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Purification and Properties of the Phosphotriesterase from *Pseudomonas diminuta**

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The phosphotriesterase produced from the *opd* cistron of *Pseudomonas diminuta* was purified 1500-fold to homogeneity using a combination of gel filtration, ion exchange, hydrophobic, and dye matrix chromatographic steps. This is the first organophosphate triesterase or organophosphofluoridate hydrolyzing enzyme to be purified to homogeneity. The enzyme is a monomeric, spherical protein having a molecular weight of 39,000. A single zinc atom is bound to the enzyme and is required for catalytic activity. Incubation with metal chelating compounds, *o*-phenanthroline, EDTA, or 2,6-pyridine dicarboxylate inactivate the enzyme. The kinetic rate constants, k_{cat} and k_{cat}/K_m, for the hydrolysis of paraoxon are 2100 s^{-1} and 4 × 10^7 M^{-1} s^{-1}, respectively. The enzyme is inhibited competitively by dithiothreitol, dithioerithritol, and β-mercaptoethanol. In addition to paraoxon the phosphotriesterase was found to hydrolyze the commonly used organophosphorus insecticides, dursban, parathion, coumaphos, diazinon, fensulfothion, methyl parathion, and cyanophos.

An enzyme capable of hydrolyzing the acetylcholinesterase inhibitor diisopropylfluorophosphate (DFP)[1] was first reported by Mazur (1) in the same year the synthesis of DFP was published (2). DFPases have since been discovered in *squid* (3), *bacteria* (4), *protozoa* (5), *fresh water clam* (6), and many mammals (7). While none of these enzymes will catalyze the hydrolysis of the related acetylcholinesterase inhibitors parathion and paraoxon, several parathionases and paraoxonases have been described. Among other sources, these enzymes are found in mammals (8), insects (9), bacteria (10), and fungi (11). Despite over 40 years of study, no organophosphate hydrolyzing enzyme[2] has ever been purified to homogeneity. One paraoxonase, the phosphotriesterase from *Pseudomonas diminuta*, has recently been shown to hydrolyze DFP (12) and a wide variety of organophosphorus triesters (12–14). The hydrolysis of these compounds can be generalized by Equation 1:

$$R-\underset{R'}{\overset{X}{\overset{\|}{\underset{|}{P}}}}-Z \xrightarrow{H_2O} HO-\underset{R'}{\overset{X}{\overset{\|}{\underset{|}{P}}}}-R + ZH \qquad (1)$$

where X = oxygen or sulfur (13), R is an alkoxy group ranging in size from methoxy to butoxy, R' is an alkoxy or a phenyl group (14), and Z is either a phenoxy group or a fluorine (12). Donarski *et al.* (13) showed the rate of the enzyme catalyzed hydrolysis to be inversely proportional to the pK_a of the leaving phenol (β_{lg} = −0.8). Furthermore, Lewis *et al.* (14) have shown that only the S_P entantiomer of *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate (S_P-EPN) is hydrolyzed by the phosphotriesterase, and this reaction occurs through the net inversion of stereochemistry at the phosphorus center. This result was interpreted as evidence for a general base mechanism having a single in line displacement at the phosphorus center with an activated water molecule.

The gene for the phosphotriesterase (*opd*, organophosphorus degrading) from *P. diminuta* has been shown to be plasmid borne (15), which has allowed the cloning of the gene into various expression systems using standard techniques. McDaniel *et al.* (16) have expressed this gene in *Escherichia coli* and a partially purified protein preparation has been obtained (12). Recently, it has been shown by Harper *et al.*[3] that overexpression of the *opd* gene product could be achieved in a baculovirus expression system when the gene is inserted behind the polyhedron protein promoter. Insect tissue cultures infected with this recombinant baculovirus produce phosphotriesterase that represents about 0.03% of the total cell mass.

This report details the purification and physical characterization of the phosphotriesterase from the baculovirus expression system. This is the first time that any DFPase, paraoxonase, parathionase, or organophosphate triesterase has ever been purified to homogeneity and characterized.

