Potential Roles of Conserved Amino Acids in the Catalytic Domain of the cGMP-binding cGMP-specific Phosphodiesterase (PDE5)∗

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The known mammalian 3′:5′-cyclic nucleotide phosphodiesterases (PDEs) contain a conserved region located toward the carboxyl terminus, which constitutes a catalytic domain. To identify amino acids that are important for catalysis, we introduced substitutions at 23 conserved residues within the catalytic domain of the cGMP-binding cGMP-specific phosphodiesterase (cGB-PDE; PDE5). Wild-type and mutant proteins were compared with respect to \( K_m \) for cGMP, \( k_{cat} \), and IC\(_{50}\) for zaprinast. The most dramatic decrease in \( k_{cat} \) was seen with H643A and D754A mutants with the decrease in free energy of binding (\( \Delta G_F \)) being about 4.5 kcal/mol for each, which is within the range predicted for loss of a hydrogen bond involving a charged residue. His\(_{643}^{\text{a}}\) and Asp\(_{714}^{\text{a}}\) are conserved in all known PDEs and are strong candidates to be directly involved in catalysis. Substitutions of His\(_{603}^{\text{a}}\), His\(_{607}^{\text{a}}\), His\(_{647}^{\text{a}}\), Glu\(_{672}^{\text{a}}\), and Asp\(_{714}^{\text{a}}\) produced marked changes in their binding and catalytic properties.

**EXPERIMENTAL PROCEDURES**

Materials—[\(^{3}H\)]cGMP was purchased from Amersham Corp. cGMP, histone VIII-S, Crotalus atrox snake venom, 3-isobutyl-1-methylxanthine, and zaprinast were obtained from Sigma. Hydroxyapatite was from Bio-Rad.

Site-directed Mutagenesis—cGB-8/14 clone encodes a full-length bovine lung cGB-PDE (11). The QuikChange site-directed mutagenesis kit (Strategene) has been used to make point mutations in the cGB-8/14 clone in pBacPAK9 expression vector (CLONTECH) according to the protocol from Strategene. The following pairs of mutagenic oligonucleotides were used: 1) Y596A, 5'-G TCT CCA ATT ATG AAA GGC GAC GTT CTT CC-3' and 5'-AAT TGG AGA GCT GCC TTT AAT ACA GC-3'; 2) H603A, 5'-G TCT CCA ATT ATG AGC GGC GAC GTT CTT CC-3' and 5'-G TCT CCA ATT ATG AGC GGC GAC GTT CTT CC-3'; 3) Y602F, 5'-G TCT CCA ATT ATG AGC GGC GAC GTT CTT CC-3' and 5'-AAT TGG AGA GCT GCC TTT AAT ACA GC-3'; 4) H603A, 5'-G TCT CCA ATT ATG AAA GGC GAC GTT CTT CC-3' and 5'-AAT TGG AGA GCT GCC TTT AAT ACA GC-3'; 5) N604A, 5'-G TCT CCA ATT ATG AAA GGC GAC GTT CTT CC-3' and 5'-AAT TGG AGA GCT GCC TTT AAT ACA GC-3'; 6) H603A, 5'-G TCT CCA ATT ATG AAA GGC GAC GTT CTT CC-3' and 5'-AAT TGG AGA GCT GCC TTT AAT ACA GC-3'; 7) N604A, 5'-G TCT CCA ATT ATG AAA GGC GAC GTT CTT CC-3' and 5'-AAT TGG AGA GCT GCC TTT AAT ACA GC-3'; 8) H603A, 5'-G TCT CCA ATT ATG AAA GGC GAC GTT CTT CC-3' and 5'-AAT TGG AGA GCT GCC TTT AAT ACA GC-3'; 9) N604A, 5'-G TCT CCA ATT ATG AAA GGC GAC GTT CTT CC-3' and 5'-AAT TGG AGA GCT GCC TTT AAT ACA GC-3'.

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The abbreviations used are: PDE, 3′:5′-cyclic nucleotide phosphodiesterase; cGB-PDE, cGMP-binding cGMP-specific phosphodiesterase; MOPS, 3-\(N\)-morpholino)propanesulfonic acid.

