The Catalytic and GAF Domains of the Rod cGMP Phosphodiesterase (PDE6) Heterodimer Are Regulated by Distinct Regions of Its Inhibitory γ Subunit*

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The central effector of visual transduction in retinal rod photoreceptors, cGMP phosphodiesterase (PDE6), is a catalytic heterodimer (αβ) to which low molecular weight inhibitory γ subunits bind to form the nonactivated PDE holoenzyme (αβγ). Although it is known that γ binds tightly to αβ, the binding affinity for each γ subunit to αβ, the domains on γ that interact with αβ, and the allosteric interactions between the regulatory and catalytic domains on αβ are not well understood. We show here that the γ subunit binds to two distinct sites on the catalytic αβ dimer (K_D1 < 1 ps, K_D2 = 3 ps) when the regulatory GAF domains of bovine rod PDE6 are occupied by cGMP. Binding heterogeneity of γ to αβ is absent when cAMP occupies the noncatalytic sites. Two major domains on γ can interact independently with αβ with the N-terminal half of γ binding with 50-fold greater affinity than its C-terminal, inhibitory region. The N-terminal half of γ is responsible for the positive cooperativity between γ and cGMP binding sites on αβ but has no effect on catalytic activity. Using synthetic peptides, we identified regions of the amino acid sequence of γ that bind to αβ, restore high affinity cGMP binding to low affinity noncatalytic sites, and retard cGMP exchange with both noncatalytic sites. Subunit heterogeneity, multiple sites of γ interaction with αβ, and positive cooperativity of γ with the GAF domains are all likely to contribute to precisely controlling the activation and inactivation kinetics of PDE6 during visual transduction in rod photoreceptors.

The extent and lifetime of activation of the photoreceptor cGMP PDE6 (PDE6; EC 3.1.4.35) must be precisely regulated in rod and cone cells to control the exquisite sensitivity, speed, and adaptational properties of the visual transduction pathway in vertebrate photoreceptors. The membrane-associated rod photoreceptor PDE6 consists of a dimer of two homologous catalytic subunits (Pαβ) to which two low molecular weight inhibitory subunits (Pγ) bind (holoenzyme stoichiometry, αβγγ). The catalytic subunits contain GAF domains that are responsible for high affinity, noncatalytic binding of two cGMP molecules/holoenzyme. It is well established that relief of the inhibitory constraint on PDE6 arises from the binding of activated heterotrimeric G protein (transducin) to Pγ following photoactivation of the visual pigment, rhodopsin (reviewed in Refs. 1–4). However, the strength of the interaction between Pγ and Pαβ has been difficult to quantitate, and K_D values vary widely (from picomolar (5–7) up to nanomolar values (8, 9)). In addition, it has not been conclusively demonstrated whether both Pγ molecules bind with equal affinity to Pαβ to form the nonactivated holoenzyme (although two different binding sites on Pαβ have been inferred using mutant Pγ (10)). Finally, recent evidence suggests that binding of activated transducin to PDE6 relieves inhibition at only one of the two active sites, further supporting the idea of catalytic subunit heterogeneity with respect to Pγ binding (11, 12).

Use of synthetic peptides to defined regions of Pγ and mutagenesis of the Pγ subunit have revealed that Pγ has multiple sites of interaction with rod Pαβ, with the α subunit of activated transducin (α_b*) and with the RGS-9 (regulator of G protein signaling 9). The C-terminal residues of Pγ (amino acids 77–87) have been shown to interact directly with the catalytic sites of Pαβ to inhibit catalysis (7, 13–15). A second major site of interaction between Pγ and Pαβ has been identified in the lysine-rich central portion (amino acids 24–45) of the Pγ sequence (13, 16–19), but the function of this interaction is unclear. α_b* has been shown to interact with Pγ at two distinct regions: one in its C-terminal domain (amino acid residues 65–87) and one in the central, lysine-rich region of Pγ (13, 14, 16, 20–22). Finally, Pγ also serves to potentiate the GTPase-activating protein function of RGS-9 by interacting with the protein in the neighborhood of Trp^70 (21–23).

In this paper, we show that the K_D of Pγ binding to rod PDE6 is in the picomolar to subpicomolar range. Furthermore, Pγ does not bind with equal affinity to the two sites on Pαβ when cGMP occupies the noncatalytic regulatory sites located on the Pαβ dimer. We also demonstrate that the central region of Pγ stabilizes high affinity cGMP binding to the noncatalytic sites, and the affinity of the central region of Pγ exceeds by 50-fold the affinity of its C-terminal region. Finally, using a series of synthetic peptides, we identify important residues in the central region that contribute to the stabilization of Pγ binding to Pαβ.

