenner, 1989), these results suggest that the quenching of the IAF–PDE fluorescence by the EITC-labeled α_TGTPγS is a reflection of the α_T–PDE interaction. Additional support for this suggestion comes from the results of competition studies carried out using the fluorescence quenching assay where an excess of either unmodified PDE or α_TGTPγS, added prior to the fluorescence titration, prevented the quenching of IAF–PDE fluorescence by EITC–α_TGTPγS (data not shown). Functional evidence for the specificity of EITC–α_TGTPγS binding is provided by the inhibition of α_TGTPγS-stimulated PDE activity, where the EITC-labeled α_TGTPγS subunits appear to compete with the native α_TGTPγS for the effector enzyme (Figure 3).

Finally, the energy transfer between the labeled α_T and PDE molecules appears to be specific for the GTPγS-bound form of the α_T subunit (Figure 2). We find that the EITC–α_TGDP is unable to quench the IAF–PDE fluorescence (Figure 2), as would be predicted if the quenching by the EITC–α_TGTPγS were due to a specific interaction between the GTPγS-bound α_T subunit and the effector enzyme. On the basis of the extents of energy transfer observed in these experiments (typically ≥30%), coupled with the determined stoichiometries of labeling of the α_T subunits (~1:1) and the PDE (~8:1),

it appears that the EITC–α_TGTPγS remains tightly associated with the IAF–PDE during the course of the fluorescence measurements. Furthermore, given the fact that there is one reactive (cysteine) site for IAF on the γ_{PDE} subunit, and assuming that there are two functionally equivalent and independent interactions between α_T and the γ_{PDE} subunit, per enzyme molecule [Deterre et al., 1988; Wensel & Stryer, 1990; however, see Whalen et al. (1990)], a maximum energy-transfer quenching of 20% would be predicted if the EITC–α_TGTPγS–γ_{PDE} complex completely dissociates from the core of the effector enzyme (i.e., following the dissociation of the γ_{PDE}, only 2 out of 10 donor fluorophores would be in the proximity of the acceptor molecule on the EITC–α_TGTPγS subunit). Whether the apparent inability of the EITC–α_TGTPγS complex to elicit the dissociation of the γ_{PDE} subunit is directly related to the observation that the EITC–α_TGTPγS complex will not stimulate cyclic GMP hydrolysis remains to be determined. Although it has been suggested that the stimulation of PDE activity by activated α_T subunits is the direct outcome of an α_T-induced dissociation of the γ_{PDE} subunit(s) from the core of the enzyme [cf. Hurley and Stryer (1982), Wensel and Stryer (1986), and Fung and Griswold-Prenner (1989)], various results also have pointed to the possibility that the α_TGTPγS stimulation of enzyme activity occurs within an intact α_T–PDE complex [cf. Sitaramayya et al. (1986) and Kroll et al. (1989)].

Irrespective of the molecular mechanism by which α_TGTPγS stimulates the PDE, the results presented above indicate that the attachment of the EITC group to α_TGTPγS does not prevent the first step in the regulation of the PDE, i.e., the specific binding of a GTPγS-bound α_T subunit to the effector. The observed K_d values for the EITC–α_TGTPγS–PDE interaction estimated from the resonance energy-transfer titrations (~20 nM) are somewhat lower than those estimated from functional assays. Specifically, when the α_TGTPγS-stimulated PDE activity is measured in solution (i.e., in the absence of lipid vesicles) as a function of [α_TGTPγS], under conditions similar to those used to make the fluorescence measurements, the apparent K_d values for α_TGTPγS range from 100 to 200 nM [cf. Phillips et al. (1989)]. These results would be consistent with the suggestion that the stimulation of the PDE activity by the α_TGTPγS subunit represents at least a two-step process, i.e., the first step would represent a tight, specific binding by the activated α_T subunit to the effector enzyme while the second step would represent an α_T-induced stimulatory conformational change in the effector enzyme which results in the ability of the enzyme to hydrolyze cyclic GMP. The situation appears to become more complicated in the presence of lipid vesicles, as, under these conditions, the α_TGTPγS dose response profiles obtained from PDE assays suggest that at least two classes of α_TGTPγS-binding sites are present on the enzyme, i.e., both high- and low-affinity sites, with the latter [K_d > 500 nM; cf. Bennett and Clerc (1989) and Phillips et al. (1989)] being responsible for eliciting the majority of the stimulation of PDE activity. Thus far, we have not characterized the apparent low-affinity interaction between the PDE and α_TGTPγS, using fluorescence approaches, although in the future we will attempt to do so using high levels of PDE in reconstituted phospholipid vesicle systems.

