

Hydrolysis of Phosphodiester through Transformation of the Bacterial Phosphotriesterase*

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The phosphotriesterase from *Pseudomonas diminuta* catalyzes the hydrolysis of a wide array of phosphotriesters and related phosphonates, including organophosphate pesticides and military nerve agents. It has now been shown that this enzyme can also catalyze the hydrolysis of phosphodiester, albeit at a greatly reduced rate. However, the enzymatic hydrolysis of ethyl-4-nitrophenyl phosphate (compound I) by the wild-type enzyme was $>10^8$ times faster than the uncatalyzed reaction ($k_{\text{cat}} = 0.06 \text{ s}^{-1}$ and $K_m = 38 \text{ mM}$). Upon the addition of various alkylamines to the reaction mixture, the k_{cat}/K_m for the phosphodiester (compound I) increased up to 200-fold. Four mutant enzymes of the phosphotriesterase were constructed in a preliminary attempt to improve phosphodiester hydrolysis activity of the native enzyme. Met-317, which is thought to reside in close proximity to the pro-S-ethoxy arm of the paraoxon substrate, was mutated to arginine, alanine, histidine, and lysine. These mutant enzymes showed slight improvements in the catalytic hydrolysis of organophosphate diesters. The M317K mutant enzyme displayed the most improvement in catalytic activity ($k_{\text{cat}} = 0.34 \text{ s}^{-1}$ and $K_m = 30 \text{ mM}$). The M317A mutant enzyme catalyzed the hydrolysis of the phosphodiester (compound I) in the presence of alkylamines up to 200 times faster than the wild-type enzyme in the absence of added amines. The neutralization of the negative charge on the oxygen atom of the phosphodiester by the ammonium cation within the active site is thought to be responsible for the rate enhancement by these amines in the hydrolytic reaction. These results demonstrate that an active site optimized for the hydrolysis of organophosphate triesters can be made to catalyze the hydrolysis of organophosphate diesters.

The bacterial phosphotriesterase from *Pseudomonas diminuta* catalyzes the hydrolysis of a wide range of organophosphate nerve agents with high efficiency (1, 2). Paraoxon, the best characterized substrate for the zinc-substituted phosphotriesterase, is hydrolyzed with a k_{cat}/K_m of $4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and a k_{cat} of 2100 s^{-1} . The active site of this enzyme contains a coupled binuclear metal center, which is absolutely essential for catalytic activity (1). The native enzyme contains two Zn^{2+} ions, but these metal ions can be replaced with Co^{2+} , Ni^{2+} , Mn^{2+} , or Cd^{2+} with retention of full catalytic activity. From chemical, kinetic, and genetic studies, it has been demon-

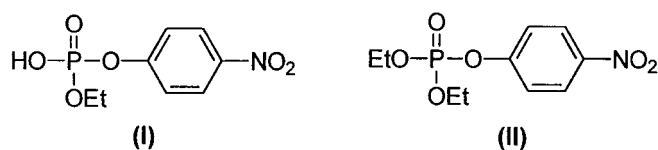
strated that the reaction proceeds via an $\text{S}_{\text{N}}2$ -like associative mechanism in which a metal-bound hydroxide ion attacks the electrophilic phosphorus center of the substrate (2–16). The role of one of the two metal ions within the active site is thought to involve the activation of the hydrolytic water molecule, whereas the companion metal ion is most likely involved in the polarization of the phosphoryl oxygen bond of the substrate to increase the electrophilicity of the substrate for nucleophilic attack (16). Not surprisingly, the substrate-binding site pocket consists predominantly of hydrophobic residues (12) that can readily accommodate a variety of nonpolar organophosphate triesters, which explains, in part, the relatively broad substrate specificity of this enzyme.

Phosphodiester are chemically more resistant to hydrolysis than are phosphotriesters. For example, it has been estimated that ethyl-4-nitrophenyl phosphate (Structure 1, compound I) spontaneously hydrolyzes with $k_{\text{obs}} \leq 10^{-10} \text{ s}^{-1}$ at 25°C and pH 8 (17), whereas under the same reaction conditions, the base-catalyzed hydrolysis of paraoxon, diethyl-4-nitrophenyl phosphate (Structure 1, compound II), proceeds with a rate constant of $\sim 10^{-7} \text{ s}^{-1}$ (1). The exceptional chemical stability of the phosphodiester backbone in molecules such as RNA and DNA facilitates the conservation of genetic information. An array of model compounds that promote the catalytic hydrolysis of phosphodiester bonds has been designed and synthesized (17–23). Nearly all of the model compounds constructed to date utilize metal ions as essential cofactors to activate the substrate and nucleophile while holding the reactants together in close proximity (17–21). However, the enzymatic hydrolysis of the phosphodiester bond is typically much faster than the small molecule mimics. Many enzymes are known to catalyze the hydrolysis of the phosphodiester bond, with k_{cat} values ranging from 10^{-2} to 10^3 s^{-1} . This class of enzymes includes, for example, the hammerhead ribozyme, which catalyzes the site-specific hydrolysis of a phosphodiester bond with $k_{\text{cat}} \sim 1 \text{ min}^{-1}$ (24); ribonuclease A ($k_{\text{cat}} = 1400 \text{ s}^{-1}$ for UpA) (25); ribonuclease T1 ($k_{\text{cat}} = 350 \text{ s}^{-1}$ for GpC) (26); *EcoRI* endonuclease ($k_{\text{cat}} = 0.9 \text{ s}^{-1}$ for GpAATTC) (27); and staphylococcal nuclease ($k_{\text{cat}} = 150 \text{ s}^{-1}$ for calf thymus DNA) (28).

