Studies of the Molecular Mechanism of Discrimination between cGMP and cAMP in the Allosteric Sites of the cGMP-binding cGMP-specific Phosphodiesterase (PDE5)*

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The regulatory domain of the cGMP-binding cGMP-specific 3′:5′-cyclic nucleotide phosphodiesterase (PDE5) contains two homologous segments of amino acid sequence that encode allosteric cyclic nucleotide-binding sites, referred to as site a and site b, which are highly selective for cGMP over cAMP. The possibility that the state of protonation in these sites contributes to cyclic nucleotide selectivity was investigated. The binding of cGMP or cAMP was determined using saturation and competition kinetics at pH values between 5.2 and 9.5. The total cGMP binding by PDE5 was unchanged by variation in pH, but the relative affinity for cGMP versus cAMP progressively decreased as the pH was lowered. Using site-directed mutagenesis, a conserved residue, Asp-289, in site a of PDE5 has been identified as being important for cyclic nucleotide discrimination in this site. It is proposed that deprotonation of Asp-289 enhances the number and strength of bonds formed with cGMP, while concomitantly decreasing the interactions with cAMP.

Discrimination between cAMP and cGMP is an essential feature of most mammalian cyclic nucleotide-regulated or cyclic nucleotide-metabolizing proteins, namely the cAMP- and cGMP-dependent protein kinases, the cyclic nucleotide-gated channels, and the 3′:5′-cyclic nucleotide phosphodiesterases (PDEs). However, the degree of discrimination and the possible molecular mechanisms that are responsible for separation of cAMP and cGMP signaling pathways vary widely (1–6).

PDEs belong to a diverse superfamily of proteins that catalyze the hydrolysis of 3′:5′-cyclic nucleotides to the corresponding nucleoside 5′-monophosphates. The multiple PDEs differ in their tissue distributions, substrate specificities, sensitivities to inhibitors, and modes of regulation. The PDEs are multidomain chimeric proteins that possess both catalytic and regulatory domains (7). The cGMP-binding PDEs comprise a group of PDEs that contains allosteric cyclic nucleotide-binding sites; two homologous amino acid sequences within each PDE monomer encode these sites, referred to as site a and site b. All of these allosteric cyclic nucleotide-binding sites that have been characterized are highly selective for cGMP. This group of PDEs consists of several families, including the cGMP-stimulated PDE (PDE2), the photoreceptor PDEs (PDE6s), and the cGMP-binding cGMP-specific PDE (PDE5). Recently, one more family (PDE10) whose amino acid sequence includes two regions that are homologous to the allosteric cyclic nucleotide-binding sites in cGMP-binding PDEs has been described (8). The results of mutagenesis (9, 10) and cyclic nucleotide analog studies have partially defined the topology of the cyclic nucleotide-binding pocket in the allosteric binding sites of these PDEs, but the molecular mechanisms that provide for discrimination between cGMP and cAMP in these sites remain unclear.

Selectivity of ligand binding to many proteins can be profoundly influenced by pH (4, 13–15) because ionizing residues are usually involved in the interactions. For the PDE5, site-directed mutagenesis has identified the ionizing residues Asp-289 (site a) and Asp-478 (site b) as important participants in cGMP binding (16). If ionization of these amino acid residues is important, the interaction with cyclic nucleotides should be sensitive to changes in pH. Studies of this variable combined with site-directed mutagenesis have been used in the present work to probe the molecular mechanism for cyclic nucleotide discrimination in the allosteric high affinity binding site of PDE5.

EXPERIMENTAL PROCEDURES

Materials—[8–3H]cGMP and [5,8–3H]cAMP were purchased from Amersham Pharmacia Biotech. cGMP, cAMP, Crotalus atrox snake venom, and 3-isobutyl-1-methylxanthine were obtained from Sigma. Hydroxyapatite was from Bio-Rad.

Site-directed Mutagenesis—The cGB-8/14 clone encodes a full-length bovine lung PDE5 (9). The QuikChange site-directed mutagenesis kit (Stratagene) has been used to make a D289N mutation in the cGB-8/14 clone in pBacPAK9 expression vector (CLONTECH) according to the protocol from Stratagene. The following pair of mutagenic oligonucleotides was used: 5′-ACA TTC ACT GAA AAA AAC GAA AAG GAC TT-3′ and 5′-AAA GTC CTT TTC GTT TTT TTC AGT GAA TGT-3′. The altered bases are underlined.

