Expression of an Active, Monomeric Catalytic Domain of the cGMP-binding cGMP-specific Phosphodiesterase (PDE5)*

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Phosphodiesterases (PDEs) comprise a superfamily of phosphohydrolases that degrade 3',5'-cyclic nucleotides. All known mammalian PDEs are dimeric, but the functional significance of dimerization is unknown. A deletion mutant of cGMP-binding cGMP-specific PDE (PDE5), encoding the 357 carboxyl-terminal amino acids including the catalytic domain, has been generated, expressed, and purified. The $K_m$ of the catalytic fragment for cGMP ($5.5 \pm 0.51 \mu M$) compares well with those of the native bovine lung PDE5 ($5.6 \mu M$) and full-length wild type recombinant PDE5 ($2 \pm 0.4 \mu M$). The catalytic fragment and full-length PDE5 have similar IC$_{50}$ values for the inhibitors 3-isobutyl-1-methylxanthine (20 $\mu M$) and sildenafil (Viagra$^{TM}$)(4 nM). Based on measured values for Stokes radius (29 Å) and sedimentation coefficient (2.9 S), the PDE5 catalytic fragment has a calculated molecular mass of 35 kDa, which agrees well with that predicted by amino acid content (43.3 kDa) and with that estimated using SDS-polyacrylamide gel electrophoresis (39 kDa). The combined data indicate that the recombinant PDE5 catalytic fragment is monomeric, and retains the essential catalytic features of the dimeric, full-length enzyme. Therefore, the catalytic activity of PDE5 holoenzyme requires neither interaction between the catalytic and regulatory domains nor interactions between subunits of the dimer.

Intracellular levels of cyclic nucleotide are determined both by their rates of formation by adenylyl cyclases and guanylyl cyclases and their rates of breakdown by cyclic nucleotide phosphodiesterases (PDEs)$^1$ (1). Both the cyclases and the PDEs are highly regulated (2–8). Mammalian PDEs form a superfamily of enzymes comprised of 10 families of PDEs which differ in cellular localization, and perhaps for other functions as well (12, 16, 17). For mammalian PDEs, the regulatory domains appear to reside mainly in the amino-terminal portions of the proteins. The carboxyl-terminal portion of each PDE contains a highly conserved region of ~250 amino acids (4), which comprises the catalytic domain.

The cGMP-binding cGMP-specific PDE (PDE5 or cG-BPDE), which is the focus of this study, is abundant in lung, platelets, and vascular smooth muscle (18–23). It is potently inhibited by sildenafil (Viagra$^{TM}$), which has been proven to be therapeutically efficacious in the treatment of male erectile dysfunction (24). The PDE5 is highly specific for cGMP, both in its catalytic site, and in the two cGMP-binding allosteric sites within the amino-terminal half of the protein (14, 25). The regulation of the PDE5 catalytic activity is not well understood, although evidence has been presented that catalytic function is influenced by elements within the regulatory domain (26, 27). Cyclic GMP binding to the allosteric cGMP-binding sites is required for phosphorylation of serine 92 by cGMP-dependent protein kinase (28), and elements that contribute to the dimerization of this protein are located in or near the allosteric cGMP-binding sites (29); the function of dimerization is unknown.

Several groups have generated catalytically active fragments of PDEs using either limited proteolysis or truncation mutagenesis, but few have addressed the oligomeric state of these fragments (14, 30–33). Studies of several PDEs have suggested that the isolated catalytic domain alone is sufficient for catalytic activity. However, the importance of dimerization to the catalytic function of the PDE remained in question (31, 32, 34, 35). An amino-terminal truncation mutant of PDE1 was CaM-independent but maintained catalytic properties ($K_m$ and $V_{max}$) that were similar to those of wild type. The sedimentation coefficient and Stokes radius of this mutant (subunit $M_r \approx 50,000$) were used in combination to determine that this fragment was dimeric ($\Delta M_5$-PDE1A mutant (13)). Active recombinant catalytic fragments of PDE3 have been studied (36–40), but the oligomeric states of these mutants, when addressed, were apparently dimeric (38). Several PDE4 amino-terminal deletion mutants (15) were also shown to be dimeric (41). More recently, Kovala et al. (42) reported a series of amino- and carboxyl-terminal mutants of PDE4; some constructs were active as monomeric PDEs, while others were dimers; however, these molecular weight estimations were based solely on Stokes radius determinations, which can be misleading.