EXPERIMENTAL PROCEDURES

Materials—Bovine retinas were purchased from W. L. Lawson, Inc. The Pγ mutant, Pγ1–45C (consisting of the first 45 amino acids of

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1 The abbreviations used are: PDE, cyclic nucleotide phosphodiesterase; LY, Lucifer Yellow; GTPγS, guanosine 5′-O-(thio)triphosphate; Pαβ, catalytic heterodimer of PDE6; Pγ, 10-kDa inhibitory γ subunit of rod PDE6; K_D, dissociation constant; K_m, Michaelis constant; K_cat, catalytic; K_i, inhibition constant; GAF, a protein domain named for its occurrence in cGMP-regulated phosphodiesterases, Adenylyl cyclases, and the E. coli protein, FlhA.
bovine rod PDE6 plus a C-terminal cysteine residue (24) was a kind gift of Dr. N. P. Skiba, whereas zaprinast was generously supplied by Rhone-Poulenc Rorer (Dagenham, UK). Crude synthetic peptides were prepared at New England Peptide or at the protein facility at the University of New Hampshire. The radiochemicals were from PerkinElmer Life Sciences. Gel electrophoresis and immunoblotting supplies were from Bio-Rad. Ultima Gold scintillation fluid was from Packard Instrument Co., filtration and ultrafiltration products were from Millipore, protein assay reagents were from Pierce, and all other chemicals were from Sigma.

Preparation of PDE6—Membrane-associated bovine rod PDE6 was purified to >90% homogeneity from frozen bovine retinas as described previously (25). The resulting nonactivated PDE holoenzyme (PDE6) was prepared by limited proteolysis of the PDE subunits followed by Mono Q ion-exchange chromatography to remove proteolytic fragments of PDE6 (25). The PDE6 preparation was >90% pure as judged from Coomassie-stained SDS-polyacrylamide gel electrophoresis. No shift in the apparent molecular weight of the α or β subunit was observed following this treatment. (Attempts to prepare PDE6 without resorting to proteolytic digestion of PDE were unsuccessful.) Immunoblot analysis with an anti-peptide PDE6 antibody (UNH9710) directed to the C-terminal region (amino acids 63–87) of PDE6 revealed no detectable full-length PDE6, a 5-kDa band representing the major proteolytic product of PDE6 exhibited <5% of the original PDE6 immunoreactivity (25, 26). To remove endogenous cGMP bound to the noncatalytic regulatory sites on PDE6, the PDE6 was incubated at 37 °C for 30 min prior to use.

Preparation and Purification of PDE6 and P1-45C—Full-length bovine rod PDE6 or the N-terminal fragment, P1-45C, was expressed in *Escherichia coli* using the pET11a expression vector (21) and purified as described previously (4, 25). The total PDE6 concentration was initially determined spectrophotometrically (ε₄₉₀ = 7550 cm⁻¹) and verified by assaying its inhibitory activity (25); these two estimates varied by <10%. The concentration of P1-45C was measured with a colorimetric protein assay (27). To fluorescently label P1-45C with Lucifer Yellow (P1-45C-LY), the procedure of Artemyev et al. (20) was used, followed by purification of P1-45C-LY by reverse-phase high pressure liquid chromatography.

Peptide Purification—Following automated peptide synthesis, peptides were released from the resin with acetic acid-95% and lyophilized. Crude, acidic peptides (P55-75 and P63-87) were first purified by anion-exchange chromatography using DEAE Sephadex A-25 and a linear NaCl gradient at pH 7.5. Other peptides were initially purified by cation-exchange chromatography on CM Sephadex C25 using a linear NH₄HCO₃ gradient at pH 8.0. All peptides were further purified by reverse-phase high pressure liquid chromatography on a 22 × 250-mm, 300 angstrom C₁₈ column (Vydac), using a linear gradient of 30–70% acetonitrile in 0.1% trifluoroacetic acid. After lyophilization, each peptide was resuspended in 10 mM Tris, pH 7.5, and its concentration was determined with the bicinchoninic acid protein assay (27) using bovine serum albumin as a standard.

Analytical Procedures and Data Analysis—The concentration of PDE6 and its catalytic activity were determined as described in detail elsewhere (25, 26). Bovine serum albumin (250 μg/ml) was added when assaying enzyme concentrations in the subnanomolar range to prevent loss of activity. A membrane filtration assay was used to determine the equilibrium and kinetic properties of cGMP binding to high affinity noncatalytic sites on PDE6 (25, 26). The PDE inhibitor, zaprinast (0.1–1.0 mM), was included in the assay solution to ensure that <10% hydrolysis of [³H]cGMP occurred. Nonspecific binding was determined as described previously (28). The fluorescence assay of P1-45–45C-LY binding to PDE6 was conducted in an Amino-Bowman Series 2 spectrofluorometer using an excitation wavelength of 430 nm and an emission wavelength of 520 nm (20).

Except where noted, experiments were performed three times, and the results are presented as the means ± S.E. Nonlinear regression analysis of the data was performed using Sigmaplot 2000 (SPSS, Inc.).

Fig. 1A and B, two different curve-fitting equations were used to fit the PDE binding data. The first equation represents the standard equilibrium binding equation for a single class of noninteracting sites.

\[
B = \frac{B_{\text{max}} [P\gamma]}{K_{D} + [P\gamma]} \quad \text{(Eq. 1)}
\]

where \(B\) is the amount of Pγ bound, \(B_{\text{max}}\) is the maximum extent of binding, \(K_{D}\) is the dissociation constant, and the free Pγ concentration, [Pγ].

