

Bacterial detoxification of organophosphate nerve agents

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Bacterial enzymes have been isolated that catalyze the hydrolysis of organophosphate nerve agents with high-rate enhancements and broad substrate specificity. Mutant forms of these enzymes have been constructed through rational redesign of the active-site binding pockets and random mutagenesis to create protein variants that are optimized for the detoxification of agricultural insecticides and chemical warfare agents. In this review, the catalytic properties of two bacterial enzymes, phosphotriesterase and organophosphorus anhydrolase, are examined for their ability to hydrolyze organophosphate nerve agents.

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Abbreviations

AChE	acetyl cholinesterase
k_{cat}	catalytic rate constant
K_{M}	Michaelis–Menten equilibrium constant
OPAA	organophosphorus acid anhydrolase
PTE	phosphotriesterase

Introduction

Organophosphate nerve agents are toxic, owing primarily to their inherent ability to inactivate the enzyme acetyl cholinesterase (AChE) [1]. Because this enzyme plays a critical role in the proper functioning of nerve cells, through the rapid hydrolysis of acetylcholine, most multicellular organisms are susceptible in various degrees to the presence of organophosphate triesters. The reaction mechanism for the enzymatic hydrolysis of acetylcholine by AChE is shown in Figure 1. During this transformation, an active-site serine residue initiates a nucleophilic attack on the carbonyl carbon of acetylcholine to form a covalent acetyl-acyl-enzyme intermediate, concurrent with the release of choline from the active site. The free enzyme is regenerated in the second step via a hydrolytic attack by water and the release of acetate. Organophosphates have been demonstrated to mimic the natural substrate, as illustrated in the chemical transformation presented in Figure 1 for the insecticide paraoxon (see Figure 2a for chemical structure). Kinetic studies have demonstrated that the first step (formation of the phosphorylated enzyme intermediate) is fast but that the regeneration of the free enzyme through the nucleophilic attack by water on the phosphoenzyme intermediate is extremely slow [2]. The phosphorylated enzyme is, thus, unable to function as an effective catalyst for the hydrolysis of acetylcholine.

Organophosphate triesters (and the related organophosphonates) have been globally distributed only since the end of World War II. There are apparently no confirmed reports for the existence of naturally occurring organophosphate triesters. However, this class of compounds has achieved substantial commercial success as a key component in the arsenal of agricultural insecticides. Unfortunately, the toxic properties of organophosphonates have also been exploited in the development of chemical warfare agents such as sarin, soman and VX [3]. The structures of these and related compounds are shown in Figure 2.

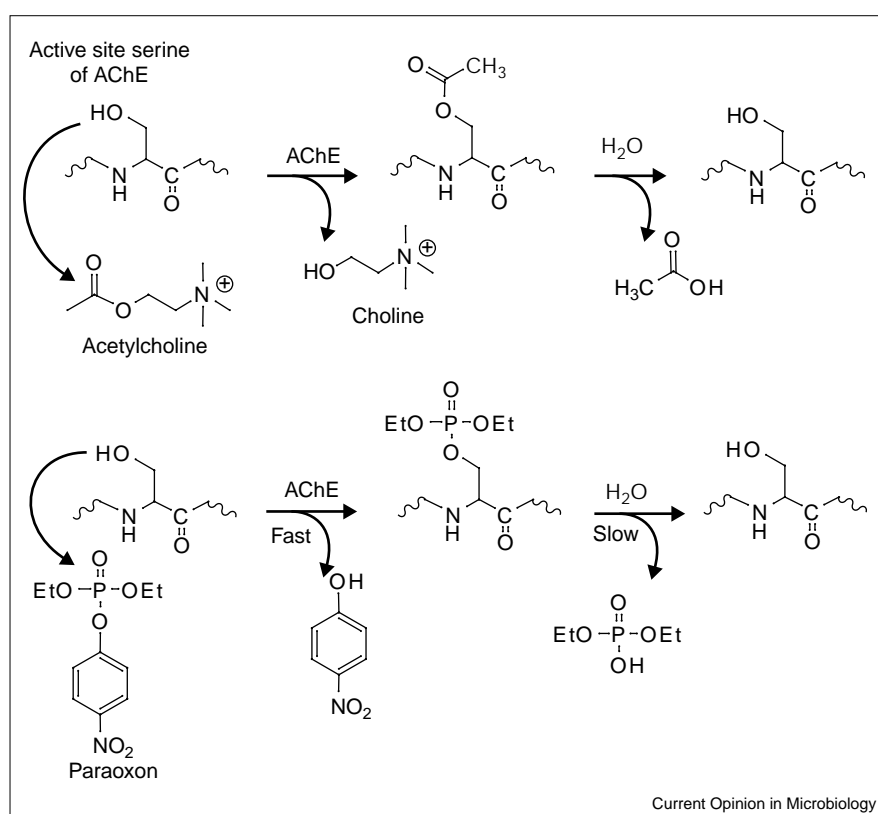
In general, bacterial systems are not affected by the presence of organophosphates. However, bacterial isolates have been identified that have the capacity to hydrolyze and, thus, detoxify a wide range of organophosphates, including the most toxic of the chemical warfare agents. The best characterized of these bacterial isolates include the phosphotriesterase (PTE) from *Pseudomonas diminuta* [4] and *Flavobacterium* sp. [5], in addition to the organophosphorus acid anhydrolase (OPAA) from *Alteromonas* sp. [6]. A natural substrate for the phosphotriesterase has not been identified, whereas the OPAA from *Alteromonas* appears to be a hydrolase that can catalyze the cleavage of dipeptides containing proline. In this review, the catalytic properties of two bacterial enzymes, PTE and OPAA, are examined for their ability to hydrolyze organophosphate nerve agents. The chemical mechanisms are presented in terms of the three-dimensional structures of the active site. The catalytic cores of PTE and OPAA have been utilized as templates for the construction of protein variants with enhanced activities toward the very toxic organophosphate nerve agents.

