

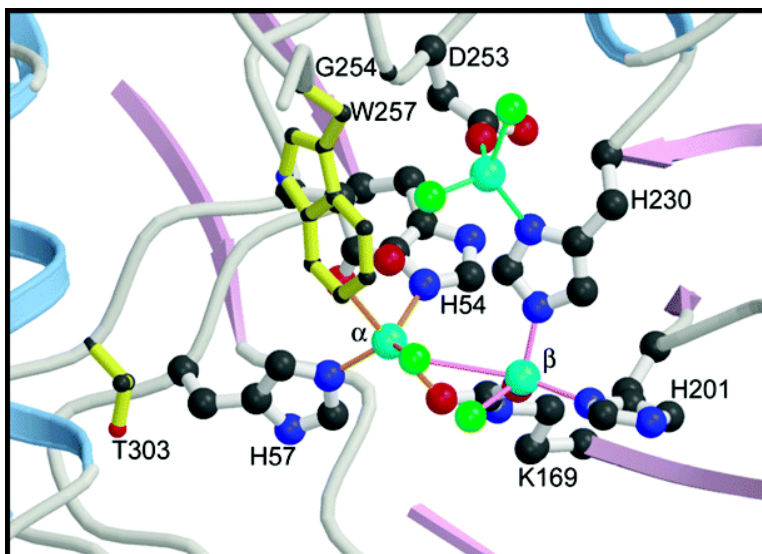
Communication

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J. Am. Chem. Soc., **2003**, 125 (30), 8990-8991 • DOI: 10.1021/ja0358798 • Publication Date (Web): 08 July 2003

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Enhanced Degradation of Chemical Warfare Agents through Molecular Engineering of the Phosphotriesterase Active Site

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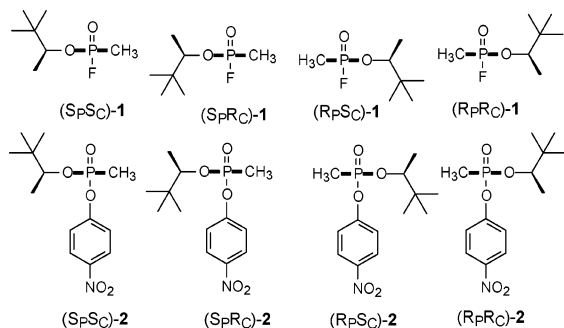
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Organophosphate triesters and the related phosphonates are among the most toxic compounds ever synthesized. The lethal properties of this class of chemicals have been harnessed as agricultural insecticides and exploited as key ingredients in the worldwide arsenal of chemical warfare (CW) agents. Recent events have emphasized the need for novel methods of detection, decontamination, and destruction of these easily prepared threat agents. The catalytic properties of enzymes are ideally suited for these purposes. However, because organophosphonate esters such as sarin, soman, and VX are completely unknown as natural products, existing proteins must be structurally modified to exploit their full catalytic potential. In this Communication, we describe an integrated approach toward the directed evolution of mutant enzymes that are specifically enhanced for the catalytic detoxification of organophosphorus nerve agents and apply this methodology toward the hydrolysis of a structural analogue of the most lethal stereoisomer of the CW agent, soman.

The chemical structures of the four stereoisomers of soman are presented in Chart 1. It has been shown previously that the two diastereomers of soman possessing the *S_P*-configuration at the phosphorus center are substantially more toxic than the *R_P*-pair.¹ To employ high throughput screening methods for the identification of mutant protein leads with enhanced catalytic properties, we synthesized the analogous soman stereoisomers bearing a *p*-nitrophenolate group as a replacement for fluoride (Chart 1). Several enzymes have been reported to hydrolyze the P–F bond in the racemic forms of both sarin and soman.² Of these proteins, only phosphotriesterase (PTE) has demonstrated that the active site is readily amenable to enhancements in the substrate- and stereo-selectivity for organophosphate hydrolysis.³