Expression of Wild-type and Mutant PDE5s—Sf9 cells were cotransfected with Bsu36I-digested BacPAK9 viral DNA (CLONTECH) and either wild-type or mutated cGB-8/14 clones in the pBacPAK9 expression vector by the lipofection method according to the protocol from CLONTECH. At 3 days postinfection, the transfection supernatant was collected, amplified twice in Sf9 cells, and then used directly as virus stock for expression without additional purification of recombinant viruses. High Five™ cells (Invitrogen) grown at 27 °C in complete Grace’s insect medium (Invitrogen) with 10% fetal bovine serum and 10 μg/ml gentamycin (Life Technologies, Inc.) in T-185 flasks were infected by 5 ml of virus stock per flask. The culture medium was harvested at 96 h postinfection.

Partial Purification of Wild-type and Mutant PDE5s—240–260 ml of the culture medium was fractionated by sequential ammonium sulfate precipitation of the culture medium was fractionated by sequential ammonium sulfate precipitation of
pH Dependence of the Binding of Cyclic Nucleotides to PDE5—The cGMP binding assay was conducted in the presence of 0.5–100 μM \([^3H]cGMP\) in a total volume of 60 μl 50 mM Tris, 50 mM bis-Tris, 100 mM sodium acetate buffer, pH 5.2–9.5. The buffer was supplemented with 1 mM EDTA, 0.25 mM 3-isobutyl-1-methylxanthine, and 2 mM dithiothreitol. The pH was adjusted with acetic acid. The ionic strength of this buffer system is constant over the pH range used in this study (17). Direct \([^3H]cAMP\) binding in this buffer system was measured at a pH value of 5.2. Competition experiments were conducted in the presence of a wide range of \([^3H]cGMP\) and 100 μM cAMP as the competing ligand. The reaction was initiated by addition of an aliquot of enzyme. Under these conditions the amount of \([^3H]cGMP\) bound to PDE5 remained constant from 7 to 30 min on ice. A 30-min incubation was selected for all measurements. Following this incubation, the assay was filtered. Therefore, the pH dependence of cAMP binding was practical to directly measure cAMP binding using Millipore filtration. Therefore, the pH dependence of cAMP binding was determined by assessing the efficacy with which unlabeled cAMP (100 μM) competes with \([^3H]cGMP\) binding to PDE5 (Fig. 1). At pH 5.2, the \(K_d\) values for \([^3H]cGMP\) binding increased 13-fold in the presence of cAMP. The increased competition by cAMP at lower pH values suggested that protonation within the binding sites could create more favorable interactions for cAMP binding. We attempted to directly measure \([^3H]cAMP\) binding at pH 5.2 (Fig. 2). Despite the clear dependence on cAMP concentration, the \([^3H]cAMP\) binding level was very low and precluded an estimation of \(K_d\). Even at a very high concentration (100 μM), the binding of cAMP to the allosteric binding sites was still at least 20 times less than that for cGMP (Fig. 2). The affinity of cAMP was so low that it could not be ruled out that some of the bound cAMP was lost during Millipore filtration.

**Rationale for Mutagenesis**—Recently, studies of site-directed mutagenesis within the high affinity binding site \(a\) of PDE5, combined with modeling, suggested that Asp-289 forms a hydrogen bond with the proton of the N-1 endo-nitrogen of the guanine base of cGMP (9). Crystallographic analysis indicated that this type of interaction, which is very specific for the guanine ring, occurs in GTP-binding proteins such as transducin-α, EF-Tu, and Ha-Ras p21 (20). In contrast, an unfavorable electrostatic effect would be expected between the negatively charged side chain of Asp and the unshared pair of electrons in the sp\(^2\) orbital at N-1 of the adenine ring of cAMP. If deprotonation (ionization) of Asp-289 fosters cGMP binding and discriminates against cAMP binding, then the cyclic nucleotide selectivity in this site should be sensitive to changes in pH. By this same reasoning, substitution of an Asn at this position would be predicted to render the cyclic nucleotide selectivity

