Enhancement, Relaxation, and Reversal of the Stereoselectivity for Phosphotriesterase by Rational Evolution of Active Site Residues†

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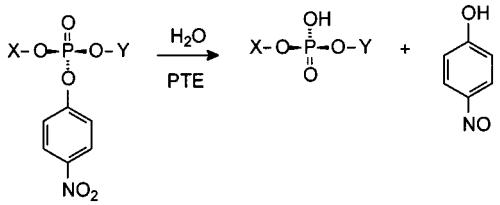
ABSTRACT: The factors that govern the substrate reactivity and stereoselectivity of phosphotriesterase (PTE) toward organophosphotriesters containing various combinations of methyl, ethyl, isopropyl, and phenyl substituents at the phosphorus center were determined by systematic alterations in the dimensions of the active site. The wild type PTE prefers the S_P-enantiomers over the corresponding R_P-enantiomers by factors ranging from 10 to 90. Enlargement of the *small* subsite of PTE with the substitution of glycine and alanine residues for Ile-106, Phe-132, and/or Ser-308 resulted in significant improvements in k_{cat}/K_a for the R_P-enantiomers of up to 2700-fold but had little effect on k_{cat}/K_a for the corresponding S_P-enantiomers. The kinetic preferences for the S_P-enantiomers were thus relaxed without sacrificing the inherent catalytic activity of the wild type enzyme. A reduction in the size of the *large* subsite with the mutant H257Y resulted in a reduction in k_{cat}/K_a for the S_P-enantiomers, while the values of k_{cat}/K_a for the R_P-enantiomers were essentially unchanged. The initial stereoselectivity observed with the wild type enzyme toward the chiral substrate library was significantly reduced with the H257Y mutant. Simultaneous alternations in the sizes of the *large* and *small* subsites resulted in the complete reversal of the chiral specificity. With this series of mutants, the R_P-enantiomers were preferred as substrates over the corresponding S_P-enantiomers by up to 500-fold. These results have demonstrated that the stereochemical determinants for substrate hydrolysis by PTE can be systematically altered through a rational reconstruction of the dimensions of the active site.

The bacterial phosphotriesterase (PTE)[1] catalyzes the cleavage of P−O, P−F, or P−S bonds in a variety of insecticides and organophosphate nerve agents (*1*, *2*). A generalized reaction for the hydrolysis of a simplified organophosphate substrate is illustrated in Scheme 1. Three binding pockets (*small*, *large*, and *leaving group*) within the active site of PTE, which interact directly with the primary substituents attached to the phosphorus center of substrates and inhibitors, have been identified by X-ray crystallography (*3*). Previous investigations with chiral and achiral organophosphate triesters have shown that the reaction rates for various substrates with the wild type PTE depend to a large extent on the size and stereochemical arrangement of the substituents attached to the phosphorus core (*4*, *5*). For example, the kinetic constants with the wild type enzyme for the two enantiomers of chiral organophosphate triesters can differ by up to 2 orders of magnitude with a clear preference for the S_P-enantiomer over the R_P-enantiomer (*4*). For the generic substrate depicted in Scheme 1, substituent Y would be bound within the *large* subsite while substituent X would be bound within the *small* subsite prior to product formation.

Scheme 1



For chiral compounds, the faster isomer is the one where substituent Y is physically larger than substituent X.

An investigation into the origin of the stereoselectivity of PTE by site-directed mutagenesis has demonstrated that the kinetic preference for chiral substrates is dictated to a large extent by the size of the *small* subsite (*6*). This subsite enhances the preference for the S_P-enantiomer by sterically hindering the binding and/or orientation of the R_P-enantiomer to the active site (*6*). The most significant residues within the small subsite are Gly-60, Ile-106, Phe-132, and Ser-308. Decreasing the size of the *small* subsite by mutation of the single glycine residue to an alanine residue significantly increased the stereoselectivity for the preferred S_P-enantiomer (*6*). Conversely, enlargement of the *small* subsite by mutation of Ile-106, Phe-132, or Ser-308 to an alanine decreased the stereoselective preference for the S_P-enantiomer (*6*). However, enlargement of the *large* subsite, which prefers the bulkier substituent of the substrate during catalysis, by replacing His-254, His-257, Leu-271, or Met-317 with an alanine residue had relatively little effect on the stereose-

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[1] Abbreviations: PTE, phosphotriesterase; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside.

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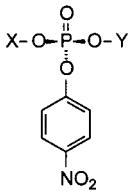
lectivity of the chiral substrates that were tested (*6*). The structural determinants of the substrate specificity and stereoselectivity for PTE are utilized in the present investigation to design a series of novel enzymes where the stereoselectivity is *enhanced*, *relaxed*, or *reversed*, relative to the catalytic properties of the wild type protein. This effort to reshape the active site of PTE is the necessary first step in the development of efficient detoxification schemes for organophosphate nerve agents and the design of catalysts that can be used in the kinetic resolution of chiral organophosphates.

In this paper, the substrate specificity and stereoselectivity of the wild type PTE for the S_P-isomers of chiral organophosphates were initially relaxed by altering the size of the small subsite via the substitution of Ile-106, Phe-132, and/or Ser-308 with dimensionally smaller side chains. Thus, the *small* subsite was enlarged by making simultaneous mutations at these residue positions with either an alanine or glycine substitution. The stereoselectivity for chiral substrates was relaxed to the point where both enantiomers were hydrolyzed at essentially the same rate. These results made it possible to reverse the stereoselectivity by making the initially faster S_P-enantiomer a significantly poorer substrate while maintaining the enhanced turnover for the initially slower R_P-enantiomer. This objective was accomplished by shrinking the *large* subsite by replacing the residues within this region (His-254, His-257, Leu-271, and Met-317) with tyrosine, tryptophan, or phenylalanine. The effects of these mutations on the substrate reactivity and stereoselectivity of PTE were analyzed with a library of organophosphate triesters possessing a common leaving group but containing various combinations of methyl, ethyl, isopropyl, and phenyl substituents at the phosphorus center.

MATERIALS AND METHODS

Materials. All buffers and chemicals were purchased from Sigma, United States Biochemical, or Aldrich. Restriction enzymes and T4 DNA ligase were acquired from New England Biolabs. Wizard Miniprep DNA purification kits were purchased from Promega. GeneClean DNA purification kits were obtained from Bio 101. The synthesis of oligonucleotides and DNA sequencing reactions were carried out by the Gene Technology Laboratory of Texas A&M University. With the exception of paraoxon, all of the other substrates (Figure 1) used for this investigation were synthesized according to previously reported protocols (*4*).

