Asymmetric Interaction between Rod Cyclic GMP Phosphodiesterase γ Subunits and αβ Subunits*

Received for publication, September 9, 2004, and in revised form, January 18, 2005
Published, JBC Papers in Press, January 24, 2005, DOI 10.1074/jbc.M410380200

Lian-Wang Guo‡, Jennifer E. Grant‡, Abdol R. Hajipour‡‡, Hakim Muradov¶, Marty Arbabian‡, Nikolai O. Artemyev¶, and Arnold E. Ruoho‡‡

From the ‡Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706, the ‡Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, and the ¶Pharmaceutical Laboratory, College of Chemistry, Isfahan University of Technology, Isfahan, 84156, I. R. Iran

Rod phosphodiesterase (PDE6) is the central effector enzyme in vertebrate visual transduction. Holo-PDE6 consists of two similar catalytic subunits (Pαβ) and two identical inhibitory subunits (Pγ). Pβ is the only heterodimer in the PDE superfamily, yet its significance for the function of PDE6 is poorly understood. An unequal interaction of Pγ with Pβ as compared with Pα in the PDE6 complex has not been reported. We investigated the interaction interface between full-length Pγ and Pαβ, by differentiating Pγ interaction with each individual Pαβ subunit through radiolabel transfer from various positions throughout the entire Pγ molecule. The efficiency of radiolabel transfer indicates that the close vicinity of serine 40 on Pγ makes a major contribution to the interaction with Pαβ. In addition, a striking asymmetry of interaction between the Pγ polycationic region and the Pαβ subunits was observed when the stoichiometry of Pγ versus the Pαβ dimer was below 2. Preferential photolabeling on Pβ from Pγ position 40 and on Pα from position 30 increased while lowering the Pγ/Pαβ ratio, but diminished when the Pγ/Pαβ ratio was over 2. Our finding leads to the conclusion that two classes of Pγ binding sites exist on Pαβ, each composed of GAF domains in both Pα and Pβ, differing from the conventional models suggesting that each Pγ binds only one of the Pαβ catalytic subunits. This new model leads to insight into how the unique Pαβ heterodimer contributes to the sophisticated regulation in visual transduction through interaction with Pγ.

Cyclic GMP (cGMP)1 phosphodiesterase in retinal photoreceptors, classified as PDE6 in the PDE families, is a key enzyme in vertebrate phototransduction. PDE6 mediates the photoreponse from rhodopsin to cGMP-gated ion channels, which eventually results in vision through neuronal activities (1). The subunit composition of rod PDE6 is unique within the PDE superfamily. The rod PDE6 holoenzyme is composed of two homologous catalytic subunits (Pαβ) and two identical inhibitory subunits (Pγ). The Pαβ catalytic subunits form the only heterodimer among the PDE families, although even the cone PDE6 has a catalytic homodimer Pαa’ (2). Under dark conditions in the rod photoreceptor cells, the two γ subunits bind Pαβ to inhibit the catalytic activity of PDE6. Upon reception of one photon of light by rhodopsin and activation of transducin, Pγ is displaced from the catalytic site of Pαβ by interaction with the transducin α subunit (Gαt), and PDE6 is thus activated (2). Following GTP hydrolysis on Gαt, which is greatly accelerated by the GTPase-accelerating protein complex of RGS9–1/Gβ5/R9AP (3), Pγ is released from Gαt and re-inhibits Pαβ, and thus Pαβ returns to an inactive state, ready for the next round of light activation (1). Therefore, because of the critical role of the Pγ/Pαβ interaction in turning on and off the rod photoreponse, the question of how Pγ binds the Pαβ heterodimer is an important issue for understanding how the activation and inactivation of PDE6 is precisely regulated in visual signal transduction. The rationale for different Pγ binding properties with Pα and Pβ arises from the following facts. 1. Although Pα and Pβ share high homology in their catalytic domains, these two subunits are 27% heterogeneous in amino acid sequence mainly in the N-terminal-half (4) containing the GAF domain (cGMP phosphodiesterases, adenylyl cyclases, and the Escherichia coli protein Fh1A) (5), which is a structural module for regulation and signal transduction (6). In accordance with the heterogeneous GAF sequences, the GAF domains of Pα and Pβ may have somewhat different conformations because mild trypsinization truncates Pβ in GAFa domain without cleaving Pα GAFa domain (7, 8). 2. The noncatalytic GAF domain plays a regulatory role in catalytic activity of PDE6, and this regulation is mediated by binding of Pγ in concert with cGMP (9). Two classes of cGMP binding sites and two classes of Pγ binding sites have been reported (10, 11). 3. Thus far, no homodimers of Pαα or Pββ has been found to be functional, although their potential presence as minor isoforms has not been ruled out, indicating the essential role of the Pαβ heterodimer in rods (12).