The present report determines physical and kinetic features of the recombinant catalytic domain of the PDE5 and investigates whether dimerization or the regulatory domain is required for catalytic domain function. Since the catalytic domain...
of PDEs is highly conserved among the species, much of the new findings for PDE5 is likely to apply to other PDEs.

**EXPERIMENTAL PROCEDURES**

The PDE5 clone was created in this laboratory as described previously (43). The initial format for this clone was the pBluescript (SK+) vector. This clone was transferred to a baculovirus expression vector, pBacPAK9.

Construction of Catalytic Fragment from Full-length Wild Type PDE5 DNA in Baculovirus Expression Vector—The PDE5 construct in the pBacPAK9 vector (described in Ref. 44) was digested with ThaI 1111, which cuts once in the amino-terminal region of PDE5 after ~17 codons, leaving a 5′ single base overhang, which was removed by digestion with mung bean nuclease using a short incubation time to prevent overdigestion. Linearized PDE5 pBacPAK9 DNA was cut again using BalIII, which cleaved just before the sequence encoding the conserved catalytic region of PDE5, leaving blunt ends and produced 2 DNA fragments of 7697 and 1462 base pairs. The 7697-base pair piece containing the catalytic domain was purified by agarose gel electrophoresis and Gelase enzyme, and then religated using T4 DNA ligase. This religated DNA was designed to maintain the frame of the original PDE5 DNA sequence, thus preserving the translated amino acid sequence and joining the amino-terminal region to the carboxy-terminal catalytic domain. However, an additional base in the DNA on the 5′ side was removed by the mung bean nuclease which should have caused the entire protein to be out of frame, but only the first 11 amino acids of the entire recombinant catalytic fragment are incorrect. This was established by AA sequence analysis of the final protein product. The correct PDE5 catalytic domain sequence begins at residue 12 (a lysine, which is amino acid 508 numbering from the wild type sequence) and is identical to that of wild type PDE5. The results were consistent with a construct that encoded a protein of the predicted size, that is, one of 368 residues including the 11 heterologous amino acids joined to the carboxy-terminal 357 residues of PDE5 beginning with the sequence YyyGln-MetVal-Thr-Leu-Glu314. In PDE5, the conserved sequence of -~250 amino acids that contains the catalytic domain begins with the sequence PDE5 discovery of the PDE5 catalytic fragment was not altered under assay conditions. In this case, the identity of the protein band corresponding to the peak of PDE activity from this column. The identity of the protein band was verified by sequential Edman degradation through 20 cycles which yielded the following sequence: X’AAATMGPGPKQMVTLEVKS20.

Phosphodiesterase Catalytic Activity—PDE catalytic activity was measured by a modified assay described for cGMP-stimulated PDE2 (46), using 40 mM MOPS (pH 7.0), 0.8 mM cGMP, and [3H]cGMP as the starting material. All purification procedures were performed at 4 °C. The thyroglobulin elution volume was taken as the void volume. The concentrated CM-Sephadex pool was applied to a Sephacryl S-100 column (0.9 × 56 cm) equilibrated in 10 mM potassium phosphate, pH 6.8, containing 1 mM EDTA, 25 mM β-mercaptoethanol, 50 mM NaCl, and protease inhibitor mixture (as described above). Fractions from this column were assayed for PDE catalytic activity. The PDE5 catalytic fragment was at least 40% pure after this step, based on densitometry (to determine percent of total protein which was PDE5 catalytic fragment) of SDS-PAGE protein band corresponding to the peak of PDE activity from this column. The identity of the protein band was verified by sequential Edman degradation through 20 cycles which yielded the following sequence: X’AAATMGPGPKQMVTLEVKS20.
these samples, which had cGMP present initially, were handled differently from the usual PDE assays. To locate the peak of activity, the samples from gel filtration were diluted into assay mixtures which had trace amounts of [3H]cGMP and no cold cGMP.

