Identification of the γ Subunit-interacting Residues on Photoreceptor cGMP Phosphodiesterase, PDE6α’*

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Photoreceptor cGMP phosphodiesterase (PDE6) is the effector enzyme in the G protein-mediated visual transduction cascade. In the dark, the activity of PDE6 is shut off by the inhibitory γ subunit (Pγ). Chimeric proteins between cone PDE6a’ and cGMP-binding and cGMP-specific PDE (PDE5) have been constructed and expressed in Sf9 cells to study the mechanism of inhibition of PDE6 catalytic activity by Pγ. Substitution of the segment PDE5-(773–820) by the corresponding PDE6α’-(737–784) sequence in the wild-type PDE5 or in a PDE5/PDE6α’ chimera containing the catalytic domain of PDE5 results in chimeric enzymes capable of inhibitory interaction with Pγ. The catalytic properties of the chimeric PDEs remained similar to those of PDE5. Ala scanning mutational analysis of the Pγ-binding region, PDE6α’-(750–760), revealed PDE6α’ residues essential for the interaction. The M758A mutation markedly impaired and the Q752A mutation moderately impaired the inhibition of chimeric PDE by Pγ. The analysis of the catalytic properties of mutant PDEs and a model of the PDE6 catalytic domain suggest that residues Met758 and Gln752 directly bind Pγ. A model of the PDE6 catalytic site shows that PDE6α’-(750–760) forms a loop at the entrance to the cGMP-binding pocket. Binding of Pγ to Met758 would effectively block access of cGMP to the catalytic cavity, providing a structural basis for the mechanism of PDE6 inhibition.

Two regions of Pγ are principally involved in the interaction with the PDE6 catalytic subunits, the central polycationic region (residues 24–45 of rod Pγ) and the Pγ C terminus. The C terminus of Pγ constitutes the key inhibitory domain, whereas the polycationic region enhances the overall affinity of Pγ toward PDE6 catalytic subunits (11–14). A cross-linking study localized the Pγ C-terminal binding site on PDE6α to residues 751–763 (residues 749–761 of PDE6β or PDE6α’) within the broader PDE6 catalytic domain (15). Our further analysis of the interaction between fluorescently labeled Pγ and PDE6αβ suggests that the C terminus of Pγ inhibits PDE6 activity by physically blocking the PDE catalytic site (16).

Progress in the investigation of the structure/function of PDE6 and the mechanism of PDE6 inhibition by Pγ has been slowed by the lack of an efficient expression system for PDE6 (17, 18). Our approach to developing a system for PDE6 expression and mutagenesis included the construction of chimeras between PDE6α’ and cGMP-binding, cGMP-specific PDE (PDE5 family) (19). PDE5 and PDE6 share a common domain organization, i.e. two noncatalytic cGMP-binding sites located N-terminally to the conserved PDE catalytic domain (20). Furthermore, PDE5 and PDE6 display a high homology (45–48% identity) between catalytic domains, a strong substrate preference for cGMP, and similar patterns of inhibition by competitive inhibitors such as zaprinast, dipryridamole, and sildenafil (20–23). Unlike PDE6, PDE5 is readily expressed using the baculovirus/insect cell system (24, 25). Earlier, we reported (19) the functional expression and characterization of a chimeric PDE6α’/PDE5 enzyme containing the PDE6α’ noncatalytic cGMP-binding sites and the PDE5 catalytic domain. In this study, we generated chimeric PDE6α’/PDE5 enzymes that contain the Pγ C-terminal binding site and that are potently inhibited by Pγ. Ala scanning mutational analysis of the Pγ-binding site, using chimeric PDE as a template, revealed the key interaction residues and provided structural justification for the mechanism of PDE6 inhibition.

EXPERIMENTAL PROCEDURES

Materials—cGMP was obtained from Roche Molecular Biochemicals. 3H[cGMP] was a product of Amersham Pharmacia Biotech. All restriction enzymes were purchased from New England Biolabs. AmpliTaq® DNA polymerase was a product of PerkinElmer Life Sciences, and Pfu DNA polymerase was a product of Stratagene. Rabbit polyclonal His probe (H-15) antibodies were purchased from Santa Cruz Biotechnology. Zaprinast and all other reagents were purchased from Sigma.

