

Inactivation of Organophosphorus Nerve Agents by the Phosphotriesterase from *Pseudomonas diminuta*¹

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The phosphotriesterase from *Pseudomonas diminuta* was tested as a catalyst for the hydrolysis of phosphofluoridates. The purified enzyme has been shown to hydrolyze the phosphorus–fluorine bond of diisopropyl fluorophosphate, isopropyl methylphosphonofluoridate, and 1,2,2-trimethylpropylmethylphosphonofluoridate at pH 7.0, 25°C, with turnover numbers of 41, 56, and 5 s⁻¹, respectively. The enzymatic rate enhancement for the hydrolysis of sarin at pH 7.0 is 2.2 × 10⁷. The turnover number for paraoxon hydrolysis is 2100 s⁻¹. The enzyme does not hydrolyze methanesulfonyl fluoride, phenylmethylsulfonyl fluoride, or *O-p*-nitrophenyl phenylsulfonate nor do these compounds inactivate or inhibit the ability of the enzyme to hydrolyze diethyl *p*-nitrophenyl phosphate. The breadth of substrate utility and the efficiency of the hydrolytic reaction exceed the more limited abilities of other prokaryotic and eukaryotic enzymes that catalyze similar reactions. The substantial rate enhancement exhibited by this enzyme for the hydrolysis of a wide variety of organophosphorus nerve agents make this enzyme the prime candidate for the biological detoxification of insecticide and mammalian acetylcholinesterase inhibitors. © 1990 Academic Press, Inc.

Mazur was the first to describe the enzymatic hydrolysis of the phosphorous–fluorine bond of diisopropyl flu-

orophosphate (DFP)³ by a crude preparation of rabbit tissue (1). Several different enzymes from sources as diverse as hog kidney (2), nervous tissue and salivary glands of cephalopods (3), microorganisms (4), and several strains of bacteria (5) have since been shown to detoxify DFP or other related organophosphorus neurotoxins (6–8). Depending upon the particular substrate tested, these enzymes have been labeled as paraoxonases (EC 3.1.1.2), parathion hydrolases, somanases, organophosphorus triesterases, and DFPases (EC 3.8.2.1). The hydrolyses of these organophosphate neurotoxins have been, for the most part, studied as crude enzyme preparations or cell extracts.

The *opd* genes isolated from large plasmids of *Pseudomonas diminuta* (pCS1, 51kb) and *Flavobacterium* species ATCC 27551 (pSM55, 43kb) encode a phosphotriesterase (9) that has been shown to hydrolyze parathion, paraoxon, and other structurally related insecticides such as coumophos, methyl-parathion, and diazinon (10). Lewis *et al.* (11) have shown the mechanism of hydrolysis to occur through the net inversion of stereochemistry at the phosphorus center due to the direct attack of an activated water molecule, making the existence of a phosphoenzyme intermediate unlikely. The DNA sequences for the *opd* genes have been determined (12) and the genes have been expressed in a variety of vectors using host-specific promoters. Among these expression systems, it has recently proven possible to produce native enzyme in insect tissue cultures derived

³ Abbreviations used: DFP, diisopropyl fluorophosphate; sarin, isopropylmethylphosphonofluoridate; soman, 1,2,2-trimethylpropylmethylphosphonofluoridate; paraoxon, diethyl *p*-nitrophenylphosphate; mipafox, *N,N'*-diisopropylphosphorodiamidate; NPPS, *p*-nitrophenylphenylsulfonate; DMF, dimethylformamide; NPEPP, *p*-nitrophenylethylphenylphosphinate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; PMSF, phenylmethylsulfonyl fluoride; alpha, 1-ethylpropylmethylphosphonofluoridate.

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from the Fall Armyworm, *Spodoptera frugiperda* (sf9 cells) from the polyhedron promoter of a baculoviral expression vector (13). The purification and initial characterization of the homogeneous enzyme have been reported previously (10).

