Phosphotriesterase variants with high methylphosphonatase activity and strong negative trade-off against phosphotriesters

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The most lethal organophosphorus nerve agents (NA), like sarin, soman, agent-VX and Russian-VX, share a methylphosphonate moiety. Pseudomonas diminuta phosphotriesterase (PTE) catalyses the hydrolysis of methylphosphonate NA analogues with a catalytic efficiency orders of magnitude lower than that towards the pesticide paraaxon. With a view to obtaining PTE variants that more readily accept methylphosphonate NA, ~75 000 PTE variants of the substrate-binding residues Gly-60, Ile-106, Leu-303 and Ser-308 were screened with fluorogenic analogues of the NA Russian-VX and cyclosarin. Seven new PTE variants were isolated, purified and their kcat/KM determined against five phosphotriesters and five methylphosphonate analogues of sarin, cyclosarin, soman, agent-VX and Russian-VX. The novel PTE variants exhibited as much as a 10-fold increase in activity towards the methylphosphonate compounds—many reaching a kcat/KM of 10⁶ M⁻¹ s⁻¹—and as much as a 29 000-fold decrease in their phosphotriesterase activity. The mutations found in two of the variants, SS0.5 (G60V/I106L/S308G) and SS4.5 (G60V/I106A/S308G), were modelled into a high-resolution structure of PTE-wild type and docked with analogues of cyclosarin and Russian-VX using Autodock 4.2. The kinetic data and docking simulations suggest that the increase in activity towards the methylphosphonates and the loss of function against the phosphotriesters were due to an alteration of the shape and hydrophobicity of the binding pocket that hinders the productive binding of non-chiral racemic phosphotriesters, yet allows the binding of the highly asymmetric methylphosphonates.

Keywords: nerve-agents/phosphotriesterase/screening/trade-off

Introduction

Organophosphate compounds (OP) have been extensively used as pesticides over the last 50 years (Sultatos, 1994). Some OP are efficient inhibitors of cholinesterases, key regulators of nerve impulse transmission at cholinergic synapses and neuromuscular junctions (Tõugu, 2001). OP are derivatives of phosphoric acids with different types of alkyl/alkoxy and leaving groups that surround a central phosphorus atom [phosphorous centre (P-centre)]. In addition to their application in agriculture, compounds with an optimised toxicity have been weaponised as poisonous nerve agents (NA) for their use in chemical warfare. The most toxic NA, such as sarin, soman, cyclosarin and the particularly pernicious agent-VX and Russian-VX, share a methylphosphonate moiety (Supplementary Fig. S1); the unusually high reactivity of methylphosphonate NA towards cholinesterases is largely due to the efficient accommodation of this moiety in the enzymes’ active site (Ordentlich et al., 2005).

A number of enzymes have been identified that catalyse the hydrolysis of toxic OP (DeFrank and Cheng, 1991; Mackness et al., 1998; Vilanova and Sogorb, 1999). Among these, Pseudomonas diminuta phosphotriesterase (PTE) gained particular popularity due to its ease of production and its catalytic power towards the man-made chemical compounds. Against the OP insecticide paraoxon, PTE exhibits a turnover number (kcat) of at least 2000 s⁻¹ and a diffusion-limited catalytic efficiency (kcat/KM) of ~1 × 10⁶ M⁻¹ s⁻¹ (Dumas et al., 1989). In addition to its paraoxonase activity, PTE-wild type (wt) exhibits high catalytic efficiencies of 9.6 × 10⁴ and 1.1 × 10⁵ M⁻¹ s⁻¹ towards the racemic mixtures of sarin and Russian-VX, respectively (Dumas et al., 1990). PTE is likely to have recently evolved from lactonases with a lower promiscuous PTE activity (Afiati et al., 2006).

