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## Structure-Activity Relationships in the Hydrolysis of Substrates by the Phosphotriesterase from *Pseudomonas diminuta*<sup>†</sup>

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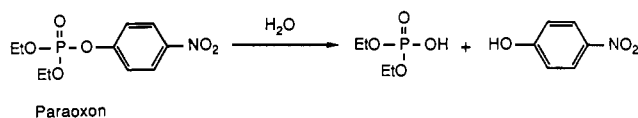
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**ABSTRACT:** The mechanism and substrate specificity of the phosphotriesterase from *Pseudomonas diminuta* have been examined. The enzyme hydrolyzes a large number of phosphotriester substrates in addition to paraoxon (diethyl *p*-nitrophenyl phosphate) and its thiophosphate analogue, parathion. The two ethyl groups in paraoxon can be changed to propyl and butyl groups, but the maximal velocity and  $K_m$  values decrease substantially. The enzyme will not hydrolyze phosphomonoesters or -diesters. There is a linear correlation between enzymatic activity and the  $pK_a$  of the phenolic leaving group for 16 paraoxon analogues. The  $\beta$  value in the corresponding Brønsted plot is -0.8. No effect on either  $V_{max}$  or  $V_{max}/K_m$  is observed when sucrose is used to increase the relative solvent viscosity by 3-fold. These results are consistent with rate-limiting phosphorus-oxygen bond cleavage. A plot of  $\log V$  versus pH for the hydrolysis of paraoxon shows one enzymatic group that must be unprotonated for activity with a  $pK_a$  of 6.1. The deuterium isotope effect by  $D_2O$  on  $V_{max}$  and  $V_{max}/K_m$  is 2.4 and 1.2, respectively, and the proton inventory is linear, which indicates that only one proton is "in flight" during the transition state. The inhibition patterns by the products are consistent with a random kinetic mechanism.

Over 30 million kilograms of organophosphorus pesticides are used annually in the United States (FAO, 1983). The usefulness of these compounds is due to their inhibition of acetylcholinesterase in insects. This is also the primary cause of toxicity in nontarget organisms—including man. Exposure to excess levels of organophosphorus compounds not only has immediate consequences but has been shown to exert delayed cholinergic toxicity (Abou-Donia et al., 1979; Ali & Fukuto, 1983) and delayed neurotoxicity (Metcalf, 1982). Smart (1987) has shown organophosphorus residues could be found in fresh vegetables and fruits taken from grocers throughout Europe, and Muhammad and Kwar (1985) have shown parathion to be present in ketchup 6 months after processing. Thus, an enzyme capable of hydrolyzing paraoxon (diethyl *p*-nitrophenyl phosphate), parathion (diethyl *p*-nitrophenyl thiophosphate), and several other related organophosphorus compounds (Scheme I) is of scientific as well as environmental value. One such enzyme was reported by Munneke (1976), but the enzymatic mechanism of this phosphotriesterase has only recently been studied.

Scheme I



Lewis et al. (1988) demonstrated that the phosphotriesterase from *Pseudomonas diminuta* would only hydrolyze the  $S_P$  isomer of the common pesticide ethyl *p*-nitrophenyl phenylthiophosphonate (EPN) and that this hydrolysis occurred through the inversion of stereochemistry at phosphorus. These results suggest a general base mechanism for the activation of a water molecule and the subsequent nucleophilic attack by the oxygen directly at the phosphorus center. A phosphoenzyme intermediate in this reaction mechanism is highly unlikely because of the net inversion of configuration (Knowles, 1980).

This paper further details some aspects of the enzymatic hydrolysis mechanism of the *Ps. diminuta* phosphotriesterase. The proton-transfer process and the participation of the putative active-site base are probed through the measurement of solvent deuterium isotope effects and an analysis of the effects of pH on the kinetic parameters  $V_{max}$  and  $V_{max}/K_m$ . A systematic variation of the leaving group ability of substituted phenols and the effect of solvent viscosity on the kinetic parameters are used to elucidate the rate-limiting step and

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transition-state structure for the hydrolysis of phosphotriesters by this enzyme.