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**FIG. 1. Inhibition of catalytic activity by Pγ at various concentrations of Pδβ.** Pδβ and Pγ were prepared as described under "Experimental Procedures." A, the ability of increasing amounts of Pγ to bind to and inhibit hydrolysis of cGMP was determined at the following Pδβ concentrations: 1.0 pm (●), 5.0 pm (▲), 10.0 pm (▲), 50.0 pm (▼), 200 pm (▲), and 500 pm (○). The substrate concentration was 100 μM cGMP at ±50 pm Pδβ or 2.0 μM at 200 and 500 pm Pδβ. The curves represent the fit of the data to Equation 2. B, the affinity of Pγ binding to Pδβ (apparent \(K_{D}\)) was estimated using either Equation 1 (○) or Equation 2 (▲) at each indicated Pδβ concentration. The points represent individual determinations except for 1 pm (mean ± S.E., n = 6).

[Pγ], is assumed to be approximated by the total added Pγ concentration (i.e. \([P\gamma] = [P\gamma]_{T}\)). Because the free Pγ concentration was not experimentally measured, Equation 1 can only be used to estimate the \(K_{D}\) when ligand depletion from solution is not significant. For high affinity binding reactions, this condition is met when the total number of binding sites (\(P_{D}\)) is near the \(K_{D}\). However, if we substitute the conservation of mass equation ([Pγ]ₜ = \(B + [P\gamma]ₜ\)) into Equation 1, we can estimate the \(K_{D}\) for any \(P\gamma\) without having to assume that \([P\gamma] = [P\gamma]ₜ\).

\[
B = \frac{B_{\text{max}}([P\gamma] - x + \sqrt{x^2 - 4P_{D}[P\gamma]ₜ})}{2} \quad \text{(Eq. 2)}
\]

where, \(x = -K_{D} - P_{D} - [P\gamma]ₜ\). This analytical approach was previously used to study high affinity binding of GTPγS to transducin (29).

**RESULTS**

Quantitative Analysis of Pγ Binding to Pδβ—Given the >100-fold range of reported \(K_{D}\) values for Pγ binding to PDE6 in the literature (see the Introduction), we suspected that stoichiometric binding of Pγ to Pδβ was occurring in those instances where the Pδβ concentration exceeded the intrinsic \(K_{D}\) value for the binding reaction. To directly test this, we examined the ability of Pγ to inhibit catalysis as a function of the Pδβ concentration (Fig. 1A). At Pδβ concentrations >±50 pm, there was a linear increase in the extent of inhibition up to the plateau as the Pγ concentration was increased; the concentra-
tion of Py needed to attain >90% inhibition was approximately twice the concentration of Pab. This behavior represents a titration phenomenon in which added Py stoichiometrically binds to Pab with very high affinity until essentially all of the binding sites are occupied (2 mol Py/mol Pab). At Pab concentrations ≈10 pM, the inhibition curve departed from stoichiometric behavior, and an excess of Py was needed to fully inhibit the enzyme (Fig. 1A).

The Py binding data in Fig. 1A were analyzed using two equations for equilibrium binding of Py to Pab. The first approach (Equation 1) assumed that the concentration of free Py, [Py], was approximately equal to the total added Py concentration, [Py]tot (i.e. no ligand depletion). When Equation 1 was used to estimate the binding affinity of Py at each concentration of Pab tested, we found that the value of the apparent Kp increased in direct proportion to the concentration of Pab (Fig. 1B, circles). For example, at 1 pM Pab, the calculated Kp for Py binding was 1.6 pM, whereas at a concentration of 500 pM Pab, the curve fitting to Equation 1 gave an apparent Kp of 3.0 ± 0.1 pM.

We re-examined the Py binding data with Equation 2 (which is not constrained by the assumption that [Py] = [Py]tot) to estimate the apparent Kp for Py binding. Within the concentration range of 1–500 pM Pab, the calculated Kp showed little dependence on the Pab concentration. The convergence of the two estimates for the apparent Kp as the Pab concentration was lowered to 1 pM indicated that both Equation 1 and Equation 2 provided similar estimates when P < Kp and when the free Py concentration could be approximated as the total Py concentration. We conclude that the most reliable condition for determining the binding affinity of Py is when the Pab concentration is in the low pM range.

Heterogeneity in Py Binding to Pab—Closer examination of the results at the lowest Pab concentration we could easily test (1 pM) indicated that the two Py binding sites on Pab were not identical when we assayed Py inhibition of cGMP hydrolysis (Fig. 2, circles). When 1 pM Pab was incubated with up to 1 mol Py/mol Pab, stoichiometric binding of Py to Pab was observed. This is evident from the fact that the data fit a straight line for the first 50% of the binding curve (Fig. 2, dotted line). After binding 1.0 mol Py/mol Pab, the remaining Py binding sites required an excess of free Py to fully inhibit Pab. Attempts to fit the entire binding curve to Equation 1 (Fig. 2, upper dashed line) or Equation 2 failed to generate statistically valid regression coefficients; therefore we fit the data to a modified form of Equation 1 that included two independent classes of binding sites. This approach gave the best fit to the experimental data (Fig. 2, solid line) and resolved two classes of Py binding sites with Kp values of 0.3 ± 0.1 pM and Kp2 = 6.6 ± 2.3 pM.