Phosphotriesterase

The phosphotriesterase from *Flavobacterium* was first identified from samples taken from a Philippine rice patty [5]. The rice patty had been treated with the organophosphate diazinon (see Figure 2e for chemical structure) as a means of insect control. At about the same time, a strain of the soil bacterium *P. diminuta* was isolated through its ability to hydrolyze parathion (see Figure 2f for chemical structure) [7]. The genes encoding the active enzymes (*opd*, for organophosphate-degrading) were localized to extrachromosomal plasmids in both cases. The gene sequences for the enzyme from the two sources were identical. Database searches have identified other proteins that appear to be close relatives of PTE. However, the ability of these proteins to function as authentic phosphotriesterases has not, as yet, been determined. The gene from *Pseudomonas* has been subcloned into a variety of expression vectors, including *Escherichia coli* [8] and *Streptomyces* [9], and insect cells [10], among others.

Figure 1

Reaction mechanism for the hydrolysis of acetylcholine by acetyl cholinesterase (AChE) and the inactivation of AChE by the organophosphate triester, paraoxon. See text for full details.



Reaction profile

The recombinant enzyme from the *E. coli* expression system has been used to extract the details of the chemical transformation and to provide an explanation for why the bacterial phosphotriesterase is able to hydrolyze this class of substrates with extremely high turnover numbers, whereas these same compounds are extremely potent inactivators of acetyl cholinesterases. Much of the mechanistic evaluation of PTE has been conducted with derivatives of the insecticide paraoxon (Figure 2a), as the *p*-nitrophenol leaving group is relatively easy to assay spectrophotometrically. The purified protein is able to hydrolyze paraoxon with a k_{cat} of $\sim 3,000 \text{ s}^{-1}$ and a value for $k_{\text{cat}}/K_{\text{M}}$ of $5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 3) [11]. The stereochemical course of the reaction was ascertained using the chiral insecticide EPN (see Figure 2g for chemical structure). When the S_{P} -enantiomer of EPN was hydrolyzed by PTE, only the S_{P} -enantiomer of the thiophosphonic acid product was formed (Figure 3). This result indicated clearly that the enzymatic transformation occurred with an inversion of configuration [12]. Therefore, cleavage of the P–O bond occurs via the direct attack of solvent water on the phosphorus center, unlike the reaction mechanism of AChE, in which a stable phosphoenzyme intermediate is formed.