Site-Directed Mutagenesis. The plasmid pJW01[2] containing the *opd* gene that encodes the mature phosphotriesterase was utilized as the initial template for the cassette mutagenesis experiments. The single-site mutations at residues Gly-60, Ile-106, Phe-132, His-254, His-257, Leu-271, Ser-308, and Met-317 were constructed as previously described (*6*). The single-site mutant plasmids were then utilized as the templates for the construction of the double, triple, and quadruple mutations at these residue positions. In each case, unique restriction sites were selected on each side of the codon of the specific amino acid to be mutated. The digested plasmid (pJW01 and derivatives) fragments were purified by agarose gel electrophoresis and concentrated using the GeneClean system. Each pair of overlapping mutagenic



Substrate	X	Y
I	CH_3	CH_3
R_p-II	CH_2CH_3	CH_3
S_p-II	CH_3	CH_2CH_3
R_p-III	$CH(CH_3)_2$	CH_3
S_p-III	CH_3	$CH(CH_3)_2$
R_p-IV	C_6H_5	CH_3
S_p-IV	CH_3	C_6H_5
V	CH_2CH_3	CH_2CH_3
R_p-VI	$CH(CH_3)_2$	CH_2CH_3
S_p-VI	CH_2CH_3	$CH(CH_3)_2$
R_p-VII	C_6H_5	CH_2CH_3
S_p-VII	CH_2CH_3	C_6H_5
VIII	$CH(CH_3)_2$	$CH(CH_3)_2$
R_p-IX	C_6H_5	$CH(CH_3)_2$
S_p-IX	$CH(CH_3)_2$	C_6H_5
X	C_6H_5	C_6H_5

FIGURE 1: Structures of the organophosphates used in the substrate library for this investigation.

oligonucleotides was annealed for 5 min at 72 °C in the presence of 2 μL of T4 DNA ligase buffer, and then incubated for 1 h at 25 °C. The annealed oligonucleotides were ligated into pJW01 or mutant plasmids using T4 DNA ligase. The ligated portion of the phosphotriesterase gene was completely sequenced to ensure that only the fragment containing the desired base changes was utilized.

Expression, Growth, and Preparation of Mutant Enzymes. The mutant plasmids were transformed into *Escherichia coli* strain BL-21 cells (*7*). The transformed BL-21 cells were grown in Luria-Bertani (LB) broth overnight at 37 °C. The overnight cultures were then used to inoculate Terrific Broth (TB) containing 50 μg/mL ampicillin and 1.0 mM $CoCl_2$. After inoculation, the cultures were incubated at 30 °C for 20−24 h and then induced with IPTG, followed by incubation for 24−30 h at 30 °C to reach the stationary phase. The cells were harvested by centrifugation at 4 °C. The wild type and mutant enzymes were purified from BL-21 cells according to the previously reported protocol (*8*). SDS−polyacrylamide gel electrophoresis indicated that all of the mutant enzymes were of the same size as the wild type enzyme and the purity of the proteins was greater than 95%. The apoenzymes were prepared and reconstituted with Co^{2+} as described previously (*9*).

Enzyme Assays and Data Analysis. The kinetic constants for each substrate were determined by varying the concentra-

[2] Unpublished work of J. Wohlschlegel, Texas A&M University.

Table 1: Maximum Velocity (s^{-1}) for the Wild Type and Mutants of PTE Constructed in the *Small* Subsite[a]

substrate	WT	I106A/ F132A	I106A/ S308A	F132A/ S308A	I106G	F132G	S308G	I106G/ F132G	I106G/ S308G	F132G/ S308G	I106G/ F132G/ S308G
I	1.8e4	2.4e3	1.2e3	1.5e3	5.9e3	1.0e4	2.0e3	4.9e3	2.0e3	2.5e3	1.5e3
(R_P)-**II**	1.4e4	2.8e4	7.3e3	1.7e4	8.0e3	2.3e4	2.6e3	1.1e4	2.9e3	5.6e3	1.7e3
(S_P)-**II**	1.5e4	1.3e4	4.9e3	1.5e4	3.5e4	2.3e4	3.9e3	2.5e4	2.2e4	1.0e4	7.5e3
(R_P)-**III**	5.0e2	6.9e3	1.5e3	2.4e3	3.3e2	1.8e2	2.4e2	8.6e1	6.7e1	2.0e2	1.3e2
(S_P)-**III**	7.1e3	1.7e4	6.6e3	8.9e3	6.4e3	1.1e4	5.3e3	8.8e3	1.4e4	6.5e3	1.6e4
(R_P)-**IV**	3.7e2	2.1e4	3.4e4	1.7e4	3.4e4	1.1e4	3.7e3	9.9e4	1.8e4	2.4e3	9.1e3
(S_P)-**IV**	4.1e4	2.5e4	3.5e4	2.4e4	4.9e4	2.5e4	2.5e4	6.4e4	4.7e4	2.2e4	2.3e4
V	7.1e3	2.2e4	5.7e3	1.0e4	2.7e4	1.6e4	7.1e3	2.2e4	3.9e4	3.4e4	3.2e4
(R_P)-**VI**	7.1e2	6.4e3	3.7e3	8.6e2	2.2e3	8.3e2	1.2e3	3.1e3	1.2e3	1.1e3	2.7e2
(S_P)-**VI**	3.0e3	1.1e4	7.8e3	4.6e3	1.1e4	3.6e3	4.5e3	1.5e4	1.5e4	7.6e3	9.8e3
(R_P)-**VII**	1.0e3	9.4e3	4.2e3	5.0e3	6.1e3	4.1e3	1.4e3	8.0e3	1.3e4	4.5e3	5.3e3
(S_P)-**VII**	9.5e3	9.2e3	4.8e3	1.2e4	4.7e3	1.9e3	4.4e3	1.5e4	4.1e3	6.0e3	3.9e3
VIII	2.2e2	1.6e3	4.1e2	7.4e1	1.3e3	4.8e2	2.2e2	1.0e3	5.6e2	6.0e2	4.4e2
(R_P)-**IX**	5.3e2	5.6e3	6.7e3	3.0e3	3.4e4	1.9e3	1.1e3	2.8e4	7.8e3	2.1e3	7.3e3
(S_P)-**IX**	2.6e3	4.7e3	5.3e3	1.3e3	1.9e4	2.7e3	1.5e3	1.5e4	5.5e3	1.8e3	8.9e3
X	3.4e3	3.0e3	1.4e3	1.6e3	2.3e3	1.3e3	1.4e3	2.0e3	1.0e4	2.7e3	4.4e3

[a] The values were obtained by a fit of the data at pH 9.0 and 25 °C to eq 1. The standard error was less than 20% of the stated values. The kinetic constants for the wild type enzyme were taken from ref *6*.

tions of the substrate with a constant enzyme concentration. The rate of hydrolysis of each substrate by the wild type and mutant enzymes was measured by monitoring the appearance of *p*-nitrophenol at 400 nm ($\epsilon = 17\,000\ M^{-1}\ cm^{-1}$) in an assay mixture containing 100 mM CHES buffer, 100 μM $CoCl_2$, and various concentrations of substrates at pH 9.0 and 25 °C, using a Gilford model 260 spectrophotometer. Because of the limited solubility of some compounds, a 20% methanol/water mixture was used in the kinetic analysis of diphenyl *p*-nitrophenol phosphate (**X**), and a 5% mixture was used for methyl phenyl *p*-nitrophenyl phosphate (**IV**), ethyl phenyl *p*-nitrophenyl phosphate (**VII**), and isopropyl phenyl *p*-nitrophenyl phosphate (**IX**). The kinetic constants (V_m and K_a) were obtained by fitting the data to eq 1 using the computer program provided by Savanna Shell Software

$$v = V_m A/(K_a + A) \quad (1)$$

where v is the initial velocity, V_m is the maximum velocity, K_a is the Michaelis constant, and A is the substrate concentration.