Preparation of Pγ—The Pγ subunit was expressed in Escherichia coli and purified on a SP-Sepharose fast flow column and on a C-4 high pressure liquid chromatography column (Microsorb-MW, Rainin) as described (26). Purified Pγ was lyophilized, dissolved in 20 mM HEPES buffer, pH 7.5, and stored at −80 °C until use.

Cloning of Chi16 and Chi17—the construct for expression of Chi16 (Fig. 1) was obtained using the pFastBacHTbChi4 vector containing cDNA coding for a PDE6α/PDE5 chimera, Chi4 (19). A silent SpeI restriction site (codons for PDE5-Glu775-Leu777-Val779) was introduced into the Chi4 cDNA using a QuickChange® kit (Stratagen) and a pair

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of complementary oligonucleotides encoding for a T → A substitution. The pFastBacHTbChi4 plasmid was used as a template for PCR using a Pfu DNA polymerase. The PCR product was digested with DpnI-specific for methylated and hemimethylated DNA and transformed into E. coli DH5α to generate Chi16, the PDE6α DNA fragment coding for PDE6α-(737–784) was PCR-amplified using a pBluescript-PDE6α vector (8, 19) as a template. The PCR product was cut with SpeI and StuI and ligated into the SpeI/StuI-digested pFastBacHTbChi4-SpeI. To obtain Chi17, the PvuII/SphI fragment from pFastBacHTbChi16 was subcloned into pFastBacHTbPDE5 (19).

Site-directed Mutagenesis of Chi16—A unique NheI site was introduced into a Chi16 cDNA using a QuickChange™ kit (Stratagene). Single amino acid substitutions corresponding to PDE6α residues at positions 750–760 were generated in Chi16 by PCR-directed mutagenesis. To facilitate the screening procedure, mutant primers were designed to either introduce or eliminate a suitable restriction site. For each mutant, the PCR product was obtained using a forward primer containing a mutated codon and a reverse primer carrying the mutant His-Bind resin (Novagen) equilibrated with 20 mM Tris-HCl buffer, pH 7.8, containing 50 mM NaCl and 25 mM imidazole. After sonication using 30-s pulses for a total duration of 3 min, the supernatant (100,000 × g, 45 min) was loaded onto a column with a His-Bind resin (Novagen) equilibrated with 20 mM Tris-HCl buffer, pH 7.8, containing 10 mM imidazole. The resin was washed with a 5× volume of the same buffer containing 500 mM NaCl and 25 mM imidazole. Proteins were eluted with the buffer containing 250 mM imidazole. β-Mercaptoethanol (2 mM) was added to the mixture. Purified proteins were passed against 40% glycerol and stored at -20 °C.

Other Methods—PDE activity was measured using [3H]GMP as described (27, 28). Less than 15% of cGMP was hydrolyzed during these reactions. The K<sub>v</sub> values for inhibition of PDE activity by P<sub>v</sub> and zaprinast were measured using 0.5 μM cGMP (i.e., <0.35% of K<sub>m</sub> value for chimeric and mutant PDE5s). Protein concentrations were determined by the method of Bradford (29), using IgG as a standard, or by using calculated extinction coefficients at 280 nm. The molar concentration of Chi16 and mutant PDEs, [PDE], were calculated based on the fraction of PDE protein in preparations and the molecular mass of 93.0 kDa. The fractional concentrations of PDE were determined from analysis of SDS-polyacrylamide gel electrophoresis (10–12% acrylamide gels) by comparing the migration of PDE protein in preparations and the molecular mass of 93.0 kDa. The fractional concentrations of PDE were determined from analysis of SDS-polyacrylamide gel electrophoresis (10–12% acrylamide gels) by comparing the migration of PDE protein in preparations and the molecular mass of 93.0 kDa. The fractional concentrations of PDE were determined from analysis of the Coomassie Blue-stained SDS gels using a Hewlett-Packard ScanJet II CXT scanner and Scion Image Beta 4.02 software. A typical fraction of PDE in partially purified preparations was 10–15%. The K<sub>cat</sub> values for c-GMP hydrolysis were calculated as k<sub>cat</sub> = V/[PDE]. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (30) in 10–12% acrylamide gels. For Western immunoblotting, proteins were transferred to nitrocellulose (0.1 μm, Schleicher & Schuell) and analyzed using rabbit His probe (H-15) antibodies (19). The antibody-antigen complexes were detected using anti-rabbit or anti-goat/sheep IgG conjugated to horseradish peroxidase and ECL reagent (Amersham Pharmacia Biotech). Fitting the experimental data to equations was performed with nonlinear least squares criteria using GraphPad Prizm Software. The K<sub>v</sub>, K<sub>m</sub>, and I<sub>C50</sub> values are expressed as mean ± S.E. for three independent measurements.

