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Characterization of the Zinc Binding Site of Bacterial Phosphotriesterase*

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The bacterial phosphotriesterase has been found to require a divalent cation for enzymatic activity. This enzyme catalyzes the detoxification of organophosphorus insecticides and nerve agents. In an *Escherichia coli* expression system significantly higher concentrations of active enzyme could be produced when 1.0 mM concentrations of Mn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+} were included in the growth medium. The isolated enzymes contained up to 2 equivalents of the these metal ions as determined by atomic absorption spectroscopy. The catalytic activity of the various metal enzyme derivatives was lost upon incubation with EDTA, 1,10-phenanthroline, and 8-hydroxyquinoline-5-sulfonic acid. Protection against inactivation by metal chelation was afforded by the binding of competitive inhibitors, suggesting that at least one metal is at or near the active site. Apoenzyme was prepared by incubation of the phosphotriesterase with β-mercaptoethanol and EDTA for 2 days. Full recovery of enzymatic activity could be obtained by incubation of the apoenzyme with 2 equivalents of Zn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, or Mn^{2+}. The ^{113}Cd NMR spectrum of enzyme containing 2 equivalents of $^{113}Cd^{2+}$ showed two resonances at 120 and 215 ppm downfield from $Cd(ClO_4)_2$. The NMR data are consistent with nitrogen (histidine) and oxygen ligands to the metal centers.

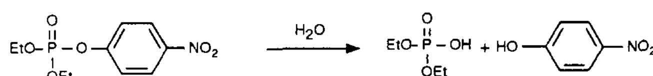
Organophosphate degrading enzymes have become the focus of recent attention because of their potential utility for the detoxification of chemical wastes, chemical warfare agents, and agricultural pesticides. Organophosphate compounds such as parathion constitute the largest class of insecticides currently used in the industrialized countries (Mulbry and Karns, 1989a). The lack of persistence of these pesticides in the soil has been attributed to their susceptibility to microbial transformation (Read, 1983). The phosphotriesterase found in the soil bacteria *Pseudomonas diminuta* and *Flavobacterium* sp. has been shown to hydrolyze a wide variety of organophosphorus triesters (Donarski *et al.*, 1989; Brown, 1980) including the acetylcholinesterase inhibitor diisopropylfluorophosphate (Dumas *et al.*, 1989).

The gene for the bacterial phosphotriesterase has been characterized and sequenced from both *P. diminuta* and *Flavobacterium* sp. (Serdar *et al.*, 1989; Mulbry and Karns, 1989b). The DNA sequences are identical, and in both cases the protein is translated as a larger precursor protein prior to cleavage of a 29-amino acid leader peptide from the N-terminal end to form the mature enzyme (Mulbry and Karns, 1989b). The gene for the bacterial phosphotriesterase has been subcloned into a variety of expression vectors (McDaniel *et al.*, 1988) and the protein purified to homogeneity using insect cells (Dumas *et al.*, 1989), *Streptomyces lividans* (Rowland *et al.*, 1991), and *Escherichia coli* (Serdar *et al.*, 1989) as hosts. Significant improvements in the levels of protein expression have been obtained by deletion of the DNA encoding the 29-amino acid leader sequence (Serdar *et al.*, 1989; Mulbry and Karns, 1989b).

The bacterial phosphotriesterase hydrolyses paraoxon to *p*-nitrophenol and diethyl phosphate as shown in Scheme 1. The enzyme from *P. diminuta* has been shown to catalyze this reaction with overall inversion of stereochemistry at the phosphorus center. This result is consistent with a chemical mechanism that utilizes an activated water molecule to directly attack the phosphorus center (Lewis *et al.*, 1988). Recent experiments, using structure-reactivity relationships and the effect of solvent viscosity, have demonstrated that the rate of hydrolysis with paraoxon as a substrate is limited by diffusion of the enzyme and substrate rather than the actual bond cleavage step (Caldwell *et al.*, 1991). This enzyme is, therefore, a highly efficient catalyst for the hydrolysis of organophosphorus insecticides.

Although the phosphotriesterase from *P. diminuta* has been purified to homogeneity and partially characterized the amount of protein available for physical studies has been severely limited. However, the enzymatic activity of the phosphotriesterase has been demonstrated to require the presence of zinc bound to the protein (Dumas *et al.*, 1989). Moreover, Serdar *et al.* (1989) reported an enhancement of the initial specific activity in whole cells when divalent metal ions were supplemented in the growth medium. Rowland *et al.* (1991), working with a *S. lividans* expression system, also observed a significant enhancement in enzyme activity when 1 mM Co^{2+} was included in the growth medium. Dumas *et al.* (1989) were able to demonstrate that the purified apoenzyme was completely inactive, but attempts to reconstitute the original activity by incubation with a variety of divalent cations were unsuccessful.