RESULTS

Selection of Mutants. The previous investigation with 14 single-alanine mutations within the active site of PTE revealed that an enlargement of the *small* subsite by substitution of Ile-106, Phe-132, or Ser-308 resulted in a general relaxation in the kinetic preference for the S_P-enantiomers of simple organophosphates (*6*). To further define the role of the *small* subsite in determining substrate reactivity and stereoselectivity of PTE, multiple mutations were constructed at these three residue positions using combinatorial substitutions with alanine and glycine. Conversely, mutations in the *large* subsite showed that substitution of His-254, His-257, Leu-271, or Met-317 with an alanine had no influence on the stereoselectivity for the chiral substrates (*6*). In this investigation, the dimensions of the *large* subsite were reduced by mutating these four residues to tyrosine, phenylalanine, or tryptophan. The information gained from the kinetic properties of these modified enzymes led to the construction of other mutants where the sizes of the *small* and *large* subsites were altered simultaneously. All of the mutant enzymes were expressed in *E. coli* and purified to homogeneity. The kinetic parameters for the hydrolysis of the entire organophosphate substrate library were determined for each mutant enzyme, and the results are presented in Tables 1−6.

Enlargement of the Small Subsite. The mutant enzymes, I106A/F132A, I106A/S308A, F132A/S308A, I106G, F132G, S308G, I106G/F132G, I106G/S308G, F132G/S308G, and I106G/F132G/S308G, were constructed and characterized for their ability to catalyze the hydrolysis of a series of achiral and chiral organophosphate substrates (**I**−**X**). With the exception of dimethyl *p*-nitrophenyl phosphate (**I**), this set of mutant enzymes displayed kinetic constants for the hydrolysis of diethyl, diisopropyl, and diphenyl *p*-nitrophenyl phosphate (**V**, **VIII**, and **X**) that were very similar to the values obtained for the wild type enzyme. Enhancements of up to 270-fold were observed in the kinetic parameters for the R_P-enantiomers containing a single phenyl substituent (**IV**, **VII**, and **IX**). However, all of the mutants exhibited kinetic constants for the S_P-enantiomers that were essentially identical with those of the wild type enzyme. Thus, the kinetic preference for the S_P-enantiomers of **IV**, **VII**, and **IX** was virtually eliminated with this set of mutants except for S308G.

The overall effects on the kinetic parameters for the R_P-enantiomer of methyl isopropyl *p*-nitrophenyl phosphate (**III**) of the glycine and alanine mutants of Ile-106, Phe-132, and Ser-308 were quite different. Reductions of up to 23-fold in the magnitude of the catalytic constants for the R_P-enantiomer of **III** were observed for I106G, F132G, S308G, I106G/F132G, I106G/S308G, F132G/S308G, and I106G/F132G/S308G, relative to those of the wild type enzyme. However, increases in the kinetic constants of up to 15-fold for the R_P-enantiomer of **III** were observed for I106A/F132A, I106A/S308A, and F132A/S308A. Thus, I106A/F132A, I06A/S308A, and F132A/S308A displayed a *reduction* in the stereoselectivity for the S_P-enantiomer of **III**, while the glycine mutants of Ile-106, Phe-132, and Ser-308 showed *enhancements* in the chiral preference for the S_P-enantiomer.

Table 2: Values of k_{cat}/K_a (M^{-1} s^{-1}) for the Wild Type and Mutants of PTE Constructed in the *Small* Subsite[a]

substrate	WT	I106A/ F132A	I106A/ S308A	F132A/ S308A	I106G	F132G	S308G	I106G/ F132G	I106G/ S308G	F132G/ S308G	I106G/ F132G/ S308G
I	1.2e7	1.5e6	4.2e5	1.4e6	1.0e7	6.4e6	1.1e6	2.5e6	1.2e6	8.1e5	2.9e5
(R_P)-**II**	3.2e7	1.6e7	7.0e6	1.1e7	1.4e7	2.9e7	3.8e6	6.2e6	2.3e6	7.1e6	9.6e5
(S_P)-**II**	3.4e7	1.6e7	5.2e6	1.5e7	4.2e7	2.4e7	7.7e6	2.2e7	1.3e7	1.2e7	4.8e6
(R_P)-**III**	8.5e5	2.1e6	4.7e5	6.5e5	3.2e5	3.0e5	1.1e5	1.3e5	3.7e4	7.0e4	5.6e4
(S_P)-**III**	2.7e7	1.5e7	8.4e6	8.7e6	2.5e7	1.5e7	6.1e6	1.1e7	1.3e7	7.8e6	2.5e6
(R_P)-**IV**	1.0e6	3.4e7	6.6e7	3.0e7	7.2e7	9.1e6	6.6e6	4.3e7	3.0e7	5.7e6	1.5e7
(S_P)-**IV**	9.3e7	1.0e8	2.0e8	1.0e8	1.2e8	3.3e7	3.2e7	6.1e7	6.5e7	3.7e7	2.7e7
V	6.4e7	4.0e7	1.3e7	2.2e7	5.5e7	7.1e7	3.1e7	4.1e7	3.3e7	4.3e7	1.6e7
(R_P)-**VI**	4.1e6	1.5e7	8.2e6	1.0e6	3.1e6	1.9e6	1.1e6	2.8e6	7.3e5	1.1e6	6.1e5
(S_P)-**VI**	4.2e7	3.6e7	2.5e7	1.0e7	3.9e7	1.6e7	1.4e7	2.4e7	1.5e7	1.9e7	7.6e6
(R_P)-**VII**	3.7e6	1.0e8	4.7e7	6.5e7	1.0e8	4.6e7	3.6e6	2.9e8	1.9e8	8.2e6	1.3e8
(S_P)-**VII**	7.6e7	1.5e8	1.5e8	1.5e8	1.2e8	7.4e7	1.1e8	8.2e7	3.3e8	9.4e7	1.0e8
VIII	4.8e6	1.8e7	4.4e6	9.5e5	2.6e6	1.5e6	4.0e5	2.9e6	8.3e5	6.1e5	5.5e5
(R_P)-**IX**	5.2e6	9.0e7	7.3e7	3.7e7	7.0e7	8.7e6	1.6e6	7.6e7	2.2e8	4.1e6	1.3e8
(S_P)-**IX**	1.8e8	5.4e7	4.6e7	3.0e7	7.3e7	3.5e7	3.0e7	1.5e7	5.2e7	2.2e7	2.9e7
X	1.6e7	3.7e7	5.7e7	4.2e7	2.3e7	2.2e7	1.4e7	4.2e7	6.1e7	1.3e7	4.2e7

[a] The values were obtained by a fit of the data at pH 9.0 and 25 °C to eq 1. The standard error in every case was less than 20% of the stated values. The kinetic constants for the wild type enzyme were taken from ref *6*.

