Metal−Substrate Interactions Facilitate the Catalytic Activity of the Bacterial Phosphotriesterase†

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ABSTRACT: The bacterial phosphotriesterase from *Pseudomonas diminuta* is a zinc metalloenzyme which catalyzes the hydrolysis of a variety of organophosphorus nerve agents with high efficiency. The active site of the enzyme consists of a coupled binuclear metal center embedded within a cluster of histidine residues. Potential protein−substrate interactions at the active site were probed by a systematic variation of metal identity, leaving group potential, phosphate host, and amino acid replacement. In order to determine the roles of these metal ions in binding and catalysis, the microscopic rate constants and kinetic parameters were obtained with various divalent cations. The divalent cations that were utilized in this investigation consisted of Co^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Mn^{2+}, and the mixed-metal Zn^{2+}/Cd^{2+} hybrid. The leaving group potential and phosphate host were varied by altering the pK_a of the departing substituted phenol or thiophenol in either a diethyl phosphate or a diethyl thiophosphate substrate. The Brϕnsted plots for the nonenzymatic hydroxide catalyzed hydrolysis of these substrates showed a linear dependence between the pseudo-first-order rate constant and the pK_a of the leaving group. Enzymatic activities of the wild-type enzyme with these same substrates varied by over 7 orders of magnitude over the entire experimental pK_a range (4.1−10.3), and the corresponding Brϕnsted plots were nonlinear. Those substrates with leaving groups with high pK_a values were limited by the rate of bond cleavage while those substrates having leaving groups with low pK_a values were limited by a conformational change or binding event. Thiophosphate substrates having leaving groups with high pK_a values were better substrates than the corresponding phosphate analogues. These results are consistent with the direct coordination of one or both metal ions with the phosphoryl sulfur or oxygen atom of the substrate. A large dependence of the rate on the leaving group rules out the possibility of protonation of the leaving group or electrostatic interaction of the leaving group oxygen (or sulfur) with a metal ion or cationic group at the active site. The large differences in the size of the β_{lg} over the range of metal ions utilized by the enzyme indicate that the metal ions polarize the phosphoryl group and alter the structure of the transition state. The values of V/K_m for the enzyme-catalyzed hydrolysis for a series of substituted thiophenol analogues were 10^2−10^3-fold smaller than those obtained for the hydrolysis of the corresponding phenolic substrates, suggesting that the bulkier sulfur substituent in the leaving group may induce conformational restrictions at the active site. With the zinc-substituted H201N mutant enzyme, there was a large decrease in the rate of phosphotriester hydrolysis but essentially no change in the rate of thiophosphotriester hydrolysis relative to the values observed for the zinc-substituted wild-type enzyme. These results suggest that a direct perturbation in the ligand structure of the binuclear metal center induces alterations in the mechanism of substrate hydrolysis.

The zinc metalloenzyme phosphotriesterase from *Pseudomonas diminuta* is a highly efficient catalyst for the hydrolysis of an extensive variety of organophosphorus nerve agents (Dumas *et al*., 1989; Donarski *et al*., 1989). Two divalent metal ions are critical for maximal catalytic activity of the enzyme (Dumas *et al*., 1989; Omburo *et al*., 1992). The two native Zn^{2+} ions can be substituted with either Co^{2+}, Ni^{2+}, Cd^{2+}, or Mn^{2+} with the restoration of full catalytic activity. The structure and mechanism of phosphotriesterase have been previously investigated using kinetic (Donarski *et al*., 1989; Caldwell *et al*., 1991a,b), spectroscopic (Lewis *et al*., 1988; Omburo *et al*., 1993; Chae *et al*., 1993), genetic (Kuo & Raushel, 1994), and X-ray diffraction methods (Benning *et al*., 1994, 1995; Vanhooke *et al*., 1996) in addition to chemical modification and inactivation (Dumas & Raushel, 1990; Banzon *et al*., 1995a,b). From these studies, the hydrolytic reaction catalyzed by phosphotriesterase has been postulated to proceed via an S_N2-like associative mechanism with activation of a water molecule by the metal center, followed by the direct hydrolysis of the substrate resulting in an inversion of configuration at the phosphorus center and dissociation of products. However, previous studies have demonstrated that the electronic changes in the leaving group of the substrates significantly affects the catalytic reactivity of the enzyme (Donarski *et al*., 1989; Caldwell *et al*., 1991a). Brϕnsted plots have indicated that the rate-determining step changes from hydrolysis of the P−O bond to a conformational change and/or binding events as the pK_a value of the leaving group is altered. The active site of this enzyme has been

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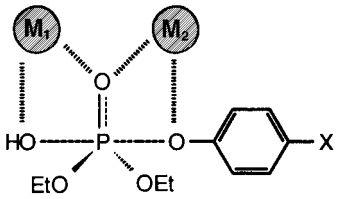
Scheme 1



shown by X-ray crystallography to consist of a coupled binuclear metal center embedded within a cluster of histidine residues (Benning *et al*., 1995; Vanhooke *et al*., 1996). The precise roles for each of the two metal ions in catalysis remain unknown although these metals are believed to catalyze the hydrolysis of organophosphates by decreasing the pK_a of the activated water molecule and polarizing the phosphoryl oxygen bond during turnover of the enzyme.