The mechanism behind the catalytic prowess of PTE has been studied extensively. The catalysis occurs via nucleophilic attack by a metal-bound hydroxide in an S N2 mechanism with a net stereo inversion of the P-centre (Lewis et al., 1988; Aubert et al., 2004). Crystallographic data have unambiguously established that the activating metal ions are coordinated through a cluster of His and Asp residues within the core of an α/β-barrel fold (Benning et al., 1995, 2000, 2001). Structural and kinetic studies had originally proposed that the reactive nucleophilic molecule is the μ-hydroxyl that bridges the metal ions (Aubert et al., 2004; Kim et al., 2008); however, work done in the Agrobacterium radiobacter PTE homologue proposed that an hydroxide molecule terminally coordinated to the α-metal is in fact the nucleophilic agent, having the bridging μ-hydroxyl in a role of a general base (Jackson et al., 2005, 2008). In the case of the natural PTE (PTE-wt), a pair of Zn(II) ions was found bound through a cluster of four histidines (His-55, His-57, His-201 and His-230), one aspartate (Asp-301) and bridged together by one carboxylated lysine (Lys-169; Kuo et al., 1997). It is possible to replace the naturally occurring Zn(II) with Co(II), Cd(II) or Mg(II) ions and still retain—or even further improve—the enzyme’s function (Omburo et al., 1993; Benning et al., 2001).
Crystal structures of PTE-wt have been solved in complex with non-hydrolysable OP substrate analogues (Vanhooke et al., 1996; Benning et al., 2000). In all the structures, the inhibitors were oriented in such a way that the double-bonded oxygen was placed within interacting distance of the β-metal, the Ne1 of Trp-131 and the Nδ1 of His-201. These electrostatic interactions apart, substrate recognition by PTE is mainly determined by geometrical constraints and hydrophobic interactions (Benning et al., 1995, 2000, 2001; Vanhoeke et al., 1996; Aubert et al., 2004; Kim et al., 2008). Vanhoeke et al. (1996) suggested that the binding site of PTE could be functionally divided into three pockets, each binding different substituents of the P-centre; these sites were called ‘small’ (Gly-60, Ile-106, Leu-303 and Ser-308), ‘large’ (His-254, His-257, Leu-271 and Met-317) and ‘leaving group’ (Trp-131, Phe-132, Phe-306 and Tyr-309; Fig. 1A). Several studies have shown that it is possible to alter PTE specificity for NA and analogues by substituting residues within the substrate-binding site (Watkins et al., 1997; Di Sioudi et al., 1999; Gopal et al., 2000); in addition, it has been possible to manipulate PTE’s inherent stereoselectivity for chiral NA chromogenic analogues by mutating residues within the ‘small’ pocket (Hong and Raushel, 1999; Chen-Goodspeed, et al., 2001a,b).

The present paper describes the selection of PTE variants from a library of the substrate-binding residues of the ‘small’ pocket (Fig. 1B), using as probes racemic fluorogenic analogues of Russian-VX and cyclosarin (Fig. 2A). Seven new PTE variants were purified and their catalytic properties determined in vitro against a panel of phosphotriester and methylphosphonate surrogates of NA. The selected set displayed an up to 10-fold increase in their $k_{cat}/K_M$ levels towards the methylphosphonate substrates tested, but showed an acute loss of catalytic power towards the phosphotriesters. Models of two variants were constructed and used in in silico docking experiments. These docking experiments, kinetic data and analysis of the mutations obtained suggest that, in the new PTE variants, the increase in methylphosphonatase

![Fig. 1](image1.png)  
**Fig. 1** (A) *Pseudomonas diminuta* PTE co-crystallised with Zn(II)/Zn(II) and the sarin analogue DIMP (Benning et al., 2001). (B) The PTE active site comprises two zinc ions (magenta), a set of metal-binding residues (grey thin lines) and substrate-binding residues clustered into ‘small’ (red), ‘large’ (green) and ‘leaving group’ (yellow) pockets. The water molecule bridging the two metal ions is shown as a small red sphere. In the library screened, the residues of the small pocket Gly-60, Ile-106, Leu-303 and Ser-308 were randomised by saturation mutagenesis.

![Fig. 2](image2.png)  
**Fig. 2** Chemical surrogates of pesticides and nerve agents (NA). (A) The fluorogenic analogues of phosphotriesters (compounds 1–5) and methylphosphonate (compounds 6–10) NA. The ‘X’ denotes the place of attachment of the fluorogenic leaving group, 3-chloro-7-oxy-4-methylcoumarin. The probes used during the screening were Russian-VX analogue (compound 8; IBCPn) and cyclosarin analogue (compound 10; CHCPn). (B) Ligands with acetylphenyl leaving groups used in the docking experiments, Russian-VX analogue (IBAPn), cyclosarin analogue (CHAPn) and phosphotriester (DEAP). The two other compounds used in the modelling study—EPO and DIMP—are also depicted. The structure of the pesticide paraoxon used in the kinetic experiments is also shown.
activity and difference in selectivity between phosphotriesters and methylphosphonates are due to the highly asymmetric P-centre of the latter.

Materials and methods

Chemicals, enzymes and primers

All enzymes and corresponding buffers were from New England BioLabs (Beverly, MA, USA), apart from Turbo Pfu-polymerase that was from Stratagene (La Jolla, CA, USA) and BpiI from Fermentas Inc. (Hanover, Germany). M 280 Streptavidin Dynabeads were from Invitrogen (Paisley, UK). Antifoam 204 was from Sigma (St Louis, MO, USA). BugBuster™ was from Novagen (Lutterworth, UK). PROTRAN Nitrocellulose Transfer membrane (pore size 0.45 μm) was from Schleicher and Schuell BioScience Inc. (Dassel, Germany). The fluorogenic NA analogues were synthesised as described previously (Briseño-Roa et al., 2006).