Fig. 2. Py binding affinity at 1 pM Pab reveals two classes of binding sites in the presence of cGMP. Pab (1.0 pM) was incubated with various concentrations of Py at 4 °C for 10 min, and then the extent of inhibition of cGMP (●, 100 μM concentration; n = 7) or cAMP (▲, 500 μM concentration; n = 6) hydrolysis was determined at 22 °C. The cAMP data were well fit as a single class of Py binding sites using either Equation 1 (dashed line, Kp = 3.7 ± 0.3 pM, Bmax = 1.80 ± 0.03 mol Py bound per mol Pab) or Equation 2 (Kp = 2.6 ± 0.3 pM, Bmax = 1.76 ± 0.04 Py/Pab; not shown). The cGMP data did not fit a single site model using either Equation 1 (dashed line) or Equation 2 (not shown), as judged by statistical analysis of the regression. For values of total added Py of ≤1.0, the binding curve fit a linear model (dotted line), consistent with stoichiometric binding (Kp < 1 pM) of 1 mol Py/mol Pab in the presence of cGMP. A two-site model was also used (by extension of Equation 1) to fit the entire data set (solid line) and resolved the binding curve into two classes of sites with Kp1 = 0.3 ± 0.1 pM and Kp2 = 6.6 ± 2.3 pM.

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Fig. 3. cAMP binding affinity for the noncatalytic sites of bovine rod Pab in the absence of Py or upon addition of Py or Py1–45. 1 μM [3H]cGMP and the indicated concentrations of cAMP were added to 10 nM Pab in the absence of Py (▲) or following preincubation with 20 nM Py (●) or 10 μM Py1–45 (●), mean ± range for n = 2. The ability of cAMP to compete with [3H]cGMP binding to noncatalytic sites was determined by filter binding, and the data were fit to a single class of noninteracting sites to obtain the following Kc and Bmax values, respectively: Pab, 4.0 mM, 0.9 cGMP/Pab; Pab+Py, 0.4 mM, 2.0 cGMP/Pab; Pab+Py1–45, 0.3 mM, 2.0 cGMP/Pab. The Kc for cAMP binding was calculated from the Kc value (59) using Kc = 60 nm for cGMP binding.

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**TABLE I**

| Condition | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | Hill coefficient |
|-----------|-------|----------|---------------|------------------|
| cGMP substrate | 14 ± 1.0 | 5440 ± 80 | 3.9 × 10^6 | 0.9 ± 0.1 |
| cAMP substrate | 910 ± 100 | 3060 ± 100 | 3.4 × 10^6 | 0.9 ± 0.1 |
| cAMP substrate + cGMP | 830 ± 60 | 3160 ± 70 | 3.8 × 10^6 | 1.0 ± 0.1 |

*To load cGMP on the noncatalytic sites of P66, 200 nM P66 was first incubated with 5 μM cGMP, 100 μM zaprinast, and 10 μM P1–45. To remove unbond low molecular weight compounds, the P66 was concentrated by ultrafiltration. The P66 was washed three times by resuspending in buffer (containing P1–45) and reconstituting. The final P66 preparation contained 1.1–1.6 mol cGMP bound per mol P66.*

The presence of 500 μM cAMP, we found that Pγ binds to P66 with a single $K_m$ value of 3 μM (Fig. 2, triangles). No evidence of binding site heterogeneity or cooperativity was evident when Pγ interaction with P66 was assayed based on its inhibition of cAMP hydrolysis. This implies that cAMP binding to noncatalytic sites does not affect Pγ binding to P66. In contrast, binding of cGMP to noncatalytic sites induces an allosteric transition that increases Pγ binding affinity ≈10-fold to one site on P66. The other Pγ binding site maintains an affinity for P66 similar to the $K_m$ observed when cAMP is present.

Lack of Direct Allosteric Communication between the Catalytic and Noncatalytic Sites on Bovine P66—Because cGMP binding induces a conformational change in P66 that enhances Pγ affinity, we hypothesized that the GAF domain might also allosterically regulate hydrolytic activity in the catalytic domain (in analogy to the cGMP-stimulated PDE, PDE2 (31, 32)). To test this, we compared the kinetic parameters of P66γ using cGMP or cAMP as the substrate. Table I shows that when cGMP is the substrate, P66γ achieves a catalytic efficiency ($k_{cat}/K_m = 4 × 10^{-6}$ μM$^{-1}$ s$^{-1}$) approaching the diffusion-controlled limit for a bimolecular collision. PDE6 has a 100-fold greater specificity for cGMP compared with cAMP, as judged by the decrease in the $k_{cat}/K_m$ value when cAMP is the substrate. Most of the reduction in substrate specificity for cAMP can be ascribed to the 65-fold increase in its $K_m$ value (Table I). For both cGMP and cAMP, no cooperativity could be detected (Table I), but this is not unexpected because the high affinity noncatalytic site on P66γ is occupied over most of the concentration range we were able to test.