RESULTS

Functional Analysis of Chimeric PDE6α/PDE5 Proteins Containing the Py-binding Site—Previously we demonstrated (19) a functional expression of chimeric PDE6α/PDE5 protein, Chi4, using Baculovirus/Sf9 system. Chi4 contained the regulatory, noncatalytic cGMP-binding domain of PDE6α and the catalytic domain of PDE5 (Fig. 1A). Chi4 was used as a basic template for the generation of new chimeras in which various portions of the PDE5 catalytic domain were replaced by corresponding sequences from PDE6α. Chi16, containing a segment of 48 residues from PDE6α (PDE6α-(737–784)) (Fig. 1), was functionally expressed in Sf9 cells with a yield of soluble protein at ~100 μg/100 ml of culture. Chi16 hydrolyzed cGMP with a K<sub>v</sub> value of 2.8 μM and a k<sub>cat</sub> value of 9.0 s<sup>-1</sup> (Fig. 2A and Table I). Both kinetic parameters of Chi16 were comparable to those of PDE5 and Chi4 (Table I). In addition, Chi16 was potently inhibited by zaprinast, a PDE5/PDE6- specific competitive inhibitor (IC<sub>50</sub> 0.12 μM) (Fig. 2B).

The PDE6α-(737–784) insert includes a segment PDE6α-(749–761) that was previously identified as a binding site for the Py C terminus. The sequence corresponding to PDE6α-(749–761) is unique for photoreceptor PDEs, which show a strong conservation at this site (15). In contrast to PDE5 and Chi4 (19), the catalytic activity of Chi16 was effectively inhibited by Py. The K<sub>v</sub> value of 3.6 nM indicates that Py binds to Chi16 with only a 20-fold lower affinity than the affinity of its interaction with native PDE6α (3) and the IC<sub>50</sub> value for the Chi17 inhibition by zaprinast (0.77 μM) was similar to the IC<sub>50</sub> value for PDE5 but somewhat higher than the IC<sub>50</sub> value for Chi16 (Fig. 2B and Table I). Py inhibited the cGMP hydrolysis by Chi17 less potently than the catalytic activity of Chi16. The maximal inhibition was up to 70% of Chi17 activity, and the K<sub>v</sub> value was 142 nM (Fig. 3). These results suggest that the noncatalytic cGMP-binding domain of PDE6α contributes to the high affinity interaction with Py.

Ala-scanning Mutagenesis of the Py-binding Region—An Ala-scanning mutagenesis of the Py C-terminal binding site in Chi16 was performed to identify the Py-binding residues of PDE6α. Eleven consecutive residues starting at position 750 were substituted with alanine. The Chi16 mutants were expressed in Sf9 cells and partially purified from the soluble fractions using affinity chromatography on a His-Bind resin. The expression levels of soluble Chi16 mutants were 50–100 μg/100 ml culture, i.e., comparable to that of Chi16. All Chi16 mutants were analyzed for their ability to hydrolyze cGMP. Two mutants, L751A and D760A, were catalytically inactive. Two other mutants, P755A and I756A, displayed notably reduced catalytic rates (Table I). In addition to lowering the k<sub>cat</sub>...
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FIG. 2. Catalytic properties of Chi16 and Chi17. A, kinetics of cGMP hydrolysis by Chi16 (▲) and Chi17 (■). PDE activities were determined using 0.1 μCi of [3H]cGMP and increasing concentrations of unlabeled cGMP. The rates of cGMP hydrolysis are expressed as percentage of maximal activity of PDE5 (9.6 mol of cGMP·mol PDE−1·s−1) (19). The kinetic characteristics for Chi16 ($K_m^c = 2.8 \pm 0.5 \mu M, k_{cat} = 9.0 \pm 1.3 s^{-1}$) and Chi17 ($K_m^c = 1.9 \pm 0.3 \mu M, k_{cat} = 9.8 s^{-1}$) were calculated from the fitting curves. B, inhibition of Chi16 and Chi17 activity by zaprinast. Activities of Chi16 (▲) and Chi17 (■) were determined in the presence of 0.5 μM cGMP and increasing concentrations of zaprinast and were expressed as a percentage of respective PDE activity in the absence of zaprinast. The calculated IC₅₀ values for Chi16 and Chi17 were 0.12 ± 0.01 and 0.77 ± 0.02 μM, respectively.