The substitution of sulfur for oxygen in organophosphorus compounds has been used in a variety of nonenzymatic and enzymatic reactions to demonstrate the coordination of metal to sulfur and oxygen. There are several reports of studies on rate effects upon substitution of sulfur for oxygen counterparts of phosphotriesters (Ketelaar *et al*., 1952; Fukuto & Metcalf, 1956; Heath, 1956a,b; Cox & Ramsay, 1964; Murdock & Hopkins, 1968; Fanni *et al*., 1986; Farschtschi *et al*., 1990). These investigations have demonstrated that for the simple chemical hydrolysis of thiophosphates, the substitution of a sulfur atom for the nonbridging phosphoryl oxygen results in a slower rate of hydrolysis. However, the phosphorothiolates, with sulfur substitution for a bridging oxygen atom, hydrolyze much more rapidly than the corresponding phosphate analogues. In rationalizing the relative reactivity of sulfur vs oxygen analogues, polarizability, electronegativity, and lability of the bridging bond have been put forth as major factors for these observations (Cox & Ramsay, 1964; Frey & Sammons, 1985).

Recent investigations have shown that the two metal atoms bound to phosphotriesterase are in close proximity to one another and the water molecule that attacks the phosphoryl center is bound directly to the binuclear metal center (Chae *et al*., 1993; Benning *et al*., 1995; Vanhooke *et al*., 1996). This report presents a detailed investigation of the potential protein−substrate interactions between the binuclear metal center and the available polarizable sites of the substrate as shown in Scheme 1. These potential interactions at the active site have now been addressed by a systematic variation of metal identity, leaving group potential, and amino acid replacement. In order to evaluate the roles of the binuclear metal center, the microscopic rate constants and other kinetic parameters for binding, hydrolysis, and product dissociation were obtained with various metal-substituted enzyme derivatives. In addition, the effect of substrate structure on catalytic activity was analyzed with a systematic variation of both the leaving group and phosphate host by altering the pK_a of the departing phenol or thiophenol within either a diethyl phosphate or a diethyl thiophosphate substrate. The very sluggish mutant enzyme (H201N), substituted with Zn^{2+} or Co^{2+}, was utilized with the same series of phosphate and thiophosphate analogues to examine in detail the effects on catalysis by altering one of the direct ligands to the binuclear metal center.

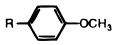
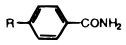
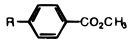
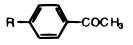
MATERIALS AND METHODS

Materials. Paraoxon (*O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphate) was purchased from Sigma, and parathion (*O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate) was obtained from Chem Service. 2- and 3-fluoro-4-nitrophenols and 2,6-difluorophenols were from Lancaster. Unless noted otherwise, the other chemicals for the syntheses of phosphotriester analogues were obtained from Aldrich. All solvents used for the syntheses were purified and dried according to published procedures (Perrin & Armarego, 1988). The synthesis of 4-cyanothiophenol was accomplished by the conversion of 4-cyanophenol according to the method of Sheley (1978). 4-Mercaptobenzamide was made from 4-cyanothiophenol by slightly modifying the procedure of Hall and Gisler (1976). A series of paraoxon and parathion analogues and substituted thiophenol derivatives were prepared as previously described (Donarski *et al*., 1989; Caldwell *et al*., 1991a), and further purified by flash column chromatography. All structures of the synthesized triesters were verified by ^{1}H NMR and ^{13}C NMR spectroscopy; mass spectral data were also consistent with the expected molecular weights of all compounds. All buffers were purchased from Sigma except for *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES),[1] which was obtained from United States Biochemical. The cell growth, purification, and reconstitution of the wild-type phosphotriesterase with Co^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Mn^{2+}, and the mixed-metal Zn^{2+}/Cd^{2+} hybrid, in addition to the mutant enzyme, H201N, with Co^{2+} and Zn^{2+}, were performed as previously described (Omburo *et al*., 1992; Kuo & Raushel, 1994).

Kinetic Measurements. The pK_a values and extinction coefficients of the leaving group phenols and thiophenols of the substrates were obtained from the literature (Donarski *et al*., 1989; Caldwell *et al*., 1991a) or determined spectrophotometrically as described previously (Caldwell *et al*., 1991a) using a Varian Cary 2200 spectrophotometer and are presented in Tables 1 and 2. The catalytic activities of metal-substituted wild-type and mutant enzymes were assayed by routinely measuring the release of 4-nitrophenol ($\epsilon = 1.7 \times 10^4$ M^{-1} cm^{-1}) spectrophotometrically at 400 nm in 100 mM CHES buffer, pH 9.0, with 1.0 mM paraoxon as a substrate. Reaction rates were determined spectrophotometrically at 25 °C with a Gilford Model 260 spectrophotometer. Rates of chemical hydrolysis were determined at 25 °C by spectrophotometrically following the release of the phenol or thiophenol product over 7 half-lives at an appropriate wavelength upon addition of potassium hydroxide to a final concentration of 1.0 N. For the enzyme-catalyzed hydrolysis, the cuvettes containing paraoxon analogues (20−1000 μM), parathion analogues (20−200 μM), or phosphorothiolates (100−3300 μM) in 100 mM CHES, pH 9.0 or 10.0, were preincubated at 25 °C for 10 min. Owing to the limited solubility in water, all thiophosphates or phosphorothiolates were tested as substrates in 15 or 20% methanol/water mixtures, respectively (Donarski *et al*., 1989). Various concentrations of the enzyme were added to initiate the reaction. The kinetic parameters, V_{max} and K_m, were determined spectrophotometrically by measuring the initial rate of product formation at an appropriate wavelength.