Table 3: Maximum Velocity (s^{-1}) for the Wild Type and Mutants of PTE Constructed in the *Large* Subsite[a]

substrate	WT	H254Y	H254F	H257Y	H257F	H257W	H257L	L271Y	L271F	L271W	M317Y	M317F	M317W
I	1.8e4	8.7e2	1.7e2	2.4e4	1.5e4	1.4e4	7.8e3	3.1e2	9.2e3	1.2e3	7.2e2	4.3e3	1.1e3
(R_P)-**II**	1.4e4	4.4e2	1.2e2	7.0e3	9.6e3	8.2e3	4.8e3	1.1e3	1.4e4	2.5e3	2.2e3	4.4e3	6.2e3
(S_P)-**II**	1.5e4	5.0e2	7.6e1	1.1e4	1.3e4	4.5e3	5.7e3	8.7e2	1.2e4	4.5e3	1.5e3	3.7e3	8.2e3
(R_P)-**III**	5.0e2	1.5e2	4.6e1	1.6e3	5.4e2	8.9e1	1.5e3	3.6e1	6.9e2	4.1e2	2.0e2	6.8e2	8.1e2
(S_P)-**III**	7.1e3	3.8e2	5.9e1	3.3e3	8.4e3	1.2e3	2.9e3	7.6e2	5.3e3	1.8e3	6.0e3	8.2e3	6.0e3
(R_P)-**IV**	3.7e2	1.6e1	1.8e1	9.4e2	3.6e2	2.3e0	5.1e1	2.1e1	3.0e2	9.4e1	2.3e2	8.1e1	9.0e1
(S_P)-**IV**	4.1e4	2.9e2	2.2e2	1.2e3	3.7e3	2.4e2	1.5e3	3.6e3	2.5e4	8.8e3	4.2e3	1.6e4	4.7e3
V	7.1e3	1.3e3	6.0e1	3.4e3	5.1e3	3.6e3	5.3e3	2.8e3	1.4e4	6.4e3	4.0e3	3.4e3	1.0e4
(R_P)-**VI**	7.1e2	4.8e1	4.5e1	1.1e3	4.8e2	1.6e1	5.1e2	1.5e2	3.0e2	1.7e2	1.7e2	1.7e2	2.4e2
(S_P)-**VI**	3.0e3	2.1e2	1.6e2	2.2e3	1.5e3	9.7e1	1.9e3	1.4e3	2.5e3	3.8e3	2.5e3	1.5e3	3.6e3
(R_P)-**VII**	1.0e3	1.4e1	1.3e2	2.1e2	1.9e2	2.0e1	2.4e2	5.4e1	3.9e2	6.7e2	3.0e2	6.2e2	6.2e2
(S_P)-**VII**	9.5e3	4.2e2	1.8e2	3.6e3	5.9e3	1.1e3	6.2e3	1.0e3	7.5e3	1.9e3	8.0e3	5.8e3	2.4e3
VIII	2.2e2	1.2e1	4.6e0	3.1e2	2.9e2	2.2e1	2.6e2	2.4e1	1.3e2	6.7e2	7.8e1	1.5e2	1.1e2
(R_P)-**IX**	5.3e2	1.1e1	1.8e1	6.7e1	4.7e1	4.4e0	1.5e2	2.4e1	9.6e1	5.9e1	3.3e1	7.9e2	7.0e1
(S_P)-**IX**	2.6e3	2.0e2	1.2e2	2.2e2	2.7e3	7.2e1	1.3e3	2.8e2	1.8e3	7.1e2	5.1e2	1.7e3	3.7e2
X	3.4e3	3.9e1	2.7e1	9.8e1	8.6e1	1.2e0	3.4e2	6.0e1	9.1e1	2.2e2	5.0e1	3.8e2	9.8e1

[a] The values were obtained by a fit of the data at pH 9.0 and 25 °C to eq 1. The standard error in every case was less than 20% of the stated values. The kinetic constants for the wild type enzyme were taken from ref *6*.

Table 4: k_{cat}/K_a Values (M^{-1} s^{-1}) for the Wild Type and Mutants of PTE Constructed in the *Large* Subsite[a]

substrate	WT	H254Y	H254F	H257Y	H257F	H257W	H257L	L271Y	L271F	L271W	M317Y	M317F	M317W
I	1.2e7	4.8e5	2.2e5	1.7e7	1.5e7	2.9e7	2.4e6	2.0e5	4.8e6	1.2e6	3.2e5	6.0e6	1.0e6
(R_P)-**II**	3.2e7	1.2e6	1.0e6	3.8e7	2.9e7	4.3e7	6.1e6	1.2e6	2.3e7	7.2e6	2.7e6	1.0e7	8.4e6
(S_P)-**II**	3.4e7	1.2e6	5.0e5	2.6e7	2.3e7	2.2e7	7.3e6	5.5e5	1.8e7	4.0e6	1.7e6	1.0e7	8.1e6
(R_P)-**III**	8.5e5	3.9e5	2.3e5	3.8e6	2.0e6	1.1e6	1.2e6	1.2e4	4.5e5	7.7e4	1.3e5	5.5e5	2.0e5
(S_P)-**III**	2.7e7	1.4e6	8.7e5	1.7e7	2.3e7	3.4e6	5.1e6	6.4e5	5.0e6	3.1e6	1.8e6	2.0e7	4.9e6
(R_P)-**IV**	1.0e6	8.2e4	1.9e5	2.8e6	1.4e6	6.6e3	1.6e5	5.7e4	2.3e5	1.1e5	3.8e5	3.5e5	2.9e5
(S_P)-**IV**	9.3e7	3.0e6	5.3e6	4.4e6	2.3e7	1.6e6	2.3e7	3.1e6	7.4e6	1.1e7	8.2e6	2.2e7	6.4e6
V	6.4e7	4.8e6	1.3e6	6.4e7	4.3e7	4.4e7	9.0e6	5.9e6	6.2e7	3.5e7	9.4e6	2.7e7	2.0e7
(R_P)-**VI**	4.1e6	1.5e5	1.9e5	4.8e6	2.6e6	5.5e4	2.5e6	1.2e5	2.5e5	4.3e5	3.8e5	1.0e6	8.2e5
(S_P)-**VI**	4.2e7	1.0e6	1.5e6	3.4e7	1.3e7	6.2e5	7.9e6	2.3e6	7.3e6	1.3e7	1.2e7	9.8e6	1.4e7
(R_P)-**VII**	3.7e6	1.7e5	1.2e6	2.8e6	1.5e6	8.1e4	1.6e6	4.5e5	4.6e6	3.8e6	1.6e6	3.8e6	1.6e6
(S_P)-**VII**	7.6e7	1.1e7	3.0e6	1.5e7	3.9e7	1.4e7	1.3e7	1.9e7	3.6e7	6.9e7	3.0e7	1.0e8	4.0e7
VIII	4.8e6	2.9e5	1.4e5	9.1e5	1.7e6	8.3e4	3.2e6	3.1e4	3.1e5	2.0e5	1.1e5	2.8e6	4.3e5
(R_P)-**IX**	5.2e6	1.1e5	3.5e5	5.0e5	3.3e5	5.0e4	1.7e6	2.8e4	1.3e5	1.2e5	9.6e4	1.1e6	4.0e5
(S_P)-**IX**	1.8e8	2.4e6	1.6e6	1.3e6	5.9e6	4.5e5	1.6e7	1.2e6	6.5e6	5.4e6	1.7e6	3.7e7	8.6e6
X	1.6e7	3.8e6	3.3e6	1.7e6	1.4e6	6.2e4	6.4e6	6.0e5	1.9e6	2.9e6	1.1e6	2.7e6	6.8e5

[a] The values were obtained by a fit of the data at pH 9.0 and 25 °C to eq 1. The standard error in every case was less than 20% of the stated values. The kinetic constants for the wild type enzyme were taken from ref *6*.

