# Limits of Diffusion in the Hydrolysis of Substrates by the Phosphotriesterase from *Pseudomonas diminuta*[†]

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ABSTRACT: The catalytic mechanism for the enzymatic hydrolysis of a series of paraoxon analogues by the phosphotriesterase from *Pseudomonas diminuta* has been determined. The Brønsted plots relating the pK_a of the leaving group to the observed kinetic parameters, V_{max} and V/K_m, are both nonlinear. This observation is consistent with a change in the rate-limiting step from chemical to physical events as the pK_a of the leaving group is decreased. This conclusion is confirmed by the effects of solvent viscosity on V_{max} and V/K_m for the same series of analogues. The data were fitted to the scheme

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \xrightarrow{k_3} EP \xrightarrow{k_7} E'P \xrightarrow{k_9} E + \text{products}$$
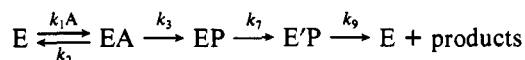
where EA is the enzyme–substrate complex, EP is the enzyme–product complex, E'P is the enzyme–product complex after a viscosity-independent unimolecular reaction, and the values for k_1, k_2, k_7, and k_9 are 4.1 $\times 10^7$ M^{-1} s^{-1}, 2550 s^{-1}, 3370 s^{-1}, and 5940 s^{-1}, respectively. The magnitude of the chemical step, represented by k_3, is dependent on the pK_a of the leaving group phenol as predicted by the Brønsted equation (log k_3 = $\beta pK_a + C$) where $\beta = -1.8$ and the constant $(C) = 17.7$. The magnitude of β indicates that the transition state for substrate hydrolysis is very product-like.

Phosphotriesterase, an enzyme that catalyzes the hydrolysis of many organophosphates (Donarski et al., 1989), hydrolyzes paraoxon with a reported V_{max}/K_m of $>4 \times 10^7$ M^{-1} s^{-1} (Dumas et al., 1989). Because of this high catalytic rate, this enzyme has generated significant interest in its ability to detoxify organophosphate pesticides and mammalian acetylcholinesterase nerve agents (Dumas et al., 1989, 1990). Equally interesting, this observed rate is very close to some other enzymatic processes approaching the diffusion-controlled limits (Albery & Hammes, 1958; Dunford & Hewson, 1977; Blacklow et al., 1988). However, these other evolutionarily optimized processes all have well-defined and specific substrates. Although this phosphotriesterase has demonstrated the ability to hydrolyze a variety of phosphotriesters, a "natural" substrate for this enzyme has remained unidentified. Thus far, the synthetic compound paraoxon remains the best substrate.

Phosphotriesterase activity was first detected in the soil microbe *Pseudomonas diminuta*, where parathion hydrolysis was observed (Munnecke & Hsieh, 1974), and this activity was later found to be associated with a protein encoded by a plasmid-borne gene (Serdar et al., 1982; Harper et al., 1989). This enzyme activity has also been detected in another soil microbe, *Flavobacterium sp.*, and a gene of identical sequence was found on a slightly smaller plasmid (Harper et al., 1989). The functional significance of the plasmid-borne gene expressing the phosphotriesterase is presently unknown. However, the evolutionary pressure on these microbes to develop an enzyme for the sole purpose of hydrolyzing organophosphate pesticides would be remarkable, given the relative short exposure of these soil bacteria to the pesticides (Schrader, 1963). Nonetheless, many organophosphate pesticides are transformed

Scheme I
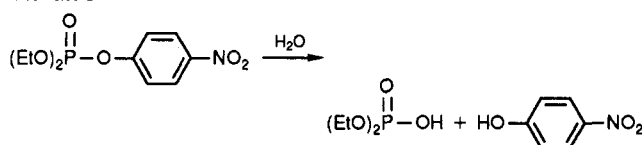
enzymatically in the soil and the resultant metabolites have been characterized (Eto, 1974; Laveglia & Dahm, 1977). These pesticides can serve as nutrient sources or be cometabolized, conjugated to naturally occurring compounds, or be accumulated within the organism. Transformation of these unnatural compounds by a preexisting enzyme may be brought about via minor changes in a gene expressing an enzyme catalyzing a related chemical reaction. The enzymatic hydrolysis of organophosphate pesticides is widespread in nature, and this capability has been identified in bacteria, yeast, fungi, and mammals (Dumas, 1989, and references cited therein). However, except for the phosphotriesterase from *P. diminuta*, none of these enzyme systems have been fully characterized.

The gene encoding the phosphotriesterase has been expressed in *Escherichia coli* and the protein purified to homogeneity (Dumas, 1989). Earlier studies have demonstrated that the mechanism of phosphotriesterase hydrolysis using paraoxon as a substrate (Lewis et al., 1988) involves the base-catalyzed attack of water at the phosphorus center resulting in the expulsion of *p*-nitrophenol and stereochemical inversion (Scheme I). On the basis of the pH rate profile (Donarski et al., 1989) and chemical modification studies (Dumas & Raushel, 1990), an unprotonated histidine is believed important in catalysis. The chemical step for substrate hydrolysis has been proposed as the rate-determining step on the basis of a substantial β value of -0.8 from a Brønsted plot with use of various phenyl-substituted phosphotriesters (Donarski et al., 1989). However, the pK_a range of this plot was limited to values between 7.1 and 8.5. The extrapolation of the existing Brønsted plot to lower pK_a values, (i.e., 4–5) would predict faster hydrolysis rates and second-order rate constants more

closely approaching diffusion-controlled limits (10^8–10^9 M^{-1} s^{-1}). In this work, several novel phosphotriesters were synthesized and their kinetic constants were measured. With these new compounds we have been able to demonstrate that the rate-limiting step for either V_{max} or V/K_m can be systematically titrated from chemical to physical events depending solely on the pK_a of the leaving group phenol. These experiments have also suggested that paraoxon is enzymatically hydrolyzed at very nearly diffusion-controlled rates.

