# Histidine-607 and Histidine-643 Provide Important Interactions for Metal Support of Catalysis in Phosphodiesterase-5†

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ABSTRACT: Class I cyclic nucleotide phosphodiesterases (PDEs) share a catalytic domain containing 18 invariant residues. In cGMP-binding cGMP-specific PDE (PDE5), we showed previously that point mutation of nine of these profoundly decreases $k_{cat}$ when the assay is conducted in the presence of $Mg^{2+}$; seven of these are in the prototypical metal-binding motifs A and B ($HX_3HX_nE$) that we identified earlier. Tandem arrangement of two of these metal-binding motifs in PDEs is novel, and whether residues within these motifs are involved in metal support of catalytic activity is a fundamental question in this field. This report shows that mutation of either His-607 (A motif) or His-643 (B motif) to alanine profoundly diminishes support of PDE catalysis by $Mn^{2+}$ or $Mg^{2+}$, but mutation of His-647 in B motif or of Glu in either motif does not. H607A and H643A mutants have much greater maximum catalytic rates supported by $Mn^{2+}$ than that by $Mg^{2+}$; catalytic activity of H603A mutant is supported weakly by either. In H607A and H643A, $K_a$s for $Mn^{2+}$ and $Mg^{2+}$ are increased, but the effect of $Mn^{2+}$ is 2-fold greater than that of $Mg^{2+}$ in each. Mutation of any of the other conserved residues (Asn-604, Asp-644, His-675, Asp-714, and Asp-754) causes unremarkable changes in $Mn^{2+}$ or $Mg^{2+}$ support of catalysis. This study identifies specific residues in PDE5 that contribute to interactions with catalytically relevant metals. The combined data suggest that despite a high degree of sequence similarity between each $HX_3HX_nE$ motif in PDEs and certain metallo-endopeptidases, PDEs employ a distinct complement of residues for interacting with metals involved in catalysis.

Cellular levels of cAMP and cGMP are determined by the relative activities of adenylyl and guanylyl cyclases which synthesize these nucleotides and cyclic nucleotide phosphodiesterases (PDEs)[1] which hydrolyze them to 5′-nucleoside monophosphates. Both cyclases and PDEs are highly regulated superfamilies of enzymes. Despite the critical catalytic requirement for divalent cation(s) in PDE function, nothing is known about the residues that interact with metal, whether there are sites that select for different metals, or the mechanism by which these cations participate in the catalytic process. In 1994, we recognized that the catalytic domains of all mammalian PDEs known at that time contained two metal-binding motifs ($HX_3HX_nE$) arranged in tandem and separated by 10 amino acids (Figure 1) (*1*). Each motif closely resembles the canonical catalytic $Zn^{2+}$-binding motif found in certain metallo-endopeptidases such as thermolysin (*2*−*4*). Since the tandem arrangement of two metal-binding motifs in PDEs is unique and the stoichiometry of $Zn^{2+}$ binding to PDE5 is ∼3 mol/PDE5 monomer, it was thought that metal could possibly bind independently at each motif or that the two motifs could join to form a novel metal-binding site (*1*). We proposed that these motifs in PDEs could provide for interaction with catalytically relevant metal(s) and that both motifs are likely to be important for catalysis (*1*). This prediction is supported by the fact that individual mutation of five of the six residues in the two motifs of PDE5 (His-603, His-607, His-643, His-647, and Glu-672) causes profound loss of catalytic activity measured in the presence of $Mg^{2+}$ [$k_{cat}(Mg^{2+})$]; this implies that a single one of these metal-binding motifs is insufficient to support normal catalysis in PDEs (*5*). This result along with a lack of a substantial effect of mutation of Glu-632 in motif A on catalysis suggests that metal binding in this region is unlikely to involve two discrete $HX_3HX_nE$ sites of the metallo-endopeptidase configuration.

Traditionally, $Mg^{2+}$ has been used as the divalent cation in PDE assays (*6*); however, $Zn^{2+}$, $Mn^{2+}$, or $Co^{2+}$ can also support catalysis, and in certain instances, these cations are more potent than $Mg^{2+}$ (*1*, *7*, *8*). Several enzymes that utilize $Zn^{2+}$ and/or $Mn^{2+}$ are known to contain metal-binding sites that are created by multiple histidines and acidic residues (*9*−*16*). We demonstrated that $Zn^{2+}$, closely followed by $Mn^{2+}$, is the most potent cation in supporting catalysis by PDE5 (*1*). In contrast, quite high concentrations of $Mg^{2+}$ are required. Metal-binding sites such as that in the active

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[1] Abbreviations: PDEs, cyclic nucleotide phosphodiesterases; PDE5, cGMP-binding cGMP-specific cyclic nucleotide phosphodiesterase; EDTA, ethylenediaminetetraacetic acid.