To directly test whether cGMP occupancy of the noncatalytic sites affected catalysis, we first preincubated P66γ with cGMP and the N-terminal half of Pγ (P1–45) to load cGMP onto both noncatalytic sites. (P1–45 has no effect on catalysis of P66γ (data not shown) but stabilizes high affinity cGMP binding at both noncatalytic sites on P66γ; see below.) The complex of P66γ with bound P1–45 and cGMP was then incubated with increasing concentrations of cAMP to determine its kinetic parameters. No significant change in $K_m$, $k_{cat}$, or the Hill coefficient could be discerned when compared with P66γ incubated with cAMP alone (Table I). These results confirm and extend previous observations with amphibian PDE6 (33, 34) that no direct allosteric mechanism regulates catalysis via the state of occupancy of the GAF domains on PDE6, as is the case for PDE2 (31, 32). Thus, the differences in the $K_m$ and $k_{cat}$ values for cAMP and cGMP are due to differences in substrate specificity at the active site not on the state of occupancy of the GAF domain.

The N-terminal Half of Pγ Binds to P66γ with Much Greater Affinity than the C-terminal Region—Previous work has documented that two major domains of interaction with P66γ exist on Pγ: the C-terminal residues and the polycationic central region (see the Introduction). To understand how distinct binding domains on Pγ contribute to the very high affinity binding of Pγ to P66γ, we assayed the ability of these individual domains of Pγ to bind to P66γ. We first assayed the ability of the C-terminal peptide, P63–87, to bind to and inhibit cyclic nucleotide hydrolysis at the active site. Based on previous work (15), we expected that P63–87 would act as a simple competitive inhibitor with respect to cyclic nucleotides. When cAMP is used as substrate, P63–87 acts as a simple competitive inhibitor of catalysis with an inhibition constant ($K_I$) equal to 3.5 μM (Fig. 4). The affinity of this inhibitory domain is 6 orders of magnitude weaker than the entire Pγ molecule ($K_D = 3$ μM), indicating that the N-terminal 62 amino acids most likely contain the major site(s) of high affinity interaction.

To directly measure the binding affinity of the N-terminal half of Pγ to P66γ, we assayed the interaction of P1–45 covalently labeled with Lucifer Yellow (P1–45-LY) with P66γ. Fig. 5A demonstrates that either in the absence of cyclic nucleotides or in the presence of cAMP, P66γ binds to P1–45-LY as a single class of binding sites with a $K_D$ of 68 ± 15 or 62 ± 14 nM, respectively. In both cases, the maximum extent of binding was the same.

To test how the fluorescent probe altered the binding affinity of P1–45, we also tested the effectiveness of P1–45 in restoring high affinity cGMP binding to the low affinity site on Pγ (25). In the absence of Pγ or P1–45, P66γ is able to bind 1 mol cGMP/mol P66γ at micromolar levels of [3H]cGMP (Fig. 3, triangles). The addition of increasing amounts of P1–45 in the presence of 600 nM [3H]cGMP stimulates cGMP binding to a second site on P66γ (Fig. 5B) with a $K_D$ of 80 nM, a value in good agreement with the measured $K_D$ for binding of P1–45-LY to P66γ.

We conclude that the N-terminal half of Pγ (P1–45) binds to P66γ about 50-fold more tightly than the C-terminal region (P63–87). Furthermore, if each binding domain were to interact with P66γ independently, the sum of the interaction energies of P1–45 and P63–87 ($K_D = 10^{-13}$ M) would account reasonably well with the measured $K_D$ for full-length Pγ.

**Relationship between Occupancy of the GAF Domain and Pγ Binding Affinity—**In Fig. 2, we showed that heterogeneity in Pγ binding to P66γ (assayed by inhibition of cyclic nucleotide hydrolysis) was dependent on the state of occupancy of the noncatalytic sites. The cGMP-dependent enhancement of full-length Pγ binding affinity is also observed with the fluorescently labeled N-terminal half of Pγ. Fig. 5A shows that P1–45-LY undergoes a 2.3-fold increase in binding affinity, with no significant change in maximum binding, when the GAF domains are occupied by cGMP compared with empty or cAMP-filled sites. Thus, occupancy of the noncatalytic site by cGMP is required to induce the conformational change in P66γ that enhances Pγ binding affinity.