Next, all catalytically active Chi16 mutants were examined for inhibition by Pγ. Most of the mutants retained a functional interaction with Pγ with the $K_i$ values of −0.8 to 5 nM (Table I). Two mutants, Q752A and M758A, were defective in Pγ binding. The Q752A mutation had a moderate effect on interaction with Pγ. Pγ was capable of full inhibition of the Q752A catalytic activity, but the $K_i$ value was increased to 29 nM (Fig. 4C). A major impairment of the Pγ interaction was observed for the M758A mutant. The inhibition of M758A by Pγ was incomplete (−75%) with the $K_i$ value of 97 nM (Fig. 4C). Since the catalytic properties of Q752A and M758A were similar to those of Chi16 (Fig. 4), the defects of Pγ binding are not likely to be caused by alterations in overall folding of the catalytic domain in these mutants.

DISCUSSION

The vertebrate visual transduction cascade is among the most studied and best understood G protein signaling systems. Yet, PDE6, the key enzyme of vision, remains arguably one of the most obscure G protein effectors in terms of understanding its structure/function relationship. Difficulties in the development of an efficient expression system for PDE6 have precluded the systematic mutational analysis of the enzyme (17–19). Our attempts to express functionally wild-type PDE6a' and co-express PDE6a' with Pγ using the Baculovirus/Sf9, COS7, or retinoblastoma Y79 cell systems have also been unsuccessful.2 A construction of chimeric enzymes between PDE6a' and related PDE5 has been proven as a useful tool for the study of PDE6. Previously, we demonstrated that a fully functional chimeric PDE6a'/PDE5 enzyme, containing the PDE6a'/noncatalytic cGMP-binding sites and the PDE5 catalytic domain, can be efficiently expressed in the Baculovirus/insect cell system (19). This chimeric enzyme showed catalytic properties and noncatalytic cGMP-binding characteristics analogous to those of PDE5 and PDE6a', respectively. Chimeric PDE6a'/PDE5 proteins containing the PDE6a'-active site were catalytically inactive, suggesting that the catalytic domain contains specific sequences preventing its functional folding in insect cells. Based on these findings, we generated and analyzed a number of chimeric PDE6a'/PDE5 proteins with replacements of various PDE5 catalytic domain segments by corresponding PDE6a' sequences. A sequence, PDE6a'-(737–784), containing the Pγ C-terminal binding site Pa'-(749–761) (15), has been introduced in one of these chimeras, Chi16 (Fig. 1). Not only was Chi16 catalytically active with $K_m$ and

![Image](86x188 to 261x309)

**FIG. 3. Inhibition of the catalytic activity of Chi16 and Chi17 by Pγ.** The activities of Chi16 (▲) and Chi17 (■) were determined upon addition of increasing concentrations of Pγ, using 0.5 μM cGMP as a substrate. The $K_i$ values from the inhibition curves were 3.6 ± 0.4 nM for Chi16 and 142 ± 13 nM for Chi17.

value for cGMP hydrolysis, the P755A substitution also resulted in an increase in the $K_m$ value from 2.8 to 42 μM (Table I). The catalytic properties of P755A indicate that this mutation likely affected the overall folding of the catalytic site in Chi16. The $K_m$ values for cGMP hydrolysis for the remaining Chi16 mutants were within the 4–15 μM range (Table I). Inhibition of Chi16 mutants by zaprinast revealed no large variations in their IC₅₀ values, which were comparable to the IC₅₀ value for Chi16 (Table I).

