Structural Characterization of the Divalent Cation Sites of Bacterial Phosphotriesterase by ^{113}Cd NMR Spectroscopy†

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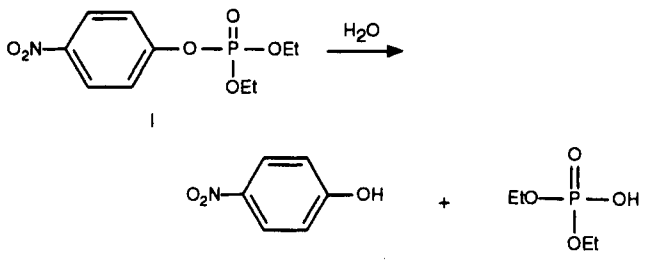
ABSTRACT: The phosphotriesterase from *Pseudomonas diminuta* catalyzes the hydrolysis of organophosphate esters. The isolated native protein contains zinc, and removal of this metal abolishes the enzymatic activity. Reconstitution of the apoenzyme requires 2 mol of cadmium per mol of protein for full catalytic activity. The k_{cat} and K_m values for the hydrolysis of paraoxon for the cadmium-substituted enzyme are 4300 s^{-1} and 390 μM, respectively. These values compare favorably with the kinetic constants observed for the zinc-substituted enzyme (2300 s^{-1} and 78 μM). A hybrid enzyme containing one zinc and one cadmium ion is catalytically active, and the kinetic constants are nearly identical to the values obtained with the all-zinc-containing enzyme. The NMR spectrum of protein reconstituted with two $^{113}Cd^{2+}$ ions per enzyme molecule exhibits cadmium resonances at 212 and 116 ppm downfield from $Cd(ClO_4)_2$. The two metal ions are, therefore, in significantly different chemical environments. These two binding sites have been designated the M_α and M_β sites for the low- and high-field signals, respectively. Protein substituted with a single cadmium ion also shows two cadmium resonances, and thus one site is not completely filled prior to the binding of metal to the other site. The Cd/Zn hybrid protein shows a single cadmium resonance at 115 ppm, and thus the cadmium is occupying the M_β site while zinc is occupying the M_α site. The positions of the observed chemical shifts for the two cadmium signals indicate that the ligands to both metals are composed of a mixture of oxygen and nitrogen atoms. Ligation by sulfur and a ligand set composed exclusively of oxygen are excluded. On the basis of observed chemical shifts of $^{113}Cd^{2+}$ bound to metalloenzymes of known structure it is likely that the metal bound at the M_α site is ligated by three nitrogens and one oxygen, while the M_β site is ligated by two nitrogens and two oxygen atoms. The binding of dithiothreitol shifts each of the two cadmium resonances downfield by 100–150 ppm, and thus both metal sites are accessible to external ligands.

The phosphotriesterase from *Pseudomonas diminuta* is the most thoroughly characterized protein for the enzymatic hydrolysis of the ester bonds common to organophosphorus pesticides and chemical warfare agents (Caldwell & Raushel, 1991). The enzymatic hydrolysis of the phosphorus–oxygen bond of the insecticide paraoxon (I) is illustrated below.



The enzymatic reaction catalyzed by the phosphotriesterase is known to proceed with inversion of stereochemistry at the phosphorus center, and thus the overall reaction mechanism does not involve the formation of a phosphorylated-enzyme intermediate (Lewis *et al.*, 1988). Caldwell *et al.* (1991) demonstrated that the hydrolysis of substrates with good leaving groups ($pK_a < 7$), such as *p*-nitrophenol, occurs at the diffusion-controlled rate. Relatively little is known about the structure of the active site or the role of individual amino acids in the hydrolysis mechanism. However, the protein appears to require a functional group with a pK_a of ~6.1 to be unprotonated for full catalytic activity (Dumas & Raushel, 1990). This residue has been tentatively identified as a histidine on the basis of pH-rate profiles and inactivation studies with diethyl pyrocarbonate.

The purified enzyme has been found to contain zinc, and removal of this metal results in the loss of enzymatic activity (Dumas *et al.*, 1989). Recently, this laboratory has described the preparation of the apophosphotriesterase and the reconstitution of full enzymatic activity with 2 equiv of divalent cations (Omburo *et al.*, 1992). These active metal ions have included Ni^{2+}, Cd^{2+}, Co^{2+}, and Mn^{2+}, in addition to the native Zn^{2+}. The catalytic turnover for the Co-substituted enzyme approaches 10^4 s^{-1}, and thus these metal-substituted derivatives can potentially serve as useful spectroscopic signals for structure–function analyses of the active site.

The metal ions in several zinc metalloenzymes have been explored by ^{113}Cd nuclear magnetic resonance (NMR) spectroscopy [for a review, see Summers (1988)]. ^{113}Cd has a nuclear spin of 1/2 and is therefore suitable for high-resolution NMR studies of metal ion sites. The chemical shift of ^{113}Cd is also sensitive to small differences in the coordination environment, and ^{113}Cd-substituted metalloenzymes display a wide range of chemical shift values extending from −100 to 800 ppm relative to $Cd(ClO_4)_2$ (Drakenberg *et al.*, 1978; Bobsein & Myers, 1980). The deshielding of the ^{113}Cd nucleus, due to the coordinating ligands, has been established to increase for the series O < N < S. The NMR properties of $^{113}Cd^{2+}$ have now been utilized to probe the environment of the metal centers in the phosphotriesterase. $^{113}Cd^{2+}$ has been used to reconstitute the apophosphotriesterase with full catalytic activity, and this has allowed the direct

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observation of the protein-bound metal by ^{113}Cd NMR spectroscopy. The number of metal binding sites and the nature of metal binding ligands have been identified in an attempt to further refine the structural features of the active site with regard to metal–protein, metal–substrate, and metal–metal interactions.