The focus of this paper is a determination of the environment and the role that divalent cations play in the catalytic activity of the phosphotriesterase. We now report a significant improvement in the purification protocol for the bacterial



SCHEME 1

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phosphotriesterase and a biosynthetic method for replacing the naturally occurring zinc ion with cobalt, manganese, cadmium, and nickel is presented. These metal-substituted enzymes have been purified and the metal ion stoichiometry and catalytic parameters determined. We also have succeeded in preparing apophosphotriesterase that can be fully reconstituted with a variety of divalent cations that are suitable for biophysical characterization.

EXPERIMENTAL PROCEDURES

Materials—EDTA, 1,10-phenanthroline, 8-hydroxyquinoline-5-sulfonic acid (HQSA),[1] paraoxon (95%), and all buffers were purchased from Sigma. Solutions of the various metal ions were prepared from spectrophotometrically pure sulfate or chloride salts obtained from Aldrich.

Enzymatic Activity Measurements—Enzymatic activity was routinely measured by monitoring the change in absorbance at 400 nm when 1.0 mM paraoxon was hydrolyzed to diethyl phosphate and *p*-nitrophenolate anion (ϵ_{400} = 17,000 M^{-1} cm^{-1}) in 150 mM CHES buffer, pH 9.0, using a Gilford model 260 spectrophotometer regulated at 25 °C. One unit of activity is defined as the hydrolysis of 1.0 μmol of paraoxon/min. Paraoxon was purified by dissolving 1–2 ml of material in 200 ml of dichloromethane and extracting with 300 ml of 10 mM CHES buffer, pH 9.0. The extraction process was repeated several times until the aqueous phase remained colorless. The organic solvent was then removed by rotary evaporation and the oily paraoxon residue dissolved in deionized water to give a final concentration of 4–6 mM. Purified paraoxon solutions were stored frozen at −20 °C.

Metal Ion Analysis—The distilled water was made metal-free using the method of Veillon and Vallee (1978). The glassware used for metal ion analysis was washed with 30% nitric acid and then thoroughly rinsed with deionized water. Dialysis tubing (Spectrapor 2, Spectrum Medical Industries, Los Angeles, CA) was rendered metal-free by first rinsing in deionized water followed by heating to 70 °C in water containing 0.05 mM EDTA. The chelator solution was discarded, replaced by water, and heated as before. Two such changes were required before final rinsing with cold water and storage at 4 °C. The Centricon-10 microconcentrator devices (Amicon Corp.) used for diafiltration or concentration of protein samples were soaked overnight in 1.0 mM EDTA and rinsed thoroughly in deionized water. Metal analyses were performed with a Perkin-Elmer model 2380 atomic absorption spectrophotometer with an acetylene-air flame.

Purification of Phosphotriesterase—The phosphotriesterase was purified from an *E. coli* expression system using the plasmid pJK33 obtained from Dr. Jeffrey S. Karns (USDA, Beltsville, MD). The use of this plasmid produces a protein in which the first 33 amino acids of the putative precursor protein are replaced with Met-Ile-Thr-Asn-Ser- (Mulbry and Karns, 1989b). The *E. coli* cells were grown to stationary phase (35–38 h) at 30 °C in a medium containing 12 g/liter Bactotryptone, 24 g/liter Bactoyeast, 80 mM K_2HPO_4, and 20 mM KH_2PO_4, and 4 ml/liter glycerol. The cells were harvested by centrifugation at 8,000 × *g* for 10 min at 4 °C. From 7 liters of cell culture 100–120 g of wet cell paste was isolated and stored at −78 °C until needed. All subsequent steps were carried out at 4 °C (or on ice bath) in 50 mM HEPES buffer, pH 8.5. The bacterial cells (60 g) were suspended in 120 ml of buffer and lysed with a 5-s pulsed sonication for 30 min at 0 °C at medium power setting using a Heat System-Ultrasonics, Inc. (Farmington, NY) model W830 ultrasonic processor. The lysed cell suspensions were combined and centrifuged at 13,000 × *g* for 15 min. The supernatant fluid was decanted, and then a solution of protamine sulfate (2% w/v) was added dropwise over a 30-min period while stirring until the protamine sulfate concentration reached 0.4%. The mixture was allowed to stir an additional 30 min before centrifugation as above. The supernatant solution was then subjected to ammonium sulfate fractionation by the addition of solid ammonium sulfate to 45% saturation (258 mg/ml of protein solution) while stirring for 30 min and then kept stirring for an additional 30 min. The pellet was collected by centrifugation at 13,000 × *g* for 20 min and the protein recovered by dissolving the precipitate in 40 ml of buffer. The protein was loaded onto a 5.0 × 150-cm gel filtration column containing Ultrogel AcA 54 (IBF, Columbia, MD) and then eluted at a flow rate of 1.0 ml/min. The fractions were pooled based on enzymatic activity and absorbance at 280 nm and then applied to a 5 × 25-cm column, containing DEAE-Sephadex A-25 previously equilibrated with 50 mM HEPES buffer, pH 8.5. The progress of the purification procedure was monitored by determining the specific activity and analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12.5% denaturing gels.