Materials and Methods

General. The phosphotriesterase used in these experiments was isolated as previously described (Dumas, 1989) from *E. coli* carrying the cloned gene on a plasmid. The enzyme preparation was purified to a specific activity of 3000 μmol/(min·mg). All the chemicals used in this study were purchased from Aldrich Chemical Co., Sigma Chemical Co., of Fisher Scientific except for the 2- and 3-fluoro-4-nitrophenols and 2,6-difluorophenol, which were purchased from Alpha Chemical Co. Compounds II–V were synthesized as previously described (Donarski et al., 1989).

Synthesis of Phosphotriesters. Compounds I and VII–IX were synthesized by the condensation of diethyl chlorophosphate with the corresponding phenol in the presence of triethylamine. The phenol was dissolved in toluene and cooled to 4 °C under nitrogen, and a 10% molar excess of diethyl chlorophosphate was added dropwise. This was followed by the dropwise addition of a 10% molar excess of trimethylamine, and the reaction was allowed to stir overnight at room temperature. The toluene solution was washed with either 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) at pH 10.0 for compound I or water for compounds VII–IX to remove any unreacted phenol. The organic layer was dried over magnesium sulfate, filtered, and concentrated. Compound I was isolated as a white crystalline solid (mp 96–98 °C) (16.3% yield). The yields for compounds VII, VIII, and IX were between 50 and 60%. Structure determination for each of the synthesized phosphotriesters was accomplished with ¹H NMR, ¹³C NMR, and accurate resolution mass spectrometry. All spectral data were in complete agreement with the expected structure of each novel compound. The molecular ion for each phosphotriester is reported (measured/calculated): I, 273.07669/273.22786; VII, 293.04857/293.1876; VIII, 293.04857/293.1876; IX, 311.03517/311.1784.

The synthesis of 2,6-difluoro-4-nitrophenyl was accomplished by dissolving 2,6-difluorophenol (1–2 g) in 2 mL of concentrated sulfuric acid, cooling the acidic mixture to –10 °C, and adding a mixture of ammonium nitrate (a 50% molar excess) in 2 mL of sulfuric acid dropwise. The temperature was maintained at –10 °C during the addition. After stirring for 10 min, the reaction was quenched with the addition of ice. The phenol was isolated by extraction with diethyl ether, and the combined organic extracts were dried over magnesium sulfate, filtered, and concentrated. The residues were further purified by florisil chromatography (dichloromethane) to give a yellow solid (mp 99–101 °C). The ¹H NMR, ¹³C NMR, and mass spectral analysis were consistent with the desired structure.

Kinetic Measurements. The enzyme activity was routinely measured by monitoring the appearance of *p*-nitrophenol produced by the hydrolysis of 1.0 mM paraoxon at pH 9.0. All spectrophotometric determinations were made at 25 °C with a Gilford Model 260 spectrophotometer. The pK_a values and extinction coefficients for each phenol were obtained from the literature (Donarski et al., 1989) or determined spectrophotometrically and are presented in Table I. The difference

Table I: Physical Parameters for the Phosphotriesters at pH 9.0

compound[a]	structure	pK_a	λ_{max} (nm)	Δ(OD/mM)[b]
I	R—⬡—CONH₂	8.56	293	11.3
II	R—⬡—CO₂CH₃	8.47	295	18.0
III	R—⬡—COCH₃	8.05	322.5	20.6
IV	R—⬡—CN	7.95	276	19.4
V	R—⬡—CHO	7.66	329	20.5
VI	R—⬡—NO₂	7.14	400	17.0
VII	R—⬡(F)—NO₂	5.94	387	17.5
VIII	R—⬡(F)—NO₂	5.45	398	16.7
IX	R—⬡(F,F)—NO₂	4.1	395	15.2

[a] $R = (EtO)_2P(O)O-$. [b] Measured with a 10-mm cell path length.

absorbance spectrum for each of the phosphotriesters and the corresponding phenol was obtained at pH 9.0 in 50 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES), and the absorbance difference at the maximum wavelength for each phenol was used to calculate the extinction coefficient for each phenol. The phosphotriesters (20–2000 μM) were tested as substrates for the phosphotriesterase (0.01–1.0 μg/3 mL assay mixture) at pH 9.0 in 50 mM CHES, and the kinetic parameters, V_{max} and V/K_m, were measured spectrophotometrically by monitoring the production of phenol from the hydrolysis at its optimum wavelength for a period of up to 10 min. The rates of chemical hydrolysis for these compounds were determined spectrophotometrically by monitoring the absorbance change resulting from the production of the phenol upon addition of 1.0 M potassium hydroxide.