The existence of phosphotriesters in nature is decidedly quite rare, and thus, it is still unclear as to the origin of the metabolic pressure for the selection and enhancement of the enormous catalytic activity exhibited by the bacterial phosphotriesterase from *P. diminuta*. Since the catalytic machinery required for the hydrolysis of phosphodiester is apparently similar to the binuclear metal center found within the active site of phosphotriesterase, we anticipated that phosphotriesterase would possess an inherent ability to hydrolyze phosphodiester substrates. However, the active site of phosphotriesterase is filled predominantly with hydrophobic residues, and thus, this site may not be suitable for the accommodation of the negatively charged phosphodiester. In this paper, we provide direct experimental evidence to support the conclusion that the bacte-

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STRUCTURE 1. Compounds I and II.

rial phosphotriesterase has a significant amount of phosphodiesterase activity. Moreover, the rate enhancement can be intensified by the incorporation of a positive charge within the active site, either through site-directed mutagenesis or the addition of alkylamines to the aqueous medium.

MATERIALS AND METHODS

General—The bacterial phosphotriesterase and the metal-substituted derivatives were isolated as described previously (29). The site-directed mutagenesis of the phosphotriesterase variants (M317A, M317H, M317K, and M317R) was carried out as described previously (8, 9, 30). Cell growth and purification of the mutant enzymes were performed according to published procedures (29). All of the chemicals used in these experiments were purchased from Sigma, Aldrich, Fisher, or U. S. Biochemical Corp. Biochemical supplies were purchased from Promega, Amersham Pharmacia Biotech, Bio 101, Inc., Perkin-Elmer, or Hoffmann-La Roche. The synthesis of oligonucleotides and DNA sequencing reactions were carried out in the Gene Technology Laboratory of the Biology Department at Texas A&M University.

Synthesis of Phosphodiesters—Compound I was synthesized according to the procedure of Hendry and Sargeson (17). Compounds III–VI (Structure 2) were synthesized by a similar procedure with the following modifications. Ethyl dichlorophosphate (12.3 mmol, 1.46 ml) was mixed with triethylamine (12.3 mmol, 1.72 ml) in dry ethyl ether (10 ml). One equivalent of the substituted phenol, dissolved in dry ethyl ether (20 ml), was added dropwise to this solution over a period of 30 min at 0 °C. The mixture was stirred for 1 h; triethylamine (1.72 ml) was added to the mixture; and the stirring was continued for an additional 15 min. Finally, water (5 ml) was added, and the mixture was stirred for 30 min. The product was extracted three times with 30-ml portions of a solution of 0.2 M aqueous triethanolamine hydrochloride, and then the water was removed *in vacuo*. The resulting white solid was resuspended in tetrahydrofuran, and then the insoluble salt was removed by filtration. The solution was dried over anhydrous $MgSO_4$, and the solvent was removed. The resulting crude product was dissolved in dichloromethane and subsequently washed with 30 ml of 2 N hydrochloric acid to remove the phosphomonoester impurity. The organic layer was dried over anhydrous $MgSO_4$, and the solvent was evaporated *in vacuo* to obtain the desired phosphodiester. The structures of compounds I–VI were confirmed by 1H and ^{13}C NMR spectroscopy.

Kinetic Measurements—Spectroscopic determinations were made using a Gilford 260 UV-visible spectrophotometer. The reactions were followed by monitoring the appearance of the substituted phenol from the hydrolysis of the phosphodiester (1–20 mM) upon the addition of the phosphotriesterase at 25 °C. The pK_a values, extinction coefficients at pH 9.0, and λ_{max} values of the leaving group phenols were obtained from the literature (3). Extinction coefficients at different pH values were calculated using Equation 1,

$$\epsilon = \epsilon_o / (10^{(pK_a - pH)} + 1) \quad (\text{Eq. 1})$$

where ϵ_o is the extinction coefficient of the substituted phenolate anion. Stock solutions of the various amines were made at 2.5–5.0 M concentrations, and pH values were adjusted using hydrochloric acid or sodium hydroxide.