Preparation of Apoenzyme—Phosphotriesterase at concentrations of less than 2 mg/ml was dialized at 4 °C against four changes (8 h between each change) of 50 mM HEPES buffer, pH 8.5, containing 0.2 M EDTA and 0.2 M β-mercaptoethanol. The enzyme solution was then either extensively dialyzed to remove excess chelator or concentrated and thoroughly diafiltered with an Amicon diafiltration device using 50 mM HEPES buffer, pH 8.5, made from a 1.0 M stock solution that had been passed through Chelex-100.

Metal Substitution Experiments—The native zinc ion in the phosphotriesterase was replaced with other divalent cations by biosynthetic and *in vitro* methods. With the biosynthetic method, the bacterial cells were grown in a medium supplemented with a 1.0 mM concentration of the desired divalent metal ion. The divalent metal salts (0.05–0.10 mM) were also added to the buffer used in the purification protocol. For the *in vitro* method, apoenzyme (<1.0 mg/ml), prepared as described above, was incubated at 25 °C for up to 48 h with various amounts of the different divalent metal ions.

Inactivation by Metal Chelators—The second-order rate constants of inactivation by the metal chelating agents 1,10-phenanthroline, EDTA, and HQSA were determined according to the method described by Dumas *et al.* (1989). Enzyme (0.05 mg/ml) was incubated with various amounts of chelator, and the remaining activity was determined by removing aliquots at specified times. The data were fit to the following equation

$$\ln(V/V_0) = -kt \quad (1)$$

where V/V_0 is the fraction of remaining activity at time *t*, and *k* is the pseudo-first-order rate constant. The pseudo-first-order rate constants were plotted *versus* the initial chelator concentration to obtain the second order rate constants.

Measurement of Catalytic Constants—The kinetic parameters, V_{max} and K_m, were measured at 25 °C in the presence of 150 mM CHES buffer at pH 9.0. The kinetic data were fit to the following equation using the computer programs of Cleland (1979).

$$v = V_m A/(K_m + A) \quad (2)$$

The pH rate profiles for *V* were determined using paraoxon as a substrate (4 mM) for the enzyme at pH values ranging from 5.0 to 11.0. The following buffers (100 mM) were used for these experiments: sodium acetate/acetic acid, pH 5.0–5.5; MES, pH 6.0; PIPES, pH 6.5–7.5; HEPES, pH 8.0; TAPS, pH 8.5; CHES, pH 9.0–9.5; CAPS, pH 10–11.0. The apparent extinction coefficient at 400 nm for *p*-nitrophenolate anion at any pH was calculated from Equation 3, where the pK_a is 7.14 and ϵ is 1.7×10^4 M^{-1} cm^{-1}. The pH rate profiles were fit to Equation 5

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

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

where *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 Nuclear Magnetic Resonance Measurements—The ^{113}Cd NMR spectra were obtained on a Varian XL-400 multinuclear NMR spectrometer operating at a frequency of 89 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 $Cd(ClO_4)_2$. Samples consisted of 0.7 mM phosphotriesterase containing 2 equivalents of ^{113}Cd in 50 mM phosphate buffer at pH 8.2.

RESULTS

The addition of divalent metal ions to the standard growth medium was found to increase the overall expression of enzyme activity substantially as shown in Fig. 1 for the addition of 1.0 mM $CoCl_2$. At stationary phase the total number of

[1] The abbreviations used are: HQSA, 8-hydroxyquinoline-5-sulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; TAPS, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid.