MATERIALS AND METHODS

Materials. Cadmium chloride, Lawesson's reagent, and other metal salts were obtained from Aldrich Chemical Co. (Milwaukee, WI). Chelex-100 and diethyl *p*-nitrophenyl phosphate (paraoxon) were purchased from Sigma Chemical Co. (St. Louis, MO). Paraoxon, obtained as a 95% pure oil, was purified according to the procedure described previously (Omburo *et al.*, 1992). Diethyl 4-methoxy-benzylphosphonate was obtained from Lancaster Chemical Co. (Windham, NH). The cadmium-113 metal (95 atom %) was obtained from Cambridge Isotope Laboratory (Woburn, MA). All other buffers and chemicals were purchased from either Sigma or Aldrich unless otherwise stated.

Synthesis of Diethyl (4-Methoxybenzyl)thiophosphonate. Diethyl (4-methoxybenzyl)phosphonate (1.0 g, 0.04 mol) was dissolved in 50 mL of freshly dried dioxane, and 0.7 g (0.02 mol) of Lawesson's reagent, a bis(phenylthionophosphine sulfide), was added and refluxed at 100 °C overnight. The reaction mixture was poured over ice/water (150 mL), and after all the ice had melted, 100 mL of diethyl ether was added. The organic layer was separated, washed twice with water, and dried over anhydrous magnesium sulfate. The ether was removed by rotary evaporation, and the residue was distilled under vacuum to yield 0.35 g (33%) of the desired compound. The ^{31}P NMR spectrum of the product, diethyl 4-(methoxybenzyl)thiophosphonate, showed a single resonance at 95 ppm downfield from phosphoric acid. ^{1}H NMR (200 MHz): δ 1.3 (d, t, 6 H, J = 1.5, 7.1 Hz, CH_3), 3.3 (d, 2 H, J = 18.3 Hz, CH_2), 3.8 (s, 3 H, OCH_3), 4.1 (d, q, 4 H, J = 3.4, 7.0 Hz, CH_2), 6.8 (d, 2 H, J = 8.7 Hz, CH), 7.2 (d, 2 H, J = 8.7 Hz, CH). Electron impact mass spectroscopy (EI/MS): Calcd mass for $C_{11}H_{17}O_4PS$, 274.25; obsd m/z (relative intensity) 274 (M^+, 13), 121 ($C_8H_9O^+$, 100).

Enzyme Preparation. The phosphotriesterase used in this study was purified from an *Escherichia coli* expression system containing the plasmid pJK01.[1] The cells were grown at 30 °C in TB medium consisting of 12 g/L Bactotryptone, 24 g/L yeast extract, 4 mL/L glycerol, 100 mM potassium phosphate, pH 7.4, and 1 mM cobalt chloride. The cells were grown for 20 h before induction with 0.4 mM isopropyl β-D-thiogalactopyranoside and then allowed to grow for an additional 34 h. The purification protocol of the phosphotriesterase from this expression system is essentially the same as that previously described (Omburo *et al.*, 1992). The purification buffers were supplemented with 0.05–0.10 mM cobalt chloride.

Preparation and Reconstitution of Apoenzyme. Phosphotriesterase (~1 mg/mL) in 50 mM HEPES[2] buffer, pH 8.2, was dialyzed against metal-free buffer and then incubated with 2.0–3.0 mM 1,10-phenanthroline until the enzymatic activity dropped to less than 1% of the initial value. The metal–chelator complex and excess phenanthroline were removed by diafiltration with an Amicon ultrafiltration device fitted with a PM10 membrane (Amicon Corporation, Danvers, MA). The progress of the diafiltration procedure was monitored by following the disappearance of absorbance at 327 nm due to the 1,10-phenanthroline. The apoenzyme and final filtrate solutions were assayed for enzymatic activity and analyzed by atomic absorption spectroscopy for zinc, cobalt, and iron content. Reconstitution of apophosphotriesterase (~1 mg/mL) was achieved by adding the metal salt solution to obtain the desired ratio of metal to protein. The mixed metal derivatives were prepared by adding both metal ions simultaneously. The solutions were incubated at 4 °C for at least 24 h, and then the specific activity and metal content were determined as previously described (Omburo *et al.*, 1992).

Metal Ion Analysis. Double distilled and deionized water was used throughout this study. Glassware, dialysis tubing, and the Amicon Centricon-10 microconcentration devices (Amicon Corp., Danvers, MA) for handling the protein were treated as previously described (Omburo *et al.*, 1992). The PM10 ultrafiltration membranes (Amicon Corporation, Danvers, MA) were soaked overnight in a 1.0 mM EDTA solution and then rinsed thoroughly in deionized water before use. Metal analyses were achieved by acetylene/air flame atomic absorption spectroscopy using a Perkin-Elmer Model 2380 spectrometer. Perkin-Elmer Intensitron lamps for atomic absorption were used at the following wavelengths in nm: 213.9 (zinc), 228.8 (cadmium), 240.7 (cobalt), 324.7 (copper), and 248.3 (iron). The phosphotriesterase concentration ranges used for the metal analyses by atomic absorption spectroscopy were 2–15 μM for Zn, Cd, and Fe and 15–50 μM for Co and Cu.

