



STEREOSPECIFIC ENZYMATIC HYDROLYSIS OF PHOSPHORUS-SULFUR BONDS IN CHIRAL ORGANOPHOSPHATE TRIESTERS

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Abstract: The bacterial phosphotriesterase has been shown to be capable of catalyzing the hydrolysis of a P-S bond in organophosphate triesters. The V_{\max} values for the hydrolysis of the insecticides, acephate (O, S-dimethyl N-acetylphosphoramidothioate) and methamidophos (O, S-dimethyl phosphoramidothioate) are 140 and 6 min^{-1} , respectively. The (S_P)-enantiomers are hydrolyzed at a rate that is 100-fold faster than the (R_P)-enantiomers.

Introduction

The bacterial phosphotriesterase from *Pseudomonas diminuta* and *Flavobacterium* sp. is a monomeric metallo-enzyme with a molecular weight of 36,000.^{1,2} It is known that this enzyme can catalyze the cleavage of the phosphorus-oxygen bond in a variety of organophosphate triesters such as paraoxon and parathion^{3,4} and

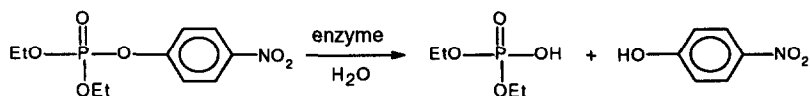


Figure 1. Enzymatic Cleavage of Phosphorus-Oxygen Bond in Paraoxon

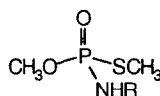
of the phosphorus-fluorine bond in the acetylcholinesterase inhibitor, DFP (diisopropyl fluorophosphate).⁵ Two divalent metal ions are essential for full catalytic activity. Zn^{2+} is present in the native enzyme, however, this metal can be replaced with other divalent metal ions such as Mn^{2+} , Cd^{2+} , Co^{2+} , or Ni^{2+} .¹ The X- and Q-band EPR spectra of the Mn-substituted enzyme have provided evidence to indicate that the two metal ions are bound to the bacterial phosphotriesterase in close proximity and are most likely bridged via a common ligand.⁶ A site-directed mutagenesis study has indicated that histidine residues are the primary metal binding ligands and that they also have a critical role in the catalytic activity of the phosphotriesterase.⁷

The reaction mechanism catalyzed by the phosphotriesterase has been shown to involve the base-catalyzed attack of water directly at the phosphorus center resulting in the inversion of stereochemistry.⁸

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Therefore, the formation of a phosphorylated-enzyme intermediate in the reaction mechanism is highly unlikely.⁹ The bacterial phosphotriesterase has been found to hydrolyze only the (*S_P*)-isomer of the common pesticide, ethyl p-nitrophenyl phenylthiophosphate (EPN).⁸ The (*R_P*)-EPN enantiomer is not hydrolyzed by the enzyme at an appreciable rate.

Acephate (O,S-dimethyl N-acetylphosphoramidothioate, (I)), and methamidophos (O,S-dimethyl phosphoramidothioate, (II)) are chiral organophosphates that have a wide range of insecticidal activity.¹⁰ Optically



I. R = COCH₃

II. R = H

pure enantiomers of both compounds have been previously prepared by Miyazaki *et al.*¹⁰ The (*R_P*)-enantiomers have been found to be more potent insecticides against houseflies than either the racemate or the other stereoisomer. Against the German cockroach, however, the (*S_P*)-enantiomers are more toxic at shorter incubation times. In the present paper, it has been demonstrated that the bacterial phosphotriesterase selectively hydrolyzes one of the two enantiomers of acephate and methamidophos by specific cleavage of the phosphorus-sulfur bond in these insecticides.