The studies reported in this publication have been directed toward determining whether the phosphotriesterase from *P. diminuta* is capable of hydrolyzing many of the alkyl fluorophosphates which inhibit mammalian acetylcholinesterases. Soman and sarin, two of the most highly toxic nerve agents ($LD_{50} \sim 0.01$ mg/kg), have now been demonstrated to be rapidly hydrolyzed by this phosphotriesterase. No other enzyme has ever been shown to detoxify these compounds in addition to many of the commonly used pesticides such as paraoxon and parathion.

MATERIALS AND METHODS

The phosphotriesterase used in this study was purified to homogeneity as described previously (10). Enzymatic activity was routinely monitored by spectrophotometrically measuring the rate of hydrolysis of paraoxon (Sigma) at 400 nm using either a Gilford, Model 260, or a Milton Roy, Spectronic 601, spectrophotometer. One unit of enzymatic activity is defined as that amount of enzyme capable of catalyzing the hydrolysis of 1 μ mol of paraoxon per minute at 25°C.

O-p-Nitrophenylphenylsulfonate (NPPS) was synthesized by mixing 20 mmol of phenylsulfonil chloride (Aldrich) with 21 mmol *p*-nitrophenol in 15 ml dimethylformamide (DMF) at 0°C under nitrogen. To this mixture, 21 mmol triethylamine (Aldrich) was added dropwise with stirring, and the final mixture was allowed to slowly warm to room temperature. After 2 h, the reaction was essentially complete as determined by thin-layer chromatography. The triethanolamine hydrochloride was removed by filtration, and the yellow filtrate was dried with a rotary evaporator. The resulting pale yellow syrup was redissolved in DMF and dried as before, and this syrup was then dissolved in a minimal amount of warm DMF. Methanol was added dropwise to induce crystallization. The pale yellow crystals (yield 79%) were filtered and washed with several quick rinses of cold methanol. The melting point of the dry product is 83°C. The structure of the product was verified by ^1H and ^{13}C NMR spectroscopy. The high resolution mass of the parent molecule was 279.020 as compared to the calculated value of 279.018 for a molecular formula of $\text{C}_{12}\text{H}_9\text{NO}_5\text{S}$.

Soman, sarin, alpha, and NPEPP were provided by the U.S. Army Chemical Research, Development, and Engineering Center, Aberdeen Proving Grounds. Mipafox was purchased from Ash Stevens, Inc. Stock solutions of soman and sarin (100 mM) were made in water containing 1.0 mM isopropanol and used within 2 days and stored at 4°C. Under these conditions, an insignificant amount of substrate (<1%) was hydrolyzed before use. Mipafox solutions were made fresh daily. The enzymatic hydrolysis of these compounds was measured in 150 mM Hepes, pH 7.0, buffer containing 500 mM KCl using a fluoride selective electrode (Orion Model 96-09-00) and an Orion pH/mV meter (Model 901 or 601A). Data from the soman and sarin experiments were collected using an analog to digital converter and stored on a floppy disk using a MacIntosh SE computer. These compounds are very toxic and should be used with extreme caution.

The enzymatic hydrolyses of two sulfonyl fluorides, methanesulfonyl fluoride (Aldrich) and phenylmethanesulfonyl fluoride (Aldrich), and NPPS were investigated in a reaction mixture containing 10% (v/v) methanol in 150 mM Hepes, pH 7.0, buffer. The hydrolysis of the sulfonyl fluorides was monitored with the fluoride ion electrode, while NPPS was assayed spectrophotometrically at 400 nm. The rates of

base-catalyzed hydrolysis for NPPS and paraoxon were determined spectrophotometrically in 10% methanol with 10 mM KOH.

