

Multiple Zinc Binding Sites in Retinal Rod cGMP Phosphodiesterase, PDE6 $\alpha\beta$ *

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The photoreceptor cGMP phosphodiesterase (PDE6) plays a key role in vertebrate vision, but its enzymatic mechanism and the roles of metal ion co-factors have yet to be determined. We have determined the amount of endogenous Zn²⁺ in rod PDE6 and established a requirement for tightly bound Zn²⁺ in catalysis. Purified PDE6 contained 3–4 g atoms of zinc/mole, consistent with an initial content of two tightly bound Zn²⁺/catalytic subunit. PDE with only tightly bound Zn²⁺ and no free metal ions was inactive, but activity was fully restored by Mg²⁺, Mn²⁺, Co²⁺, or Zn²⁺. Mn²⁺, Co²⁺, and Zn²⁺ also induced aggregation and inactivation at higher concentrations and longer times. Removal of 93% of the tightly bound Zn²⁺ by treatment with dipicolinic acid and EDTA at pH 6.0 resulted in almost complete loss of activity in the presence of Mg²⁺. This activity loss was blocked almost completely by Zn²⁺, less potently by Co²⁺ and almost not at all by Mg²⁺, Mn²⁺, or Cu²⁺. The lost activity was restored by the addition of Zn²⁺, but Co²⁺ restored only 13% as much activity, and other metals even less. Thus tightly bound Zn²⁺ is required for catalysis but could also play a role in stabilizing the structure of PDE6, whereas distinct sites where Zn²⁺ is rapidly exchanged are likely occupied by Mg²⁺ under physiological conditions.

The cGMP-specific phosphodiesterases of rod and cone photoreceptor cells (PDE¹ 6 family) play a central role in visual signal transduction (reviewed in Ref. 1). They are responsible for rapid hydrolysis of cGMP in response to light activation of rhodopsin or cone pigment G protein-coupled receptors. This rapid reduction in cGMP concentration is the critical biochem-

ical event for the electrical response of rod and cone photoreceptors to light, because it leads to closure of plasma membrane cGMP-gated channels. Similar PDE-mediated transduction cascades may operate in other neurons as well, for example, some taste receptors (2) and depolarizing photoreceptors in lizards (3).

PDE isolated from low salt extracts of retinal rod outer segments contains two large catalytic subunits, α and β (4) as well as smaller inhibitory γ subunits in what appears to be an $\alpha\beta\gamma\gamma$ stoichiometry (5). The catalytic subunits contain regions of amino acid sequence that constitute catalytic domains and are conserved throughout a large superfamily of cyclic nucleotide-specific phosphodiesterases, PDE1–PDE10 (6–9).

It has been suggested that PDE isozymes are Zn²⁺ metalloenzymes (10), and a catalytic role for Zn²⁺ in phosphodiester hydrolysis is plausible, as it has already been observed for other enzymes catalyzing ester hydrolysis, including phosphate mono- and diesters (reviewed in Ref. 11). However, the experimental evidence on the role of Zn²⁺ in PDE isozymes remains incomplete. A study of a cGMP binding cGMP-specific PDE (PDE5 (10)) reported inhibition by EDTA or 1,10-phenanthroline, partial restoration of activity by Zn²⁺, and Zn²⁺ binding sites of moderate affinity ($K_d \sim 0.6 \mu\text{M}$). Zn²⁺ was found in PDE solutions by atomic absorption analysis, but the samples analyzed had been treated with high concentrations of exogenous Zn²⁺. Another study of a cGMP-inhibited PDE (PDE3 (12)) reported that 1,10-phenanthroline inhibited PDE, whereas nonchelating isomers did not, but only inhibitory effects of Zn²⁺ on PDE activity were found, as reported previously (13). More recently, substitution of serine for some histidine residues proposed to act as ligands for catalytic Zn²⁺ (from one of two His-Xaa₃-His-Xaa_{24–26}-Glu motifs highly conserved throughout the PDE superfamily (10)) was found to abolish catalytic activity in PDE4A (14), and Zn²⁺ was found to have both activating and inhibiting activities toward recombinant PDE4A (15). In two studies (10, 15), ⁶⁵Zn²⁺ was found to bind PDE4A and PDE5, but in neither case did the concentration dependence of binding correlate well with the concentration dependence of activation. Taken together these previous results suggest that PDE isozymes in general have more than one class of Zn²⁺ binding sites, which can either activate or inhibit catalytic activity. In no case, however, has it been demonstrated that any PDE loses catalytic activity when Zn²⁺ bound *in vivo* is removed but Mg²⁺ is present and then regains activity when Zn²⁺ is replaced.

Rod PDE activity has a well established requirement for micromolar Mg²⁺ (16). A study of the Mg²⁺ requirement (17) revealed effects of [Mg²⁺] on apparent K_m for cGMP and of [cGMP] on the dependence of activity on [Mg²⁺], consistent with sequential but random binding of Mg²⁺ and cGMP to form a competent substrate complex at the active site(s). The apparent presence of two inhibitory subunits and two catalytic sub-

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¹ The abbreviations used are: PDE, phosphodiesterase; MOPS, 4-morpholinepropanesulfonic acid; DPA, dipicolinic acid; mfpPDE, PDE from which adventitious metal ions were removed by repeated concentration and re-dilution in a Centricon with metal-free buffer; mftPDE, mfpPDE prepared by treatment with metal-free buffer after activation by limited trypsin digestion; MES, 4-morpholineethanesulfonic acid; zfPDE, PDE treated extensively with DPA/EDTA pH 6.0 to remove tightly-bound Zn²⁺; TPEN, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine.

units in each heterotetrameric holoenzyme complex suggests that there may be two active sites for cGMP hydrolysis, but it is not clear whether both are functional or whether there are any interactions between them. Noncatalytic sites for cGMP have been identified for both rod and cone PDE isozymes. The affinity of cGMP for these noncatalytic sites is much higher than the apparent affinity of cGMP for the active site, and cGMP dissociation from the mammalian rod isozyme is very slow (18, 19). Extrinsic metals do not appear necessary for binding to the noncatalytic sites; cGMP binding to frog rod PDE (20) was observed without added metals and with EGTA, whereas bovine cone PDE bound cGMP with high affinity in the presence of 10 mM EDTA (18).