Substrate specificity

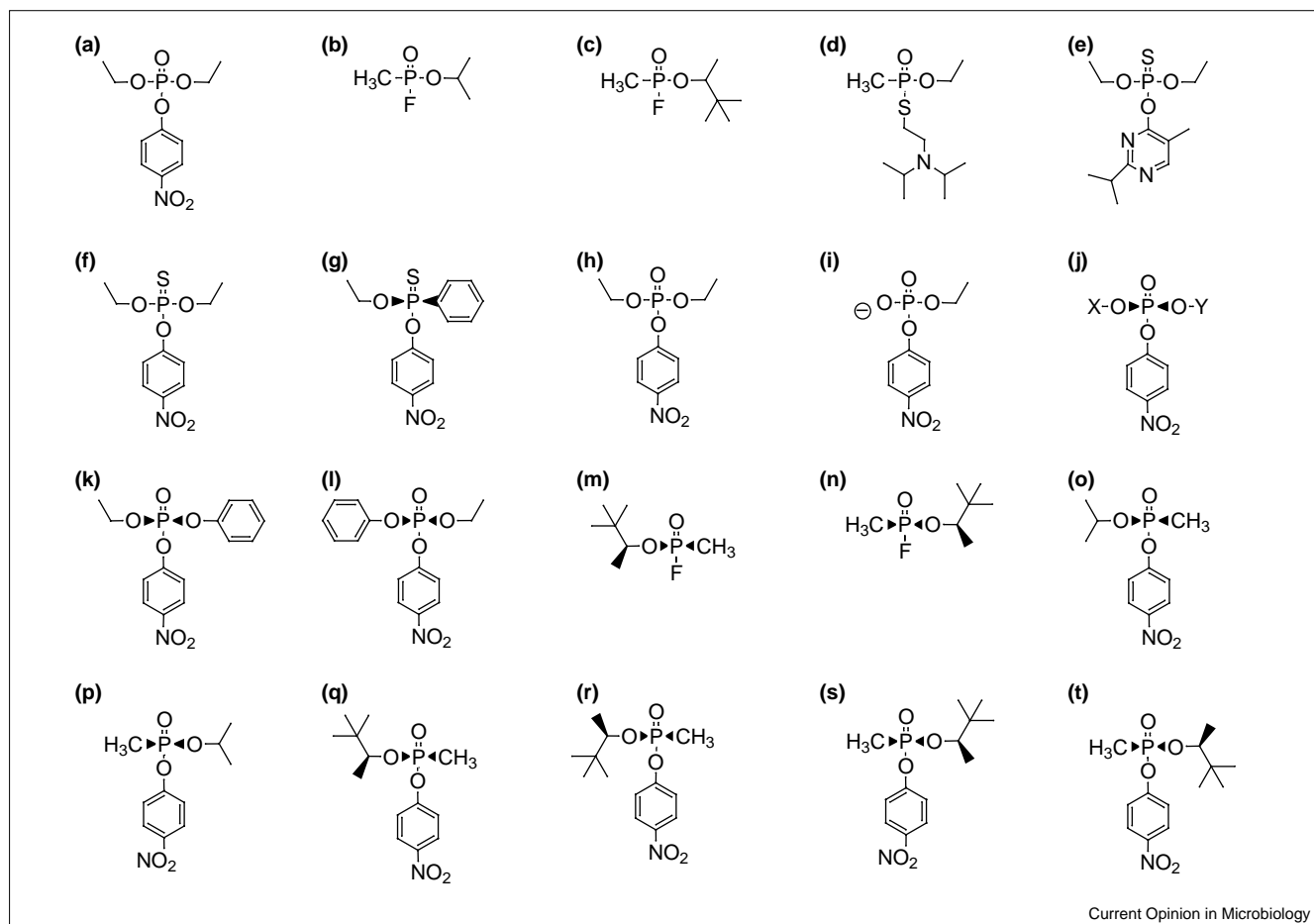
The reaction specificity is relatively broad. For PTE, the best substrate identified to date is paraoxon. However, a

significant number of perturbations can be made to the phosphorus core while retaining substrate turnover [13]. The phosphoryl oxygen can be replaced with sulfur, as exemplified with the insecticide parathion (Figure 2f). A variety of leaving groups can be cleaved from the phosphorus center, although the rate of hydrolysis is very much dependent on the pK_{a} of the leaving group [14]. For example, an unsubstituted phenol is cleaved at 10^{-5} times the rate of a substrate bearing a *p*-nitrophenol leaving group. The enzyme will cleave insecticides that have P–S bonds, as in the case of malathion (see Figure 2h for chemical structure). However, the enzyme does not catalyze the cleavage of carbonyl groups such as those found in *p*-nitrophenyl acetate. Organophosphate diesters (see Figure 2i for chemical structure) are very poor substrates ($k_{\text{cat}} = \sim 10^{-3} \text{ s}^{-1}$) [15].