Despite extensive studies of PDE6, heterogeneity of the Pα and Pβ catalytic subunits, in terms of their interactions with the Pγ in the PDE6 complex, has been poorly understood. It has been reported that the N-terminal Pγ binding regions on Pα and Pβ were different, but the binding was investigated using short Pα and Pβ peptides (13, 14). A direct demonstration of different Pγ/Pαβ interactions in the PDE6 complex has not been shown. The difficulty for obtaining differentiated struc-
ture/function information for Pα and Pβ is because of the lack of atomic structure and the further lack of an efficient expression system to obtain recombinant Pαβ heterodimers for analysis by mutagenesis. Additionally, these two subunits are always tightly associated with each other to achieve their functions. Once separated, their function is lost. The PDE6 activity assay is a very popular method used for the study of Pγ/Pαβ interactions (2), but there is no readily available approach by which Pα and Pβ functions can be differentiated. The recent electron microscopy (EM) structures of rod Pαβ associated with δ subunit (15) and Pαβ with bound Pγ (16) have revealed the arrangement of the distinct domains of PDE6, the tandem two GAF domains and the catalytic domain. However, details of the association between Pγ and the Pα and Pβ subunits remain vague because of the low resolution (2.8 nm) of the EM-derived structures.

In our approach, the reversible Pγ photoprobes, prepared with $^{125}$I-labeled ACTP (N-[3-iodo-4-azidophenylpropioamido-S-(2-thiopyridyl)] cysteine) (17), provide powerful tools to differentiate Pα and Pβ in their interactions with Pγ. In this study, 13 Pγ photoprobes that were generated as mixed disulfides with single cysteine substitutions at various positions throughout the entire Pγ molecule enabled us to systematically explore the Pγ interaction interface with Pγ. Strikingly, we found a preference of radiolabel transfer to one subunit over the other from the polycationic region of Pγ following DTT reduction of the photocrosslinked Pγ/Pαβ complex, when we lowered the Pγ/Pαβ molar ratio to less than 2. Our data support a new view of interaction between the Pγ and Pαβ subunits in the rod PDE6 holoenzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—All the restriction enzymes and chitin beads were purchased from New England Biolabs. Pfu-Turbo DNA polymerase was from Stratagene, TPCK-treated trypsin was from Promega. Oligonucleotides were from Integrated DNA Technologies. BDM (2-(Na-benzoylbenzamido)-Pγ6(6-biotinamidocaproyl)-t-(lysylamido) ethyl methylamiothanesulfonate) was from Toronto Research Chemicals. Na$^{+}$-V1 was from New England Nuclear. Antibodies against Pα and Pβ are products of Affinity Bioreagents. Anti-Pα is against the bovine Pα-N terminal sequence 1–16 (MGEVTAEEVEKFLDSN); Anti-Pγ is against the mouse Pγ N-terminal sequence 20–36 (HQQFGKKSLSPVANGA). All other reagents were purchased from Sigma unless stated elsewhere.

**Preparation of the Single Cysteine Pγ Construct**—The full-length Pγ-intein fusion construct was prepared using the IMPACT system obtained from New England Biolabs. The Pγ sequence was ligated into pTXB1 at SapI and NdeI restriction sites, in-frame with the intein domain. Site-directed mutagenesis was performed by the QuikChange method using Pfu-Turbo DNA polymerase to mutate the single cysteine at position 68 on wild-type Pγ into alanine, and this cysteine-less construct was used as a template to substitute amino acids at various positions on Pγ with single cysteines. All the constructs were confirmed by DNA sequencing, which was carried out in the Biotechnology Center at University of Wisconsin, Madison. The plasmids were transformed into E. coli strain BL21 DE3 cells (Novagen) for overexpression. The cells in LB culture were induced for 4–5 h at 30 °C after adding 0.5 mm isopropyl-1-thio-β-D-galactopyranoside at A$_{600}$ = 1. The cells were then lysed by sonication, and the lysate cleared by centrifugation was applied to the chitin affinity column. After extensive washing, Pγ was eluted with 140 mM β-mercaptoethanol by incubation at room temperature overnight. The eluted Pγ was ~90% pure as assessed by SDS-PAGE. This purification was further purified by reverse-phase HPLC (Waters 616/626 LC System) using a PEEK column (4.6 mm D/100 mm L, Applied Biosystems) self-packed with POROS 20 R2 Perfusion Chromatography Resin (PerSeptive Biosystems). Anti-Pγ/H9251 and the PDE6-containing extract was concentrated by ultrafiltration using a YM-30 Amicon membrane. The PDE6 catalytic heterodimer (Pαβ) was prepared by mild tryptic proteolysis of holo-PDE6 followed by Mono Q ion exchanger chromatography to remove proteolytic Pγ fragments. This preparation was >95% pure as judged from Coomassie Blue-stained SDS-PAGE.