Effect of pH on \([^3H]cGMP\) Binding—Saturation analyses were performed as described at different pH values over the range from 5.2 to 9.5 using a Tris/bis-Tris acetate buffer that maintains a constant ionic strength throughout the entire pH range used in these studies (17). The binding affinity for \([^3H]cGMP\) to the allosteric sites of PDE5 declined at lower pH values (Fig. 1, inset). The \(K_d\) for \([^3H]cGMP\) binding was 2.4 ± 0.3 μM at a pH value of 5.2, which was 16-fold lower affinity than the \(K_d\) of 0.15 μM measured at a pH value of 9.5. To address possible denaturation of PDE5 at the more extreme pH values, the values for total \([^3H]cGMP\) binding were calculated and expressed as nanomoles of cGMP bound per mg of total protein used in the assay (Fig. 1, inset). These values were essentially unaffected by alteration of pH. This indicated that the number of available binding sites does not depend on \([H^+]\) and that the decrease in affinity for cGMP could be because of protonation of a residue(s) within the binding sites.

Because affinity for cAMP binding to the allosteric sites of PDE5 is considerably lower than that for cGMP, it is not practical to directly measure cAMP binding using Millipore filtration. Therefore, the pH dependence of cAMP binding was determined by assessing the efficacy with which unlabeled cAMP (100 μM) competes with \([^3H]cGMP\) binding to PDE5 (Fig. 1). At pH 5.2, the \(K_d\) values for \([^3H]cGMP\) binding increased 13-fold in the presence of cAMP. The increased competition by cAMP at lower pH values suggested that protonation within the binding sites could create more favorable interactions for cAMP binding. We attempted to directly measure \([^3H]cAMP\) binding at pH 5.2 (Fig. 2). Despite the clear dependence on cAMP concentration, the \([^3H]cAMP\) binding level was very low and precluded an estimation of \(K_d\). Even at a very high concentration (100 μM), the binding of cAMP to the allosteric binding sites was still at least 20 times less than that for cGMP (Fig. 2). The affinity of cAMP was so low that it could not be ruled out that some of the bound cAMP was lost during Millipore filtration.

**RESULTS**

**Effect of pH on \([^3H]cGMP\) Binding**—Saturation analyses were performed as described at different pH values over the range from 5.2 to 9.5 using a Tris/bis-Tris acetate buffer that maintains a constant ionic strength throughout the entire pH range used in these studies (17). The binding affinity for \([^3H]cGMP\) to the allosteric sites of PDE5 declined at lower pH values (Fig. 1, −cAMP). The \(K_d\) for \([^3H]cGMP\) binding was 2.4 ± 0.3 μM at a pH value of 5.2, which was 16-fold lower affinity than the \(K_d\) of 0.15 μM measured at a pH value of 9.5. To address possible denaturation of PDE5 at the more extreme pH values, the values for total \([^3H]cGMP\) binding were calculated and expressed as nanomoles of cGMP bound per mg of total protein used in the assay (Fig. 1, inset). These values were essentially unaffected by alteration of pH. This indicated that the number of available binding sites does not depend on \([H^+]\) and that the decrease in affinity for cGMP could be because of protonation of a residue(s) within the binding sites.

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**FIG. 1. pH dependence of the binding of \([^3H]cGMP\) to the allosteric sites of PDE5.** Saturation binding analyses in the absence (○) or presence (□) of 100 μM cAMP were performed at different pH values as described under "Experimental Procedures." The total \([^3H]cGMP\) binding (inset) and \(K_d\) values are the means ± range for two experiments with points determined in triplicate.

**FIG. 2. \([^3H]cyclic nucleotide binding to the allosteric sites of PDE5 at pH 5.2.** The data for the wild-type PDE5 were obtained with increasing concentrations of \([^3H]cGMP\) (○) or \([^3H]cAMP\) (□) as described under "Experimental Procedures." The 60% efficiency of counting and specific activities of 11.1 Ci/mmol for \([^3H]cGMP\) and 42 Ci/mmol for \([^3H]cAMP\) were used to recalculate counts/min obtained in experiment into the nanomoles of cyclic nucleotide bound per mg of total protein used in assay. The data are given as means ± range for two experiments with points determined in triplicate.
insensitive to changes in pH. Both Asp and Asn have the potential to provide a hydrogen bond to the proton of the N-1 position of cGMP, so it is expected that cGMP binding properties of wild-type and mutant PDE5s could be similar. Also, this replacement of an acid with its corresponding amide should have the additional advantage of minimizing potential structural alterations within the recombinant protein.