Three-dimensional structure

High-resolution X-ray structures have been obtained for the recombinant enzyme [16••]. The protein folds into a $(\alpha\beta)_8$ -barrel motif, as illustrated in Figure 4. This is a common fold for enzymes and, in every case, the active site is found at the carboxy-terminal end of the central β -sheet core. With PTE, there is a binuclear metal center at which two Zn^{2+} atoms are bound within a cluster of histidine residues. One of the divalent cations is bound to two histidine residues and an aspartic acid, whereas the other divalent cation is bound to two histidines. In addition,

Figure 2



Structures of the compounds discussed in this review: (a) paraoxon; (b) sarin; (c) soman; (d) VX; (e) diazinon; (f) parathion; (g) EPN; (h) malathion; (i) organophosphate diester; (j) general structural template for substrate library; (k) S_P -enantiomer of ethyl phenyl

p -nitrophenyl phosphate; (l) R_P -enantiomer of ethyl phenyl p -nitrophenyl phosphate; (m) S_P,S_C -enantiomer of soman; (n) R_P,R_C -enantiomer of soman; (o) S_P -enantiomer of sarin; (p) R_P -enantiomer of sarin; (q–t) enantiomers of the soman analogs.

the two metal ions are bridged by a solvent hydroxide and an unusual carbamate functional group formed from the post-translational carboxylation of a lysine side chain with carbon dioxide. A cartoon of the binuclear metal center is presented in Figure 5. The apo-enzyme can be prepared and the native zinc atoms can be replaced with Co^{2+} , Cd^{2+} , Ni^{2+} or Mn^{2+} , with retention of full catalytic activity. The structure of the binuclear metal center in PTE is identical to the ones found in urease [17] and dihydroorotase [18].

Catalytic mechanism

X-ray structures of PTE have been obtained in the presence of non-hydrolyzable substrate mimics [19^o]. These results have enabled the visualization of potential enzyme–substrate interactions. A working model for an enzymatic reaction mechanism for organophosphate triester hydrolysis is provided in Figure 6. In this mechanism, the organophosphate binds to the binuclear metal center within the active site via coordination of the phosphoryl oxygen to the β -metal ion (more solvent exposed in this

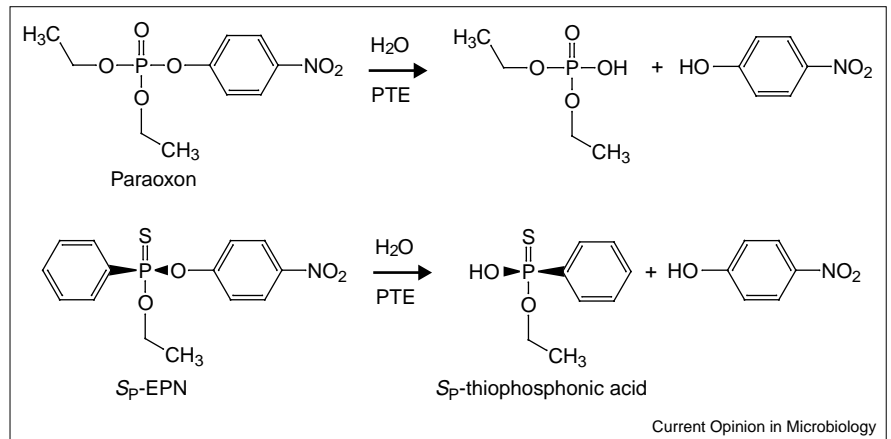
model). This interaction weakens the binding of the bridging hydroxide to the β -metal (as evidenced by the longer oxygen–metal distance in the complex relative to the unbound state). The metal oxygen interaction polarizes the phosphoryl oxygen bond and makes the phosphorus center more electrophilic. Nucleophilic attack by the bound hydroxide is assisted by proton abstraction from Asp301. As the hydroxide attacks the phosphorus center, the bond to the leaving group weakens, although ^{18}O isotope effects [20] and Brønsted analyses [21] support the notion that the transition state is late. His354 may facilitate the transfer of a proton from the active site to the bulk solvent.

Structural determinants of substrate specificity

If PTE has evolved within the past half-century to recognize and hydrolyze organophosphate nerve agents, then the active site may exhibit a certain degree of flexibility for the tolerance of structural perturbations to the size and shape of substrates that are turned over. This substrate promiscuity was tested with a small substrate library, using the general

Figure 3

Reaction products for the hydrolysis of paraoxon and EPN by the bacterial phosphotriesterase.



structural template shown in Figure 2j, in which all possible combinations of methyl, ethyl, isopropyl and phenyl substituents were permitted to be substituted for the ligands X and Y. The wild-type enzyme was able to accept all of these variations, although some combinations of substituents were better than others (the range for k_{cat}

varied from a high of 18 000 s⁻¹ to a low of 220 s⁻¹). The most interesting feature to be observed in this study was the emergence of stereoselectivity [22,23^{••}]. When the substituents X and Y are not the same, a pair of chiral enantiomers is formed. With PTE, the preferred enantiomer had the physically larger group in the relative position

Figure 4

Ribbon diagram for the structure of the bacterial phosphotriesterase [16^{••}].