Data Analysis—The values of k_{cat} and K_m were determined by fitting the kinetic data to Equation 2,

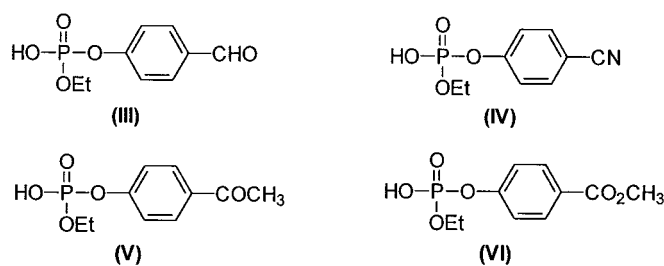
$$v = V_{max}A / (K_m + A) \quad (\text{Eq. 2})$$

where v is the initial velocity, k_{cat} is the maximum velocity, K_m is the Michaelis constant, and A is the substrate concentration. The pH-rate profiles were analyzed by fitting the data to Equations 3–5 to obtain the pK values.

$$\log(V/K_m) = \log[c / (1 + [H^+]/K_a)] \quad (\text{Eq. 3})$$

$$\log(V/K_m) = \log[c / (1 + K_b/[H^+])] \quad (\text{Eq. 4})$$

$$\log(V/K_m) = \log[c / (1 + [H^+]/K_a + K_b/[H^+])] \quad (\text{Eq. 5})$$



STRUCTURE 2. Compounds III–VI.

The Brønsted plots were fit to straight lines, and the β values were obtained directly from the slopes of these lines. The kinetic data from the experiment where the phosphodiester concentration was varied at different levels of methylamine were fit to Equation 6,

$$v = \frac{V_{max}(K_A + \beta/\alpha[B])[A]}{K_mK_A + K_A[A] + K_m[B] + [A][B]/\alpha} \quad (\text{Eq. 6})$$

where A is the concentration of phosphodiester, B is the concentration of amine, K_m is the Michaelis constant of the phosphodiester in the absence of added amine, V_{max} is the maximum velocity in the absence of added amine, K_A is the equilibrium constant for the formation of an enzyme-amine complex, and α and β are the ratios of K_m (or K_A) and V_{max} in the presence and absence of added methylamine, respectively.

RESULTS

Enzymatic Hydrolysis of Ethyl-4-nitrophenyl Phosphate (Compound I)—Compound I was tested as a substrate for the cobalt-substituted bacterial phosphotriesterase. The rate of hydrolysis of compound I catalyzed by the phosphotriesterase was very slow ($k_{cat} = 0.06 \pm 0.01 \text{ s}^{-1}$ and $k_{cat}/K_m = 1.6 \pm 0.3 \text{ M}^{-1} \text{ s}^{-1}$ at pH 9.0) compared with that of paraoxon ($k_{cat} = 8600 \text{ s}^{-1}$ and $k_{cat}/K_m = 4.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at pH 9.0), but was $>10^8$ times faster than the uncatalyzed reaction under similar reaction conditions. The phosphotriesterase used for this study was purified by gel filtration. The phosphodiesterase and phosphotriesterase activities for compounds I and II were measured for each fraction during the purification in addition to the absorbance readings at 280 nm. A plot of these activities *versus* the fraction number is presented in Fig. 1 and serves to illustrate the coincidence of the two activities during the purification. Four mutant enzymes were also prepared in a preliminary attempt to enhance the overall rate of phosphodiesterase activity exhibited by the native enzyme. Of the four mutant enzymes, M317K catalyzed the hydrolysis of the phosphodiester test substrate (compound I) with the highest kinetic constants ($k_{cat} = 0.34 \pm 0.03 \text{ s}^{-1}$ and $k_{cat}/K_m = 11 \pm 1 \text{ M}^{-1} \text{ s}^{-1}$ at pH 9.0). The kinetic constants for the other mutants (M317A, M317R, and M317H) are listed in Table I.

The slow hydrolysis rate of compound I was accelerated up to 200-fold when various alkylamines (0.1–2.0 M) were added to the reaction mixture. The kinetic constants for these rate enhancements are summarized in Table II. In general, a greater rate acceleration was observed for the wild-type enzyme when amines with shorter and/or branched alkyl chains were added. Secondary amines showed a larger enhancement than primary amines. The greatest rate enhancement was observed with 2.0 M dimethylamine ($k_{cat} = 0.23 \pm 0.01 \text{ s}^{-1}$ and $k_{cat}/K_m = 260 \pm 30 \text{ M}^{-1} \text{ s}^{-1}$ at pH 9.0). For the M317A mutant enzyme, amines with longer alkyl chains yielded greater enhancements (Table III). *t*-Pentylamine was the best activator for this mutant enzyme ($k_{cat} = 3.8 \pm 0.1 \text{ s}^{-1}$ and $k_{cat}/K_m = 210 \pm 10 \text{ M}^{-1} \text{ s}^{-1}$ at pH 9.0). No enhancement of the phosphodiesterase activity of the wild-type phosphotriesterase was observed in the presence of Ca^{2+} or Mg^{2+} at concentrations of $<100 \text{ mM}$.