The investigations described here were undertaken to determine whether the photoreceptor PDE is a Zn^{2+} metalloenzyme and also to try to resolve some of the confusing issues concerning the role of Zn^{2+} in the general class of cyclic nucleotide-specific PDEs. They have revealed that each PDE molecule contains close to four tightly bound and very slowly dissociating Zn^{2+} ions and that at least some of these tightly bound Zn^{2+} ions are essential for catalysis. They also reveal the presence of additional sites where Zn^{2+} affects function; these may not be of physiological significance because of their low affinity, but they introduce considerable confusion into experiments addressing the role of Zn^{2+} in PDE. These sites include the one(s) at which Mg^{2+} is normally thought to act but at which Zn^{2+} and other metal ions can substitute at micromolar concentrations and lower affinity inhibitory sites, where Zn^{2+} destroys activity and induces aggregation.

EXPERIMENTAL PROCEDURES

Protein Purification—PDE was purified by hydroxylapatite chromatography from hypotonic extracts of bovine retinal rod outer segments as described (21), except that some extractions were from unbleached membranes. Additional purification was achieved by ion exchange high pressure liquid chromatography using a Waters Protein Pak 5PW-DEAE column and a linear gradient of 10–500 mM NaCl in a buffer containing 10 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol. Protein concentrations were routinely determined by dye binding (22), using bovine serum albumin as the standard, and correcting the resulting values by dividing by the factor 1.2 (23). Concentrations determined in this way agreed well with those determined by amino acid analysis. For quantitation of PDE in some samples analyzed for zinc content, amino acid analysis using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate pre-column derivatization (24) was used, and in others, concentrations were verified by UV absorbance, using a calculated extinction coefficient of $\epsilon_{280\text{ nm}}$ of $211,510\text{ M}^{-1}\text{ cm}^{-1}$ (25).

Metal-free Buffers—Metal-free solutions were made by dissolving dry reagents of the highest purity commercially available in water purified to 18 megohms of resistance with a Milli-Q reverse osmosis system. Because these solutions were routinely found to contain readily detectable levels of contaminating metal ions, they were further purified by passing them through columns of Chelex-100 resin that had previously been washed with the buffer to be purified. Because Chelex-100 is not a sufficiently strong chelator to remove contaminating metal ions from chelators like Fura-2, solutions of Fura-2 were passed through the “calcium sponge” resin described previously (26); this material is very similar to the resin formerly available from Molecular Probes under the name “polymetal ion sponge®.” A closed loop system was used to recirculate the Fura-2 solutions multiple times through the column to remove metal leached from pump tubing, plastic containers, etc. Metal-free solutions were stored in plastic containers that had been extensively washed with Mill-Q water and handled only with extensively washed plastic labware.

Metal Ion Addition—Metal ions were added to metal-free buffers as small volumes of stock solutions containing the metal ion and citric acid in a 2:1 (M^{2+} :citrate) ratio. These were prepared by dilution of stocks prepared with 10 mM M^{2+} (as the dichloride salt for Mg^{2+} , Zn^{2+} , Cu^{2+} , and Mn^{2+} and as the diacetate salt for Co^{2+}), and 5 mM dipotassium citrate (pH 5.0–5.5).

Fluorescence Metal Ion Assays— Zn^{2+} and other metal ions were routinely detected by their effects on the excitation spectra of Fura-2 (27, 28). Typically 1–2 ml of Fura-2 at a final concentration of 0.5–2.6

μM was monitored in an acrylic cuvette using either an instrument described previously (29) or an ISS PC1 photon counting spectrofluorimeter. Titrations of metal-free Fura-2 with standard Zn^{2+} solutions were used to standardize the assay, which displayed a linear response in the range of 100–1000 nM total Zn^{2+} . Buffers were tested for metal ion contamination by adding them to Fura-2 in a 1:1 (v/v) ratio and measuring the excitation spectrum with emission detected at 485 nm. Estimations of free $[Zn^{2+}]$ were calculated using a K_d for Zn^{2+} -Fura-2 of 3 nM (27, 30). For estimating free Zn^{2+} in dipicolinic acid (DPA)-buffered samples, intensity at 346 nm excitation was measured as a function of added $ZnCl_2$ with 10 μM DPA and 1 μM Fura-2. The signal was plotted as the raw metal-induced intensity increase (raw intensity minus the intensity measured in the presence of saturating EDTA) divided by the maximum intensity change induced by 10 μM total Zn^{2+} .