None of the glycine and alanine mutants had significant effects on the kinetic constants or stereoselectivity for chiral substrates **II** and **VI**. The entire set of kinetic constants for this set of mutants is presented in Tables 1 and 2.

Reduction of the Large Subsite. The cavity size of the *large* subsite was reduced by the introduction of phenylalanine, tyrosine, or tryptophan as replacements for His-254, His-257, Leu-271, or Met-317. The kinetic constants for the

Table 5: Maximum Velocity (s^{-1}) for the Mutants of PTE Constructed in the *Small* and *Large* Subsites Simultaneously[a]

substrate	WT	I106A/ H257Y	F132A/ H257Y	I106A/ F132A/ H257Y	I106A/ H257Y/ S308A	I106A/ F132A/ H257W	F132A/ H257Y/ S308A	I106G/ H257Y	F132G/ H257Y	I106G/ F132G/ H257Y	I106G/ H257Y/ S308G	I106G/ F132G/ H257Y/ S308G
I	1.8e4	5.0e4	1.9e4	3.3e4	2.3e4	4.8e3	4.5e3	2.2e4	5.4e3	2.8e4	2.7e4	7.4e3
(R_P)-**II**	1.4e4	2.7e4	8.1e3	3.5e4	3.3e4	7.8e3	2.8e3	1.5e4	1.2e4	6.4e4	7.9e3	1.6e4
(S_P)-**II**	1.5e4	2.3e4	7.9e3	3.5e4	2.7e4	7.0e3	3.1e3	1.4e4	6.8e3	1.8e4	8.2e3	7.3e3
(R_P)-**III**	5.0e2	7.8e3	1.3e3	5.7e3	6.8e3	3.1e3	9.3e2	1.5e3	6.0e3	7.9e3	1.3e3	2.0e3
(S_P)-**III**	7.1e3	9.2e3	2.5e3	5.7e3	5.5e3	1.6e3	2.5e3	4.6e3	7.6e3	6.9e3	3.7e3	2.3e3
(R_P)-**IV**	3.7e2	2.5e4	2.1e3	1.1e4	2.0e4	4.0e3	2.1e3	2.6e4	2.5e3	8.6e3	2.0e4	2.7e4
(S_P)-**IV**	4.1e4	4.2e3	1.1e3	2.5e3	8.8e3	1.4e3	1.6e3	1.1e3	1.2e4	1.7e2	6.0e2	4.3e2
V	7.1e3	2.1e4	2.9e3	1.0e4	1.9e4	8.2e3	1.5e3	1.6e4	7.6e3	4.5e4	3.1e4	4.5e4
(R_P)-**VI**	7.1e2	3.2e3	7.3e2	2.2e3	1.9e3	9.7e2	5.4e2	7.5e2	6.0e2	2.0e3	7.5e2	3.1e2
(S_P)-**VI**	3.0e3	3.9e3	1.0e3	1.6e3	3.5e3	1.4e3	1.1e3	3.3e3	2.7e3	3.8e3	5.4e3	2.4e3
(R_P)-**VII**	1.0e3	8.0e3	3.1e3	7.5e3	1.3e4	4.1e3	2.9e3	1.5e4	1.8e3	9.3e3	1.9e4	5.9e3
(S_P)-**VII**	9.5e3	1.2e3	1.1e3	6.5e2	8.1e2	7.3e2	1.8e3	2.6e2	4.4e3	3.2e2	2.5e2	4.1e1
VIII	2.2e2	4.6e2	1.6e2	3.6e2	8.0e2	1.2e2	1.1e2	1.1e3	2.6e2	1.5e3	7.1e1	4.0e2
(R_P)-**IX**	5.3e2	2.2e3	5.2e2	7.2e3	7.7e3	3.6e2	1.2e3	1.1e4	1.2e3	1.4e4	9.2e3	1.1e4
(S_P)-**IX**	2.6e3	1.5e2	8.1e1	1.0e2	8.6e1	1.4e2	1.0e3	1.0e1	2.4e3	3.7e0	6.6e0	3.3e0
X	3.4e3	2.4e2	2.3e1	5.3e2	6.9e2	1.8e1	2.3e2	2.4e2	1.0e3	1.7e2	8.7e2	4.2e2

[a] The values were obtained by a fit of the data at pH 9.0 and 25 °C to eq 1. The standard error in every case was less than 20% of the stated values. The kinetic constants for the wild type enzyme were taken from ref *6*.

Table 6: k_{cat}/K_a Values (M^{-1} s^{-1}) for Mutants of PTE Constructed in the *Small* and *Large* Subsites Simultaneously[a]

substrate	WT	I106A/ H257Y	F132A/ H257Y	I106A/ F132A/ H257Y	I106A/ H257Y/ S308A	I106A/ F132A/ H257W	F132A/ H257Y/ S308A	I106G/ H257Y	F132G/ H257Y	I106G/ F132G/ H257Y	I106G/ H257Y/ S308G	I106G/ F132G/ H257Y/ S308G
I	1.2e7	2.0e7	8.4e6	1.1e7	1.3e7	1.5e7	2.5e6	1.8e7	2.1e6	8.4e6	6.0e6	2.8e6
(R_P)-**II**	3.2e7	3.1e7	1.8e7	1.8e7	2.4e7	1.4e7	6.5e6	2.8e7	1.3e7	2.3e7	7.2e6	4.5e6
(S_P)-**II**	3.4e7	2.4e7	1.1e7	1.1e7	1.3e7	5.0e6	4.3e6	1.9e7	3.6e6	1.8e7	4.2e6	2.8e6
(R_P)-**III**	8.5e5	7.2e6	3.4e6	5.3e6	3.2e6	8.4e6	7.1e5	1.6e6	1.2e6	1.2e6	4.5e5	2.0e5
(S_P)-**III**	2.7e7	7.4e6	7.0e6	3.5e6	3.9e6	4.0e5	2.2e6	4.4e6	7.9e6	1.8e6	1.2e6	6.0e5
(R_P)-**IV**	1.0e6	5.8e7	8.4e6	5.2e7	1.1e8	2.2e7	1.6e7	7.4e7	6.0e6	5.8e7	6.4e7	5.2e7
(S_P)-**IV**	9.3e7	1.0e7	2.3e6	4.5e6	1.1e7	1.6e6	2.5e6	1.9e6	3.9e7	4.9e5	7.3e5	2.8e5
V	6.4e7	5.7e7	2.4e7	2.5e7	3.2e7	2.4e7	7.4e6	3.7e7	3.0e7	3.8e7	9.6e6	7.3e6
(R_P)-**VI**	4.1e6	1.1e7	8.3e5	5.0e6	2.9e6	2.7e6	6.0e5	1.5e6	5.0e5	7.9e5	3.3e5	2.7e5
(S_P)-**VI**	4.2e7	3.8e7	3.7e6	1.3e7	1.4e7	2.2e6	5.6e6	8.1e6	5.1e6	5.0e6	3.1e6	1.5e6
(R_P)-**VII**	3.7e6	4.6e7	2.3e7	6.2e7	8.6e7	8.8e6	3.4e7	1.0e8	6.6e6	7.0e7	9.1e7	1.2e8
(S_P)-**VII**	7.6e7	8.0e6	5.0e6	6.1e6	4.4e6	1.6e6	7.1e6	5.1e6	3.6e7	1.2e6	1.7e6	1.5e6
VIII	4.8e6	2.3e6	3.1e5	3.2e6	1.3e6	2.1e5	1.2e5	7.3e5	1.2e6	4.8e5	2.3e5	1.4e5
(R_P)-**IX**	5.2e6	1.2e7	5.1e6	2.9e7	3.0e7	4.5e6	1.1e7	5.0e7	8.4e6	3.2e7	5.1e7	3.8e7
(S_P)-**IX**	1.8e8	6.8e6	4.1e6	2.5e6	1.7e6	4.7e5	3.7e6	3.4e5	2.3e7	1.8e5	1.1e5	8.3e4
X	1.6e7	1.3e6	7.1e5	2.6e6	6.0e6	1.5e6	1.7e6	4.0e6	2.5e7	2.3e6	5.2e6	4.7e6