In conclusion, the results of this study demonstrate that resonance energy-transfer approaches, employing labeled protein components from a G-protein-mediated signaling pathway, provide a sensitive monitor of the binding of a G-protein to an effector enzyme and should allow detailed analysis of the kinetics of this binding interaction. This is of interest since thus far it has not been possible to directly compare the rates for the dissociation of a GTP (or GTPγS)-bound α_T subunit from either the $\beta\gamma_T$ complex and/or the receptor with the rates for the initial binding of α_T to its effector protein. The fluorescence method developed in this study can provide the required sensitivity that will allow the application of rapid kinetic techniques. In addition to the development of a direct monitor for retinal G-protein–effector interactions, the results of the present study, together with the earlier work of Hingorani and Ho (1987), indicate that the EITC (or FITC) modification of the α_T subunit inhibits their ability to stimulate PDE activity despite the observation that the initial binding of these two components appears to be unaffected by the labeling. The observed inhibitory effects may be attributable to changes in the tertiary structure of the α_T subunit induced by the isothiocyanate modifications. These changes, in turn, may preclude the necessary activating conformational changes in one or more of the PDE subunits which normally occur subsequent to the initial binding of α_T. Future studies, using a combination of molecular biology, fluorescence, and reconstitution approaches, will be aimed at delineating the details of this process.

References

Bennett, N., & Clerc, A. (1989) *Biochemistry 28*, 7418–7424.

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

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

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

Fung, B., & Nash, C. (1983) *J. Biol. Chem. 258*, 10503–10510.

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

Fung, B., & Stryer, L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2500–2504.

Gierschik, P., Simons, C., Woodward, C., Somers, R., & Spiegel, A. (1984) *FEBS Lett. 172*, 321–325.

Gilman, A. (1987) *Annu. Rev. Biochem. 56*, 615–650.

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

Hingorani, V., & Ho, Y. (1987) *Biochemistry 26*, 1633–1639.

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

Kroll, S., Phillips, W., & Cerione, R. (1989) *J. Biol. Chem. 264*, 4490–4497.

Lochrie, M., Hurley, J., & Simon, M. (1985) *Science 228*, 96–99.

Miki, N., Baraban, J. M., Keirns, J. J., Boyce, J. J., & Bitensky, M. W. (1975) *J. Biol. Chem. 250*, 6320–6327.

Oprian, D., Molday, R., Kaufman, R., & Khorana, H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8874–8878.

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

Phillips, W. J., & Cerione, R. A. (1988) *J. Biol. Chem. 263*, 15498–15505.

Phillips, W. J., Trukawinski, S., & Cerione, R. A. (1989) *J. Biol. Chem. 264*, 16679–16688.

Pines, M., Gierschik, P., Milligan, G., Klee, W., & Spiegel, A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4095–4099.

Pugh, E. (1987) *Annu. Rev. Physiol. 49*, 715–742.
Sitaramayya, A., Harkness, J., Parkes, J., Gonzalez-Olivia, C., & Liebman, P. (1986) *Biochemistry 25*, 651–656.
Wensel, T., & Stryer, L. (1986) *Proteins: Struct., Funct., Genet. 1*, 90–99.
Wensel, T., & Stryer, L. (1990) *Biochemistry 29*, 2155–2161.
Whalen, M., Bitensky, M., & Takemoto, D. (1990) *Biochem. J. 265*, 655–658.
Yee, R., & Liebman, R., (1978) *J. Biol. Chem. 253*, 8902–8909.