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\[ \text{GAC GCA GGA GAT AGA GAG AGG}^{-3} \]

Equation 1.

and centrifuged at 48,000 rpm for 30 min at 4 °C. The supernatant was diluted with six volumes of ice-cold deionized water and concentrated to approximately 1 ml using an Amicon filtration cell equipped with a PM-30 membrane. All purification steps were performed at 4 °C. The final preparation was stored in 20% glycerol at −70 °C.

Catalytic Activity of cGB-PDE—PDE activity was measured using a modification of the assay procedure described previously (12). Incubation mixtures contained 40 mM MOPS, pH 7.5, 0.5 mM EGTA, 15 mM magnesium acetate, 0.15 mg/ml bovine serum albumin, 20 μg/ml Zaprinast (unless otherwise stated), [3H]cGMP (100,000–150,000 cpm/assay), and one of the cGB-PDE samples, in a total volume of 250 μl. The incubation time was 10 min at 30 °C. The reaction was stopped by placing the tubes in a boiling water bath for 3 min. After cooling, 20 μl of 10 mg/ml C. atrox snake venom was added, followed by a 20-min incubation at 30 °C. Nucleoside products were separated from unreacted nucleotides on the columns with DEAE Sephadex A-25 equilibrated with 20 mM Tris-HCl buffer, pH 7.5, and counted. In all studies, less than 15% of the total [3H]cGMP was hydrolyzed during the reaction. The apparent \( K_m \) and \( V_{max} \) values were determined from Lineweaver-Burk plots after assaying PDE activity in duplicate at 1–250 μM cGMP. \( K_m \) was obtained by dividing \( V_{max} \) by the molar enzyme concentration. The molar enzyme concentration was calculated as described below.

Mutagenesis Strategy—The sequence alignments of the conserved catalytic domain of different PDEs have been published (13). The conserved residues in the catalytic domain of PDE5 were identified using the program MINSQ II (Micromath Scientific Software, Salt Lake City, UT) to obtain the dissociation constant \( K_d \), which was then rinsed four times with a total of 4 ml of cold 10 mM sodium phosphate buffer, pH 6.8, with 1 mM EDTA, and then dried and counted. The data were corrected by subtracting nonspecific binding, which was defined as either the [3H]cGMP bound in the absence of cGB-PDE or the [3H]cGMP bound in the presence of a 100-fold excess of unlabeled cGMP. A similar 2–4% of nonspecific binding was obtained with each method. The data were subjected to nonlinear least squares analysis using the program MINOS II (Micromath Scientific Software, Salt Lake City, UT) to obtain the dissociation constant \( K_d \).

Other Methods—SDS-electrophoresis in 10% polyacrylamide gels and Western blot analysis were done as described previously (12). Total protein concentrations were determined by the method of Bradford (14) using bovine serum albumin as the standard. To determine the cGB-PDE protein concentration, the Coomassie Brilliant Blue-stained SDS-PAGE bands were scanned with a densitometer (Hoeffer). The cGB-PDE protein concentration was calculated from the ratio of the cGB-PDE band densities to the total protein concentration determined by Bradford assay. To convert the cGB-PDE protein concentration into the molar cGB-PDE concentration, the value of the molecular weight of cGB-PDE of 98.5 kDa (calculated from the amino acid sequence of cGB-PDE) was used.

RESULTS

Mutagenesis Strategy—The sequence alignments of the conserved catalytic domain of different PDEs have been published (2, 10, 15, 16). These studies revealed two blocks of conserved amino acid residues (Try706, His767 and Asp764, Gly768, in the case of cGB-PDE) separated by a variable sequence containing two invariant residues (Thr713 and Asp714 in the case of cGB-PDE) located approximately in the middle of this sequence (Fig. 1). It has been suggested that the first block is responsible for Zn\(^{2+}\) binding and could be part of the catalytic machinery of PDEs (13). Mutational studies on one of the PDE4 isozymes have shown that replacement of invariant His788, His311, or Thr349 (corresponding to His643, His675, or Thr713 in cGB-PDE) decreased the \( V_{max} \) of this enzyme, but \( K_m \) measurements for substrate were not reported (7). The second block possesses...
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Fig. 1. The sequences of cGB-PDE targeted for mutagenesis. Residues that are conserved in the catalytic domain of all known mammalian PDEs are shown in bold. The residues that were individually substituted in this study are indicated with asterisks. The numbers on the top indicate the position of the residue in the cGB-PDE sequence.

