Available online at www.sciencedirect.com

SCIENCE DIRECT®

Toxicology and Applied Pharmacology 207 (2005) S459 – S470

Toxicology and Applied Pharmacology

www.elsevier.com/locate/ytaap

Review

Detoxification of organophosphate nerve agents by bacterial phosphotriesterase

Eman Ghanem, Frank M. Raushel*

Department of Chemistry, Texas A&M University, P.O. Box 30012, College Station, TX 77843-3012, USA

Received 15 July 2004; revised 1 February 2005; accepted 15 February 2005
Available online 27 June 2005

Abstract

Organophosphates have been widely used as insecticides and chemical warfare agents. The health risks associated with these agents have necessitated the need for better detoxification and bioremediation tools. Bacterial enzymes capable of hydrolyzing the lethal organophosphate nerve agents are of special interest. Phosphotriesterase (PTE) isolated from the soil bacteria *Pseudomonas diminuta* displays a significant rate enhancement and substrate promiscuity for the hydrolysis of organophosphate triesters. Directed evolution and rational redesign of the active site of PTE have led to the identification of new variants with enhanced catalytic efficiency and stereoselectivity toward the hydrolysis of organophosphate neurotoxins. PTE has been utilized to protect against organophosphate poisoning in vivo. Biotechnological applications of PTE for detection and decontamination of insecticides and chemical warfare agents are developing into useful tools. In this review, the catalytic properties and potential applications of this remarkable enzyme are discussed.

Keywords: Phosphotriesterase; Detoxification; Organophosphates

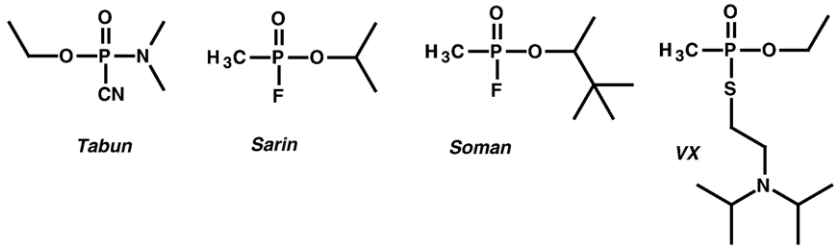
Contents

Introduction

Organophosphate triesters have been employed in the agricultural industry as pesticides and insecticides for several decades. Approximately 40,000 metric tons of various organophosphates are applied annually to agricul-

* Corresponding author. Fax: +1 979 845 9452.
E-mail address: raushel@tamu.edu (F.M. Raushel).

0041-008X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2005.02.025



Scheme 1.

ture crops in the US. The most toxic organophosphorus compounds known are the lethal nerve agents, tabun (GA), sarin (GB), soman (GD), and VX as shown in Scheme 1. The toxicity of these compounds is due primarily to the practically irreversible inhibition of acetylcholine esterase (AChE), the enzyme responsible for hydrolysis of the neurotransmitter acetylcholine (Ecobichon, 2001). Enzymes that are capable of hydrolyzing and detoxifying such agents are of significant utility.

Several organophosphate degrading enzymes have been isolated and characterized. The best characterized among them is the phosphotriesterase (PTE) that was initially isolated from the soil bacterium *Flavobacterium* sp. strain ATCC 27551 identified in Philippines (Raushel and Holden, 2000). The *opd* (organophosphate degrading) gene encoding the active hydrolase was located on an extrachromosomal plasmid.

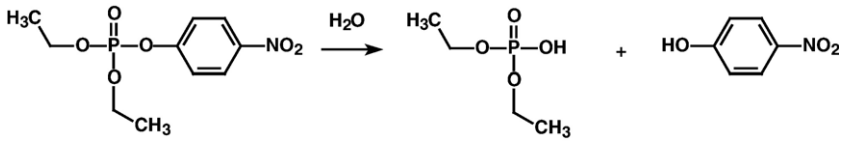
The gene for the bacterial phosphotriesterase was subcloned into several expression systems, including *Escherichia coli* (Dumas et al., 1989), insect cells (Dumas et al., 1990a), and in vitro compartmentalization (Griffiths and Tawfik, 2003). The natural substrate for PTE remains unknown. However, the purified enzyme is capable of hydrolyzing the insecticide paraoxon at a rate that approaches the diffusion-controlled limit as presented in Scheme 2. The turnover number of the zinc-substituted PTE for paraoxon hydrolysis is 2100 s^{-1}, while the corresponding value for k_{cat}/K_m is 4×10^7 M^{-1} s^{-1}. Since the synthesis of paraoxon was first reported in 1950 (Schrader, 1950), it is remarkable that PTE could have evolved to such a catalytic efficiency over a relatively short period of time. PTE also possesses the ability to catalyze the hydrolysis of a wide spectrum of organophosphate insecticides including parathion, methyl parathion, fensulfothion, among others (Dumas et al., 1989). In addition, PTE is capable of hydrolyzing the nerve agents sarin, soman, and VX, but the catalytic efficiencies for these substrates are significantly lower than the less toxic insecticides (Dumas et al., 1990b; Kolakowski et al., 1997).

Three-dimensional structure of phosphotriesterase

Phosphotriesterase is a member of the amidohydrolase superfamily (Holm and Sander, 1997), which also includes urease (Jabri et al., 1995) and dihydro-orotase (Thoden et al., 2001) among others. Members of this superfamily utilize one or two divalent metal ions to activate a hydrolytic water molecule for a nucleophilic attack at tetrahedral phosphorus or trigonal carbon centers. PTE is a homodimeric metalloprotein with a molecular weight of ~36 kDa/monomer (Benning et al., 1994). A high resolution X-ray structure has been solved for the bacterial PTE (Benning et al., 2001). The active site of the native enzyme contains two zinc ions per monomer. Incubation of PTE with various metal chelators such as EDTA and 1,10-phenanthroline renders the enzyme inactive (Omburo et al., 1992). The enzyme retains catalytic activity when the native Zn^{2+} is replaced by Co^{2+}, Cd^{2+}, Ni^{2+}, or Mn^{2+}. The cobalt-substituted enzyme is the most active form (Omburo et al., 1992).

Like other members of the amidohydrolase superfamily, PTE adopts a TIM-barrel $(\alpha\beta)_8$ fold where the binuclear metal center is located at the C-terminal end of the β-barrel (Fig. 1). The more buried metal ion in the active site, also known as the α-metal, is ligated to His-55, His-57, and Asp-301. The more solvent exposed metal ion (β-metal) is coordinated by His-201, His-230, and a water molecule. The two metal ions are bridged by the carboxylated Lys-169 and a water/hydroxide molecule, which is the apparent nucleophilic species during the hydrolysis reaction (Fig. 2).

The X-ray structure of phosphotriesterase bound with the prochiral substrate analog, diethyl 4-methylbenzylphospho-



Scheme 2.