Enzyme Assays. The enzyme concentration was determined spectroscopically using an $\epsilon_{280} = 2.9 \times 10^4$ M^{-1} cm^{-1}. The enzymatic activity of the phosphotriesterase was routinely determined by monitoring the change in absorbance at 400 nm when 4.0 mM paraoxon was hydrolyzed to diethyl phosphate and *p*-nitrophenol (ϵ_{400} = 17 000 M^{-1} cm^{-1}) in 150 mM CHES buffer, pH 9.0. A Gilford UV-vis spectrophotometer (Model 260) was used and regulated at 25 °C. Kinetic parameters were determined by fitting the kinetic data to eq 1 using the computer programs of Cleland (1979). In this

$$v = V_m A/(K_m + A) \quad (1)$$

equation, v is the initial velocity, V_m is the maximal velocity, K_m is the Michaelis constant, and A is the initial substrate concentration. The competitive inhibition constants were similarly determined from a fit of the data to eq 2, where I

$$v = V_m A/(K_m(1 + I/K_{is}) + A) \quad (2)$$

is the inhibitor concentration and K_{is} is the slope inhibition constant. The pH rate (V_{max}) profiles using saturating paraoxon (4.0 mM) as a substrate were determined over the pH range 5–11. The following buffers (100 mM) were utilized over the indicated pH range: sodium acetate/acetic acid, pH 5.0–5.5; MES, pH 6.0–6.5; PIPES, pH 6.5–7.5; HEPES, pH 7.5–8.0; TAPS, pH 8.5–9.0; CHES, 9.0–9.5; and CAPS, pH 10.0–11.0. The kinetic constants for the pH rate profiles were determined by obtaining the apparent extinction coefficients at 400 nm for *p*-nitrophenolate/*p*-nitrophenol at any pH using eq 3 and the initial velocity data fit to eq 4. A pK_a of 7.14 and an extinction coefficient of 1.7 × 10^4 M^{-1} cm^{-1} for

[1] The details for the construction of this plasmid will be reported at a later date (Jane Kuo, Texas A&M University, personal communication).

[2] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid; NMR, nuclear magnetic resonance.

$$\epsilon_{app} = \epsilon(10^{pK_a - pH} + 1) \quad (3)$$

$$\log V_m = \log(C/(1 + (H/K)) \quad (4)$$

p-nitrophenolate anion were used in the calculation. In eq 4, C is the pH-independent value of V_m, H is the hydrogen ion concentration, and K is the dissociation constant of the ionizable group.

^{113}Cd NMR Spectroscopy. $^{113}CdCl_2$ was prepared by dissolving 65 mg of 95 atom % cadmium-113 metal in 2 mL of concentrated hydrochloric acid. After 48 h the metal was completely dissolved, and the excess acid was removed by repeated evaporation using a Speed Vac concentrator (Savant Instrument Corporation, Farmingdale, NY). The solid residue was dissolved in 1 mL of metal-free water and evaporated as before. This procedure was continued until the pH of the solution obtained a value of ~4, after which the volume was adjusted to 2.0 mL with H_2O, and then the final concentration of the cadmium was determined by atomic absorption spectroscopy as described above. The enzyme for the ^{113}Cd NMR spectroscopy experiments was prepared by reactivation of the apoenzyme (50 mL of a 1 mg/mL solution) as described above but using the enriched cadmium-113. The pH of the reconstituted enzyme was adjusted to 9.0, and the solution was filtered and concentrated to 1.5 mL; then 0.5 mL of 50 mM HEPES buffer (pD 9.0) in D_2O was added. Mixed-metal derivatives of the phosphotriesterase were similarly prepared by adding equal concentrations of the two metal salts to the apoenzyme. The ^{113}Cd NMR spectra were obtained with either a Varian XL-400 multinuclear NMR spectrometer, operating at a frequency of 89 MHz, or a Varian Unity 500 NMR spectrometer operating at a frequency of 109 MHz. Typical acquisition parameters were 40 000-Hz sweep width (digital resolution, 1.0 Hz/point), 0.5-s acquisition time, 1.25-s delay between pulses, 19-μs pulse width (90° pulse width = 25 μs), and no proton decoupling. Chemical shifts are reported relative to the resonance position of 0.1 M $Cd(ClO_4)_2$. Typical sample concentration was 36–54 mg/mL (1.0–1.5 mM).

RESULTS

Preparation of Metal–Phosphotriesterase Derivatives. The preparation of apophosphotriesterase was accomplished in high yield by incubation of the holoenzyme with 1,10-phenanthroline until the enzymatic activity was reduced to less than 1% of the original value. The mixture was diafiltered using metal-free buffer, and complete removal of the excess 1,10-phenanthroline was ascertained by the disappearance of the absorbance at 327 nm due to the chelator. The procedure yielded apophosphotriesterase having less than 1% of the original enzymatic activity. Reactivation of the apoenzyme with increasing amounts of $CdCl_2$ is shown in Figure 1. The titration demonstrates that the maximum catalytic activity is obtained when the cadmium concentration is twice the enzyme concentration, and addition of excess divalent metal ions, up to 4 equiv, has no further influence on the catalytic activity of the phosphotriesterase. Henceforth, the enzyme consisting of two metal ions/protein will be denoted as M/M–protein, e.g., the protein containing 2 Cd/protein is presented as Cd/Cd–phosphotriesterase.

Kinetic Analyses of Metal–Substituted Enzymes. The catalytic constants, k_{cat} and K_m, for the hydrolysis of paraoxon and parathion by the various metal derivatives of the phosphotriesterase are presented in Table I. The enzyme samples used in these experiments were prepared by reacti-

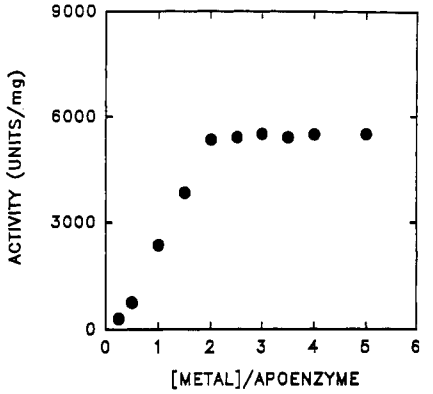


FIGURE 1: Reactivation of apophosphotriesterase with varying amounts of cadmium chloride. The apoenzyme (25 μM) was incubated at 4 °C for 48 h with the indicated molar equiv of Cd^{2+} in 50 mM HEPES buffer, pH 8.2. The enzyme activity was measured as described in the text.