**ACTP Derivatization of the Single Cysteine Pγ Constructs and Activity Assay**—The radiosynthesis of 460 Ci/mmol $^{125}$I-ACTP was accomplished by the reaction of 125I-3-iodo-4-azidophenylpropionyl succinimide (AIPPS) and cysteine thioglytropyl (CTP) at room temperature for 12 h, based on the method described by Dhanasekaran et al. (17). 125I-ACTP was purified by silica gel thin layer chromatography.

The derivatization reactions for the single Pγ cysteine constructs were carried out by incubating purified Pγ constructs with 46Ci/mmol $^{125}$I-ACTP (1:20 molar ratio) 20 h overnight at room temperature in a solution containing 10 mM Na$_2$PO$_4$, pH 6.5 and 100 mM NaCl. The derivatized Pγ constructs were purified with AutoSeq G-50 spin column (Amersham Bioscience). The radioactivity of each derivatized Pγ was determined by measuring the radioactivity with PhosphorImager (445 SI, Molecular Dynamics), and protein amount from scanned Coomassie Blue-stained gels.

To assess function of the ACTP-modified Pγ probes, the nonradioactive Pγ derivatives were prepared using $^{127}$I-ACTP. The derivatization reaction included 50 mM Na$_2$PO$_4$ (pH 6.7), 50 mM acetonitrile, and 0.2% 2-fold more than Pγ. The reaction mixture was incubated under argon and at room temperature for 3 h in the dark, and then subjected to reversed phase HPLC for purification of the $^{127}$I-ACTP-derivatized Pγ using a Vydac C4 column (214MS510). An acetonitrile gradient of 0.125% per minute was applied to separate $^{127}$I-ACTP-Pγ (eluted at ~45% acetonitrile) from underivatized Pγ at a flow rate of 1 ml/min. The Pγ probe derivatized with the benzophenone capped CTP at position 40 was prepared under similar conditions.

To characterize the ACTP-Pγ derivatives, electrospray ionization mass spectrometry was carried out in Department of Chemistry at University of Wisconsin, Madison. The mass spectra were obtained with a Micromass (Beverly, MA) LTC mass spectrometer using a time-of-flight analyzer.

The inhibition of cyclic GMP hydrolysis by the $^{127}$I-ACTP-Pγ derivatization was determined using PDE assay that utilizes [3H]cGMP, based on the method previously described (2). Briefly, 5 μl Pαβ subunits were incubated in 80 μl of 20 mM Tris-HCl (pH 8.0) buffer containing 50 mM NaCl, 1 mM MgSO$_4$, and 2 mM β-mercaptoethanol, 0.1 units of bacterial alkaline phosphatase, 200 μl [3H]cGMP (100,000 cpm) in the presence of increasing concentrations of the ACTP-Pγ derivatives at 25 °C. After addition of [3H]cGMP, the reaction was allowed to proceed for 10 min, and was stopped by the addition of AG1-X2 cation exchange resin (0.5 ml of 20% bed volume suspension). Samples were incubated for 10 min at 25 °C with occasional mixing and spun at 9,000 × g for 2 min. Aliquots of 0.25 ml were removed for counting in a scintillation counter. To determine Kᵢ values, the data were fit to the equation $Y = B_{max} × (X/Kᵢ + X)$ where $X$ is the free Pγ concentration, and $B_{max}$ is the maximal inhibition.

**Photocrosslinking of ACTP-Pγ Derivatives with Pβ**—The photocrosslinking reactions were performed in the buffer UB (10 mM HEPES, pH 7.5, 120 mM NaCl, 5 mM MgCl$_2$) containing 3 μM Pαβ and $^{127}$I-ACTP-Pγ. The reaction mixture was incubated on ice for 15 min and then photolyzed for 6 s in ice water at a distance of 10 cm from a water-jacketed AH-6 1-kilowatt high pressure mercury lamp. To reverse the disulfide bond, the sample buffer was added to a final concentration of 50 mM DTT and 0.5% SDS immediately after photolysis or after subsequent Pγ truncation with trypsin. The proteins were separated by SDS-PAGE 16.5% gel, and the $^{127}$I-label transfer was detected by PhosphorImager.

To separate the Pα and Pβ subunits by SDS-PAGE, the N terminus of Pαβ was truncated by limited trypsinization on ice following a photocrosslinking reaction. Typically, ~4 μg of Pαβ was incubated with 2 μl of TPCK-treated trypsin on ice for 2.5 h in buffer UB, and then 5 μl soybean trypsin inhibitor was added, and the reaction maintained on ice for 10 min to terminate the trypsin digestion.