Removal of Adventitious Zn^{2+} from PDE Solutions—PDE solutions, stored at -20°C in 40% (v/v) glycerol, were diluted to 2.0 ml (1.2 mg/ml) with metal-free pH assay buffer (see below) and concentrated to 400 μl in a Centricon 30 ultrafiltration device. The flow-through from the concentrator was saved for fluorescence assays, and the concentrated protein was again diluted in metal-free pH assay buffer. This process was continued until no further reduction in metal ion concentration was detected in the flow-through samples (typically 10–16 cycles, Fig. 1A). PDE prepared this way is referred to here as mfpDE. PDE treated the same way after activation by limited trypsin digestion to remove the inhibitory PDE γ subunit followed by the addition of soybean trypsin inhibitor as described (21) is termed mftPDE.

Removal of Tightly Bound Zn^{2+} from PDE—The procedure for removal of Zn^{2+} from adenosine deaminase (31) was used with modifications. Because exposure to buffers of pH below 6 was found to inactivate PDE irreversibly, a pH 6.0 buffer was used containing MES (25 mM), NaCl (50 mM), DPA (20 mM), and EDTA (10 mM). For experiments described in Fig. 5, PDE concentration at its most dilute was 0.01–0.02 mg/ml, whereas for those described in Table I and Fig. 6, the concentration at greatest dilution in the centricons was 0.2 mg/ml. The combination of DPA and EDTA was much more effective than treatment with EDTA alone. PDE was repeatedly diluted and reconcentrated ~13-fold in a Centricon-30, until its trypsin inducible activity, assayed at pH 8.0 in the presence of 2 mM $MgCl_2$, was reduced to less than 10% of its initial value (7–24 cycles depending on protein concentration). It was then washed as described above for mfpDE using pH 7.6 buffer (150 mM NaCl, 20 mM MOPS, metal-free), in parallel with a control (no chelator treatment) PDE sample, until the flow-through from both showed no further change in metal ion content (typically 14 cycles of 13-fold dilution). PDE prepared this way is referred to as zpPDE. In metal protection experiments, metal ions were present at the indicated total concentrations (typically 20 mM) during chelator treatment. Fura-2 measurements indicated that 20 mM total Zn^{2+} in the DPA/EDTA solution yielded an excitation spectrum intermediate between that of metal-free and Zn^{2+} -saturated Fura-2, implying that free $[Zn^{2+}]$ under these conditions was on the order of its K_d for Fura-2 (3 nM).

Zinc Analysis—PDE samples from which adventitious metal ions had been removed, as well as the final flow-through solution from ultrafiltration, were analyzed by atomic absorption spectrometry at the Center for Biochemical and Biophysical Sciences and Medicine at Harvard Medical School. Analyses were carried out at 213.9 nm in duplicate on multiple dilutions using a Perkin-Elmer model 2280 flame atomic absorption spectrophotometer or a Perkin-Elmer 4100 ZL instrument equipped with a transverse heated graphite furnace, Zeeman background correction, and an AS-70 autosampling device. Protein concentration was determined by amino acid analysis (24) or by UV absorption spectrophotometry, as described above.

PDE Assays—PDE catalytic activity was monitored using the pH recording method (32) as described previously (21) using PDE concentrations of 15–20 nM, initial cGMP concentration of 2.0 mM, and initial pH 8.0 at 23°C . The extent of cGMP hydrolysis was monitored continuously, and only hydrolysis rates obtained at cGMP concentrations above 400 μM ($\sim 10 \times K_m$ (1)) were used; when necessary additional cGMP was added at the end of reaction time courses to ensure that substrate was not limiting. In all cases PDE was activated by treating with trypsin (before or after various treatments to remove or add metal ions) to remove the inhibitory PDE γ subunits. No effects of metal ions on trypsin susceptibility were detected, nor did we observe any effects of trypsin treatment on sensitivity to metal ions or chelators. For assessing the role of divalent metal ions, metal-free pH assay buffer (20 mM MOPS, 150 mM KCl, adjusted to pH 8.0 with KOH) was prepared without the addition of $MgCl_2$ and passed through Chelex-100 resin. Alternatively, standard pH assay buffer was prepared with the same composition except for the inclusion of 2 mM $MgCl_2$. To minimize inter-

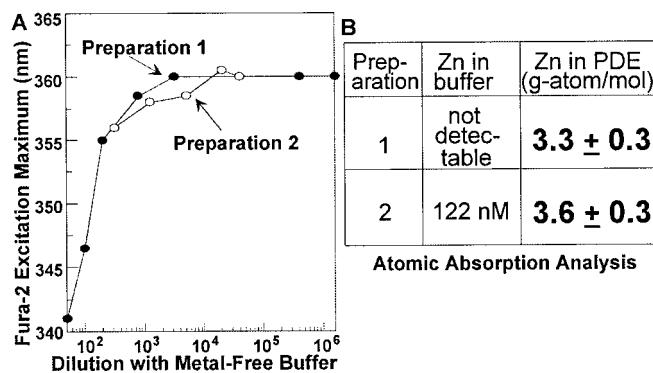


FIG. 1. Atomic absorption analysis of holopDE. A, PDE was treated as described under "Experimental Procedures" to remove free metal ions, and the flow-through solutions were assayed by mixing with Fura-2 and measuring the excitation spectra. The wavelengths of maximum excitation are plotted as a function of the dilution of the original low molecular weight buffer components for two different preparations of PDE. B, concentrated PDE samples from the last steps shown in A were analyzed by atomic absorption analysis and amino acid analysis to determine the stoichiometries of bound Zn^{2+} as shown.

