A 5000-Fold Increase in the Specificity of a Bacterial Phosphotriesterase for Malathion through Combinatorial Active Site Mutagenesis

Tatheer Naqvi1,2, Andrew C. Warden2, Nigel French2, Elena Sugrue3, Paul D. Carr3, Colin J. Jackson3, Colin Scott2*

1 Department of Environmental Sciences, COMSATS Institute of Information Technology, Abbottabad, Pakistan, 2 Ecosystem Sciences, Commonwealth Scientific and Industrial Research Organisation, Canberra, Australian Capital Territory, Australia, 3 Research School of Chemistry, Australian National University, Canberra, Australian Capital Territory, Australia

Abstract

Phosphotriesterases (PTEs) have been isolated from a range of bacterial species, including *Agrobacterium radiobacter* (PTEAr), and are efficient enzymes with broad substrate ranges. The turnover rate of PTEAr for the common organophosphorous insecticide malathion is lower than expected based on its physical properties; principally the pKa of its leaving group. In this study, we rationalise the turnover rate of PTEAr for malathion using computational docking of the substrate into a high resolution crystal structure of the enzyme, suggesting that malathion is too large for the PTEAr binding pocket. Protein engineering through combinatorial active site saturation testing (CASTing) was then used to increase the rate of malathion turnover. Variants from a CASTing library in which Ser308 and Tyr309 were mutated yielded variants with increased activity towards malathion. The most active PTEAr variant carried Ser308Leu and Tyr309Ala substitutions, which resulted in a ca. 5000-fold increase in $k_{cat}/K_M$ for malathion. X-ray crystal structures for the PTEAr Ser308Leu/Tyr309Ala variant demonstrate that the access to the binding pocket was enhanced by the replacement of the bulky Tyr309 residue with the smaller alanine residue.

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* E-mail: colin.scott@csiro.au

Introduction

The World Health Organization has estimated that there are > 3,000,000 cases of pesticide poisonings annually, which result in approximately 200,000 deaths. Many of these cases are due to accidental or deliberate intoxication with neurotoxic organophosphate pesticides (OPs) [1].

OPs are potent cholinesterase inhibitors used extensively for the control of a variety of invertebrate pest species, but which also effect acute intoxication in humans [2]. The OPs share a phosphotriester structure, which is closely related to chemical warfare agents, such as VX and Sarin (Fig. 1). Enzymes that hydrolyse, and consequently detoxify, these phosphotriesters have been isolated from diverse origins [3,4]. The best described of these enzymes are the bacterial phosphotriesterases (PTEs).

PTEs have been isolated from a range of bacterial species, including *Pseudomonas diminuta* (PTEpd) and *Agrobacterium radiobacter* (PTEAr) [5,6]. They are binuclear metalloenzymes [7], highly efficient catalysts and display broad substrate specificities that include most phosphotriesters [5,6,8,9]. PTEs have therefore been investigated for applications in environmental monitoring, pesticide decontamination, nerve agent detoxification and in clinical applications [9–15].

The $S_N2$ mechanism of PTE, using a metal-activated water as the nucleophile, is well documented [16–18]. This mechanism results in a biphasic dependence of the rate of hydrolysis upon the pKa of the leaving group of the substrate (Fig. 2). For OPs with leaving groups that have pKₐ values of $\sim$8.0 or lower the $k_{cat}/K_M$ for the reaction is near the diffusion limit, while at pKₐ values of greater than $\sim$8.0 there is a linear relationship between pKₐ and log ($k_{cat}/K_M$). Outliers to this trend have been documented, with their lower than expected turnover rates typically resulting from physical barriers to correct substrate binding, such as steric hindrance or non-productive binding [8,19,20].

The active site and substrate-binding pocket of the PTEs are also well defined, with an iron-zinc binuclear metal center coordinated by four histidine residues (His55, His57, His201 and His230) and asparagine (Asp301) and an unusual carbamylated lysine (Lys196), which bridges the center by co-coordinating both metals [7] (numbering for PTEAr). The substrate-binding pocket is comprised of a number of largely hydrophobic residues: Gly60, Ser61, Ile106, Thr131, Phe132, Arg254, Tyr257, Leu271, Leu303, Phe306, Ser308 and Phe309 in PTEAr [6,17–20]. The large, hydrophobic substrate-binding pocket can accommodate the majority of anthropogenic phosphotriesters [3,10,19], including insecticides and nerve agents, and is responsible for the broad substrate range of the enzyme.
The efficiency of PTE against many such substrates, including chlorpyrifos, demeton-S, chlorfenvinphos, disopropyl fluorophosphate and others, has been improved by directed (laboratory) evolution, rational design and incorporation of unnatural amino acids [18,20–24]. However, there have been no reports of substantial improvements in the turnover rate of PTEs towards malathion, the most widely used OP insecticide in the US [25] with significant applications in the control of West Nile virus and fruit infestation [26,27].