Library construction

The PTE library was synthesised as described previously (Griffiths and Tawfik, 2003) with modification. Using the template pLVEX-S5 and a standard PCR using PFU (Stratagene), DNA fragments were created up and downstream from the regions corresponding to residues Gly-60 and Leu-303/Ser-308 using the pair of primers LMB2-1(3× biotin)/P010704B, PIVB-1/P010704A, P010704K/LMB2-1(3× biotin) and P010704I/PIVP-1. These products were treated with 20 U of BpiI enzyme in a final volume of 80 μl for 3 h at 37°C. After purification, the digested fragments were mixed in equimolar ratios (typically 10 pmol in 50 μl) and incubated with T4 Ligase for 20 h at 10°C. Ligation products were purified by capture through the biotin tags with streptavidin-coated Dynabeads M 280 (Invitrogen). Fifty microlitres (500 ng) of the DNA-bead conjugates was used for two additional PCRs with the primer pairs P090804A/PIVB-3 and P090804B/LMB2-3(3× biotin). The procedure of BpiI-digestion, T4-ligation and bead-capture was repeated. The whole reconstituted gene was obtained using the DNA-bead conjugates as templates for a PCR with the primer pair P220404A/P220404B. Once synthesised, the libraries were further digested with NcoI and SacI endonucleases and purified by gel electrophoresis. Ligation into pLVEX were performed using 2 U T4 DNA ligase in a final volume of 50 μl. Typically, 10–35 μg of insert and 4–18 μg of vector were used. The ligation mixtures were left at 4°C for 3 h. One hundred microlitres of Escherichia coli MC1061 (F-araD139 Δ(lac-leu)7696 galE15 galK16 Δ(lac)X74 rpsL(Strr) hsdR2(rk-mk+) mcrA mcrB1) cells (7.8 × 10⁸ cells ml⁻¹) was transformed with 9–12 μg (total) of ligated library DNA. After recovering at 37°C for 20–25 min, the cells were plated on pre-dried 23 x 23 cm plates with 2xTY, 1.0% (w/v) D(+)glucose, 100 μg mL⁻¹ ampicillin, 1.5% (w/v) agar, covered with a 22 x 22 cm Nitrocellulose Transfer membrane and incubated at 37°C for 14 h.

Screening

The selection in bacterial cells was performed as described elsewhere (Briseño-Roa et al., 2010). Briefly, E. coli C41 cells were transformed with 100–180 ng of library plasmid DNA. Cells were plated in 23 x 23 cm plates containing 2xTY, 1.0% (w/v) D(+)glucose, 100 μg mL⁻¹ ampicillin, 1.5% (w/v) agar, covered with a 22 x 22 cm Nitrocellulose Transfer membrane and incubated at 37°C for 14 h. Membranes were detached and inversely placed over 23 x 23 cm plates containing 2xTY, 1 mM IPTG, 100 μg mL⁻¹ ampicillin and 1.5% (w/v) agar. The plates were incubated at room temperature for ~ 20 h. Twenty-five microlitres of 0.5% (w/v) agarose, 2 × BugBuster (Invitrogen), 50 mM HEPES, pH = 8.5 (per plate), was prepared and equilibrated at 45°C. The substrate used as a probe was added to a final concentration of 50 μM. The formulation was poured over the plate containing the induced bacterial colonies. All the substrate manipulation was carried out in a fume hood. NA analogues are toxic and should be handled with care (Briseño-Roa et al., 2006). Plates were illuminated from above with a 365-nm UV lamp, and colonies with the highest activity were picked with sterile wooden toothpicks and recovered in LB solid media. Several colonies from each selected clone were transferred into 384-well microplates and in vivo activity determined against the probes. Plasmid DNA was extracted and sequenced with PTE-1 and PTE-2 primers.