ference by contaminating heavy metals in assays of Mg^{2+} effects on activity, the standard ($MgCl_2$ -containing) pH assay buffer was supplemented with either EDTA (e.g. 0.1 mM EDTA) or TPEN. Whereas removal of all free metal ions greatly reduced PDE activity, it did not completely abolish it, even when high concentrations of EDTA or TPEN were used. When cGMP (metal-free) was first added to PDE in the absence of free metal ions, there was a burst of activity, followed by a rapid decline to a plateau level of activity that was typically 5–10% of the maximum activity observed with saturating Mg^{2+} . Activities reported here were all measured after this plateau level of activity was reached, i.e. at least 2 min after cGMP was added to PDE in metal-free buffer. The basis for the initial burst of activity is currently being investigated; preliminary results are consistent with some metal ion(s) bound initially being released from PDE by cGMP addition. Effects of prolonged incubation with TPEN were studied by incubating mftPDE on ice with 10 μM TPEN, 10 μM TPEN plus 10 μM Zn^{2+} (added as Zn^{2+} /citrate solution), or a control solution containing the same trace of ethanol (0.01%) as the TPEN-containing samples. At various times, aliquots of PDE were removed and assayed in metal-free pH assay buffer supplemented with Mg^{2+} (2.0 mM) and TPEN (10 μM).

RESULTS AND DISCUSSION

Endogenous Zn^{2+} Tightly Bound to PDE—Two independent preparations of PDE were purified and rendered free of adventitious metals. The results from analyses by graphite furnace atomic absorption (with protein quantification by amino acid analysis) revealed $3.3 (\pm 0.3)$ g atoms of Zn^{2+} /mole of PDE in one preparation and $3.6 (\pm 0.3)$ g/mole in the other (Fig. 1). The buffer controls (flow-through samples from the Centricon filters) contained in one case too little Zn^{2+} to be detected (<50 nM or <0.002 g atoms of adventitious Zn^{2+} /g of PDE-bound Zn^{2+}) and in the other case, 122 nM Zn^{2+} (0.018 g atoms of adventitious Zn^{2+} /g of PDE-bound Zn^{2+}). Because these PDE samples had been extensively washed with metal-free buffers for many hours, these sites are very likely of high affinity or, at least, release bound Zn^{2+} very slowly. Because there are two catalytic subunits with highly conserved sequences, it seems likely that there are an even number of high affinity sites, i.e. four, and that the stoichiometry of 3.3–3.6 represents partial occupancy of one (or more) site as a result of the extensive washing. If it assumed that the solutions analyzed had reached equilibrium and that the total number of high affinity sites is four, then single site K_d values estimated from the analyses would be <10.6 nM for the first preparation and 13.6 nM for the second. Because these sites exchange metal ions very slowly and no exogenous Zn^{2+} had been added at any stage of PDE preparation from bovine retinas, it is almost certain that the Zn^{2+} ions we detected were bound to PDE *in vivo*. Disc mem-

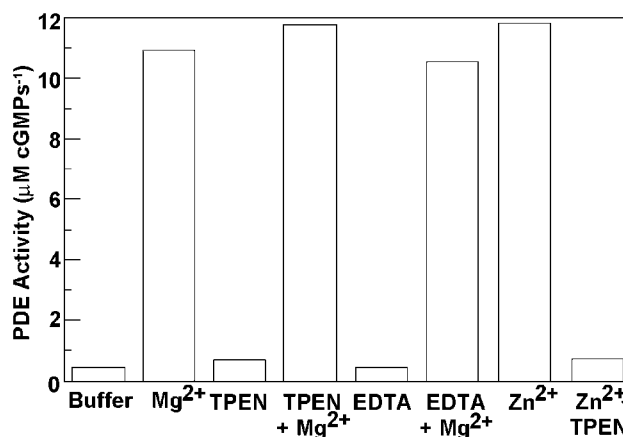


FIG. 2. Effects of divalent metal ions and chelating agents on PDE activity. Samples of mftPDE were assayed for catalysis of cGMP hydrolysis in the presence of the chelators and metal ions shown using metal-free buffer. Where indicated, concentrations were: 5 μM TPEN, 200 μM Mg^{2+} (no EDTA) or 300 μM (with EDTA), 100 μM EDTA, 1 μM Zn^{2+} , 0.0029 μM PDE. Zn^{2+} and Mg^{2+} were added as the 2:1 M^{2+} :citric acid solutions described under "Experimental Procedures."

branes from bovine rod outer segments have been reported to contain 0.15 g atom of Zn^{2+} /mole rhodopsin, corresponding to about 15 g atom of Zn^{2+} /mole of PDE, based on particle induced x-ray emission analysis (33).

Effects on Catalysis of Removal of Free Zn^{2+} by Chelators or Washing with Metal-free Buffers—When PDE from which adventitious Zn^{2+} had been removed (mftPDE), the same sample of holopDE used for atomic absorption analysis, containing <50 nM free Zn^{2+} before >1000-fold dilution for the assays) was assayed in the presence of 2 mM Mg^{2+} and 0.1 mM EDTA, the specific activity observed with saturating substrate (2 mM cGMP) was 2381 moles of cGMP hydrolyzed/mole of PDE/second after trypsin treatment, in reasonable agreement with typical values observed for rod PDE (reviewed in Ref. 1), although somewhat lower than maximum values occasionally observed of ~ 7000 mol of cGMP-PDE $^{-1}$ s $^{-1}$ (18).² The addition of Zn^{2+} did not significantly increase the activity of PDE after free metal ions had been removed and Mg^{2+} added, nor did the chelator TPEN significantly reduce the activity. Fig. 2 shows results from PDE freed of adventitious metals after trypsinization (mftPDE). Again, neither low concentrations of Zn^{2+} nor TPEN had much effect on activity, as long as Mg^{2+} was present and the Zn^{2+} concentration was not so high as to inhibit PDE (see below). Thus we can conclude with certainty that free Zn^{2+} (as opposed to that tightly bound to PDE) is not required for robust PDE activity.