pH-rate Profiles—The pH-rate profiles of $\log(V/K_m)$ *versus* pH were made without any amine added (Fig. 2A) and also with either guanidine (Fig. 2B) or dimethylamine (Fig. 2C) in the

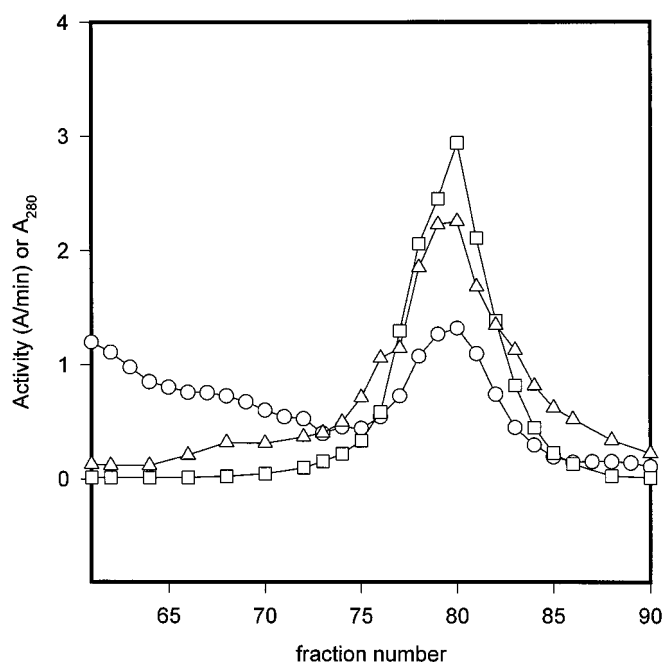


Fig. 1. Gel filtration chromatography of the phosphotriesterase. \circ , A_{280} ; \square , phosphotriester hydrolysis activity; \triangle , phosphodiester hydrolysis activity. The phosphodiesterase activity was normalized to adjust for the relative specific catalytic activities. Additional details are given under "Results."

TABLE I
Kinetic constants for the mutant enzymes with phosphodiester and paraoxon

Conditions were pH 9.0 and 25 °C for the cobalt-substituted phosphotriesterase. The data were fit to Equation 2, and the standard errors for each of the kinetic constants reported below were <25% of the stated values.

	Phosphotriester (II)			Phosphodiester (I)		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	mM	$M^{-1} s^{-1}$	s^{-1}	mM	$M^{-1} s^{-1}$
Wild-type	8600	0.20	4.3×10^7	0.06	38	1.6
M317A	2750	0.29	9.5×10^6	0.18	35	5.1
M317H	3650	0.65	5.6×10^6	0.05	43	1.1
M317K	1970	0.66	3.0×10^6	0.34	30	11
M317R	120	1.1	1.1×10^5	0.09	29	3.1

reaction mixtures. In general, the reactions were very slow and the K_m values were very large when no amine was added, and thus, only V/K_m values could be determined with sufficient confidence. In the pH profile without added amine, it can be seen that protonation of a single group accelerates the catalytic reaction. The pK_a value of this functional group could not be determined from the experimental data obtained over the pH range of 6.4–9.6. When guanidine was added, protonation of a single group diminished the reaction rate. From a least-square fit of the data to Equation 3, the pK_a value of this group was determined to be 6.5 ± 0.2 . Two ionizable groups are involved in the enzymatic reaction in the presence of dimethylamine. Fitting of the data to Equation 5 yielded pK_a values of 5.8 ± 0.2 and 9.1 ± 0.1 for these two groups. The pH-rate profiles for phosphodiester hydrolysis by the M317H and M317R mutant enzymes were also obtained. The profiles of $\log(V/K_m)$ versus pH for both enzymes show a drop in activity above pH ~ 7 (data not shown). From a fit of these data to Equation 4, pK_a values of 7.7 ± 0.1 and 7.0 ± 0.1 were obtained for the M317H and M317R mutants, respectively.

Variation of Metal Substitution—The effects of substitution of the binuclear metal center of the wild-type phosphotriesterase with Co^{2+} , Zn^{2+} , and Cd^{2+} at pH 7 and 9 are presented in

TABLE II
Effect of added amines on phosphodiester (compound I) hydrolysis by phosphotriesterase

These assays were conducted at 25 °C and pH 9.0. The data were fit to Equation 2. The standard errors for each of the kinetic constants reported below were <20% of the stated values.