Sacrose Density Gradient Centrifugation—Sacrose density gradient centrifugation was performed by the method of Martin and Ames in 10 mM potassium phosphate buffer containing 1 mM EDTA, 25 mM β-mercaptoethanol, 150 mM NaCl, and 5–20% sucrose. Samples were centrifuged for 48 h at 36,000 rpm in a Beckman SW41 rotor. Internal standards included for calculating the sedimentation coefficient were aldolase (7.35 S), CAMP-dependent protein kinase catalytic subunit (3.14 S), and cytochrome c (1.71 S). Sedimentation coefficient of PDE was determined by assaying the PDE catalytic activity in the sucrose gradient fractions. Absorbance at 280 nm was used to locate internal protein standards except cAMP-dependent protein kinase catalytic subunit, which was followed by assaying protein kinase activity (49). Attempts to determine the m<sub>obs</sub> of the PDE5 catalytic fragment in the absence of internal standards were unsuccessful due to loss of catalytic activity.

Molecular Weight Calculation—The molecular weight of the PDE5 catalytic fragment was calculated using the experimentally determined Stokes radius and sedimentation coefficient of PDE5 catalytic fragment in the Siegel and Monty equation (48),

\[
M = \frac{(6 \pi n N a s)}{(1 - \nu \rho)}
\]

where \(N\) = Avogadro’s number; \(n\) = viscosity of medium, assumed to be 1; \(a\) = Stokes radius; \(s\) = sedimentation coefficient; \(\rho\) = density of medium, assumed to be 1, and \(\nu\) = partial specific volume, assumed to be 0.725 ml/g.

K<sub>50</sub> Values for Recombinant PDE5 Catalytic Fragment and Wild Type Recombinant PDE5—The K<sub>50</sub> values were determined using standard PDE assay conditions, except that the cGMP substrate concentration was varied from 1 to 1000 μM cGMP. The value for the K<sub>50</sub> of PDE5 catalytic fragment was compared in parallel assays to that of native and wild type recombinant PDE5. The PDE5 catalytic fragment (mean K<sub>50</sub> = 5.52 ± 0.51 (S.E.) μM cGMP), native PDE5 (K<sub>50</sub> = 5.6 μM cGMP) (28), and wild type PDE5 (mean K<sub>50</sub> = 2 ± 0.4 (S.E.) μM cGMP) (50) were assayed in the presence of standard assay conditions (as described above).

Determination of IC<sub>50</sub> Values—When indicated, PDE inhibitors were added at various concentrations to the standard reaction mixtures containing 20 μM cGMP as substrate. IC<sub>50</sub> was the concentration of inhibitor that produced 50% inhibition of PDE activity.

Materials—The PDE5 inhibitor, sildenafil (Viagra<sup>(34)</sup>), was a generous gift from Pfizer, United Kingdom. Many molecular biology reagents were obtained via the Vanderbilt Diabetes Center molecular biology core. The Vanderbilt Diabetes Center tissue culture core facility provided many media and competent cells. Sequenase Kit version 2.0 was from U. S. Biochemical Corp. Protease inhibitor mixture tablets (Complete) were from Roche Molecular Biochemicals. Baculovirus expression vector pBacPAK9, viral DNA pBacPAK6, and transfection reagents and protocols were obtained from CLONTECH. Mung bean nuclease and some restriction enzymes were obtained from Promega. Other restriction enzymes were obtained from New England Biolabs. Gelase enzyme was purchased from Epicentre Laboratories. Maxiprep and miniprep kits were purchased from Qiagen. Sf9 log phase cells were purchased from Invitrogen. Grace’s medium/TMN/FH was purchased from either Life Technologies, Inc. or PharMingen. Fetal bovine serum was purchased from Life Technologies, Inc. Tissue culture supplies were made by Falcon and Nunc. DEAE-Sepharcl, column chromatography columns, and SDS-PAGE unstained molecular weight markers, SDS-PAGE mini-gel apparatus, Transblot apparatus, and protein assay reagent were from Bio-Rad. Crotalus atrox venom 5'-nucleotidase was from Sigma. SDS-PAGE (PROTOGEL) reagents were purchased from National Diagnostics. Aqueous scintillant was Beckman Ready-Safe. [8-3H]cGMP was purchased from Amersham Pharmacia Biotech.