The position of the residue in the cGB-PDE sequence.

some general sequence similarity with the allosteric cGMP-binding sites (12) and could be involved in substrate binding. These findings prompted us to systematically assess the functional role of individual conserved amino acids using scanning mutagenesis.

Twelve amino acid residues in the first block were substituted singly by alanine. Thr and Asp of the TD dyad and seven residues in the second block were also replaced by alanine. Lys and Phe in the second block were replaced by Met and Leu, respectively.

Expression and Purification of Wild-type and Mutated Forms of cGB-PDE—Wild-type and mutants of the bovine lung cGB-PDE were expressed in High Five cells as described under "Experimental Procedures." The levels of expression of most of the mutants were comparable to that of the wild-type enzyme. The total production of recombinant cGB-PDEs was approximately 1–6 mg/100 ml of culture. The wild-type and mutant cGB-PDEs were partially purified similarly 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 these proteins from the hydroxyapatite column compared with that for the wild-type enzyme. Fig. 2 shows a Coomassie Blue-stained SDS-polyacrylamide gel of partially purified mutants obtained following the hydroxyapatite column step. All mutated cGB-PDEs migrated with essentially the same mobility as that of the wild-type enzyme. The identity of the recombinant proteins was verified by Western blot analysis (data not shown).

Kinetic Analysis of Mutants—The kinetic parameters, $K_m$ for cGMP and $k_{cat}$ (Table I), were determined from Lineweaver-Burk plots. The contribution of the substituted amino acid side chain to binding energy in enzyme-transition state complexes was calculated from values of the catalytic efficiency ($k_{cat}/K_m$) using Equation 2.

$$\Delta G_T = -RT \ln \left( \frac{k_{cat}}{K_m} \right)_{mutant} / \ln \left( \frac{k_{cat}}{K_m} \right)_{wild-type}$$ (Eq. 2)

$\Delta G_T$ is the change in the free energy of binding in enzyme-transition state complexes attributable to the substituted group (17). $R$, the ideal gas constant, is equal to $1.98 \times 10^{-3}$ kcal/degree/mol, and $T$, the temperature at which the assay was done, is equal to 303 K. The effect of substitution of the amino acid side chain that interacts with a substrate may be manifested in terms of $k_{cat}$, $K_m$, or both (17). In the present study, the binding of substrate in the transition state was chosen because the intrinsic binding energy of groups on the enzyme and substrate may not be fully realized until the enzyme-transition state complex is formed, whereby some of the binding energy may be diverted to stabilize the transition state.

Previous studies have determined the magnitude of the changes in transition state binding expected for the disruption of particular interactions between enzymes and substrates (18, 19). Deletion of a charged group to disrupt a hydrogen bond between the enzyme and a substrate weakened the binding energy by 3.5–4.5 kcal/mol (19), whereas the disruption of an electrostatic interaction between a charged group in the enzyme and substrate weakened binding by 2.0 kcal/mol (18). It is important to emphasize that the values for the calculated $\Delta G_T$ are maximum values that include any loss of binding energy due to small perturbations of the overall conformation of the enzyme. Therefore, only the amino acid positions whereby substitutions cause large loss of function can be considered essential. Alternatively, the residues whose substitution lead to moderate changes may be involved in the general arrangement of the catalytic site.