[a] The values were obtained by a fit of the data at pH 9.0 and 25 °C to eq 1. The standard error in every case was less than 20% of the stated values. The kinetic constants for the wild type enzyme were taken from ref *6*.

hydrolysis of diphenyl *p*-nitrophenyl phosphate (**X**) were diminished significantly with all of the single-site mutants constructed at these four residue positions. Most of the mutants also displayed lower catalytic activities for the hydrolysis of the three other achiral substrates (**I**, **V**, and **VIII**). Mutation of His-257 to a tyrosine virtually eliminated the observed stereoselectivity for chiral substrates **IV**, **VII**, and **IX**. The H257Y mutant exhibited decreases in the kinetic parameters for the S_P-enantiomers of **IV**, **VII**, and **IX**, but had little effect on the kinetic constants for the corresponding R_P-enantiomers. Reductions in substrate turnover were observed for most of the other single-site mutants constructed within the *large* subsite when either enantiomer of **IV**, **VII**, or **IX** was utilized as a substrate. However, none of these other mutants caused an appreciable effect on the stereoselectivity for the chiral substrates.

For the wild type enzyme, the S_P-enantiomer of methyl isopropyl *p*-nitrophenyl phosphate (**III**) is a better substrate than the R_P-enantiomer. Significant effects on the stereoselectivity for **III** were observed with H254F, H254Y, H257W, and H257Y. The H257Y mutant exhibited 3−4-fold increases in the kinetic parameters for the R_P-enantiomer, coupled with decreases in these constants for the S_P-enantiomer. Losses in the enantiomeric selectivity of **III** were also observed with H257W, H254F, and H254Y. The value of k_{cat}/K_a with H257W for the S_P-enantiomer of **III** was reduced by a factor of 8, but k_{cat}/K_a for the R_P-enantiomer (**III**) varied little from the value obtained with the wild type enzyme. Therefore, mutation of His-257 to a tryptophan resulted in the virtual elimination of the stereoselectivity for substrate **III**.

Mutation of Leu-271 to phenylalanine, tyrosine, or tryptophan reduced the magnitude of the kinetic constants for nearly all of the substrates that were tested, while mutation of Met-317 had virtually no effect on the catalytic properties. The mutants designed to reduce the size of the large subunit have demonstrated that the mutation of His-257 to a tyrosine residue can selectively diminish the rate of hydrolysis for the initially faster S_P-enantiomer while maintaining the wild

type levels of activity for the initially slower R_P-enantiomer. All of the kinetic constants determined for this set of mutations within the *large* subsite are presented in Tables 3 and 4.

Enlarging the Small Subsite and Shrinking the Large Subsite Simultaneously. Those mutations found within the *large* subsite that were able to reduce the catalytic constants for the S_P-enantiomers were combined with those mutations identified within the *small* subsite that were able to enhance the kinetic constants for the R_P-enantiomers. These experiments were carried out in an attempt to reverse the kinetic preference from the S_P-enantiomers to the R_P-enantiomers while maintaining the inherent catalytic power of the wild type enzyme. The mutant enzymes, I106A/H257Y, F132A/H257Y, I106A/F132A/H257Y, I106A/H257Y/S308A, F132A/H257Y/S308A, I106A/F132A/H257W, I106G/H257Y, F132G/H257Y, I106G/F132G/H257Y, I106G/H257Y/S308G, and I106G/F132G/H257Y/S308G, were tested for their ability to catalyze the hydrolysis of the organophosphate triesters (**I**−**X**).

With the native enzyme, the S_P-enantiomers of **IV**, **VII**, and **IX** are better substrates than the R_P-enantiomers. However, for most of the mutants in this series, the rate of hydrolysis for the R_P-enantiomer is now faster than those for the corresponding S_P-enantiomer. The kinetic constants for the S_P-enantiomers of **IV**, **VII**, and **IX** were reduced by up to 3 orders of magnitude. The most severe losses in catalytic activity were observed with I106G/H257Y, I106G/F132G/H257Y, I106G/H257Y/S308G, and I106G/F132G/H257Y/S308G, while smaller reductions in rate were observed for I106A/H257Y, F132A/H257Y, I106A/F132A/H257Y, I106A/H257Y/S308A, and I106A/F132A/H257W. However, significant improvements in the substrate turnover for the R_P-enantiomers of **IV**, **VII**, and **IX** were observed for all of the mutants, relative to the values obtained for the native enzyme. The kinetic constants for some substrates were increased by up to 2 orders of magnitude. Consequently, I106G/H257Y, I106G/F132G/H257Y, I106G/H257Y/S308G, and I106G/F132G/H257Y/S308G exhibited a 40−500-fold kinetic preference for the R_P-enantiomers of **IV**, **VII**, and **IX** relative to the S_P-enantiomer. The remaining mutants in this series displayed an up to 20-fold stereoselectivity for the R_P-enantiomers.

A significant effect on the stereoselectivity for methyl isopropyl *p*-nitrophenyl phosphate (**III**) was observed with I106A/F132A/H257W. This mutant displayed a 68-fold decrease in k_{cat}/K_a for the S_P-enantiomer of **III** but exhibited a 10-fold increase in the k_{cat}/K_a for the R_P-enantiomer of **III**. Thus, the 32-fold preference for the S_P-enantiomer of **III**, observed with the native enzyme, was reversed to a 21-fold preference for the R_P-enantiomer with this mutant. All of the other mutants displayed an increase in the kinetic parameters for the initially slower R_P-enantiomer and a decrease in the magnitude of the kinetic constants for the S_P-enantiomer of **III**. The kinetic constants for the enzymes constructed for this series of mutants are presented in Tables 5 and 6.