MATERIALS AND METHODS

General—Enzymatic activity was routinely measured by monitoring the absorbance at 400 nm as 0.75 mM paraoxon was hydrolyzed to diethylphosphate and *p*-nitrophenol (ϵ_{400} = 17,000 M^{-1} cm^{-1}) in 150 mM CHES, pH 9.0, buffer using a Gilford model 260 spectrophotometer regulated at 25 °C. One unit of activity is defined as the hydrolysis of 1 μmol of paraoxon/min. Protein concentration in crude samples was determined by measuring the absorbance at 280 nm or by the

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[1] The abbreviations used are: DFP, diisopropyl fluorophosphate; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; DTE, dithioerythritol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; FPLC, fast protein liquid chromatography; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[2] "Organophosphate hydrolyzing enzyme" is used as a general term to describe those enzymes which catalyze the hydrolysis of organophosphate triesters and organophosphofluoridates. This description is not meant to include the many organophosphate monoesterases or diesterases.

[3] L. L. Harper, F. M. Raushel, V. Luckow, M. Summers, and J. R. Wild, *Virology*, manuscript submitted for publication.

bicinchoninic acid assay method developed by Smith *et al.* (18) (Pierce) with bovine serum albumin as a standard. Denaturing polyacrylamine gel electrophoresis was carried out by the method of Laemmli (19), and protein was visualized by silver staining according to the method of Wray *et al.* (20). Unless otherwise stated, the chemicals used in this report were supplied by either Sigma or Aldrich.

Purification of Phosphotriesterase—The phosphotriesterase from *P. diminuta* was purified from sf9 cells (fall armyworm) infected with the recombinant baculovirus (A5B) containing the *opd* gene. All cell lines and media used were as described previously.[3] The cells were infected at a cell density of 2–2.5 × 10^6 cells/ml with 0.2 ml virus/ml cells. The virus generally had a titer of 1 × 10^8 (plaque forming units)/ml. This infection was allowed to proceed at 27 °C for 4 days before the cells were harvested by centrifugation at 6100 × *g* for 30 min at 4 °C. All subsequent steps in the purification were carried out at 4 °C. The baculoviral infected sf9 cells (5–6 g/liter of cell culture) were suspended in 50 mM triethanolamine, pH 9.0, buffer containing 0.1 mM $ZnCl_2$ (buffer A) and gently stirred for 1 h. Cell lysis was achieved by 5-s pulsed sonication for 5 min at a medium power setting using a Heat Systems-Ultrasonics, Inc. model W830 ultrasonic processor with a macro-probe tip. This suspension was centrifuged at 25,000 × *g* for 30 min. The supernatant fluid was decanted, and the cells were resuspended in buffer A and centrifuged as before. This supernatant fluid was combined with the previous supernatant fraction. DEAE-cellulose (DE52, Whatman), washed and equilibrated in buffer A, was added to the combined supernatant fractions at a concentration of 1 ml of settled gel/500 mg of protein. This slurry was swirled for 30 min and filtered through a coarse scintered glass funnel, retaining the filtered solution for application to a 2.5 × 48-cm Green A dye matrix column (Amicon Corp.) equilibrated in buffer A. The phosphotriesterase was applied at a rate of 1 ml/min. The column was extensively washed with buffer A before initiating a 800-ml KCl gradient (0–700 mM) in buffer A at a rate of 1.0 ml/min. The fractions containing phosphotriesterase activity were pooled and loaded onto a phenyl-Sepharose column (2.5 × 15 cm) equilibrated in buffer A containing 700 mM KCl. After loading at a rate of 1 ml/min, the column was thoroughly washed with buffer A. Phosphotriesterase was eluted in a 800-ml ethylene glycol gradient (0–60%) in buffer A at 1.0 ml/min. The fractions containing phosphotriesterase were pooled and aliquots of 30 ml were loaded on a 2.5 × 90-cm G-75 Sephadex column equilibrated in buffer A and chromatographed.

Fast Protein Gel Filtration Chromatography—Purified phosphotriesterase, 15 μg, was injected onto a Pharmacia Superose 12 column using buffer A at a flow rate of 0.5 ml/min. The absorbance of the eluant was monitored continuously at 280 nm and aliquots of the 1-ml fractions were assayed for phosphotriesterase activity. The Stoke's radius was determined graphically after calibrating the Superose column with proteins of known radii (21).

Isoelectric Focusing—Purified phosphotriesterase was dialyzed overnight against water at 4 °C. This enzyme solution was focused in a Bio-Rad Rotofor isoelectric focusing apparatus using the method described by the manufacturer in 2% Bio-Lyte 7/9 ampholyte (Bio-Rad). The phosphotriesterase was refractionated three times. The pI was recorded as the mean pH of the three fractionations.