Protein expression and purification

The selected alleles were cloned into a pRSET-A (Novagen) modified vector. The DNA inserts were positioned downstream in frame with a sequence that encoded a His tag followed by the soluble Lipoyl Domain and a TEV protease cleavage recognition site. The constructs were cloned into E. coli C41 (Miroux and Walker, 1996) and grown in an auto-induction medium [2xTY, 1 mM MgSO₄, 0.5% (v/v) glycerol, 0.05% (v/v) D(+)glucose, 0.2% (w/v) α-lactose, 50 mM KH₂PO₄, 50 mM NaH₂PO₄, 25 mM (NH₄)₂SO₄, 0.5% (v/v) Antifoam-204 and 1 mM ZnCl₂] at 37°C, shaking at 240 rpm. After 8 h, the temperature was decreased to 25°C for a further incubation of 16 h. Cells were harvested, lysed by sonication and the soluble fraction obtained by centrifugation. The filtered (0.2 μm) supernatants were loaded onto a 25-ml Nickel-NTA (Qiagen) column equilibrated with 50 mM potassium phosphate, 300 mM NaCl, 10 mM imidazole, pH = 8.0, using a Pharmacia low-pressure system. The protein was eluted using 50 mM potassium phosphate, 300 mM NaCl, 250 mM imidazole, pH = 8.0. Fractions containing the target construct were pooled and treated typically with 1 mg of TEV protease per 50–200 mg of protein and dialysed against 50 mM potassium phosphate, 300 mM NaCl, 50 mM imidazole, pH = 8.0, at 4°C for 16 h. The dialysed samples were filtered (0.2 μm) and loaded onto a 25-ml Nickel-NTA column equilibrated with the dialysis buffer. The flow-through fractions were pooled, concentrated and subsequently run in a Superdex-75 column (Phamacia, Pfizer) pre-equilibrated with 50 mM NaCl, 50 mM HEPES, pH = 8.5. Fractions were analysed by SDS–PAGE and pooled based on absorbance at 280 nm and enzymatic activity. The protein concentration was determined by spectrophotometry. The purity of the enzyme preparations was determined to be >95% by SDS–PAGE. Aliquots were
prepared, frozen in liquid N₂ and stored at −80°C until further use.

**Determination of kinetic parameters**

It was determined as described previously (Briseño-Roa et al., 2006), with the addition that the enzymes were pre-incubated for ~16 h at 4°C in freshly prepared 50 mM HEPES, pH = 8.5, BSA 0.1 mg mL⁻¹, 10 μM ZnCl₂ and 100 μM KHCO₃. The hydrolysis of the NA analogues was followed by the change in fluorescence at 460 nm (ex. = 350 nm, cut-off = 455 nm) using a SpectraMAX GeminiXS Microplate reader (Molecular Devices) at 25°C. Calibration endpoints were obtained by incubating the substrates with 0.1 M NaOH.

**Structure modelling and docking**

Ground states of the enantiomers of O-isobutyl O-acetylphenyl methylphosphonate (R⁺-IBAPn and S⁻-IBAPn) and O-cyclohexyl O-acetylphenyl methylphosphonate (R⁺-CHAPn and S⁻-CHAPn) were modelled, energy minimised (MM2 force field) and the partial charges added (extended Huëckel method) using ChemBio3D Ultra v.11.0 (CambridgeSoft, UK). The high-resolution PTE structure 1HZY (Benning et al., 2008) was first prepared using a text editor: non-polypeptide atoms were removed with the exception of the Zn atoms (residues 401 and 402), catalytic water (residue 876) and the carboxyl group of the post-translationally modified Lys-169. Covalent bonds were defined between Zn (401) and D301-062, His55-Ne2, His57-Ne2, Lys-169-CBXO1; and between Zn (402) and His201-Ne2, His230-Ne2, Lys-169-CBXO2. The catalytic oxygen was covalently linked to both Zn atoms. Polar hydrogens and Kollman partial charges were added using AutodockTools 1.5.2 (Scripps Research Institute, USA). The charges of the Zn ions were set at +1.40 each, and the remaining charge was transferred to the coordinating residues as described previously (Irwin et al., 2005). The partial charge of the catalytic oxygen was set to −1.0. Mutations at the active site of PTE were done using the program Chimera (University of California, USA) and minimised with a GROMOS 43B1 force field (Van Gunsteren et al., 1996). Dockings were done using the programs Autogrid and Autodock 4.2 (Scripps Research Institute, USA) running in an AutodockTools 1.5.2 (Scripps Research Institute) environment (Morris et al., 2008). A 40 × 40 × 40 grid with a 0.375-Å spacing was set centred in the PTE active site. Dockings experiments were done using a Lamarckian genetic algorithm with 100 docks, an initial population of 300 individuals and performing 500 000 energy evaluations or 27 000 generations. These docking parameters were obtained from preliminary tests using the inhibitor disopropyl methylphosphonate (DIMP); the results were evaluated by comparing them against the structure of PTE co-crystallised with DIMP (PDB code 1EZ2; Benning et al., 2000). The substrate diethyl 4-methoxophenyl phosphate (EPO) co-crystallised with Agrobacterium PTE (Jackson et al., 2008) was set to have five free torsion angles and with random values in the initial states; all side chains were kept fixed in the macromolecular template. Docking cluster analysis was done with a 1.75-Å RMSD. Each docking experiment took ~2.5 h to be completed in a 1.86-GHz Intel Pentium M processor. Figures were prepared using PyMOL (Delano Scientific, PaloAlto).