Amine	[Amine] ^a	k_{cat}	K_m	k_{cat}/K_m
	M	s^{-1}	mM	$M^{-1} s^{-1}$
Ammonia	0.75	0.28	30	9.2
Methyl	0.50	0.30	8.7	34
Ethyl	0.38	0.20	7.8	25
Propyl	0.25	0.12	25	4.9
Butyl	0.10	0.06	16	3.9
<i>i</i> -Propyl	0.50	0.33	11	30
<i>i</i> -Butyl	0.25	0.18	28	6.5
<i>t</i> -Butyl	0.50	3.5	36	97
<i>t</i> -Pentyl	0.50	1.5	24	62
Dimethyl	2.0	0.23	0.87	260
Diethyl	0.35	0.14	20	6.9
Trimethyl	0.50	5.1	38	135
Guanidine	0.40	0.60	18	35

^a Concentration of amine used to enhance the phosphodiesterase activity of phosphotriesterase.

TABLE III
Effect of added amines on the phosphodiester hydrolysis by the M317A mutant enzyme

These assays were conducted at 25 °C and pH 9.0. The data were fit to Equation 2. The standard errors reported for each of the kinetic constants were <10% of the stated values.

Amine	[Amine] ^a	k_{cat}	K_m	k_{cat}/K_m
	M	s^{-1}	mM	$M^{-1} s^{-1}$
Methyl	1.75	0.31	19	16
Ethyl	0.50	0.29	24	12
Propyl	0.20	0.24	16	21
Butyl	0.10	0.33	4.9	67
Hexyl	0.01	0.2	7.6	26
<i>i</i> -Propyl	1.00	0.48	18	27
<i>i</i> -Butyl	0.15	0.20	13	16
<i>t</i> -Butyl	0.30	2.8	29	96
<i>t</i> -Pentyl	0.20	3.8	18	214
Dimethyl	0.30	0.33	4.4	76
Diethyl	0.30	0.15	23	6.6
Trimethyl	1.0	0.94	39	24
Guanidine	0.20	0.17	19	9.2

^a Concentration of amine used to enhance the phosphodiesterase activity of M317A phosphotriesterase.

Table IV. The kinetic constants for paraoxon (compound II) hydrolysis are presented for comparison. The Co^{2+} -substituted enzyme shows the highest k_{cat} value for phosphodiester (compound I) hydrolysis as well as for paraoxon (compound II) hydrolysis. The relative magnitude of the k_{cat} values ($Co^{2+} > Cd^{2+} > Zn^{2+}$) at pH 9 is similar to that of paraoxon hydrolysis.

Variation of Amine Concentration—The added amine and phosphodiester concentrations were varied together in order to elucidate the kinetic mechanism for the enhancement reaction. The overall reaction rate was increased with increasing amounts of methylamine (Fig. 3), and these data were fit to Equation 5. The values of k_{cat} and K_m are $0.06 \pm 0.01 s^{-1}$ and $38 \pm 10 mM$, respectively. The α and β values are 0.03 ± 0.02 and 7 ± 2 , respectively. These values demonstrate that the addition of the amine decreases K_m and increases k_{cat} .

Brønsted Plots for the Hydrolysis of Phosphodiesters—Kinetic constants were determined for compounds I and III–VI in the presence and absence of trimethylamine. Trimethylamine was selected because primary and secondary amines form imine or iminium adducts with the carbonyl groups of compounds III and V. In the absence of added amine, only V/K_m values could be determined because the K_m values were too large to be accurately measured. The Brønsted plots (Fig. 4) show a linear relationship between the pK_a values of the leav-

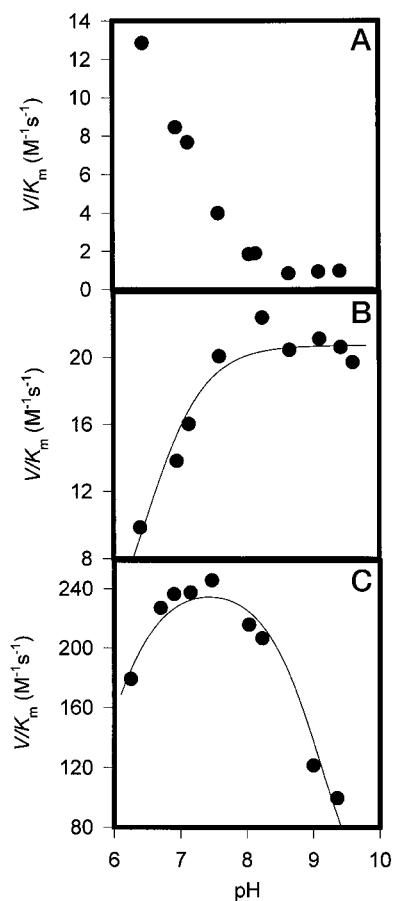


FIG. 2. The pH-rate profiles of the phosphodiester hydrolysis catalyzed by the phosphotriesterase. A, in the absence of added amine; B, in the presence of 0.4 M guanidine; C, in the presence of 1.0 M dimethylamine. Additional details are given under "Results."

TABLE IV

Hydrolysis of phosphodiester (compound I) and paraoxon by the metal-substituted enzymes

These assays were conducted at 25 °C. The data were fit to Equation 2. The standard errors are <25% of the stated values.