Substitution of Zn^{2+} at the Mg^{2+} Binding Sites—When mft-PDE was assayed without added Mg^{2+} , its activity was very low (Figs. 2 and 3A), as previously observed (16, 17, 34, 35). The addition of Zn^{2+} to PDE in the absence of added Mg^{2+} increased the activity (Figs. 2 and 3A). The activity observed after Zn^{2+} addition varied with time and was highest immediately after Zn^{2+} was added, so the values observed immediately after Zn^{2+} addition are plotted in Figs. 2 and 3. The addition of TPEN prior to Zn^{2+} addition prevented the activity enhancement by Zn^{2+} (Fig. 2). PDE activity was also stimulated by Co^{2+} or Mn^{2+} (Fig. 3A). The approximate concentrations (as total added metal ion) for half-maximal activity were 0.2 μM Zn^{2+} , 2.2 μM Co^{2+} , 7 μM Mn^{2+} , 21 μM Mg^{2+} .

Inhibition of PDE by Zn^{2+} —At higher concentrations (5 μM), especially at longer incubation times, Zn^{2+} exhibited an inhibitory effect (Figs. 3B and 4, A and B). The kinetics of inhibition

² J. A. Malinski and T. G. Wensel, unpublished observations.

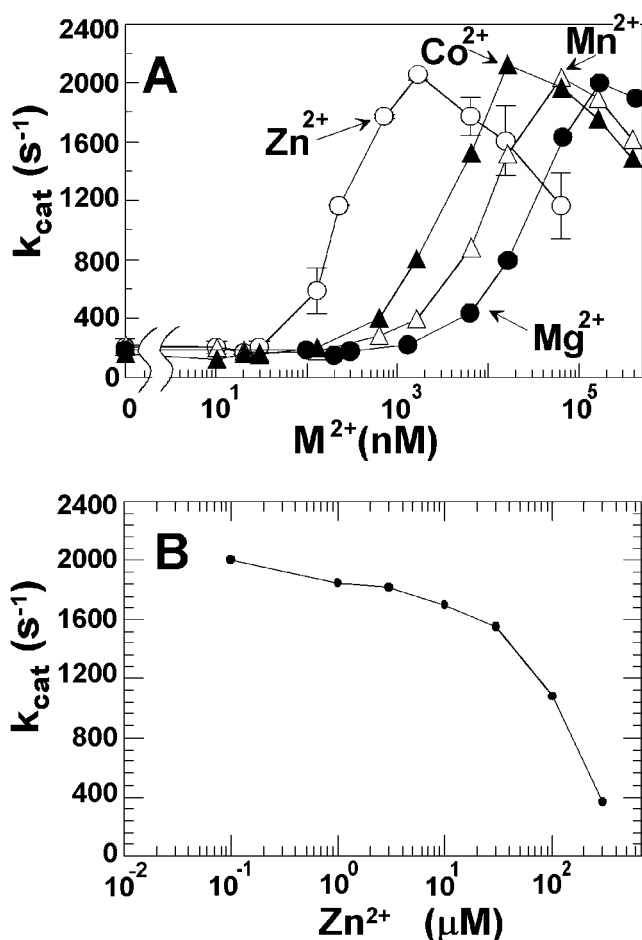


FIG. 3. Activation and inhibition of mftPDE by divalent metal ions. Metal ions were added as the 2:1 M^{2+} :citric acid solutions described under "Experimental Procedures." PDE activities were measured within 20 s of the addition of metal ions, at a PDE concentration of 6 nM (maximum hydrolytic rate of $12.0 \mu M$ cGMP s^{-1}). A, open circles, Zn^{2+} ; filled circles, Mg^{2+} ; open triangles, Mn^{2+} ; filled triangles, Co^{2+} . The Zn^{2+} samples were performed in duplicate, and the averages \pm S.D. are plotted; other points represent individual experiments. B, inhibition of mftPDE by Zn^{2+} in the presence of Mg^{2+} . Activity plotted is that observed at $200 \mu M$ $MgCl_2$ immediately upon the addition of Zn^{2+} to the indicated concentrations.

are much slower than activation. Fig. 4A shows the slow loss of activity observed when Zn^{2+} is added in the presence of saturating Mg^{2+} . Similar slow inhibition is observed in the absence of Mg^{2+} but is revealed as a biphasic activity response (Fig. 4B). Thus, depending on how much Zn^{2+} is added and how long after addition activity is assayed, Zn^{2+} can appear to have a stimulatory effect, an inhibitory effect, or no effect at all on PDE activity. This complex behavior may help to explain some of the differences observed with different PDE isozymes in different laboratories. Although we did not characterize in detail the concentration dependence of the inhibition kinetics, we did observe that inhibition occurs faster with increasing Zn^{2+} concentrations. An interesting feature of the inhibition by Zn^{2+} is that it is readily prevented by chelating agents (either EDTA or TPEN), but once it occurs, the simple addition of chelating agents does not restore activity, even in the presence of sufficient Mg^{2+} for full activity. At high protein concentrations, visible precipitates were observed to form in the presence of Zn^{2+} , suggesting that aggregation may account for all or part of the inhibition.