RESULTS AND DISCUSSION

Expression of Wild Type PDE5 and PDE5 Catalytic Fragment—The baculovirus/Sf9 cell expression system was used to express the wild type full-length PDE5 (44) and the catalytic fragment of PDE5 (Fig. 1). The catalytic fragment of PDE5 was expressed at an average level of 2 mg/liter infected cell media, which was comparable to the level of expression of the wild type enzyme. Both the wild type PDE5 and catalytic fragment of PDE5 were catalytically active. Catalytic activity was used to monitor the purification of the wild type PDE5 and the PDE5...
catalytic domain. Studies were performed to determine the optimal time of Sf9 cell harvest after infection with wild type or catalytic fragment recombinant baculovirus stocks. Wild type PDE5 and PDE5 catalytic fragment were harvested from infected Sf9 cells on days 4 and 6 post-infection, respectively. Both were found primarily in the media incubated with the infected cells. It is unclear if this was due to lysis of cells infected with this recombinant virus or due to active extrusion. The media were used as source of enzymes for purification and characterization in all of the experiments described herein.

**Purification**—Recombinant wild type PDE5 from Sf9 cells (44) and native PDE5 from bovine lung (29) were purified as described previously in this laboratory.

The catalytic fragment exhibited chromatographic properties that were dramatically different from those of full-length PDE5. The baculovirus/Sf9 cell preparations of PDE5 catalytic fragment were subjected to sequential purification using DEAE-Sephacel, hydroxyapatite, CM-Sephadex, and S-100 gel filtration chromatographies in order to attain the highest level of purification. In the most purified samples, one main protein band (39 kDa) was observed by Coomassie Blue staining after SDS-PAGE (Fig. 1). Based on amino acid sequencing of the SDS-PAGE protein band which corresponded to the PDE catalytic fragment construct. The molecular mass determined by SDS-PAGE mobility (39 kDa) of the native PDE5 was 178 kDa (Table I) as described in Francis et al. (33). The actual molecular mass of the native PDE5, based on amino acid sequence, is 198 kDa or 98.6 kDa per monomer.

**Physical Properties**—In nondenaturing conditions, gel filtration on Sephacryl S-200 was used to determine a Stokes radius of 29 Å for the PDE5 catalytic fragment (Fig. 2 and Table I). This was smaller than the Stokes radius of 44 Å for full-length, wild type PDE5. As described under “Experimental Procedures,” the Stokes radius was not altered by performing the gel filtration chromatography under conditions which mimicked those of the PDE assay, i.e. high substrate (1 mM cGMP) and Mg$^{2+}$ (10 mM). Thus, the presence of assay constituents did not alter the apparent oligomeric state of the catalytic fragment (data not shown). Analysis by sucrose gradient centrifugation in nondenaturing conditions determined that the sedimentation coefficient of the PDE5 catalytic fragment is 2.9 S (Fig. 3 and Table I). In similar experiments, the sedimentation coefficient of wild type full-length PDE5 was 9.35 S (33). The values for the sedimentation coefficients and Stokes radii were used to calculate a molecular weight for each protein (Table I) using the Siegel and Monty equation (48) as described under “Experimental Procedures.” The calculated molecular mass of the catalytic fragment was 35 kDa, which was similar to the monomeric molecular mass, 42.2 kDa, predicted by the amino acid content of the PDE5 catalytic fragment construct. The molecular mass determined by SDS-PAGE mobility (39 kDa) of PDE5 catalytic fragment, which was confirmed by amino acid sequencing, also approximated these values. An active monomeric PDE was further supported by the fact that under assay conditions, dilution of the enzyme by as much as 100-fold produced no decrease in the rate of catalysis (data not shown) when the activity was normalized to the new enzyme concentration. Had activity dropped disproportionately to the enzyme dilution, such as being lower than expected, this might have suggested that catalysis depends on an active multimeric enzyme which dissociates into inactive monomers with progressively greater dilution. It is concluded from these cumulative lines of evidence that the PDE5 catalytic fragment is active as a monomeric species. This contrasts with the dimeric state of the native PDE5. Using the same approach described here, the calculated molecular mass of full-length, native bovine lung PDE5 was 178 kDa (Table I) as described in Francis et al. (33). The actual molecular mass of the native PDE5, based on amino acid sequence, is 198 kDa or 98.6 kDa per monomer.