**Results**

A PTE library of the residues forming the ‘small’ substrate-binding pocket (Gly-60, Ile-106, Leu-303 and Ser-308; Fig. 1A) was synthesised and screened as described elsewhere (Briseño-Roa et al., 2010). Briefly, the selected residues were randomised by saturation mutagenesis using the PTE-S5 variant as a template for the library. PTE-S5 contains non-active site mutations that confer a 20-fold increase in expression in E. coli cells while maintaining identical catalytic properties against paraaxon and 2-naphthyl acetate (Roodveldt and Tawfik, 2005). The library was transformed into E. coli C41 cells and screened using a top-agarose formulation that contained non-denaturing detergents and fluorogenic probes. Two probes were separately used: O-cyclohexyl O-3-chloro-7-oxo-4-methylcoumarin methylphosphonate (CHCPn) and O-isobutyl O-3-chloro-7-oxo-4-methylcoumarin methylphosphonate (IBCPn), the former being an analogue of the NA cyclosarin and the latter an analogue of Russian-VX (Fig. 2A). After screening between 25 000 and 50 000 colonies with each probe, the selected clones (26 in total) were grown in microplates and their activity against either of the probes measured. The top eight clones with the highest in vivo activity (normalised by the A₅₀₀nm) were recloned into an expression vector containing in its N-terminus a His tag and sequenced for a second time. Proteins were expressed and purified using a combination of NTA-Nickel and gel filtration columns. The catalytic efficiency (k_{cat}/K_M) towards a panel of NA analogues (Fig. 2A) was determined for a set of seven final variants, PTE-wt and PTE-S5. The panel of NA analogues tested included fluorogenic surrogates of the phosphotriester pesticides methyl-parathion (compound 1), parathion (compound 2), methyl-paraoxon (compound 3), paraoxon (compound 4), the phosphate NA DFP (compound 5), and the methylphosphonate NA: agent-VX (compound 6), sarin (compound 7), Russian-VX (compound 8), soman (compound 9) and cyclosarin (compound 10). The analogues for Russian-VX and cyclosarin corresponded to the probes IBCPn and CHCPn, respectively (Fig. 2A).

The PTE variant S5 exhibited similar kinetic properties to those of PTE-wt towards most of the compounds tested (Fig. 3). The main differences were found with methyl-paraoxon (k_{cat}/K_M = 1.4 ± 0.7 × 10⁸ M⁻¹ s⁻¹) and parathion (6.8 ± 0.4 × 10⁸ M⁻¹ s⁻¹) as PTE S5 hydrolysed them ~3-fold faster than PTE-wt (Table I). The selected PTE variants exhibited different kinetic properties from PTE-S5 and PTE-wt. A final set of four PTE variants were obtained when the library was probed with IBCPn: SS0.5, SS1.6, SS1.7 and SS2.8. When compared with PTE-S5, PTE variant SS0.5 showed a 2- and 10-fold increase in its catalytic efficiency against the Russian-VX analogue/probe IBCPn (5.1 ± 0.3 × 10⁸ M⁻¹ s⁻¹) and the soman analogue (7.8 ± 0.1 × 10⁷ M⁻¹ s⁻¹), respectively. Mutants SS1.6 and SS1.7 exhibited a similar 6-fold increased towards the cyclosarin analogue (5 × 10⁸ M⁻¹ s⁻¹). When the library was probed with the cyclosarin analogue CHCPn, a final set of three PTE variants were obtained: SS4.1, SS4.5 and SS4.10. PTE variant SS4.1 exhibited a 4-fold increase in its k_{cat}/K_M against
Russian-VX analogue \( (8.6 \pm 1.0 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \). Variant SS4.5 showed 6- and 7-fold increases in its catalytic efficiency towards soman \( (4.4 \pm 0.2 \times 10^5 \text{ M}^{-1} \text{s}^{-1}) \) and cyclosarin \( (5.5 \pm 0.6 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \), respectively. PTE mutant SS4.10 proved to be 3-fold more active towards the Russian-VX analogue/probe IBCPn \( (5.9 \pm 0.6 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \). In contrast with their activity increase against the methylphosphonate substrates (compounds 6–10), all seven of the characterised PTE variants showed a considerable decrease in their activity towards the phosphotriester substrates tested (compounds 1–5). In the case of PTE mutants SS0.5—selected with the probe IBCPn—the activity against the methyl-paraoxon \( (6.3 \pm 0.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}) \) and paraoxon \( (9.0 \pm 0.5 \times 10^3 \text{ M}^{-1} \text{s}^{-1}) \) analogues dropped 20- and 300-fold, respectively. Similarly, for mutant SS4.5—selected with the probe CHCPn—the activity against methyl-paraoxon \( (k_{\text{cat}}/K_M = 3.7 \pm 0.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}) \) and paraoxon \( (k_{\text{cat}}/K_M = 680 \pm 63 \text{ M}^{-1} \text{s}^{-1}) \) analogues was reduced \( \sim 120 \)-fold and 29 000-fold, respectively. The affinity and turnover number towards the pesticide paraoxon were also severely compromised (Table I).