Table I: Kinetic Constants for Paraoxon and Parathion Hydrolysis by Metal-Substituted Phosphotriesterase[a]

metal–enzyme	substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	pK_a
Zn/Zn	paraoxon	78 ± 8	2300 ● 150	30 ± 2	5.8 ± 0.1
	parathion	40 ± 3	450 ± 30	11 ± 1	
Co/Co	paraoxon	150 ± 15	6990 ± 450	47 ● 3	6.5 ± 0.1
	parathion	25 ± 4	500 ± 25	20 ± 2	
Cd/Cd	paraoxon	480 ± 30	4300 ± 320	9 ± 1	8.5 ± 0.2
	parathion	11 ± 2	120 ± 7	11 ± 1	
Cd/Co	paraoxon	390 ± 26	4700 ± 350	12 ± 1	7.6 ± 0.1
	parathion	20 ± 3	325 ± 22	17 ± 2	
Cd/Zn	paraoxon	80 ± 6	2600 ± 180	33 ± 2	6.0 ± 0.1
	parathion	25 ± 4	400 ± 30	16 ± 1	

[a] From fits of the data to eqs 1 and 4 at pH 9.0, 25 °C.

vating the apoenzyme either with 2 equiv of a single divalent metal ion (Zn, Cd, and Co) or with 1 equiv each of two different divalent metal ions. The results indicate that the k_{cat} value for paraoxon hydrolysis is highest for the Co/Co–enzyme, followed by the Cd/Cd–enzyme. The Zn/Zn–enzyme has only 1/3 the value observed for the cobalt-substituted enzyme. The K_m values, however, are highest for the Cd/Cd–enzyme, while somewhat lower values are observed for the Co/Co– and Zn/Zn–enzymes. The enzyme consisting of one cadmium and one cobalt ion per protein molecule has k_{cat} and K_m values that are intermediate between the homogeneously substituted enzymes, whereas the protein containing one Cd and one Zn ion has k_{cat} and K_m values very similar to those observed for the Zn/Zn–enzyme. When parathion (II) is used as the substrate, the Zn/Zn– and Co/Co–enzymes show nearly identical k_{cat} values, and the kinetic constants for the Cd/Zn–enzyme still resemble those of the Zn/Zn–enzyme. The Cd/Co–enzyme displays kinetic constants intermediate between those of the Co/Co– and the Cd/Cd–enzyme. The kinetic pK_a values, determined from the pH rate activity profiles, for these metal-substituted enzymes are also shown in Table I. The relative values are strikingly similar to the other kinetic parameters. The kinetic pK_a for the Cd/Co–phosphotriesterase is approximately midway between the values obtained for the Cd/Cd– and Co/Co–phosphotriesterase derivatives, while the kinetic pK_a value measured for the Cd/Zn–phosphotriesterase is nearly the same as that of the Zn/Zn–phosphotriesterase.

The change in K_m and k_{cat} values for the enzyme samples, prepared by titration of the apophosphotriesterase with varying ratios of cadmium/zinc and cadmium/cobalt, is shown in Figure 2. The plot in Figure 2A illustrates that as the ratio

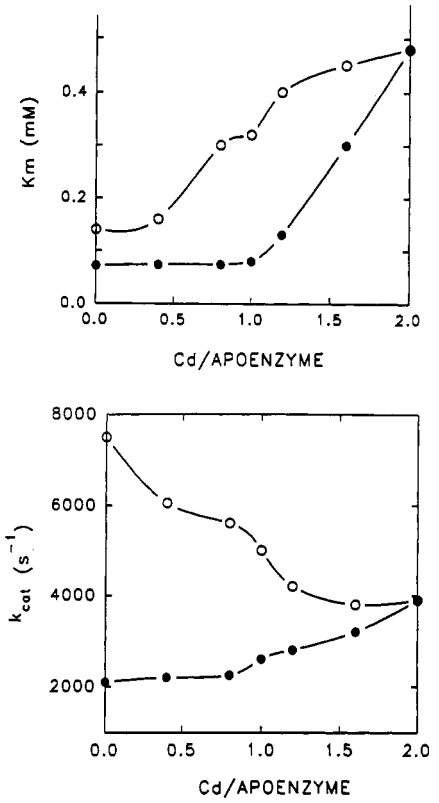


FIGURE 2: Variation of catalytic constants, K_m and k_{cat}, for the apophosphotriesterase titrated with various ratios of cadmium, zinc, and cobalt. The ratio of total metal to enzyme was maintained at 2:1. The apoenzyme (30 μM) in 50 mM HEPES buffer, pH 8.2, was activated with a mixture of Cd^{2+} and Zn^{2+} (●) or Cd^{2+} and Co^{2+}(○) for 48 h at 4 °C. The change in K_m values is illustrated in panel A (top), while panel B (bottom) shows the variation in the value of k_{cat}.

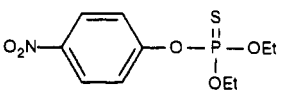
Table II: Competitive Inhibition Constants for Dithiothreitol (III), Diethyl 4-Methoxybenzylphosphonate (IV), and Diethyl 4-Methoxybenzylthiophosphonate (V)[a]

	competitive inhibition constants (μM)		
metal–phosphotriesterase	III	IV	V
Zn/Zn	14 ± 1	410 ± 20	310 ± 15
Cd/Cd	2 ± 1	290 ± 15	200 ± 10
Zn/Cd	13 ± 1	350 ± 18	250 ± 12

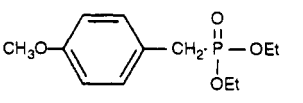
[a] From fits of the data to eq 2 at pH 9.0, 25 °C.

of Cd to Zn is increased, the resulting metalloenzyme maintains the characteristics of the Zn/Zn–phosphotriesterase until the metal ion ratio becomes 1 Cd and 1 Zn per protein molecule. At this point, the hybrid enzyme exhibits a K_m value of 80 μM, whereas the value observed for Zn/Zn–phosphotriesterase is 78 μM. These values are significantly different from the K_m value of 480 μM observed for the Cd/Cd–phosphotriesterase. Titration of the apophosphotriesterase with a mixture of Co and Cd shows that the k_{cat} for the mixed-metal enzyme derivatives progressively decreases as the Cd/Co ratio is elevated (Figure 2B). When this ratio is 1:1, the mixed-metal hybrid enzyme has a k_{cat} of approximately 5000 s^{-1}, a value that is intermediate between those observed for the Cd/Cd– and Co/Co–phosphotriesterase derivatives. Similar trends are also observed for the K_m values, which progressively increase as the ratio of Cd is increased relative to Co (Figure 2A).