Kinetic data were fit to either Eq. [1] or [2] using the computer programs of Cleland (14):

$$v = \frac{VA}{K + A} \quad [1]$$

$$v = \frac{VA}{K(1 + I/K_i) + A} \quad [2]$$

RESULTS AND DISCUSSION

Enzymatic hydrolysis of phosphorus-fluorine bond. The phosphotriesterase from *P. diminuta* was tested as a catalyst for the cleavage of the phosphorus-fluorine bond that exists in a wide variety of highly toxic mammalian acetylcholinesterase inhibitors. The purified enzyme was found to efficiently hydrolyze the P-F bond of soman, sarin, DFP, and a number of other structurally related compounds. The kinetic constants from fits of the data to Eq. [1] are presented in Table I. Sarin is the most rapidly hydrolyzed phosphofluoridate identified to date. The k_{cat} for sarin hydrolysis is 56 s^{-1} , which is also very close to the values observed for DFP and alpha hydrolyses. This catalytic constant represents a rate enhancement of 2.2×10^7 over the rate constant of $2.6 \times 10^{-6} \text{ sec}^{-1}$ for sarin hydrolysis in water at pH 7.0 (15). We obtained no evidence to indicate that the mixtures of stereoisomers present with each of these compounds were hydrolyzed at kinetically different rates. Soman is hydrolyzed approximately 1 order of magnitude more slowly than either sarin or alpha. Nonetheless, the phosphotriesterase catalyzes the hydrolysis of soman 1.5×10^6 times faster than the uncatalyzed reaction at pH 7.0 ($k = 3.1 \times 10^{-6} \text{ s}^{-1}$) (16). Since the P-F bonds in soman and sarin are approximately of equal chemical reactivity this difference must be due to an unfavorable interaction of soman with the enzyme. Similar observations were made by Donarski *et al.* (17) with an extensive series of dialkyl *p*-nitrophenyl phosphates. They found that large and bulky groups attached to the central phosphorus atom reduced the hydrolytic reaction catalyzed by the phosphotriesterase. For example the di-*s*-butyl triester was hydrolyzed 50 times more slowly than the diethyl triester (paraoxon).

Mipafox is hydrolyzed very slowly by the enzyme. This result is probably due to the reduced electrophilic character of the phosphorus center caused by the two isopropylamine substituents. As expected, mipafox was found to be a competitive inhibitor versus either paraoxon ($K_i = 7 \pm 2$ mM) or DFP ($K_i = 2.5 \pm 0.4$ mM).

Enzymatic hydrolysis of sulfur-fluorine bond. The ability of the phosphotriesterase from *P. diminuta* to catalyze the hydrolysis of sulfonates was tested with

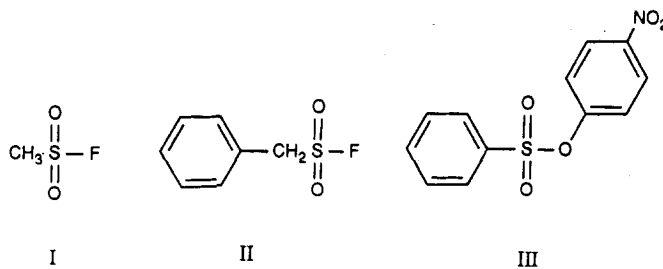
TABLE I
Kinetic Constants for the Hydrolysis of Nerve Agents^a

Compound	Common name	K_m (mM)	V/K (mM ⁻¹ s ⁻¹)	k_{cat} (s ⁻¹) ^a
	Paraoxon	0.05	41400	2070
	DFP	0.1	410	41
	Soman	0.5	9.6	4.8
	Sarin	0.7	80	56
	Mipafox	0.9	0.4	0.33
	NPEPP	1.1	610	670
	Alpha	1.7	14	23

^a At pH 7.0 and 25°C.

methane sulfonyl fluoride (I), PMSF (II), and NPPS (III) (Scheme I). We were unable to detect any hydrolytic activity with any of these compounds (<10⁻³% of the rate of paraoxon hydrolysis) at concentrations levels of 0.1–5.0 mM. Moreover, these compounds did not inhibit or inactivate the enzyme activity when paraoxon was utilized as a substrate. The lack of hydrolytic activity is not due to a decreased chemical reactivity of the sulfonate analogs relative to a typical phosphotriester since the pseudo-first-order rate constant for NPPS hydrolysis (9 × 10⁻³ min⁻¹) by 10 mM KOH is faster than that for paraoxon hydrolysis (5 × 10⁻³ min⁻¹). A significant contributing factor for the lack of substrate activity and binding is the absence of an alkyl group attached to

the central sulfur atom. Donarski *et al.* (17) demonstrated that when one or both of the ethyl groups of paraoxon are replaced by methyl groups the binding to the