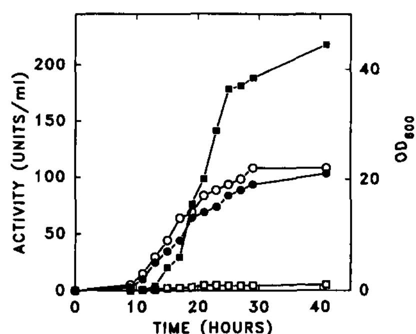

FIG. 1. **The effect of added Co^{2+} on the growth and expression of enzymatic activity of *E. coli* cells containing plasmid pJK33.** The *closed circles* (●) and *closed squares* (■) represent the growth and expression of enzymatic activity in medium supplemented with 1.0 mM Co^{2+}. The *open circles* (○) and *open squares* (□) indicate the growth and expression of enzymatic activity in unsupplemented medium. Additional details are given under "Experimental Procedures."

TABLE I
Metal analysis and activity of phosphotriesterase

Metal ion	Initial specific activity	Protein isolated[a]	Final specific activity[b]	Metal analysis	Purification medium
	units/g cells	*mg*	*units/mg*	*mol ratio*	
None	860	44	1,760	1.1 Zn	Metal free
Zn	1,530	47	2,080	1.4 Zn	Metal free
			2,540	1.8 Zn	0.05 mM Zn
Co	37,500	183	8,020	1.0 Co	Metal free
				0.2 Zn	
				0.2 Fe	
			8,120	1.7 Co	0.05 mM Co
				0.2 Zn	
				0.1 Fe	
Ni	4,750	179	3,320	0.6 Ni	0.1 mM Ni
				0.3 Zn	
				0.8 Fe	
Cd	2,500	78	4,100	1.8 Cd	0.05 mM Cd
				0.2 Zn	
Mn	6,000	216	3,020	1.0 Mn	0.1 mM Mn
				0.3 Zn	
Cu	1,900	70	1,890	0.3 Cu	0.05 mM Cu
				0.3 Fe	
				1.0 Zn	
Fe	250	115	50	0.7 Fe	0.05 mM Fe
				0.2 Zn	

[a] The amount of protein isolated has been normalized for 100 g of cells.
[b] pH 9.0, 25 °C.

units of phosphotriesterase activity was approximately 40 times greater than the unsupplemented medium, but the cell density was approximately the same. Other divalent metal ions (zinc, manganese, cadmium, nickel, iron, and copper) showed varying increases in enzyme activity relative to the unsupplemented medium.[2] The phosphotriesterase activities, determined after cell lysis and recorded as units of activity/g of wet cell paste, are summarized in Table I.

The phosphotriesterase from the *E. coli* expression system was purified to homogeneity in four steps with an overall yield of 65%. A summary of a typical purification from 160 g of cells, grown in the presence of 1 mM cobalt and isolated in metal-free buffer at pH 8.5, is shown in Table II. The purification protocol included protamine sulfate (2% w/v) precipi-

TABLE II
Purification of cobalt phosphotriesterase

Purification step[a]	Volume	Total protein	Total activity	Specific activity	Purification	Yield
	ml	*mg*	*10^6 units*	*units/mg*	*-fold*	*%*
Lysate	375	54,000	3.7	69	1.0	100
Protamine sulfate	445	11,600	3.1	270	3.9	84
Ammonium sulfate	46	3,000	2.8	950	14	76
Ultrogel	290	580	2.6	4,480	65	70
DEAE-Sephadex	346	298	2.4	8,020	122	65

[a] Starting from 160 g of bacterial cells.

TABLE III
Inactivation of phosphotriesterase by metal chelators

Metal phosphotriesterase	Second-order rate constant of inactivation		
	1,10-Phenanthroline[a]	HQSA[a]	EDTA[a]
	$M^{-1}\,s^{-1}$		
Zn	1.6	2.4	0.8×10^{-3}
Co	133	32	2.7×10^{-3}
Mn	4.2	2.5	1.0×10^{-3}
Cd	1.2	1.5	0.8×10^{-3}
Ni	4.0	3.4	0.4×10^{-3}

[a] pH 8.5, 25 °C.

tation to remove nucleic acids and fractionation with ammonium sulfate. This was followed by gel filtration chromatography using Ultrogel AcA 54 and anion exchange fractionation with DEAE-Sephadex A-25. Only a single band was observed using sodium dodecyl sulfate-gel electrophoresis. Nearly 300 mg of homogeneous phosphotriesterase was isolated from 160 g of wet cell paste. Similar results were obtained for the purification of the enzyme from cells grown in media supplemented with other divalent metal ions, and the results are summarized in Table I.