**A Putative Leucine Zipper Motif in the Regulatory Domain Is Not Required for Dimerization of PDE5**—The demonstration that the PDE5 catalytic fragment is monomeric makes it unlikely that the sequence in this fragment of the protein contributes significantly to the dimerization of PDE5. This sequence includes not only that of the catalytic domain per se (amino acid residues 578–812), but also the 53 residues at the extreme carboxyl terminus of PDE5 and the 69 residues (residues 508–
This table lists a number of values comparing the physical properties of these two proteins. The calculated values for Stokes radius and sedimentation coefficient each represent the mean of three separate experiments which were each assayed in duplicate. The numbers are given as the mean ± S.E. Values for PDE5 holoenzyme were described previously (33). The combination of Stokes radii and sedimentation coefficient for each protein determines that the wild type protein exists as a dimer under nondenaturing conditions, while the PDE5 catalytic fragment exists as a monomer under the same conditions.

<table>
<thead>
<tr>
<th>Method</th>
<th>Catalytic fragment</th>
<th>Holoenzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stokes radius</td>
<td>Sephacryl-200 gel filtration</td>
<td>29 Å ± 1.31</td>
</tr>
<tr>
<td>Sedimentation coefficient</td>
<td>Sucrose density gradient centrifugation</td>
<td>2.9 S ± 0.12</td>
</tr>
<tr>
<td>Subunit molecular weight</td>
<td>SDS-PAGE mobility</td>
<td>39 kDa</td>
</tr>
<tr>
<td>Undenatured molecular weight</td>
<td>Predicted by amino acid sequence</td>
<td>42.2 kDa</td>
</tr>
<tr>
<td>Assigned quaternary structure</td>
<td>Estimated from Stokes radius and sedimentation coefficient</td>
<td>35 kDa</td>
</tr>
</tbody>
</table>

This figure shows the sucrose density gradient centrifugation of PDE5 catalytic fragment. The Sucrose gradient (5–20%) centrifugation of enzyme from the hydroxyapatite step of purification was performed as described under "Experimental Procedures." The PDE activity (indicated by the filled circles) and protein content (indicated by the open squares) of fractions collected from a sucrose density gradient representative of three separate experiments, which were assayed in duplicate, are indicated. The specific activity of the applied sample was 0.63 μmol/min/mg, and 4.5 μg of this sample was applied. The fractions containing the highest percent sucrose, 20%, are shown at the left. Elution positions of the internal protein standards, aldolase (7.35 S) and cytochrome c (1.71 S), used for determining the sedimentation coefficient of PDE5 catalytic fragment are indicated by arrows. The elution position of another internal standard, the purified catalytic subunit of cAMP-dependent protein kinase (3.14 S), which was determined by kinase activity, is also indicated by an arrow. For comparison, the position at which PDE5 holoenzyme elutes is also indicated by an arrow.

Using site-directed mutagenesis, three residues in the hep-tad repeat of PDE5 (Leu159, Leu253, and Ile330) were converted to alanine in a single recombinant PDE5. This approach of altering the multiple positions in a zipper sequence has been employed to successfully block the dimerization function of a number of proteins (51–55). The mutant PDE5, as well as wild type PDE5, were expressed in the baculovirus/Sf9 cell system and characterized. The mutant PDE5 was catalytically active, immunoreactive with wild type antibody, and co-migrated with wild type PDE5 on SDS-PAGE. Furthermore, the sedimentation coefficient and Stokes radius of the PDE5 triple mutant were the same as those of the wild type protein (data not shown). The triple mutant PDE5 remained dimeric even under stringent conditions: preincubation and gel filtration in varied ionic strengths (0–1 M NaCl), varied temperatures (4–22 °C), high concentration of β-mercaptoethanol (100 mM), high substrate/inhibitor concentration (20 μM cGMP, 250 μM IBMX), and chaotropic salt (200 mM potassium isothiocyanate) did not disrupt the dimerization of the mutant PDE5. Therefore, since we find a monomeric PDE5 catalytic fragment but a dimeric holoenzyme zipper mutant, we conclude that: (a) the leucine zipper motif near the end of the cGMP-binding site α in the regulatory domain of PDE5 either does not contribute to dimerization of the enzyme, or the zipper sequence is only part of a multiple-faced dimerization mechanism, (b) the catalytic domain does not provide for dimerization of PDE5, and (c) the nonconserved sequences located at the carboxyl terminus or that located between the catalytic domain and the allosteric site b do not contribute significantly to PDE5 dimerization. It appears likely that dimerization of PDE5 is provided primarily
coefficient of 0.53 was obtained and there was a poor fit (data not shown). When the data were analyzed for negative cooperativity, a Hill coefficient of 0.75 was obtained. In this case, the non-saturable component at the higher substrate concentrations suggests heterogeneity, al-