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

Table 1: Physical Constants of Diethyl-Substituted Phenyl Phosphates at pH 9 or 10

	compound[a]	pK_a	λ (nm)	Δ(OD/mM)
I		10.29	295	2.08[b]
II		10.00	280	1.13[b]
III		9.91	282	1.82[b]
IV		9.38[c]	296.5	1.91[d]
V		9.28[c]	282.5	1.87[d]
VI		8.56[e]	293	11.3[f]
VII		8.47[c]	295	18.0[f]
VIII		8.05[c]	322.5	20.6[f]
IX		7.95[c]	276	19.4[f]
X		7.66[c]	329	20.5[f]
XI		7.14[c]	400	17.0[f]
XII		5.94[e]	387	17.5[f]
XIII		5.45[e]	398	16.7[f]
XIV		4.10[e]	395	15.2[f]

[a] R = EtOP(O)(Et*O*)*O*- (XV) or EtOP(S)(Et*O*)*O*- (XVI). [b] Values determined at pH 10 (this work). [c] Values taken from Donarski *et al.* (1989). [d] Values determined at pH 10 (Donarski *et al*., 1989). [e] Values taken from Caldwell *et al*. (1991a). [f] Values determined at pH 9 (Caldwell *et al*., 1991a).

Data Analysis. All kinetic data were analyzed by fitting to the appropriate equations with the computer programs supplied by Savanna Shell Software. The values of V_{max} and K_m were determined from a fit of the data to the eq 1

$$v = V_{max}A/(K_m + A) \quad (1)$$

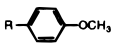
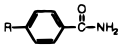
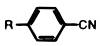
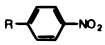
where v is the initial velocity, V_{max} is the maximum velocity, K_m is the Michaelis constant, and A is the substrate concentration. In analyzing the Brϕnsted plots, a simplified kinetic scheme was used for the enzymatic hydrolysis of phosphotriesters, and is presented in Scheme 2, where k_1 and k_2 are the rate constants for the formation of the Michaelis complex, k_3 is the rate constant for the chemical transformation, and k_5 is the rate constant for product dissociation. The

Scheme 2

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \xrightarrow{k_3} EPQ \xrightarrow{k_5} E + \text{products}$$

expressions for the kinetic constants, V_{max} and V/K_m, can be described by eqs 2 and 3, where the microscopic rate constant, k_3, is dependent on β and the pK_a of the leaving group as described in eq 4 (Jencks & Carriuolo, 1961). The pseudo-first-order rate constants obtained for the chemical hydrolysis of phosphotriesters, thiophosphotriesters, or phosphorothiolates by 1.0 N KOH were obtained by a fit of the data to eq 5 where A is the concentration of the substrate, k is the pseudo-first-order rate constant, and t is time.

Table 2: Physical Constants of Diethyl *S*-*p*-Substituted Phenyl Phosphorothiolates at pH 9

	compound[a]	pK_a[b]	λ (nm)	Δ(OD/mM)[b]
I		6.95	261	14.4
II		6.68	263.5	13.3
III		6.54	259	12.3
IV		6.11	271	11.4
V		5.42	314	17.8
VI		4.86	314	18.9
VII		4.42	414	17.3

[a] R = EtOP(O)(Et*O*)*S*- (XVII). [b] Values determined in 10% MeOH.

$$V_{max} = k_3k_5/(k_3 + k_5) \quad (2)$$

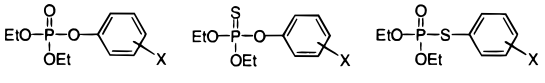
$$V/K_m = k_1k_3/(k_2 + k_3) \quad (3)$$

$$\log k_3 = (\beta pK_a) + C \quad (4)$$

$$A = A_o e^{-kt} \quad (5)$$

RESULTS

Chemical Hydrolysis of Phosphotriester Analogues. The Brϕnsted plots for the hydrolysis of paraoxon analogues (XV), parathion analogues (XVI), and *S*-aryl phosphorothiolates (XVII) with 1.0 N KOH show a linear dependence



XV XVI XVII

between the pseudo-first-order rate constant and the pK_a of the leaving group over the whole experimental range of pK_a values as illustrated in Figure 1. It has been determined that the chemical hydrolysis of phosphotriesters is approximately an order of magnitude faster than that of thiophosphotriesters. The observed values of β_{lg} for the chemical hydrolysis of thiophenol and phenol derivatives of phosphotriesters are comparable over the same pK_a range. The slopes of the lines in Figure 1 give values of $\beta_{lg} = -0.43$, -0.35, and -0.42 for the hydrolysis of paraoxon analogues, parathion analogues, and the *S*-aryl phosphorothiolates, respectively.

Hydrolysis Catalyzed by Zinc-Substituted Wild-Type Enzyme. The values of V_{max} and K_m were measured for the enzymatic hydrolysis of 13 paraoxon analogues, 13 parathion analogues, and 7 diethyl *S*-aryl phosphorothiolates catalyzed by the zinc-substituted wild-type enzyme at 25 °C and pH 9.0 or 10.0 (Table 3). The Brϕnsted plots of log V_{max} vs the pK_a of the leaving group phenol in a diethyl phosphate or diethyl thiophosphate substrate show nonlinear correlations, as illustrated in Figure 2A. The dependence of log (V/K_m)