	Ethyl-4-nitrophenyl phosphate								Paraoxon, pH 9	
	pH 7				pH 9					
	No amine		Me ₂ NH		No amine		Me ₂ NH		k _{cat}	K _m
	k _{cat}	K _m	k _{cat}	K _m	k _{cat}	K _m	k _{cat}	K _m		
s ⁻¹	mM	s ⁻¹	mM	s ⁻¹	mM	s ⁻¹	mM	s ⁻¹	mM	
Co ²⁺	0.13	18	0.19	1.7	0.06	38	0.23	0.87	8600	0.2
Zn ²⁺	0.008	6.3	0.08	7.1	ND ^a	ND	0.12	6.5	1700	0.05
Cd ²⁺	0.014	13	0.04	1.5	ND	ND	0.16	26	3900	0.3

^a These constants could not be determined because the reaction rates were too slow to measure accurately.

ing group and $\log(V_{\max})$ or $\log(V/K_m)$. The β values are -1.3 ± 0.3 and -1.7 ± 0.1 for $\log V/K_m$ in the absence and presence of added amine, respectively. The β value for V_{\max} in the presence of added amine is -1.9 ± 0.4 .

DISCUSSION

Hydrolysis of Phosphodiesters—The full negative charge within the phosphodiester substrate is thought to be primarily responsible for the slow rate of catalytic hydrolysis of these compounds by the phosphotriesterase. The active site of the phosphotriesterase is largely hydrophobic (12), and thus, it would not be expected to accommodate the negative charge on the substrate very well. Furthermore, the nucleophile in the active site, *i.e.* metal-bound hydroxide ion, may not be able to attack the anionic substrate effectively. As a result, the phos-

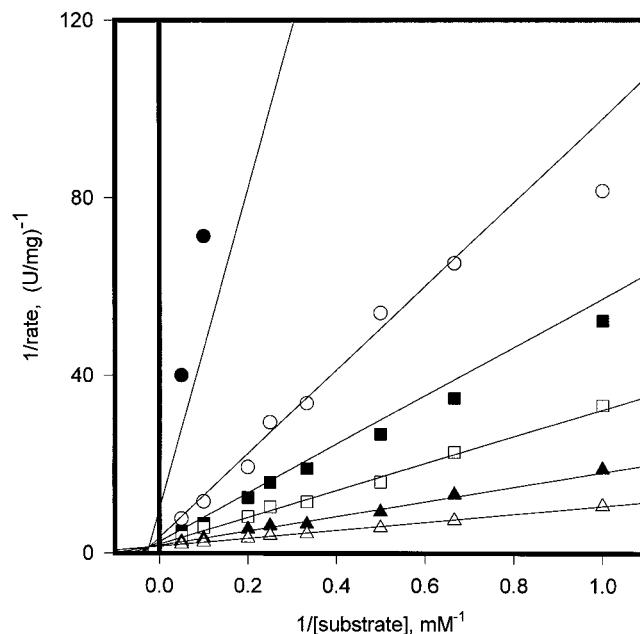


FIG. 3. Double-reciprocal plot of the phosphodiester hydrolysis catalyzed by the phosphotriesterase in the presence of 0 (●), 0.063 (○), 0.125 (■), 0.25 (□), 0.50 (▲), and 1.0 (△) M methylamine. The data were fit to Equation 6. Additional details are given under "Results."

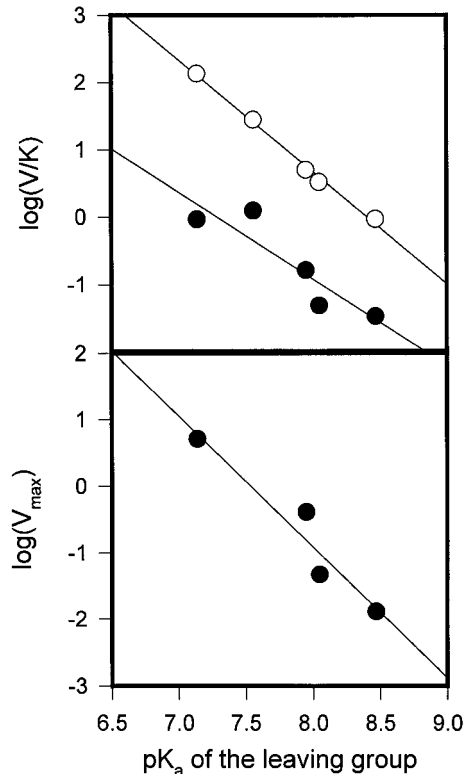


FIG. 4. Brønsted plots for the hydrolysis of phosphodiester compounds I and III–VI. Upper panel, $\log(V/K_m)$ versus pK_a of the leaving group in the absence (●) and presence (○) of trimethylamine; lower panel, $\log(V_{\max})$ versus pK_a of the leaving group in the presence of trimethylamine. Additional details are given under "Results."