Chart 1. Stereoisomers of Soman (1) and Chromogenic Analogue (2)



The three-dimensional structure of wild-type PTE has been determined to high resolution in the presence or absence of substrate analogues bound within the active site.⁴ The catalytic properties of

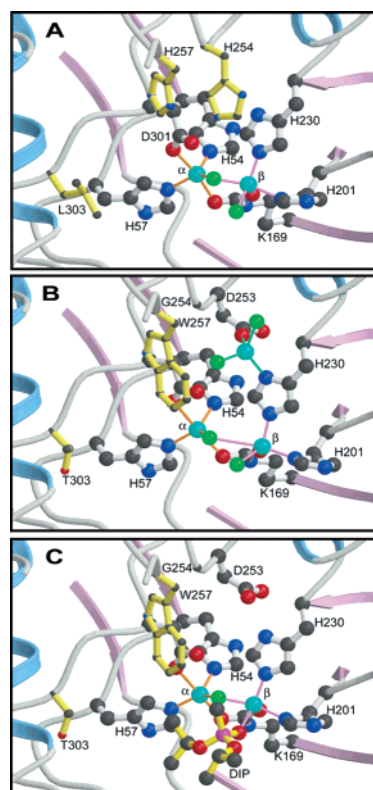


Figure 1. Active site structures of wild-type PTE (a), H254G/H257W/L303T (b), and triple mutant bound with inhibitor (c).

PTE originate from a binuclear zinc metal center that functions to activate the bridging water molecule for nucleophilic attack and to enhance the electrophilic character of the substrate (Figure 1a). Substrate recognition by the enzyme is dictated by the relative positioning of those amino acid residues surrounding the ligands to the metal center.⁵ Several of these amino acid residues were targeted for combinatorial mutagenesis through the substitution of all possible permutations of the 20 naturally occurring amino acids. In particular, the region of the substrate-binding site in the vicinity of H254 and H257 (Figure 1a) has been shown to alter the substrate- and stereoselectivity for several organophosphate triesters.³ These two residue positions were randomized via cassette mutagenesis to create a PTE mutant library with a potential for 400 unique combinations of amino acids at these two sites.⁶ Nearly 1200 colonies were isolated and screened for their ability to hydrolyze the individual stereoisomers of the soman analogue.⁷ Shown in Figure 2a is a representative bar graph for one of the 96-well microtiter plates used to assess the catalytic prowess of the H254X/H257X library toward the hydrolysis of (*S_PS_C*)-2. Two members contained within this library clearly displayed a higher level of

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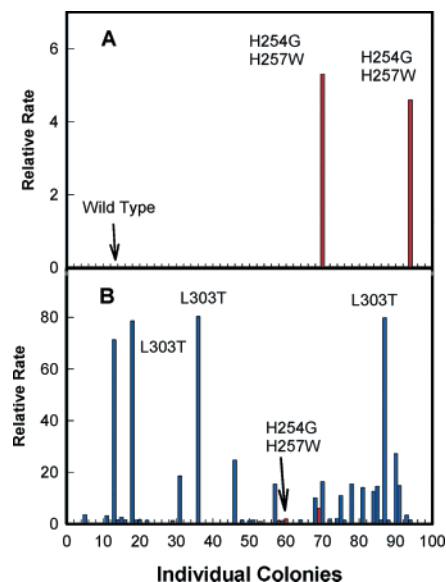


Figure 2. Screening of the mutant libraries for hydrolysis of (S_pSc)-2: (a) Randomization of wild-type PTE at positions H254 and H257. (b) Randomization of H254G/H257W mutant at position L303.

activity than any of the other mutant proteins, including the wild-type control. The genes for both isolates were found to have the same mutations, H254G/H257W.