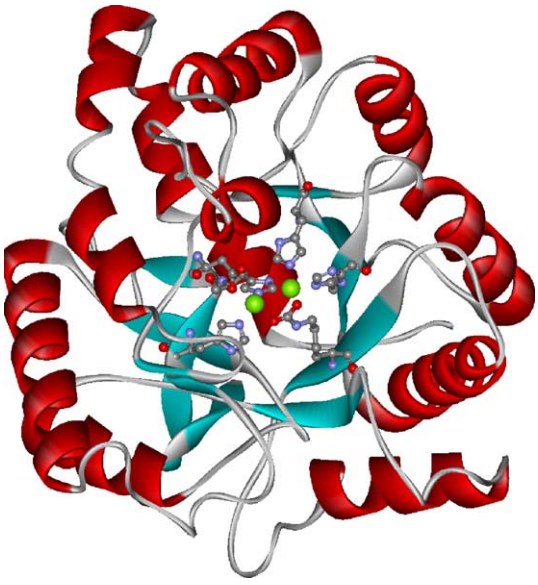


Fig. 1. Crystal structure of phosphotriesterase featuring a TIM barrel fold. Coordinates taken from PDB file 1 HZY.

nate, revealed the presence of three subsites for substrate binding: the small, large, and leaving group pockets accommodate the three substituents attached to the phosphorus center (Vanhooke et al., 1996). The large subsite contains residues His-254, His-257, Leu-271, and Met-317. The small binding pocket is defined by the residues Gly-60, Leu-303, Ser-308, and Ile-106. The hydrophobic leaving group pocket contains the residues Phe-306, Phe-132, Trp-131, and Tyr-309 (Fig. 3). It has been shown that the side chains of these 12 residues dictate the substrate and stereoselectivity of PTE.

Reaction mechanism

The catalytic mechanism for the hydrolysis of organophosphate triesters has been extensively studied. The proposed mechanism of the bacterial PTE is presented in Fig. 4. The pH-rate profiles for the hydrolysis of paraoxon indicate that a single group must be deprotonated with a pK_a of ~6.1 (Donarski et al., 1989). The ionization observed in the pH-rate profiles must be associated with the enzyme since the substrate does not ionize in this pH range. In addition, it has been demonstrated that the kinetic pK_a is dependent on the identity of the divalent cation bound to the enzyme (Aubert et al., 2004). These results are consistent with the utilization of a bridging hydroxide between the two divalent metal ions as the nucleophile during substrate turnover. The reactivity of the bridging hydroxyl is apparently enhanced by proton transfer to Asp-301. Nucleophilic attack of the activated hydroxyl at the phosphorus center occurs with the expulsion of the leaving group. His-254 is currently believed to assist the reaction by the shuttling of the proton from Asp-301 to the bulk solvent and away from the active site (Aubert et al., 2004). Theoretical studies have suggested that Tyr-309 could stabilize the *p*-nitrophenol leaving group through a hydrogen bonding interaction (Koca et al., 2001). However, the mutation of Tyr-309 to a phenylalanine did not significantly affect the hydrolysis of organophosphate triesters by PTE and thus this potential interaction does not contribute to substrate turnover (Aubert et al., 2004).

The chiral insecticide *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate (EPN) was utilized to determine the net stereochemistry at the phosphorus center during organophosphate hydrolysis by PTE. It was determined that

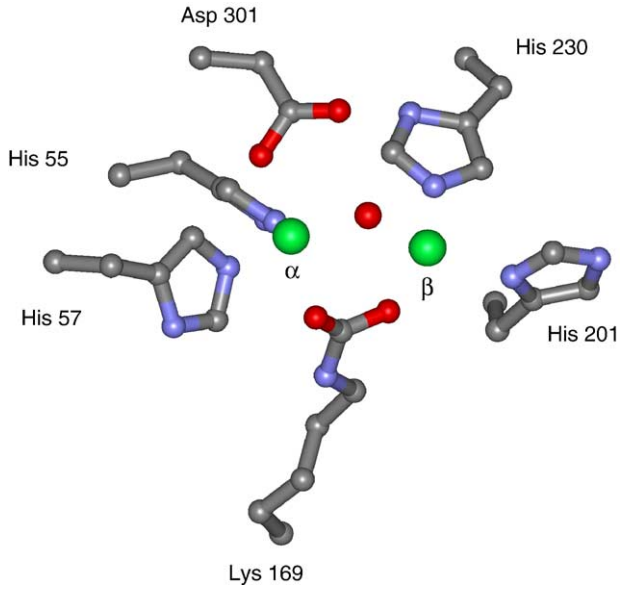


Fig. 2. Binuclear metal center of phosphotriesterase representing the α and β metals bridged by the catalytic solvent molecule. Coordinates taken from PDB file 1 HZY.

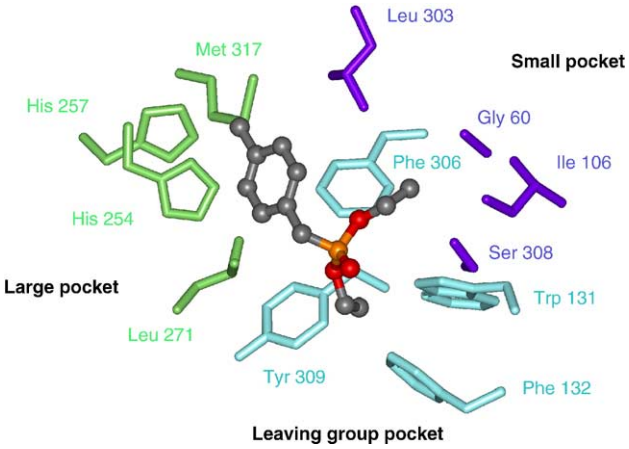


Fig. 3. The substrate binding site of phosphotriesterase bound to the inhibitor diethyl 4-methylbenzyl phosphonate. Residues in the small binding pocket are Gly60, Ile106, Ser308, and Leu303. The large binding pocket is defined by residues His254, His257, Met317, and Leu271. Residues in the leaving group pocket are Phe132, Trp131, Phe306, and Tyr309. Coordinates taken from PDB file 1 DPM.

hydrolysis of the S_P-enantiomer of EPN by PTE results in the formation of the S_P-enantiomer of the corresponding thiophosphonic acid (Lewis et al., 1988). This result demonstrates that the reaction proceeds via an S_N2-like mechanism with a net inversion of stereochemistry at the phosphorus center. Therefore, the reaction mechanism does not involve the formation of a phosphorylated enzyme intermediate. The X-ray structure of PTE bound to the sarin analog, diisopropyl methyl phosphonate, has provided evidence for the proposed orientation of the substrate in the active site. In this complex, the distance between the phosphoryl oxygen of the substrate and the more solvent exposed metal ion (β-metal) was 2.5 Å (Benning et al., 2000). These results support the role of the β-metal to polarize the phosphoryl oxygen bond and enhance the electrophilicity of the phosphorus center.

Substrate and stereochemical specificity

Although paraoxon is the best substrate known for PTE, the substrate specificity is very broad and covers a wide range of organophosphates, thiophosphates, and phosphorothiolates. For substrates with different leaving groups, the catalytic efficiency and the rate limiting step are dependent on the pK_a of the leaving group (Hong and Raushel, 1996). The substrate specificity of PTE was probed using a small library of paraoxon analogs as illustrated in Scheme 3. All combinations of methyl, ethyl, isopropyl, and phenyl groups were substituted for the substituents X and Y, resulting in 16 potential organophosphate substrates. All of these paraoxon analogs were found to be substrates for PTE with turnover values ranging from 18,000 s^{-1} for dimethyl *p*-nitrophenyl phosphate to 220 s^{-1} for the diisopropyl *p*-nitrophenyl