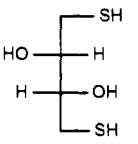
The competitive inhibition constants, K_i, for dithiothreitol (III), diethyl (4-methoxybenzyl)phosphonate (IV), and diethyl (4-methoxybenzyl)thiophosphonate (V) are shown in Table II. Dithiothreitol (III) is a strong competitive inhibitor of the phosphotriesterase, with a K_i value of less than 15 μM. The K_i is lowest for the Cd/Cd–enzyme, whereas the Zn/Zn– and the Zn/Cd–phosphotriesterase exhibit nearly the same value. The substrate analogs are also competitive inhibitors of the enzyme but with higher K_i values (200–410 μM).



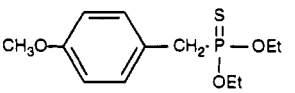
II



IV



III



V

^{113}Cd NMR Spectroscopic Analysis. The ^{113}Cd NMR spectrum of the Cd–phosphotriesterase, reactivated with 2 equiv of $^{113}Cd^{2+}$ per protein, is shown in Figure 3A. The spectrum taken at 25 °C in 50 mM HEPES buffer, pH 9.0, shows two separate resonances centered at 212 and 116 ppm downfield from 0.1 M $Cd(ClO_4)_2$. Reconstitution of the apophosphotriesterase with 1 equiv of $^{113}Cd^{2+}$ per protein molecule gave a nearly identical spectrum except for the reduction in amplitude for the two resonances. The two resonances are centered at 211 and 117 ppm as shown in Figure 3B. Reconstitution of the apophosphotriesterase with a combination of two different metal ions results in ^{113}Cd NMR spectra in which the intensity of the resonance at 212 ppm was greatly reduced relative to that observed at 116 ppm. The spectrum in Figure 4A is of the phosphotriesterase prepared by reconstitution of the apoenzyme with 1 equiv each of $^{113}Cd^{2+}$ and Zn^{2+} per protein (added simultaneously). The ^{113}Cd NMR spectrum of Cd/Zn–phosphotriesterase shows only a single resonance at 115 ppm. The ^{113}Cd NMR spectrum of the Cd/Co–phosphotriesterase (Figure 4B) shows one major resonance at 117 ppm and a much broader resonance at 216 ppm. The Cd/Mn–phosphotriesterase also displays one primary resonance centered at 117 ppm and a minor but fairly broad resonance centered at 215 ppm (Figure 4C).

When 2 equiv of dithiothreitol (III) per metal site were added to the Cd/Cd–phosphotriesterase, the two original resonances, initially at 116 and 212 ppm, completely disappear and are replaced with two new resonances centered at 225 and 368 ppm (Figure 5A). When the Zn/Cd–enzyme sample was similarly subjected to 2 equiv of dithiothreitol (III) per metal site, the resonance that was originally observed at 115 ppm is shifted to 241 ppm and extensively broadened (Figure 5B). In this spectrum there may also be a broadened residual resonance at approximately 115 ppm, but the poor signal-to-noise ratio precludes a more detailed evaluation. When 1 equiv of diethyl (4-methoxybenzyl)phosphonate (IV) was added to the Cd/Cd–enzyme, two very weak resonances were observed at 211 and 118 ppm (data not shown). Attempts to observe the ^{113}Cd NMR spectrum for the Cd/Cd–phosphotriesterase in the presence of the substrate analog (V) were unsuccessful.

DISCUSSION

We have previously demonstrated that the enzymatic activity of the phosphotriesterase from *P. diminuta* requires at least one divalent cation for full catalytic activity (Dumas *et al.*, 1989; Omburo *et al.*, 1992). Removal of the native zinc

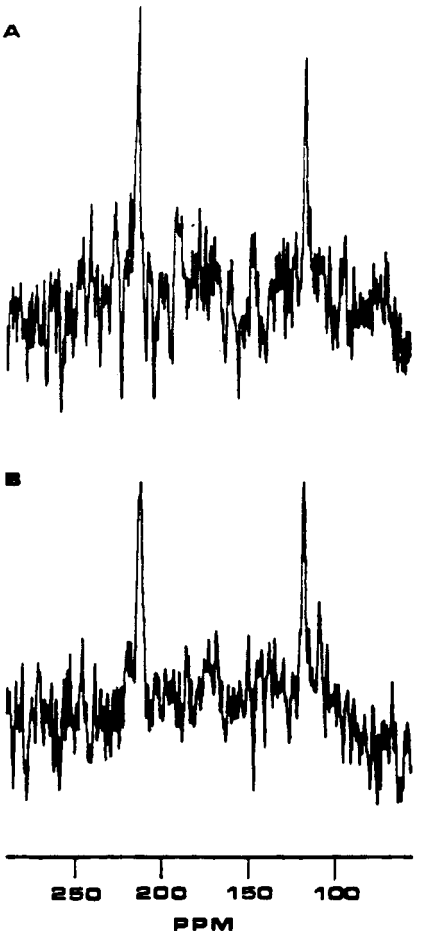


FIGURE 3: ^{113}Cd NMR spectra of cadmium-113-substituted phosphotriesterase. (A) The ^{113}Cd NMR spectrum of Cd–phosphotriesterase reconstituted with 2 equiv of $^{113}Cd^{2+}$ per protein molecule (51 938 transients). (B) The ^{113}Cd NMR spectrum of Cd–phosphotriesterase containing 1 equiv of $^{113}Cd^{2+}$ (47 360 transients). The phosphotriesterase concentration was approximately 1.0 mM in 50 mM HEPES, pH 9.0. The spectra are shown with 75-Hz line broadening. Additional details are given in the text.