The PDE5 catalytic fragment was determined as described under "Experimental Procedures" using enzyme from the hydroxyapatite step of purification (protein concentrations of samples being assayed for the plot shown above was 0.4 mg/ml with <5% of that being PDE5 catalytic fragment). The $K_m$ of PDE5 catalytic fragment ($n = 3$) was 5.5 ± 0.51 (S.E.) μM cGMP. The curve was fitted based on the Michaelis-Menten model plus a non-saturable component (see text). The $y$ axis of the plot is given as nanomoles of cGMP substrate converted per minute assay per ml of PDE5 catalytic fragment protein sample. The data points shown represent the mean of three duplicate assays of samples from one protein purification step from a single baculoviral infection and S9 cell harvest. Error bars, which are given for each data point, represent the standard error for the three assays. This figure was drawn using Fig. Perfect™ for Windows™ and standard nonlinear regression curve-fitting to the data points. A similar result from the calculation of $K_m$ of the PDE5 catalytic fragment was found when these data were plotted using the Eadie-Hofstee format.

Kinetic Properties—Since the quaternary structure of this catalytically active PDE5 fragment differs so dramatically from that of the full-length PDE5, the catalytic properties of this PDE5 catalytic fragment were examined in order to determine whether enzyme function was substantially altered. Comparisons of PDE5 native enzyme, wild type and catalytic fragment substrate affinities revealed similar $K_m$ values for cGMP (5.6 μM cGMP (28), 2 ± 0.4 (S.E.) μM cGMP (50), and mean 5.52 ± 0.51 (S.E.) μM cGMP, respectively), when prepared in the same buffers and assayed similarly. The calculations for the $K_m$ of the PDE5 catalytic fragment were determined from data which were also used to create the rate plot shown in Fig. 4. The curve was fitted based on the Michaelis-Menten model plus a non-saturable component. The non-saturable component at the higher substrate concentrations suggests heterogeneity, although we cannot completely rule out negative cooperativity. When the data were analyzed for negative cooperativity, a Hill coefficient of 0.53 was obtained and there was a poor fit (data not shown). A similar value for the catalytic fragment $K_m$ was obtained when applying the Eadie-Hofstee or Lineweaver-Burk plot formats to the same data (graphs not shown).

The $k_{cat}$ of the catalytic fragment was determined using only the most purified preparations of PDE5 catalytic fragment; the concentration of the PDE5 catalytic fragment of these preparations was quantified by combining the measured value for the total protein in the sample and the density of the PDE5 catalytic domain band from SDS-PAGE (such as the gel lane seen in Fig. 1B) relative to other contaminating proteins in the samples in order to determine the most accurate value for the catalytic fragment possible. The best estimates for the $k_{cat}$ of the wild type PDE5 and catalytic fragment, as derived from a Lineweaver-Burk plot of these data (x axis = reciprocal of substrate; y axis = reciprocal of enzyme turnover rate), were comparable: wild type $k_{cat} = 4.3 \text{s}^{-1}$ (substrate turnover rate per mole of dimeric PDE (56)); PDE5 catalytic fragment $k_{cat} = 2.9 \text{s}^{-1}$ (substrate turnover rate per mole of monomeric catalytic fragment). The $k_{cat}$ value reported here for this enzyme reflects the combination of this protein concentration with the value at which the Lineweaver-Burk plot line intersects the y axis.