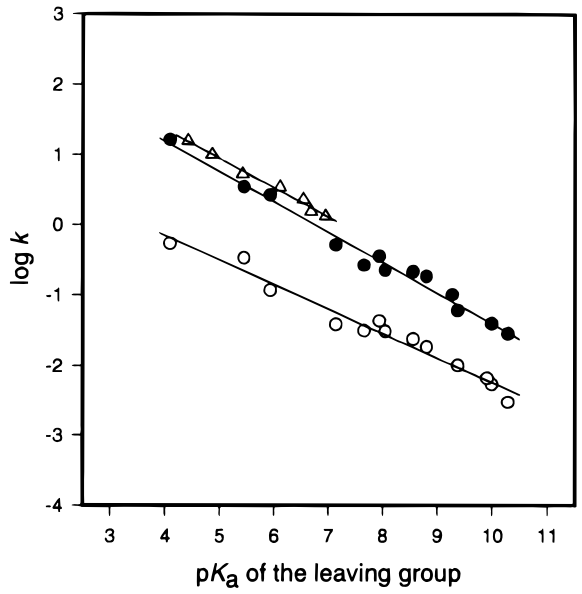


FIGURE 1: Brϕnsted plots of log k versus the pK_a of the leaving group for the hydroxide-catalyzed hydrolysis of a series of phosphates (●), thiophosphates (○), and phosphorothiolates (△). The respective β_{lg} values are −0.43, −0.35, and −0.42.

on the pK_a of the leaving group also follows a similar nonlinear Brϕnsted-type relationship (figure not shown). The linear segments of each Brϕnsted plot intersect at a pK_a of approximately 8.0 for the hydrolysis of paraoxon analogues and 8.5 for the hydrolysis of the parathion analogues with this enzyme. Those substrates with leaving groups in the lower range of pK_a values are hydrolyzed at the same rate while those substrates in the higher range of pK_a values show an enhanced sensitivity to the specific substituent on the leaving group. The Brϕnsted-type relationships shown in Figure 2A give large negative values of β_{lg} of −2.2, −2.0, and −1.0 for the reactions of zinc-substituted wild-type enzyme with phosphates, thiophosphates, and phosphorothiolates, respectively, and the data are presented in Table 3.

Hydrolysis Catalyzed by Cadmium-Substituted Wild-Type Enzyme. The plots of log V_{max} versus the pK_a of the departing group phenol or thiophenol for reactions of the cadmium-substituted wild-type enzyme with substrates display nonlinear Brϕnsted correlations (Figure 2B). Nonlinear Brϕnsted plots of log (V/K_m) vs the pK_a of the leaving group were also observed (figure not shown). The crossover pK_a values are 8.1 and 8.5 for the phosphate and thiophosphate derivatives, respectively. The small dependence on log V_{max} for the catalytic hydrolysis of thiophosphates by the cadmium-substituted wild-type enzyme with increasing pK_a of the leaving group ($\beta_{lg} = -1.4$) significantly differs from the large dependence on the pK_a of the leaving group for reactions of phosphates with the same enzyme, where the value for $\beta_{lg} = -3.0$. The Brϕnsted slope for the enzymatic hydrolysis of the phosphorothiolate substrates gives a value of $\beta_{lg} = -1.5$. The data are presented in Table 4.

Hydrolysis Catalyzed by Manganese-Substituted Wild-Type Enzyme. The nonlinear Brϕnsted plots of log V_{max} vs the pK_a of the leaving group for catalysis of the hydrolysis of phosphates and thiophosphates by the manganese-substituted wild-type enzyme are shown in Figure 2C. The Brϕnsted plots of log (V/K_m) versus the pK_a of the leaving group for reaction of the manganese-substituted wild-type enzyme with phosphates and thiophosphates have nonlinear correlations (figure not shown). The crossover points from the Brϕnsted plots are found at pK_a values of 8.2 and 9.1 for phosphates and thiophosphates, respectively. The thiophosphate series gives a large negative value of $\beta_{lg} = -4.3$ compare to a value of $\beta_{lg} = -3.2$ for the paraoxon analogues. Identical experiments were also conducted with the nickel- and cobalt-substituted phosphotriesterase for the series of paraoxon and parathion analogues, and the zinc/cadmium hybrid enzyme was tested in the same manner (figures not shown).

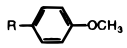
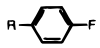
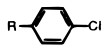
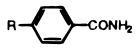
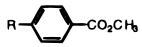
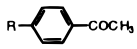
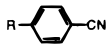
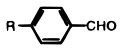
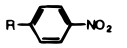
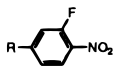
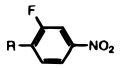
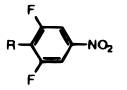
Hydrolysis Catalyzed by Mutant H201N Enzyme. The reaction of substrates with the mutant enzyme (H201N) reconstituted with either Co^{2+} or Zn^{2+} also shows nonlinear Brϕnsted-type relationships of log V_{max} versus the pK_a of the leaving group as illustrated in Figure 2D. The values of V_{max} and V/K_m for the hydrolysis of the series of phosphotriesters by the zinc-substituted mutant are smaller than those measured for the reaction catalyzed by the wild-type enzyme with these same substrates. However, the rate constants for the hydrolysis of the thiophosphate triesters are nearly the same for the zinc-substituted wild-type protein and the H201N mutant.

Calculation of Rate Constants for the Kinetic Model. The nonlinear Brϕnsted plots for V_{max} and V/K_m with all eight metal derivatives of phosphotriesterase were fit to the kinetic model that appears in Scheme 2. The microscopic rate constants for the binding (k_1) and dissociation (k_2) of the substrate to the Michaelis complex in addition to the rate constants for the dissociation of the enzyme−product complex (k_5) and the β_{lg} values are listed in Table 5.

