From the Three-Dimensional Structure of Phosphotriesterase

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Cite This: Biochemistry 2021, 60, 3413−3415

Organophosphate nerve agents are among the most toxic compounds known. In 1994, Raushel and Holden, along with Jane Kuo and Matt Benning, published the first three-dimensional crystal structure of an enzyme capable of hydrolyzing organophosphate triesters (PDB id: 1PTA) in Biochemistry.1 Although this first structure was of the apo-enzyme and determined in the absence of bound divalent cations, additional structures of the protein complexed with a binuclear metal center (PDB id: 1PSC) and then a non-hydrolyzable substrate analogue bound in the active site (PDB id: 1DPM) soon followed. These structures have left a significant impact on our understanding of the evolution of enzyme active sites and the design of new enzymes for the stereoselective hydrolysis of chiral organophosphate esters.

Equally important for us, this publication initiated a fantastic collaboration between our two laboratories that has endured for nearly three decades and has resulted in more than three dozen joint publications, including 17 that have appeared in Biochemistry.

Based on the common structural features of phosphotriesterase (PDB id: 1PTA), adenosine deaminase (PDB id: 1FKX), and urease (PDB id: 2KAU), Sander and Holm first identified the amidohydrolase superfamily of enzymes (AHS).2 The AHS is currently one of the most extensive enzyme superfamilies, encompassing 24 clusters of orthologous groups (COGs) and approximately 0.5% of the known enzyme universe. Subsequent investigations have shown that members of the AHS bind anywhere from zero to three divalent cations in the active site and catalyze the hydrolysis of P−O (mono-, di-, and triesters of phosphate), C−O (lactone), and C−N (amide) bonds in addition to the deamination of guanine, cytosine, and adenine based nucleic acids. Other enzymes in this broad superfamily have been shown to catalyze the decarboxylation of hydroxy benzoic acids, the addition of water to carbon−carbon double bonds, and the isomerization of aldose and ketose carbohydrates.3

The three-dimensional structure of PTE is illustrated in Figure 1, and the zinc binuclear metal center is provided in Figure 2. The overall protein fold is that of a distorted (β/α)₈-barrel where the binuclear metal center is perched at the C-terminal end of the eight β-strands. To date, the best substrate for the bacterial PTE is paraoxon (1) (Scheme 1), where we have shown that the $k_{cat}$ of PTE approaches $10^4$ s$^{-1}$ and $k_{cat}/K_m$ is nearly $10^8$ M$^{-1}$ s$^{-1}$. This is quite remarkable since paraoxon is not a naturally occurring compound and has existed in the environment for less than 70 years. In addition to paraoxon, PTE catalyzes the hydrolysis of fully functionalized phosphonate and phosphinate substrates, with at least one leaving group having a $pK_a$ less than $\sim 10$. Remarkably, the active site of PTE is quite tolerant of changes to the substrate structure, particularly with...
regard to the size and shape of the two nonhydrolyzed ethyl esters.

When these two substituents are different, the phosphorus center is chiral, and wild-type PTE catalyzes the hydrolysis of the individual enantiomers at different rates. For example, with 4-acetophenyl methyl phenyl phosphate \((2)\), the \(S_P\)-enantiomer is hydrolyzed \(\sim100\) times faster than the \(R_P\)-enantiomer. This difference in rate is currently thought to occur through a preferential binding of one isomer in the active site where the leaving group is optimally positioned for nucleophilic attack by the hydroxide that bridges the two divalent cations in the active site. Using the three-dimensional structure of PTE as a guide, individual residues of PTE were selected for mutation with the overall aim of creating modified forms of PTE where the stereoselectivity could be enhanced, relaxed, or reversed relative to the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme. In one noteworthy example, Gly60 was mutated to alanine (G60A), and the stereoselectivity for the wild-type enzyme.

 Conversely, when Ile106 and Phe132 were simultaneously mutated to glycine (I106G/F132G), the stereoselectivity was reduced to less than 5:1. However, with one additional mutation to His257 (I106G/F132G/H257Y), the stereoselectivity reversed, and the \(R_P\)-enantiomer was preferentially hydrolyzed by a factor of 1000. Thus, the stereoselectively for G60A and I106G/F132G/H257Y differ by a factor of more than 10\(^3\), and yet these two proteins differ by only 4 amino acids out of more than 330.\(^4\)

The fact that the active site of PTE was amendable to manipulation while retaining the catalytic power for the hydrolysis of organophosphate esters led to the utilization of this enzyme for the detoxification of organophosphate nerve agents (Scheme 1). Compounds such as sarin (3), soman (4), and VX (5) are toxic because they rapidly react with the active site serine residue in acetylcholinesterase. In collaboration with Steve Harvey from the Edgewood Chemical Biological Center at the Aberdeen Proving Ground, we have shown that PTE has the ability to hydrolyze the \(P\sim F\) bond of sarin and soman in addition to the \(P\sim S\) bond in VX. As expected, the two enantiomers of sarin and VX and the four diastereomers of soman are differentially toxic, where the \(S_P\)-enantiomers are significantly more toxic than the \(R_P\)-enantiomers. Variants of PTE have been identified through rational and random mutational strategies to create an enzyme that can rapidly hydrolyze the most toxic enantiomers of these organophosphate nerve agents. For example, the simple H257Y/L303T variant of PTE hydrolyzes the \(S_P\)-enantiomer of sarin with a value of \(k_{\text{cat}}/k_{\text{m}}\) of \(6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\). This variant, after encapsulation and injection into guinea pigs, provides protection from death after repeated doses of sarin over a period of 8 days.\(^5\)

The ability to reshape the active site of PTE has also been used to differentially prepare chiral precursors for the synthesis of antiviral prodrugs such as remdesivir and sofosbuvir. We have shown that variants of PTE can be used to differentially hydrolyze the two enantiomers of compound 6. This compound has been used to prepare various antiviral prodrugs via the displacement of the \(p\)-nitrophenyl substituent by a modified nucleotide. Using this methodology, the \(R_P\)-enantiomer of remdesivir (7), an antiviral produg that has been used in the treatment of Covid-19, has been synthesized.\(^6\)

From the initial structure of PTE determined more than 27 years ago, the active sites of related enzymes have helped to unveil the remarkable breadth of the amidohydrolase superfamily and have provided insights into the evolution of novel enzymes for the emergence of new metabolic transformations. These insights have paved the way for the rational and random mutation of active site residues for the creation of modified forms of PTE with finely tuned substrate profiles. We have exploited this information to create enzymes that are significantly enhanced in their ability to recognize and hydrolyze highly toxic chemical warfare agents such as sarin, soman, and VX. Other efforts have identified how modifications to the active site of PTE can lead to the stereoselective synthesis of precursors to important antiviral therapeutics.

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**Funding**

Our research programs are supported by grants from the National Institutes of Health (GM122825, GM139428, and GM134643) and the Robert A Welch Foundation (A-840).

**Notes**

The authors declare no competing financial interest.

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