SCHEME I

TABLE II
A Comparison among Several Organophosphate Hydrolyzing Enzymes^a

Source	DFP ($\mu\text{mol}/\text{min}/\text{mg}$)	Soman ($\mu\text{mol}/\text{min}/\text{mg}$)	Paraoxon ($\mu\text{mol}/\text{min}/\text{mg}$)	Reference
<i>P. diminuta</i>	64	7.3	3200	(10)
Squid Ganglia	108	27	—	(20)
<i>E. coli K₁₂sr</i>	1	N.R. ^b	—	(21)
<i>Tetrahymena</i>	0.2	0.9	—	(22)
<i>Rangia cuneata</i>	1	15	—	(23)
Hog kidney	0.2	1	—	(2)
Rabbit serum	—	—	10	(24)
Sheep serum	—	—	1	(25)
Hamster liver	—	—	0.01	(26)
Locust	—	—	0.2	(27)

^a These data have been calculated from the literature sources cited. In some cases, these references contained ambiguous information which may cause a slight variation from the actual values. These data are not from homogeneous enzyme preparations and the specific activities would be expected to improve upon purification.

^b Not reported.

enzyme is greatly diminished. Furthermore, when either of these groups is removed altogether to form a phosphodiester all activity and binding is lost. The precise bond angles and lengths of the phosphotriesters relative to the sulfonates may also be contributing factors.

The sizable range of substrates for this phosphotriesterase, including the variety of widely used pesticides (10), is unprecedented among the organophosphate hydrolyzing enzymes. None of the reported DFPases have been shown to hydrolyze paraoxon or the related pesticides, and none of the so-called paraoxonases have been shown to hydrolyze DFP or other phosphofluoridates. As illustrated in Table II, the maximal rate of DFP and soman hydrolysis by this phosphotriesterase is comparable to the best reported values for squid DFPase, and up to 60 times better than any other DFPase. None of these DFPases are capable of hydrolyzing the related acetylcholinesterase inhibitors paraoxon and parathion, while this phosphotriesterase hydrolyzes these compounds 300–3000 times faster per milligram protein than any other paraoxonase preparation. The ability of only one of these enzymes to hydrolyze sarin has been cited in the literature. The squid type DFPase is reported to hydrolyze sarin at 80% the rate of soman hydrolysis (18). This would give a maximal estimated rate of hydrolysis of 22 $\mu\text{mol}/(\text{min}\cdot\text{mg protein})$. The phosphotriesterase from *P. diminuta* hydrolyzes sarin with a specific activity of 73 $\mu\text{mol}/(\text{min}\cdot\text{mg})$ and is thus the fastest and best characterized catalyst for sarin hydrolysis. Sarin is the major organophosphate deterrent stockpiled by the United States (19) and is thought to be a major component of the Soviet arsenal (19).

The phosphotriesterase from *P. diminuta* has the broadest substrate range and is capable of hydrolyzing the organophosphates DFP, soman, and sarin at rates per milligram protein equal to or greater than any other

known DFPase. In addition, many other organophosphate substrates for this phosphotriesterase are not substrates for the other DFPases. Moreover, the mechanism and active site structure of this enzyme are quite well understood and provide a well-defined target for site-directed mutagenesis. Production of large quantities of the phosphotriesterase is now possible from insect tissue culture or bacterial expression systems. These characteristics make the phosphotriesterase from *P. diminuta* the leading candidate for applications in the detoxification and treatment of organophosphate pesticides and mammalian acetylcholinesterase inhibitors.

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