Results and Discussion

Enzymatic Hydrolysis of the Phosphorus-Sulfur Bond. The bacterial phosphotriesterase was tested as a catalyst for the hydrolysis of the phosphorus-sulfur bond in acephate and methamidophos. The ³¹P NMR spectrum of a reaction mixture initially containing 50 mM acephate (or 50 mM methamidophos) in 50 mM HEPES buffer, pH 8.0, with Co-phosphotriesterase exhibited two resonances after 12 h of incubation. One resonance originated from the substrate (35 ppm for acephate; 42 ppm for methamidophos), while the other was assigned to the corresponding hydrolysis product (-3.1 ppm for O-methyl N-acetylphosphoramidate; 11 ppm for O-methylphosphoramidate). The reaction mixture containing dithionitrobenzoic acid (DTNB) produced a strong yellow color that arose from the reaction of the liberated CH₃SH with DTNB. After one day of incubation, other small resonances (< 10%) in the ³¹P NMR spectrum were apparent (16 and 26 ppm for acephate; 25 and 26 ppm for methamidophos). However, these new products were the result of the non-enzymatic decomposition of either substrate. This conclusion was confirmed by incubation of the reaction mixture without enzyme under otherwise identical reaction conditions. These results clearly indicate that the phosphotriesterase can specifically cleave the P-S bond of these substrates.

Table I. Kinetic Constants for Racemic Acephate and Methamidophos Hydrolysis by Metal Substituted Phosphotriesterase at pH 6.95 and 25 °C

Substrate	Metal-phosphotriesterase	K_m (mM)	V_{max} (min ⁻¹)	V_{max}/K_m (mM ⁻¹ min ⁻¹)
Acephate	Zn-	45 ± 2	11 ± 0.3	0.24 ± 0.01
Acephate	Mn-	75 ± 40	4.1 ± 1.6	0.06 ± 0.01
Acephate	Co-	97 ± 6	140 ± 7	1.4 ± 0.03
Methamidophos	Co-	88 ± 19	6.0 ± 1.0	0.068 ± 0.005
Paraoxon ^a	Co-	0.05	1.2 × 10 ⁵	2.5 × 10 ⁶
DFP ^a	Co-	0.1	2.5 × 10 ³	2.5 × 10 ⁴

^aAt pH 7.0 and 25 °C. The kinetic constants were obtained by converting the units from the data in reference 4.

Kinetic Analysis. The kinetic constants, K_m , V_{max} , and V/K_m , for the different metal phosphotriesterase-catalyzed hydrolysis of racemic acephate and methamidophos are presented in Table I. All kinetic analyses for acephate and methamidophos were performed using DTNB as a coupling reagent. At concentrations of DTNB of <1.0 mM, the hydrolysis rates were not affected by the DTNB concentration. At higher concentrations, the rates were somewhat reduced (approximately half of the maximal rates are observed at 3.33 mM DTNB concentration).

The kinetic constants, V_{max} and V_{max}/K_m , for the enzymatic hydrolysis of acephate, observed for the Co-phosphotriesterase, are approximately 20 times higher than those observed for methamidophos. However, the K_m values are similar. The V_{max}/K_m values for acephate are approximately 6 and 4 orders of magnitude lower than those previously observed for paraoxon and DFP, respectively. Moreover, the K_m values for acephate and methamidophos are approximately 3 orders of magnitude higher than those obtained for paraoxon and DFP. For the hydrolysis of acephate, the K_m values are lowest for the Zn-enzyme while somewhat higher values are observed for the Co- and Mn-enzymes. The V_{max}/K_m value, however, is highest for the Co-enzyme (25 times higher than the Mn-enzyme) followed by the Zn-enzyme. The V_{max}/K_m and K_m values for acephate and methamidophos indicate that these substrates are hydrolyzed very slowly and are binding very weakly to the enzyme relative to paraoxon and DFP. Thus, the cleavage rate of the P-S bond is very much slower compared to those rates previously observed for the hydrolysis of P-O and P-F bonds in other substrates.