Based on calculated $\Delta G_T$ (Table I), the mutants could be arbitrarily placed into two groups. The first group includes Y596A, E632A, H674A, H675A, S756A, K760M, F776L, G780A, D781A, and E783A mutants. The changes found for these mutants are not sufficient to suggest an essential role for these residues. The second group of 13 mutants have $\Delta G_T$ values in the range expected for important roles for these amino acid residues in the wild-type enzyme. This group could be divided into three categories: those defective mainly in $k_{cat}$, those defective mainly in $K_m$, and those defective in both of these parameters.

Mutants Defective in $k_{cat}$—Nine mutants have $k_{cat}$ that is less than 15% of the wild-type value (Fig. 3), including two mutants (H643A and D754A) that retain only 0.4% of wild-type $k_{cat}$. Substitution within the second block of conserved amino acid residues (Fig. 1) had little effect on $k_{cat}$ value, except for substitution of the invariant Asp754. Mutations with markedly decreased $k_{cat}$ were primarily clustered around the conserved HX2-HX2-E motifs of the putative Zn$^{2+}$-binding site (13). The mutation of the Asp754 in the invariant TD dyad also displayed significantly reduced PDE activity.

Five mutants (H603A, N604A, H607A, D644A, and D714A) were defective in $k_{cat}$ only (Table I). These residues may be
directly involved in catalysis, or they may provide important structural features that allow for effective catalysis. Substitution of these residues may perturb the configuration of the active site. H643A and D754A mutants have a 7-fold increase in $K_m$; however, this defect is insignificant in comparison to the large decrease (270- and 280-fold, respectively) in $k_{cat}$. One possible interpretation of such large changes by mutation of His$^{643}$ and Asp$^{754}$ is that these residues represent a catalytic dyad.

Mutants Defective in $k_{cat}$ and $K_m$—H647A and E672A mutants possess a 10- and 14-fold increase in $K_m$ for cGMP, and an 8- and 13-fold decrease in $k_{cat}$, respectively. The role of these residues cannot be interpreted unambiguously. They may be involved in catalysis, important for recognition of substrate, or provide a structural role.

Mutants Defective in $K_m$—Four mutants (Y602A, T713A, E775A, and Q779A) were defective mainly in $K_m$ (Table I). Two of these (Thr$^{713}$ and Glu$^{779}$) are uncharged amino acids and, despite the moderate changes in $K_m$ when these are substituted with alanine, the $\Delta \Delta G_T$ for each of these mutants was in the range that is predicted for loss of a hydrogen bond between an enzyme polar side chain and the substrate (19). Two mutants, Y602A and E775A, exhibited profound losses in affinity for cGMP with $K_m$ values of 65 and 70 $\mu M$, respectively, compared with a $K_m$ of 2 $\mu M$ for wild-type cGB-PDE. The $\Delta \Delta G_T$ for each of these mutants is 2.9 or 2.8 kcal/mol, respectively, which is within the range expected for the loss of a salt bridge (electrostatic interaction) (18) and approaching the range (3.5–4.5 kcal/mol) expected for the loss of a hydrogen bond involving a charged residue (19).

To further probe the possible function of Tyr$^{602}$ and Glu$^{775}$ in cGMP binding to the substrate site, three additional mutants (Y602F, E775D, and E775Q) were generated, expressed and partially purified using the experimental procedures described for the major set of mutants. Y602F, E775D, and E775Q possessed the same level of expression, the same chromatographic behavior on hydroxyapatite columns, and exhibited the same mobility on the SDS-polyacrylamide gel as did wild-type enzyme. The identity of these mutants was verified by Western blot analysis (data not shown). Kinetic parameters of the Y602F mutant were indistinguishable from those of wild-type enzyme (Table II). Similarly, insignificant effect (3-fold) on $k_{cat}$ for cGMP and on $K_m$ was found for the E775D mutant. In contrast, the E775Q mutant was clearly defective in substrate binding.