Herein we have used Combinatorial Active-Site Saturation Testing (CASTing) [28] to improve the efficiency of malathion turnover by reducing the rate limitation caused by steric hindrance of substrate binding.

**Methods and Materials**

**Chemicals and reagents**
All chemicals and reagents were obtained from Sigma-Aldrich. Malathion and its metabolites were of analytical grade and >99% pure. Synthetic oligonucleotides were obtained from GeneWorks (Australia). Restriction enzymes were obtained from New England Biolabs (Australia).

**Bacterial growth**
*E. coli* Bl21 (DE3) was used for screening and production of variant enzymes and was cultured in LB, on LB supplemented with 1.5% w/v agar (LBA) or in Terrific Broth [29]. The media were supplemented with 100 μg.mL⁻¹ ampicillin as required.

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**Figure 1. Hydrolytic activity of PTE₄₉₄.** A) Schematic showing the PTE₄₉₄-mediated hydrolysis of malathion. B) Structure of the OP insecticides demeton, chlorpyrifos, parathion and diazinon.

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**Figure 2. Brønsted plot of leaving group pKa values vs log(kcat/Km) for a range of substrates.** The pKₐ values of the leaving groups 2,6-difluoro-4-nitrophenol; quinoxalin-2-ol; 2-fluoro-4-nitrophenol; 2-isopropyl-6-methylpyrimidin-4-ol; 3-fluoro-4-nitrophenol; 4-nitrophenol; 4-hydroxybenzaldehyde; 2,2-dichloroethenol; 4-hydroxybenzonitrile; 1-(4-hydroxyphosphonil)ethanone; methyl 4-hydroxybenzoate; 4-hydroxybenzamide; 3-chloro-7-hydroxy-4-methyl-2H-chromen-2-one; 2-(ethylthio)ethanethiol; 2-(diethylamino)ethanethiol; 2-(diisopropylamino)ethanethiol; and 4-(methoxymethyl)phenol plotted (left to right) against their log(kcat/Km) values. pKₐ values were as published elsewhere [8,17,20] or as calculated using the SPARC online pKₐ calculator (http://ibmlc2.chem.uga.edu/sparc/) [38]. The biphasic dependence of the enzyme on pKₐ as described elsewhere [8,20] is shown: the curve flattens below a pKₐ of ~8.0 and there is a linear dependence on pKₐ at values below ca. 8.0.

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Site saturation libraries

pETMCS1-opdA was used as a template for the site saturation mutagenesis libraries. The method of Ho et al. [30] was used to make libraries of opdA mutants in which codons were replaced with the NNS degenerate codon. Mutant libraries targeted either single amino acid substitutions at position 106, 271 or 303 or two simultaneous amino acid substitutions at positions 60 and 61, 131 and 132, 254 and 257, 306 and 309, and 308 and 309. Each of the single substitution libraries encoded 32 variants and each of the double substitution libraries encoded 1024 variants. The library ampiclons were cloned into pETMCS1 [24] using NdeI and EcoRI. The diversity of mutations within each library was ascertained by sequencing plasmid DNA obtained from transformants prior to any laboratory selection.

Screening for malathion hydrolase activity

For malathion hydrolase activity screening, competent BL21 (λDE3) were transformed with libraries cloned into pETMCS1, plated on LBA and incubated at 37 °C overnight. Ninety five transformants were screened from each library with single amino acid substitutions and 3070 transformants screened from each library with double amino acid substitutions; this resulted in each library being screened at ~3x the diversity of the library. Transformants were transferred into two 96-well growth blocks (3 mL volume in each well), with each growth block containing 94 library transformants, one BL21 (DE3) pETMCS1-opdA colony (base-line control) and one BL21 (DE3) colony (negative control).