Metal Stoichiometry—The glassware used for metal analysis was washed with nitric acid. The water was made metal-free using the method described by Veillon and Vallee (22) with Barnstead deionizer columns. The resistivity of the water was >18 MΩ-cm. Protein concentrations were determined by the bicinchoninic acid assay. Enzyme was transferred into 10 mM sodium pyrophosphate pH 9.0 buffer by gel filtration through a Superose 12 FPLC column. The enzyme was concentrated to 0.15–0.50 mg/ml in an Amicon Centricon-10 microconcentrator that had been soaked in 10 mM ethylenediaminetetraacetate (EDTA) and thoroughly washed with water. Zinc concentrations were determined by flame atomic absorption using a Perkin-Elmer model 2380 spectrophotometer and a Perkin-Elmer Zn Intensitron lamp at 213.9 nm, 0.2 mm slit width, and background correction using an air:acetylene (3:1) mixture. Samples were aspirated in 100-μl aliquots into the flame. Absorptions were recorded as the mean of four determinations for each sample.

Apoenzyme was made by dialyzing the phosphotriesterase against pyrophosphate buffer containing 1 mM *o*-phenanthroline (1 ml of phosphotriesterase in 2 liters of buffer) for 16 h with two buffer changes followed by dialysis against buffer in the absence of *o*-phenanthroline. The removal of excess *o*-phenanthroline was verified spectrophotometrically.

Inactivation with Metal Chelators—The enzymatic activity was measured at various intervals after incubation in the presence of *o*-phenanthroline (0.4–3.0 mM), EDTA (50 mM), or 2,6-pyridine dicarboxylate (50 mM) at 25 °C in 150 mM CHES, pH 9.0, buffer. The second order rate constant, k'', for inactivation was calculated using Equation 2,

$$\ln(E_t/E_0) = -k''Yt \quad (2)$$

where E_0 represents the initial enzymatic activity, E_t is the activity at time = t, and Y is the inactivator concentration. The effect of the related compounds, *m*-phenanthroline and *p*-phenanthroline, was analyzed under the same conditions. Restoration of enzyme activity was attempted by dialyzing the *o*-phenanthroline-treated enzyme (5 ml) against three changes of 4.0 liters of 50 mM triethanolamine, pH 9.0, containing 0.10 mM $ZnCl_2$ over a 24-h period.

The kinetics of inactivation during the irreversible modification of phosphotriesterase by *o*-phenanthroline was also determined using a method analogous to that of Tsou (23). The concentration of product formed over time for a one substrate reaction with simultaneous inactivation can be described by Equation 3

$$P_t = \frac{V_{max}A(1 - e^{-k''Yt})}{k''Y(K_m + A)} \quad (3)$$

where P_t is the product concentration, V_{max} is the maximal rate of hydrolysis in the absence of inactivator, A is the initial substrate concentration, and K_m is the Michaelis constant in the absence of inactivator. It can be shown that as t approaches infinity the following relationship holds.

$$\ln[(P - P_t)/P] = -k''Yt \quad (4)$$

This method allows the rapid measurement of k'' in the presence of substrate. Protection against inactivation was shown by determining the pseudo-first order rate constant, $k''Y$, at different inactivator concentrations of cysteine (8–40 mM), histidine (30–150 mM), and 2-aminoethanthiol (1–6 mM) in the presence of either saturating (2 mM) or dilute (0.05 mM) paraoxon.

Inhibition by DTT, DTE, and β-Mercaptoethanol—The inhibitory activity of DTT, DTE, β-mercaptoethanol, and oxidized DTT (24) was determined at various concentrations of paraoxon at pH 9.0 in 100 mM CHES buffer. The competitive inhibition constants for DTT were also obtained at pH 10 (100 mM CAPS) and at pH 7 (150 mM HEPES) by the least squares fit of the data to Equation 5 with the computer programs of Cleland (25).

$$v = \frac{V_{max}A}{K_m(1 + I/K_i) + A} \quad (5)$$

Substrate Specificity—In addition to paraoxon, *p*-nitrophenylacetate and several commercially available pesticides were evaluated as substrates at pH 9 in 100 mM CHES buffer. The reactions were monitored spectrophotometrically, either in the UV region or within the visible spectrum depending upon the characteristics of the substrate. All of the pesticides, *O,O*-diethyl *O*-(3-chloro-4-methyl-2-oxo-2*H*-1benzopyran-7-yl)phosphorothioate (coumaphos), *O,O*-dimethyl *p*-cyanophenyl phosphorothioate (cyanophos), *O,O*-diethyl *O*-2-isopropyl-4-methyl-6-pyrimidyl phosphorothioate (diazinon), *O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate (dursban), *O,O*-diethyl [*p*-(methylsulfinyl)phenyl] phosphorothioate (fensulfothion), *O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate (parathion), and *O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate (methyl parathion) were purchased from Chem Service, Inc., West Chester, PA. The limited solubility of these compounds necessitated the addition of 10% methanol to the reaction mixtures. There is no loss of activity at these low concentrations of methanol (13).