To estimate qualitatively the cause of the dramatic change in the selectivity of the new PTE variants, models of two selected variants and in silico docking experiments were carried out using as a template a high-resolution crystallographic structure of PTE and the program Autodock 4.2 (Benning et al., 2001; Morris et al., 2008). A detailed description of the docking experiments can be found in the ‘Materials and methods’ section. Briefly, a PTE-wt structure and models of the mutants PTE-SS4.5 (G60V/I106A/S308G) and PTE-SS0.5 (G60V/I106L/S308G) were used as rigid macromolecular templates. The substrates utilised as ligands were \( O,O \)-diethyl \( O \)-acetylphenyl phosphate (DEAP), the enantiomers of \( O \)-isobutyl \( O \)-acetylphenyl methylphosphonate \( (R_P \)-IBApn and \( S_P \)-IBApn) and \( O \)-cyclohexyl \( O \)-acetylphenyl methylphosphonate \( (R_P \)-CHAPn and \( S_P \)-CHAPn), the former pair being analogues of Russian-VX and the latter analogues of cyclosarin. Ground-state compounds bearing an acetylphenyl leaving group were used given that preliminary docking experiments using coumarin-based ligands yielded too few or no productive poses as the predicted interactions were dominated by the bulky coumarin leaving group (data not shown). Several chloropyrifos high-

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**Table I.** Catalytic efficiencies \( (k_{\text{cat}}/K_M \text{ M}^{-1} \text{s}^{-1}) \) for PTE-wt, PTE-SSS and selected variants for fluorogenic NA analogues.

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<td>( 9.4 \pm 0.7 \times 10^7 )</td>
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**References:**

Benning et al., 2001; Morris et al., 2008.
Table II. Catalytic parameters towards paraoxon for PTE-wt, PTE-S5 and selected variants

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Table III. Results from the docking experiments

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<th>SS4.5</th>
<th>SS0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAP</td>
<td>−3.24α0.14</td>
<td>−2.59α0.02</td>
<td>−2.52α0.01</td>
</tr>
<tr>
<td>R5-IBAPn</td>
<td>−3.90α0.13</td>
<td>−3.94α0.03</td>
<td>−3.55α0.04</td>
</tr>
<tr>
<td>S5-IBAPn</td>
<td>−3.20α0.02</td>
<td>−3.23α0.04</td>
<td>NF</td>
</tr>
<tr>
<td>R5-CHAPn</td>
<td>−4.59α0.02</td>
<td>−4.63α0.08</td>
<td>−4.10α0.01</td>
</tr>
<tr>
<td>S5-CHAPn</td>
<td>NF</td>
<td>−4.56α0.01</td>
<td>NF</td>
</tr>
</tbody>
</table>

First figure indicates predicted binding energy (in kcal mol$^{-1}$) of the best-ranked productive pose; second figure indicates fraction of productive poses found from 100 docking experiments. NF, no productive poses found.

When the paraoxon analogue DEAP was docked into the PTE-wt binding pocket, productive poses grouped together in the best-ranked and most-populated clusters (with 14% of the total poses; Table III, Fig. 4). The pose with the lowest binding energy was therefore productive, with a binding constant of $-3.24$ kcal mol$^{-1}$. Similarly, docking with the enantiomer $R_5$-IBAPn showed the three best-ranked and most-populated clusters containing productive poses (with 13% of the total); the best-fitted pose showed a binding affinity of $-3.90$ kcal mol$^{-1}$. With the enantiomer $S_5$-IBAPn, the best-ranked poses within the PTE-wt binding pocket were not productive (having the leaving group occupying the central cavity of the pocket); however, productive solutions were found in a cluster containing 2% of the total poses, with the best having a binding constant of $-3.20$ kcal mol$^{-1}$. As for the cyclosarin analogues, the best-ranked pose with the enantiomer $R_5$-CHAPn was found to be productive with a binding constant of $-4.59$ kcal mol$^{-1}$ (2% productive poses). No productive poses were found with the enantiomer $S_5$-CHAPn.