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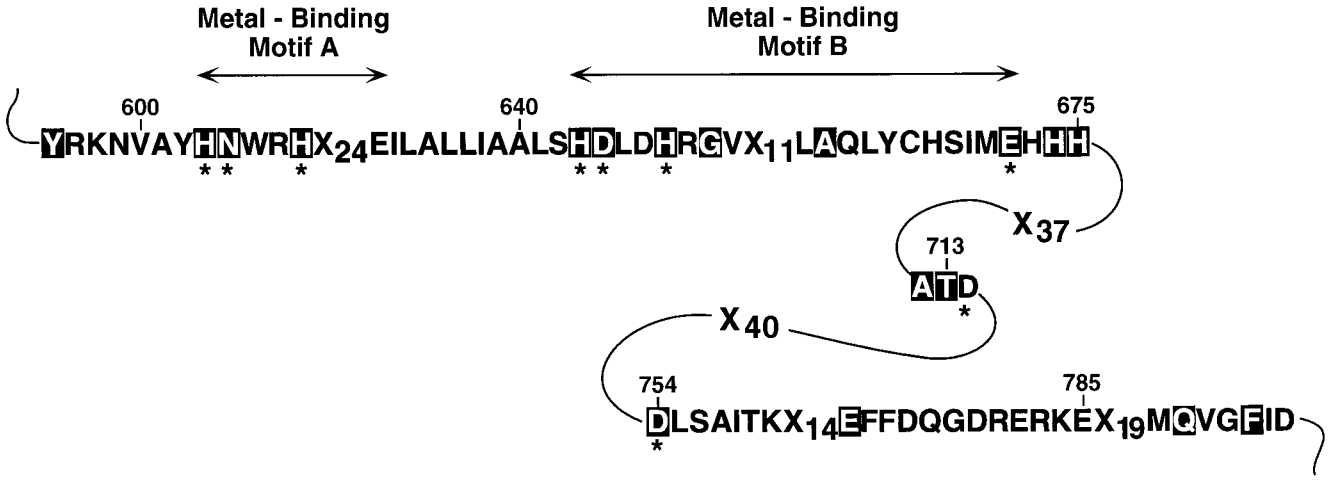


FIGURE 1: Sequence containing conserved catalytic domain residues that were mutated in PDE5. Residues that are conserved in catalytic domains of all known class 1 PDEs are shown in black boxes. Each of these (with exception of Ala-640, Ala-663, Ala-712, Gln-806, and Phe-808) was individually mutated in full-length PDE5 and characterized for changes in $K_m$ for cGMP and $k_{cat}(Mg^{2+})$ as previously described (*5*). Mutations that caused a ~10-fold or greater decrease in $k_{cat}(Mg^{2+})$ of PDE5 are indicated by asterisks, and these same mutations are the subject of this report. Numbers above residues indicate positions of those amino acids in bovine PDE5 A1 sequence. The metal-binding motifs A and B denote the two canonical $HX_3HX_nE/D$ sequences in all known mammalian PDEs that closely resemble the single metal-binding motif that coordinates the catalytic metal in certain metallo-endoproteases, such as thermolysin (*2*−*4*).

site of thermolysin, which use a nitrogen-rich $HX_3HX_nE$ motif to coordinate a catalytic $Zn^{2+}$, can also bind $Mn^{2+}$ (*4*, *17*), and a site containing two histidines and a glutamic acid has been described for binding $Mn^{2+}$ in the active site of 3,4-dihydroxyphenylacetate 2,3-dioxygenase (*18*). Coordination of $Mg^{2+}$ more typically involves a site rich in oxygen atoms derived from residues such as Asp, Glu, Thr, Ser, and Tyr (*2*, *3*, *19*). The study presented here identifies specific conserved residues within the catalytic domain of PDE5 that contribute importantly to interaction with $Mn^{2+}$ or $Mg^{2+}$ in supporting catalysis and compares the importance of each of the conserved histidines and glutamates in metal-binding motifs A and B in PDEs to that of homologous residues in the single metal-binding sites of certain metallo-endopeptidases.

## EXPERIMENTAL PROCEDURES

*Materials.* [$^3$H]cGMP was purchased from Amersham Life Science Inc. Cyclic GMP, histone VIII-S, *Crotalus atrox* snake venom 5′-nucleotidase, and 3-isobutyl-1-methylxanthine were obtained from Sigma. Puratronic highly pure $MgCl_2$ and $MnCl_2$ (99.999% metals basis) were purchased from John Matthey Co. QAE-Sephadex-25 was from Pharmacia Biotech Inc.