## MATERIALS AND METHODS

**General.** The bacterial phosphotriesterase was isolated either from *Ps. diminuta* by the method described by Lewis et al. (1988) or from *Escherichia coli* carrying the cloned gene on a plasmid as outlined by Dumas et al. (1989). Both of these preparations appear to be kinetically identical. All kinetic measurements were performed at 25 °C by using a Gilford Model 260 spectrophotometer. Enzymatic activity was routinely assayed by monitoring the absorbance of *p*-nitrophenol at 400 nm generated upon the hydrolysis of 0.5 mM paraoxon at pH 9.0. The chemicals used in this study were purchased from either Aldrich or Sigma.

**Solvent Deuterium Isotope Effects.** The solvent deuterium isotope effect was determined for the phosphotriesterase reaction at pH 10 in 99% D<sub>2</sub>O. An inventory of the protons involved in catalysis was made in accordance to the method described by Venkatasubban and Schowen (1984). The initial velocity of the enzyme-catalyzed hydrolysis of 0.1 mM paraoxon at pH 10.0 in 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer was determined at various D<sub>2</sub>O concentrations.

**pH Profile.** The kinetic constants  $V_{\max}$  and  $V_{\max}/K_m$  were measured with paraoxon as a substrate for the phosphotriesterase at pH values ranging from 5.5 to 10.0. In each case, the reported pH was the measured value at the end of the enzymatic reaction. The buffers 2-(*N*-morpholino)ethanesulfonic acid (MES), piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid (TAPS), 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) were each used at a concentration of 50 mM.

**Structure-Activity Relationships.** Several substituted diethyl phenyl phosphates (compounds I-XVI) were synthesized by the general method of Rossi and Bunnett (1973) using diethyl phosphochloridate and substituted phenols. The structure for each of the synthesized triesters was verified by <sup>1</sup>H, <sup>13</sup>C, and <sup>31</sup>P NMR spectroscopy. Low-resolution mass spectrometric analysis agreed with the expected molecular weight of each of the newly synthesized compounds. The purity of all compounds was >95% as determined by multinuclear NMR spectroscopy. The  $pK_a$  and extinction coefficients of the substituted phenols and their respective diethyl phosphate esters were determined spectrophotometrically and are presented in Table I. The rate of base-catalyzed hydrolysis in 1 M KOH was determined for each phosphotriester as well as the  $K_m$  and the relative  $V_{\max}$  for the enzyme-catalyzed hydrolysis at pH 10.0 in 50 mM CAPS buffer.

Other substrate analogues were synthesized from *p*-nitrophenyl phosphodichloridate and various alcohols according to the general method of Steurbaut et al. (1975). The structures were verified in all cases by <sup>1</sup>H, <sup>13</sup>C, and <sup>31</sup>P NMR spectroscopy. These compounds were tested as substrates and/or inhibitors of the phosphotriesterase reaction at pH 10.0 in 50 mM CAPS buffer. Because of limited solubility, some of these compounds were tested as substrates in methanol/water mixtures. All of the above compounds are potential acetylcholinesterase inhibitors and should therefore be handled with caution.

**Inhibition Studies.** The inhibition by products and product analogues versus paraoxon was examined at pH 9.0 in 100 mM CHES buffer. Diethyl phosphate and diethyl thiophosphate

were synthesized by the base hydrolysis of the respective phosphochloridates. The structures and purity were confirmed by <sup>1</sup>H and <sup>31</sup>P NMR spectroscopy. The inhibitory effect of three organic solvents, methanol, dimethylformamide, and 1,4-dioxane, versus paraoxon at pH 10.0 was quantitated.

**Solvent Viscosity.** The viscosity of several sucrose solutions (15.6, 21.3, and 26.7% by weight) relative to water was measured at 25 °C with an Ostwald viscometer. The kinetic parameters  $V_{\max}$  and  $V_{\max}/K_m$  were determined for the hydrolysis of paraoxon by the phosphotriesterase in each of these sucrose solutions at pH 9.0.