The metal content of the isolated enzymes was found to vary depending on whether the purification was conducted in a buffer system containing added divalent cations. The enzyme isolated from the unsupplemented medium and purified in metal-free buffer contained 1.1 zinc/protein, and no cobalt, magnesium, calcium, or nickel was detected by atomic absorption spectroscopy. However, a ratio of 1.4 zinc/protein was found for enzyme expressed in the presence of 1 mM zinc. When this enzyme was purified in buffer supplemented with 0.05 mM zinc a ratio of 1.8 zinc/protein was found. Similar results were obtained for the enzyme purified from cells grown in the presence of other divalent cations. An increase in the ratio of metal to protein was always observed when the buffer used in the purification was supplemented with added divalent metal ions. The cobalt-containing enzyme had the highest specific activity followed by cadmium, nickel, manganese, zinc, copper, and iron. The results are summarized in Table I.

The purified phosphotriesterase was found to be inactivated by metal chelators in a time- and concentration-dependent manner. The enzyme was inactivated by 1,10-phenanthroline, HQSA, and EDTA, although inactivation by EDTA required significantly higher concentrations and β-mercaptoethanol to completely inactivate the enzyme. The second-order rate constants for inactivation of the phosphotriesterase by these chelators are summarized in Table III. The metal chelators 1,10-phenanthroline and HQSA inactivated the zinc-, manganese-, cadmium-, and nickel-substituted enzymes with second-order rate constants that did not differ by more than 3-fold. The cadmium phosphotriesterase was inactivated by the two chelators, the slowest, followed by the zinc enzyme, while the cobalt enzyme was the most sensitive to these chelators.

[2] The concentrations of zinc and iron in the unsupplemented medium were 50 and 25 μM, respectively, as determined by atomic absorption spectroscopy.

The pseudo-first-order rate constants of inactivation by 1,10-phenanthroline and HQSA were found to increase at lower pH values (data not shown). The phosphotriesterase activity is fully protected against inactivation by these metal chelators when the substrate analogs diethyl *p*-fluorophenylphosphate and diethyl phenylphosphate were included in the incubation mixture. The pseudo-first-order rate constant for inactivation of phosphotriesterase with 1.0 mM 1,10-phenanthroline was reduced by a factor of 14 in the presence of 1.0 mM diethyl *p*-fluorophenylphosphate or diethyl phenylphosphate. These substrate analogs were found to be competitive inhibitors of the enzyme with K_i values of 0.02 and 0.04 mM, respectively.

Preparation of a stable apoenzyme was achieved by dialysis of the protein against 0.2 M EDTA in the presence of 0.2 M β-mercaptoethanol. The process required several days of incubation because of the insensitivity of the enzyme to chelation by EDTA. After dialysis the final enzyme preparations were devoid of metal ions as determined by atomic absorption spectroscopy. The apophosphotriesterase was stable for a minimum of 2 months when stored at 4 °C at low concentrations (~0.5 mg/ml) in 50 mM HEPES buffer, pH 8.5; and when frozen at −78 °C, the apoenzyme was stable for greater than 6 months. Full enzymatic activity was regained by the addition of zinc at less than 10 stoichiometric equivalents of metal to protein. Addition of higher levels of zinc (millimolar) had either an inhibitory effect or failed to achieve any significant reactivation. A titration curve for the reactivation of apoenzyme with cobalt, cadmium, and zinc is shown in Fig. 2 as a plot of the observed specific activity *versus* the ratio of the metal ion added per apoprotein. Other divalent metal cations were also successful in reactivating the apophosphotriesterase with maximal activity being attained between 1 and 2 metal/apoprotein. In nearly all the cases, the reactivated enzyme showed a higher specific activity than the protein prepared by growing the bacterial cells in metal-supplemented medium. These results are summarized in Table IV. Reactivation of apoenzyme at various pH values was found to be pH-dependent with an optimum pH being around 8, as illustrated in Fig. 3. However, after dialysis to remove excess metal, the metal content was generally greater at higher pH values as shown in Fig. 4. The high metal content at pH values above 9 does not correlate with specific activity. The kinetic pK_a values of the metal-substituted phosphotriesterase derivatives, determined from the pH-rate profiles, are presented in Table IV. The cadmium phosphotriesterase showed the highest pK_a (8.1), whereas the pK_a for the zinc enzyme was 5.8.