Angiotensin-Converting Enzyme: Zinc- and Inhibitor-Binding Stoichiometries of the Somatic and Testis Isozymes†

Mario R. W. Ehlers and James F. Riordan*

Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, Massachusetts 02115

Received March 18, 1991; Revised Manuscript Received May 1, 1991

ABSTRACT: The blood pressure regulating somatic isozyme of angiotensin-converting enzyme (ACE) consists of two homologous, tandem domains each containing a putative metal-binding motif (HEXXH), while the testis isozyme consists of just a single domain that is identical with the C-terminal half of somatic ACE. Previous metal analyses of somatic ACE have indicated a zinc stoichiometry of 1 mol of Zn^{2+}/mol of ACE and inhibitor-binding studies have found 1 mol of inhibitor bound/mol of enzyme. These and other data have indicated that only one of the two domains of somatic ACE is catalytically active. We have repeated the metal and inhibitor-binding analyses of ACE from various sources and have determined protein concentration by quantitative amino acid analysis on the basis of accurate polypeptide molecular weights that are now available. We find that the somatic isozyme in fact contains 2 mol of Zn^{2+} and binds 2 mol of lisinopril (an ACE inhibitor) per mol of enzyme, whereas the testis isozyme contains 1 mol of Zn^{2+} and binds 1 mol of lisinopril. In the case of somatic ACE, the second equivalent of inhibitor binds to a second zinc-containing site as evidenced by the ability of a moderate excess of inhibitor to protect both zinc ions against dissociation. However, active site titration with lisinopril assayed by hydrolysis of furanacryloyl-Phe-Gly-Gly revealed that 1 mol of inhibitor/mol of enzyme abolished the activity of either isozyme, indicating that the principal angiotensin-converting site likely resides in the C-terminal (testicular) domain of somatic ACE and that binding of inhibitor to this site is stronger than to the second site. The second zinc- and inhibitor-binding site, instead, may be involved in a previously reported, unusual endoproteolytic cleavage of luteinizing hormone releasing hormone [Skidgel, R. A., & Erdös, E. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1025–1029], which we find is performed >300-fold faster by somatic compared to testis ACE. Taken together, these data indicate that the somatic enzyme may have an additional substrate specificity distinct from that for conventional substrates.

Angiotensin-converting enzyme (ACE;[1] peptidyl dipeptide hydrolase, EC 3.4.15.1) is a zinc-metallopeptidase that typically cleaves C-terminal dipeptides from oligopeptide substrates, as in its best-known substrate angiotensin I (Ehlers & Riordan, 1990). On the basis of numerous kinetic and chemical modification studies and on measured zinc stoichiometries of 1 mol of Zn^{2+}/mol of enzyme, ACE was inferred to contain a single carboxypeptidase A like active site [reviewed in Patchett and Cordes (1985); Ehlers & Riordan, 1990]. Indeed, the design of the current generation of potent, active site directed, zinc-coordinating inhibitors was based on this model (Ondetti et al., 1977; Cushman et al., 1977; Cushman & Ondetti, 1980; Patchett et al., 1980). These compounds are so effective that inhibition of the ACE-catalyzed conversion of angiotensin I to angiotensin II has become an important strategy in the treatment of hypertension and congestive cardiac failure (Gavras, 1990).

Recent results from molecular cloning have predicted that the somatic isozyme of ACE, the predominant isozyme that is widely distributed in mammalian tissues, consists of two homologous domains, each containing a putative metal-binding site of the type HEXXH (Soubrier et al., 1988), a motif that is common to many metallopeptidases exemplified by thermolysin but that differs from that present in carboxypeptidases (Vallee & Auld, 1990). There is also a unique testis isozyme of ACE that consists of and is essentially identical with the second, i.e., C-terminal domain of the somatic isozyme and thus contains only one of the two metal-binding motifs (Ehlers et al., 1989; Lattion et al., 1989; Kumar et al., 1989).

† This work was supported in part by Grant HL 34704 from the National Institutes of Health.

* Address correspondence to this author at the Center for Biochemical and Biophysical Sciences and Medicine, Seeley G. Mudd Building, 250 Longwood Avenue, Boston, MA 02115.

[1] Abbreviations: ACE, angiotensin-converting enzyme; hTACE, recombinant human testis ACE; HK-ACE, ML-ACE, RL-ACE, and RT-ACE, human kidney, mouse lung, rabbit lung, and rabbit testis ACE, respectively; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Fa-FGG, 2-furanacryloyl-L-phenylalanylglycylglycine; Hepes, N-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LH-RH, luteinizing hormone releasing hormone; Dnp-F, 1-fluoro-2,4-dinitrobenzene; Dnp, dinitrophenyl; OP, 1,10-phenanthroline; TFA, trifluoroacetic acid; buffer A, 50 mM Hepes, pH 7.5/0.3 M NaCl; HPLC, high-performance liquid chromatography.

0006-2960/91/0430-7118$02.50/0