These results and our previous study with bovine rod P66γ (25) are in accord with a simple reciprocal relationship: cGMP occupancy of the noncatalytic sites enhances Pγ affinity to one of its binding sites and, conversely, Pγ binding to P66γ enhances cGMP binding affinity to its low affinity noncatalytic site. Un-
expectedly, this correlation does not apply when cAMP occupies the noncatalytic sites. Fig. 3 shows that the addition of Py or Py1–45 to Pαβ enhances 10-fold the ability of cAMP to compete with cGMP for binding to both noncatalytic sites. This suggests that Py binding induces a conformational change in Pαβ that enhances the affinity of both cGMP and cAMP for Pαβ. However, Fig. 5A reveals that cAMP occupancy of the noncatalytic sites is not sufficient to induce an increase in Py1–45-LY binding to Pαβ. It appears that cGMP, but not cAMP, is needed to induce a conformational change in Pαβ that enhances Py binding affinity. This conclusion is supported by experiments with cGMP analogs that show intermediate effects on the relationship between cyclic nucleotide occupancy and enhanced Py binding affinity.2

Defining the Amino Acid Residues Responsible for High Affinity Interactions of Py with Pαβ—We prepared a set of synthetic peptides corresponding to various regions of the Py primary sequence to identify regions of Py that stabilized high affinity binding of Py. We first measured the ability of these peptides to compete with full-length, endogenous Py bound to nonactivated PDE6, as judged by the increase in catalytic activity at the active site. Fig. 6A shows that two peptides, Py1–45 and Py18–41, were able to compete with full-length Py to activate PDE6 to 70–80% of its maximal rate and with \( K_{D1} \) values of 1 and 11 \( \mu M \), respectively. The Py21–46 peptide showed a significant drop in its ability to compete with Py when compared with Py18–41, suggesting that amino acid residues 18–20 may stabilize a peptide conformation of Py18–41 that enhances its interaction with Pαβ. Several other peptides were able to stimulate cGMP hydrolysis to a limited extent (20–40% activation) and with low affinity (\( K_{D2} \approx 400–500 \mu M \)), including Py1–18, Py10–30, and Py35–56. The peptide Py55–75 was completely ineffective in competing with Py. These results indicate that the interaction of Py with Pαβ may consist of multiple low affinity sites of interaction along the N-terminal half of the Py molecule, with high affinity interactions occurring in the region of amino acid residues 18–41.

We showed above that the N-terminal half of Py, Py1–45, could restore high affinity cGMP binding to a low affinity class of noncatalytic sites on Pαβ (Figs. 3 and 5B). To better define this region on Pαβ, we tested the ability of peptides to bind to Pαβ and restore high affinity cGMP binding to this class of sites. Fig. 6B shows that Py18–41 and Py21–46 both fully restored cGMP binding to Pαβ with a binding affinity reduced ~10-fold compared with Py1–45. Py10–30 was also able to fully restore cGMP binding, but its interaction with Pαβ was 10-fold further reduced. Even though Py15–26 failed to interact with Pαβ to restore cGMP binding, the overall results with several other peptides suggest that residues 18–30 of Py are important in stabilizing Py binding to Pαβ.

We also tested peptides covering amino acids 35–56 of the Py sequence (Fig. 6B). Although full restoration of cGMP binding could not be observed with Py35–56 or smaller peptides, all three peptides showed \( K_{D} \) values in the 20–30 \( \mu M \) range. The effectiveness of the shortest peptide, Py35–41, to stimulate cGMP binding to Pαβ indicates that these residues are important in direct binding to Pαβ and in stabilizing a high affinity conformation of the second noncatalytic site on PDE6.

Although Py1–45 has 10-fold higher affinity for Pαβ than Py18–41, we could not detect any interaction of the peptide Py1–18 with Pαβ in terms of restoring high affinity cGMP binding (Fig. 6B). It is possible that some of the N-terminal

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2 H. Mou and R. H. Cote, unpublished observations.
residues may stabilize a conformation of Pγ1–45 that favors its binding to Paβ compared with Pγ18–41. Two other peptides, Pγ55–75 and Pγ63–87, completely lacked the ability to restore cGMP binding even at 300 μM peptide concentrations.

The binding affinity of cGMP for the noncatalytic sites was determined in the presence of 200 μM of each peptide to examine whether the low and high affinity noncatalytic sites could be distinguished (data not shown). All Pγ peptides that were competent to restore cGMP binding to the low affinity sites failed to alter the Kd for cGMP binding (Kd range: 51–63 nM). No evidence for binding site heterogeneity could be detected. We conclude that binding of Pγ or central region Pγ peptides induces a conformational change in the low affinity noncatalytic site that enhances its affinity for cGMP to a value indistinguishable from the high affinity noncatalytic site.

The restoration of cGMP binding shown in Fig. 6B describes the ability of Pγ peptides to act on the low affinity noncatalytic sites on Paβ. To examine both high and low affinity noncatalytic sites, we examined whether Pγ peptides could alter the rate of [3H]cGMP dissociation from the noncatalytic sites of Paβ. Fig. 6C shows that most of the peptides were able to retard the rate of [3H]cGMP dissociation from the noncatalytic sites. In all instances, the dissociation kinetics were fit to a single exponential loss of [3H]cGMP binding; no evidence for two distinct classes of sites was evident (not shown). As expected, Pγ1–45 most effectively retarded cGMP exchange at both noncatalytic sites on Paβ, causing a 16-fold increase in the half-time for [3H]cGMP release from noncatalytic sites. The Pγ peptides Pγ18–41 and Pγ10–30 also retarded cGMP exchange by greater than 10-fold. Comparison of Pγ18–41 (t1/2 = 84 min) with Pγ21–46 (t1/2 = 41 min) supports the importance of amino acid residues 18–20 in stabilizing binding of Pγ to low affinity sites (Fig. 6B). The Pγ1–18 and Pγ15–26 peptides slowed cGMP dissociation—3-fold from the high affinity noncatalytic sites of Paβ (Fig. 6C) without being able to restore cGMP binding to the low affinity site (Fig. 6B) suggests that this region of Pγ may affect only the high affinity GAF domain of PDE6.