![Image](86x188 to 261x309)

**FIG. 4.** Functional properties of Chi16 mutants

| Mutant | $K_m^c$ (μM) | $k_{cat}$ (s⁻¹) | IC₅₀ for zaprinast (nM) | $K_i$ for Pγ (nM) |
|--------|-------------|----------------|----------------------|-----------------|
| PDE6a' | 23 ± 2      | 3500 ± 1       | 0.28 ± 0.05          | 0.17 ± 0.02     |
| PDE5a' | 3.0 ± 0.5   | 9.6 ± 0.75     | NA                   | NA              |
| Chi4a  | 1.5 ± 0.3   | 10.0 ± 0.65    | NA                   | NA              |
| Chi16a | 2.8 ± 0.5   | 9.0 ± 0.12     | 3.6 ± 0.4            | NA              |
| Chi17a | 1.9 ± 0.3   | 9.8 ± 0.77     | 142 ± 13             | NA              |
| V750Aa | 8.7 ± 0.9   | 4.8 ± 0.40     | 0.01 ± 0.07          | 0.78 ± 0.05     |
| L751Aa | NA          | NA             | NA                   | NA              |
| Q752Aa | 12 ± 2      | 8.0 ± 0.20     | 29 ± 4               | NA              |
| Q753Aa | 7.3 ± 0.8   | 9.5 ± 0.27     | 4.2 ± 0.7            | NA              |
| Q754Aa | 11 ± 1      | 7.3 ± 0.15     | 2.1 ± 0.4            | NA              |
| Q755Aa | 42 ± 6      | 0.8 ± 0.19     | 0.72 ± 0.1           | NA              |
| L756Aa | 4.6 ± 0.8   | 1.4 ± 0.27     | 2.3 ± 0.3            | NA              |
| P757Aa | 15 ± 2      | 9.0 ± 0.18     | 4.1 ± 0.5            | NA              |
| M758Aa | 9.5 ± 0.9   | 8.9 ± 0.26     | 97 ± 10              | NA              |
| M759Aa | 7.9 ± 0.6   | 6.8 ± 0.23     | 5.1 ± 0.4            | NA              |
| D760Aa | NA          | NA             | NA                   | NA              |

* Data from Granovsky et al. (19).

* NA, not applicable.
**k\text{cat}** values similar to PDE5, but it also acquired sensitivity to P\text{y}. The K value of Chi16 for P\text{y} (3.6 nm) was just 10–20-fold higher than the K values of native PDE6a reported previously (19, 32). Contacts between P\text{y} and the PDE6a catalytic domain outside of PDE6a-(737–784) may account for the lower K value of the native enzyme. The noncatalytic C-GMP-binding sites are allosterically coupled with the P\text{y}-binding sites and may regulate P\text{y} affinity for the PDE catalytic subunits (33–35). To test the role of the C-GMP-binding domain, PDE6a-(737–784) was also replaced into the wild-type PDE5 sequence (Chi17). P\text{y} inhibited Chi17 (K of 142 nM) less potently than Chi16, indicating that the noncatalytic C-GMP-binding domain of PDE6a, allosterically or due to additional contacts, enhances the P\text{y} interaction with the catalytic domain.

Previously we demonstrated (16) that binding of the P\text{y} C terminus to the PDE6 catalytic domain blocks the access of C-GMP to the catalytic site. The P\text{y} C-terminal binding was also competitive with zaprinast. We concluded that residues that participate in the binding/hydrolysis of C-GMP and the binding of competitive inhibitors are in a very close proximity to the P\text{y} C-terminal binding residues in a three-dimensional structure of PDE6 (16). In this study, an introduction of the P\text{y}-binding site into the PDE5 catalytic domain did not appreciably alter the catalytic properties. Therefore, the residues that bind P\text{y} are not directly involved in binding/hydrolysis of C-GMP by PDE6, and they likely form a domain distinct from the catalytic pocket. Both conclusions, proximity of the P\text{y} site to and its structural independence from the catalytic pocket, are supported by the model of PDE6 catalytic site (Fig. 5). The model was generated based on the recently determined structure of PDE4 catalytic domain, the first crystal structure of a PDE enzyme (36). According to this model, the P\text{y}-binding site, PDE6a-(749–761), forms a loop near the entrance to the catalytic C-GMP-binding pocket. However, PDE6a-(749–761) residues do not participate in the formation of the catalytic cavity itself. The latter is primarily assembled by residues conserved in the PDE superfamily. These residues include two histidines, His\text{461} and His\text{597} (His\text{238} and His\text{274} in PDE4), critical for coordination of two metal ions (36) (Fig. 5). The two metal atoms, apparently a tightly bound Zn\text{2+} and a more loosely associated Mg\text{2+}, are central to the hydrolysis of cyclic nucleotides by PDE6 (37). Corresponding residues, His\text{607} and His\text{643}, are necessary for the metal support of catalysis in PDE5 (38). Another important residue within the PDE6a catalytic pocket is conserved Gln\text{771} (Fig. 5). The docking of CAMP into the PDE4 structure shows that a side chain of an analogous Gln\text{443} hydrogen bonds with the 1-N and 6-NH\text{2} groups of the adenine base, but if the Gln\text{443} amide group is rotated by 180° it may