When the enzyme, isolated with 1.1 zinc/protein, was incubated for 34 h with varying concentrations of cobalt and zinc, an enhancement in activity was observed for the addition of cobalt, whereas depression of activity was observed for the addition of zinc, as shown in Fig. 5. After dialysis the cobalt added samples were determined to have 1.0 cobalt and 1.1

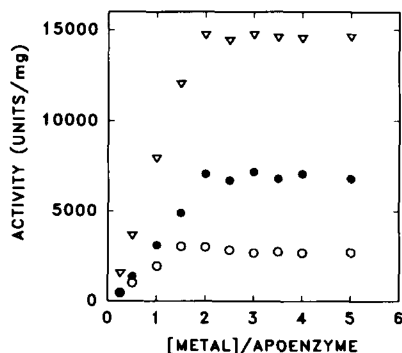

FIG. 2. **Reactivation of apophosphotriesterase with varying amounts of divalent cations.** The apoenzyme (20 μM) was incubated with the indicated amounts of Zn^{2+} (○), Cd^{2+} (●), and Co^{2+} (▽) in 50 mM HEPES buffer at pH 8.5. The specific enzyme activity was determined after 48 h of incubation at room temperature.

TABLE IV

Kinetic constants for isolated and reactivated phosphotriesterase

Metal phosphotriesterase	K_m	k_{cat}	k_{cat}/K_m	pK_a
		μM	s^{-1}	$10^7\ M^{-1}\ s^{-1}$
Zn				
Isolated[a]	45 ± 5	1,520 ± 40	3.4 ± 0.3	
Reconstituted[b]	91 ± 5	2,430 ± 40	2.7 ± 0.1	5.8 ± 0.1
Co				
Isolated	130 ± 10	4,870 ± 90	3.8 ± 0.2	
Reconstituted	195 ± 20	7,750 ± 270	4.0 ± 0.3	6.5 ± 0.1
Mn				
Isolated	65 ± 4	1,800 ± 40	2.8 ± 0.2	
Reconstituted	80 ± 6	1,750 ± 40	2.2 ± 0.1	7.0 ± 0.1
Cd				
Isolated	360 ± 50	2,460 ± 150	0.7 ± 0.1	
Reconstituted	400 ± 60	4,620 ± 270	1.2 ± 0.1	8.1 ± 0.1
Ni				
Isolated	97 ± 4	1,990 ± 30	2.1 ± 0.1	
Reconstituted	150 ± 10	5,970 ± 150	3.9 ± 0.2	7.4 ± 0.1

[a] The enzyme preparations used for these studies are described in Table I.

[b] The enzymes used for these studies were made by incubation of the apoenzyme with 2 equivalents of the indicated metal.

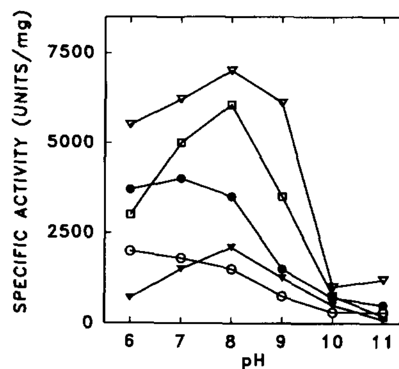

FIG. 3. **Reactivation of apophosphotriesterase at varying pH values.** The apoenzyme (20 μM) was incubated with 1 equivalent of Zn^{2+} (○), Mn^{2+} (▼), Cd^{2+} (●), Ni^{2+} (□), and Co^{2+} (▽), and then enzymatic activity was measured 24 h later at pH 9.0.

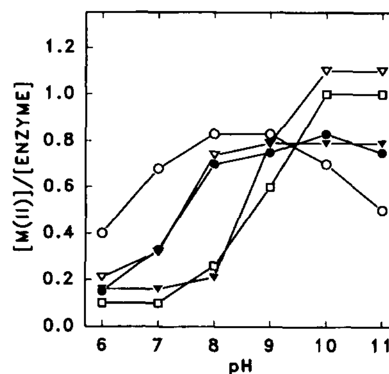

FIG. 4. **Binding of metal ions to apophosphotriesterase at varying pH values.** The apoenzyme (20 μM) was incubated with 1 equivalent of Zn^{2+} (○), Mn^{2+} (▼), Cd^{2+} (●), Ni^{2+} (□), and Co^{2+} (▽) at the indicated pH values for 24 h. The samples were dialyzed at the same pH to remove unbound ligands, and then the metal content was determined by atomic absorption spectroscopy.