Kinetic Resolution of Reaction Products. The time course for the enzymatic hydrolysis of acephate is shown in Figure 2. There is a distinct change in the hydrolysis rate after approximately half of the acephate has been consumed. Similar results (data not shown) were observed for the hydrolysis of methamidophos. Thus, it appears that one enantiomer is hydrolyzed at a much faster rate than the other

isomer. The relative rate of hydrolysis for the two isomers (at 25 mM each) of acephate and methamidophos are 150 and 90, respectively. In order to determine the enantiomeric preference exhibited by the phosphotriesterase the enzymatic reaction was allowed to proceed halfway and then the reaction mixture was extracted with chloroform.

The ^{31}P NMR spectrum indicated that the chloroform extracted material from the acephate hydrolysis mixture was predominantly unreacted starting material rather than hydrolysis products (95/5). For the hydrolysis of methamidophos the ratio of reactant to product was 88/12 from the integration of the two resonances in the ^{31}P NMR spectrum. The specific optical rotations obtained were as follows: acephate hydrolysis reaction, $[\alpha]_{\text{D}} +62^\circ$ (CHCl_3); methamidophos hydrolysis reaction, $[\alpha]_{\text{D}} +47^\circ$ (CHCl_3). The literature values¹⁰ for the pure enantiomer of each compound have the following specific optical rotations in chloroform: (+)-acephate, $[\alpha]_{\text{D}} +64^\circ$; (-)-acephate, $[\alpha]_{\text{D}} -62^\circ$; (+)-methamidophos, $[\alpha]_{\text{D}} +55^\circ$; (-)-methamidophos, $[\alpha]_{\text{D}} -54^\circ$. Thus, it can be concluded that the (-)-stereoisomers of acephate and methamidophos react at least two orders of magnitude faster than their enantiomers. The absolute configurations of the phosphorus centers for both (+)-enantiomers of acephate and methamidophos are known to be R_{P} . Therefore, the bacterial phospho-triesterase stereospecifically hydrolyzes the (S_{P})-enantiomers in preference to the (R_{P})-enantiomers. These results are presented graphically in Figure 3.

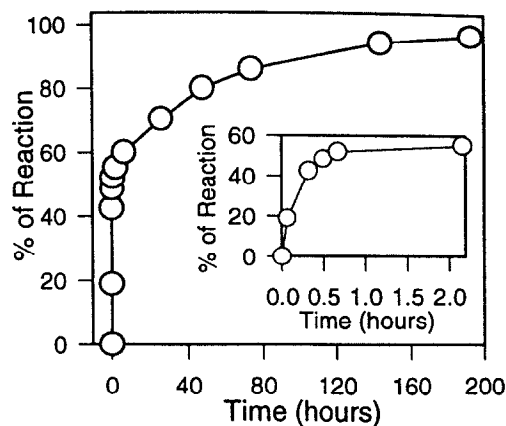


Figure 2. Time course for the hydrolysis of acephate catalyzed by the Co-phosphotriesterase. The reaction was followed by ^{31}P NMR spectroscopy. The initial conditions in the NMR tube were as follows: racemic acephate, 50 mM; 150 mM HEPES buffer, pH 8.0; Co-phosphotriesterase, 1.29 mg/mL.

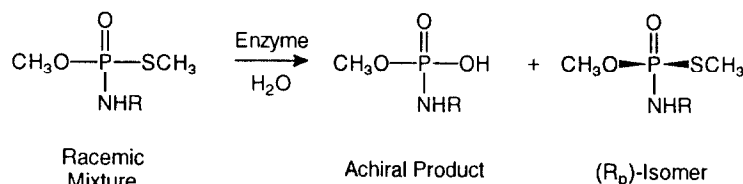


Figure 3: Enzymatic Hydrolysis of Chiral Organophosphate Triester

Experimental Methods

Methods. ^1H NMR and ^{31}P NMR spectra were obtained on a Varian XL-200 spectrometer. Typical acquisition parameters for the phosphorus spectra were 17,953 Hz sweep width, 0.836 s acquisition time, and 10 μs pulse width. Specific optical rotations were obtained using a Jasco DIP-360 digital polarimeter with a cell path length of 10 mm.