Solvent Viscosity. Sucrose and poly(ethylelene glycol) (PEG, molecular weight = 8000) were used as the viscosogens. The relative viscosity ($\eta_{rel} = \eta/\eta_0$) of the sucrose and PEG solutions was measured relative to the 150 mM CHES solution at pH 9.0 with no added viscosogen with use of an Ostwald viscometer at 25 °C. The viscosities for the sucrose solutions were similar to those of previously published values (Bazelyansky et al., 1986; Brouwer & Kirsch, 1984; Pocker & Janjic, 1987). The values with sucrose solutions were (w/w%, η_{rel}) 13.3, 1.2; 15.6, 1.4; 21.3, 1.8; 26.7, 2.1; and 30, 2.4. The values measured with poly(ethylene glycol) were (w/w%, η_{rel}) 1.7, 1.6; 3.3, 2.0; and 6.7, 3.6. An effect of the sucrose on the extinction coefficient was only observed for 4-hydroxybenzamide and was corrected for in the analysis of V_{max} and V/K_m.

Data Analysis. The kinetic data were analyzed according to the least-squares method of Cleland (1970) and applied to the appropriate equation to give the kinetic parameters and estimates of the standard error. The values for V/K_m and V_{max} were calculated from a fit of the data to

$$v = VA/(K_m + A) \tag{1}$$

where v is the initial velocity, V is the maximum velocity, K_m is the Michaelis constant, and A is the substrate concentration. The pseudo-first-order rate constants for chemical hydrolysis

Table II: Kinetic Parameters and Effect of Sucrose Viscosity on V/K and V_{max} for Substrate Hydrolysis by the Phosphotriesterase at pH 9.0

compound	V_{max} (s^{-1})	K_m (μM)	V/K_m (μM^{-1} s^{-1})	k^a (min^{-1})	$(V/K)_\eta{}^b$	$V_\eta{}^b$
I	69 ± 3	62 ± 7	1.1 ± 0.1	0.21 ± 0.02	0.007 ± 0.001	0.005 ± 0.005
II	99 ± 2	30 ± 2	3.3 ± 0.2		0.020 ± 0.002	0.011 ± 0.008
III	347 ± 11	42 ± 4	8.3 ± 0.6	0.22 ± 0.02		
IV	704 ± 31	69 ± 4	10.4 ± 0.4	0.35 ± 0.03	0.24 ± 0.02	0.19 ± 0.02
V	1575 ± 50	90 ± 7	17.9 ± 0.1	0.26 ± 0.01	0.68 ± 0.02	0.29 ± 0.03
VI	2100 ± 84	43 ± 4	48.8 ± 3.9	0.51 ± 0.03	0.97 ± 0.05	0.32 ± 0.03
VII	1972 ± 69	48 ± 5	42.5 ± 2.3	2.6 ± 0.3	0.96 ± 0.01	0.32 ± 0.01
VIII	2260 ± 52	58 ± 3	41.0 ± 2.0	3.4 ± 0.3	0.96 ± 0.06	0.36 ± 0.02
IX	1991 ± 50	52 ± 5	40.0 ± 2.0	16.2 ± 0.5	0.84 ± 0.04	0.37 ± 0.03

^{a} Pseudo-first-order rate constant for hydrolysis in 1 M KOH based on an average of at least two determinations. ^{b} The effect of solvent viscosity of the kinetic parameters, V/K_m and V_{max}, were determined from the slopes of the plots as presented in Figure 3 for paraoxon.

of VII–IX were determined with use of data collected over time for the hydrolysis of a single concentration of the substrate and fit to

$$A = A_0 e^{-kt} \qquad (2)$$

where A represents the concentration of the phosphotriester, k is the pseudo-first-order rate constant, and t is time.

X-ray Structure Determination. Compound I was crystallized from dichloromethane and diethyl ether to give colorless plates suitable for X-ray structure determination. Data collection was performed on a Nicolet R3m/V X-ray diffractometer at Texas A&M University. With use of an ω (Wyckoff) scanning technique (Wyckoff et al., 1967), 1349 unique reflections were used in the calculations. The structure was solved by Direct Methods (SHELXS, SHELXTL-PLUS program) (Sheldrick, 1988). For full-matrix least-squares anisotropic refinement, convergence of 164 least-squares parameters gave $R = 0.114$, $\omega R = 0.168$, and $S = 4.07$. The cell parameters [Triclinic, PT; $a = 8.665(2)$ Å, $b = 9.512(2)$ Å, $c = 10.137(3)°$, $\alpha = 66.71(2)°$, $\beta = 70.44(2)°$, $\gamma = 64.979(16)°$] and a volume of 681.2(3) Å^3 were calculated for the least-squares fitting of the setting angle for 25 reflections.