Growth blocks were incubated at 37 °C over night then centrifuged at 5,300×g for 30 minutes to sediment the cultures. The pellets were resuspended and lysed in 3 mL Bugbuster solution (Novagen), according to the manufacturer’s instructions. 40 µL of cell-free extract (CFE) from each well was transferred to a 96-well microtitre plate.

The rate of malathion hydrolysis by the CFEs was followed by measuring the rate of thiol group liberation (i.e. diethyl 2-mercaptosuccinate formation) using Ellman’s reagent modified for use in 96-well microtitre plate format: 140 µL of 5 mM Ellman’s reagent containing 20 mM malathion was added to 40 µL of CFE. The change in absorbance at 412 nm was measured for 30 min using a SpectraMax M2 spectrophotometer (Molecular Devices, CA).

Plasmids were obtained from transformants that possessed greater malathion hydrolase activity than the BL21 (λDE3) pETMCS1-opdA control using a plasmid DNA purification kit (Macherey-Nagel, Germany), and the sequences of the OpdA mutants were obtained (Micromon, Melbourne).

Protein expression, purification and crystallization

PTE<sub>AR</sub> and variants were expressed in BL21 (λDE3) grown in TB supplemented with 100 µM CoCl<sub>2</sub> at 30 °C for 48 hours. The cells were harvested after 48 hours by centrifugation (5,000×g for 15 min), pellets were then resuspended in 5 mL of 50 mM HEPES with 1 mM CoCl<sub>2</sub> (pH 8) per gram of cells. Cells were lysed using an EmulsiflexC3 homogeniser (Avestin Inc., Germany) according to the manufacturer’s instructions. Cell debris was removed by centrifuging at 20,000×g for 30 minutes and the supernatant was recovered.

PTE<sub>AR</sub> and variants were purified as described elsewhere [17,19]. All columns and chromatographic media were purchased from GE Healthcare. Protein concentration was determined using a nanodrop ND-1000 spectrophotometer (Thermofisher Scientific, Australia), assuming an extinction coefficient of 29,280 M<sup>-1</sup>·cm<sup>-1</sup> [19]. Protein purity was monitored by using reducing pre-cast SDS-PAGE gels (NuSep, Australia) stained with Coomasie brilliant blue. PTE<sub>AR</sub> was crystallised as previously described [7,18].

Crystal soaking and X-ray data collection

PTE<sub>AR</sub> crystals were serially transferred to cryoprotectant solutions consisting of 40% PEG 3350 and 0.2 M NaNO<sub>3</sub> with or without 2 mM malathion for 2 minutes before data collection. The crystals were flash-cooled to 100 K in a cryogenic nitrogen gas stream. Diffraction data were collected on a Marresearch mar345 system, comprising a Xenonics Genix<sup>3D</sup> Cu high flux generator and a mar345 image plate detector. All data reduction was performed using XDS and CCP4 [31,32].

Structure determination

Crystals were isomorphous to those previously solved (space-group P<inf>3</inf>121, a = 108.9, c = 62.4) [7,18]; accordingly, this model was used to calculate the initial protein phases. REFMAC as implemented in the CCP4 suite of program [33], was employed for refinement. The structure and restraints for diethyl thiophosphoate were taken from previous work [17]. Difference Fourier maps obtained from soaked PTE<sub>AR</sub> crystals in the absence of bound substrate/products were obtained initially, followed by inclusion of the substrates/products in the models and real-space refinement against the positive density using restraints and the COOT

Figure 3. Docking of malathion into the crystal structures of wild-type PTE<sub>AR</sub> and PTE<sub>AR</sub> Ser308Leu/Tyr309Ala. A. The substrate-binding pocket of PTE<sub>AR</sub> (2R1N) with bound substrate. The amino acids that were randomised in this experiment are labelled. B. Superimposed structures of malathion docked in the active site of PTE<sub>AR</sub> in two different conformations. The branched leaving group of the substrate/products in the models and real-space refinement against the positive density using restraints and the COOT.
These were further refined using REFMAC. Ligand occupancy was adjusted until the B-factors of the ligands refined to values comparable to the interacting metal ions/amino acids.