Inspection of the crystal structure of PTE suggested that L303 could act as a potential locale for substrate/protein interactions.⁸ A H254G/H257W/L303X library was constructed using the H254G/H257W mutant as the parent. Screening of this 20-member library identified a significant improvement upon the mutation of L303 to threonine as illustrated in Figure 2b. The three single mutant proteins, H254G, H257W, and L303T, and the two other double mutant proteins, H254G/L303T and H257W/L303T, were constructed by standard methods to assess the additivity and synergy among the three positional sites within the active site of PTE. These mutant proteins were purified to homogeneity, and their catalytic properties were ascertained for the hydrolysis of the S_pSc -soman analogue. The absolute values for k_{cat}/K_m for the eight proteins are presented in Figure 3. The triple mutant protein, H254G/H257W/L303T, has been enhanced in catalytic turnover for the S_pSc -soman analogue by nearly 3 orders of magnitude, relative to the wild-type enzyme. Of the three single mutant proteins, only H257W is significantly more active relative to the wild-type enzyme. The three double mutant proteins are activated by 1–2 orders of magnitude over their respective single site variants. There is clearly a significant amount of synergism among the three mutated sites that would have been missed had the mutagenesis protocol proceeded sequentially.

The molecular perturbations within the active site of the H254G/H257W/L303T mutant protein were examined by determining the X-ray crystal structure to a resolution of 1.9 Å.⁹ While the overall α -carbons between the wild-type enzyme and the triple mutant protein superimpose with a RMS-deviation of 0.47 Å, there are several significant differences between the two forms of the enzyme, which occur specifically at the H254G substitution and, via propagation, at the binuclear metal center. The substitution of H254 with a glycine results in a cavity that allows for the introduction of a third metal binding site. This third metal ion is coordinated in a tetrahedral arrangement by Asp 253, His 230, and two water molecules (Figure 1b). This additional metal coordination results

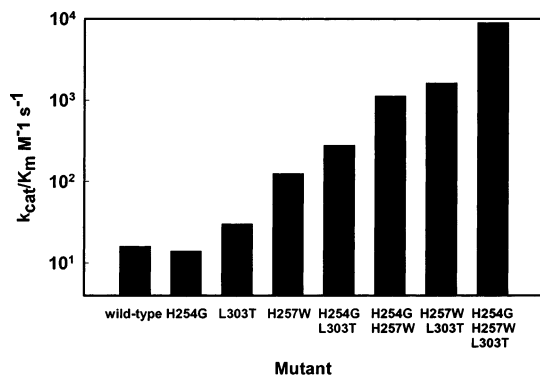


Figure 3. Activity of wild-type protein and additional mutants toward the hydrolysis of (S_pSc)-2.

in a distortion of the symmetry of the binuclear metal center. Specifically, in wild-type PTE, the bridging solvent molecule lies within 2.0 Å of both zinc ions. In the unbound form of the triple mutant protein, however, this bridging solvent is no longer equidistant to the two metals but rather is positioned at 2.1 and 3.2 Å from the α - and β -metals, respectively. When the triple mutant protein structure is solved in the presence of diisopropyl methyl phosphonate (to a resolution of 2.0 Å), the third zinc is expelled from the binding site. The binuclear metal center resumes its typical geometry with the bridging solvent molecule located at approximately the same distance from both the α - and β -metals (\sim 2.0 Å). The inhibitor binds to the triple mutant protein in a fashion nearly identical to that observed for the wild-type enzyme/diisopropyl methyl phosphonate complex.

In this Communication, we have experimentally demonstrated that a significant expansion of the substrate specificity can be achieved through a rather limited number of amino acid mutations that are focused directly on an enzyme active site. Three amino acid changes in PTE have enhanced the turnover rate for an analogue of the most toxic stereoisomer of soman by nearly 3 orders of magnitude. In the mutagenic process, a new metal binding site has been created. A precise structural explanation for the alteration in substrate specificity is being developed.

Acknowledgment. This work was supported in part by the ONR (N00014-99-0235) and the NIH (GM 33894 and GM 68550).

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JA0358798