Kinetics of Metal Ion Exchange at the Activation Sites—In contrast to the very slow exchange kinetics observed at the high affinity sites and at the inhibitory sites, Zn^{2+} ions at the

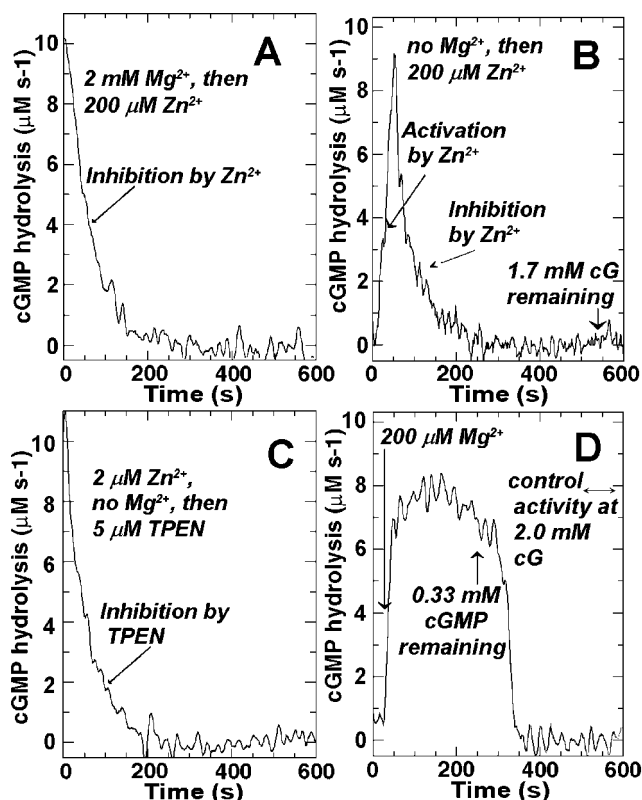


FIG. 4. Time course of mftPDE inhibition and activation. Activity was monitored continuously, and the activity plotted by taking the time derivative of the voltage output. A, time course of mftPDE inhibition by $200 \mu M$ Zn^{2+} in the presence of 2.0 mM Mg^{2+} . B, biphasic effects of $200 \mu M$ Zn^{2+} on PDE activity in metal-free buffer. At time 0, Zn^{2+} was added to $200 \mu M$ in a solution of mftPDE and cGMP in metal-free buffer. At the time indicated by a small vertical arrow the remaining cGMP concentration was 1.7 mM. C, PDE activity was measured after the addition of 5 μM TPEN in the presence of 1 μM Zn^{2+} in otherwise metal-free buffer. Note that A, B, and C are plotted on identical time scales to facilitate the comparison of kinetics of three different processes: Zn^{2+} release from Mg^{2+} sites (A), Zn^{2+} binding to Mg^{2+} sites (rising phase of B), and Zn^{2+} inhibition (A and decay phase of B). D, Mg^{2+} control. At the indicated time, Mg^{2+} was added to mftPDE to $200 \mu M$. Activity decline after the vertical upward arrow, indicating the time at which cGMP concentration had declined to 330 μM , resulted from substrate depletion, as indicated by the horizontal double arrow indicating the activity measured upon the addition of cGMP to 2.0 mM at 800 s.

Mg^{2+} site undergo fairly rapid exchange. The initial activation phase seen in Fig. 4B shows that binding of Zn^{2+} to this site is rapid (seconds), and the fairly rapid inhibition observed when TPEN was added to Zn^{2+} -activated PDE (Fig. 4C) indicates that dissociation from this site is fairly rapid (tens of seconds) as well.

Effects of Phenanthrolines—The Zn^{2+} chelating agent 1,10-phenanthroline is commonly used to determine the effects of removing free Zn^{2+} on enzyme activities. We observed inhibition of PDE by 1,10-phenanthroline. However, we found that two nonchelating phenanthroline isomers (1,7 and 4,7) inhibited PDE with comparable, and even slightly higher, potency. Coupled with the failure of TPEN to inhibit PDE when Mg^{2+} was present, these results indicate that phenanthrolines inhibit PDE by a mechanism not involving Zn^{2+} chelation. Similar results were observed with yeast PDE (36).

Effects of TPEN—As discussed above, mild treatment with metal-free buffers did not efficiently remove the endogenous Zn^{2+} that is tightly bound, and chelating agents did not reduce PDE activity significantly when Mg^{2+} was present. Even prolonged incubation (24 h) of mftPDE with the Zn^{2+} chelating