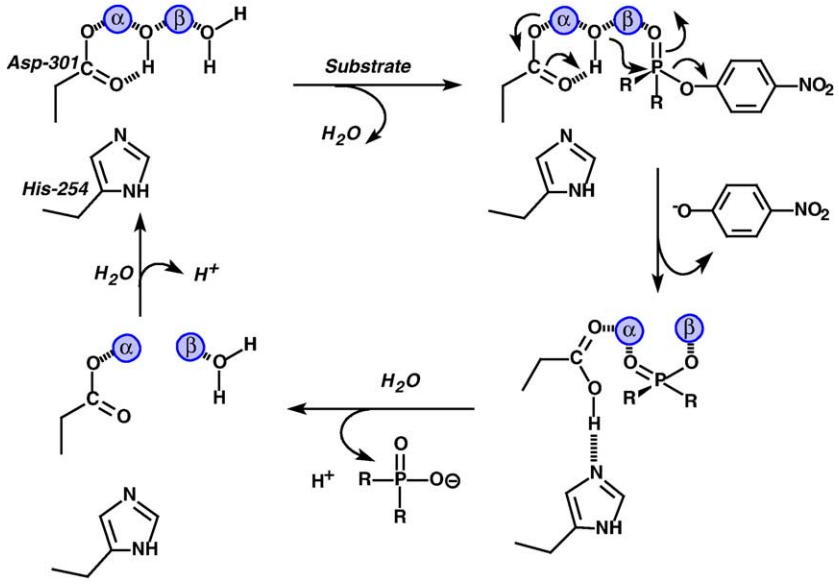
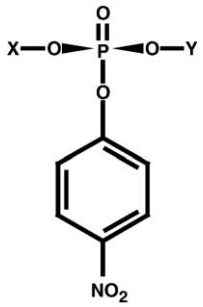


Fig. 4. Proposed catalytic mechanism for the hydrolysis of paraoxon by phosphotriesterase (Aubert et al., 2004).



Scheme 3.

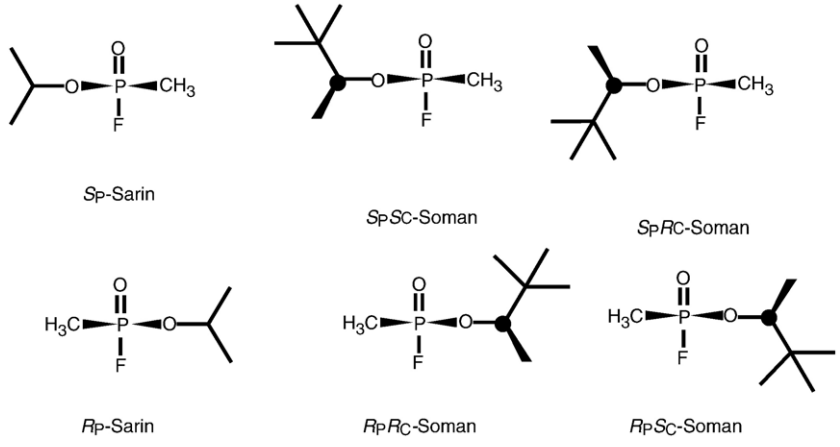
phosphate (Hong and Raushel, 1999a). The most interesting feature that emerged from this study was the inherent stereoselectivity of PTE. The enzyme was found to preferentially hydrolyze the S_P-enantiomer within a racemic mixture of chiral organophosphate triesters (Hong and Raushel, 1999b; Lewis et al., 1988). The stereoselectivity was more pronounced for the methyl isopropyl *p*-nitrophenyl phosphate, for which the efficiency of hydrolyzing the S_P-enantiomer was 100-fold greater than for the corresponding R_P-enantiomer (Hong and Raushel, 1999a). This catalytic property is of significant interest since the toxicity of most organophosphate nerve agents is dependent on the stereochemistry at the phosphorus center (Benschop and De Jong, 1988). From the crystal structure of PTE with a bound substrate analog, it was demonstrated that the substituents attached to the phosphorus center bind in a specific orientation within the active site, which is apparently dictated by the space available within the various subsites (Vanhooke et al., 1996). In all cases, the preferred stereoisomer is the one with the bulkier group represented by Y and the smaller group represented by X.

When these paraoxon analogs were screened for their ability to inactivate AChE, the R_P-enantiomer was always a more potent inhibitor of AChE than the corresponding S_P-enantiomer (Hong and Raushel, 1999a, 1999b). From a comparison of the absolute configuration of the nerve agent sarin and methyl diisopropyl *p*-nitrophenol phosphate, it was predicted that PTE would preferentially hydrolyze the least toxic R_P-enantiomer of sarin over the more toxic S_P-enantiomer. The same trend in relative stereoselectivity was obtained for the hydrolysis of chiral organophosphates with a thiolate leaving group. When a racemic mixture of the insecticide acephate was subjected to hydrolysis by PTE, the rate of hydrolysis of the S_P-enantiomer was significantly higher than the R_P-enantiomer (Chae et al., 1994). However, the overall rates of hydrolysis of the P-S bond are substantially slower than the rates reported for the cleavage of P-O and P-F bonds (Chae et al., 1994).

Inactivation of acetylcholinesterase

Acetylcholinesterase (AChE) is a remarkably efficient serine hydrolase. It catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic synapses. The hydrolysis reaction is initiated by the nucleophilic attack of Ser-200 on the carbonyl center of ACh to form an acetylated enzyme intermediate that is coupled with the release of the first product, choline. The free enzyme is regenerated by hydrolysis of the acetylated enzyme intermediate and release of the second product, acetate. In the presence of organophosphates, the active site Ser-200 is phosphorylated to form a phosphorylated enzyme intermediate. In this case, the hydrolysis of the phosphorylated enzyme intermediate is very slow. If AChE is not reactivated by an oxime nucleophile such as 2-PAM, the phosphorylated enzyme intermediate can undergo a base-assisted dealkylation reaction resulting in formation of “aged” AChE that is permanently inactivated (Millard et al., 1999). The half-time for aging is dependent on the nerve agent bound to the active site, pH, and ionic strength (Berman and Decker, 1986).

The toxicity of nerve agents is dependent on the stereochemistry at the phosphorus center. Since sarin and VX have a single chiral center there are two stereoisomers in



Scheme 4.

Table 1
Toxicity of individual stereoisomers of organophosphate nerve agents from Schemes 1 and 4 (Benschop and De Jong, 1988)

Nerve agent stereoisomer	LD_{50} (mouse) μg/kg	Inhibition rate constant ($M^{-1}min^{-1}$)
R_P-sarin		$<3 \times 10^3$
S_P-sarin	41	1.4×10^7
R_PR_C-soman	>5000	$<5 \times 10^3$
R_PS_C-soman	>2000	$<5 \times 10^3$
S_PR_C-soman	99	2.8×10^8
S_PS_C-soman	38	1.8×10^8
R_P-VX	165	2×10^6
S_P-VX	12.6	4×10^8

the racemic mixture (see Scheme 4). However, in soman there is an additional stereogenic center within the pinacolyl group and thus soman consists of a mixture of four stereoisomers. Although the racemic mixture of soman is a potent inhibitor of AChE, studies with mice have indicated that the two S_P-diastereomers of soman are 100-fold more toxic than the corresponding R_P-enantiomers (Benschop et al., 1984). A similar trend has been observed for sarin and VX where the S_P-enantiomer is a more potent AChE inhibitor than the R_P-enantiomer. Table 1 lists the toxicity of individual isomers of sarin, soman, and VX in terms of LD_{50} values and rate constants for the inhibition of AChE (Benschop and De Jong, 1988).