These results support the interpretation that the PDE5 catalytic fragment is catalytically active as a monomer, which indicates that the basic components that provide for catalysis result from interactions within a single catalytic domain rather than involving both the regulatory and catalytic domains or multimeric catalytic domains. We further conclude that the structural components provided by the residues within a single catalytic domain of PDE5 are sufficient to form a site that efficiently interacts with substrate, cGMP, and that catalyzes the hydrolysis of cGMP with reasonable efficiency.

Comparison of PDE5 Catalytic Fragment with the Catalytic Fragments of Other Phosphodiesterase Families—Like PDE5 catalytic fragment, CaM-PDE deletion mutants displayed $K_m$ and $V_{max}$ values similar to those of the wild type enzyme (13). However, PDE3 amino-terminal deletion mutants had $K_m$ values for cAMP and cGMP that were significantly lower than those for intact enzyme, and the $K_m$ for cGMP progressively decreased with larger amino-terminal deletions of PDE3 (37). Another study with PDE3 showed similar $K_m$ values with amino-terminal deletion mutants, but the $V_{max}$ values increased with larger amino-terminal deletions (40). Similarly, several studies involving PDE4 amino-terminal deletion mutants determined that these enzymes had similar $K_m$ values as did wild type PDE4, but increased specific enzyme activity (15, 42). These results reveal a similar pattern of properties shared by PDE catalytic domains, and support the notion that results from our studies with PDE5 catalytic fragment may apply to those of other mammalian PDEs.

The kinetic data comparing PDE5 catalytic fragment and full-length PDE5 suggest but do not prove that the amino-terminal sequence extending through the allosteric cGMP-binding domain does not serve an autoinhibitory function under the conditions used in this study. Neither the PDE activity nor the substrate affinity of the catalytic domain changes significantly in the absence of the regulatory domain under the conditions used in this study. The results reported are also supported by independent studies of the catalytic properties of a chimeric PDE6α/PDE5 enzyme which contains the PDE6α amino terminus including the allosteric cGMP-binding sites and the PDE5 catalytic domain (57). The catalytic properties of this chimeric PDE were indistinguishable from those of the wild type PDE5. Caution must be used in drawing such conclusions since purification, assay conditions, or other handling of these holoenzymes could alleviate or obscure effects of the regulatory domain. Nevertheless, the similarities in the kinetic properties of these various enzymes (native PDE5, wild type PDE5, chimeric PDE6α/PDE5, and the PDE5 catalytic domain) are remarkable given the fact that they are derived from different sources using quite different purification protocols. However, if preparations of either of these forms of PDE5 contain inactive (denatured) forms of protein, then quantitative comparisons cannot be drawn. The possibility cannot be excluded that the so-called hinge region of the PDE5, which is located between the allosteric cGMP-binding region and the catalytic domain, contains an autoinhibitory element. This region is included in the construct of the recombinant catalytic fragment (Fig. 1) and could influence catalytic activity since it is present in both the recombinant catalytic fragment and the
full-length PDE5.

Effects of PDE Inhibitors—The effects of two PDE inhibitors on the PDE5 catalytic fragment were examined in order to determine whether the site of interaction with inhibitors is located exclusively within the catalytic domain of PDE5, i.e. whether components of the PDE5 regulatory domain are involved in producing the wild type inhibitory profiles. The catalytic fragment and full-length wild type PDE5 had similar IC_{50} values (20 μM) for 3-isobutyl-1-methylxanthine (IBMX), a classical PDE inhibitor (Fig. 5A). Sildenafil (Viagra™), a much more potent and specific inhibitor of PDE5, also potently inhibited the PDE5 catalytic fragment (IC_{50} = 4 nM) (Fig. 5B) in agreement with the value for native PDE5 (3.9 nM) (24) or full-length wild type recombinant PDE5 (3.6 nM). Since no differences in inhibitory profiles were revealed by the comparison of PDE5 holoenzyme and catalytic fragment, it is concluded that the monomeric catalytic domain of PDE5 contains a binding site for either IBMX or sildenafil (Viagra™), which is sufficient for high affinity of PDE5 interaction with these inhibitors without PDE5 oligomerization or regulatory domain interaction. The PDE families and subfamilies are very different in inhibitor selectivities, and this selectivity is often used as a method of distinguishing PDEs in a mixture. The catalytic domain of PDEs is relatively conserved (25–40% (4)), so it is possible that the more variant regulatory domains contribute to the variability of inhibitor action for the different PDEs. Therefore, it is important to assess the IC_{50} profiles carefully when studying PDEs with the missing regulatory domains. This was especially true in the case of PDE5, for which the influence of the regulatory domain on catalytic activity is not well understood.