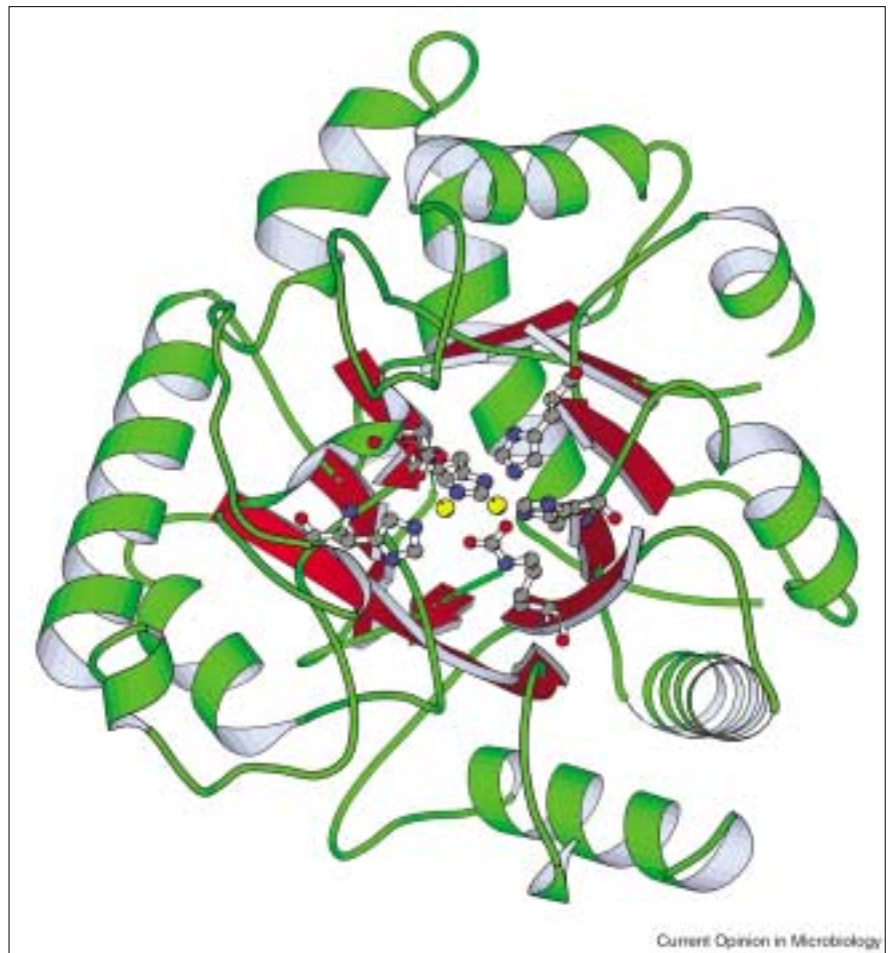
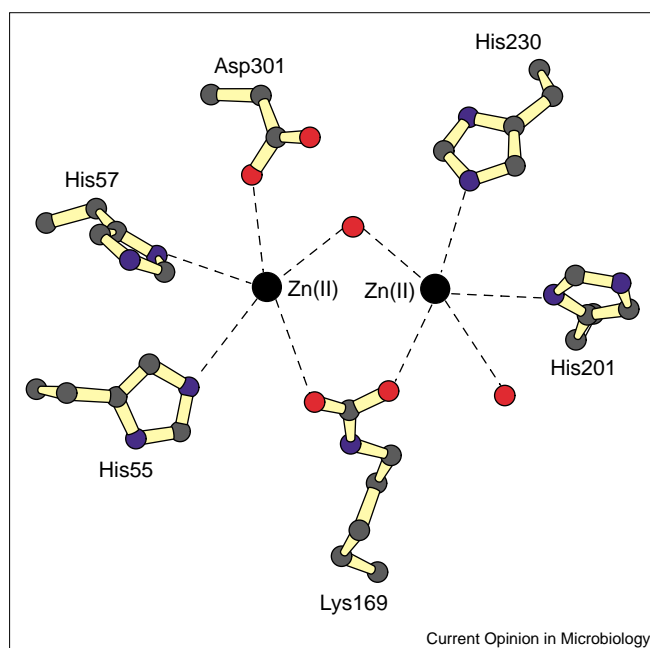