DISCUSSION

The Brϕnsted analysis for the phosphotriesterase-catalyzed hydrolysis of paraoxon analogues exhibited a biphasic dependence on the values for V_{max} and V/K_m with the pK_a of the leaving group (Caldwell *et al*., 1991a). The independence on the enzymatic rate of hydrolysis for those substrates with leaving groups having pK_a values of <8 was in significant contrast to the rather large dependence ($\beta_{lg} = -1.8$) on the kinetic constants found with those analogues with leaving groups having pK_a values >8. Caldwell *et al*. (1991a) demonstrated that the actual bond cleavage step (k_3) limited the overall rate for the enzymatic hydrolysis when the pK_a of the leaving group was >8, whereas the rate-limiting step was an associated conformational change or diffusion-controlled dissociation when the pK_a value of the leaving group was <8. These results indicated that the alteration in the effective charge on the leaving group oxygen resulted in a change in the rate-limiting step. In contrast, the Brϕnsted-type correlation for log k_{hyd} with the pK_a of the leaving group for the hydroxide catalyzed hydrolysis of these same substrates showed a strictly linear relationship with $\beta_{lg} = -0.44$ throughout the whole pK_a range. The present investigation has now included additional substrates with leaving groups that further extend the original pK_a range from a high of 8.5 to 10.3. Corresponding Brϕnsted plots for the hydrolysis of these substrates by the phosphotriesterase reconstituted with either Zn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, Mn^{2+}, or the mixed-metal Zn^{2+}/Cd^{2+} hybrid were obtained and the microscopic rate constants calculated. Moreover, two additional sets of substrate analogues were synthesized by substitution of a sulfur for an oxygen atom in either the nonbridging or the bridging phosphoryl positions of the

Table 3: Kinetic Constants for the Zn/Zn-Phosphotriesterase

	compound[a]	V_{max} (s^{-1})			K_m (mM)		
		XV	XVI	XVII	XV	XVI	XVII
I		0.012	0.33	3.3	0.31	0.53	20
II		0.018	0.61	18	0.22	0.50	40
III		nd	0.82	36	nd	0.34	74
IV		1.2	1.9	89	0.13	0.17	23
V		7.4	nd	nd	0.33	nd	nd
VI		100	680	300	0.065	0.17	40
VII		120	380	nd	0.031	0.15	nd
VIII		420	730	nd	0.030	0.18	nd
IX		680	600	1800	0.042	0.16	47
X		1500	500	nd	0.044	0.24	nd
XI		2200	450	240	0.045	0.13	29
XII		2600	430	nd	0.049	0.17	nd
XIII		2900	600	nd	0.063	0.08	nd
XIV		3100	460	nd	0.075	0.10	nd

[a] R = EtOP(O)(Et*O*)*O*- (XV), EtOP(S)(Et*O*)*O*- (XVI), or EtOP(O)(Et*O*)*S*- (XVII).

paraoxon analogues. This was done to probe for specific metal−substrate interactions in the transition state with the wild-type and H201N mutant enzymes substituted with various divalent metal ions.

Transition-State Structure for Chemical Hydrolysis of Phosphotriester Analogues. The linear free energy relationships for the chemical hydrolysis of phosphate, thiophosphate, and phosphorothiolate triesters are presented in Figure 1. The slopes from these Brϕnsted plots reflect the charge associated with the leaving group in the transition state structure and are directly related to the degree of bond formation and cleavage (Jencks & Gilchrist, 1968). The results obtained here are therefore consistent with very similar transition-state structures for each of the three series of triester analogues. However, the value of β_{lg} obtained for the thiophosphate triesters is less negative than those obtained for the series of phosphate and phosphorothiolate analogues. This difference indicates that the extent of phenolic oxygen bond cleavage with the thiophosphate triesters in the transition state is less developed than the extent of bond cleavage in the other two series of substrates. Moreover, when sulfur is substituted for the phosphoryl oxygen (P═O), the corresponding triesters are hydrolyzed by KOH about an order of magnitude more slowly. This result is consistent with the reduced electronegativity for sulfur (relative to oxygen) that diminishes the electrophilic character of the phosphorus center and thus the rate of hydrolysis for the thiophosphates decreases accordingly. Substitution of sulfur for the bridging oxygen has the opposite effect since the rate of hydrolysis for the phosphorothiolate series is approximately an order of magnitude faster than that observed for the phosphate triesters. This increase in reactivity, however, can be directly attributed to the increase in the acidity of the thiophenol leaving group relative to the parent phenol. When the reactivities are compared based on the pK_a of the leaving group, the rate constants for hydrolysis are essentially the same.