DISCUSSION

Previous studies of the stereoselectivity of phosphotriesterase toward a series of asymmetric organophosphate triesters indicated that the wild type enzyme prefers the S_P-enantiomer over the corresponding R_P-enantiomer by up to 90-fold (*4*). Therefore, the proper positioning of substrates bound within the active site of PTE must be essential for optimal catalysis. The availability of enantiomerically pure organophosphate triesters, coupled with the three-dimensional structure of PTE containing a bound substrate analogue, offers a unique way of assessing the configurational and spatial orientation of individual enantiomers within the active site of this enzyme. Three distinct hydrophobic binding regions (*small*, *large*, and *leaving group* subsites) have been identified within the active site of PTE that are important in dictating the substrate reactivity and stereoselectivity of the wild type enzyme (*3*). Mutational studies have now clearly demonstrated that the chiral selectivity can be modulated by specific adjustments to active site residues within these subsites. The derived structural determinants that dictate the substrate specificity can now be programmed to *enhance*, *relax*, or *reverse* the stereoselective properties of this enzyme. Therefore, derivatives of PTE can be rationally created as catalysts for the kinetic resolution of chiral organophosphates and for enhancing the specific destruction of highly toxic acetylcholinesterase inhibitors.

Enlargement of the Small Subsite. Previous studies have shown that single-site mutations of Ile-106, Phe-132, or Ser-308 to alanine residue resulted in increases in the k_{cat} and k_{cat}/K_a values for the hydrolysis of the initially slower R_P-enantiomers from the chiral substrate library (*6*). These results suggest that structural constraints within the *small* subsite dictate, to a large extent, the chiral preference for the S_P-enantiomer by sterically hindering or perturbing the binding of the R_P-enantiomer to the active site of PTE. It was surmised that these physical constraints within the *small* subsite could be further relaxed by enlarging this subsite even more with multiple glycine and alanine mutations at these three residue positions. These expectations were borne out by the catalytic properties of the 10 glycine and alanine mutants constructed within the small subsite. Of the six R_P-configuration organophosphate triesters synthesized for this investigation, all of them were more rapidly hydrolyzed by one or more members of the new mutant library. The smallest increase (2-fold) in k_{cat} was obtained for (R_P)-**II** with I106A/F132A, while an increase of 270-fold was observed for (R_P)-**IV** with I106G/F132G. Increases in turnover rates of 1 order of magnitude were obtained for the R_P-stereoisomers of **III**, **VI**, **VII**, and **IX**. These increases in the rate of substrate turnover for the initially slower R_P-stereoisomers were achieved without sacrificing the high rate of turnover for the initially faster S_P-stereoisomers. This is a highly desirable property when a single enzyme is to be used for the catalytic detoxification of a racemic mixture of an organophosphate nerve agent.

Shrinking the Large Subsite. The crystal structure of PTE indicates that the side chains of His-254, His-257, Leu-271, and Met-317 are involved in the formation of the *large* subsite (*3*). Since the larger substituent of the preferred S_P-enantiomer for PTE is positioned in the *large* subsite during catalysis, this subsite is apparently better able to accommodate the bulkier substituents than does the *small* subsite. Previous studies showed that enlargement of this subsite with H254A, H257A, L271A, and M317A did not result in any significant improvement in the rates of hydrolysis for the S_P-enantiomers of the six chiral substrates. In addition, these

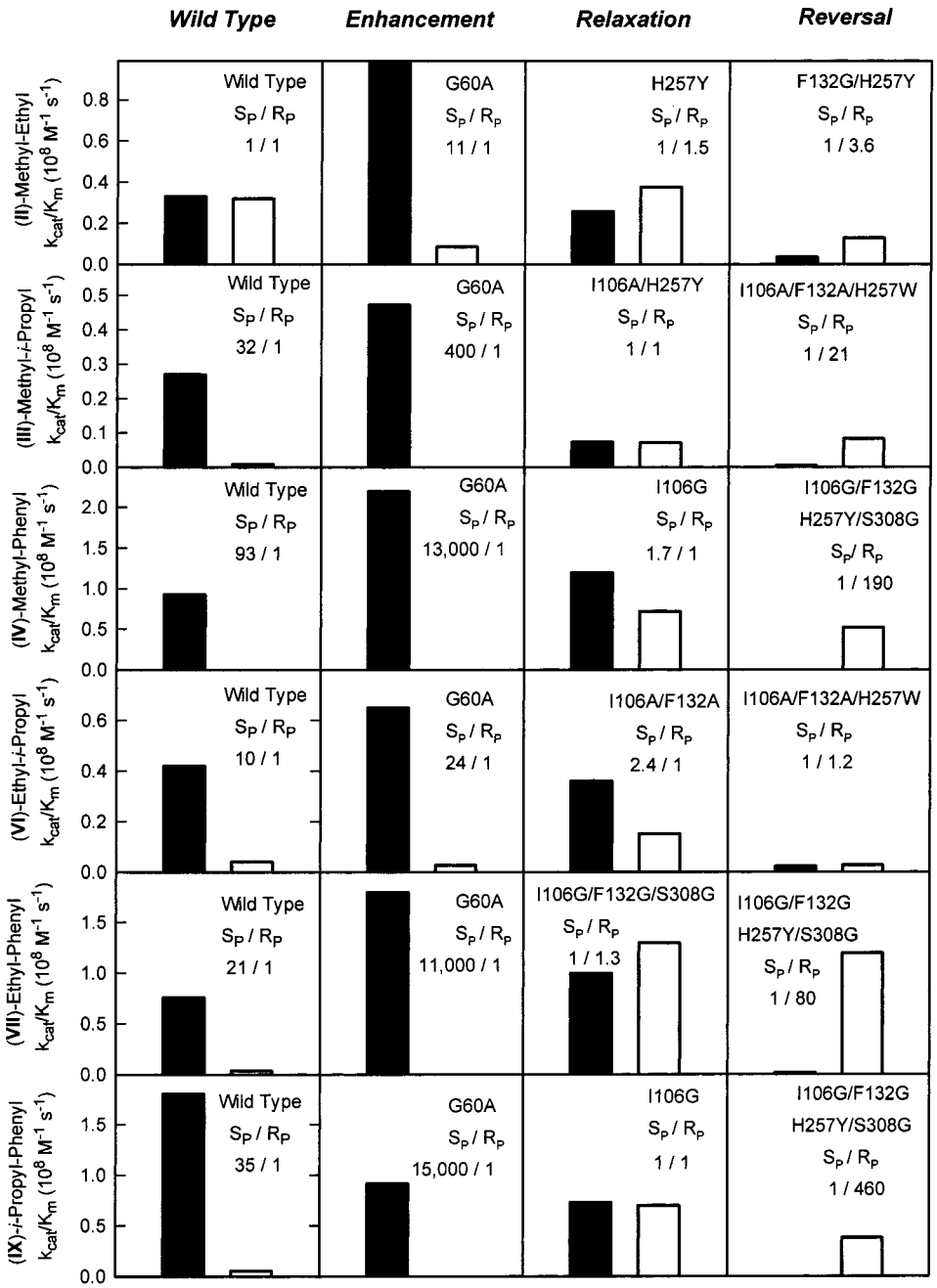


FIGURE 2: Bar graphs representing the values of k_{cat}/K_a for the S_P- and R_P-stereoisomers with the six pairs of chiral organophosphates synthesized for this investigation (Figure 1). In column 1, the ratios of the kinetic constants are presented for the wild type enzyme. In column 2, the ratios of the kinetic constants are presented for the best mutant enzyme with each pair of enantiomers, where the rate constants for the S_P-enantiomer have been maximized relative to the R_P-enantiomer. In column 3, comparisons are presented for the best mutant enzymes where the stereoselectivity for each pair of enantiomers has been relaxed by increasing the kinetic constants for the initially slower R_P-enantiomer. In column 4, the best examples of mutant enzymes are presented for each pair of chiral enantiomers where the initial stereoselectivity has been reversed.