Enzyme assays

Rates of hydrolysis of malathion and demeton were measured by quantifying the formation of thiol groups using the modified Ellman’s assay [35], essentially as described above, 140 μl of 5 mM Ellman’s reagent containing malathion or demeton (0, 5, 10, 25, 50, 100, 150, 300, 400 and 600 μM) was added to 40 μl of 50 mM HEPES buffer containing 0.22–0.43 nM enzyme, as appropriate. Each assay was conducted in triplicate. An extinction coefficient of 14,140 M⁻² cm⁻¹ [35] was used. Hydrolysis rates for diazinon, chlorpyrifos and parathion were obtained spectrophotometrically, as described elsewhere [10]. Rates were determined over 30 minutes. Values for kcat and KM were estimated using “Hyper32” hyperbolic regression software [20].

Computational procedures

Docking of malathion to the PTEAr native structure with a water molecule bridging the metal centres was performed using CDOCKER as implemented in Accelrys Discovery Studio [36]. The top 10 poses were taken from a run docking 40 conformers of malathion with simulated annealing for each pose.

Results and Discussion

Malathion is a poor substrate for PTEAr

PTEAr has a kcat/KM value for malathion of 4×10² s⁻¹ M⁻¹. The pKₐ of the leaving group of malathion (diethyl 2-mercaptosuccinate) is predicted to be 7.7, suggesting that malathion is turned over by PTEAr with a kcat/KM that is at least several order of magnitude lower than other phosphotriesters with leaving groups that have similar pKₐ values (Fig. 2). This suggests that another parameter, such as substrate binding or formation of the Michaelis complex, may limit the rate of this reaction.

Table 1. Kinetic parameters of purified PTEAr and most active variant against malathion for a range of OP insecticides.

| Substrate | Wild Type | PTEAr | Ser308Leu | /Tyr309Ala |
|-----------|-----------|-------|-----------|------------|
|           | kcat(s⁻¹) | KM(μM) | kcat/KM(s⁻¹ M⁻¹) | kcat(s⁻¹) | KM(μM) | kcat/KM(s⁻¹ M⁻¹) |
| Malathion | 3.9×10⁻²  | 1100  | 3.5×10⁴   | 7.6×10⁴   | 410    | 1.9×10⁶   |
|           | (2.1×10⁻²) | (97) | (3.3×10⁴) | (35)      |        |          |
| Parathion | 6.2×10⁴   | 330   | 1.9×10⁷   | 9.1×10⁷   | 340    | 2.7×10⁸   |
|           | (9.3×10⁵) | (25) | (8.2×10⁷) | (31)      |        |          |
| Demeton   | 1.4×10⁻²  | 490   | 2.9×10⁵   | 1.1×10⁻²  | 490    | 2.2×10¹   |
|           | (1.4×10⁻²) | (39) | (9.3×10⁻⁴) | (17)      |        |          |
| Diazinon  | 1.0×10⁵   | 480   | 2.1×10⁶   | 1.1×10⁴   | 430    | 2.5×10⁷   |
|           | (9.6×10⁵) | (46) | (8.4×10⁶) | (39)      |        |          |
| Chlorpyrifos | 3.8×10¹  | 290   | 4.3×10⁵   | 2.3×10⁰   | 220    | 1.0×10⁸   |
|           | (2.6×10¹) | (27) | (2.1×10⁵) | (13)      |        |          |

Standard deviations for the kcat and KM values are given in parentheses below the mean values obtained for triplicate experiments.

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Figure 4. Screening for improved malathion hydrolase activity. The activity of three cell-free extracts from transformants isolated from the Ser308Xxx/Tyr309Xxx libraries are shown alongside that of the cell-free extract from a transformant obtained using the unmodified gene. The library transformants were subsequently shown to carry the Ser308Leu substitution and an Ala, Ser or Gly at 309. The data presented here are the averages of three independent assays, replicates varied by less than 10%.

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particular clashes near Ser308 and Tyr309 (Fig. 3B). This revealed that the substrate binding pocket was too small to accommodate the branched leaving group of malathion, with previous work having shown that PTE$_{Ar}$ Ser308Leu/Tyr309Ala produced a docked pose that was essentially superimposable with a crystal structure of an PTE$_{Ar}$-diethyl 4-methoxyphenyl phosphate complex [17](Fig. 3A). Forty orientations of forty conformers of malathion were docked, with simulated annealing for each pose. The results did not yield any poses that were productively bound with the substrate appropriately aligned for nucleophilic attack from the metal-ion coordinated nucleophile. To investigate further, we superimposed various conformers of malathion onto the substrate in a crystallographic PTE$_{Ar}$-substrate complex (Fig. 3). This revealed that the substrate binding pocket was too small to accommodate the branched leaving group of malathion, with particular clashes near Ser308 and Tyr309 (Fig. 3B).