*Expression and Purification of Wild-Type and Mutant PDE5s.* The cGB-8/14 clone encodes a full-length bovine lung PDE5 (*20*). The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) has been used to introduce point mutations in the cGB-8/14 clone in the pBacPAK9 expression vector (Clontech, Palo Alto, CA) as previously described (*5*). Wild-type PDE5 and the PDE5 mutants were expressed in Sf9 cells and partially purified as previously described (*5*). Fractions containing PDE5 activity were diluted with 6 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, and all reagents and solutions were made using water (electrical resistivity of ~18 MΩ) that had been deionized using a three-cartridge water purification system. The final preparation was stored in 20% glycerol at −70 °C (*5*). For all subsequent assays, metal-free Eppendorf tips (USA Scientific) were used throughout, and reaction tubes were prerinsed four times with deionized water, dried, and stored in airtight containers prior to being used. All general glassware was hand-cleaned and reserved exclusively for these studies.

*Phosphodiesterase Catalytic Activity.* PDE catalytic activity was measured as previously described (*1*, *21*). An assay mixture containing a final concentration of 50 mM Tris buffer (pH 7.5), 40 μM unlabeled cGMP, and 0.4 μM [$^3$H]cGMP (~400000 cpm/assay) in a final volume of 150 μL was used. The assay was initiated by addition of PDE5 which had been diluted in ice-cold 50 mM Tris (pH 7.5); the concentration of various PDE5 proteins in assays ranged from 0.05 to 0.6 nM. The assay proceeded for ~15−30 min at 30 °C. Less than 15% of the cGMP was hydrolyzed per assay.

*Allosteric cGMP-Binding Activity.* The level of cyclic GMP binding was determined in a total volume of 60 μL containing 20 mM potassium phosphate buffer (pH 6.8), 2 mM EDTA, 25 mM 2-mercaptoethanol, 0.25 mM 3-isobutyl-1-methylxanthine, 0.5 mg/mL histone VIII, and 5 μM [$^3$H]-cGMP. The reaction was initiated by addition of enzyme followed by a 60 min incubation on ice. Assay mixtures were then filtered onto premoistened Millipore HAWP filters (0.45 μm pore size) and then rinsed four times with a total of 4 mL of cold 10 mM $KH_2PO_4$ (pH 6.8) and 1 mM EDTA. The filters were then air-dried and counted in a nonaqueous scintillant. Data were corrected by subtraction of the level of nonspecific binding, which was defined as the amount of [$^3$H]cGMP bound in the absence of PDE5.

*Data Presentation and Analysis.* Each of the experimental protocols was performed as indicated. Values presented are means ± the standard error.

## RESULTS

*Effectiveness of $Mn^{2+}$ and $Mg^{2+}$ in Supporting PDE Catalytic Activity.* We recently reported results of studies in

which each of the conserved amino acids in the catalytic domain of full-length recombinant bovine PDE5A was replaced using site-directed mutagenesis (Figure 1), and catalytic properties as well as cGMP binding activities were characterized (*5*). The structural integrity of each of these proteins was judged to be functionally intact since properties of cGMP binding to their allosteric cGMP-binding sites were essentially the same as those of wild-type (WT) PDE5 (*5*), but certain mutants exhibited profound changes in $k_{cat}$ or $K_m$ for cGMP. These results suggested that changes in catalytic activity resulted from specific perturbations introduced into the catalytic domain. Kinetic properties, including $K_m$ for cGMP, $k_{cat}$, and $IC_{50}$ for zaprinast of each mutant PDE5, were characterized; nine mutations dramatically lowered catalytic activity to less than 15% of that of WT PDE5. Those earlier analyses were conducted under standard assay conditions, which included 15 mM $Mg^{2+}$ acetate, but effects of these mutations on specificity and/or potency for $Mg^{2+}$ versus another cation, such as $Mn^{2+}$, were not studied. In the investigation presented here, we have used mutants from this series to examine whether the reduction in catalytic activity of these mutants results from alterations in the mutants' interaction with divalent cations at the active site.