RESULTS

Structure–Reactivity Relationships. The kinetic constants, V/K_m and V_{max}, for the phosphotriesterase-catalyzed hydrolysis of substrates with different leaving group abilities are presented in Table II. The Brønsted plot of log V_{max} versus the pK_a of the leaving group is shown in Figure 1 and is composed of two linear segments that intersect at an approximate pK_a of 7.2. Those substrates for which the pK_a of the leaving group is <7 are all hydrolyzed at the same rate, whereas those substrates with pK_a values >7 are hydrolyzed more slowly with an increase in the pK_a. A nonlinear Brønsted relationship is also observed for the plot of log V/K_m versus the pK_a of the leaving group as illustrated in Figure 2. In contrast, the Brønsted plot for the chemical hydrolysis of these same phosphotriester analogues with potassium hydroxide shows a linear relationship between the pseudo-first-order rate constant and the pK_a of the leaving group over the entire experimental pK_a range with a β of -0.44 ± 0.03.

Effect of Solution Viscosity. The effect of solution viscosity on the kinetic parameters of several different substrates for the phosphotriesterase was determined with the use of sucrose and PEG as viscosogens. Sucrose affects both the macroviscosity and the microviscosity of the solution, whereas the addition of PEG, a polymeric viscosogen, affects only the macroviscosity (Blacklow et al., 1988). Figure 3 illustrates the effect of relative sucrose or PEG solution viscosity on the relative kinetic parameters, V/K_m and V_{max}, for the enzymatic hydrolysis of paraoxon. Since PEG does not alter either V/K_m or V_{max}, the macroviscosity effects are very small. The effect
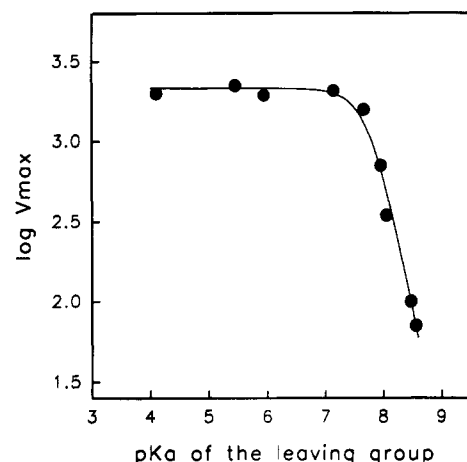

FIGURE 1: The Brønsted plot for the enzymatic hydrolysis of compounds I–IX using data from Tables I and II. The solid line represents a computer fit of the data to eqs 4 and 6 where $k_5 = 2150$ s^{-1}, $\beta = -1.84$, and $C = 17.7$.
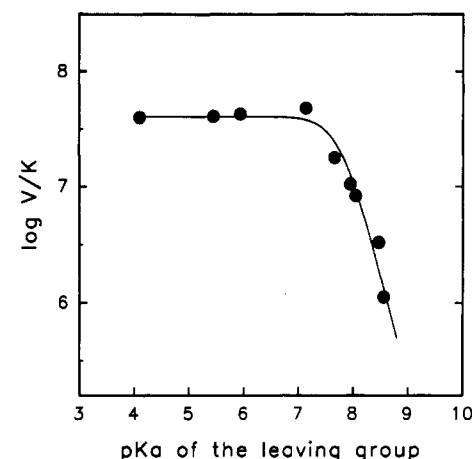

FIGURE 2: The Brønsted plot for the enzymatic hydrolysis of compounds I–IX using data from Tables I and II. The pK_a of the leaving group is plotted versus the value for log V/K. The solid line represents a computer fit of the data to eqs 5 and 6 where $k_1 = 4.1 \times 10^7$ M^{-1} s^{-1}, $k_2 = 2550$, $\beta = -1.84$, and $C = 17.7$.

of sucrose is characterized by a linear increase in the relative kinetic parameters with increasing solvent viscosity. The slopes [$(V/K)_\eta$ and V_η] obtained from Figure 3 for the sucrose effect on paraoxon hydrolysis are 0.98 and 0.32, respectively.[1] The values for $(V/K)_\eta$ and V_η for the enzymatic hydrolysis of the other substrates are presented in Table II. The observed relationship between $(V/K)_\eta$ and the pK_a of the leaving group
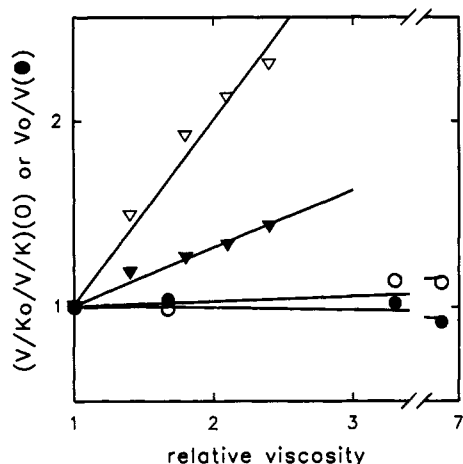
FIGURE 3: The effect of sucrose (▼) and PEG (●) as viscosogens on the relative values of V_{max} (filled) and V/K_m (open) in the hydrolysis of paraoxon.
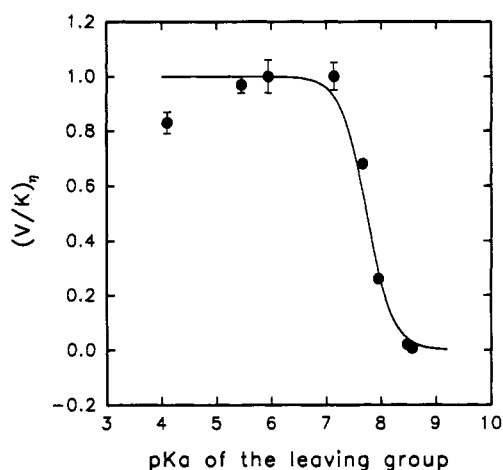

FIGURE 4: The change in the effect of solvent viscosity on the V/K kinetic values as a function of the pK_a of the leaving group. The solid line represents a fit to eqs 6 and 8 where $k_2 = 2150$ s^{-1}, $\beta = -1.84$, and $C = 17.7$.

for each substrate is graphically depicted in Figure 4 where values for $(V/K)_\eta$ vary between 0 and 1.0 for the range of compounds examined. A similar relationship is observed for the values of V_η and the pK_a of the leaving group, but as illustrated in Figure 5 the maximum value for V_η is only 0.37.