RESULTS

Purification—Table I summarizes the results of a typical purification procedure. The elution profile of phosphotriesterase with the dye matrix Green A column is shown in Fig. 1. Elution of phosphotriesterase with 700 mM KCl results in a 20-fold purification. Moreover, the relatively high salt concentration aids in the hydrophobic interaction between the enzyme and the phenyl-Sepharose media (Fig. 2) used in the subsequent column. The introduction of ethylene glycol into the phenyl-Sepharose column allowed elution of the enzyme without the denaturing side effects often observed with other

TABLE I
Purification of phosphotriesterase

Step	Volume	Activity	Protein	Specific activity	-Fold	Yield
	ml	*mmol/min*	*mg*	*units/mg*		*%*
Sonicate	226	11,400	5,400	2.1	1.0	100
DE-52	450	11,800	1,550	7.6	3.6	104
Green A	124	9,390	62.4	150	71	82
Phenyl-Sepharose	117	10,100	4.2	2,400	1,140	89
G-75	390	8,520	2.7	3,200	1,500	75

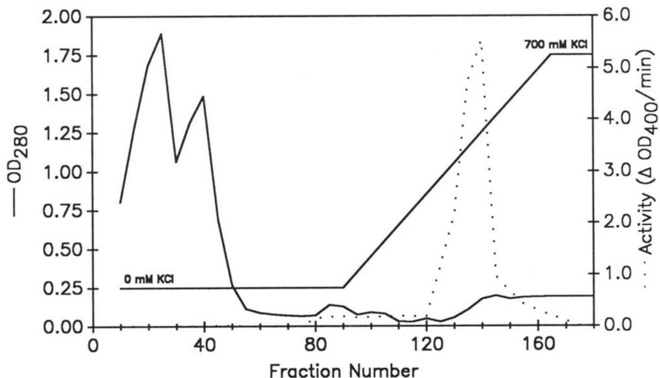

FIG. 1. **The elution of phosphotriesterase from a Green A dye matrix column.** While a substantial amount of protein (*solid line*) did not bind to the medium, all enzymatic activity (*dotted line*) was associated with a single peak that eluted only in the presence of greater than 500 mM KCl.

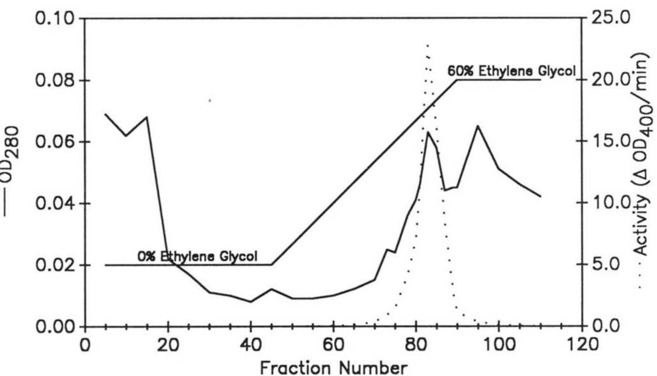

FIG. 2. **The elution profile of phosphotriesterase from the phenyl-Sepharose column.** All enzymatic activity (*dotted line*) was associated with a single peak that eluted in the presence of greater than 50% ethylene glycol.

organic solvents. This purification step provided an additional 16-fold purification. The phosphotriesterase was further purified by gel filtration to give a homogeneous preparation as shown by SDS-PAGE (Fig. 3). In addition, high resolution gel filtration (Superose 12 FPLC) shows a single absorbance peak at 280 nm with which all enzymatic activity is associated. From the 8 g of A5B-infected sf9 cells, 2.7 mg of homogeneous enzyme was obtained with an overall yield of 75% after a 1500-fold purification.