phodiesters have diminished k_{cat} and elevated K_m values. To enhance the hydrolytic reaction rate, cations were added to the reaction medium in an attempt to neutralize the negative charge of the substrate. Alkylamines were selected because they are cationic in the pH range used for this investigation, and the size and hydrophobicity could easily be controlled by

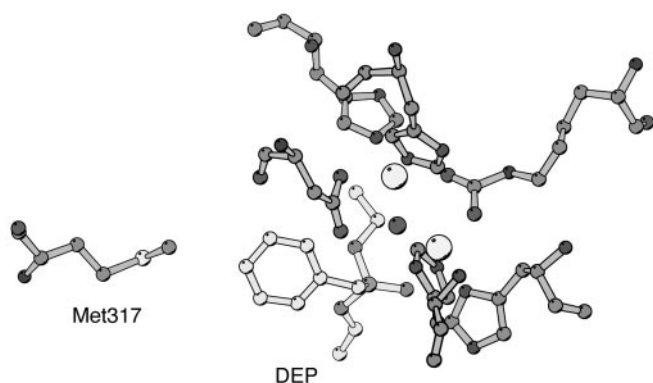


FIG. 5. Active-site structure of the phosphotriesterase with the substrate analogue diethylphenyl phosphonate (12). The distance between the terminal methyl group of the side chain of methionine 317 and the phosphorus atom of the bound substrate analogue is 8.2 Å. DEP, diethylphenyl phosphonate.

varying the number and the length of the alkyl chains. For the wild-type enzyme, faster reaction rates were observed when amines with shorter alkyl chains were added, whereas the M317A mutant enzyme preferred amines with longer alkyl chains. Met-317 is located within the pro-S-binding pocket of the enzyme active site (12). It was thought that by mutating the methionine residue to a smaller alanine residue, larger amines could be accommodated more effectively. Amines with branched alkyl chains accelerated the reaction faster than amines with straight chains, and secondary amines were more effective than primary amines in enhancing the reaction. The reasons for these preferences are not yet clear. The other three mutant enzymes, M317H, M317K, and M317R, were designed to incorporate a cationic group within the enzyme active site without adding amines from the external medium. The rate enhancements were not very large, and the pH-rate profiles showed a pattern similar to that of the wild-type enzyme without added amine. It appears that these mutant enzymes catalyze the phosphodiesterase reaction with little help from the substituted cationic residues, with the possible exception of M317K, which showed a 7-fold enhancement of k_{cat}/K_m . The small rate enhancement by these mutations could, in part, be attributed to the distance between Met-317 and a substrate analogue bound in the enzyme active site (Fig. 5). The distance between the thiomethyl group of the side chain of Met-317 and the phosphorus center of the bound substrate analogue is 8.2 Å (12). When this methionine residue is replaced with other amino acid residues with cationic side chains (arginine and lysine), the distance between the positive charge of the new side chains and the negative charge of a bound phosphodiester substrate was estimated to be ~ 6 Å using Insight II (Biosym Technologies) to model the approximate locations of the substrate and mutated amino acid.

Many previous attempts have been made to construct mutant enzymes with altered substrate specificity. For example, the arginine residue of aspartate aminotransferase that ion pairs with the side chain carboxylate of the substrate was mutated to aspartate in an attempt to change the substrate specificity of this enzyme from aspartate to the cationic amino acids (lysine and arginine). Rate enhancements of 9–16-fold of the k_{cat}/K_m values for lysine and arginine were observed (31). In a subsequent study using the same enzyme, the k_{cat}/K_m value for the transamination of phenylalanine was increased 1500-fold after mutation of six active-site residues (32).

pH-rate Profiles—The pH profile for diester hydrolysis in the absence of added amine shows that a particular group needs to be protonated for catalytic activity. This group could be either the P-O⁻ group of the phosphodiester substrate or the metal-

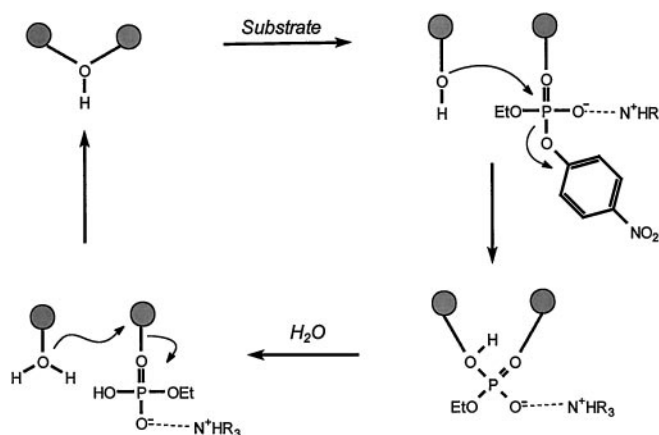
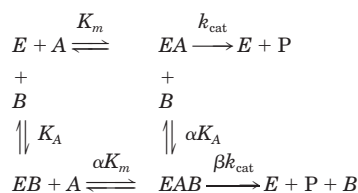