Other PDE catalytic fragments have also been analyzed for their interaction with PDE inhibitors. In one study, amino-terminal deletion mutants of PDE3 produced 3-fold higher IC_{50} values for standard inhibitors lixazinone and milrinone (37). However, another group found that the values for IC_{50} of their PDE3 deletion mutants with cGMP and milrinone were similar to that of wild type. Deletion mutants of PDE4 (deletion of 97 amino acids at the amino terminus or 99 amino acids at the carboxyl terminus) did not abolish PDE inhibition by rosiglitazone, RO 20–1724, or milrinone (15). However, the regulatory domains of the PDEs are very divergent, and the lack of influence of the regulatory domains of a few other PDE families on PDE inhibitor effects might not have represented the mechanism in all PDEs. The PDE subfamilies are very different in inhibitor selectivity, and the mechanism of inhibition may vary depending on the PDE and the inhibitor being studied. It seems from the experimental data presented in this paper and those of other laboratories that the sites of PDE inhibitor interaction and specificity must reside, for the most part, within the catalytic domain.

Concluding Remarks—In conclusion, we have successfully constructed and expressed a recombinant deletion mutant of PDE5. The PDE5 catalytic fragment (368 amino acids) contains the conserved sequence that was proposed to represent the catalytic domain of PDEs. Since this mutant, which comprises the entire conserved catalytic domain and carboxyl terminus of PDE5, lacks almost all of the putative regulatory domain and is hydrolytically active, the essential structural requirements for hydrolysis of cyclic nucleotide must reside within this region of PDE5. Therefore, the regulatory domain of PDE5 is not required for the catalytic process. Removal of the regulatory domain does not significantly alter either the enzyme affinity for cGMP as substrate or the enzyme rate under the conditions used. These studies do not rule out the possibility that the regulatory domain of PDE5 can, under certain conditions, influence PDE5 catalytic function: modulate activity, localize the protein, or some other mechanism. Two known PDE inhibitors, IBMX and sildenafil (Viagra™), inhibit the recombinant catalytic fragment with IC_{50} values that closely approximate those of wild-type PDE5. Therefore, inhibition of PDE5 by IBMX or sildenafil (Viagra™) apparently does not involve elements of the regulatory domain. Physical characterization of the PDE5 recombinant catalytic fragment reveals that this enzyme is monomeric. The experiments described in this paper are the first to unequivocally demonstrate that a monomeric PDE catalytic domain is sufficient for catalytic activity. Thus, the process of catalysis, the inhibitor potency, and affinity of the wild type PDE5 for cGMP do not require the interaction of two catalytic domains across its dimeric structure or the interaction of a regulatory domain and a catalytic domain within a single subunit.

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I. V. Turko, unpublished results.
Cyclic Nucleotide Phosphodiesterase Catalytic Fragment

core facility. Amino acid sequencing of the PDE5 catalytic fragment was performed by Eric Howard and Dr. Masaaki Tamura in the Vanderbilt Protein Chemistry Laboratory (P30 CA68455). We also thank Richard Whitesell for the gift of his time and help in the graphing and analyses of kinetic data. Many reagents were obtained via the Vanderbilt Diabetes Center core facilities with the aid of Kris Ellis, Shelley Wright, Scott Wright, and Dr. Rob Hall. Computer analyses and densitometry was performed in part in the Vanderbilt University Medical Center Cell Imaging Resource (CA68455 and DE20593), under the patient direction of Jonathan Sheehan. Wild type PDE5 was provided by Dr. Lark Turko and Toni Renee Dawson.

REFERENCES


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