**Fig. 4. Functional properties of Chi16 mutants, Q752A and M758A.** A, kinetics of C-GMP hydrolysis by Q752A (●) and M758A (▲). PDE activities were determined using 0.1 μCi of [3H]C-GMP and increasing concentrations of unlabeled C-GMP. The rates of C-GMP hydrolysis are expressed as percentage of maximal activity of PDE5 (9.6 mol of C-GMP mol PDE\text{−}\text{α}−1 s\text{−1}) (19). The kinetic characteristics for Q752A (K\text{m} 12 ± 2 μM, k\text{cat} 8.0 s\text{−1}) and M758A (K\text{m} 9.5 ± 0.9 μM, k\text{cat} 8.9 s\text{−1}) were calculated from the fitting curves. B, inhibition of Q752A and M758A activity by zaprinast. Activities of Q752A (●) and M758A (▲) were determined using 0.1 μCi of [3H]C-GMP and increasing concentrations of zaprinast and were expressed as a percentage of respective PDE activity in the absence of zaprinast. The calculated IC\text{50} values for Q752A and M758A were 0.20 ± 0.01 and 0.26 ± 0.01 μM, respectively. C, inhibition of the catalytic activity of Q752A and M758A by P\text{y}. The activities of Q752A (●) and M758A (▲) were determined upon addition of increasing concentrations of P\text{y}, using 0.5 μM C-GMP as a substrate. The K\text{p} values from the inhibition curves were 29 ± 4 nM for Q752A and 97 ± 10 nM for M758A.
interact with the 1-NH and 6-CO groups of cGMP (36). Gln<sup>443</sup> in PDE4 is constrained by the interaction with Tyr<sup>403</sup> (36). The Tyr residue is substituted by Gln<sup>729</sup> and Gln<sup>765</sup> in PDE6 and PDE5, respectively, which appears to contribute to the cGMP substrate specificity. The Gln<sup>765</sup>→Tyr substitution was among several mutations that shifted the cGMP/cAMP selectivity of PDE5 (39).

Ala-scanning mutational analysis of PDE6α<sup>2</sup>-(750–760) in Chi16 identified two mutants, Q752A and M758A, with impaired inhibition by P<sub>y</sub>. The M758A substitution resulted in a particularly profound defect of P<sub>y</sub> binding. Both mutants retained the catalytic properties (K<sub>m</sub> and k<sub>cat</sub>) for cGMP hydrolysis and the IC<sub>50</sub> values for inhibition by zaprinast similar to those of Chi16, suggesting their intact overall folding. The model of the PDE6α<sup>2</sup> catalytic domain shows that the side chains of Gln<sup>752</sup> and Met<sup>758</sup> are solvent-exposed and are similarly orientated on the surface of the molecule. Hence, in all probability, these residues directly interact with P<sub>y</sub>. If the Pro<sup>755</sup> terminus is lined up along the plane formed by the side chains of Gln<sup>752</sup> and Met<sup>758</sup>, it may also make a contact with Pro<sup>755</sup>. Our data do not rule out the possibility of this contact. The Pro<sup>755</sup>A mutant had a significantly reduced rate of cGMP hydrolysis, and therefore, its inhibition by P<sub>y</sub> might not be directly compared with that for Chi16. Out of the residues, Met<sup>758</sup> is located at the very tip of the P<sub>y</sub>-binding loop facing the opening of the catalytic cavity. Such a location of the Pro<sub>y</sub>-binding residue would allow P<sub>y</sub> to effectively block the entry of cGMP into the catalytic pocket.