**Data Analysis.** The least-squares method using the FORTRAN programs of Cleland (1970) was applied to the appropriate equations to determine the values of kinetic constants. The values for  $K_m$  and  $V_{\max}$  were calculated from a fit of the data to

$$v = VA/(K + A) \quad (1)$$

where  $v$  is the initial velocity,  $V$  is the maximal velocity,  $K$  is the Michaelis constant, and  $A$  is the substrate concentration. The  $pK_a$ s from the pH profile were determined from the fit of the data to

$$\log y = \log [c/(1 + H/K_a)] \quad (2)$$

where  $y = V_{\max}$  or  $V_{\max}/K_m$ ,  $c$  is the pH-independent value of  $y$ ,  $H$  is the hydrogen ion concentration, and  $K_a$  is the dissociation constant of the group that ionizes. Inhibition data were fit to the equations for competitive inhibition (eq 3), noncompetitive inhibition (eq 4), or uncompetitive inhibition (eq 5)

$$v = \frac{VA}{K[1 + (I/K_{is})] + A} \quad (3)$$

$$v = \frac{VA}{K[1 + (I/K_{is})] + A[1 + (I/K_{ii})]} \quad (4)$$

$$v = \frac{VA}{K + A[1 + (I/K_{ii})]} \quad (5)$$

where  $K_{is}$  is the slope inhibition constant and  $K_{ii}$  is the intercept inhibition constant. The concentration of un-ionized phenol at pH 9.0 was determined by

$$pH = pK_a + \log ([A^-]/[HA]) \quad (6)$$

where  $[A^-]$  is the concentration of the ionized species and  $[HA]$  is the concentration of the protonated species.

## RESULTS

**pH Profiles.** The pH profiles for the enzymatic hydrolysis of paraoxon indicate that a single group must be unprotonated for catalytic activity. The dependence of the kinetic parameters  $V_{\max}$  and  $V_{\max}/K_m$  with pH are characterized by a single ionization with slopes of +1 below pH 6.0 and plateaus above pH 7.0 (Figure 1). From a least-squares fit of the data to eq 2, the  $pK_a$  value from the  $\log V_{\max}$  plot is  $6.14 \pm 0.09$ , and for the  $\log V_{\max}/K_m$  relationship, the  $pK_a$  is equal to  $6.01 \pm 0.06$ .

**Solvent Deuterium Isotope Effects.** The solvent deuterium isotope effect of D<sub>2</sub>O was determined at pH 10. The effect on  $V_{\max}$  and  $V_{\max}/K_m$  using paraoxon as a substrate was found to be 2.4 and 1.2, respectively. The solvent deuterium isotope effect for the base hydrolysis (1.0 M KOH) of paraoxon was 1.0.

In a proton inventory experiment, where the initial velocity of the enzymatic hydrolysis was determined at 10 different concentrations of D<sub>2</sub>O, the relation between D<sub>2</sub>O concentration

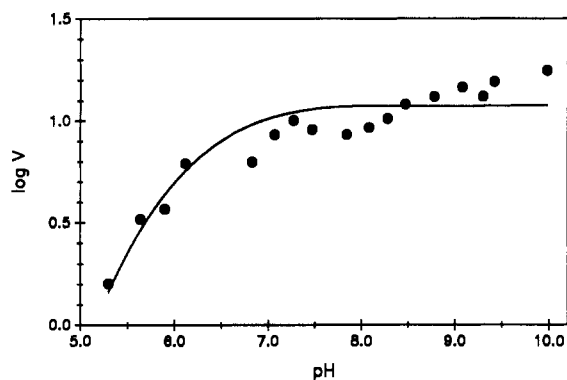


FIGURE 1: pH-rate profile for the hydrolysis of paraoxon by the phosphotriesterase. The fit of the data to eq 2 indicates that a single group must be ionized with a  $pK_a$  value of 6.14.