All cyclic nucleotide PDEs require divalent cation to support catalysis, and $Mg^{2+}$ has been most commonly used to supplement PDE assays. Even in the absence of added divalent cation, WT PDE5 has a basal activity that is ~30% of that measured in the presence of saturating levels of either $Mn^{2+}$ or $Mg^{2+}$ (Figure 2A). This basal activity is most likely attributable to metal retained by WT PDE5 throughout purification since addition of EDTA abolishes basal activity (not shown). Maximum catalytic activity of either native bovine PDE5 or recombinant bovine WT PDE5 using either $Mn^{2+}$ or $Mg^{2+}$ is essentially the same [$V_{max}(Mn^{2+})/V_{max}(Mg^{2+}) = 1.2 \pm 0.1$, $n = 14$] (*22*), and at optimum concentrations of each metal, they do not produce additive effects on catalytic rate. The apparent affinity of either native or recombinant WT PDE5 for $Mn^{2+}$ is consistently higher than that for $Mg^{2+}$ (Figure 2A), but this relative affinity varies depending on the enzyme preparation and purification protocol. In these studies, recombinant WT PDE5 exhibits a ~3-fold higher apparent affinity for $Mn^{2+}$ ($K_a \sim 0.6 \pm 0.1$ mM) than for $Mg^{2+}$ ($K_a \sim 2.0 \pm 0.3$ mM).

The effectiveness with which either $Mn^{2+}$ or $Mg^{2+}$ supports PDE catalytic activity has been examined for nine mutants (H603A, N604A, H607A, H643A, D644A, H647A, E672A, D714A, and D754A) which have previously been shown to have profoundly deleterious effects on catalysis in the presence of $Mg^{2+}$ (*8*). Two other mutants (E632A and H675A), which had been found to have slight effects on catalysis, and WT PDE5 have also been analyzed to serve as controls. The most marked difference between the relative effectiveness of $Mn^{2+}$ and $Mg^{2+}$ in supporting maximum catalytic activity occurs in the H607A and H643A mutants (panels B and C of Figure 2), which have a 13-fold ± 1 SEM ($n = 6$) and a 26-fold ± 0.7 SEM ($n = 6$) preference for $Mn^{2+}$, respectively. Furthermore, catalytic activity present in the H607A and H643A mutants in the absence of added metals is typically very low (<4% of maximum catalytic activity achieved in the presence of a high $Mn^{2+}$ concentration) (panels B and C of Figure 2 and Table 1). The maximum catalytic activity that can be achieved using these