Characterization—The molecular weight of denatured phosphotriesterase as determined by SDS-PAGE is 39,000 which is identical to the value obtained for the native enzyme using high resolution gel filtration chromatography. Therefore, the purified enzyme exists as a monomer in solution. The Stoke's radius is calculated as 22 Å (data not shown). Pure enzyme had a single protein peak with which all enzymatic activity was associated upon isoelectric focusing. The isoelectric point is 8.3 ± 0.1, and a 0.1% solution of enzyme has an absorbance of 1.86 at 280 nm.

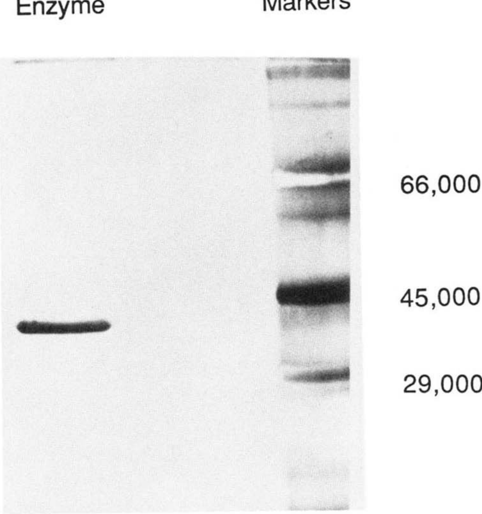

FIG. 3. **SDS-PAGE with 10 mg of purified phosphotriesterase.** The molecular weight markers included bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000).

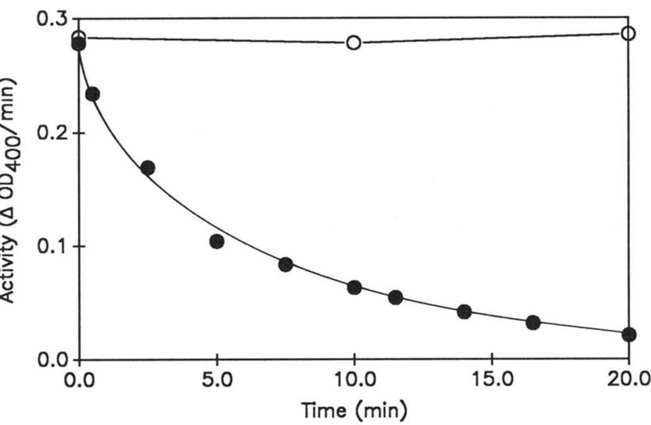

FIG. 4. **The inactivation of phosphotriesterase in the presence of 2 mM *o*-phenanthroline (*closed circles*).** This process occurs with a second order rate constant of 5.3 M^{-1} s^{-1}. A second phosphotriesterase sample to which only water was added (*open circles*) showed little change in activity over the observed time period.

TABLE II
Competitive inhibition constants
Determined from fits of the data to Equation 5.

Inhibitor	K_{is}
	μM
DTT (pH 7)	3.5 ± 0.3
DTT (pH 9)	8.8 ± 0.5
DTT (pH 10)	14.3 ± 0.9
DTE (pH 9)	7.0 ± 0.4
Cysteine	2500 ± 200
β-Mercaptoethanol	140 ± 10
3-Mercaptopropanol	230 ± 20
Cysteine methyl ester	130 ± 10

Two independent phosphotriesterase preparations (specific activity of 2400 units/mg) were used to determine whether zinc was bound to the protein as isolated. The first sample had a phosphotriesterase concentration of 12.9 μM based upon a molecular weight of 39,000. The zinc concentration was found to be 11.8 μM. The second sample had an enzyme concentration of 4.6 μM and a zinc concentration of 4.5 μM.

TABLE III

Kinetic constants for the hydrolysis of organophosphorus insecticides

Structure	Common name	λ_{max}	Δ	K_m	V_{rel}	$(V/K)_{rel}$
		nm	*OD/mM*	*mM*		
$(EtO)_2P(=O)-O-C_6H_4-NO_2$	Paraoxon	400	17	0.09	100	100
$(EtO)_2P(=S)-O-$(3,5,6-trichloropyridin-2-yl)	Dursban	276	5.3	0.11	0.08	0.07
$(EtO)_2P(=S)-O-C_6H_4-NO_2$	Parathion	400	17	0.24	30	11.25
$(EtO)_2P(=S)-O-$(Cl-substituted coumarin)	Coumaphos	348	9.1	0.39	29	6.70
$(EtO)_2P(=S)-O-$(Me, iPr-substituted pyrimidine)	Diazinon	228	3.3	0.45	8.4	1.68
$(EtO)_2P(=O)-O-C_6H_4-S(=O)-Me$	Fensulfothion	284	8.0	0.46	3.2	0.63
$(MeO)_2P(=S)-O-C_6H_4-NO_2$	Methyl-parathion	400	17	0.84	2.4	0.26
$(MeO)_2P(=S)-O-C_6H_4-CN$	Cyanophos	274	18	2.1	7.5	0.32