Potential Roles of Metal Ions in Binding and Catalysis. Phosphotriesterase requires two divalent metal ions for enzymatic activity. Model studies show that metal ions can effectively contribute to an increase in the rate of hydrolysis of phosphate triesters (Ketelaar *et al*., 1956; Mortland & Raman, 1967; Gellman *et al*., 1986; Menger *et al*., 1987; Breslow & Singh, 1988; Morrow & Trogler, 1989; Sharma *et al*., 1990; Hay & Govan, 1990; Vitarius & Sultatos, 1995). Possible roles of metal ions in these studies include the following: (1) The metal ions may decrease the pK_a of the bound water molecule and increase the nucleophilic character of the attacking hydroxide. (2) The metal ions can increase the polarization of the P═O (or P═S) bond, and thereby

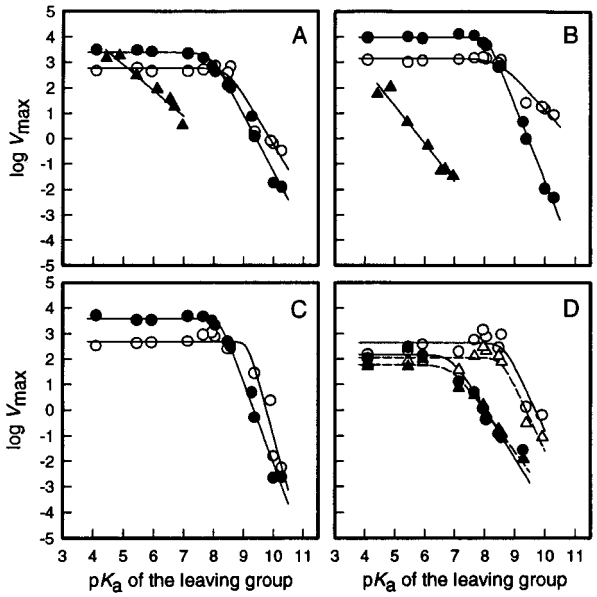


FIGURE 2: Brϕnsted plots for the dependence of log V_{max} on the pK_a of the leaving group. (A−C) Brϕnsted plots for the hydrolysis of a series of phosphates (●), thiophosphates (○), and phosphorothiolates (▲) catalyzed by the Zn^{2+}-substituted phosphotriesterase (A), Cd^{2+}-substituted phosphotriesterase (B), and Mn^{2+}-substituted phosphotriesterase (C). (D) Brϕnsted plots of log V_{max} for the hydrolysis of a series of phosphates (●), and thiophosphates (○) by the Zn^{2+}-substituted H201N mutant enzyme (the solid lines), and a series of phosphates (▲), and thiophosphates (△) by the Co^{2+}-substituted H201N mutant enzyme (the dashed lines) versus the pK_a of the leaving group.

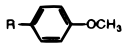
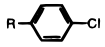
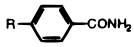
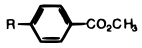
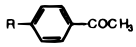
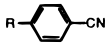
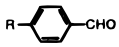
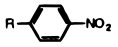
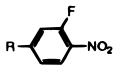
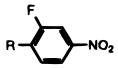
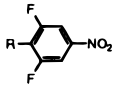
accelerate the approach of an attacking hydroxyl ion by increasing the electrophilic character of the phosphorus center. (3) The metal ions may neutralize the development of negative charge on the leaving group. All three contributions may operate in the functioning of phosphotriesterase. The pH−rate profiles for the wild-type phosphotriesterase reconstituted with Zn^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, and Cd^{2+} all indicate that a single ionizable group from the enzyme must be unprotonated for enzymatic activity. However, the kinetic pK_a value is the lowest (pK_a = 5.8) with the zinc-substituted enzyme and the highest (pK_a = 8.1) with the cadmium-substituted enzyme (Omburo *et al*., 1992). These results are consistent with the direct ligation of the hydrolytic water molecule (or hydroxide) with one or both of the metal ions in the active site.[2] The X-ray crystal structures of the Cd^{2+}- and Zn^{2+}-substituted enzymes show a solvent water molecule (or hydroxide) bridging the two metal ions (Benning *et al*., 1995; Vanhooke *et al*., 1996). An obvious general base has not been identified. To date, there has been no evidence to support the charge neutralization of the leaving group during the hydrolysis of phosphotriester substrates by the enzyme since there is no loss of activity at high pH (potential proton donor) and the β values for the hydrolysis of triesters with various leaving groups are so large (>∼2). Therefore, the focus of this investigation is directed at whether one or both metal ions in the binuclear metal center of phosphotriesterase are directly involved in the polarization of the P═O or P═S bond during catalysis.

Structure−Reactivity Relationships in Enzymatic Hydrolysis of Substrates by Wild-Type Phosphotriesterase. Brϕnsted plots for the hydrolysis of substrates by the Zn^{2+}/Zn^{2+}-phosphotriesterase in Figure 2A indicate that the rate-limiting step changes from hydrolysis of the P−O bond to a conformational change or binding event as the pK_a value of the leaving group for the substituted phosphates and thiophosphates is lowered. The β_{lg} for V_{max} is −2.2 for the series of paraoxon analogues with pK_a values >7.9 and −2.0 for the series of thiophosphate analogues with pK_a values >8.5. The large negative β_{lg} for the enzymatic reactions strongly suggests that the chemical step is rate-limiting for phosphate and thiophosphate analogues with elevated pK_a values. The rather large dependence on the rate with the leaving group ability of the product would appear to exclude the possibility of a direct electrostatic interaction with the binuclear metal center or proton donor at the active site. In addition, these high β_{lg} values strongly suggest that the transition state of the enzymatic reaction with substrates having poor leaving groups is significantly dissociative and quite product-like, implying a very late transition state. Figure 2A also shows that the thiophosphate analogues are enzymatically hydrolyzed significantly faster than the corresponding phosphate analogues as the pK_a of the leaving group becomes >∼8. The hydroxide-catalyzed reactions show the opposite trend. This observation supports the proposal that one or both of the metal ions in the binuclear metal center directly coordinates the phosphoryl group by polarization of the P═O (or P═S) bond. It has been previously demonstrated that the Cu^{2+}-catalyzed hydrolysis of thiophosphotriesters is much more effective than with the corresponding oxygen analogues. For example, the hydrolyses of EPN [*O*-ethyl-*O*-(*p*-nitrophenyl)phenyl phosphonothioate] and parathion proceed 48 and 20 times faster in the presence of the Cu^{2+} ions, respectively, whereas the rate enhancement with paraoxon under similar conditions is only 2-fold (Ketelaar *et al*., 1956). This observation can be explained by the direct coordination of the metal ions with the sulfur or oxygen atom. This potential interaction is apparently much more effective with sulfur than with oxygen, and thus the formation of this complex greatly increases the electrophilicity of the phosphorus center.