**Western and Far-Western Blotting**—For Western blotting, the protein samples were prepared in 5% SDS sample buffer, and subjected to 12% SDS-PAGE. The secondary antibodies were rabbit anti-Pγ (5,000× dilution) and anti-Pβ (1,000× dilution) antibodies. The antibody-antigen complexes were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (50,000× dilution) and enhanced chemiluminescence (ECL) reagents obtained from Pierce.

Biotin label was detected by far-Western blotting using streptavidin-conjugated horseradish peroxidase (Kirkegaard & Perry Laboratories).
and ECL reagents. The separated proteins were transferred to the same brand of polyvinylidene difluoride membranes as were used for Western blotting. Horseradish peroxidase was diluted up to 200,000-fold.

**Analytical Methods**—The Py concentration was measured using an extinction coefficient of 6990 at 280 nm, or by the Bradford method using the Bio-Rad reagent and then corrected based on the 280 nm spectrophotometric measurement. Poβ concentration was measured by the Bradford method using IgG as a standard. Proteins on the scanned Coomassie Blue-stained gels were quantitated using NIH Image 1.62.

**RESULTS**

The Vicinity of Py Ser^10 Is a Major Site of Interaction with Poβ—In our study, two major Py binding regions with Poβ were revealed by detection of the direct interaction between Py and Poβ using the full-length Py probes. The region on Py that provided a major contribution to the interaction with Poβ, as determined by label transfer, was defined to be in close vicinity to serine 40.

To map the whole spectrum of interaction of full-length Py with Poβ subunits, we generated 13 Py constructs with single cysteines at various positions from N-terminal position 3 through the very C-terminal position 87. The single cysteine substitutions occurred in the Py molecule approximately every 5–10 residues (Fig. 1A). The radiiodinated, photoreactive, and disulfide-reversible cross-linker 125I-ACTP (Fig. 1B) was used to derivatize each single cysteine through a mixed disulfide bond. Fig. 1C shows that the 125I-ACTP-Py derivatives were effectively purified free of excess unreacted 125I-ACTP by use of AutoSeq G50 spin columns. The mixed disulfide linkage formed between 125I-ACTP and single cysteine on Py was confirmed by DTT reversal (data not shown). The relatively uniform derivatization of Py constructs by 125I-ACTP (Table I) and the high sensitivity of the 125I radiolabel made the detection and quantitation of label transfer from Py to Poβ quite convenient and accurate.

To assess the functional activity of the derivatized Py photoprob, the nonradioactive Py derivatives were synthesized using 127I-ACTP. These derivatives were purified and estimated to be at least 90% pure by HPLC. Their masses were confirmed by electrospray ionization mass spectrometry (ESI/MS) (Table I), and the functional activity was determined by inhibition of PDE6 cGMP hydrolysis. The 127I-ACTP derivatives of Py have a PDE6 inhibitory activity similar to that of the wild-type (wt) Py (Table I).

Using the radiolabeled ACTP derivatives of single cysteine Py constructs, we were able to compare the interactions of various sites on Py with Poβ on the same SDS-PAGE after photocrosslinking and DTT reversal (Fig. 2). The specificity of Py-Poβ cross-linking was demonstrated by the fact that no 125I-label transfer occurred in the dark control and that very low background label transfer was observed in the photocrosslinking reaction with the holoenzyme of PDE6, which contained two endogenous Py molecules prebound (Fig. 2A).

This observed basal label transfer was very likely caused by the exchange of 125I-ACTP-Py and the endogenous Py during the dark incubation prior to photolysis. Furthermore, the fact that photolysis did not cause label transfer to BSA, which was included in the photocrosslinking reactions as an internal control (Fig. 2B), also strongly demonstrates the specificity of radiolabel transfer to Poβ from 125I-ACTP-Py after photocrosslinking and disulfide reversal.

Taking into consideration the specific radioactivity of the Py photoprobe (Table I), the profiling of photolabel transfer from various positions on Py to Poβ (Fig. 3A) indicates two major regions on Py that interacted with Poβ, the central polycationic region and the C-terminal region. The validity of the label transfer approach in this study is further supported by the consistency of our data with the previous observations by others. It is well established that there are two major regions of Py that interact with Poβ, the C-terminal region that primarily includes the Py residues from Cys^86 to the very C-terminal residue Ile^87, and the central polycationic region spanning the residues from Val^21 to Lys^45. It has been suggested that the polycationic region enhances overall Py affinity to Poβ by binding to the GAF domain while the C terminus of Py binds to the catalytic site of Poβ thus maintaining the enzyme in an activated state (18, 21, 22). Through kinetic measurements of PDE6 activity using synthetic peptides corresponding to various regions in the Py molecule, Mou and Cote (9) showed that the Py central region mainly accounted for the binding affinity with the Poβ GAF domains. Our data obtained from full-length Py (Fig. 3) are consistent with the two-region interaction pattern and also revealed that the Py polycationic region provided a major contribution to the affinity of Py-Poβ interaction. Moreover, a probable site on Py effecting the strongest interaction with Poβ was further defined to the vicinity around position 40. Since the spacer arm from cysteine on Py to the photoreactive moiety (estimated to be 9–12 Å) confers flexibility to some extent, the site on wild-type Py that