Other organophosphate hydrolyzing enzymes

Another group of enzymes involved in the detoxification of chemical warfare agents is the squid-type DFPases that have the ability to hydrolyze diisopropylfluorophosphate (DFP), sarin, soman, tabun, but not VX. The crystal structure of the DFPase from *Loligo vulgaris* shows that the protein exists as a monomer and adopts a 6-fold β-propeller-like motif (Scharff et al., 2001). DFPase contains high and low affinity Ca^{2+} binding sites that have been suggested to be involved in the hydrolytic reaction. DFPase is very efficient for the detoxification of DFP with a k_{cat}/K_m value of 1.3×10^6 M^{-1} s^{-1} (Hartleib and Rüterjans, 2001).

An organophosphorus acid anhydrolase (OpaA) from *Alteromonas* sp. JD6.5 was cloned and sequenced at the Edgewood Research, Development, and Engineering Center (Cheng et al., 1996). The natural function of OpaA has been proposed to be a dipeptidase that catalyzes the hydrolysis of dipeptides with a proline residue at the C-terminus (Cheng et al., 1996). OpaA also displays catalytic activity with organophosphate triesters including the nerve agents sarin and soman. Unlike PTE, OpaA is unable to hydrolyze the nerve agent VX (Cheng et al., 1999). The stereoselectivity of OpaA was surveyed with the same library of paraoxon analogs that has been used for PTE. Like PTE, OpaA preferentially hydrolyzes the S_P-enantiomers of the organophosphate triesters shown in Scheme 3, although the overall catalytic efficiency is significantly lower than for PTE (Hill et al., 2000). Similarly, OpaA displays a stereoselective preference toward the hydrolysis of the least toxic R_P-enantiomers of sarin and soman analogs (Hill et al., 2001).

Another prolidase that was identified as an enzyme capable of degrading organophosphates is the *E. coli* aminopeptidase P (PepP). PepP is a metalloprotein that requires two Mn^{2+} cations for catalysis. The enzyme functions as a tetramer that adopts a "pita-bread" fold similar to methionine aminopeptidase and creatinase (Wilce et al., 1998). The ability of PepP to hydrolyze organophosphate triesters was probed with a small library of racemic paraoxon analogs. The highest rates were observed for the substrates containing the substituents methyl isopropyl and methyl isobutyl (Jao et al., 2004).

PTE-catalyzed detoxification of organophosphorus compounds

Currently, decontamination of organophosphates relies on bleach treatment, alkaline hydrolysis, or incineration. In all cases, the conditions are harsh and the byproducts can be toxic. Therefore, efforts have been directed toward enzyme-catalyzed decontamination and bioremediation. The use of bio-scavengers, such as the exogenous administration of acetyl cholinesterase (Saxena et al., 1997) or human butyryl cholinesterase (Raveh et al., 1997), has proven effective for protection against organophosphate poisoning. These reagents exert their function by binding to the nerve agents and preventing them from reaching their target. The disadvantage associated with this approach is the large amount of scavenger required for optimum protection, since these enzymes have a binding stoichiometry of unity. Therefore, attention has been drawn to enzymes that can catalytically hydrolyze organophosphate nerve agents.

Due to a broad substrate specificity and stereoselectivity, PTE is a promising candidate for in vivo and in vitro detoxification of organophosphate nerve agents. Animal studies in mice showed that intravenous injection with 1.5 IU (paraoxonase activity of 1.5 μmol/min) of PTE before or immediately after the administration of paraoxon protected brain AChE activity against inhibition by paraoxon (Kaliste-Korhonen et al., 1993). When PTE was injected 10 min before administration of paraoxon, the concentration required to decrease the activity of brain AChE to 40% of the control was increased from 0.5 mg paraoxon/kg to 20 mg paraoxon/kg (Kaliste-Korhonen et al., 1993).

The slower rates of sarin, soman, and DFP hydrolysis by PTE were reflected in the in vivo study. In the case of DFP exposure, PTE treatment protected slightly the activity of brain AChE, but not the serum AChE

(Tuovinen et al., 1994). However, when the two Zn^{2+} ions in the binuclear metal center were replaced by Co^{2+}, the protection of AChE against DFP inhibition was enhanced (Tuovinen et al., 1996a). The total plasma paraoxon hydrolyzing activity for the Co^{2+}-reconstituted PTE was approximately 10-fold higher than the Zn^{2+} enzyme. The intravenous injection of 6.9 μg PTE/g 10 min after exposure to 1.8 mg DFP/kg retained AChE activity of 90% of the control for up to 6 h, which was better than the protection effect of the carbamate, eptastigmine (Tuovinen et al., 1996b). If the same dose of PTE was injected 30 min after exposure, the protection of AChE dropped to 40% after 6 h and 15% after 24 h (Tuovinen et al., 1996c). These data are similar to the protection rates obtained for eptastigmine. Therefore, it was concluded that PTE treatment is much more effective during the first few minutes after exposure.

PTE pretreatment did not significantly protect brain or serum AChE against sarin or soman toxicity (Tuovinen et al., 1994). However, it increased the sarin dose required to induce inactivation of AChE. A relatively high dose of PTE (104 U/g body weight) increased the LD_{50} of sarin toxicity from 1.4 mg/kg to 4.7 mg/kg (Tuovinen et al., 1999). When the same dose of PTE was combined with 0.09 mg/kg body weight of the carbamate, physositigmine, the LD_{50} was further increased to 6.0 mg/g (Tuovinen et al., 1999). When PTE was incubated in vitro with mouse serum that was treated with paraoxon, the AChE activity was recovered from 25–31% to 76–100% of control activity in 24 h (Tuovinen et al., 1994). The overall results from animal studies indicated that PTE-pretreated animals can tolerate 50-fold higher doses of paraoxon than the untreated animals.

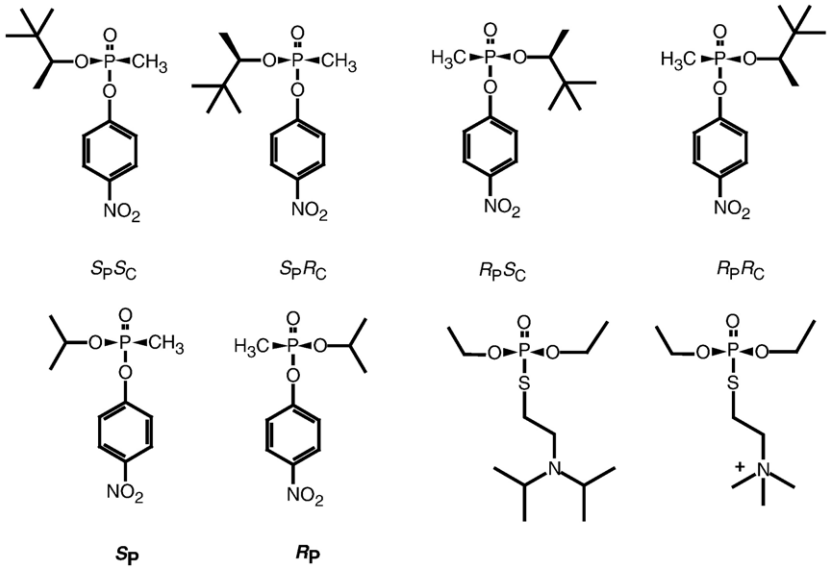
The biological distribution of PTE using [^{125}I] labeling indicated that the enzyme was found in the liver, kidney, and lungs, but not in the nervous system. This suggests that PTE hydrolyzes organophosphates in circulation before reaching the nervous system (Tuovinen et al., 1996a). The in vivo therapeutic application of PTE is restricted by its half-life in plasma, which was reported to be approximately 5 h (Tuovinen et al., 1996a). To overcome this limitation, attempts have been made to encapsulate PTE in biodegradable carriers. PTE was successfully encapsulated within murine erythrocytes by hypotonic dialysis (Pei et al., 1994) and within sterically stable liposomes (Petrikovics et al., 1999) to antagonize paraoxon toxicity.