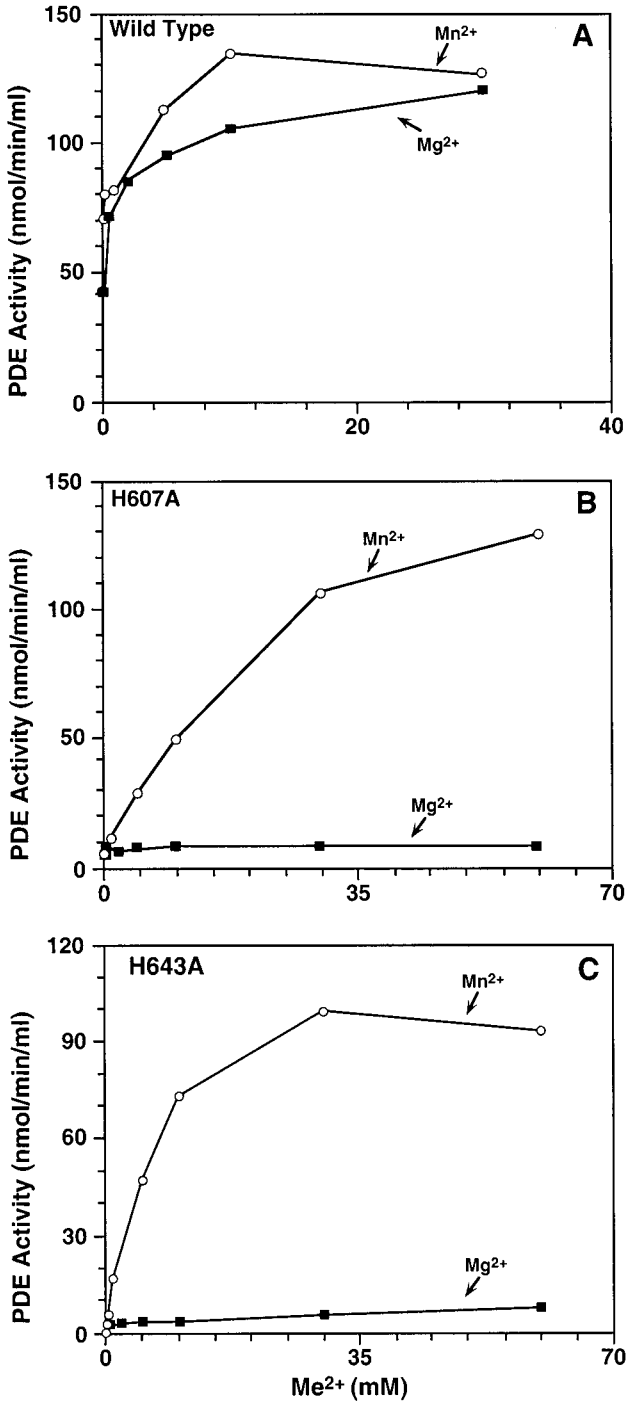


FIGURE 2: Comparison of effects of $Mn^{2+}$ or $Mg^{2+}$ in supporting PDE catalytic activity in WT PDE5 and in the H607A and H643A mutants. PDE activity was determined as described in Experimental Procedures, and the results used to generate the respective curves for $Mn^{2+}$ or $Mg^{2+}$ were generated in the same assay. The results for WT PDE5 (A) are representative of 10 separate assays performed in duplicate. Results shown in panels B (H607A) and C (H643A) are representative of four separate assays performed in duplicate and in conjunction with WT PDE5 for comparison. Note the difference in the *X*-axes in panel A vs panels B and C.

mutants at high concentrations of $Mg^{2+}$ is ~2- and 8-fold greater, respectively, than basal activity. In contrast, the maximum catalytic activity that can be achieved in these same mutants in the presence of high concentrations of $Mn^{2+}$ is 26- and 200-fold greater, respectively, than basal activity. Responsiveness of catalytic activity in the H607A mutant

Table 1[a]

| enzyme | $V_{max}(Mn^{2+})/V_{max}(Mg^{2+})$ | basal activity (% of maximum) | |
|---|---|---|---|
| | | $Mn^{2+}$ | $Mg^{2+}$ |
| wild type | 1.2 ± 0.1 | 22 ± 3 | 30 ± 4 |
| H603A | 0.47 ± 0.02 | 38 ± 10 | 22 ± 8 |
| N604A | 0.51 ± 0.01 | <2 | <2 |
| H607A | 13 ± 0.1 | 3.9 ± 1 | 13 ± 1 |
| E632A | 0.9 ± 0.04 | 20 ± 1 | 16 ± 2 |
| H643A | 26 ± 0.7 | 3 ± 1 | <1 |
| D644A | 0.5 ± 0.05 | 10 | 4 |
| H647A | 3.7 ± 0.5 | <1 | <1 |
| E672A | 3.6 ± 0.7 | 3 ± 0.4 | 8 ± 0.5 |
| H675A | 0.25 ± 0.04 | 9 ± 2 | 4 ± 2 |
| D714A | 1.6 ± 0.1 | 33 ± 14 | 22 ± 5 |
| D754A | 0.3 ± 0.03 | 59 ± 9 | 28 ± 8 |

[a] $V_{max}$ values in the presence of $Mn^{2+}$ or $Mg^{2+}$ and basal activities (no added metal) were determined for each PDE5 in the same sets of assays according to the protocol described in Experimental Procedures; in most instances, WT PDE5 activity was analyzed concomitantly. Metal concentrations required to support maximum catalytic activity for each protein varied due to altered metal affinities, and onset of inhibition of catalysis by higher concentrations of $Mn^{2+}$ varied. Therefore, $V_{max}(Mg^{2+})$ and $V_{max}(Mn^{2+})$ values were each determined from assays in which the concentration of the respective metal was varied from zero to 250 mM. The maximum catalytic rates for each mutant with the respective metals were derived from these concentration curves. Catalytic turnover rates for the respective proteins in the presence of $Mg^{2+}$ were comparable to $k_{cat}$ values previously reported for these proteins (*5*). The concentration of various PDE5 proteins in PDE assays ranged from 0.05 to 0.6 nM (based on cGMP binding stoichiometry). Results derived from data derived from *n* iterations: WT ($n = 14$), H603A ($n = 6$), N604A ($n = 8$), H607A ($n = 6$), E632A ($n = 9$), H643A ($n = 24$), D644A ($n = 4$), H647A ($n = 6$), E672A ($n = 4$), H675A ($n = 4$), D714A ($n = 8$), and D754A ($n = 7$).

to varied concentrations of $Mg^{2+}$ is not altered by including 5 mM $Mn^{2+}$ in the same assay (not shown). Other mutants show modest, if any, changes in the relative efficacy of $Mn^{2+}$ and $Mg^{2+}$ in supporting catalysis; the E672A and H647A mutants exhibited a ~4-fold difference in the maximum catalytic activity that can be achieved by $Mn^{2+}$ versus $Mg^{2+}$. Our earlier study has shown that the E672A mutation causes a 28-fold increase in $K_m$ for cGMP (*5*); therefore, catalytic activity has been determined using both 40 and 80 $\mu$M cGMP. Activity is the same at the two concentrations, indicating that the maximum catalytic rate is being measured. Two mutants (E632A and D714A) have a pattern like that of WT PDE5, with essentially equal catalytic activities with the two cations (Table 1); five mutants (H603A, N604A, D644A, H675A, and D754A) show small differences in the efficacy of the two cations and have ~2−3-fold higher activity in the presence of high $Mg^{2+}$ concentrations than in the presence of high $Mn^{2+}$ concentrations.