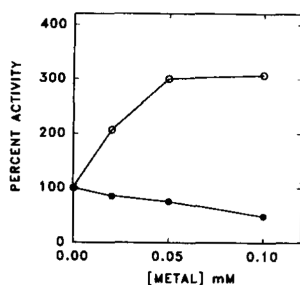

FIG. 5. **The effect of added metal ion on the enzymatic activity of phosphotriesterase containing 1.0 zinc/protein.** The activities upon the addition of Zn^{2+} (●), and Co^{2+} (○) are indicated relative to the initial activity. The enzyme (40 μM) was incubated with varying amounts of Zn^{2+} and Co^{2+} for 34 h at room temperature in 50 mM HEPES, pH 8.5. Additional details are given under "Experimental Procedures."

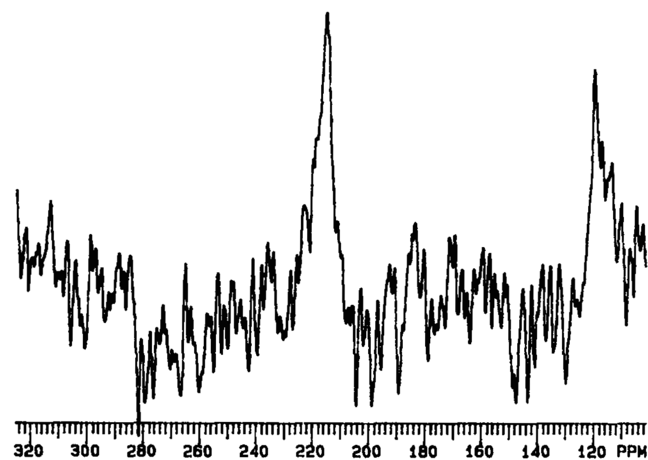

FIG. 6. **Cadmium-113 NMR spectrum of phosphotriesterase containing 2 equivalents of ^{113}Cd.** The two observed resonances are 215 and 120 ppm downfield from $Cd(ClO_4)_2$. The protein concentration was 0.7 mM, and 116,000 transients were collected at 5.4 °C and transformed with a line broadening of 100 Hz. Additional details are given under "Experimental Procedures."

zinc/protein. A similar enhancement of specific activity by the other metal enzyme preparations (determined to contain approximately 1 metal/protein as isolated) was also observed upon addition of cobalt, nickel, cadmium, and manganese but not zinc. The specific activity of an enzyme preparation containing ~1 manganese/protein could be stimulated more than 3-fold after the addition of 0.10–0.50 mM manganese in the presence of 0.2 M β-mercaptoethanol. The activity of the native zinc enzyme, purified in the presence of extra zinc ions, could be enhanced by the addition of substoichiometric amounts of 1,10-phenanthroline or HQSA. Attempts were made, however, to displace directly zinc from the zinc-saturated enzyme (1.8 zinc/protein) by other divalent metal ions. Only cadmium was found to displace zinc. However, zinc was found to displace all other metal ions from the protein except cadmium. One cadmium and one zinc were bound when the enzyme initially known to contain 1.8 cadmium/protein was incubated for 3 days with 2 equivalents of zinc. A similar result was obtained when the zinc enzyme was treated with cadmium. In every case the specific activity of the enzyme was always reduced whenever excess zinc was added.

The ^{113}Cd NMR spectrum of the labelled phosphotriesterase is shown in Fig. 6. Two ^{113}Cd resonances are observed at 215 and 120 ppm downfield from an external standard of $Cd(ClO_4)_2$.

DISCUSSION

Biosynthetic incorporation of various divalent metal ions into the zinc sites of proteins has been achieved for yeast alcohol dehydrogenase (Curdel and Iwatsubo, 1968) and *E. coli* RNA polymerase (Speckhard *et al.*, 1977). We have now successfully incorporated a variety of divalent metal ions into the bacterial phosphotriesterase without the necessity of removing traces of zinc from the metal-enriched growth medium and have found that bacterial cell growth was not significantly inhibited despite the presence of the added divalent metal ions. Furthermore, the phosphotriesterase was overexpressed in media enriched with manganese, cobalt, nickel, iron, cadmium, or copper. However, the addition of 1 mM zinc to the growth medium afforded only a nominal increase in the amount of phosphotriesterase isolated compared with the unsupplemented experiment. The significant increase (40-fold) in total enzymatic activity when the cells are grown in the presence of Co^{2+} is in part a result of the higher specific activity (units/mg) of the cobalt-enzyme relative to the zinc-enzyme. However, it is readily apparent that more protein is expressed as well and thus the concentration of divalent metal ions in the growth medium may be regulating the overall biosynthesis of the phosphotriesterase. This increase in net protein production may be a result of either a specific increase in the rate of translation or a corresponding decrease in the rate of protein degradation.