DISCUSSION

The previous kinetic analysis of the phosphotriesterase from *P. diminuta* has shown a linear correlation between the pK_a of the leaving group and the experimental values for both V_{max} and V/K_m (Donarski et al., 1989). The kinetic parameters for the hydrolysis of the insecticide paraoxon (pK_a of *p*-nitrophenol = 7.14) are 2100 s^{-1} and 4.8×10^7 M^{-1} s^{-1}, respectively. The present study was conducted in order to ascertain the absolute limits of the ability of this enzyme to hydrolyze phosphotriesters. Extrapolation of the previous Brønsted relationship for V/K_m predicts that the kinetic constants would eventually reach diffusion-controlled limits (i.e., 10^8–10^9 M^{-1} s^{-1}) with substrates having leaving group pK_a values of approximately 4–5. The reduction in the pK_a of the phenols was accomplished by substituting fluorine for hydrogen on the *p*-nitrophenyl ring of paraoxon. The data in Tables I and II indicate that the substitutions did reduce the pK_a of the phenolic leaving group but did not affect the K_m of the corresponding phosphotriester. The rate of chemical hydrolysis for these fluorine-substituted analogues (VII–IX)
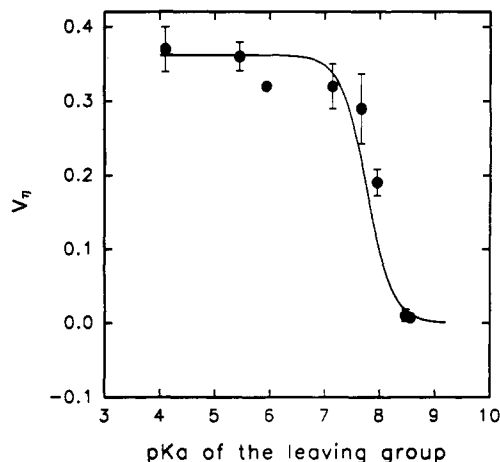

FIGURE 5: The change in the effect of solvent viscosity on the V_{max} kinetic values as a function of the pK_a of the leaving group. The solid line represent a fit to eqs 6 and 12 where $\beta = -1.84$, $C = 17.7$, $k_7 = 3370$ s^{-1}, and $k_9 = 5940$ s^{-1}.

using hydroxide anion as a nucleophile fit the extrapolated linear relationship of the Brønsted plot (Donarski et al., 1989), yielding a slope of -0.44 ± 0.03.

However, unlike the Brønsted plot for chemical hydrolysis, the Brønsted plots for the enzymatic kinetic parameters, V/K_m and V_{max}, are nonlinear. This observation strongly suggests a change in the rate-limiting step for V/K_m and V_{max} with this series of substrates for the phosphotriesterase. The linear portions for both plots, encompassing those substrates whose leaving group pK_a values are > 7 reflect a dependence of V_{max} and V/K_m on the pK_a value of the leaving group. This dependence is not observed with leaving group pK_a values < 7. The limiting values for V_{max} or V/K_m in these plots do not appear to be the result of some steric effects since the K_m values are all very similar. The linear dependence of substrates with leaving groups of pK_a > 7 suggests that chemical events are rate-limiting for these substrates. The hydrolysis of those substrates whose leaving group pK_a values are < 7 may then be limited by some physical step. This may involve a common conformational step or diffusional limitations imposed by substrate association and/or product dissociation from the enzyme surface.

Mechanistic Model for Substrate Hydrolysis. The Brønsted plots have been analyzed by use of the simplified kinetic scheme for the enzymatic hydrolysis of phosphotriesters. In this model (Scheme II), k_1 and k_2 represent the binding of the

Scheme II

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \xrightarrow{k_3} EPQ \xrightarrow{k_5} E + products$$
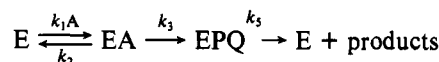
substrate to the enzyme, k_3 represents the chemical step, and k_5 is the rate constant for product dissociation. The chemical step, k_3, is assumed to be essentially irreversible because of the large free energy of hydrolysis for these reactions and the lack of any evidence for a kinetically significant reverse reaction. According to the net rate constant method (Cleland, 1975), rate expressions for the kinetic constants, V_{max} and V/K_m, can be derived where

$$V_{max} = (k_3 \ k_5)/(k_3 + k_5) \qquad (4)$$

and

$$V/K_m = (k_1 \ k_3)/(k_2 + k_3) \qquad (5)$$

The absolute value of k_3 is dependent on the β and the pK_a of the leaving group phenol as expressed by the Brønsted