The results emphasize that, unlike WT PDE5, effects of $Mn^{2+}$ and $Mg^{2+}$ in promoting catalysis are not interchangeable in these PDE5 mutants. This conclusion is based on the observation that the catalytic activity in two mutants (H607A and H643A) is supported more effectively by $Mn^{2+}$ than by $Mg^{2+}$. The results are also consistent with a particularly important role for His-607 and His-643 in providing for high-affinity interaction with metal in PDE5, whereas His-647 and Glu-672 may contribute to a lesser extent. The effectiveness of high concentrations of $Mn^{2+}$ in supporting significant catalytic activity in the H607A, H643A, and E672A mutants suggests that multiple residues contribute energetically to coordinate the catalytically relevant metal in PDE5 and that none of these residues is absolutely required for catalysis. The profound effects observed with the H607A and H643A mutants implicate these histidines as direct participants in coordinating the metal(s) required for catalysis. At relatively high concentrations of $Mn^{2+}$, PDE catalytic activity declines in WT PDE5 as well as in the catalytic domain mutants described herein, with the exception of H603A (results not shown). The low catalytic activity of this latter mutant, which is quite unresponsive to stimulation of catalysis by $Mn^{2+}$, is not inhibited even at 250 mM $Mn^{2+}$. At this same concentration of $Mn^{2+}$, WT PDE5 is inhibited by >90%. Previous studies have shown that the effect of $Zn^{2+}$ on PDE5 catalytic activity is biphasic; activity is stimulated at low concentrations of $Zn^{2+}$ and inhibited at higher concentrations (*1*). Overall, previous results and those reported herein are consistent with the presence of more than one class of cation-binding sites in PDE5. This interpretation is supported by two observations in this report: (a) the differential responsiveness of certain mutants (H607A and H643A) to $Mn^{2+}$ or $Mg^{2+}$ and (b) characteristics of $Mn^{2+}$ inhibition in WT PDE5 and H603A, H607A, and H643A mutants.

*Effect of Catalytic Domain Mutations on the Apparent Affinity for $Mn^{2+}$ and $Mg^{2+}$.* Three of the catalytic domain mutations that have the largest impact on the relative effectiveness of $Mn^{2+}$ and $Mg^{2+}$ in supporting maximum catalytic activity also produce the largest decreases in the apparent affinities for these metals. For H607A, H643A, and E672A, $K_a$ for $Mn^{2+}$ is increased ~17-, 20-, and 8-fold (10, 12, and 5 mM), respectively, with respect to WT PDE5 value (0.6 mM). In these same mutants, the $K_a$ for $Mg^{2+}$ is increased somewhat less: ~10-, 9-, and 6-fold, respectively (20, 18, and 12 mM). The contribution of these residues to the binding energy for $Mn^{2+}$ and $Mg^{2+}$ is calculated using the equation

$$\Delta\Delta G_T = -RT \ln[K_a(\text{mutant})/K_a(\text{wild type})]$$

On the basis of the change in the $Mn^{2+}$ $K_a$ value in mutants versus the wild type, substitution of His-607, His-643, or Glu-672 weakens the interaction between PDE5 and $Mn^{2+}$ by ~1.8, 1.7, and 1.2 kcal/mol, respectively. The $\Delta\Delta G_T$ values for the H607A and H643A mutants are in the range expected if these amino acids play important roles in the interaction of WT PDE5 with $Mn^{2+}$. This energy change approaches that resulting from disruption of an electrostatic interaction between enzyme and ligand (2.0 kcal/mol) (*23*, *24*). For $Mg^{2+}$, the change in free energy of binding for these same mutants is 1.4, 1.3, and 1.1 kcal/mol, respectively. By contrast, E632A and H647A mutations have no discernible effect on $K_a$s for either $Mn^{2+}$ or $Mg^{2+}$, and H675A and D714A mutations have slight effects (2- and 3-fold increases, respectively) in $K_a$s for both $Mn^{2+}$ and $Mg^{2+}$.