Altering substrate specificity and directed evolution of PTE

PTE has been subjected to several rational design and directed evolution studies to optimize the active site for enhanced detoxification of organophosphates and the related organophosphonate nerve agents. The stereospecificity displayed by PTE initiated a series of investigations to reshape the substrate binding site by utilizing site directed mutagenesis for enhanced, relaxed, or reversed stereoselectivity. The first set of experiments was based on the mutation of all residues that define the hydrophobic substrate binding site to alanine, and a subsequent investigation of the impact of these alterations on the stereoselectivity of PTE. For the residue Gly-60, which is located in the small binding pocket, mutation to alanine significantly enhanced the substrate stereoselectivity (Chen-Goodspeed et al., 2001a). For example, the ratio for hydrolysis of the chiral methyl phenyl *p*-nitrophenyl phosphate by wild-type PTE is 90 in favor of the S_P-enantiomer over the R_P-enantiomer (Hong and Raushel, 1999a). The G60A mutant of PTE displayed a 100-fold reduction in k_{cat}/K_m for the R_P-enantiomer and therefore the ratio of stereoselectivity was enhanced to 13,000 in favor of the S_P-enantiomer (Chen-Goodspeed et al., 2001a). Mutations of the large subsite residues His-245, His-257, Leu-271, and Met-317 to alanine had no impact on the stereospecificity. These data suggest that the substrate specificity is mainly dictated by the steric constraints within the small binding pocket (Chen-Goodspeed et al., 2001a).

These studies were further extended to include other mutations that can reconstruct the active site of PTE. The mutation I106G in the small subsite relaxed the stereoselectivity for hydrolysis of chiral methyl phenyl *p*-nitrophenyl phosphate from 90:1 to 1.7:1 (Chen-Goodspeed et al., 2001b). This result was achieved by increasing the k_{cat}/K_m for the hydrolysis of the R_P-enantiomer 100-fold over the wild-type enzyme. The stereoselectivity was reversed by simultaneously enlarging the small subsite and shrinking the large subsite. While the wild-type enzyme prefers the S_P-enantiomer of methyl phenyl *p*-nitrophenyl phosphate by a factor of 90, the mutant I106G/F132G/H257Y/S308G prefers the R_P-enantiomer by a factor of 190. The same mutant displays a preference for the R_P-enantiomer of chiral ethyl phenyl *p*-nitrophenyl phosphate over the S_P-enantiomer with a ratio of 80:1 (Chen-Goodspeed et al., 2001b). A novel application based on these findings was to utilize PTE variants with enhanced and reversed stereospecificity for efficient synthesis of chiral organophosphate triesters. The mutants G60A and I106A/F132A/H257Y were successfully used to kinetically resolve racemic mixtures of chiral paraoxon analogs with an enantiomeric excess of approximately 95–99% (Wu et al., 2000).

PTE is of interest owing to its ability to catalytically hydrolyze the nerve agents sarin, soman, and VX. Since these agents are not readily available for research use, analogs have been synthesized to help screen for optimized variants of PTE toward the detoxification of organophosphonates (see Scheme 5). For sarin and soman, chromogenic analogs were obtained by substituting the fluoride leaving group with a *p*-nitrophenyl group. For VX, prochiral analogs were synthesized with slight



Scheme 5.

modifications in the thiolate leaving group. The toxicity of these analogs was assessed in terms of minimum effective concentration (MEC) using a simple and cost efficient bioassay in *Hydra attenuata* (Lum et al., 2003). The individual toxicity of sarin, soman, and VX was reflected in the toxicity of their analogs with two of the VX analogs being the most toxic (Table 2). For the chiral sarin analogs, the S_P-enantiomer was approximately 4 orders of magnitude more toxic than the R_P-enantiomer (Lum et al., 2003).

Very promising and interesting results were obtained when the chiral sarin and soman analogs were used to evaluate the potential of PTE for stereoselective detoxification of nerve agents. As expected from previous stereoselectivity profiles, wild-type PTE preferentially hydrolyzes the least toxic R_P-enantiomer of the sarin analog 10-fold better than the more toxic S_P-enantiomer. This preference was reversed by the mutant I106A/F132A/H257Y, which showed an enhanced rate of hydrolysis of the S_P-enantiomer by a factor of 30 (Li et al., 2001). A similar trend was observed for the soman analogs. These data clearly demonstrate that the active site of PTE can be manipulated for optimum decontamination and decomposition of the neurotoxins, sarin and soman.

A semi-rational approach was taken to expand the search for PTE mutants with enhanced activity for the hydrolysis of chemical warfare agents. The active site residues were randomly mutated by saturation mutagenesis and variants were screened using a crude cell assay. Screening with the most toxic *p*-nitrophenyl soman analog resulted in the isolation and identification of the mutant H254G/H257W/L303T, which has a rate enhancement of approximately 3 orders of magnitude over the wild-type enzyme (Hill et al., 2003). It was noted from the mutagenesis experiments on PTE that the large subsite residue, His-257, is significant for modifying the substrate specificity. Another PTE homolog (opdA) was recently isolated from *Agrobacterium radiobacter* P 230 (Horne et al., 2002) that shares a 90% sequence identity with PTE from *Pseudomonas diminuta*. The major difference between PTE and *opd*A is the presence of Arg and Tyr at positions 254 and 257, respectively, instead of His in PTE. In a directed evolution study, the *opd* gene was shuffled in search for enhanced variants for methyl parathion hydrolysis. The mutation H257Y was found in all isolates that displayed higher activities (Cho et al., 2002). The highest activity was obtained by introducing 7 different mutations, with only the mutation H257Y located in the active site. The large subsite was target for a rational design experiment that led to the identification of the mutant H254R/H257L, which had 20-fold enhancement over wild type for the VX analog demeton-S (Di Sioudi et al., 1999).

Table 2
Toxicity of organophosphate analogs that appear in Scheme 5 (Lum et al., 2003)

Compound	MEC_{92th} (mg/L)
p-nitrophenol	9.4×10^{1}
paraoxon	2.5×10^{1}
(R_P)-GB *p*-NP	1.0×10^{2}
(R_P/S_P)-GB *p*-NP	2.0×10^{-2}
(S_P)-GB *p*-NP	1.3×10^{-2}
(R_PR_C/R_PS_C/S_PR_C/S_PS_C)-GD *p*-NP	2.5×10^{-4}
(S_PR_C/S_PS_C)-GD *p*-NP	2.0×10^{-4}
(S_PS_C)-GD *p*-NP	8.5×10^{-5}
Ecothiophate iodide	1.0×10^{-4}
Diethyl VX	3.0×10^{-6}
Tetriso	3.1×10^{-6}