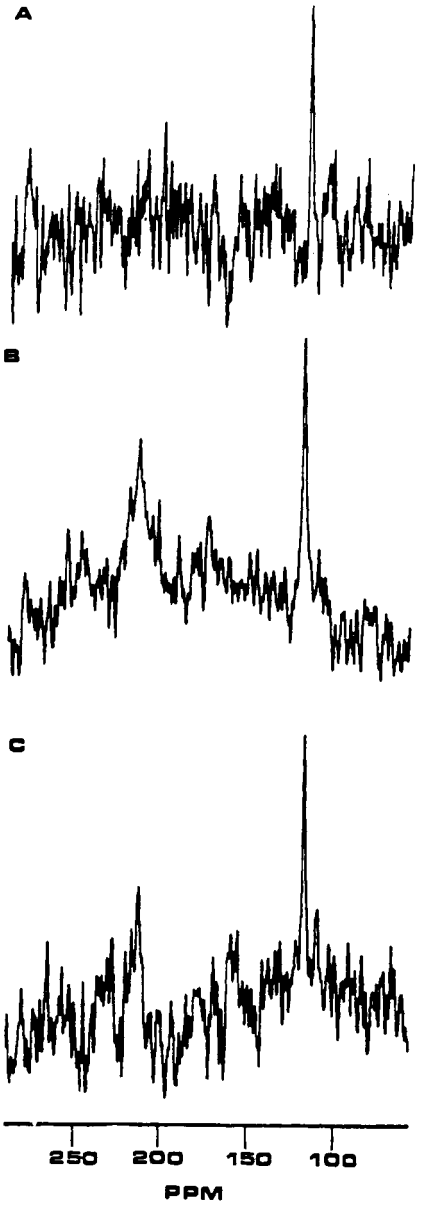


FIGURE 4: ^{113}Cd NMR spectra of mixed-metal complexes of phosphotriesterase. (A) NMR spectrum of 1.2 mM ^{113}Cd/Zn–phosphotriesterase (44 800 transients). (B) NMR spectrum of 1.5 mM ^{113}Cd/Mn–enzyme (48 128 transients). (C) NMR spectrum of 1.3 mM ^{113}Cd/Co–enzyme (55 040 transients). The mixed-metal complexes were prepared by simultaneous addition of the two metal ions to the apoenzyme. All spectra were transformed with a line broadening of 75 Hz. Additional details are given in the text.

from the purified protein with metal chelators results in the loss of enzymatic activity (>99%), but full catalytic activity can be restored upon reconstitution of the apoenzyme with a variety of other divalent cations (Omburo *et al.*, 1992). The focus of this investigation has been to replace the spectroscopically silent zinc found in the native phosphotriesterase with cadmium in order to more fully characterize the structural and functional features of the metal binding site(s) of this unusual hydrolytic enzyme. The ^{113}Cd isotope is NMR active, and this property has been previously exploited with great success by a number of other investigators for the characterization of divalent cation sites of metalloproteins (Armitage & Otvos, 1982; Summers, 1988). These spectroscopic studies have been initiated with the bacterial phosphotriesterase in order to provide new information regarding the nature of the metal–protein, metal–substrate, and metal–metal interactions.

Preparation and Reconstitution of Apophosphotriesterase. The divalent cations bound to the purified phosphotriesterase can be efficiently removed by incubation of the protein with metal chelators. Reconstitution of the apoprotein with increasing amounts of Cd^{2+} (Figure 1) clearly indicates that two metal ions per enzyme molecule are required to fully restore the catalytic activity. Since the enzymatic activity (as measured by k_{cat}) of the cadmium-substituted enzyme is greater than that exhibited by the native Zn–enzyme, it appears that the structure of the active site has not been adversely affected by the change in metal ion. Therefore, the conclusions obtained from the ^{113}Cd NMR investigations should be relevant to the structure of the native Zn-containing protein. Previous experiments have demonstrated that significant activity can also be obtained with Zn, Cd, Mn, Ni, and Co (Omburo *et al.*, 1992). This demonstrates that the two metal ion sites of this enzyme are quite flexible since metals of different ionic radii and preferred coordination number are readily accommodated.

^{113}Cd NMR Studies. When the phosphotriesterase is substituted with 2 ^{113}Cd/protein, the NMR spectrum clearly shows two resonances of nearly equal intensity at 116 and 212 ppm (Figure 3A). The observed chemical shift difference (~100 ppm) between these two resonances supports the conclusion that the local environment of the two metal binding sites within this enzyme are significantly different. These two sites have been arbitrarily designated as the M_α and M_β

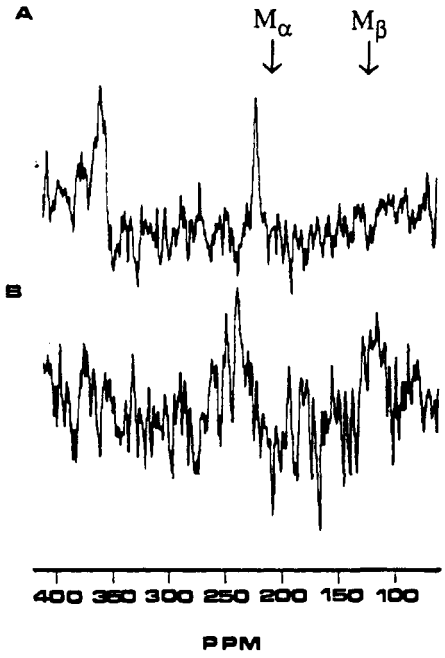
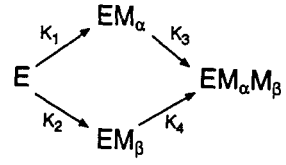


FIGURE 5: Effect of dithiothreitol and substrate analogs on ^{113}Cd NMR spectra of bacterial phosphotriesterase. (A) NMR spectrum of Cd/Cd–phosphotriesterase with 2 equiv of dithiothreitol per metal site. (B) NMR spectrum of the Cd/Zn–enzyme plus 2 equiv of dithiothreitol per metal site.