The ratios of zinc to enzyme are 0.91 and 0.98, respectively. The atomic absorption of zinc standards was identical whether the measurements were made in water or pyrophosphate buffer. There was also no effect upon absorption in the presence of 15 μM bovine serum albumin, and thus there are no measurable matrix effects caused by either the buffer or the protein. When the zinc containing phosphotriesterase was dialyzed against *o*-phenanthroline and then dialyzed against buffer to remove the *o*-phenanthroline, the specific activity decreased to 0.4 unit/mg, and the zinc concentration was reduced to 0.4 μM (0.1 zinc/enzyme).

Metal Chelators—The kinetics for inactivation of phosphotriesterase with various metal chelators was found to be first order with respect to the chelator concentration. Of the three compounds tested, *o*-phenanthroline inactivated phosphotriesterase the fastest. The second order rate constant for inactivation with *o*-phenanthroline (*I*) at pH 9 is 5.3 $M^{-1} s^{-1}$ (Fig. 4). EDTA and 2,6-pyridine dicarboxylate inactivate at the much slower rates of 0.0016 $M^{-1} s^{-1}$ and 0.0022 $M^{-1} s^{-1}$, respectively. The second order rate constant for inactivation by *o*-phenanthroline decreased by a factor of 2.6 when the paraoxon concentration was increased from 0.05 to 2.0 mM. Neither 20 mM *m*-phenanthroline (II) nor 20 mM *p*-phenanthroline (III) caused any inactivation when incubated with phosphotriesterase for 1 h.

I II III

Three other compounds which may act as metal chelators have also been shown to inactivate phosphotriesterase. Cysteine and histidine inactivate phosphotriesterase with second order rate constants of 0.19 and 0.041 $M^{-1} s^{-1}$, respectively, and the related compound 2-aminoethanethiol has a second order rate constant for inactivation of 1.47 $M^{-1} s^{-1}$. Enzymatic activity can be partially restored after inactivation with *o*-

TABLE IV
Amino acid composition of phosphotriesterase

Residue	Number/enzyme[a]	Predicted/enzyme[b]
Ala	38.1 ± 1.5	41
Asx	27.9 ± 2.3	24
Arg	25.0 ± 0.1	33
Cys	3.8 ± 0.2	4
Gly	29.9 ± 0.7	26
Glx	27.7 ± 1.2	24
His	7.9 ± 0.7	7
Ile	23.3 ± 0.7	23
Leu	32.6 ± 1.0	36
Lys	9.0 ± 0.5	10
Met	4.7 ± 0.2	7
Phe	15.4 ± 0.4	14
Pro	14.6 ± 0.5	15
Ser	26.8 ± 0.6	26
Thr	23.8 ± 1.2	22
Tyr	6.4 ± 0.3	7
Val	22.6 ± 0.4	21

[a] Residue amounts were calculated by normalizing peak areas to the sum of the predicted number of residues for six composition determinations. The value for cysteine was determined as cysteic acid from a performic acid oxidation of phosphotriesterase for three analyses.

[b] The predicted values were calculated from the gene sequence for phosphotriesterase.

phenanthroline. Dialyzing inactivated enzyme against a buffer containing 1 mM Zn^{2+} resulted in 12% of the original activity being recovered.

Inhibition by Thiols—β-Mercaptoethanol, DTT, and DTE were found to be potent competitive inhibitors versus paraoxon (Table II). The inhibition constants for DTT *versus* paraoxon at pH values of 7, 9, and 10 were obtained and also presented in Table II.

Kinetic Parameters for Paraoxon Hydrolysis—The Michaelis constant for paraoxon hydrolysis with freshly prepared enzyme was 0.048 ± 0.004 mM. Using a specific activity of 3200 units/mg and a molecular weight of 39,000, the calculated values for k_{cat} and k_{cat}/K_m are 2100 s^{-1} and 4×10^7 M^{-1} s^{-1}. The second order rate constant for the chemical hydrolysis of paraoxon by KOH is 7.5×10^{-2} M^{-1} s^{-1}. From this, the catalytic rate enhancement for the phosphotriesterase-catalyzed hydrolysis of paraoxon relative to KOH at pH 7.0 is calculated to be 2.8×10^{11}.