relationship (Jencks & Carriuolo, 1961)

$$\log k_3 = (\beta p K_a) + C \qquad (6)$$

These equations predict that as the chemical step, k_3, becomes very fast, the maximum value for V_{max} will be limited by product release, k_5. Conversely, the relative rates of slow substrates will be inversely proportional to the pK_a of the phenol. Similarly, the limiting value for V/K will approach k_1 as the pK_a of the phenol becomes small. Utilizing the Marquardt Levenberg algorithm (Marquardt, 1963), a fit of the experimental data for V_{max} in Figure 1 to a combination of eqs 4 and 6 gives values for $k_5 = 2150 \pm 190$ s^{-1}, $\beta = -1.84 \pm 0.15$, and $C = 17.7 \pm 1.3$. The fit of these data to eqs 4 and 6 is very good as indicated by the solid line drawn in Figure 1. A similar fit of the experimental V/K_m data for substrate hydrolysis to a combination of eqs 5 and 6 using the determined values for β and C provides values for $k_1 = 4.1$ (± 0.5) $\times$ 10^7 M^{-1} s^{-1} and $k_2 = 2550 \pm 560$ s^{-1}. The solid line shown in Figure 2 represents a fit with these constants.[2]

According to Scheme II, k_1, k_2, and k_5 represent diffusional events between the enzyme and the substrate and products, and thus these rate constants will be affected by changes in solution viscosity. It can be demonstrated for the kinetic parameter, V/K_m, that

$$(V/K)_0/(V/K) = (k_3 \eta_{rel} + k_2)/(k_2 + k_3) \qquad (7)$$

Equation 7 predicts a linear relationship between the relative second-order rate constant, $(V/K)_0/(V/K)$, and the relative viscosity as demonstrated in Figure 3 for paraoxon and in studies of other enzymatic reactions (Blocklow et al., 1988; Bazelyansky et al., 1986; Brouwer & Kirsch, 1982).[3] The slope of this line provides a measure of the sensitivity on V/K_m to viscosity changes. From eq 7, the slope is defined by

$$(V/K)_\eta = k_3/(k_2 + k_3) \qquad (8)$$

Therefore, the value of $(V/K)_\eta$ can vary from 0 to 1 depending on the relative magnitude of k_2 and k_3. As k_3 becomes infinitely large, $(V/K)_\eta$ approaches 1, and as k_3 becomes very small, $(V/K)_\eta$ approaches zero. Figure 4 depicts the relationship between the pK_a of the leaving group and the measured value for $(V/K)_\eta$. The above evaluation of the nonlinear Brønsted plot (Figure 2) predicts the enzymatic hydrolysis rates of those substrates with leaving groups whose pK_a values are < 7 are limited by physical diffusion. Therefore, this analysis predicts that the value for $(V/K)_\eta$ at one extreme should approach a limiting value of 1.0 when k_3 is very large and at the opposite extreme should approach 0 when k_3 is relatively small. Figure 4 is drawn with use of the values for k_2 and k_3 calculated above the eq 8. The excellent fit of the viscosity data to the predicted values, based on the rate constants obtained from the Brønsted plot analysis, provides strong support for this kinetic analysis.

Product dissociation, represented by k_5 in Scheme II, is also affected by solution viscosity, and therefore, the magnitude of the product dissociation step, relative to the maximal velocity, can be measured. The effect of relative viscosity on the relative V_{max}, V_0/V, can be related by the equation

$$V_0/V = (k_3 \eta_{rel} + k_5)/(k_3 + k_5) \qquad (9)$$

where the "slope effect" is defined as

$$V_\eta = k_3/(k_3 + k_5) \qquad (10)$$

This relationship predicts extremes from 0 to 1.0 depending on the relative magnitude of k_3 and k_5 and assumes no other unimolecular transformations preceding the actual product release step. However, as illustrated in Figure 5, a limiting value of 0.37 is observed and suggests that the simple model proposed in Scheme II is not valid and that a more complicated model must be formulated. If a viscosity-independent unimolecular step is added, a new mechanistic model can be proposed (Scheme III) where k_3 is substrate-dependent, k_7 is

Scheme III

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \xrightarrow{k_3} EPQ \xrightarrow{k_7} E'PQ \xrightarrow{k_9} E + \text{products}$$
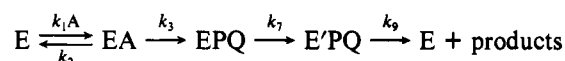
substrate- and viscosity-independent, and k_9 is viscosity-dependent. Inclusion of this extra step requires the expansion of eq 9 where

$$V_0/V = (k_3 k_7 \eta_{rel} + k_7 k_9 + k_3 k_9)/(k_3 k_7 + k_7 k_9 + k_3 k_9) \qquad (11)$$

and the viscosity effect on V_{max} is defined as

$$V_\eta = k_3 k_7/(k_3 k_7 + k_7 k_9 + k_3 k_9) \qquad (12)$$

From this equation, limits on V_η can be predicted. As k_3 becomes very small, V_η approaches zero, but as k_3 becomes infinitely large

$$V_\eta = k_7/(k_7 + k_9) \qquad (13)$$

A fit of eq 12 to the experimental data for V_η is shown as a solid line in Figure 5 with use of the previously obtained values for β and C and operating under the constraint where