*Catalytic Activity Supported by $Mn^{2+}$ Compared to the cGMP-Binding Activity of Mutants.* Since the catalytic activity in several mutants is significantly improved when different assay conditions are used, the ratio of maximum catalytic activity determined in the presence of either $Mn^{2+}$ or $Mg^{2+}$ to cGMP binding activity was calculated for seven of the mutant PDEs and compared to that for WT PDE5 (Figure 3). Previous studies have shown that the cGMP-binding properties of these PDE5 mutants are essentially the

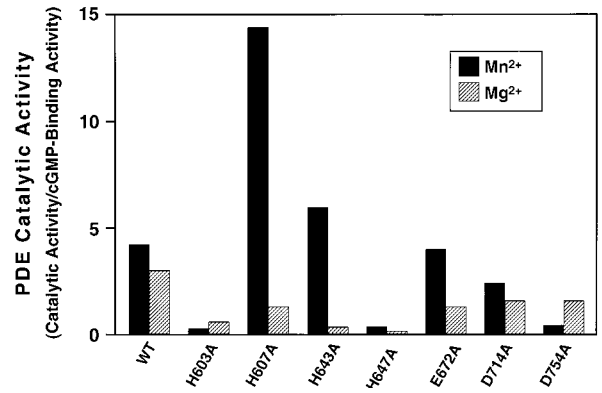


FIGURE 3: Ratio of PDE catalytic activity supported by either $Mn^{2+}$ or $Mg^{2+}$ compared to cGMP-binding activity in WT PDE5 and in PDE5 catalytic domain mutants. Cyclic GMP-binding activity and PDE catalytic activity were determined using the [$^3$H]cGMP binding assay as described in Experimental Procedures. Maximum catalytic activity was determined in presence of $Mn^{2+}$ or $Mg^{2+}$ (determined from assays conducted from zero added metal to 250 mM added metal as described in footnote of Table 1), and cGMP-binding activity was calculated. The level of cyclic GMP-binding was determined from replicate analyses, and WT PDE5 and mutant PDE5s were analyzed concomitantly. Ratios approaching that of WT PDE5 indicate that a mutant PDE exhibits a pattern comparable to that of WT PDE5. A very low ratio indicates that cGMP binding greatly exceeds catalytic activity and suggests that the defect in catalytic function cannot be significantly restored by high concentrations of either $Mn^{2+}$ or $Mg^{2+}$. The partially purified WT PDE5 catalyzed cGMP hydrolysis at a rate of 0.1 $\mu$mol min$^{-1}$ mL$^{-1}$ and cGMP binding activity was estimated to bind 100 pmol of cGMP/mL. On the basis of determinations using purified native and WT PDE5, calculations for cGMP binding stoichiometry assumed 2 mol of cGMP per PDE5 dimer and cGMP hydrolytic rate was taken to be 2.5 $\mu$mol min$^{-1}$ mg$^{-1}$ (*20*, *36*).

same as those of WT PDE5 (*5*). [$^3$H]cGMP binding has been assessed as described in Experimental Procedures. Therefore, if high concentrations of divalent cation can support catalytic activity to a level approaching that of WT PDE5, then the ratio between PDE catalytic activity and cGMP binding activity in a particular mutant should resemble that for WT PDE5. If high concentrations of divalent cation cannot support catalytic activity to nearly wild-type levels, then the amount of PDE5 estimated by catalytic turnover rates should be quite low compared to the estimate based on WT PDE5 cGMP binding (which is not altered), and the ratio will be quite low. The results are shown in Figure 3. The ratio of PDE catalytic activities in the presence of $Mn^{2+}$ to cGMP-binding activities for the H643A, E672A, and D714A mutants is quite comparable to that for WT PDE5, and the H607A mutant has a 3-fold higher ratio than does WT PDE5. Whether the higher relative value for the H607A mutant is an important difference remains to be determined. These results indicate that high concentrations of $Mn^{2+}$ support a catalytic turnover rate in H607A, H643A, E672A, and D714A mutants that approximates that of WT PDE5. The ratios of maximum catalytic activities for H607A, H643A, and E672A determined in the presence of high $Mg^{2+}$ concentrations to cGMP binding activities are significantly lower than that for WT PDE5; this emphasizes the fact that certain mutants are particularly defective in catalysis supported by $Mg^{2+}$. However, for H603A and H647 mutants, catalytic activities are not restored by high concentrations of either divalent cation, and ratios of catalytic activity to [$^3$H]cGMP-binding activity are quite low. Thus, perturbations introduced by these latter mutations profoundly alter catalytic activity in a manner that is not corrected by changing the cation or by utilizing high concentrations of either $Mn^{2+}$ or $Mg^{2+}$. For the D754A mutant the ratio of $Mg^{2+}$-supported catalytic activity to cGMP-binding activity is significantly greater than that of the $Mn^{2+}$-supported activity.