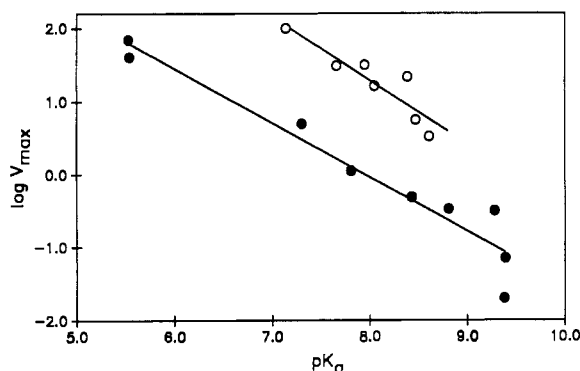
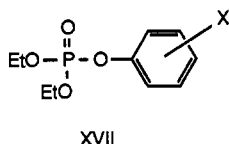


FIGURE 2: Brønsted plot for the hydrolysis of paraoxon analogues by the phosphotriesterase. The  $pK_a$  of the leaving phenol is plotted versus the value for  $\log V_{\max}$ . The slope of the upper line (O) is  $-0.8$  and the slope of the lower line is  $-0.7$  (●). The data for compounds III, V, VII-IX, XI, and XII appear on the upper line, while the data for compounds I, II, IV, X, XIII-XVI appear on the lower (see also Table I).

and initial velocity is linear with a least-squares correlation constant of 0.997.

**Structure-Activity Relationships.** The values of  $K_m$  and  $V_{\max}$  (rel) were determined for 16 paraoxon analogues (XVII) that differ only in the structure of the leaving group phenol (Table I). A plot of  $\log V_{\max}$  versus the  $pK_a$  of the leaving



phenol shows two groups of compounds that exhibit linear correlations of activity and the  $pK_a$  of the leaving group (Figure 2). The slope of the upper line is  $-0.8$ , while the slope of the lower line is  $-0.7$ . Two sets of correlations are also observed for the relationship between  $\log V_{\max}/K_m$  and  $pK_a$  (data not shown), with the upper line slope of  $-1.1$  and the lower line slope of  $-0.7$ . Less than 0.01% of the maximal activity for paraoxon hydrolysis could be detected with the *p*-fluoro, *p*-methyl, and *p*-methoxy derivatives. The chemical hydrolysis of these same paraoxon analogues gives a single line with a slope of  $-0.5$  for the plot of  $\log k$  versus  $pK_a$ , where  $k$  is the pseudo-first-order rate constant for hydrolysis in 1 M KOH (Figure 3).

**Effect of Solvent Viscosity.** The kinetic parameters for the hydrolysis of paraoxon by the phosphotriesterase in solutions of increasing solvent viscosity were determined. Shown in Figure 4 is the effect on  $V_{\max}$  and  $V_{\max}/K_m$  when sucrose is used to increase the relative viscosity. Also shown in this figure

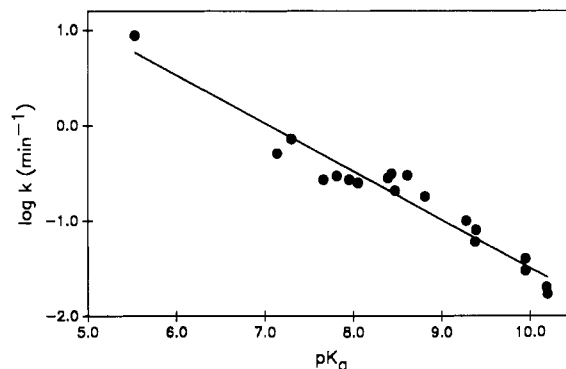


FIGURE 3: Brønsted plot for the chemical hydrolysis by 1.0 N KOH of 19 paraoxon analogues. The solid line is drawn for a slope of  $-0.5$  (see also Table I).