**DISCUSSION**

This paper shows that Pγ binds to two distinct high affinity binding sites on Paβ when cGMP occupies the noncatalytic sites. Our work also describes the reciprocal allosteric regulation of PDE6 resulting from Pγ binding to Paβ and cGMP binding to high affinity sites in the GAF domains of Paβ. Because no direct allosteric communication between the GAF domains and the active sites is detected, we conclude that the Pγ subunit is required to facilitate allosteric communication between the regulatory and catalytic domains of PDE6. Finally, we have mapped regions within the N-terminal half of the molecule that interact with Paβ, some of which induce conformational changes at the GAF domain.

**Implications of High Affinity, Two-site Pγ Binding to Paβ for PDE Function**—Our quantitative analysis of Pγ binding to Paβ helps to make sense of the wide range of apparent binding affinities reported in the literature. The majority of studies of the Pγ affinity for Paβ have been carried out with concentrations of enzyme much greater than the Kd value for Pγ binding. Under these conditions, Pγ will bind in a stoichiometric manner (Fig. 1). Even when the appropriate model is applied to the binding data (i.e. Equation 2), it is difficult to extract meaningful values for the Kd values. For example, we were unable to satisfactorily fit data obtained at >800 pM Paβ concentrations to Equation 2, primarily because the estimate of the free Pγ concentration is very uncertain. In previous reports where the PDE concentration was lowered into the picomolar range (5, 6, 34), the binding affinities of Pγ for Paβ were much closer to the values we report. Previous evidence supporting two distinct Pγ binding sites on Paβ has mostly been obtained indirectly, based on heterogeneity in transducin activation of PDE6 (11, 12, 35, 36). However, Berger et al. (10) have reported two classes of Pγ binding sites on Paβ when probed with a fluorescently labeled mutant of Pγ.

The functional heterogeneity in Pγ binding to Paβ is a consequence of the state of occupancy of the noncatalytic sites with cGMP (Fig. 2)—but not cAMP. Because the membrane-associated bovine rod PDE6 holoenzyme has one exchangeable and
one nonexchangeable cGMP binding site (25), we hypothesize that the very high affinity \((K_D < 1 \text{ pm})\) Pγ-binding site may correlate with this nonexchangeable cGMP site. This Pγ-binding site associated with the nonexchangeable cGMP site is unlikely to function during visual transduction. Instead, it may serve a structural role in stabilizing the native conformation of the PDE6 holoenzyme. This idea is supported by studies showing that mutations in the GAF domains of PDE6 (Ref. 37; reviewed in Ref. 38) or a disrupted or mutated Pγ gene (39) can affect the levels of expression and/or activation of PDE6. It might also explain the difficulty in expressing functional PDE6 in various expression systems (40–42) if co-expression of catalytic and Pγ subunits must occur to properly fold the nascent polypeptide chains of PDE6.

**Allosteric Regulation of PDE6 Requires Pγ to Communicate between the GAF Domain and the Catalytic Domain**—In addition to a catalytic domain near the C terminus that is conserved in all members of the vertebrate PDE superfamily, most PDE families contain N-terminal domains that serve regulatory functions. It has been proposed (43) that a common regulatory feature of the N-terminal domain is to alter the inhibitory constraint on catalysis via conformational changes in the catalytic dimer. Almost half of the known PDE families (PDE2, PDE5, PDE6, PDE10, and PDE11) contain two tandem GAF domains (44) that in most cases code for functional, noncatalytic cGMP binding sites. For PDE2 and PDE5, binding of cGMP to the GAF domain induces a conformational change in the catalytic dimer that either directly or indirectly stimulates catalysis (31, 32, 45, 46). For PDE6, we show that loading the GAF domain with cGMP has no direct influence on PDE6 kinetic parameters (Table I).

Nonetheless, cGMP binding to the GAF domain of PDE6 does induce a conformational change in the catalytic dimer. Occupancy of the noncatalytic sites enhances the interaction of Pγ (Fig. 2) or Pγ1–45 (Fig. 5A) to the catalytic dimer. This effect is specific for cGMP (Fig. 5A). Conversely, the addition of Pγ to bovine rod Pαβ enhances cyclic nucleotide binding affinity to the noncatalytic sites (Fig. 3) in a reciprocal manner. For cGMP, Pγ acts to restore high affinity binding to a low affinity class of sites on bovine Pαβ; the other, high affinity class of cGMP sites undergoes no change in affinity (25). For cAMP, addition of Pγ or Pγ1–45 to Pαβ increases the binding affinity 10-fold to both noncatalytic sites on Pαβ (Fig. 3).