![Fig. 1. 125I-ACTP derivatization at various single cysteine positions throughout the Py molecule.](http://www.jbc.org/)
Asymmetric Interaction between Pγ and Pβ

The predicted masses of the Pγ constructs were calculated using PeptideMass of ExPASy (Swiss Institute of Bioinformatics). Each measured mass represents an average of at least 5 ESI MS peaks (± S.D.). The constant difference (±8) between the predicted and measured masses is likely caused by the calibration of mass spectrometry.

Table I
Characterization of the ACTP-Pγ derivatives

<table>
<thead>
<tr>
<th>Single cysteine positions</th>
<th>125I-ACTP-Pγ-specific radioactivity</th>
<th>ESI mass</th>
<th>125I-ACTP-Pγ derivatives</th>
<th>PDE inhibitory activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPM/µg Pγ</td>
<td></td>
<td>Predicted</td>
<td>Measured</td>
</tr>
<tr>
<td>Wt</td>
<td>N/A</td>
<td></td>
<td>9,997.4 ± 0.6b</td>
<td>10,042.0 ± 0.2</td>
</tr>
<tr>
<td>Leu3</td>
<td>19,457</td>
<td>10,050</td>
<td>10,056.8 ± 0.3c</td>
<td>10,056.8 ± 0.3</td>
</tr>
<tr>
<td>Leu60</td>
<td>19,910</td>
<td>10,016</td>
<td>10,084.2 ± 0.2</td>
<td>10,084.2 ± 0.2</td>
</tr>
<tr>
<td>Val16</td>
<td>16,569</td>
<td>10,076</td>
<td>10,068.6 ± 0.2</td>
<td>10,068.6 ± 0.2</td>
</tr>
<tr>
<td>Ser40</td>
<td>19,264</td>
<td>10,016</td>
<td>10,099.4 ± 0.3</td>
<td>10,099.4 ± 0.3</td>
</tr>
<tr>
<td>Trp70</td>
<td>6,572</td>
<td>9,971</td>
<td>9,970.9 ± 2.1</td>
<td>9,970.9 ± 2.1</td>
</tr>
<tr>
<td>Cys68</td>
<td>13,837</td>
<td>10,066</td>
<td>10,414.1 ± 0.1</td>
<td>10,414.1 ± 0.1</td>
</tr>
<tr>
<td>Val21</td>
<td>19,910</td>
<td>10,044</td>
<td>10,043.6 ± 0.2</td>
<td>10,043.6 ± 0.2</td>
</tr>
<tr>
<td>Leu76</td>
<td>12,639</td>
<td>10,043</td>
<td>10,414.1 ± 1.5</td>
<td>10,414.1 ± 1.5</td>
</tr>
</tbody>
</table>

Table footnote:
- a The wt-Pγ (68C) sample that is not ACTP-modified.
- b A mass adduct of 328 Da has been constantly observed with the wt-Pγ construct, which has a single cysteine at position 68.
- c Not measured.
- d These masses were measured at a different time than the other constructs.

**FIG. 2.** Specific photolabeling of Pβ from various positions on the Pγ molecule. A, photolabeling of Pβ is compared on the autoradiogram of the photocrosslinking reactions with 125I-ACTP-40C and Pβ (a and b) or holo-PDE6 (c) after photolysis (a and c) or dark incubation (b). Samples of Pγ and Pβ that contained the same amount of protein were used for the three conditions, and all the reactions were subjected to the same SDS-PAGE. B, Coomassie Blue-stained SDS-PAGE (upper panel) and autoradiogram (lower panel) showing Pβ labeling by 125I-ACTP derivatized Pγ from various positions. The positions are listed between the panels corresponding to the lanes. The first lane is BSA as a size standard. In the other lanes, the upper band is Pβ, the lower band is BSA, which was included in the photocrosslinking reactions as an internal control. The photocrosslinking reactions were performed with an approximate 2:1 stoichiometry of Pγ/Pβ dimer.

**FIG. 3.** Profile of Pβ labeling from various positions on the Pγ molecule. A, profile of Pβ labeling by 125I-ACTP-Pγ derivatives. The percentage of Pβ labeling by 125I-ACTP-Pγ from various positions is expressed relative to labeling from position 40. The specific radioactivity of each 125I-ACTP-Pγ was determined (Table I), and the amount of label transfer to Pβ was normalized. The data are presented as an average of four separate experiments (± S.D.). B, Kd for inhibition of cGMP phosphodiesterase activity by the 125I-ACTP-Pγ derivatives. The data are from Table I. The dotted line indicates the Kd of wt-Pγ.

interacts with Pβ is likely close to but may not be precisely at the covalent derivatization position.