Potential applications

The current methods for the detoxification of organophosphorus compounds are harmful and possess serious environmental consequences. Therefore, utilizing enzymes for the detection and decontamination of organophosphate agents has received considerable attention. PTE has been recognized as a potential candidate for extending the process of organophosphate detoxification. The insecticides paraoxon and parathion were successfully hydrolyzed by PTE that was immobilized on a trityl agarose matrix (Caldwell and Raushel, 1991a). The immobilization resulted in a relative increase in the stability of PTE and the maximum efficiency of the system was about 40%, relative to the free enzyme. The immobilized enzyme exhibited a similar kinetic profile to the free enzyme with a 3-fold higher K_m for paraoxon (Caldwell and Raushel, 1991a). Since immobilization of PTE on trityl agarose was based on hydrophobic interactions, the use of organic solvents to solubilize the pesticides was problematic and weakened the interaction between the enzyme and the solid support. This limitation was overcome by covalently immobilizing PTE on nylon tubing, powder, and membranes (Caldwell and Raushel, 1991b). In this case, methanol was used as a solvent at a concentration up to 40% with no adverse effect on the activity of PTE, which allowed hydrolysis of very high substrate concentrations. The amount of PTE immobilized was dependent on the surface area of the membrane and was limited to 390 U for a 48-cm^2 nylon 66 membrane. This amount was significantly increased to 9000 U for a 2000-cm^2 nylon 6 membrane. The catalytic efficiency of the 11-U nylon 66 membrane reactor in the presence of 0%, 10%, and 40% methanol were reduced to 4% of the soluble enzyme activity under the same reaction conditions (Caldwell and Raushel, 1991b). This low efficiency could be attributed to the internal diffusional effect since hydrolysis of paraoxon by soluble PTE achieves the diffusion control limits. PTE immobilized on nylon powder (240 U/750 mg nylon 11 powder) was stable for as long as 20 months at 5 °C. A reactor of 900 PTE units immobilized onto 2.5 g of the nylon 11 powder could hydrolyze 18.3 mM paraoxon in 30% methanol at a rate of 76 μmol/min (Caldwell and Raushel, 1991b).

Another study to develop a delivery method for use of PTE in the decontamination of organophosphates involved incorporating PTE within fire fighting foams. Foams containing PTE displayed high capacity for surface decontamination. A 1.2-cm foam height containing 11.4 nM PTE could detoxify a surface contaminated with 1.15 μmol paraoxon/cm^2 with 43% conversion (LeJeune and Russell, 1999). When the foam height was increased to 3.0 cm, the conversion was enhanced to 70%. To test the ability of PTE foams to decontaminate soil, two sand columns were contaminated with 2.3 μmol/g paraoxon. While soluble PTE was applied to one column, an equal amount of enzyme activity was immobilized onto the foam and applied to the second column. By analyzing the products, it was determined that the column treated with the PTE foam had a 85% conversion in 1 h, while the column treated with soluble PTE displayed less than 50% conversion (LeJeune and Russell, 1999). In addition, nanocomposites of PTE adsorbed on isocyanatopropyl-silica and poly hydroxymethylsiloxane (PHOMS) were shown to be effective in detoxifying liquid and gas phase organophosphates (Gill and Ballesteros, 2000).

The extensive use of organophosphates necessitated the need to develop devices for early detection and quantification of organophosphates in contaminated areas. AChE inhibition-based biosensors have been used to monitor organophosphate exposure. Although AChE-biosensors are very sensitive, they are not selective since AChE is inhibited by a wide range of compounds including carbamates. In addition, AChE sensors are based on very complicated procedures and cannot be regenerated because of irreversible inhibition. PTE-based devices were developed based on the products of the hydrolysis reaction. For example, the hydrolysis of paraoxon by PTE releases two protons and a chromophoric leaving group, which can be utilized for the design of potentiometric and optical biosensors, respectively. To develop a PTE-based potentiometric biosensor, a pH glass electrode was modified by immobilizing purified PTE. Concentrations of paraoxon, parathion, and methyl parathion as low as 2 μM were detected using a 500-U PTE electrode (Mulchandani et al., 1999a). Based on the same principle, a PTE potentiometric biosensor was developed by immobilizing recombinant *E. coli* cells harboring the *opd* gene instead of purified protein (Rainina et al., 1996). A fluorescent biosensor was developed based on the change of pH resulting from hydrolysis of organophosphates by using fluorescein isothiocyanate (FITC) modified PTE. The FITC-PTE was adsorbed on polymethyl methacrylate beads, and the decrease in the fluorescent intensity of FITC was measured as the enzymatic hydrolysis proceeds (Roger et al., 1999). A broad scope of organophosphates could be detected by this methodology including parathion, diazinon, methylparathion, and coumaphos.

A fiber-optic enzyme biosensor (FOEB) of PTE was designed based on the reaction products that can be detected spectrophotometrically. The biosensor contained a monochromator with a wavelength cutoff of 400 nm (for the *p*-nitrophenol leaving group of paraoxon and parathion) and a cutoff of 348 nm for the hydrolysis product of coumaphos (Mulchandani et al., 1999b). PTE was immobilized by covalent cross-linking with glutaraldehyde on a 0.45-μm pore size nylon membrane. The detection limit for this FOEB was 2 μM paraoxon, which is comparable to the potentiometric biosensors.

Despite their great potential, the detection limit of PTE biosensors was much higher than the AChE-based devices. An amperometric biosensor was developed based on electrochemical oxidation of the *p*-nitrophenol (*p*NP)

released from PTE-catalyzed hydrolysis (Mulchandani and Mulchandani, 1999). The electrode was composed of purified PTE immobilized onto a printed carbon surface. The oxidation current was directly proportional to the level of *p*NP released. Unlike the potentiometric and optical biosensors, the detection limits for this device were significantly low, 0.7 nM and 0.9 nM for methyl parathion and paraoxon, respectively (Mulchandani and Mulchandani, 1999). The same principle was applied to develop a microbial amperometric biosensor based on the immobilization of whole cells expressing PTE anchored to the outer membrane by fusion to the ice nucleation protein from *Pseudomonas syringae* (Mulchandani et al., 2001). It was determined that the microbial biosensor offered sufficient selectivity and that other cellular or environmental chemicals did not interfere with the sensor detection. This technology provides a cost efficient, simple, reliable, and rapid detection of organophosphate pesticides and nerve agents.

Conclusion

Phosphotriesterase, an enzyme isolated from the soil bacteria *P. diminuta*, has displayed great potency in hydrolyzing a broad range of the extremely toxic organophosphate pesticides and nerve agents with a well-characterized stereoselectivity. In addition, PTE is the only enzyme known to hydrolyze the V-series of nerve agents including VX. Modifications of the active site were shown to be promising in altering the substrate specificity and enhancing the catalytic efficiency for slower substrates. In vivo studies demonstrated that PTE can be used for protection and treatment of organophosphates toxicity. PTE represents a plausible target for future mutagenesis studies to optimize the hydrolysis of organophosphate neurotoxins.

Acknowledgment

This work was supported in part by the National Institutes of Health (GM 33894 and GM 68550).

References

Aubert, S.D., Li, Y., Raushel, F.M., 2004. Mechanism for the hydrolysis of organophosphates by the bacterial phosphotriesterase. Biochemistry 43, 5707–5715.

Benning, M.M., Kuo, J.M., Raushel, F.M., Holden, H.M., 1994. Three-dimensional structure of phosphotriesterase: an enzyme capable of detoxifying organophosphate nerve agents. Biochemistry 33, 15001–15007.

Benning, M.M., Hong, S.B., Raushel, F.M., Holden, H.M., 2000. The binding of substrate analogs to phosphotriesterase. J. Biol. Chem. 275, 30556–30560.

Benning, M.M., Shim, H., Raushel, F.M., Holden, H.M., 2001. High resolution X-ray structures of different metal-substituted forms of phosphotriesterase from *Pseudomonas diminuta*. Biochemistry 40, 2712–2722.