**D289N Mutant**—The D289N mutant was expressed in High Five™ cells and partially purified from culture medium using ammonium sulfate precipitation and hydroxyapatite chromatography as described under “Experimental Procedures.” There was no noticeable difference in binding to and subsequent elution of this protein from the hydroxyapatite column compared with that for the wild-type enzyme. The catalytic and cyclic nucleotide binding properties of the D289N mutant were recently described (21), and they did not differ significantly from those of wild-type PDE5. When analyzed by SDS-polyacrylamide gel electrophoresis this protein also migrated with essentially the same mobility as that of the wild-type enzyme (21). These combined results indicated that the D289N mutant is expressed as a correctly folded, catalytically active, full-length protein.

The pH dependence of [3H]cGMP binding to the D289N mutant in the presence or absence of 100 μM cAMP is shown in Fig. 3. As with the wild-type PDE5, the total [3H]cGMP binding was not appreciably altered over the pH range studied (Fig. 3, inset). This indicated that the number of available binding sites did not depend on hydrogen ion concentration. The $K_d$ for [3H]cGMP binding to the D289N mutant was 1.4 μM at pH 5.2, which was 7-fold lower than the $K_d$ of 0.2 μM measured at pH 9.5. The pH profiles for the D289N mutant showed small improvement in cAMP binding, and the $K_d$ value for [3H]cGMP binding in the presence of 100 μM cAMP at pH 5.2 increased 3-fold only compared with that in the absence of cAMP. To compare wild-type and mutant enzymes, the value of $K_d$ for cAMP/$K_d$ for cAMP was plotted versus pH (Fig. 4). These plots show that substitution of asparagine for the aspartic acid residue in the high affinity site strongly reduces the pH-dependent improvement in cAMP binding that is observed for the wild-type enzyme.

Results of pH titrations combined with those using site-directed mutagenesis indicated that ionization of Asp-289, which occurs at physiological pH, contributes importantly to the high affinity binding of cGMP and to the discrimination of this site against cAMP. Even though protonation significantly altered cyclic nucleotide selectivity, the effect was not absolute.

Results of this report indicate that the ionization state of Asp-289 in the high affinity allosteric binding site of PDE5 affects the apparent affinity for cGMP and that cGMP binds more tightly to the enzyme when this residue is in the unprotonated state. Asp-289 in the unprotonated state apparently plays an important role not only in high affinity cGMP binding but also in discrimination against cAMP. The proposed molecular mechanism of this discrimination is shown in Fig. 5 and is
based on the different chemical properties of the N-1 position of the corresponding guanine and adenine bases of cGMP and cAMP. In this model, a hydrogen bond can be formed between the carbonyl of aspartic acid and the N-1 hydrogen of cGMP at physiological pH. For cAMP at the same pH value, a repulsion would be predicted between the negatively charged side chain and the unshared pair of electrons in the sp² orbital at the N-1 position of adenine. Protonation of aspartic acid at lower pH values should reduce the strength of the interaction with cGMP, while eliminating the repulsive forces with cAMP.

Results similar to those described in this report were obtained earlier for cyclic nucleotide-gated ion channels, which also apparently utilize an aspartic acid residue for bonding with cGMP (4, 22). Because other members of cGMP-binding PDEs, PDE2 and PDE6, possess the conserved aspartic acid residue in the position corresponding to Asp-289 of PDE5, the mechanism of discrimination against cAMP in these PDEs is likely to be similar to that in PDE5.

Despite a very high degree of cyclic nucleotide discrimination in vitro (6, 11, 12), could cAMP interaction with the allosteric sites of cGMP-binding PDEs occur in vivo? At least two points must be considered when addressing this issue. First, the apparent pKₐ values for ionizable groups within amino acids obtained from kinetic measurements may not represent the actual pKₐ values of these groups in intact proteins because protein folding can markedly shift the ionization state of these groups. For instance, in some proteins the carboxyl group of a buried Asp may be partially protonated even at pH 7.0 (23). Second, the intracellular concentration of cAMP in many cells exceeds the intracellular concentration of cGMP by 10–100-fold. The combination of these factors may allow cAMP interaction with the allosteric sites of cGMP-binding PDEs in some cells. This assumption is also consistent with the observation that cAMP can allosterically modulate the activity of PDE2 in vivo (24–26).

In summary, the results of the present study provide initial insights pertaining to the molecular mechanism of cyclic nucleotide discrimination in the allosteric sites of PDE5. The important role of a conserved aspartic acid residue in this mechanism could represent a general phenomenon relevant to other cGMP-binding PDEs.

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