Zaprinast-binding Site—Zaprinast selectively inhibits cGMP-specific PDEs (20). Fig. 4 shows that double reciprocal plots for wild-type cGB-PDE at various concentrations of zaprinast intersect at $1/V_{max}$; this indicates a competitive inhibitory mechanism for zaprinast. By determining the values of $K_m$ for cGMP at different concentrations of zaprinast, the value for $K_m$ was 0.15 $\mu M$ under the experimental conditions used. As a competitive inhibitor, zaprinast should directly compete with cGMP for the active site of cGB-PDE. Thus, zaprinast may be used to probe the structure of the cGB-PDE active site. IC$_{50}$ values for zaprinast using the wild-type and all of the mutant enzymes are shown in Table I. To generate each IC$_{50}$ value, the cGMP concentration in the assay was one-third the $K_m$ for each of the mutant tested. When using low substrate concentrations, IC$_{50}$ values approach the $K_m$ for a competitive inhibitor. To decipher how the replacement of conserved residues in the catalytic domain of cGB-PDE affects the affinity for zaprinast in com-
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Fig. 4. Inhibition of cGB-PDE by zaprinast. Lineweaver-Burk plots were generated for the wild-type cGB-PDE at various concentrations of zaprinast: none, 0.5 μM, 1.0 μM, and 2.0 μM.

Fig. 5. Comparison of Km for cGMP and IC50 for zaprinast of catalytic domain mutants of cGB-PDE. Km for cGMP and IC50 for zaprinast of wild-type enzyme were taken as 1.0, and the corresponding values for each mutant (from Table I) are expressed as a fold increase with respect to the wild-type cGB-PDE. The identity of the protein preparation is indicated under each bar.

The strategy of scanning mutagenesis is widely used to identify potentially important residues involved in protein function. Once identified, these residues can be analyzed more extensively to further examine their functional and structural role.
In this study, the possible role of 23 amino acid residues that are conserved in all known catalytic domain of PDEs has been evaluated by systematic substitution of these residues in cGB-PDE. All mutants were expressed as full-length enzymes with similar levels of production and the same chromatographic properties. This indicates that the substitutions did not cause gross perturbations to the tertiary conformation and subsequent destabilization or proteolysis of the enzymes.

An interesting finding was that the residues that are most important for catalysis (His643 and Asp754) and for substrate binding (Tyr602 and Glu775) are not located in two different blocks of conserved residues. Furthermore, the invariant TD dyad, which is located in the highly variable sequence between these two blocks, contributes to catalysis (Asp714) and substrate binding (Thr715). Such distribution of the functionally important residues assumes that the active site may be formed at the interface between these blocks of conserved residues. Thus, the catalytic and substrate-binding components are overlapping.

Nine residues (His603, Asn604, His607, His643, Asp644, His647, Glu672, Asp714, and Asp754) have been found to be important for catalysis in cGB-PDE. Substitution of either of two of them, His643 or Asp754, is accompanied by the largest decrease in catalytic efficiency. His643 of cGB-PDE corresponds to His278 of a cAMP-specific PDE (RNPDE4D). The latter residue was shown to be critical for PDE4 activity (7), and our results are consistent with this observation. In addition to His278, two more residues (His311 and Thr349) of PDE4 were found to be critical for catalytic activity in PDE4 (7). Substitution of the corresponding positions of cGB-PDE (His675 and Thr713) produced little effect on catalysis. Instead of a possible His/Thr catalytic dyad in PDE4, a possible His/Asp catalytic dyad was uncovered in cGB-PDE (PDE5).