Table 2. Data collection and refinement statistics for structures reported in this work.

|                      | PTE$_{Ar}$ Ser308Leu/Tyr309Ala | PTE$_{Ar}$+2 mM malathion Ser308Leu/Tyr309Ala |
|----------------------|--------------------------------|---------------------------------------------|
| Space group          | P3 12 1                        | P3 12 1                                    |
| Unit-cell parameters |                                |                                             |
| a (Å)                | 57.09                          | 109.10                                     |
| b (Å)                | 101.80                         | 109.10                                     |
| c (Å)                | 79.89                          | 63.43                                      |
| α,β,γ (°)            | 90, 90, 120                    | 90, 90, 120                                |
| Data collection      |                                |                                             |
| Resolution range (Å)* | 29.36–1.99 (2.04–1.99)        | 28.22–1.99 (2.04–1.99)                     |
| No. of unique reflections | 29386                      | 30260                                      |
| Redundancy           | 10.8 (9.9)                     | 7.2 (6.6)                                  |
| Completeness (%)     | 99.8 (97.8)                    | 99.8 (98.4)                                |
| Rmerge                | 0.122 (0.750)                  | 0.123 (0.889)                              |
| Mean <$\sigma_f$>    | 20.2 (4.0)                     | 15.4 (2.4)                                 |
| Rmerge$^2$            | 0.998 (0.877)                  | 0.997 (0.734)                              |
| Refinement           |                                |                                             |
| No. reflections (total) | 27872                       | 28317                                      |
| Resolution range      | 29.36–1.99 (2.04–1.99)        | 28.22–1.99 (2.04–1.99)                     |
| Rwork/Rmerge$^2$     | 0.158/0.186 (0.212/0.278)     | 0.206/0.251 (0.266–0.319)                  |
| R.m.s deviations     |                                |                                             |
| Bond lengths (Å)     | 0.022                          | 0.019                                      |
| Bond angles (°)      | 2.084                          | 2.036                                      |
| PDB ID               | 3WML                          | 4NP7                                       |

To rationalize the very low kinetic parameters of PTE$_{Ar}$ with malathion, we performed a series of computational docking procedures using the CDOCKER algorithm. CDOCKER has previously been verified crystallographically with PTE$_{Ar}$, producing a docked pose that was essentially superimposable with a crystal structure of an PTE$_{Ar}$-diethyl 4-methoxyphenyl phosphate complex [17](Fig. 3A). Forty orientations of forty conformers of malathion were docked, with simulated annealing for each pose. The results did not yield any poses that were productively bound with the substrate appropriately aligned for nucleophilic attack from the metal-ion coordinated nucleophile. To investigate further, we superimposed various conformers of malathion onto the substrate in a crystallographic PTE$_{Ar}$-substrate complex (Fig. 3). This revealed that the substrate binding pocket was too small to accommodate the branched leaving group of malathion, with particular clashes near Ser308 and Tyr309 (Fig. 3B).

CASTing for improved malathion hydrolysis

In accordance with the docking results, a semi-rational approach was used to improve the turnover of this substrate. CASTing was performed in the substrate-binding pocket of PTE$_{Ar}$, with each of the residues that form the substrate-binding pocket included in at least one CASTing library. CASTing is an approach wherein analysis of an enzyme’s 3D structure is used to identify groups of two or three amino acids from the binding-pocket, which are then randomized simultaneously to create relatively small libraries of mutants that can be screened with relative ease. For PTE$_{Ar}$, each CASTing library carried substitutions at one or two amino acid positions and therefore producing libraries of 32 or 1024 variants (using an NNS degeneracy). The libraries were: Gly60Xxx and Ser61Xxx, Ile106Xxx, Trp131Xxx and Phe132Xxx, Arg254Xxx and Tyr257Xxx, Leu271Xxx, Leu303Xxx, Phe360Xxx and Ser308Xxx, and Ser308Xxx and Tyr309Xxx. Cell-free extract from each library was screened for the rate of formation of free thiol (as a result of malathion hydrolysis; Fig. 1), with 95 or 3070 transformants screened per library depending upon the size of the library screened (i.e., screened at ~3 x the diversity of the library).
Although the majority of transformants from all libraries retained some hydrolytic activity against malathion, only the library in which Ser308 and Tyr309 were targeted for mutagenesis provided variants with increased activity towards malathion compared with that of the wild-type enzyme (Fig 4). Variants with increased rates of malathion activity were found to carry a Ser308Leu substitution and a substitution of Tyr309 for Gly, Ser, or Ala. Site-directed mutants of wild-type pde4 were constructed that encoded only substitutions of Tyr309 for Gly, Ser or Ala; however, the expressed variants were insoluble.