Figure 5



Representation of the structure of the binuclear metal center within the active site of the bacterial phosphotriesterase [16**].

occupied by Y (in the pro-*S* position) and the smaller group occupied by X (in the pro-*R* position), as illustrated by the structural template in Figure 2j. For example, for the enantiomeric pair of ethyl phenyl *p*-nitrophenyl phosphate, the *S_p*-enantiomer (see Figure 2k for chemical structure) is hydrolyzed 21 times faster than the *R_p*-enantiomer (see Figure 2l for chemical structure). The three-dimensional structure of a stable substrate analogue complexed with PTE revealed the relative locations of the three substituent binding sites [20,24]. These binding subsites have been labeled 'large', 'small' and 'leaving group', and are illustrated in Figure 7 (it should be noted that many of the specific residues are actually at the interface between one or more sites).

Directed evolution and rational design

There has been considerable interest in the utilization of enzymes such as PTE for the detoxification of organophosphate nerve agents [25]. Catalytic decomposition of these highly toxic materials could be utilized in the degradation of agricultural and household pesticides. There is also a considerable effort in using protein systems for the detection and destruction of chemical warfare agents by the military and homeland defense [26,27]. To harness the full catalytic potential of these enzymes, the active site must be reshaped in order to optimize the mutant enzyme for a specific class of substrate targets. These experimental efforts have demonstrated dramatically that significant changes and enhancements to the catalytic prowess of PTE can be realized through rational modifications to the substrate-binding cavities.

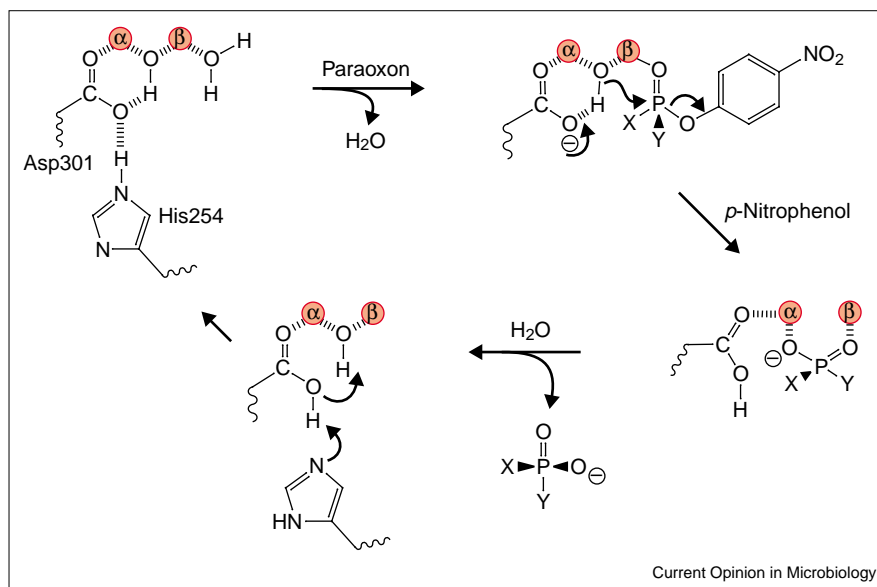
The initial probes of substrate specificity revealed that the wild-type PTE possessed a fair degree of stereoselectivity in the hydrolysis of chiral organophosphate triesters [22,23**]. This was a significant observation, as the major chemical warfare agents (sarin, soman and VX) are racemic mixtures with substantial differences in the toxic properties by the individual enantiomers. For example, the *S_pS_C*-enantiomer of soman (see Figure 2m for chemical structure) reacts with acetyl cholinesterase >10³ times faster than does the *R_pR_C*-enantiomer (see Figure 2n) [28]. The probes of the protein structural determinants of enzyme specificity revealed the locations of the binding subsites that apparently dictated the catalytic properties of PTE. The size and shape of these binding subsites were remolded through a rational restructuring via site-directed modification. Overall, these efforts demonstrated that significant changes to the substrate specificity could be achieved through a very small number of amino acid changes [23**,29**]. In the area of stereo-selectivity, the inherent selectivity of the wild-type has been 'enhanced', 'relaxed' and 'reversed'. The hydrolysis of the two enantiomers of ethyl phenyl *p*-nitrophenyl phosphate (Figures 2k and 2l) is used as an example to illustrate this.