The metal analysis and reactivation data presented in this paper indicate the presence of two divalent metal binding sites per monomer. It appears likely that at least one metal ion is incorporated with the protein during cell growth, and the second site is populated or lost during isolation of the enzyme. A large excess of zinc reversibly inhibits the enzymatic activity of the purified phosphotriesterase, suggesting the presence of an additional nonspecific binding site whose occupancy by zinc is characterized by inhibition. There are no inhibitory effects with excess cobalt, cadmium, manganese, or nickel. However, the data do not rule out the possibility of zinc binding to catalytic residues at the active site as observed by Larsen and Auld (1988) for carboxypeptidase A.

Zinc is tightly bound to the enzyme as evidenced by the insensitivity of the enzymatic activity to chelation by EDTA. Except for the cobalt-substituted phosphotriesterase, the various metal enzymes are equally sensitive to the more potent metal chelators 1,10-phenanthroline and HQSA. Further evidence for zinc being tightly bound to the protein is the inability of other divalent metal ions to displace zinc from the protein directly. Only a single zinc ion can be directly displaced by cadmium, whereas zinc displaces all other divalent metal ions except for a single cadmium ion. These same data also suggest that cadmium is tightly bound to one site of the enzyme.

The kinetic constants of the purified phosphotriesterase and the enzyme prepared by reactivating apophosphotriesterase with various metal ions differ significantly for some of the divalent metal ions utilized in this study. This phenomenon is primarily a result of the presence of contaminating levels of other less active metal ions in the purified enzyme preparations. The purified nickel phosphotriesterase had the highest iron contamination, causing the observed enzymatic activity to be approximately one-third of the value observed for the reactivated enzyme. The isolated iron enzyme had a specific acitivty of 50 units/mg, comparable with the level of activity obtained by reactivation of apoenzyme with stoichiometric amounts of iron.

Reactivation of apophosphotriesterase with stoichiometric amounts of divalent cations is pH-dependent with all of the

metal ions tested and is optimum at pH 8.0. The diminished affinity of the protein for the divalent metal ions at low pH is not surprising since zinc and other metals are known to dissociate from proteins upon protonation of metal liganding groups. This property has been used to prepare apocarboxypeptidase (Vallee and Neurath, 1954). The metal ion incorporation into the apoenzyme is slow and concentration-dependent. The noninstantaneous reactivation of apoenzyme by stoichiometric amounts of the metal ions is consistent with gross protein conformational changes when the metal ion is removed to form apoenzyme, and therefore a complete reorganization of ligands in the apoenzyme to form metal-ligand bonds may be required to regain fully active enzyme.

The data presented in this paper suggest that at least one metal ion is located at or near the active site.[3] This conclusion is consistent with the protection against inactivation of enzyme functions with metal chelators by binding of substrate analogs to the active site. The pH-rate profile for V_{max} of the phosphotriesterase using paraoxon as a substrate indicates that a single enzymatic group must be unprotonated for activity (Dumas and Raushel, 1990). With the zinc-enzyme it has been demonstrated that this group is most likely a histidine residue that acts as a general base in the abstraction of a proton from an activated water molecule (Dumas and Raushel, 1990). The results obtained in this paper now demonstrate that this kinetic pK_a is variable depending upon the exact metal composition. The observed values are lowest with zinc (5.8) and highest with cadmium (8.1). Therefore the metal ion is modulating the pK_a of the active site histidine. This most likely involves a water molecule coordinated to the active site metal center. The role of the metal ion that binds to the second site is unknown. It may be involved in the maintenance of protein structure, or perhaps it also actively participates in the catalytic process via charge neutralization. However, the enzymes isolated with either 1.0 or 1.7 cobalt/enzyme have virtually the same specific activity, and thus it appears that occupancy at the second metal binding site is not critical for activity.

The environment of the metal center was probed by preliminary measurement of the chemical shifts for the bound cadmium ions. The ^{113}Cd NMR spectrum clearly shows resonances for two separate cadmium environments, consistent with the binding studies using atomic absorption spectroscopy. The range of observed chemical shifts (120 and 215 ppm) are consistent only with nitrogen and perhaps oxygen as the ligands coordinated to the metal centers (Coleman and Gettins, 1986). Coordination to a cysteine is excluded since complexation to a sulfur would shift the observed resonances to greater than 300 ppm (Coleman and Gettins, 1986). The precise ligands to both metal centers are now being probed by site-directed mutagenesis of all 7 histidine residues.

[3] Preliminary NMR experiments using manganese as a paramagnetic probe indicate that the metal center is < 5.5 Å from the phosphorus atom of a bound inhibitor.

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