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REFERENCES
1. Chabre, M., and Deterre, P. (1989) Eur. J. Biochem. 179, 255–266
2. Yarfitz, S., and Hurley, J. B. (1994) J. Biol. Chem. 269, 14329–14332
3. Beavo, J. A. (1995) Physiol. Rev. 75, 725–748
4. Baehr, W., Devlin, M. J., and Applebury, M. L. (1979) J. Biol. Chem. 254, 11669–11677
5. Hurley, J. B., and Stryer, L. (1982) J. Biol. Chem. 257, 11094–11099
6. Deterre, P., Bigay, J., Forquet, F., Robert, M., and Chabre, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2424–2428
7. Gillespie, P. G., and Beavo, J. A. (1988) J. Biol. Chem. 263, 8133–8141
8. Li, T., Volpp, K., and Applebury, M. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 293–297
9. Hamilton, S. E., and Hurley, J. B. (1990) J. Biol. Chem. 265, 11259–11264
10. Lipkin, V. M., Khramtsov, N. V., Vasilevskaya, N. V., Atabekova, K. G., Muradov, K. G., Li, T., Johnston, J. P., Volpp, K. J., and Applebury, M. L. (1990) J. Biol. Chem. 265, 12955–12959
11. Artemyev, N. O., and Hamn, H. E. (1992) Biochem. J. 283, 273–279
12. Takemoto, D. J., Hurt, D., Oppert, B., and Cunnick, J. (1992) Biochem. J. 281, 637–643
13. Brown, R. L. (1992) Biochemistry 31, 5918–5925
14. Skiba, N. P., Artemyev, N. O., and Hamn, H. E. (1995) J. Biol. Chem. 270, 13210–13215
15. Artemyev, N. O., Natochin, M., Busman, M., Schey, K. L., and Hamn, H. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5407–5412
16. Granovsky, A. E., Natochin, M., and Artemyev, N. O. (1997) J. Biol. Chem. 272, 11686–11689
17. Piriev, N. I., Yamashita, C., Samuel, G., and Farber, D. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9340–9344
18. Qin, N., and Baehr, W. (1994) J. Biol. Chem. 269, 3265–3271
19. Granovsky, A. E., Natochin, M., McIntaffer, R. L., Haik, T. L., Francis, S. H., Corbin, J. D., and Artemyev, N. O. (1998) J. Biol. Chem. 273, 24485–24490
20. McAllister-Lucas, L. M., Sonnenburg, W. K., Kadlecak, A., Seger, D., Trong, H. L., Colbran, J. L., Thomas M. K., Walsh, K. A., Francis, S. H., Corbin, J. D., and Beavo, J. A. (1993) J. Biol. Chem. 268, 22863–22873
21. Gillespie, P. G., and Beavo, J. A. (1989) Mol. Pharmacol. 36, 773–781
22. Turko, I. V., Ballard, S. A., Francis, S. H., and Corbin, J. D. (1999) Mol. Pharmacol. 56, 124–130
23. Ballard, S. A., Gingell, C. J., Tang, K., Turner, I. A., Price, M. E., and Naylor, A. M. (1998) J. Urol. 159, 2164–2171
24. Turko, I. V., Haik, T. L., McAllister-Lucas, L. M., Burns, F., Francis, S. H., and Corbin, J. D. (1996) J. Biol. Chem. 271, 22240–22244
25. Turko, I. V., Francis, S. H., and Corbin, J. D. (1998) J. Biol. Chem. 273, 6460–6466
26. Artemyev, N. O., Arshavsky, Y. V., and Cote, R. H. (1998) Methods 14, 93–104
27. Thompson, W. J., and Appleman, M. M. (1971) Biochemistry 10, 311–316
28. Natochin, M., and Artemyev, N. O. (2000) Methods Enzymol. 315, 539–554
29. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
30. Laemmli, U. K. (1970) Nature 227, 685–688
31. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
32. Hamilton, S. E., Prusti, R. K., Bentley, J. K., Beavo, J. A., and Hurley, J. B. (1993) FEBS Lett. 318, 157–161
33. Yamazaki, A., Bartucco, F., Ting, A., and Bitensky, M. W. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3702–3706
34. Cote, R. H., Bownds, M. D., and Arshavsky, V. Y. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4845–4848
35. Mou, H., Grazio, H. J., Cook, T. A., Beavo, J. A., and Cote, R. H. (1999) J. Biol. Chem. 274, 18813–18820
36. He, F., Seryshev, A. B., Cowan, C. W., and Wensel, T. G. (2000) J. Biol. Chem. 275, 20572–20577
37. Francis, S. H., Turko, I. V., Grimes, K. A., and Corbin, J. D. (2000) Biochemistry 39, 9591–9596
38. Turko, I. V., Francis, S. H., and Corbin, J. D. (1998) Biochemistry 37, 4200–4205
39. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723