Benschop, H.P., De Jong, L.P.A., 1988. Nerve agent stereoisomers: analysis, isolation and toxicology. Acc. Chem. Res. 21, 368–374.

Benschop, H.P., Konings, C.A.G., Genderen, J.V., De Jong, L.P.A., 1984. Isolation, anticholinesterase properties, and acute toxicity in mice of the four stereoisomers of the nerve agent soman. Toxicol. Appl. Pharmacol. 72, 61–74.

Berman, H.A., Decker, M.M., 1986. Kinetic, equilibrium, and spectroscopic studies on dealkylation ("Aging") of alkyl organophosphonyl acetylcholinesterase. J. Biol. Chem. 261, 10646–10652.

Caldwell, S.R., Raushel, F.M., 1991a. Detoxification of organophosphate pesticides using an immobilized phosphotriesterase from *Pseudomonas diminuta*. Biotechnol. Bioeng. 37, 103–109.

Caldwell, S.R., Raushel, F.M., 1991b. Detoxification of organophosphate pesticides using a nylon based immobilized phosphotriesterase from *Pseudomonas diminuta*. Appl. Biochem. Biotechnol. 31, 59–73.

Chae, M.Y., Postula, J.F., Raushel, F.M., 1994. Stereospecific enzymatic hydrolysis of phosphorus-sulfur bonds in chiral organophosphate triesters. Bioorg. Med. Chem. Lett. 4, 1473–1478.

Cheng, T.C., Harvey, S.P., Chen, G.L., 1996. Cloning, expression and nucleotide sequence of a bacterial enzyme for decontamination of organophosphorus nerve agents. Appl. Environ. Microbiol. 62, 1636–1641.

Cheng, T.C., DeFrank, J.J., Rastogi, V.K., 1999. *Alteromonas* prolidase for organophosphorus G-agent decontamination. Chem. Biol. Interact. 119–120, 455–462.

Chen-Goodspeed, M., Sogorb, M.A., Wu, F., Hong, S.B., Raushel, F.M., 2001a. Structural determinants of the substrate and stereochemical specificity of phosphotriesterase. Biochemistry 40, 1325–1331.

Chen-Goodspeed, M., Sogorb, M.A., Wu, F., Raushel, F.M., 2001b. Enhancement, relaxation, and reversal of the stereoselectivity for phosphotriesterase by rational evolution of active site residues. Biochemistry 40, 1332–1339.

Cho, C.M., Mulchandani, A., Chen, W., 2002. Bacterial cell surface display of organophosphorus hydrolase for selective screening of improved hydrolysis of organophosphate nerve agents. Appl. Environ. Microbiol. 68, 2026–2030.

Di Sioudi, B.D., Miller, C.E., Lai, K., Grimsley, J.K., Wild, J.R., 1999. Rational design of organophosphorus hydrolase for altered substrate specificities. Chem. Biol. Interact. 119–120, 211–223.

Donarski, W.J., Dumas, D.P., Heitmeyer, D.P., Lewis, V.E., Raushel, F.M., 1989. Structure–activity relationships in the hydrolysis of substrates by the phosphotriesterase from *Pseudomonas diminuta*. Biochemistry 28, 4650–4655.

Dumas, D.P., Caldwell, S.R., Wild, J.R., Raushel, F.M., 1989. Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*. J. Biol. Chem. 264, 19659–19665.

Dumas, D.P., Wild, J.R., Raushel, F.M., 1990a. Expression of *Pseudomonas* phosphotriesterase activity in the Fall Armyworm confers resistance to insecticides. Experientia 46, 729–731.

Dumas, D.P., Durst, H.D., Landis, W.G., Raushel, F.M., Wild, J.R., 1990b. Inactivation of organophosphorus nerve agents by the phosphotriesterase from *Pseudomonas diminuta*. Arch. Biochem. Biophys. 277, 155–159.

Ecobichon, D.J., 2001. Toxic effects of pesticides. In: Klaassen, C.D. (Ed.), Casarett and Doull's Toxicology: The Basic Science of Poisons, 6th ed. McGraw-Hill, New York, pp. 763–810.

Gill, I., Ballesteros, A., 2000. Degradation of organophosphorus nerve agents by enzyme-polymer nanocomposites: efficient biocatalytic materials for personal protection and large-scale detoxification. Biotechnol. Bioeng. 70, 400–410.

Griffiths, A.D., Tawfik, D.S., 2003. Directed evolution of and extremely fast phosphotriesterase by in vitro compartmentalization. EMBO J. 22, 24–35.

Hartleib, J., Rüterjans, H., 2001. Insights into the reaction mechanism of the diisopropyl fluorophosphatase from *Loligo vulgaris* by means of kinetic studies, chemical modification and site-directed mutagenesis. Biochim. Biophys. Acta 1546, 312–324.

Hill, C.M., Wu, F., Cheng, T.C., DeFrank, J.J., Raushel, F.M., 2000. Substrate and stereochemical specificity of the organophosphorus acid anhydrolase from *Alteromonas* sp. JD6.5 toward *p*-nitrophenyl phosphotriesters. Bioorg. Med. Chem. Lett. 10, 1285–1288.

Hill, C.M., Li, W.S., Cheng, T.C., DeFrank, J.J., Raushel, F.M., 2001. Stereochemical specificity of organophosphorus acid anhydrolase toward *p*-nitrophenyl analogs of soman and sarin. Bioorg. Chem. 29, 27–35.

Hill, C.M., Li, W.S., Thoden, J.B., Holden, H.M., Raushel, F.M., 2003. Enhanced degradation of chemical warfare agents through molecular engineering of the phosphotriesterase active site. J. Am. Chem. Soc. 125, 8990–8991.

Holm, L., Sander, C., 1997. An evolutionary treasure: unification of a broad set of amidohydrolases related to urease. Proteins 28, 72–78.

Hong, S.B., Raushel, F.M., 1996. Metal–substrate interactions facilitate the catalytic activity of the bacterial phosphotriesterase. Biochemistry 35, 10904–10912.

Hong, S.B., Raushel, F.M., 1999a. Stereochemical constraints on the substrate specificity of phosphotriesterase. Biochemistry 38, 1159–1165.

Hong, S.B., Raushel, F.M., 1999b. Stereochemical preference for chiral substrates by the bacterial phosphotriesterase. Chem. Biol. Interact. 119–120, 225–234.

Horne, I., Sutherland, T.D., Harcourt, R.L., Russell, R.J., Oakeshott, J.G., 2002. Identification of an opd (organophosphate degradation) gene in an *Agrobacterium* isolate. Appl. Environ. Microbiol. 68, 3371–3376.

Jabri, E., Carr, M.B., Hausinger, R.P., Karplus, P.A., 1995. The crystal structure of urease from *Klibsiella aerogenes*. Science 268, 998–1004.

Jao, S.C., Huang, L.F., Tao, Y.C., Li, W.S., 2004. Hydrolysis of organophosphate triesters by *Escherichia coli* aminopeptidase P. J. Mol. Catal. B: Enzym. 27, 7–12.

Kaliste-Korhonen, E., Ylitalo, P., Hänninen, O., Raushel, F.M., 1993. Phosphotriesterase decreases paraoxon toxicity in mice. Toxicol. Appl. Pharmacol. 121, 275–278.

Koca, J., Zhan, C.G., Rittenhouse, R.C., Ornstein, R.L., 2001. Mobility of the active site bound paraoxon and sarin in zinc-phosphotriesterase by molecular dynamics simulation and quantum chemical calculation. J. Am. Chem. Soc. 123, 817–826.