Further support for direct chelation of the phosphoryl group by metal ions at the active site is apparent in Figure 2B. The Brϕnsted slope of −1.4 for the hydrolysis of thiophosphates by the Cd^{2+}-substituted wild-type enzyme is in sharp contrast to the value of −3.0 observed for the corresponding phosphate analogues. This suggests that the transition state for the reaction of thiophosphates with the Cd^{2+}-substituted enzyme has more associative character than that for the reaction with the phosphate analogues. This rather large difference in the size of the β_{lg} probably arises from the formation of a stronger chelate with the soft sulfur ligand by the soft Cd^{2+} within the active site. If this explanation is true, then it might be expected that the β_{lg} value for the hydrolysis of the thiophosphate analogues with the Mn^{2+}-substituted enzyme should be very large because

[2] The pK_a values of the metal-bound water molecule in complexes of Zn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, and Mn^{2+} are 8.8, 8.9, 9.0, 10.6, and 10.6, respectively (Basolo & Pearson, 1967), whereas the pK_a values of carbonic anhydrase containing Zn^{2+}, Co^{2+}, Cd^{2+}, and Mn^{2+} are 6.9, 6.8, 9.1, and 8.2, respectively (Lindskog, 1982). Similarly, the pK_a values of the metal-substituted phosphotriesterase determined by kinetic methods are 5.8, 6.5, 8.1, 7.4, and 7.0 for Zn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, and Mn^{2+}−enzyme (Omburo *et al*., 1992). The differences in the observed pK_a values between the metal−water complexes and the metal-substituted enzymes are likely due to changes in the coordination number and alterations in the geometry of the protein−metal complexes.

Table 4: Kinetic Constants for the Cd/Cd-Phosphotriesterase

	compound[a]	V_{max} (s^{-1})			K_m (mM)		
		XV	XVI	XVII	XV	XVI	XVII
I		0.005	8.6	0.033	3.3	0.60	0.64
II		0.010	15	0.064	3.5	0.93	0.78
III		nd	19	0.055	nd	0.33	0.38
IV		0.97	27	0.52	1.5	0.11	0.72
V		4.7	nd	nd	3.0	nd	nd
VI		730	1300	4.3	3.9	0.81	0.72
VII		660	940	nd	0.74	0.31	nd
VIII		4600	1400	nd	1.6	0.56	nd
IX		5900	1600	106	1.5	0.54	2.3
X		11300	1500	nd	1.5	0.67	nd
XI		12900	1300	58	0.71	0.61	1.1
XII		8600	1200	nd	0.57	0.64	nd
XIII		10400	1000	nd	0.84	0.58	nd
XIV		9400	1230	nd	0.77	0.98	nd

[a] R = EtOP(O)(Et*O*)*O*- (XV), EtOP(S)(Et*O*)*O*- (XVI), or EtOP(O)(Et*O*)*S*- (XVII).

Table 5: Rate Constants and Brϕnsted β Coefficients for the Hydrolysis of Phosphotriesters and Thiophosphotriesters by Metal-Substituted Phosphotriesterase at 25 °C

metal enzyme	substrate	k_1 (M^{-1} s^{-1})[a]	k_2 (s^{-1})[a]	k_5 (s^{-1})[a]	β[b]	c[b]	crossover pK_a
Zn/Zn	oxo	5.1×10^7	5.5×10^3	2.4×10^3	−2.2	20.9	7.9
	thio	4.3×10^6	1.3×10^3	5.9×10^2	−2.0	19.5	8.5
Zn/Zn (H201N)	oxo	1.8×10^6	5.8×10^1	1.5×10^2	−1.9	15.5	6.9
	thio	5.4×10^5	1.2×10^3	4.3×10^2	−2.5	24.5	8.6
Co/Co	oxo	5.5×10^7	6.4×10^3	1.2×10^4	−2.7	25.5	8.0
	thio	1.1×10^7	3.4×10^3	6.6×10^3	−2.7	26.8	8.5
Co/Co (H201N)	oxo	8.6×10^5	5.7×10^1	5.9×10^1	−1.6	13.0	6.9
	thio	3.2×10^5	1.8×10^2	1.1×10^2	−2.6	23.9	8.6
Cd/Cd	oxo	1.2×10^7	3.0×10^4	9.8×10^3	−3.0	28.5	8.1
	thio	2.3×10^6	8.9×10^2	1.4×10^3	−1.4	14.7	8.5
Ni/Ni	oxo	3.2×10^7	2.7×10^3	7.4×10^3	−2.5	22.1	7.4
	thio	2.6×10^6	6.0×10^2	9.2×10^2	−2.4	23.0	8.3
Mn/Mn	oxo	1.2×10^7	1.3×10^4	3.9×10^3	−3.2	29.8	8.2
	thio	1.5×10^6	3.4×10^2	4.8×10^2	−4.3	41.7	9.1
Zn/Cd	oxo	2.4×10^7	2.5×10^3	2.0×10^3	−2.1	19.7	7.8
	thio	2.5×10^6	9.6×10^2	3.2×10^2	−1.6	16.0	8.6

[a] Derived from a fit to eqs 2 and 3. [b] Derived from a fit to eq 4.