With the wild-type enzyme, PTE catalyzes the hydrolysis of ethyl phenyl *p*-nitrophenyl phosphate with a stereoselectivity of ~21 in favor of the *S_p*-enantiomer over the *R_p*-enantiomer. This difference in rate arises, presumably, because the bulkier phenyl substituent is better optimized in the 'large' subsite, and the ethyl group within the 'small' subsite for the *S_p*-enantiomer. In order to enhance this stereoselectivity, the 'small' subsite was diminished by increasing the size of the side chains that constituted the wall of the subsite. Of the residues attempted, the conversion of Gly60 to alanine had the most dramatic effect [23**]. With the G60A mutant, the stereoselectivity increased to a factor of 11 000:1. In this case, it is apparently more difficult for the bulkier phenyl substituent of the *R_p*-enantiomer to fit within the 'small' subsite.

If the ultimate goal is the decontamination of racemic organophosphate esters, then there is an advantage if both enantiomers are hydrolyzed simultaneously. For PTE, this objective was met through an expansion of the 'small' subsite. The rationale here was the increased likelihood that a larger 'small' subsite would be better able to accommodate groups of increased bulk. The greatest success came with the conversion of Phe132, Ser308 and Ile106 to glycine and/or alanine residues [23**,29**]. In many instances, the effects at the individual positions were found to be additive. With the mutant I106G/F132G/S308G, the stereoselectivity for the *S_p* and *R_p* enantiomers of ethyl phenyl *p*-nitrophenyl phosphate dropped from 21:1 to ~1:1.3. It should be noted that this relaxation in stereoselectivity was achieved by *increasing* the rate of hydrolysis for the initially slower *R_p*-enantiomer to the initial level of activity for the *S_p*-enantiomer, rather than diminishing the rate of the *S_p*-enantiomer to that of the *R_p*-enantiomer [29**].

Figure 6

Working model for the reaction mechanism for the hydrolysis of organophosphate nerve agents by phosphotriesterase. See text for full details.



The most difficult achievement was the actual reversal of the stereoselectivity. In order to accomplish this objective, the rate of hydrolysis of the R_p -enantiomer must be enhanced while the rate of hydrolysis of the initially faster S_p -enantiomer is diminished. This objective was met by

enlarging the 'small' subsite while simultaneously reducing the size of the initially 'large' subsite. For the target substrates (Figures 2k and 2l), the stereoselectivity for the S_p : R_p pair of enantiomers was altered to 1:460 for k_{cat}/K_M with the mutant I106G/F132G/H257Y/S308G. An

Figure 7

The relative positions of the amino acids that define the active-site structure of the bacterial phosphotriesterase.

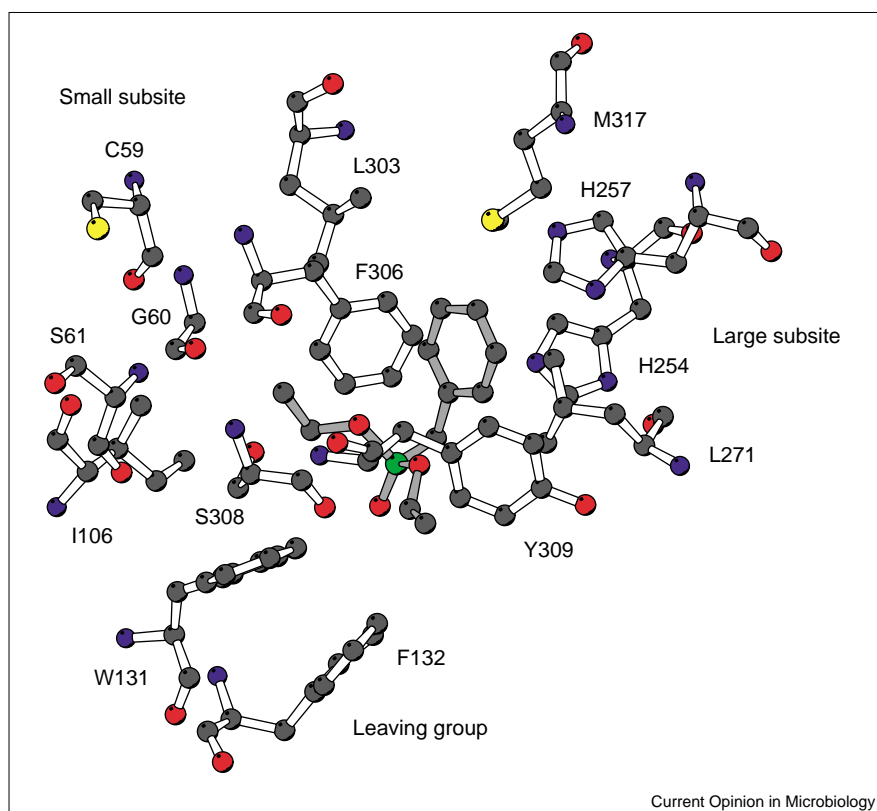
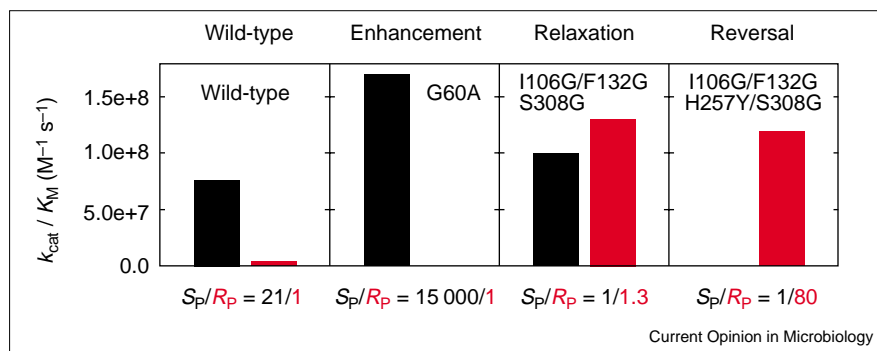