We conclude that unlike PDE2 or PDE5, the GAF domain in the PDE6 catalytic subunit is allosterically uncoupled from its catalytic domain unless Pγ is bound to bridge the two domains. Furthermore, the heterogeneity in Pγ binding to Pαβ suggests that only one Pγ binding site on Pαβ is sensitive to cGMP occupancy of the noncatalytic sites. Future efforts will identify which catalytic subunit contains the cGMP-sensitive Pγ binding site and the high affinity noncatalytic site on Pαβ.

**Pγ Is a Multi-functional Subunit with Several Sites of Interaction with the PDE6 Transduction Complex**—The Pγ subunit of PDE6 contains within its 87-amino acid sequence numerous sites of interactions with Pαβ, transducin, RGS-9, and perhaps other proteins (see the Introduction). In this study, we have focused on Pγ-Pαβ interactions and find that the N-terminal half of Pγ binds 50-fold more tightly to the catalytic dimer than to the C-terminal region (Pγ63–87). This high affinity binding domain in the region of residues 18–41 of Pγ explains why αβ* activation of the PDE6 holoenzyme sometimes displaces the inhibitory constraint of Pγ without causing complete dissociation of Pγ from Pαβ (5, 47). The correlation of Pγ dissociation from transducin-activated PDE with loss of cGMP from the GAF domain (12) also makes sense based on the reduced binding affinity of Pγ or Pγ1–45 in the absence of cGMP (Figs. 2 and 5).

Within the central region of the Pγ subunit, amino acids 18–20 and 35–41 contribute to stabilizing Pγ binding to Pαβ (Fig. 6). Other Pγ interaction sites probably exist within this polycationic region that went undetected with our selected peptides, including potential sites of regulation via phosphorylation at Thr*22 and/or Thr*35 (48–50) or via ADP-ribosylation at Arg*33 or Arg*36 (51).

Our results demonstrate that the N-terminal 18 amino acids of Pγ play no direct role in inhibiting catalysis or stimulating high affinity cGMP binding to Pαβ (Fig. 6), contrary to a previous study (52). However, the N terminus of Pγ probably contains weak sites of interaction with Pαβ and may help stabilize a high affinity conformation of the 18–41 region of Pγ.

Significantly, the one peptide that shows no detectable interaction with Pαβ, namely Pγ55–75, contains a major site of interaction with activated transducin, αβ* (see the Introduction). A recent structural determination of the interaction of the C-terminal half of Pγ with αβ* has shown that αβ* binds to several residues in the vicinity of Trp*70 of Pγ to cause the displacement of the C-terminal residues of Pγ known to block the active site (22).

**Summary**—Our results extend previous models postulating two major sites of interaction between PDE6 and activated transducin, both of which are mediated through the inhibitory Pγ subunit. The extreme C terminus of Pγ functions to block catalysis at the active site but has relatively low affinity for Pαβ. It is the high affinity interactions of the central, polycationic region of Pγ (particularly residues 18–41) that stabilize binding to Pαβ, thereby insuring that a very small fraction of PDE6 holoenzyme is catalytically active prior to light activation. High levels of cGMP in dark-adapted photoreceptor cells result in saturation of the noncatalytic sites on PDE6, further enhancing Pγ affinity to one binding site on the PDE6 holoenzyme. Upon activation of the phototransduction cascade, binding of activated transducin to the PDE6 holoenzyme, specifically in the vicinity of Trp*70 of Pγ, is sufficient to displace the Pγ C terminus and cause activation at one site on Pαβ. (The very high affinity Pγ binding site \((K_D < \text{pm})\) when cGMP is present likely prevents αβ* displacement of Pγ at the second active site (11, 12).) At early times following light stimulation, αβ* remains associated with the PDE6 holoenzyme and does not physically dissociate as an αβ*-Pγ complex. Following PDE6 activation, cGMP levels in the outer segment drop and remain low until the inactivation process reinitiates PDE6. The positive cooperativity between the GAF domains and the central region of Pγ suggests that a sustained lowering of cGMP levels (e.g., during light adaptation) will lead to cGMP dissociation and a lowered Pγ affinity for Pαβ. This cGMP-dependent allosteric transition could cause the multi-functional Pγ subunit to become available to interact with RGS-9 to facilitate an enhanced GTPase rate on αβ*, consistent with biochemical and structural studies (22, 53–55).

Although homology modeling of the GAF domain (56) and the catalytic domain (57, 58) of PDE6 complement recent structural information on Pγ binding to αβ* (22), we still lack crystal structures revealing the interactions of each Pγ molecule with the α and β subunits of the rod PDE6 heterodimer. The present study provides the biochemical basis for understanding how the central and C-terminal domains of Pγ bridge the regulatory and catalytic domains of the rod PDE6 heterodimer to control the magnitude and duration of PDE activation during visual excitation, recovery, and light adaptation.

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