A full understanding of PDE catalysis is complicated by the fact that the activities of PDEs are supported by a number of divalent metal cations (13, 21, 22). The catalytic activity of cGB-PDE is supported by Zn$^{2+}$, Mn$^{2+}$, Co$^{2+}$, and Mg$^{2+}$, but Zn$^{2+}$ promotes GTPase activity at lower concentrations than do other cations (13). Sequence alignment reveals two putative Zn$^{2+}$-binding motifs which are conserved in all mammalian PDEs (13). These motifs include His603, His607, and Glu632 followed by His643, His647, and Glu672 residues of cGB-PDE. All of these residues were replaced with alanine in the present study, and five of six of these mutants exhibited a marked decrease in catalytic efficiency (Table I). The only residue in this group which seems nonessential for catalysis is Glu632. The $k_{cat}$ values of these mutants are critical for interpretation, because it is assumed that Tyr602, Thr713, Glu775, and Glu779 residues of cGB-PDE are important for binding the cGMP substrate. Unfortunately, the magnitude of $K_m$ changes for T713A and Q779A mutants cannot be interpreted unambiguously, because the values for the calculated $\Delta G_f$ are maximum values that include any loss of binding energy due to small perturbations of the overall conformation of the enzyme. Only the amino acid positions whereby substitutions cause large loss of function can be considered essential. Alternatively, the residues whose substitution lead to moderate changes in substrate binding may be involved in the general arrangement of the substrate-binding site, and do not necessarily interact directly with substrate. For this reason, only two residues (Tyr602 and Glu775), for which substitution exhibited the largest changes in $K_m$ (Table I) were selected for further analysis, and additional mutations (Y602F, E775D, and E775Q) were generated and analyzed (Table II). The possible roles of Tyr602 and Glu775 in cGMP binding in the catalytic site of cGB-PDE are discussed below.

Tyrosine 602—There are several options for the tyrosine residue to interact with cGMP: 1) a hydrogen bond to an oxygen atom on the phosphoryl group, 2) a hydrogen bond to the 2'-OH group of the ribose ring, and 3) stacking interaction with the base. These types of interactions for a tyrosine residue have been found in the complex of ribonuclease T1 with 2'-GMP (26), in the nonphysiological complex of nucleoside diphosphate kinase with cAMP (27), in the AMP-binding site of fructose-1,6-bisphosphatase (28), and in site B of the regulatory subunit of protein kinase A (29). To scrutinize the possible functions of Tyr602 in the cGB-PDE catalytic site, results of cyclic nucleotide analogs as competitive inhibitors of cGB-PDE (30) can also be considered. The published results indicate that the 2'-OH group of cGMP is not a major requirement for binding to the catalytic site of cGB-PDE (30, 31). Tyr602 is invariant in all mammalian PDEs, but in Drosophila dunce PDE (32) and in Saccharomyces cerevisiae PDE (33), the corresponding Tyr602 position is occupied by Phe. Phe could mimic Tyr for possible participation in stacking interactions with the guanine base. The Y602F mutant was indistinguishable from wild-type enzyme in terms of $K_m$ and $k_{cat}$ values (Table II). This is a strong argument that Tyr602 stacks with the purine base of cGMP in the substrate-binding site of cGB-PDE and is likely to serve a similar role in other PDEs.

Glutamic Acid 775—There are two major chemical options for the glutamic acid residue to interact with cGMP: 1) a hydrogen bond interaction with the 2'-OH group of the ribose ring, such as that found in the catabolite gene activator protein.
for catalysis; (iii) Tyr602 and Glu775 are critical for cGMP binding to the PDE catalytic site, whereas the 2-amino group of cGMP contributes to binding at the catalytic site, whereas the 2-amino group of cGMP is not a major requirement for interaction (30, 31). Thus, a possible function of Glu775 could be for interaction with the hydrogen atom of the N-1 endo-nitrogen of the guanine base. The fact that the E775D mutant exhibits only small changes in the major contact points for these two compounds are quite different. This could explain in part the inhibitory effect of zaprinast, which might maintain a different orientation in the binding site, and could interact with a residue which is critical for catalysis (such as Asp754).

In summary, we have assessed the effect of substitution at 23 positions in the catalytic domain of the cGB-PDE which are conserved in all known mammalian PDEs. To our knowledge, this is the most comprehensive mutational analysis of any PDE reported to date. The major observations of this study are as follows: (i) His642 and Asp754 are critical for cGMP-PDE catalytic activity; (ii) His603, His607, His643, His647, Glu672, Asp714, and Asp754 may be part of a metal-binding site which is important for catalysis; (iii) Tyr602 and Glu775 are critical for cGMP binding, and Tyr602 is probably involved in stacking interactions with the guanine base of cGMP; (iv) the zaprinast-binding site is overlapping with the substrate cGMP-binding site, but some of the residues for binding these two ligands differ.

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