Figure 8



The changes in the stereoselectivity of mutants of the bacterial phosphotriesterase with the substrate ethyl phenyl *p*-nitrophenyl phosphate. The black bars are for the S_P -enantiomer (see Figure 2k for chemical structure), and the red bars are for the R_P -enantiomer (see Figure 2l for chemical structure).

illustration showing the enhancement, relaxation and reversal of stereoselectivity for the hydrolysis of the S_P -enantiomer and R_P -enantiomer of ethyl phenyl *p*-nitrophenyl phosphate is provided in Figure 8. It is interesting to note that only five amino acid changes are required to change the stereoselectivity from 11 000:1 (for G60A) to 1:460 (for I106G/F132G/H257Y/S308G). This represents a change of greater than 10^6 ! This library of PTE mutants has proven useful in the preparative scale production of chiral organophosphates that are being used as chiral building blocks in more elaborate chemical transformations [30].

Variants of PTE have been constructed with substrate profiles that are optimized for the hydrolysis of chemical warfare agents and certain agricultural insecticides. For example, the substrate and stereoselectivity of the wild-type PTE has been examined with a series of analogs of sarin and soman. Wild-type PTE hydrolyzes the S_P -enantiomer (see Figure 2o for chemical structure) and R_P -enantiomer (see Figure 2p for chemical structure) of sarin with a 10-fold preference for the least toxic enantiomer of sarin. However, the mutant I106A/F132A/H257Y prefers the hydrolysis of the more toxic S_P -enantiomer of sarin by a factor of 30 [31•]. Similar effects have been obtained for the enantiomers of the soman analogs (Figures 2q–t). The laboratories of Mulbry [32•] and Wild [33] have constructed mutants that have enhanced rates for the hydrolysis of the nerve agents sarin, soman and VX. These results suggest that even further enhancements can be tailored to the active site of PTE.

Organophosphorus acid anhydrolase

An organophosphorus acid anhydrolase (OPAA) has been isolated by the laboratory of DeFrank [34]. The protein appears to be a proline dipeptidase and is able to catalyze the hydrolysis of organophosphate triesters, including the chemical warfare agents sarin and soman, but not VX [34]. The three-dimensional structure is not known. The stereoselectivity has been probed with the same set of organophosphate triesters as PTE. The enzyme displays a similar stereoselectivity to that displayed by PTE, but the overall rate of hydrolysis is substantially reduced relative to PTE [35]. This enzyme has also received considerable

interest for use in the catalytic decomposition of chemical warfare agents. The stereoselective properties for the various stereoisomers of sarin and soman have been tested with a series of chiral analogs [36].

Conclusions

Bacterial systems have been isolated that have the ability to catalyze the hydrolysis and detoxification of organophosphate nerve agents. Given that compounds of this type are not naturally occurring materials, this property must result from an adventitious side reaction, or else these enzymes have evolved new substrate specificities from pre-existing enzymes. In the case of phosphotriesterase, new substrate specificities can be grafted onto the protein with relative ease. It is also interesting to note that atrazine chlorohydrolase, an enzyme shown to catalyze the degradation of the herbicide atrazine, is a member of the same superfamily of enzymes that includes phosphotriesterase [37,38]. It would appear that the active site of this superfamily of proteins has been recruited by Nature to catalyze the hydrolysis of some significant environmental pollutants.

Acknowledgements

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