The Pγ Polycationic Region Binds Pα and Pβ Subunits Asymmetrically—By performing radioactive photolabel transfer experiments with Pαβ and full-length Pγ photoprobe and subsequent truncation of Pβ, we were able to differentiate the binding property of each ACTP derivatization site on Pγ from various positions expressed relative to labeling from position 40. The specific radioactivity of each 125I-ACTP-Pγ was determined (Table I), and the amount of label transfer to Pβ was normalized. The data are presented as an average of four separate experiments (± S.D.). B, Kd for inhibition of cGMP phosphodiesterase activity by the 125I-ACTP-Pγ derivatives. The data are from Table I. The dotted line indicates the Kd of wt-Pγ.
The appropriate $\beta$ truncation was confirmed by immunoblotting. As shown in Fig. 5, only a very small amount ($<5\%$) of $\beta$ remained after trypsin truncation, as detected with antibody against the N terminus of $\beta$. The truncated $\beta$ of ~70 kDa (the lower Coomassie Blue band) was not detected by immunoblotting because the N terminus, which includes the antigenic sequence, was removed.

We observed a significant labeling preference to $\beta$ from $\gamma$ positions 40, 50, and 60, and a preference to $\alpha$ from positions 21 and 30, when the photocrosslinking reactions were performed with a $\beta/\alpha$ ratio at ~1 (only one $\beta$ per $\alpha$ dimer) (Fig. 4). These positions encompass the central polycationic region of $\gamma$. In contrast, $\alpha$ and $\beta$ were almost equally labeled from the positions in the C terminus. The $\alpha$ labeling from the N terminus also appeared equal, but considering the low level of label transfer, the significance of equal labeling from the N-terminal positions is not clear. No obvious labeling was found in the 17-kDa band, which is likely the truncated portion of $\beta$ (data not shown). These data also indicate that the observed labeling preference on $\alpha$ from $\gamma$ positions 30 and 21 was not due to removal of a labeled N-terminal fragment of $\beta$. This conclusion is further confirmed by the data in Fig. 6B that the labeling ratio of $\beta$ versus $\alpha$ from $\gamma$ position 30 was close to 1 (~0.93) at high $\beta/\alpha$ ratio, indicating that there was no significant loss of photolabeling because of the N-terminal truncation of $\beta$. Regarding positions 40, 50, and 60, if there were labeling on the $\beta$ N terminus that was removed by truncation, the preference of labeling on $\alpha$ would have been even more remarkable than observed here.

To address the possibility that the labeling preference by $\gamma$ photoprobes was caused by the selectivity of the nitrene-generating photoreactive moiety of ACTP toward certain $\alpha/\beta$ residues, an additional photoprobe, BBM, with benzophenone instead of phenyl azide as a photoreactive group, was used to carry out similar photocrosslinking experiments (Fig. 7). Benzophenone reacts with the nearby C-H bonds of protein via a nonspecific free radical mechanism upon photolysis. Using BBM as a photoprobe, the asymmetric labeling of $\alpha$ and $\beta$ from $\gamma$ position 40 was still observed. In addition, $\alpha$ and $\beta$ was normalized based on the specific radioactivity of each $^{125}$I-ACTP-$\gamma$ probe, and the average of all the probes, with $p$ values of <0.02.
Asymmetric Interaction between Py and Paβ

**DISCUSSION**

This work demonstrates that in the bovine rod PDE6 complex the Py polycationic region binds the Pa and Paβ catalytic subunits asymmetrically. Apparently, the vicinity of Ser40 is the key interaction site with Paβ, which shows labeling preference to Paβ over Pa, whereas position 30 shows preference to Pa over Paβ. Our finding offers a reasonable explanation for the heterogeneous nature of the GAF domains in the two catalytic subunits, indicating that Paβ may have a different GAF domain structure than Pa and thereby plays a different role with respect to interaction with Py.