$$k_5 = k_7 k_9/(k_7 + k_9) = 2150 \text{ s}^{-1} \qquad (14)$$

The fit was performed with use of the Marquardt Levenberg algorithm and yielded values for $k_7 = 3370 \pm 90$ s^{-1} and $k_9 = 5940 \pm 280$ s^{-1}. This fit is consistent with the new kinetic model and indicates that product dissociation is partially diffusion-limited with substrates whose leaving group pK_a values are < 7. As the pK_a of the leaving group increases, k_3 becomes smaller and therefore more rate-determining and the solution viscosity effect on product dissociation, V_η, becomes less pronounced. When the chemistry of substrate hydrolysis becomes totally rate-limiting, no effect on V_η is observed.

Diffusion-Controlled Rates. The Brønsted analysis and the measured effect of solution viscosity on the second-order rate constant for several substrates of phosphotriesterase have provided an upper limit for the rate of productive collisions between enzyme and substrate. However, this observed rate constant, k_1 (4.1 $\times$ 10^7 M^{-1} s^{-1}), is at least 1 order of magnitude smaller than might be expected for a diffusion-controlled process. Since there are no observable effects on either V_{max} or V/K_m by sucrose for the slowest of the substrates used in this study, sucrose must only function as a viscosogen. Therefore, the maximal effects of solvent viscosity on V/K_m with the best substrates can be utilized to support the conclusion that V/K_m is limited by diffusion.

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[2] Donarski et al. (1989) reported β values of -0.8 and -1.1 for the effect of the leaving group pK_a on V_{max} and V/K_m, respectively. These values are somewhat smaller than the value of -1.8 reported in this paper. The kinetic constants reported here for compounds II–VI are within the experimental errors of those kinetic constants determined previously except for that of compound V. The larger β value is attributed to the fact that the previous analysis was limited to a narrower range of pK_a values and no consideration was given to the apparent nonlinearity of the data as the pK_a values approach 7.

[3] Equations 7, 9, and 11 were derived by starting from the relevant equations for either V_{max} or V/K_m. The rate constants k_1, k_2, k_5, and k_9 were assumed to be viscosity-dependent. These constants were then replaced in the velocity equations with the appropriate terms (k_x/η_{rel}) to obtain the expressions for the effect of solvent viscosity on the kinetic parameters V_{max} and V/K_m.
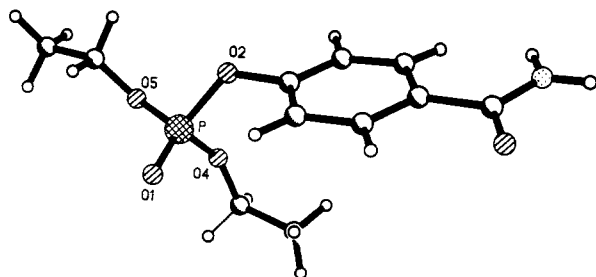
FIGURE 6: X-ray structure of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate.

Theoretical treatments (Schmitz & Schurr, 1972; Hill, 1975) and experimental results from several enzyme systems (Blacklow et al., 1988; Dunford & Hewson, 1977) indicate that diffusion-controlled enzymatic reactions can occur with rate constants of approximately 10^8 M^{-1} s^{-1} or less. On the basis of the model by Schmitz and Schurr (1972), it is theorized that the diminution of the observed diffusion-controlled rate of the reaction is a result of angular constraints between a planar surface with a target area (active site) and the mobile ligand (substrate) that lies above this planar surface. The calculated second-order rate constant for the association of substrate and the phosphotriesterase is 4.1×10^7 M^{-1} s^{-1}. This is approximately 45 times slower than the diffusion-controlled rate constant of 2×10^9 M^{-1} s^{-1} for the theoretical model. If the active site represents the target area, the size of the active site can be estimated by use of the reported radius of 22 Å for the monomeric enzyme (Dumas et al., 1990). The total surface area of the sphere would be 6100 $Å^2$ and 1/45th of this value would then represent the potential target area of approximately 135 $Å^2$ for the active site. The X-ray crystal structure of compound I (see Figure 6) has dimensions of 12.03 Å for the long axis and a maximum perpendicular width of 5.05 Å, and thus, substrates of this size can clearly be accommodated by the minimum active site dimensions calculated above. There are, however, other factors that may also result in the observed V/K_m values of less than the theoretical limit. For example, substrate binding may require a particular conformational state at the active site. Electrostatic effects and ion pairing are unlikely factors since these substrates are all neutral.

The effects of solvent viscosity on V_{max} are small relative to the effects on V/K_m. As indicated by the analysis with Scheme III, these results are consistent with a model whereby a partially rate-limiting unimolecular step precedes the final product release step(s). This last product dissociation step may involve the release of the two products and/or the transfer of a proton from a histidine ($pK_a = 6.1$) to the bulk solvent. The proton transfer rate to H_2O is estimated at approximately 10^4 s^{-1} (Fersht, 1985). This analysis is also consistent with the observed solvent deuterium isotope effects in the enzymatic hydrolysis of paraoxon where $^DV = 2.4$ and $^D(V/K_m) = 1.2$ (Donarski et al., 1989). It is interesting to note that the η_{rel} for D_2O is 1.24 (Hardy & Kirsch, 1984) and thus for paraoxon hydrolysis the $^D(V/K_m)$ effect is due entirely to viscosity changes. However, it has not been determined whether D_2O causes other changes to the protein that may be manifested in the isotope effects on V_{max}.