FIG. 6. Proposed mechanism for the phosphodiester hydrolysis catalyzed by phosphotriesterase.

bound hydroxide ion. The protonation of the substrate would accelerate the reaction by neutralizing the negative charge of the substrate and making it appear more like a phosphotriester. On the other hand, protonation of the metal-bound hydroxide would reduce the nucleophilicity of the attacking group, but charge repulsion would be reduced, and the overall reaction rate may be enhanced. The pH-rate profile in the presence of 1.0 M dimethylamine displays a bell-shaped curve, suggesting that there are two ionizable groups involved in the catalytic reaction. One of these may be the metal-bound water ($\text{p}K_a = 5.8$), whereas the other may be dimethylamine ($\text{p}K_a = 9.1$). The reaction rate decreases at low pH, where the bound hydroxide is protonated, and also at high pH, where the amine itself is deprotonated. The pH profile in the presence of 0.4 M guanidine shows a somewhat different behavior. The rate increases as the pH increases, until it is saturated at $\text{pH} \sim 8$. The $\text{p}K_a$ of the group is 6.5, and from this value and the shape of the profile, it is thought that this group is the metal-bound water molecule (16). Unlike the case with dimethylamine, the rate does not decrease at high pH because guanidinium cation cannot be deprotonated below pH 10.

The pH profiles of the M317H and M317R mutant enzymes for phosphodiester hydrolysis were also measured. The patterns of these profiles were similar to the one obtained with the wild-type enzyme reaction without added amine. The M317R profile shows a loss of activity at high pH, whereas the profile of the wild-type enzyme in the presence of 0.4 M guanidine shows a loss of activity at low pH. It appears that the guanidine group of the M317R mutant enzyme is not properly located for effective charge neutralization, and the reaction proceeds without significant assistance from the cationic residue. The relatively small 2-fold enhancement of the k_{cat}/K_m value supports this view.

Kinetic Mechanism of Phosphodiester Hydrolysis—Kinetic measurements were made at six different methylamine concentrations in an effort to elucidate the kinetic mechanism of the amine-assisted enzymatic reaction. The data and the least-square fit are shown in Fig. 3. Equation 5, to which the kinetic data were fit, was derived from the mechanism shown in Scheme 1.



SCHEME 1

In this mechanism, the amine forms a complex with the enzyme, and then the substrate adds to form an enzyme-amine-substrate complex. This ternary complex is then hydrolyzed prior to the release of the products. When $[B] = 0$, the reaction mechanism becomes a simple Michaelis-Menten-type reaction with $k_{\text{cat}} = 0.06 \text{ s}^{-1}$ and $K_m = 38 \text{ mM}$. The amine-assisted pathway has a faster k_{cat} ($\beta = 7$) and a smaller K_m ($\alpha = 0.03$) than the non-assisted pathway. An alternative mechanism in which a substrate-amine complex (AB) is formed prior to binding to the active site did not fit the experimental data.

Brønsted Plots—The Brønsted plots for the dependence of $\log(V/K_m)$ on the $\text{p}K_a$ of the leaving group in the absence and presence of trimethylamine have β_{lg} values of -1.3 and -1.7 , respectively (Fig. 4). The large negative β_{lg} values strongly suggest that the chemical step is the rate-limiting step, and the transition state of the reaction has considerable product-like character. In the presence of trimethylamine, the plot of $\log(V_{\text{max}})$ versus $\text{p}K_a$ of the leaving groups has a slope of -1.9 and was slightly more negative than the slope of the $\log(V/K_m)$ versus $\text{p}K_a$ plot of the same reaction (-1.7).

Mechanism of Phosphodiester Hydrolysis—The data presented in this paper demonstrate that the catalytic machinery that has evolved for the hydrolysis of phosphotriesters can be exploited to effectively hydrolyze phosphodiester substrates. The biggest obstacle the phosphotriesterase has in recognition of phosphodiester substrates is in accommodation of the negative charge and the loss of binding energy derived from the missing alkyl substituent. These factors can, in part, be overcome through chemical rescue via the addition of alkylamines from the external medium. Attempts to accomplish the same aim through site-directed mutagenesis were less successful. The most likely cause for this failure is the imprecise placement of the positive charge from the mutant residue. Therefore, further work is ongoing to construct and characterize mutant enzymes that can more effectively catalyze the hydrolysis of phosphodiester bonds. A working model for phosphodiester hydrolysis as catalyzed by the bacterial phosphotriesterase is presented in Fig. 6. The phosphoryl oxygen bond is polarized by the metal ions, and the negative charge is neutralized by the added amine. The suggested mechanism is similar to that of the phosphotriester hydrolysis (16), where the metal ions polarize the phosphoryl oxygen

bond and the metal-bound hydroxide attacks the phosphorus atom.

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