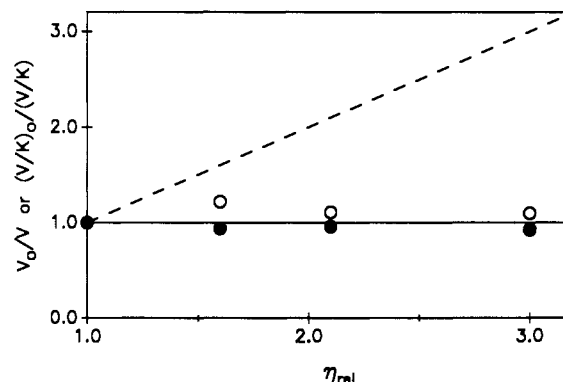


FIGURE 4: Relative kinetic parameters for the hydrolysis of paraoxon by the phosphotriesterase as a function of increasing solvent viscosity. The open (O) and solid (●) circles represent the relative values for  $V_{\max}$  and  $V_{\max}/K_m$ , respectively. The dashed line is drawn for a slope of 1.0.

is the expected correlation if the reaction rate was diffusion limited (slope = 1.0).

**Substrate Specificity.** The specificity for substrate activity exhibited by this enzyme was determined by systematically varying the substituents bonded to the central phosphorus atom. The kinetic constants for 12 analogues (III, XVIII-XXVIII) are presented in Table II. Changes were made in the length of the alkyl side chains. Other heteroatoms were substituted for the phosphoryl oxygen and the atom bridging the phosphorus and the leaving group.

**Inhibition Studies.** The elucidation of the kinetic mechanism for the enzymatic hydrolysis of phosphotriesters was attempted by determining the inhibitory effect of the products on the cleavage of paraoxon. Shown in Table III are the inhibition constants exhibited by diethyl phosphate, diethyl thiophosphate, and a variety of substituted phenols at pH 9.0. The inhibition constants for the phenols have also been corrected for the amount of ionized species. Methanol, dimethylformamide, and 1,4-dioxane are each competitive inhibitors with inhibition constants equal to 640, 126, and 63 mM, respectively. The phosphotriesterase remains active in up to 50% organic solvent (Figure 5) when the hydrolysis reaction was conducted with 0.25 mM paraoxon.

## DISCUSSION

Of the 30 analogues that have been synthesized and tested for this study, paraoxon (III) is the best substrate for the phosphotriesterase from *Ps. diminuta*. When one or both of the ethyl groups of paraoxon are removed, no activity could be detected, and thus the enzyme will not utilize phosphomonoesters or phosphodiester as substrates. If the ethyl groups are replaced with methyl substituents, then the  $K_m$

Table I: Kinetic Parameters for Hydrolysis of Phosphotriesters at pH 10

	compound <sup>a</sup>	pK <sub>a</sub>	λ <sub>max</sub> (nm)	Δ (OD/mM)	V <sub>max</sub> (rel)	K <sub>m</sub> (mM)	V/K (rel)	k <sup>b</sup> (min <sup>-1</sup> )
I		5.53	265.5	1.29	70	1.4	2.5	8.8
II		5.54	257.5	1.40	41	0.38	5.5	
III		7.14	400.0	17.0	100	0.05	100	0.51
IV		7.30	271.5	1.67	5.0	0.45	0.56	0.72
V		7.66	329.0	28.1	31	0.03	52	0.27
VI		7.81	275.5	1.49	1.1	2.70	0.02	0.03
VII		7.95	276.0	22.7	32	0.06	27	0.27
VIII		8.05	322.5	22.0	16	0.04	20	0.25
IX		8.39	253.0	5.21	22	0.41	2.7	0.28
X		8.43	291.0	2.23	0.48	0.49	0.05	0.31
XI		8.47	295.0	20.2	5.6	0.03	9.4	
XII		8.61	317.5	3.06	3.3	0.19	0.87	0.30
XIII		8.81	281.5	2.29	0.33	1.7	0.01	0.18
XIV		9.28	282.5	1.87	0.31	1.5	0.01	0.10
XV		9.38	296.5	1.91	0.02	0.21	0.005	0.06
XVI		9.39	279.5	13.0	0.07	1.8	0.002	0.08

<sup>a</sup>R = EtOP(O)(EtO)O-. <sup>b</sup>Pseudo-first-order rate constant for hydrolysis in 1 M KOH.