Scheme I



sites for the downfield and upfield signals, respectively. When apoenzyme is reconstituted with 1 equiv of cadmium, the NMR spectrum also shows two resonances of nearly equal intensity at the same chemical shift positions observed with the 2 ^{113}Cd/protein sample (Figure 3B). This result demonstrates that during the titration of the apoenzyme with increasing amounts of cadmium, one site is not completely filled prior to the binding of metal to the second site (ordered step-wise binding). These results further suggest either that the occupancy of the two sites during the addition of metal is completely random and the two metal ions bind with nearly equal affinity to independent sites or, alternatively, that the binding mechanism is highly cooperative and the two metal ions bind in pairs. A general model for the binding of two metal ions to a protein is given in Scheme I, where K_1, K_2, K_3, and K_4 are the dissociation constants for the possible metal–protein complexes (EM_α, EM_β, and $EM_\alpha M_\beta$). The highly cooperative binding mechanism is favored ($K_3 \ll K_1$ and/or $K_4 \ll K_2$) since the regain of enzymatic activity is linear with increasing amounts of cadmium up to a ratio of 2. However, these data alone cannot provide any additional information regarding the functional role for the two metal ions or determine whether both metals are required for catalytic activity.

Mixed-Metal Complexes. Catalytically active hybrid metal derivatives of the bacterial phosphotriesterase have been isolated. These derivatives were prepared to contain an equal mixture of two different metal ions bound to the protein. The most interesting properties for this class of enzymes are those observed for the Cd/Zn hybrid. The catalytic constants for this enzyme derivative most closely resemble those kinetic constants obtained for the Zn/Zn–phosphotriesterase. The values of the kinetic parameters, K_m and k_{cat}, and the kinetic pK_a for the hydrolysis of paraoxon and parathion by the Zn/Cd–phosphotriesterase are nearly identical to those measured for the Zn/Zn–enzyme (see Table I). Moreover, the K_i values for the competitive inhibition by dithiothreitol, using these two enzyme derivatives, also support this conclusion (Table II). Thus, the Zn ion in the Zn/Cd hybrid is dictating the kinetic parameters. On statistical grounds the Zn/Cd hybrid could consist of a complex mixture of the Zn/Zn–, the Cd/Cd–, and two forms of the Cd/Zn–enzyme. However, the ^{113}Cd NMR spectrum of the Zn/Cd hybrid clearly shows a single ^{113}Cd NMR signal at the high-field M_β-site. Therefore, it appears that the Zn^{2+} ion is bound preferentially to the M_α site while the Cd ion is bound predominantly to M_β site. It can also be concluded that the identity of the metal at the M_α site dictates the catalytic properties. These observations are consistent with our previous experiments which demonstrated that zinc could displace a single cadmium ion from the Cd/Cd–enzyme and that cadmium could displace a single zinc ion from the Zn/Zn–enzyme (Omburo *et al.*, 1992). The other mixed-metal proteins, Cd/Co– and Cd/Mn–phosphotriesterase, do not display as simple ^{113}Cd NMR spectra as observed for the Cd/Zn–enzyme. The spectra for the Cd/Mn– and the Cd/Co–enzyme display both the high- and low-field resonances. However, in both cases there appears to be a preference for cadmium at the high-field M_β site, but differences in relaxation times for Cd at the M_α and M_β sites may account for some of the changes. The values of the catalytic parameters, K_m and k_{cat}, and the kinetic pK_a of the Cd/Co–enzyme are also intermediate between those constants observed for the two homogeneous metal enzymes. Therefore, due to partial occupancy of the low-field M_α site by Cd, one metal ion is not dictating the hydrolytic process as observed for the Cd/Zn–enzyme.

Metal–Protein Interactions. Zinc and other divalent cations are coordinated to proteins primarily via the side chains of cysteine, histidine, aspartate, and glutamate (Vallee & Galdes, 1984). In general, catalytic zinc ions are bound to enzymes by three protein ligands, while the fourth ligand is usually H_2O. With relatively few exceptions, two of these metal ligands are separated in sequence by 1–3 amino acid residues, which are in turn separated from the third ligand by a long spacer consisting of 20–120 residues (Vallee & Auld, 1990a,b). In contrast, structural zinc sites are generally coordinated to four protein ligands. Since Armitage *et al.* (1976) first initiated the study of metalloenzymes by ^{113}Cd NMR spectroscopy, over 30 different metalloenzymes have been examined. The chemical shifts of ^{113}Cd in these cadmium-substituted proteins are highly sensitive to the type of coordinating ligands, and this property has provided a general correlation between the observed chemical shifts and the nature of the ligand donor atoms (Armitage & Otvos, 1982; Summers, 1988). Generally, for metal binding sites composed exclusively of oxygen ligands, the ^{113}Cd NMR spectra exhibit chemical shift values ranging from –125 to 0 ppm, as has been observed for concanavalin A (–120 ppm; Bailey *et al.*, 1978) and calmodulin (–100 ppm; Anderson *et al.*, 1982). When the ligands are coordinated to the cadmium exclusively through sulfur atoms, as in the metallothioneins and the structural zinc site of horse liver alcohol dehydrogenase, the observed chemical shifts are found above 400 ppm (Bobsein & Myers, 1980; Armitage *et al.*, 1982). The metal binding sites composed of a mixture of nitrogen and oxygen ligands show resonances in the range of 40–300 ppm, and such metal sites

include those found in the B site of alkaline phosphatase (1 histidine, 2 glutamates; 52 ppm; Gettins & Coleman, 1983, 1984a,b), carboxypeptidase A, (2 histidines, 1 glutamate, 1 water; 133 ppm; Armitage *et al.*, 1978; Gettins, 1986), and carbonic anhydrase (3 histidines, 1 water; 220 ppm; Armitage *et al.*, 1976; Jonsson *et al.*, 1980; Evelhoch *et al.*, 1981).