In contrast to PTE-wt, only two productive poses were found when the phosphotriester DEAP was docked into the structural model of the mutant SS4.5 (G60V/I106A/S308G; Fig. 4); the best of these showed a binding constant of $-2.59$ kcal mol$^{-1}$ (Table III). Docking $R_5$-IBAPn on the
SS4.5 model also showed fewer productive poses (3%) compared with PTE-wt; however, a cluster containing these few poses was the best ranked, having the lowest binding energy of $-3.94 \text{ kcal mol}^{-1}$. In the case of $S_P$-IBAPn, although the docking program did find productive poses, these were neither ranked-first nor belonged to the most-populated cluster. Several productive poses were found docking $R_P$-CHAPn into SS4.5, all found in the first and second ranked cluster, with a lowest binding energy of $-4.63 \text{ kcal mol}^{-1}$. Only one productive pose was obtained with upon docking the enantiomer $S_P$-CHAPn, and it was ranked first with a binding energy of $-4.56 \text{ kcal mol}^{-1}$ (Fig. 4). In the case of docking of the paraoxon analogue DEAP with the mutant SS0.5 (G60V/I106L/S308G), productive poses were not found to be the best-ranked or within significantly populated clusters. With $R_P$-IBAPn, the two poses with the lowest-affinity binding constant were found to have a productive configuration, but no productive poses were found with either $S_P$-IBAPn or $S_P$-CHAPn. In general, fewer productive poses were found in the models of the PTE mutants and to make sure that the loss of binding was not due to artefacts of the modelled residues within the active site, a PTE-wt model was re-built from the SS4.5 coordinates and then docked into DEAP. This re-built PTE-wt model showed similar docking results to those obtained using the crystallographic PTE-wt itself (data not shown).

**Discussion**

A set of new PTE variants have been isolated from a library containing the residues of the ‘small’ pocket randomised by saturation mutagenesis (Fig. 1). The library was screened by partial lysis of bacterial cells using as probes the fluorogenic analogues of Russian-VX and cyclosarin. These new seven PTE variants were purified and their catalytic efficiency ($k_{cat}/K_M$) determined for five phosphotriesters and five methylphosphonates (Fig. 2A). The mutants showed wt or higher (up to 10-fold) $k_{cat}/K_M$ levels towards the methylphosphonate substrates and acute losses of function against the phosphotriesters (Fig. 3). For instance, PTE variant SS4.5 (G60V/I106A/S308G) showed a 7-fold increase against the probe cyclosarin analogue (CHCPn) and a 120-fold decrease against the methyl-parathion analogue—and a dramatic 58 000-fold decrease for the pesticide paraoxon (Table II).

Given the steric-dominated active site, it is remarkable that such dramatic reductions were observed, in particular where the phosphotriester substrates were less bulky than the methylphosphonates (e.g. methyl-paraoxon versus soman analogues). How can such a clear resolution between methylphosphonates and phosphotriesters be achieved? It is likely that this strong trade-off between the phosphotriesterase and methylphosphonate activities is caused by the difference in the symmetry of their P-centres: the methylphosphonates have in common a P-methyl group that is smaller and more hydrophobic than the phosphotriester methoxy group, and moieties that are equal in size or bulkier than an ethoxy group; this difference in the size of the groups creates a highly asymmetric P-centre on the methylphosphonates (as opposed to the achiral P-centres on the phosphotriesters tested).

Considering that the highly asymmetric P-centres imply that the $S_P$ and $R_P$ enantiomers are geometrically very distinct from each other, that PTE-wt inherently hydrolyses $R_P$-phosphonates more readily (Nowlan et al., 2006) and that its stereo-selectivity can be significantly modified by mutagenesis of the residues within the active site (Chen-Goodspeed et al., 2001a,b), it is possible that eventual changes in the enantio-selectivity of the selected mutants contributes to the strong selectivity against phosphotriesters. To estimate qualitatively the stereo-selectivity of the selected variants, the mutations found in variants SS0.5 (G60V/I106L/S308G) and SS4.5 (G60V/I106A/S308G) were
modelled into a PTE-wt crystallographic structure and docked with four methylphosphonate enantiomers and one phosphotriester (Figs 2B and 5). Docking runs using PTE-wt qualitatively recapitulated its stereo-selectivity, as productive poses were more abundant and energetically favourable when the \( R_P \) enantiomers were used as ligands (Table III); the docking experiments also recapitulated the exclusion of phosphotriesters from the active site of SS0.5 and SS4.5 models, as conspicuous decreases in the number and affinity of ligand poses for the phosphotriester were observed (Table III, Fig. 4, Supplementary Figs S2 and S3). As seen for PTE-wt in the model of the mutants, productive poses with \( R_P \) enantiomers were also found, and in the case of SS4.5, favourable poses were also found with the \( S_P \) enantiomers (Table III, Figs 3 and 5). Given the overall decrease in the productive poses obtained for variant SS0.5 and the diverse nature of the mutations found in the other selected variants, it is not possible to draw a general conclusion on the enantio-selectivity of the new variants; however, the docking experiments suggest that at least in the case of variant SS4.5, the strong negative trade-off against phosphotriesters does not seem to reflect an acute change in the stereo-selectivity and that the \( S_P \) enantiomer could be better fitted into SS4.5 than into the PTE-wt active site.