Table II: Kinetic Constants for the Hydrolysis of Phosphotriesters

	compound <sup>a</sup>	V <sub>max</sub> (rel)	K <sub>m</sub> (mM)	V/K (rel)
XVIII	HOP(O)(OH)OR	<10 <sup>-3</sup>		
XIX	HOP(O)(EtO)OR	<10 <sup>-3</sup>		
XX	MeOP(O)(MeO)OR	120	6.4	0.94
XXI	MeOP(O)(EtO)OR	66	0.1	33
III	EtOP(O)(EtO)OR	100	0.05	100
XXII	<i>n</i> -PrOP(O)( <i>n</i> -PrO)OR	22	0.014	79
XXIII	<i>i</i> -PrOP(O)( <i>i</i> -PrO)OR	1.5	0.005	15
XXIV	<i>n</i> -BuOP(O)( <i>n</i> -BuO)OR	2.1	0.029	3.6
XXV	<i>s</i> -BuOP(O)( <i>s</i> -BuO)OR	1.3	0.003	22
XXVI	EtOP(O)(EtO)NHR	<10 <sup>-3</sup>		
XXVII	EtOP(O)(EtO)SC <sub>6</sub> H <sub>5</sub>	0.85 <sup>b</sup>	0.19 <sup>b</sup>	5.8 <sup>b</sup>
XXVIII	EtOP(S)(EtO)OR	63 <sup>c</sup>	0.26 <sup>c</sup>	8.2 <sup>c</sup>

<sup>a</sup>R = -C<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>. <sup>b</sup>In 20% methanol. <sup>c</sup>In 10% methanol.

Table III: Inhibition Constants of Products and Organic Solvents

compound	type of inhibition <sup>a</sup>	K <sub>ii</sub> (mM)	K <sub>is</sub> (mM)	K <sub>ii</sub> <sup>b</sup> (mM)	K <sub>is</sub> <sup>b</sup> (mM)
phenol	NC	6	10	5	9
4-cyanophenol	NC	76	31	6	2
2,6-difluorophenol	NC	160	390	3	7
4-methoxyphenol	NC	17	2	16	2
thiophenol	NC	2	0.5	0.2	0.06
diethyl phosphate	UC	0.52			
diethyl thiophosphate	NC	200	200		
1,4-dioxane	C		63		
DMF	C		130		
methanol	C		640		

<sup>a</sup>NC = noncompetitive; UC = uncompetitive; C = competitive.<sup>b</sup>Corrected for actual concentration of un-ionized phenol.

increases by 2 orders of magnitude but no significant change is noted for V<sub>max</sub>. The K<sub>m</sub> and V<sub>max</sub> values decrease substantially when these groups are changed to the more hydrophobic and bulkier propyl and butyl esters. These results

suggest that the active site that binds this portion of the substrate is hydrophobic and does not accept negative charges associated with the substrate.

The enzyme is quite tolerant of changes in other parts of the substrate. The phosphoryl oxygen can be replaced with

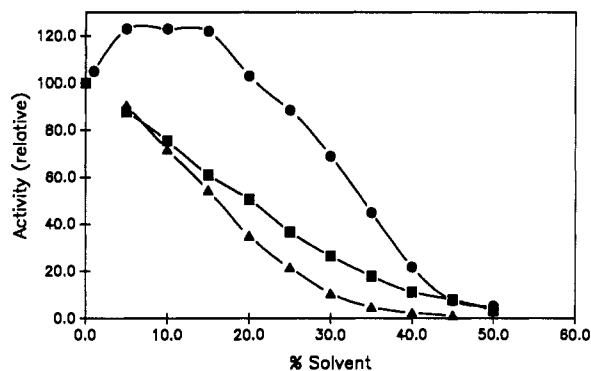


FIGURE 5: Rate of enzymatic hydrolysis of 0.25 mM paraoxon at pH 10 in the presence of increasing concentration of various organic solvents: (●) methanol; (▲) dioxane; (■) dimethylformamide.