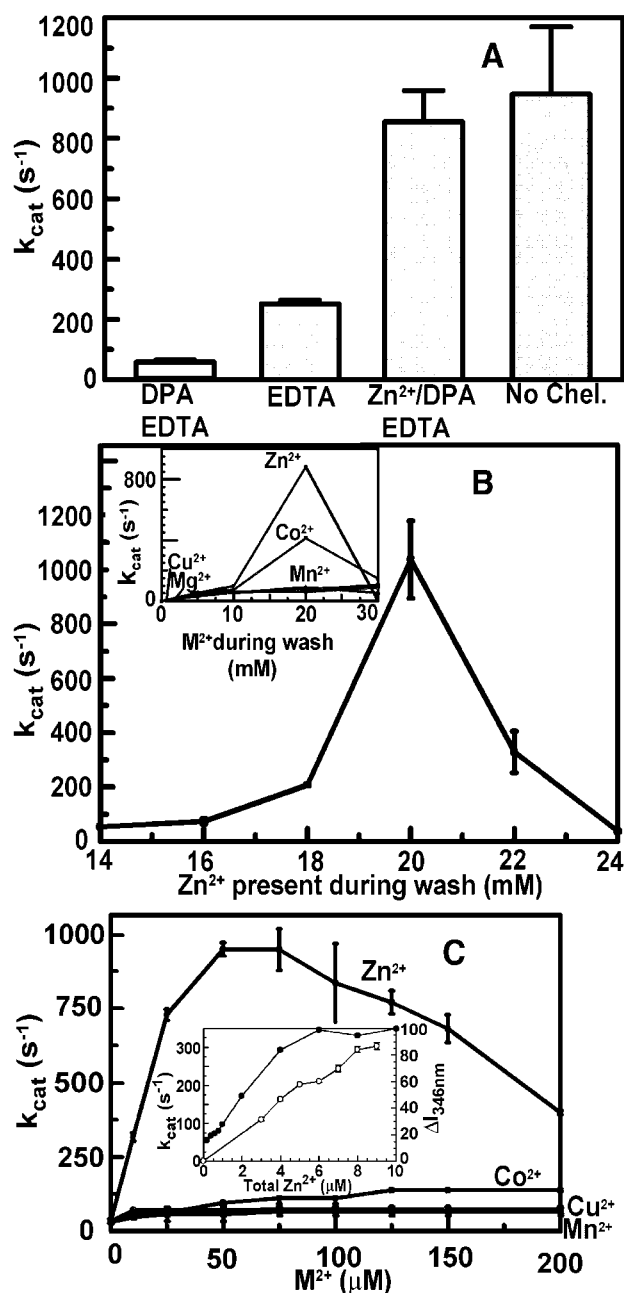


FIG. 5. Loss of PDE activity under conditions that remove tightly bound Zn^{2+} . A, prolonged treatment (9 cycles over 2 days) at pH 6.0, as described under "Experimental Procedures," with chelators or metal ions present as indicated during treatment in Centricon. Where present, concentrations were 20 mM DPA, 10 mM EDTA, 20 mM $ZnCl_2$. Mg^{2+} was not present during the ultrafiltration but was present (2.0 mM in excess over EDTA) in the assays. B, prevention of activity loss by Zn^{2+} . $ZnCl_2$ was added at the indicated concentrations during treatment with 20 mM DPA, 10 mM EDTA, pH 6.0. *Inset*, protective effects of metal ions added to the indicated concentrations during chelator treatment (DPA/EDTA). C, restoration of activity to zPDE by metal ions. Following treatment with DPA/EDTA without added metal ions as in A, chelators were removed by 14 additional cycles in the centricon, and metal ions were added to the indicated concentrations, prior to assays in the presence of Mg^{2+} . *Inset*, restoration of activity to zPDE by DPA-buffered Zn^{2+} . Activity (open circles) was monitored as a function of Zn^{2+} added to metal-free PDE assay buffer containing 10 μM DPA and 0.2 mM Mg^{2+} , and free Zn^{2+} monitored by fluorescence intensity excited at 346 nm, in separate samples containing 1 μM Fura-2, but otherwise identical to those used to monitor activity.

agent TPEN (10 μM) did not reduce PDE activity substantially as compared with control samples incubated without TPEN or with TPEN and excess Zn^{2+} , when PDE was assayed in the

TABLE I
Activity and zinc content of washed PDE

pH	Chelators	Log ₁₀ dil. ^a	Days ^b	Zn/PDE ^c	Specific Activity Mg^{2+} ^d	Specific Activity Mg^{2+} & Zn^{2+} ^e
8.0	None	4.6	2	3.6	2381 s ⁻¹	n.d.
8.0	None	6.3	3	3.3	1971 s ⁻¹	n.d.
7.6	None	15.6	4	1.49	959 s ⁻¹	n.d.
6.0	DPA/EDTA	7.8	1	n.d. ^f	142 s ⁻¹	n.d.
6.0	DPA/EDTA	10.0	2	n.d.	142 s ⁻¹	n.d.
6.0	DPA/EDTA	20.0	4	n.d.	88 s ⁻¹	n.d.
6.0	DPA/EDTA	24.7	6	n.d.	26 s ⁻¹	279 s ⁻¹
6.0 ^g	DPA/EDTA	40.3	10	0.136	9.8 s ⁻¹	187 s ⁻¹

^a Total dilution of the original buffer by repeated concentration and redilution with metal-free buffers.

^b Total days of treatment with metal-free buffers at 4 °C.

^c Determined by atomic absorption spectrophotometry.

^d Assayed in the presence of at least 1 mM free Mg^{2+} .

^e Following treatment, incubated with 20 mM Zn^{2+} , 20 mM DPA, 10 mM EDTA, then assayed with Mg^{2+} .

^f n.d., not determined.

^g 6 days, DPA/EDTA, pH 6.0, 5×10^{24} -fold dilution, then 4 days, no chelator, pH 7.6 (4×10^{15} -fold dilution).

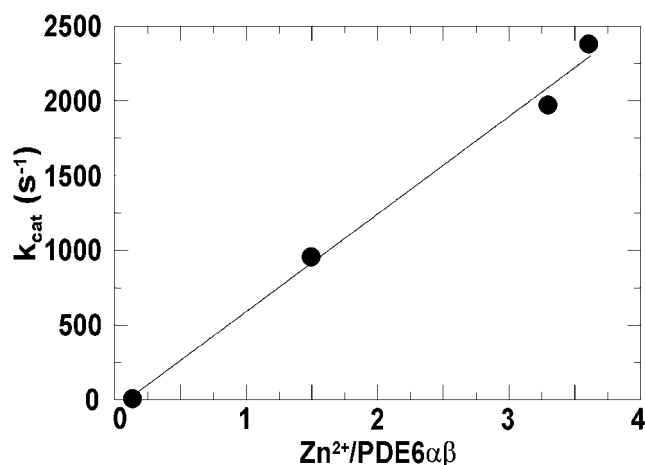


FIG. 6. Correlation of catalytic activity with Zn^{2+} content. Four separate samples, prepared as described under "Experimental Procedures" and in Table I, were assayed for catalytic activity in the presence of Mg^{2+} and for Zn^{2+} content by atomic absorption spectrophotometry. The line is a linear least squares fit.