GAF Domains May Provide the Asymmetric Binding Sites for the Polycationic Region of Py—There are several lines of evidence to support the conclusion that the positions in the Py polycationic region that showed unequal binding with Pa and Paβ have binding sites in the GAF domains. First, direct interaction of Py position 23 with the PDE6 GAFa domain was observed (23), and the C-terminal positions on Py were found to interact with the catalytic domain of PDE6 (8, 22, 24). In addition, the Py structure was found to be extended when bound to Paβ using fluorescence resonance energy transfer (FRET) (25). These observations support a linear pattern of Py/Paβ interactions, with the Py polycationic region binding to the Paβ GAF domain and the C terminus binding to the catalytic domain. Second, using synthetic Py peptides, Mou and Cote (9) found that the N-terminal half of Py bound Paβ with 50-fold greater affinity than the C-terminal inhibitory region, and the N-terminal half, particularly the polycationic region, was responsible for the positive cooperativity between Py binding and cGMP binding in the GAF domain, but it had no effect on catalytic activity. Their data suggested that the polycationic region of Py, which provides the major binding energy for Py/Paβ, interacts with the Paβ GAF domain. Consistent with their results, our data show that the major photolabeling of Paβ occurred from the Py polycationic region (Fig. 3). Third, the GAF domain may account for the major heterogeneity between the Pa and Paβ subunits. Compared with the sequence-conserved catalytic domains, the GAF domains of Pa and Paβ show high heterogeneity in their amino acid sequences. When subjected to trypsinization, Paβ is cleaved in the GAFa domain before the GAF domain in Pa is cleaved (Figs. 4 and 5) (8), indicating a different structure of the GAF domain in Paβ than Pa. Therefore, it is reasonable to propose that the heterogeneity of GAF domains in Pa and Paβ facilitates an asymmetric interaction with the two Py molecules. In agreement with this proposition, our data indicate that the central part of Py accounted for the asymmetric interaction with Paβ (Fig. 4). On the other hand, the symmetric interaction of C terminus of Py with Paβ is consistent with the high sequence homology of the catalytic domains in Pa and Paβ. In addition, the GAF domain has been found to be the regulatory region in Paβ. Several observations further indicate that within Py it is the central portion encompassing the polycationic region that plays a regulatory role. Various modifications on Py, such as phosphorylation and ADP-ribosylation, which involve a variety of regulatory activities, occur in this central region (26, 27). It is therefore reasonable to conclude that the regulatory role of the polycationic region of Py is achieved through asymmetric binding to the regulatory Paβ GAF domains.

Implication of Two Classes of Py Binding Sites, Each Made Up of Both Pa and Paβ GAF Domains—The GAF domain is considered as the major regulatory region not only in PDE6 but also in other PDE families, such as PDE2 and PDE5. For PDE2 and PDE5, binding of cGMP to the GAF domain allosterically regulates the catalytic activity of the catalytic domain (28). It has been recently reported that PDE5 was directly activated upon cGMP binding to the GAFa domain, and this effect did not require PDE5 phosphorylation (29). Unlike PDE5 and PDE2, direct effects of cGMP binding in the noncatalytic GAF domain on the catalytic activity of PDE6 have not been demonstrated. However, cGMP binding to the PDE6 GAFa domain may modulate the interaction of Paβ with Py, and thus affect PDE6 activity indirectly.

It had been previously observed that there were two classes of Py/Paβ binding sites (11), and two classes of noncatalytic cGMP binding sites with different binding affinities to Paβ (10). The recent work by Cote and co-workers (30, 31, 9) has revealed that the Py binding sites and cGMP binding sites were positively cooperative and these sites were likely all in the GAF domain.
domain, and Muradov et al. (32) have identified the Pγ binding surface and the cGMP binding pocket within the PDE6 GAFα domain. However, these studies did not differentiate Pα and Pβ functions. The composition of the two classes of binding sites has not been well defined.

Our data regarding asymmetric interaction between Pγ and Pαβ indicate that the two classes of Pγ binding sites may be actually each made up of both the Pα and Pβ GAF domains. When the Pγ concentration was lower than 1 Pγ per Pαβ dimer, there was a strong labeling preference for Pα from position 30, indicating a preferential interaction between the Pγ Phe30 region and Pα. On the other hand, the strong labeling preference for Pβ from position 40 indicates a preferential interaction between the Pγ Ser40 region and Pβ. (Fig. 6B).

The nearly equal labeling of Pα and Pβ from the Pγ C-terminal position 73 most likely resulted from the exceptionally high sequence homology between the two catalytic domains in Pα and Pβ. When the Pγ polycationic region is bound to the Pαβ GAF domains, the high catalytic domain sequence homology may confer equal interaction for the Pγ C terminus to transfer label to the two catalytic domains (assuming a flexibility in the Pγ segment between the polycationic region and the C terminus). In fact, this segment is exceptionally glycine rich (5 glycine residues over 15 amino acids from Gly39 to Gly43), which very likely confers flexibility, consistent with a FRET study indicating that the N-terminal half of free Pγ beyond position 68 has a random conformation (25). Additionally, the solution structure of the N-terminal half of the free Pγ molecule has been found to be highly disordered by nuclear magnetic resonance (NMR) analysis. Furthermore, an EM study has shown that the Pα and Pβ catalytic domains are in close proximity (15), indicating that it does not take much flexibility in this segment to facilitate a nearly equal binding of Pγ C terminus to the Pα and Pβ catalytic domains when the Pγ polycationic region binds to the GAF domain. Alternatively, Pγ position 73 may interact with the catalytic domain of one of the Pαβ subunits in a manner that allows the ACTP photoprobe to reach and photolabel the corresponding site of the other subunit, because of the flexibility of the ACTP arm and the proximity of the two catalytic domains.