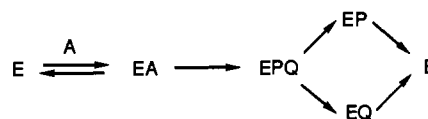
a sulfur, as in parathion (XXVIII), with only a small change in the value for  $V_{\max}$ . The 5-fold increase in the  $K_m$  for parathion is attributed primarily to the use of methanol as a cosolvent (see below). The enzyme will cleave P-S bonds, but no activity could be detected when 4-nitroaniline (XXVI) was used as the leaving group. This observation is consistent with the lack of general acid catalysis for protonation of the leaving group and the direct correlation between  $V_{\max}$  and the  $pK_a$  of substituted phenols.

**Rate-Limiting Steps.** The significance of the actual bond-breaking step in the expressed catalytic activity of the phosphotriesterase was probed by determining the correlation between the kinetic parameters for enzymatic hydrolysis and the  $pK_a$  of the substituted phenols that were used as leaving groups. The large negative  $\beta$  values in the Brønsted plot in Figure 3 clearly indicate that stabilization of the developing charge within the leaving group is the primary determinant for substrate turnover (Fersht, 1985). The effect expressed by the  $pK_a$  of the substituted phenols is actually greater in the enzymatic reaction than it is in the chemical hydrolysis by KOH. It can therefore be concluded that phosphorus-oxygen bond cleavage is the rate-limiting step in the mechanism for enzymatic phosphotriester hydrolysis.

However, the compounds used to construct the Brønsted plot for the enzyme-catalyzed reaction have segregated into two distinct groups, although the slopes are not significantly different. The group with the slower relative velocities is composed primarily of compounds with multiple fluorine substitutions, while the faster group has only electron-withdrawing substituents with  $sp^2$  hybridized atoms attached to either the meta or para position of the phenyl ring. These substituents perhaps interact with the enzyme in such a way as to induce a protein conformational state that is more favorable for catalysis. These interactions are reflected in the significantly tighter binding by those compounds found on the upper line. The average  $K_m$  for these compounds is 0.1 mM, which is an order of magnitude smaller than the average  $K_m$  for those compounds found on the lower line. Steric hindrance cannot account for the difference since the slower group is composed almost entirely of the conservative replacement of fluorine for hydrogen. This effect is not apparent in the Brønsted plot for the hydrolysis of these same compounds by KOH.

The conclusion that the bond-breaking step is rate limiting is also supported by the effect of solvent viscosity on the kinetic parameters  $V_{\max}$  and  $V_{\max}/K_m$ . When sucrose is used to increase the relative solvent viscosity approximately 3-fold, there is no significant effect on either  $V_{\max}$  or  $V_{\max}/K_m$ . If the diffusion-controlled steps were kinetically slow, the corresponding reduction in the microscopic rate constants for these steps by high levels of sucrose would have resulted in a sig-

Scheme II



nificant reduction in  $V_{\max}$  or  $V_{\max}/K_m$ <sup>1</sup> (Brouwer & Kirsch, 1982; Blacklow et al., 1988, and references cited therein).