At a more mechanistic level, how can an increase in the methylphosphonatase activity be concomitant with a decrease in the PTE activity? The ‘small’ pocket accommodates optimally the ethoxy group of pesticides 2 and 4; the decrease in the PTE activity? The ‘small’ pocket accommodates optimally the ethoxy group of pesticides 2 and 4; the decrease in the \( k_{cat}/K_M \) for the methylphosphonate analogues suggests that at least in the case of variant SS4.5, the strong negative trade-off against phosphotriesters does not seem to reflect an acute change in the stereo-selectivity and that the \( S_P \) enantiomer could be better fitted into SS4.5 than into the PTE-wt active site.

The seven PTE-S5 variants selected contained three or four mutations (in all cases at Gly-60, Ile-106 and Ser-308) and in some cases (four of the mutants) at Leu-303. Summation of the van der Waals volumes of the four amino acids comprising the small pockets in all variants indicated that the relative volume increase in the small pocket was around 2-fold (data not shown), suggesting the space available for binding there had diminished considerably, perhaps even halved. In six of the variants, Gly-60 had been replaced by valine (G60V), which along with two or three co-mutations, could account for the loss of phosphotriesterase function by hindering the binding of even small achiral phosphates (such as pesticide analogue 4) as replacement of the \( \alpha \)-hydrogen atom of glycine with the isopropyl side chain of valine considerably reduces the size of the small pocket, but increases its hydrophobicity. In PTE variant SS2.8, the Gly-60 had been replaced by alanine (G60A). Replacement of the \( \alpha \)-hydrogen atom of glycine here with the methyl side chain of alanine restricts the small pocket and increases its hydrophobicity too, but to a lesser extent than the G60V substitution. This alteration should limit or exclude binding of OP substrates with large alkoxy groups and lower the affinity for inappropriately configured enantiomers. It has been observed that G60A mutants are unable to bind racemic substrates with bulky (e.g. isopropoxy or phenoxy) substituents and \( S_P \)-phosphonate enantiomers productively (Chen-Goodspeed et al., 2001a,b; Nowlan et al., 2006). In addition to the consensus G60V mutation, in some mutants obvious large-to-small substitutions at Ile-106 and Ser-308 were co-selected, for instance mutants SS0.5 (G60V/I106A/S308G) and SS4.5 (G60V/I106A/S308G). Two zinc ions (magenta) and water molecule bridged by metals (red) are shown. Dockings were done using Autodock 4.2. Protein surfaces are coloured according to a hydrophobic scale (hydrophobic=red; Eisenberg et al., 1984). Figure prepared using PyMol (Delano Scientific, PaloAlto).
decrease in the ‘small’ pocket and the increase in hydrophobicity produced by the mutation G60V might improve binding of the methyl group of the R₉ enantiomers present in the methylphosphonate racemic substrates (Fig. 5), whereas the extra space created by mutations in the positions Ile-106 and Ser-308 could help accommodate the S₉ enantiomers. This possible explanation is in agreement with what was observed in the kinetic and docking experiments. The PTE variants also carry the mutations K185R/D208G/R319S of PTE-S5 in the background. The mutation D208G alters the equilibrium between PTE productive configurations (Jackson et al., 2009), so it could be implicated in the increases observed for the methylphosphonates and could also account for the differences seen between PTE-S5 and PTE-wt activities towards the NA analogues; however, it was thought unlikely that PTE-S5-related mutations, or a synergism with mutations in the active site, accounted for the drastic effects against the phosphotriester substrates tested.

The present study is consistent with reported selection/directed evolution experiments, where an increase in the promiscuous activities of enzymes can cause a considerable loss of the activity towards wt substrate if the mutations lie within or close to the enzyme’s active site (Khersonsky and Tawfik, 2010). Although the improvements obtained in the catalytic proficiency towards methylphosphonates are dwarfed by the drastic loss of PTE activity in SS4.5, SS0.5 and the rest of the variants described here, care needs to be taken when comparing the results to other selection/directed evolution exercises, as the starting template, PTE-wt, was already significantly active towards the probes utilised.

Future experiments will use the new PTE variants presented here in structural studies with non-hydrolysable methylphosphonate substrates (Blum et al., 2008) and kinetic experiments to determine their activity towards the pernicious methylphosphonate chemical warfare NA themselves (Briseño-Roa et al., 2006).

Supplementary data

Supplementary data are available at PEDS online.

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References