Kolakowski, J.E., DeFrank, J.J., Harvey, S.P., Szafraniec, L.L., Beaudry, W.T., Lai, K., Wild, J.R., 1997. Enzymatic hydrolysis of the chemical warfare agent VX and its neurotoxic analogues by organophosphorus hydrolase. Biocatal. Biotransform. 15, 297–312.

LeJeune, K.E., Russell, A.J., 1999. Biocatalytic nerve agent detoxification in fire fighting foams. Biotechnol. Bioeng. 62, 659–665.

Lewis, V.E., Donarski, W.J., Wild, J.R., Raushel, F.M., 1988. Mechanism and stereochemical course at phosphorus of the reaction catalyzed by a bacterial phosphotriesterase. Biochemistry 27, 1591–1597.

Li, W.S., Lum, K.T., Chen-Goodspeed, M., Sogorb, M.A., Raushel, F.M., 2001. Stereoselective detoxification of chiral sarin and soman analogues by phosphotriesterase. Bioorg. Med. Chem. 9, 2083–2091.

Lum, K.T., Huebner, H.J., Li, Y., Phillips, T.D., Raushel, F.M., 2003. Organophosphate nerve agent toxicity in *Hydra attenuata*. Chem. Res. Toxicol. 16, 953–957.

Millard, C.B., Kryger, G., Ordentlich, A., Greenblatt, H.M., Harel, M., Raves, M.L., Segall, Y., Barak, D., Shafferman, A., Silman, I., Sussman, J.L., 1999. Crystal structures of aged phosphorylated acetylcholinesterase: nerve agent reaction product at the atomic level. Biochemistry 38, 7032–7039.

Mulchandani, A., Mulchandani, P., 1999. Amperometric thick-film strip electrodes for monitoring organophosphate nerve agents based on immobilized organophosphorus hydrolase. Anal. Chem. 71, 2246–2249.

Mulchandani, P., Mulchandani, A., Kaneva, I., Chen, W., 1999a. Biosensor for direct determination of organophosphate nerve agents. 1. Potentiometric enzyme electrode. Biosens. Bioelectron. 14, 77–85.

Mulchandani, A., Pan, S., Chen, W., 1999b. Fiber-optic enzyme biosensor for direct determination of organophosphate nerve agents. Biotechnol. Prog. 15, 130–134.

Mulchandani, P., Chen, W., Mulchandani, A., Wang, J., Chen, L., 2001. Amperometric microbial biosensor for direct determination of organophosphate pesticides using recombinant microorganism with surface expressed organophosphorus hydrolase. Biosens. Bioelectron. 16, 433–437.

Omburo, G.A., Kuo, J.M., Mullins, L.S., Raushel, F.M., 1992. Characterization of the zinc binding site of bacterial phosphotriesterase. J. Biol. Chem. 267, 13278–13283.

Pei, L., Omburo, G., McGuinn, W.D., Petrikovics, I., Dave, K., Raushel, F.M., Wild, J.R., DeLoach, J.R., Way, J.L., 1994. Encapsulation of phosphotriesterase within murine erythrocytes. Toxicol. Appl. Pharmacol. 124, 296–301.

Petrikovics, I., Hong, K., Omburo, G., Hu, Q.Z., McGuinn, W.D., Sylvester, D., Tamulinas, C., Papahadjopouls, D., Jaszberenyi, J.C., Way, J.L., 1999. Antagonism of paraoxon intoxication by recombinant phosphotriesterase encapsulated within sterically stabilized liposomes. Toxicol. Appl. Pharmacol. 156, 56–63.

Rainina, E.I., Efremenco, E.N., Varfolomeyev, S.D., Simonian, A.L., Wild, J.R., 1996. The development of a new biosensor based on recombinant *E. coli* for the direct detection of organophosphorus neurotoxins. Biosens. Bioelectron. 11, 991–1000.

Raushel, F.M., Holden, H.M., 2000. Phosphotriesterase: an enzyme in search for its natural substrate. Adv. Enzymol. Relat. Areas Mol. Biol. 74, 51–93.

Raveh, L., Grauer, E., Grunwald, J., Cohen, E., Ashani, Y., 1997. The stoichiometry of protection against soman and VX toxicity in monkeys pretreated with human butyrylcholinesterase. Toxicol. Appl. Pharmacol. 145, 43–53.

Roger, K.R., Wang, Y., Mulchandani, A., Mulchandani, P., Chen, W., 1999. Organophosphorus hydrolase-based fluorescence assay for organophosphate pesticides. Biotechnol. Prog. 15, 517–522.

Saxena, A., Maxwell, D.M., Quinn, D.M., Radic, Z., Taylor, P., Doctor, B.P., 1997. Mutant acetylcholinesterase as potential detoxification agents for organophosphate poisoning. Biochem. Pharmacol. 54, 269–274.

Scharff, E.I., Lucke, C., Fritzsch, G., Koepke, J., Hartleib, J., Dierl, S., Ruterjans, H., 2001. Crystal structure of diisopropylfluorophosphatase from *Loligo vulgaris*. Structure 9, 493–502.

Schrader, G., 1950. Organische phosphor-verbindungen als neuartige insektizide (auszug). Angew. Chem. 62, 471–473.

Thoden, J.B., Phillips, G.N., Neal, T.M., Raushel, F.M., Holden, H.M., 2001. Molecular structure of dihydroorotase: a paradigm for catalysis through the use of a binuclear metal center. Biochemistry 40, 6989–6997.

Tuovinen, K., Kaliste-Korhonen, E., Raushel, F.M., Hänninen, O., 1994. Phosphotriesterase: a promising candidate for use in detoxification of organophosphates. Fundam. Appl. Toxicol. 23, 578–584.

Tuovinen, K., Kaliste-Korhonen, E., Raushel, F.M., Hänninen, O., 1996a. Protection of organophosphate-inactivated esterases with phosphotriesterase. Fundam. Appl. Toxicol. 31, 210–217.

Tuovinen, K., Kaliste-Korhonen, E., Raushel, F.M., Hänninen, O., 1996b. Eptastigmine–phosphotriesterase combination in DFP intoxication. Toxicol. Appl. Pharmacol. 140, 364–369.

Tuovinen, K., Kaliste-Korhonen, E., Raushel, F.M., Hänninen, O., 1996c. Phosphotriesterase, pralidoxime-2-chloride (2-PAM), and eptastigmine treatments and their combinations in DFP intoxication. Toxicol. Appl. Pharmacol. 141, 555–560.

Tuovinen, K., Kaliste-Korhonen, E., Raushel, F.M., Hänninen, O., 1999. Success of pyridostigmine, physostigmine, eptastigmine and phosphotriesterase treatments in acute sarin intoxication. Toxicology 134, 169–178.

Vanhooke, J.L., Benning, M.M., Raushel, F.M., Holden, H.M., 1996. Three-dimensional structure of the zinc-containing phosphotriesterase with the bound substrate analog diethyl 4-methylbenzylphosphonate. Biochemistry 35, 6020–6025.

Wilce, M.C.J., Bond, C.S., Dixon, N.E., Freeman, H.C., Guss, J.M., Lilley, P.E., Wilce, J.A., 1998. Structure and mechanism of a proline-specific aminopeptidase from *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 95, 3472–3477.

Wu, F., Li, W.S., Chen-Goodspeed, M., Sogorb, M.A., Raushel, F.M., 2000. Rationally engineered mutants of phosphotriesterase for preparative scale isolation of chiral organophosphates. J. Am. Chem. Soc. 122, 10206–10207.