Enzyme Preparation. The phosphotriesterase used in this study was purified from an *E. coli* strain containing the plasmid pJK01.⁷ The enzyme was purified as previously described.¹ Apoprotein was prepared by incubating phosphotriesterase (~1.0 mg/mL) with 2 mM 1,10-phenanthroline overnight. The solution was then diafiltered with an Amicon ultrafiltration device fitted with a PM10 membrane (Amicon Corporation, Danvers, MA) to remove the metal chelator complex and excess chelator. The process of diafiltration was followed by monitoring the disappearance of absorbance at 327 nm. The divalent metal-activated form of phosphotriesterase was prepared by incubating the apoenzyme at ~1 mg/mL in 50 mM HEPES-KOH, pH 8.3, with two equivalents of metal at 4 °C for at least 40 hr. The enzyme concentration was determined spectrophotometrically using an $\epsilon_{280} = 2.9 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$.

Enzyme Assays. The enzymatic activities of the phosphotriesterase were determined by the method of Ellman, which monitors the change in absorbance at 412 nm.^{11,12} The change in absorbance at this wavelength is caused by reaction of DTNB (5,5'-dithiobis (2-nitrobenzoic acid) with the liberated thiol from the reaction of substrates and enzyme. The molar extinction coefficient of the 4-nitrophenolate anion is $14,150 \text{ M}^{-1}\text{cm}^{-1}$ at 412 nm.¹² Typically, the concentration of DTNB used for the assays was 0.33 mM. Each reaction was initiated by adding an enzyme solution to the preincubated solution of substrate and DTNB.

A Gilford UV-Vis spectrometer (Model 260) was used and regulated at 25 °C. Kinetic parameters were determined by fitting the kinetic data to eq. (1) using the computer programs provided by Sahara Shell Software. In this equation, v is initial velocity, V_m is the maximal velocity, K_m is the Michaelis constant and A is the initial substrate concentration.

$$v = V_m A / (K_m + A) \quad (1)$$

Sample Preparations for Measurement of Specific Optical Rotation. The racemic mixture of acephate (80 mM) in 150 mM HEPES, pH 8.0, containing 0.15 mg/mL of Co-phosphotriesterase was incubated at room temperature and the reaction was followed by ^{31}P NMR spectroscopy. When the reaction was half-complete, the enzyme was denatured by the addition of 6 N HCl to obtain a pH of 3.0. The reaction mixture was then extracted with chloroform three times and the chloroform layer was dried with MgSO_4 and then evaporated. An oily liquid

(34 mg) was obtained. The purity of this compound was at least 95 % based on the integrated areas of ^{31}P NMR and ^1H NMR spectra [^{31}P NMR δ 35; ^1H NMR δ 3.86 (*d*, 3H), 2.39 (*d*, 3H), 2.18 (*d*, 3H), 2.05 (broad NH)]. The observed NMR data are identical to those obtained for the racemic acephate. The ^{31}P NMR spectrum of the aqueous layer gave two distinct peaks at 35 ppm (acephate) and -3.1 ppm (hydrolysis product).

The racemic mixture of methamidophos (70 mM) in 150 mM HEPES, containing 0.19 mg/mL Co-phosphotriesterase was incubated at room temperature until the reaction was half-completed. The hydrolysis products were extracted with chloroform as described above. A white solid (13 mg) was obtained that contained 88 % methamidophos and 12 % of the hydrolysis product as determined by ^{31}P NMR spectroscopy.

^{31}P NMR Spectroscopy for Time Course Reaction of Acephate. The racemic mixture of acephate (30.2 mg) was dissolved in a mixture of 0.3 mL of 1.0 M HEPES buffer, pH 8.5, and 0.5 mL of D_2O . The NMR acquisition was started as soon as 2.5 mL of Co-phosphotriesterase (1.7 mg/mL in 50 mM HEPES, pH 8.3) was added. The final concentrations in the NMR tube were as follow: acephate, 50 mM; buffer, 150 mM HEPES buffer, pH 8.0; Co-phosphotriesterase, 1.29 mg/mL.

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