Mn^{2+} is a hard metal ion. In fact, the observed β_{lg} value for the Mn^{2+}/Mn^{2+}-substituted enzyme is −4.3.

If it is accepted that the binuclear metal center functions, in part, to polarize the phosphoryl oxygen (or sulfur) bond, then it is now possible to address whether one or both metal ions serve in this capacity. We have previously demonstrated that a unique hybrid metal derivative of phosphotriesterase can be constructed (Omburo *et al.*, 1993). In this hybrid, the binuclear metal center is composed of an equal mixture of Zn^{2+} and Cd^{2+}. In addition, cadmium NMR has shown that each metal occupies a single site in the binuclear metal center but, as of yet, we do not

know which metal is occupying which site.[3] With the very fast substrates, paraoxon and parathion, the kinetic constants for the Zn^{2+}/Cd^{2+}-hybrid are nearly identical with the measured kinetic constants for the Zn^{2+}/Zn^{2+}-enzyme and quite distinct from the Cd^{2+}/Cd^{2+}-enzyme. Thus, when bond cleavage is not rate-limiting, it appears that the Zn^{2+} site in the hybrid is dominating the functional properties of the hybrid.

For those substrates where the bond cleavage step is rate-limiting, mixed results were obtained. With the thiophosphate analogues, the β_{lg} value for the hybrid (−1.6) is intermediate in size relative to the values obtained for the Zn^{2+}/Zn^{2+}- (−2.0) and Cd^{2+}/Cd^{2+}-enzyme (−1.3). However, for the hydrolysis of the phosphate analogues, the β_{lg} value for the hybrid is identical to the value obtained for the Zn^{2+}/Zn^{2+}-enzyme and quite different from the Cd^{2+}/Cd^{2+}-enzyme. Either the complexation scheme is distinctly different in the transition state for the phosphate and thiophosphate analogues or else the interrelationship between the closely spaced metals in the active site is more complicated than this simple model would suggest. In the absence of any further experimental evidence, our working model will depict polarization of the phosphoryl bond by both metal ions in the binuclear metal center. Further refinement of this model will have to await additional crystallization and/or spectroscopic experiments in the presence of substrate analogues.

Hydrolysis of Substrates by the H201N Mutant Phosphotriesterase. The histidine at position 201 was originally mutated to an asparagine in an attempt to determine whether the imidazole side chain was participating in the functioning of the phosphotriesterase either as a ligand to the metal center or, alternatively, as an active-site base. Mutation of this residue results in the loss of catalytic activity, and the X-ray crystal analysis by Benning *et al*. (1995) clearly indicates that this residue is a ligand to the more solvent-exposed metal within the binuclear metal center. We initially anticipated that the Brϕnsted plots for this mutant would be similar in shape to those observed for the wild-type enzyme except that the crossover point for those substrates that would be limited by conformational changes or binding events would occur at a much lower pK_a value for the leaving group. To a certain extent, this is true for the effect on V_{max} and V/K_m with the pK_a of the leaving group for the hydrolysis of the phosphate esters, but it is not true for the hydrolysis of the thiophosphate esters. With the phosphate esters, there also appears to be a general reduction in all of the calculated rate constants relative to the wild-type enzyme. And thus the maximally obtainable value for k_{cat} is lower for the mutant even with substrates having leaving groups of very low pK_a values. It is also interesting to note that the rates of thiophosphate hydrolysis by the zinc-substituted enzyme are nearly identical to the wild-type values whereas the rates of phosphate hydrolysis are significantly depressed. We anticipate that this perturbation in the structure of the binuclear metal center has not only perturbed the catalytic efficiency of the enzyme but also altered conformational changes that are required for substrate binding and/or product release.

[3] We suspect that zinc is ligated to the more buried His-55, His-57, and Asp-301 while cadmium is ligated to His-201 and His-230, but confirmation of this proposal will have to await further crystallographic or NMR experiments.

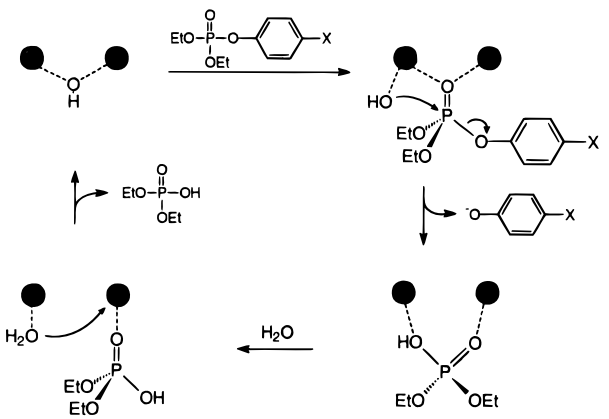


FIGURE 3: Working model for the hydrolysis of substrates by the bacterial phosphotriesterase. The metal ions are shown as black spheres and coordination bonds as dashed lines.

Mechanism of Hydrolysis. A working model for the hydrolysis of substrates by the phosphotriesterase can be constructed which utilizes both metals in conjunction with one another for the activation of the hydrolytic water molecule and the polarization of the phosphoryl oxygen bond. The model is presented in Figure 3.

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