## DISCUSSION

Although divalent cations are required to support PDE catalytic activity, the residues involved in interacting with these metals are not known. In this report, we have used PDE5 catalytic domain mutants that have been shown to drastically decrease $k_{cat}(Mg^{2+})$ to gain insight into residues that contribute to interaction with metals involved in PDE catalysis. The results clearly reveal a potentially important role for residues His-607 and His-643 in metal-binding motifs A and B for interactions with the metal(s) that supports catalysis. The catalytic activity in the His-603 mutant (A motif) is very low; substitution of an alanine for this histidine may ablate a direct contact with metals or otherwise nonspecifically perturb the topography of the catalytic site. At high concentrations of $Mn^{2+}$, the catalytic activity is largely restored in the His-607 and His-643 mutants, but $Mg^{2+}$ is relatively ineffective. The profound disruption effected by these two point mutations on the capacity of $Mg^{2+}$ to support catalytic activity clearly suggests that $Mn^{2+}$ and $Mg^{2+}$ are not entirely interchangeable. Likewise, the somewhat greater impact of these mutations on the $K_a$ for $Mn^{2+}$ compared to that for $Mg^{2+}$ further distinguishes between the interactions of these cations with PDE5 and, in combination with other results, could suggest the presence of multiple metal-binding sites in PDE5. While certain other conserved residues exhibit modest changes in potency and efficacy with which $Mn^{2+}$ or $Mg^{2+}$ supports catalysis, none of these changes appear to be energetically significant under the conditions used here.

The combined results presented herein provide the first evidence that A and B metal-binding motifs of class 1 PDEs contribute to the support of PDE catalytic activity by divalent cations and identify specific residues within these motifs that provide for this function. These results using PDE5 differ significantly from those of studies of the single $HX_3HX_nE$ motif that coordinates catalytic metal in certain metallo-endopeptidases such as thermolysin and neprilysin. In enzymes utilizing this single motif to coordinate the catalytic metal, mutation of either of the two histidines or glutamate essentially abolishes catalytic activity, which emphasizes the critical importance of each of these residues (*26*−*31*). Those results are supported by X-ray crystallographic studies showing that each of these three residues directly coordinates catalytic metal in those metallo-endopeptidases (*4*, *17*).

The results of this study suggest that $Mn^{2+}$ association with PDE5 involves contributions from multiple residues and is likely to include electrostatic interactions with His-607 and His-643, and perhaps significant contributions from His-603 and Glu-672. The observation that $Mn^{2+}$ at high concentrations supports catalytic activity in certain mutants involving residues in each of the conserved metal-binding motifs, but $Mg^{2+}$ does not, could indicate the presence of multiple metal sites in PDE5 which are selective for either $Mn^{2+}$ or $Mg^{2+}$.

Reports by several investigators also point to an important role for the four conserved histidine residues in metal-binding motifs A and B of other PDEs in maintaining catalytic activity (*32*). In some instances, the resulting mutant enzymes are rendered inactive, as in the case of mutation of the second histidine in metal-binding motif B in PDE3A (*33*), and in other instances, catalytic efficiency is dramatically decreased. Mutation of the first histidine in metal-binding motif B of PDE4D produces an inactive enzyme (*32*); mutation of any of the four histidines in metal-binding motifs A and B of human PDE4A substantially reduces or abolishes catalytic activity (*34*). In another report, mutation of either histidine to serine in metal-binding motif A of human platelet PDE4A completely inactivates that enzyme, and activity cannot be stimulated by addition of metals; in that same study, mutation of either of the two histidines in motif B reduces catalytic activity (*35*). The latter results have been interpreted to mean that conserved histidines in motif A are somehow absolutely required for PDE4A catalytic activity, whereas the histidines in motif B are needed to achieve maximum activity. These authors suggest that each histidine pair in these motifs in PDE4A could interact with a required divalent metal cation. In contrast, the results reported herein support the interpretation that three of the four histidines in the two metal-binding motifs are important determinants for interactions with metal(s) involved in the catalytic process of PDE5 and that two of these (His-607 and His-643) make a major contribution to the energy of interaction with $Mn^{2+}$. These same two mutations also profoundly alter the effectiveness with which $Mg^{2+}$ supports catalysis; this effect could result from disruption of a direct interaction with $Mg^{2+}$ or from a more general perturbation of the topology of the catalytic center, thereby also altering interactions with $Mn^{2+}$. On the basis of metal requirements and the stoichiometry of metal binding demonstrated in earlier studies (*1*), and current results using site-directed mutagenesis of PDE5, it seems likely that a significant portion of the contacts for the metal-binding sites that foster catalysis are provided by specific conserved histidines in metal-binding motifs A and B in class I PDEs.

## NOTE ADDED IN PROOF

While this report was in press, the first X-ray crystal structure of a class 1 PDE (4B2B) was reported (*37*). The results of Xu et al. confirm the conclusions of this report, i.e., that class 1 PDEs have multiple metal-binding sites and that the second histidine in metal-binding motif A and the first histidine in metal-binding motif B form critical contacts with metals in the catalytic site.

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