Substrate Specificity—The purified phosphotriesterase was unable to catalyze the hydrolysis of *p*-nitrophenylacetate ($<10^{-5}$ times the rate of paraoxon hydrolysis). There is, however, an extensive set of commercially used organophosphate pesticides that are hydrolyzed by phosphotriesterase (Table III).

Amino Acid Composition—The amino acid composition determined for a homogeneous preparation of phosphotriesterase is presented in Table IV and compared with the predicted composition from the recently determined DNA sequence of the *opd* gene.[4] The N terminus could not be sequenced, apparently due to some terminal modification of the protein.

DISCUSSION

Purification—While there have been several organophosphorus hydrolyzing enzymes (DFPases, somanases, sarinases, paraoxonases, parathionases, etc.) reported in the literature, the purification to homogeneity has never been achieved with any of these enzymes. In the absence of a pure and stable preparation of protein, little information is known about the physical and chemical characteristics of these enzymes. Mechanistic investigations of the enzyme-catalyzed reactions are equally sparse. This lack of information has led some investigators to question whether the observed hydrolysis of the organophosphorus compounds by various organisms was the result of a single enzyme or multiple enzyme systems (6). The phosphotriesterase from *P. diminuta* is readily purified from the baculovirus expression system with a procedure involving three chromatographic steps. Phosphotriesterase does not bind to the anion exchange media DE52 at pH 9 because of the relatively high pI of 8.3. The enzyme preparation is further fractionated by dye matrix chromatography on Green A. Dye matrix chromatography can be used in an affinity-like manner with the elution of desired enzymes with substrates or substrate analogs (26). However, this technique was not attempted due to the extreme toxicity of the substrates for the phosphotriesterase. After elution from the Green A column with high salt, phosphotriesterase can be loaded directly onto a phenyl-Sepharose column. Since proteins bind to this medium through hydrophobic interactions, the high ionic strength of the Green A eluant aids in the binding of phosphotriesterase to the column material. The gel filtration step removes the remaining contaminating proteins. The criteria used to judge enzyme purity included the demonstration of a single dark band on SDS-PAGE with silver staining, a single absorbance and activity peak on FPLC gel filtration, and a single protein peak with isoelectric focusing.

Physical Properties—The same apparent molecular weight of 39,000 for the phosphotriesterase is obtained from both native and denaturing experiments, and thus the enzyme exists as a single monomeric unit in solution. This value is very close to the predicted molecular weight of 37,826 based upon the DNA sequence for the *opd* gene (16). The other organophosphate hydrolyzing enzymes for which molecular weight data are available are in general larger (4–8) except for those of the squid DFPase of 26,600 (3) and the *Rangia cuneato* enzyme of 22,000 (6). There is no evidence with the purified enzyme to support the previous observation that the phosphotriesterase in cell-free extracts can exist as a dimer (16). The theoretical Stoke's radius for a spherical protein of molecular weight 39,000 is 25 Å.[5] This value is within experimental error of the value of 22 Å found for the phosphotriesterase, and thus the protein is roughly spherical in shape.

The purified phosphotriesterase from *P. diminuta* has 1 mol of zinc bound/mol of enzyme, but it is not known whether the function of this zinc is structural or catalytic. The metal ion is required for activity since the phosphotriesterase is inactivated with the zinc chelator, *o*-phenanthroline. The loss of activity is probably due to chelation of the zinc rather than to the binding of this molecule to some hydrophobic region of the enzyme since no inactivation or inhibition is observed with the structurally related isomers *m*- and *p*-phenanthroline. Furthermore, incubation with *o*-phenanthroline removes the zinc from the enzyme, producing an inactive apoenzyme. Activity is only partially restored upon dialysis of the apoprotein against $ZnCl_2$. Therefore, the removal of zinc from the enzyme is probably followed by an irreversible conformational change or a chemical modification of the active site such as

[4] Amino acid analysis was performed by the Texas Agricultural Experiment Station Protein Services Center at Texas A&M University.