presence of Mg^{2+} (data not shown). TPEN has a very high affinity for Zn^{2+} , with a reported $K_a = 10^{15.58} M^{-1}$ (37), whereas its affinity for Mg^{2+} is negligible with $K_a = 10^{1.7} M^{-1}$. Thus, any catalytically essential Zn^{2+} must not have dissociated from PDE on a 24-h time scale. In contrast, TPEN rapidly abolished the action of Zn^{2+} at the catalytically essential divalent cation binding sites usually occupied by Mg^{2+} in our assays (Figs. 2 and 4C).

Removal of Tightly Bound Zn^{2+} by DPA and Low pH—A previous study of Zn^{2+} bound to adenosine deaminase (31) reported that low pH and use of a combination of dipicolinic acid and EDTA were effective in removing very tightly bound Zn^{2+} . We used this procedure on PDE6 and found that prolonged treatment lead to loss of activity (Fig. 5 and Table I) in zPDE. Some activity was lost just from the prolonged treatment at pH 6.0, even without chelator, but the loss was much greater in the presence of EDTA and was greatest when both DPA and EDTA were used. The chelator-dependent loss of activity was prevented by including Zn^{2+} in the low pH chelator solution (Fig. 5). The optimal concentration (total) of Zn^{2+} for preventing the loss was in a very narrow range centered on 20 mM (Fig. 5B), the concentration at which nearly every Zn^{2+} is expected to be in a Zn-EDTA ($K_a = 10^{16.4} M^{-1}$) or Zn-(DPA)₂ ($K_1 = 10^{6.4} M^{-1}$ and $K_2 = 10^{5.5} M^{-1}$) complex, but at which very

little free chelator would be present (38). Measurements with Fura-2 indicate that under these conditions, free $[Zn^{2+}]$ is on the order of the Fura-2 K_d , ~ 3 nM (1.5–3.5 nM using either ratiometric or single wavelength methods (27)). The simplest explanation for the biphasic character of the concentration dependence is that at lower Zn^{2+} concentrations the excess free chelator was still able to remove Zn^{2+} from PDE, and at higher concentrations, Zn^{2+} was able to act at the inhibitory sites discussed above.

Other metal ions were much less effective at preventing the loss of activity. Co^{2+} was the most effective of these (Fig. 5B, inset), whereas Mn^{2+} , Mg^{2+} , and Cu^{2+} provided almost no protection. Fura-2 assays confirmed that over the concentration range tested sufficient Co^{2+} and Mn^{2+} were available to approach saturation of 1 μM Fura-2.

Similar results were observed when activity was restored after washing by adding back metal ions to zFPDE. Zn^{2+} was most effective in restoring activity, whereas Co^{2+} was much less effective, and other metal ions were almost without effect (Fig. 5C). Again, the Zn^{2+} effect was biphasic, because of the inhibitory sites, but at the peak, nearly complete activity was restored. Thus, it seems likely that Zn^{2+} originally bound to the enzyme is essential for catalytic activity, but the enzyme still has some catalytic activity if Co^{2+} is substituted for Zn^{2+} .

As shown in Table I, the DPA treatment that inactivated PDE was indeed effective in removing tightly bound Zn^{2+} . Comparison of activity levels over a range of treatments of increasing stringency for metal removal as well as Zn^{2+} content for those samples assayed by atomic absorption (Table I and Fig. 6) reveals a strong correlation between Zn^{2+} removal and loss of activity. As can be seen from the last column of Table I, in some cases Zn^{2+} only partially restored activity to the most stringently treated PDE, suggesting that some irreversible structural changes may have accompanied Zn^{2+} removal.

A surprising feature of the results shown in Fig. 5C is the concentration range (tens of micromolar) for Zn^{2+} required for maximal restoration of activity to zFPDE. Because it seemed likely that some chelators might be contaminating the preparation, we washed the PDE even more extensively with metal-free buffers and used DPA to buffer free Zn^{2+} . The Zn^{2+} concentrations required were then much lower (Fig. 5C, inset), and significant activation of zFPDE was observed at about the same total Zn^{2+} as that giving half-maximal saturation of the Fura-2 signal ($K_d \sim 3$ nM; note that at higher Zn^{2+} where the Fura-2 signal begins to saturate, reliable estimates of free Zn^{2+} cannot be obtained by this technique).

The loss of activity upon removal of Zn^{2+} , its selective prevention by Zn^{2+} , and its selective restoration by Zn^{2+} represent strong evidence for an essential role for tightly bound Zn^{2+} in PDE catalytic activity. Although Zn^{2+} is clearly essential for catalysis, it is not possible from our data to conclude that it plays a direct role in catalysis (e.g. by activating water) or that it does not play a structural role. Very high affinity binding is consistent with a structural as well as catalytic role, as loss of such a high affinity ligand must necessarily be accompanied by a free energy increase for the metal-free state. Further studies

of PDE structural integrity and thermal stability as a function of metal ion content, now possible due to the determination of conditions for Zn^{2+} removal, should allow the structural importance of Zn^{2+} and the Zn^{2+} binding residues to be tested.

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