For Pγ to bind the GAF domains in both Pα and Pβ simultaneously, one would expect that either the Pα or Pβ subunits or the Pγ subunits in the holo-PDE6 complex must crossover in the GAF region. A direct evidence for a crossover structure of the PDE GAF domains has been reported from the recent crystal structure of mouse PDE2 GAF homodimer (33). Therefore, based on previous studies that have suggested a linearly elongated Pγ molecule in its interaction with the Pαβ subunits (8, 22, 23, 24, 25) and the elongated organization of the domains in Pα and Pβ when Pγ is bound (16), and from the data presented in this report, we propose two probable models for Pγ/Pαβ interaction (Fig. 8) to explain our data. One model is illustrated in Fig. 8A, in which the high affinity interaction is the Phe30 region to Pα GAFα and the Ser40 region to Pβ GAFα, and the low affinity interaction is the Phe3 region to Pγ GAFα and the Ser40 region to Pγ GAFβ (that is; the complementary side, not shown). Alternatively, Pγ interacts at the Ser40 region with Pβ GAFβ (see dashed line, Fig. 8A). The other model is illustrated in Fig. 8B, in which two Pγ molecules bind to the two GAFα domains comparably, but position 30 binds to the GAFα domain of Pα with higher affinity and position 40 preferentially binds to the GAFα domain of Pβ. According to this model the polycationic region and the C terminus of the same Pγ molecule bind the opposite catalytic subunits, and an assumption of the Pγ flexibility is not necessary. Further investigation on differentiation of the asymmetric interactions of the Pγ polycationic region with the Pαβ GAF domains may solve the longheld puzzle of two classes of Pγ binding sites.

In summary, from our results a new point of view regarding Pγ/Pαβ interaction emerges. This model differs from the conventional parallel binding models, in which one Pγ binds to only one catalytic subunit. In our proposed cross-binding model, one Pγ binds to both catalytic subunits simultaneously, and two distinct Pγ binding sites on Pαβ with different affinities are thus formed. Considering the cooperation of Pγ and cGMP binding in the GAF domain, it would not be surprising if cGMP binding to the GAF domains would also be found to be asymmetric.

The synergistic and asymmetric binding of Pγ and cGMP in the GAF domain might be an important regulatory mechanism in rod visual transduction. The low affinity site may be essential for rapid activation of PDE6 upon light activation of rhodopsin, since GoT can remove Pγ from Pαβ more efficiently from the low affinity site. Once cGMP level becomes very low Because of activation of PDE6, Pγ molecules at the high affinity sites would also dissociate from Pαβ because of the lack of positive cGMP cooperativity. However, regulation of guanylate cyclase in the Ca2+ feedback loop brings cGMP concentration back to a certain level (34), and then Pγ could re-bind Pαβ.

2 Fariba Assadi-Porter, personal communication.

![Fig. 8. Proposed models for asymmetric interaction between Pγ and Pαβ.](image-url)

A. position 30 of Pγ binds to Pα GAFα domain and position 40 to Pβ GAFα while position 73 binds to the catalytic domain in Pβ. The dashed Pγ represents an alternative interaction with position 40 binding to GAFβ instead of GAFα. The other Pγ molecule is proposed to bind on the complementary side of Pαβ (not shown in the figure). B, both positions 30 and 40 of Pγ bind to GAFα domain in Pα, while position 73 binds to the catalytic domain. Cyan, Pα; red, Pβ; blue, Pγ; circle, GAFα; oval, GAFβ; square, catalytic domain; dot, Pγ positions interacting with Pαβ as pointed by the arrows.
Asymmetric Interaction between Py and Poβ

rapidly through synergistic binding of GMP at the high affinity site. In this regard, the high affinity site is likely to be essential for returning PDE6 to the inactive state quickly, thereby facilitating the next round of photoresponse. The exceptionally tight binding of PPy (Kd \( \sim 1 \) pm) (35) is important to maintain a very low background of photoresponse, which is critical for rod-supported night vision.

Acknowledgments—We thank Dr. A. E. Granovsky, who worked with N. O. A., for preparation of some of the Poβ samples. Thanks go to Dr. M. M. Vestling at University of Wisconsin-Madison for performing ESI MS. We also thank Dr. M. Sievert in the laboratory of A. E. R. for his help in preparation of the manuscript.

REFERENCES
Asymmetric Interaction between Rod Cyclic GMP Phosphodiesterase γ Subunits and αβ Subunits

doi: 10.1074/jbc.M410380200 originally published online January 24, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M410380200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 22 of which can be accessed free at
http://www.jbc.org/content/280/13/12585.full.html#ref-list-1