**Acid-Base Catalysis.** Many enzymatic hydrolytic reactions utilize general base catalysis to activate a water molecule (Jencks, 1969). The pH-rate profile for the phosphotriesterase-catalyzed hydrolysis of paraoxon shows only one group that must be unprotonated for full activity. Since paraoxon contains no dissociable protons, this ionizable group must originate with the enzyme. This observation is consistent with an active-site general base that abstracts a proton from water during the catalytic event. No loss in activity is observed at high pH, and thus it appears that general acid catalysis is not involved in protonation of the phenolic oxygen. This conclusion is supported by the magnitude of the  $\beta$  values in the Brønsted plots since concerted protonation of the phenolic oxygen would reduce the dependence of the reaction on the  $pK_a$  of the leaving group. The identity of the general base is unknown, although a carboxyl and an imidazole residue are the most likely candidates.<sup>2</sup> The proton-transfer steps were also probed by determining the solvent isotope effect with  $D_2O$ . The magnitude of this effect on the maximal velocity and the linear proton inventory is consistent with only one proton in flight during the transition state (Schowen, 1977).

**Transition-State Structure.** The mechanism for phosphotriester hydrolysis in chemical systems is thought to be primarily associative, while the mechanism for phosphomonoesters proceeds through a largely dissociative transition state (Benkovic & Schray, 1973). The large negative  $\beta$  value for the enzymatic hydrolysis of phosphotriesters reported in this paper indicates that there is a large amount of negative charge development on the phenolic oxygen in the transition state, and thus the P-O bond is substantially broken. The enzyme may stabilize this transition state by charge neutralization of the leaving phenolate oxygen and/or polarization of the single phosphoryl oxygen of the substrate. Both of these functions could be provided by enzyme-bound metals such as the  $Zn^{2+}$  which appears to be associated with this enzyme. This is in contrast to the situation for the hydrolysis of phosphomonoesters, since Herschlag and Jencks (1987) have demonstrated that electrostatic interaction of divalent metals with the phosphoryl oxygens actually inhibits these reactions. The change in bond order of the phosphoryl oxygen during enzymatic phosphotriester hydrolysis is currently being determined by measurement of the secondary oxygen-18 isotope effect (Weiss et al., 1986).

**Kinetic Mechanism.** The two extreme kinetic mechanisms for a uni bi hydrolytic reaction involve either a two-step ping-pong scheme with a phosphoenzyme intermediate or a sequential mechanism with a single chemical event. The ping-pong mechanism can be readily eliminated from consideration since the stereochemical course of the phosphotriesterase reaction has been shown to be net inversion at the phosphorus center (Lewis et al., 1988). Moreover, compounds I-XVI are all hydrolyzed at different rates even though they all have diethyl phosphate as a common product. The most

<sup>1</sup> The value of  $V_{\max}/K_m$  is  $1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  (D. Dumas, unpublished observations).

<sup>2</sup> Preliminary evidence indicates that  $Zn^{2+}$  is bound to the protein (D. Dumas, unpublished observations).

general model for a random sequential release of products is illustrated in Scheme II, where A is the phosphotriester and P and Q are the two products.

In such a kinetic mechanism where the rate-limiting step is the irreversible conversion of EA to EPQ, both products are predicted to be competitive inhibitors versus the phosphotriester substrate (Cleland, 1970). All of the phenols tested as inhibitors give noncompetitive inhibition, and thus it must be postulated that the phenols also bind in dead-end fashion with the enzyme-phosphotriester complex (this provides the necessary intercept effect). The pH dependence and the inhibition constants corrected for the amount of un-ionized phenol strongly suggest that only the protonated species is the actual inhibitor. Diethyl phosphate is an uncompetitive inhibitor versus paraoxon, and thus this compound binds predominantly in dead-end fashion with the enzyme-phosphotriester complex. However, it cannot be determined from the available data whether the preferred product release scheme is random or if diethyl phosphate is released before the phenol. Since diethyl thiophosphate is a noncompetitive inhibitor, a random release of products is probably more likely.

**Summary.** The mechanism of the phosphotriesterase from *Ps. diminuta* requires a general base with a  $pK_a$  of 6.1 to activate a single water molecule for attack at the phosphorus center. The large dependence on the enzyme reaction rate on the  $pK_a$  of the leaving group phenol and the lack of a measurable effect by high viscosity show that phosphorus-oxygen bond cleavage is rate limiting.

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