The steady-state kinetic parameters of the most active variant (Ser308Leu, Tyr309Ala) were obtained and compared with those of the wild-type enzyme. There was a 2.7-fold decrease in the $k_{\text{cat}}$ for malathion (410 vs. 1,100 μM; Table 1) and a $2 \times 10^4$-fold increase in $k_{\text{cat}}/k_{\text{m}}$ (7.7×10$^2$ vs. 3.9×10$^{-2}$ s$^{-1}$; Table 1), leading to a 5.4×10$^4$-fold increase in the $k_{\text{cat}}/K_{\text{M}}$ (1.9×10$^5$ vs. 3.5×10$^3$ s$^{-1}$M$^{-1}$; Table 1). Thus, amino acid substitutions at positions 308 and 309 alleviate the majority of the limitation on the rate of malathion hydrolysis by PTE$_{\text{Le}}$. Such large changes in turnover rates were not observed with a range of other OP insecticides including parathion (ca. 5-fold reduction in $k_{\text{cat}}/K_{\text{M}}$ value), or 2- and 9-fold increases in $k_{\text{cat}}/K_{\text{M}}$ values for chlorpyrifos and diazinon respectively, with no significant change in the rate of demeton turnover (Table 1). These data suggest that the specific interaction between PTE$_{\text{Le}}$ and malathion via amino acids at positions 308 and 309 was responsible for the low turnover of malathion by the wild-type enzyme.

**Crystallographic analysis of PTE$_{\text{Le}}$ Ser308Leu/Tyr309Ala**

In order to rationalise the effects of these mutations, we solved the crystal structure of the PTE$_{\text{Le}}$ Ser308Leu/Tyr309Ala mutant (crystallographic data in Table 2). This did not reveal any significant changes to the backbone or B-factors of the loop that these mutations are located on (Loop 7). However, the Tyr309Ala mutation did expand the size of the active site entrance substantially (Fig 3D). To investigate whether the enzyme, as constrained by the crystal packing, could still hydrolyze malathion, we performed a crystal soaking experiment, in which we soaked the crystal in 1 mM malathion for two minutes. The structural model obtained from the soaked crystal revealed density in the active site matching the product diethyl thiophosphate, in a known product binding mode [17]. This establishes that the crystal structure observed here is capable of hydrolysing malathion at rapid rates.

To investigate whether this increase in activity was a result of relieving the steric hindrance that was inhibiting substrate turnover in the wild-type enzyme, we performed substrate docking with malathion and the engineered Ser308Leu/Tyr309Ala variant (Fig 3D). These results confirmed that the widening of the active site that occurs as a result of these mutations allows productive substrate binding and vastly improved turnover rates. It also explains the reduced turnover of paraaxon (Table 1): previous work has established that the interaction between the aromatic group of Tyr309 and the aromatic group of paraaxon, which is lost in this mutant, enhances catalysis.

**Summary**

PTE$_{\text{Le}}$ has potential in a wide range of applications, due to its high turnover rates and broad substrate specificity. However, the applicability of the wild-type enzyme is limited for some substrates, such as malathion. Here we have enhanced the turnover rate for malathion by ~5,000 fold using a semi-rational approach, which has alleviated the steric hindrance responsible for the low rate of malathion turnover in the wild-type enzyme. The requirement for two amino acid substitutions, adjacent to each other in the protein, suggests that it would have been unlikely to have produced this specific variant by another, purely random, method.

**Author Contributions**

Conceived and designed the experiments: TN AGW ES PDC JJ CS. Performed the experiments: TN AGW NF ES PDC. Analyzed the data: TN AGW ES PDC JJ CS. Wrote the paper: TN AGW JJ CS.

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