Transition-State Structure. The kinetic analysis of this enzymatic mechanism yields a rather large β value of -1.8 compared to the values of -1.0 and -0.44 observed for the chemical hydrolysis using water (Khan & Kirby, 1970) and hydroxide, respectively, as the nucleophiles. Therefore, for the enzymatic reaction, there is a higher sensitivity of the

leaving group to the rate of hydrolysis. The β value of the equilibrium of transfer of acyl oxyanions has been reported as high as -1.7 (Jencks & Gilchrist, 1968), and thus the observed value for the enzymatic reaction is very near the limiting extreme. This result is consistent with a significant amount of charge transferred to the phenol in the transition-state structure and thus implies that the P–O bond is very nearly completely broken. The observed difference between the β values for chemical and enzymatic hydrolysis may be attributed to a hydrophobic environment at the active site (Jencks, 1971) and the participation of active site residues or the essential zinc atom. In the following paper the structure of the transition state is probed in finer detail by measurement of the primary and secondary oxygen-18 isotope effects.

Evolution of Catalytic Activity. According to the theory proposed by Knowles and Albery (1977), an enzyme that performs its catalytic function at or near diffusion-controlled rates has reached the end of its evolutionary development. The commercially utilized insecticide, paraoxon, is hydrolyzed by the phosphotriesterase at rates that approach the diffusion-controlled rate. Since a natural substrate and the functional significance of this enzyme are unknown, it is difficult to speculate about substrate specificity and rate of reaction in terms of evolutionary consequences. However, it does suggest that perhaps the transformation of a preexisting enzyme to one with altered specificity and reactivity can occur in a relatively short period of time. Significant primary amino acid sequence homology with other enzymes has not been detected, but the alteration (evolution) of an optimized protease or phosphodiesterase to an enzyme with phosphotriesterase activity could be accomplished with a small number of amino acid changes.

SUPPLEMENTARY MATERIAL AVAILABLE

Experimental details and seven tables listing atomic coordinates, bond lengths, bond angles, anisotropic displacement parameters, hydrogen atom coordinates, and torsion angles and nonbonded distances for the X-ray structure determination of compound I (18 pages). Ordering information is given on any current masthead page.

Registry No. I, 6376-03-0; II, 120579-20-6; III, 16906-51-7; IV, 6132-16-7; V, 22955-88-0; VI, 311-45-5; VII, 134458-80-3; VIII, 134458-81-4; IX, 134458-82-5; phosphotriesterase, 9047-01-2; sucrose, 57-50-1.

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Transition-State Structures for Enzymatic and Alkaline Phosphotriester Hydrolysis[†]

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ABSTRACT: The primary and secondary ^{18}O isotope effects for the alkaline (KOH) and enzymatic (phosphotriesterase) hydrolysis of two phosphotriesters, *O,O*-diethyl *p*-nitrophenyl phosphate (I) and *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate (II), are consistent with an associative mechanism with significant changes in bond order to both the phosphoryl and phenolic leaving group oxygens in the transition state. The synthesis of [^{15}N, *phosphoryl*-^{18}O]-, [^{15}N, *phenolic*-^{18}O]-, and [^{15}N]-*O,O*-diethyl *p*-nitrophenyl phosphate and *O,O*-diethyl *O*-(4-carbamoylphenyl)phosphate is described. The primary and secondary ^{18}O isotope effects for the alkaline hydrolysis of compound I are 1.0060 and 1.0063 ± 0.0001, whereas for compound II they are 1.027 ± 0.002 and 1.025 ± 0.002, respectively. These isotope effects are consistent with the rate-limiting addition of hydroxide and provide evidence for a S_N2-like transition state with the absence of a stable phosphorane intermediate. For the enzymatic hydrolysis of compound I, the primary and secondary ^{18}O isotope effects are very small, 1.0020 and 1.0021 ± 0.0004, respectively, and indicate that the chemical step in the enzymatic mechanism is not rate-limiting. The ^{18}O isotope effects for the enzymatic hydrolysis of compound II are 1.036 ± 0.001 and 1.0181 ± 0.0007, respectively, and are comparable in magnitude to the isotope effects for alkaline hydrolysis, suggesting that the chemical step is rate-limiting. The relative magnitude of the primary ^{18}O isotope effects for the alkaline and enzymatic hydrolysis of compound II reflect a transition state that is more progressed for the enzymatic reaction.

The mechanism of phosphoryl transfer has been studied in detail because of the importance of this reaction for biological systems. Extensive characterization of phosphoryl transfer

from phosphate monoesters and diesters in enzymatic processes has been made in terms of general chemical mechanisms (Bruice & Benkovic, 1966; Benkovic & Schray, 1973; Westheimer, 1981). However, the current limited understanding of chemical phosphotriester hydrolysis has not been applied to enzymatic hydrolytic reactions because unlike phosphate monoesters and diesters, which serve as specific substrates for well-characterized enzymes, a naturally oc-