[5] The theoretical Stoke's radius for a protein with a molecular weight of 39,000 can be calculated from the following equation

$$r_H = [(3/4\pi)(M_r/N)(V_2 + \phi_1 V_1)]^{1/3}$$

where the partial specific volume of the protein, V_2, is 0.72 cm^3/g based upon the sum of the partial specific volumes of the amino acid residues; the partial specific volume of water, V_2, is 1.0 cm^3/g; the hydration of the protein, ϕ_1, is 0.35 $g_{water}/g_{protein}$; and N is Avogadro's number.

FIG. 5. **Working model for the enzymatic hydrolysis of phosphotriesters.**

the oxidation of thiol groups to disulfides. The rate of inactivation with EDTA or 2,6-pyridine dicarboxylate is 2600 times slower than with *o*-phenanthroline. Both of these metal chelators are negatively charged at pH 9, and thus the slow inactivation may be evidence for a lack of tolerance for negatively charged molecules at the active site. This conclusion is supported by the observation that the phosphotriesterase will not catalyze the hydrolysis of phosphate monoesters or diesters (13), and the anionic forms of the products are very poor inhibitors (13).

Histidine and cysteine are two of the most common residues to act as ligands in zinc metalloproteins (27), and both of these amino acids also inactivate phosphotriesterase. Presumably, these amino acids inactivate the enzyme by complexation and removal of the zinc from the active site. Deletion of the carboxylate moiety of cysteine gives aminoethanethiol which increases the rate of inactivation 10-fold. None of the 18 other common amino acids had a measurable effect upon enzymatic activity. A decrease in the rate of inactivation by *o*-phenanthroline in the presence of saturating paraoxon suggests that the zinc is at or near the site of catalysis.

The location of the bound Zn^{2+} at the active site is also indirectly supported by the competitive inhibition patterns displayed by a variety of sulfhydryl containing compounds. DTT, DTE, and β-mercaptoethanol are rapid and reversible competitive inhibitors *versus* paraoxon. The competitive inhibition constant for DTT at pH 7 is an order of magnitude smaller than the K_m for paraoxon. The sulfhydryl groups in these compounds are perhaps coordinating with the Zn^{2+} in the active site and displaced by higher concentrations of actual substrates.

Substrate Specificity—The phosphotriesterase from *P. diminuta* will hydrolyze many of the commonly utilized organophosphorus insecticides in addition to paraoxon. The general trends in the kinetic constants, V_{max} and V/K_m, with substrate structure are similar to those previously observed by Donarski *et al.* (13). Replacement of the phosphoryl oxygen with a sulfur increases the K_m but reduces V_{max}. Substitution of methoxy for ethoxy groups produces substrates with higher K_m values and reduced catalytic rates. The size of the leaving group appears to be relatively unimportant since coumaphos is hydrolyzed at a rate comparable with parathion. This suggests that there are probably few molecular interactions between the enzyme and the leaving group. The dominant factor in the rate of substrate hydrolysis is stabilization of the anionic product (13).

The identity of any naturally occurring substrate for this enzyme is unknown at the present time. Although the substrate specificity exhibited by this enzyme is very broad (12–14), we have been unable to detect any activity with mono- or diesters of phosphoric acid. Since the triester derivatives of phosphoric acid have been in existence for only 50 years (28), enzyme activity may have evolved from a pre-existing esterase, protease, or phosphodiesterase. However, the isolated enzyme does not catalyze the hydrolysis of any of these compounds at a kinetically detectable rate. The paraoxonase activity may be an ancillary reaction of some other more important but less obvious function. However, the experimentally determined values for k_{cat} and k_{cat}/K_m with paraoxon as a substrate are quite substantial. The k_{cat}/K_m of 4×10^7 M^{-1} s^{-1} is reasonably close to the diffusion controlled limit of 10^8–10^9 M^{-1} s^{-1} (29). Thus, the phosphotriesterase activity of this protein is quite respectable.

Mechanism of Action—A working model for the mechanism of phosphotriester hydrolysis by this enzyme is presented in Fig. 5. There appears to be a single base with a pK_a of 6.1 in the active site that abstracts a proton from water (13). The activated water molecule attacks directly at the phosphorus center since the overall stereochemical outcome is one of inversion of configuration (14). The phosphoryl oxygen is polarized by the active site zinc. This representation is very similar to the hydrolysis of peptides and esters by carboxypeptidase (30). Alternatively, the zinc may participate in this reaction by serving as a template for hydroxide or as a Lewis acid catalyst of the leaving group. Metal ion replacement and isotope effect studies are currently in progress in an attempt to further refine the mechanism of enzymatic phosphotriester hydrolysis.

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