The two cadmium resonances for the cadmium-substituted phosphotriesterase are found at 116 and 212 ppm. The positions of these chemical shift values exclude the possibility for sulfur ligation and an all-oxygen ligand set. Therefore, it would appear that both of the metal ions are coordinated to the protein via a combination of nitrogen and oxygen ligands. The signal at 212 ppm is tentatively assigned to coordination by 1 oxygen and 3 nitrogen ligands, while the metal that resonates at 116 ppm would appear to be coordinated to fewer nitrogens (perhaps only two), with the remainder being oxygen ligands.

Metal–Substrate Interactions. A direct interaction between one or both metal sites and the substrates of this enzyme is indicated by the significant change in the chemical shift for the cadmium resonances upon binding of competitive inhibitors and also by the systematic changes in the kinetic constants observed with the zinc and cadmium enzymes when paraoxon and parathion are utilized as substrates. The ratio of the observed K_m values for paraoxon hydrolysis with the cadmium- and zinc-substituted enzymes is 6.9 (480/70), whereas with the corresponding sulfur analog, parathion, the apparent affinity for the substrate is reversed. The ratio of K_m values for parathion hydrolysis with the cadmium and zinc enzymes is 0.26 (11/40). The distinct preference of the cadmium–enzyme for the sulfur-containing substrate is consistent with the higher affinity expected for the softer ligand with this metal (Pearson, 1966). This preference is confirmed by the significantly tighter binding of dithiothreitol (DTT) observed with the cadmium–enzyme relative to that observed for the zinc-substituted enzyme. The direct coordination of the sulfur atoms in DTT to both metal sites in the cadmium-substituted enzyme is confirmed by the observation of a 100–150 ppm downfield shift for each of the cadmium resonances upon binding of DTT. The dithiothreitol binds to both metal centers in phosphotriesterase since both resonances are shifted by similar amounts. The diethyl (4-methoxybenzyl)phosphonate, which is also a competitive inhibitor, does not cause a significant shift in the two observed ^{113}Cd resonances of the phosphotriesterase. This is not necessarily surprising because coordination of this substrate analog to the active site metal is likely to occur through the phosphonyl oxygen by displacing a water molecule that usually occupies the fourth ligand site in catalytic metal sites.

Metal–Metal Interactions. Evidence for metal–metal interactions can potentially be obtained from the ^{113}Cd NMR spectra by the observation of ^{113}Cd–^{113}Cd homonuclear coupling or, alternatively, by line-broadening effects induced by the formation of a hybrid metal complex with a paramagnetic partner. Homonuclear coupling has been observed with the metallothioneins where two cadmium ions are bridged by cysteine thiolate groups. Coupling constants on the order of 20–50 Hz have been observed (Summers, 1988). The average line width for the two resonances observed in Figure 3A is 160 Hz. Unless the homonuclear coupling constants are substantial, it is very difficult to directly observe them with the line widths and signal-to-noise ratios inherent with this system. Furthermore, homonuclear coupling through either a carboxylate or an imidazole bridge would also be expected to be significantly smaller. Paramagnetic line broadening has been previously observed with superoxide dismutase substituted with $^{113}Cd^{2+}$ and Cu^{2+} (Armitage *et al.*, 1978; Bailey *et al.*, 1980). The two metals in superoxide dismutase are separated by 6–7 Å and bridged with an anionic imidazole side chain (Richardson *et al.*, 1975). A ^{113}Cd NMR signal is observed when the copper is removed or reduced but not when it is oxidized. The cadmium resonances of the phosphotriesterase are observed for both the Cd/Co and the Cd/Mn hybrid enzyme, but it should be noted that the line widths are broadened somewhat relative to those observed for the Cd/Zn hybrid (from 75 to 100 and 150 Hz, respectively). The Solomon–Bloembergen equation predicts a line broadening of 100 Hz for a Cd–Mn pair separated by 7.8 Å, assuming a correlation time of 2×10^{-9} s (Solomon & Bloembergen, 1956). However, the inaccuracies in the line-width measurements and the low signal-to-noise ratio make a more quantitative analysis unwarranted.

Catalytic and Structural Functions of Metal Ions. The two metal ions bound to the bacterial phosphotriesterase may function as either catalytic or structural sites. The systematic changes in the kinetic constants with the specific metal ion that occupies the M_α site clearly indicate that this metal plays a significant catalytic role in the mechanism of phosphotriester hydrolysis. On the basis of an analogy with the chemical mechanism for carboxypeptidase it would appear that this metal acts as a Lewis acid for the polarization of the phosphorus–phosphoryl oxygen bond and also as a binding site for the hydrolytic water molecule. The function of the metal that occupies the M_β site is not so clear, but we have determined that the ligands to these metal sites are composed primarily of histidine side chains. The specific role for these metal sites is currently being refined through the systematic mutagenesis of all probable metal ligands and an investigation of the EPR properties of derivatives substituted with paramagnetic metal ions. Preliminary characterization of the Mn/Mn–phosphotriesterase by EPR spectroscopy indicates that the two metals are magnetically coupled via a common ligand.[3]

[3] George Omburo, unpublished observations (1992).

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