same mutants had essentially no effect on the overall stereoselectivity for the chiral substrate library. Thus, the *large* subsite is capable of accommodating the larger substituent of the initially faster S_P-enantiomer without significant steric restrictions. To further characterize the steric constraints within the large subsite, the dimensions of this subsite were reduced by replacing His-254, His-257, Leu-271, or Met-317 with the larger aromatic residues, tyrosine, phenylalanine, or tryptophan.

The most interesting enzyme within this series of mutants is H257Y. The values of k_{cat} and k_{cat}/K_a for the S_P-enantiomers for the six chiral organophosphates were all reduced, relative to those of the wild type enzyme, with this mutation to the active site. The largest reductions were observed for those compounds containing a single phenyl substituent. For example, the k_{cat} for the S_P-isomer of **II** was reduced to 73% of the wild type value, but the k_{cat} for the S_P-isomer of **IV** was reduced to 3% of the wild type value. The other 11 mutants constructed within the large subsite of PTE were less effective in the selective reduction of the kinetic constants for the initially faster S_P-enantiomer while maintaining the basal rate for the initially slower R_P-enantiomer. Therefore, the H257Y mutation at the *large* subsite was combined with the mutations previously made within the

small subsite in the rational search for novel proteins where the stereoselectivity was the opposite of that of the wild type enzyme.

Simultaneous Enlargement of the Small Subsite and Shrinking of the Large Subsite. It has been demonstrated that enlargement of the *small* subsite with the substitution of glycine and/or alanine for Ile-106, Phe-132, and Ser-308 results in the significant improvement in the rate of hydrolysis for most of the slower R_P-enantiomers of the substrate library. However, the mutations made to the *small* subsite had much smaller effects on the rate of hydrolysis for the initially faster S_P-enantiomers. In contrast, reduction in the size of the *large* subsite with the mutant H257Y resulted in the diminution in the kinetic parameters for most of the faster S_P-enantiomers but relatively smaller effects on the kinetic parameters for the initially slower R_P-enantiomers. These results indicated that it should be possible to create variants of the native PTE that could reverse the stereoselectivity by modulation of the sizes of the *large* and *small* subsites simultaneously, if the effects at the individual sites were additive.

A total of 11 mutants were constructed in an attempt to reshape the structure of the *small* and *large* subsites simultaneously. Mutant enzymes were identified for the reversal of each pair of stereoisomers with the single exception of ethyl isopropyl *p*-nitrophenyl phosphate (**VI**). The most dramatic example is the case for the two stereoisomers of substrate **IX**. The wild type enzyme prefers the S_P-isomer by a factor of 35, while the mutant I106G/H257Y/S308G prefers the R_P-stereoisomer by a factor of 460! The enhancements in the rate of hydrolysis for the R_P-enantiomers caused by these mutants are very similar to those observed with the glycine and alanine mutants of Ile-106, Phe-132, and Ser-308 that only enlarge the *small* subsite. Comparatively, the observed reductions in the kinetic parameters for the S_P-enantiomers seen with I106G/H257Y, I106G/F132G/H257Y, I106G/H257Y/S308G, and I106G/F132G/H257Y/S308G were greater than those seen with I106A/H257Y, F132A/H257Y, I106A/F132A/H257Y, I106A/H257Y/S308A, and F132A/H257Y/S308A. These results suggest that the removal of the side chains of Ile-106, Phe-132, and Ser-308 may permit additional orientations of the bound substrate within the active site of PTE. The increased steric constraints within the *large* subsite and the addition of nonproductive complexes may also account for the significant reductions in the kinetic constants for the S_P-enantiomers from the substrate library with the mutants containing glycine substitutions for Ile-106, Phe-132, and Ser-308.

Summary. The investigation of the enantiomeric selectivity of phosphotriesterase toward chiral organophosphorus compounds is of considerable practical significance. A variety of toxic pesticides and chemical warfare agents are phosphorus compounds that contain a chiral phosphorus center. Previous studies have shown that the more toxic isomers of these acetylcholinesterase inactivators are the poorer substrates of the wild type PTE (*4*). The current investigation has clearly demonstrated that the reactivity and stereoselectivity of PTE can be *enhanced*, *relaxed*, or *reversed* by the rational evolution of active site residues. The enhancement and reversal of the stereoselectivity should make it possible to utilize variants of PTE for the kinetic resolution of racemic mixtures of chiral organophosphates and obtain either isomer with a substantial enantiomeric excess. The relaxation of the stereoselectivity is desired for bioremediation when catalysts are needed to efficiently detoxify hazardous pesticides and chemical warfare agents. The overall success of this effort, directed at the modulation of the kinetic properties of the wild type enzyme, is graphically presented in Figure 2. The relative kinetic parameters for the six pairs of chiral organophosphate substrates with the wild type enzyme and the best mutant enzyme, where the relative kinetic parameters have been *enhanced*, *relaxed*, or *reversed*, are provided. Enhancements of up to 3 orders of magnitude in the stereoselectivity for the preferred S_P-enantiomer have been achieved by the mutant G60A for all of the substrates that were tested. Multiple mutations within the active site have led to the complete reversal in the original chiral selectivity. These results suggest that further mutations within the active site could be engineered to accommodate nearly any organophosphate.[3]

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[3] Some of the mutants created for this investigation have been tested as catalysts for the hydrolysis of the military type organophosphonate nerve agents. These preliminary experiments were conducted in collaboration with Drs. Steven P. Harvey and Joseph J. DeFrank of the U.S. Army Edgewood Chemical and Biological Center. The mutant I106A/H257Y was found to hydrolyze the racemic mixture of sarin (also known as GB) with a maximal velocity of ~1600 μmol min^{-1} mg^{-1}. This rate is approximately 4 times faster than that of the wild type enzyme and apparently the highest rate of turnover for sarin ever measured. The mutant G60A was found to hydrolyze the racemic mixture of soman (also known as GD) with a maximal velocity of ~19 μmol min^{-1} mg^{-1}. This rate of turnover is greater than 3 times faster than that of wild type PTE. The H254A mutant hydrolyzed the nerve agent VX 11-fold faster than the wild type enzyme. Experiments are currently in progress to determine the stereoselective properties of these mutants with the individual stereoisomers of these organophosphonates and closely related analogues.