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Kinetic Evolution to the Catalytic Core of the Bacterial Phosphotriesterase

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1 AMIDOHYDROLASE SUPERFAMILY

The amidohydrolase superfamily is a related group of enzymes that catalyze the hydrolysis of bonds to carbonyl and phosphoryl centers. The most prominent members of this family of proteins include urease (URE), phosphotriesterase (PTE), adenosine deaminase (ADA), dihydroorotase (DHO), and atrazine chlorohydrolase (1). The reactions catalyzed by some of these enzymes are illustrated in Sch. 1. Structurally, all of these proteins have been shown to fold into a typical $(\beta\alpha)_8$-barrel motif, although the level of overall sequence identity is rather low. The hallmark for this family of enzymes is a cluster of four histidine residues that come together in three-dimensional space to form a highly structured binding site for divalent metal ions (2–4). The most common arrangement is for a binuclear metal center, as observed in the x-ray crystal structures of URE, PTE, DHO, and the phosphotriesterase homology protein (PHP), although a mononuclear metal binding site has been observed with ADA (5). Within the binuclear metal ion clusters, there

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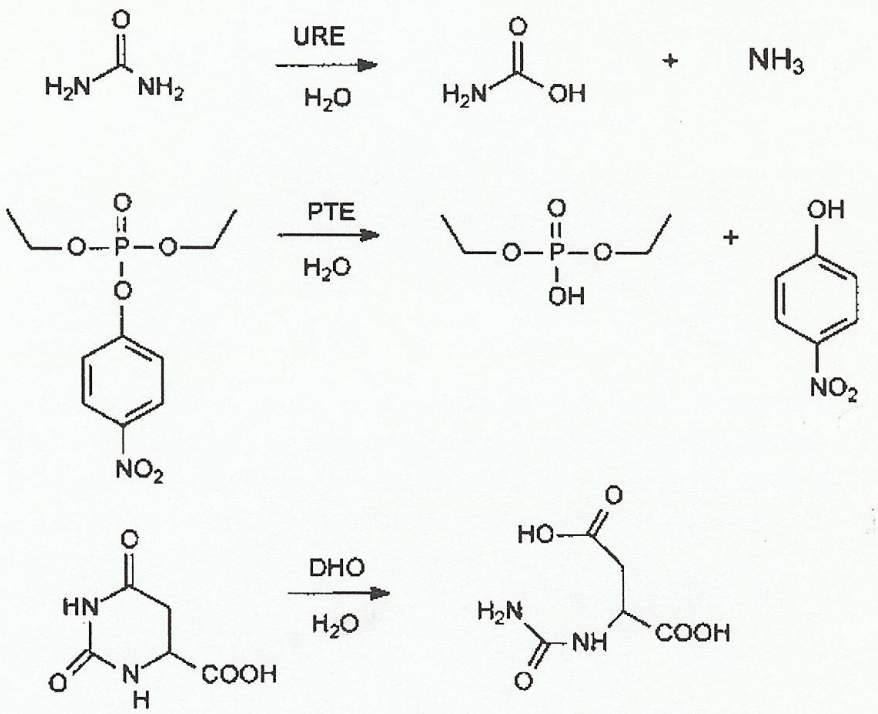

Scheme 1 Reactions catalyzed by members of the amidohydrolase super-family.

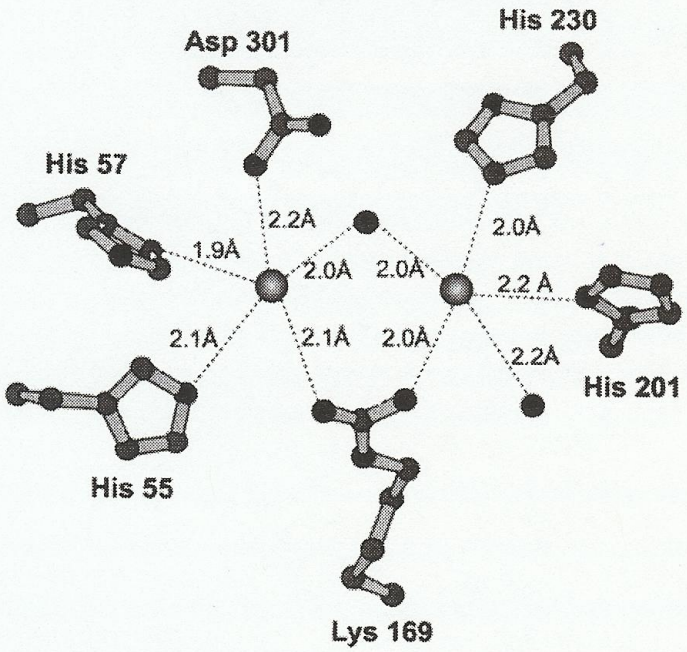

Figure 1 Representation of the binuclear metal center within the active site of phosphotriesterase. (From Ref. 10.)

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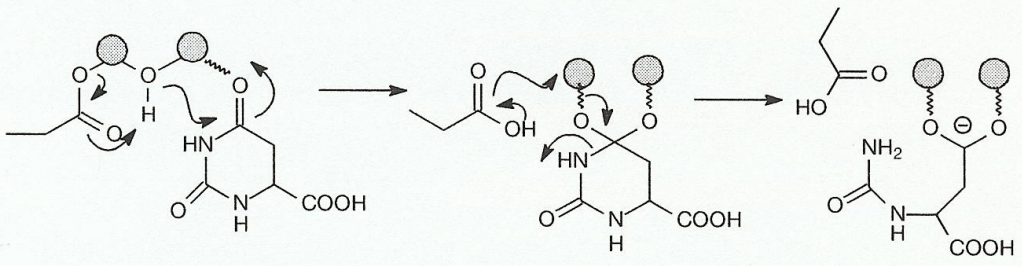
are always two ligands that bridge the two metal ions: a hydroxide from solvent and a carboxylate group. In the case of URE, PTE, and DHO, the bridging carboxylate originates from the side chain of a conserved lysine residue that has reacted with CO_2 to form a carbamate functional group (2–4). In PHP, the bridging group is contributed from the side chain of a glutamate residue (6). A cartoon of the binuclear metal center in PTE is shown in Fig. 1.

2 CHEMICAL MECHANISM

The apparent role of the metal centers within the active sites of these enzymes is to activate the hydrolytic water molecule and substrate for nucleophilic attack. The actual chemical transformation is best understood in the reaction catalyzed by DHO because an x-ray crystal structure was determined with the substrate and product bound to separate monomers within the dimeric protein (4). The proposed reaction mechanism is summarized in Sch. 2 for the hydrolytic cleavage of dihydroorotate. In this chemical mechanism, the binding of dihydroorotate to the active site polarizes the carbonyl group via ligation to the β-metal ion. This binding interaction weakens the coordination of the bridging hydroxide to the β-metal (as evidenced by the longer bond to the β-metal ion relative to the α-metal ion). The hydroxide attacks the polarized carbonyl group, with assistance from Asp250, to form a tetrahedral adduct that now bridges the two divalent cations. Proton transfer from the protonated form of Asp250 to the incipient amide nitrogen initiates the collapse of this intermediate. Carbamoyl aspartate departs the active site and the binuclear metal center is recharged with a hydroxide ion from solvent. Similar mechanisms have been proposed for other members of the amidohydrolase superfamily.

It would appear that this family of enzymes has evolved as a "delivery device" for the nucleophilic attack of hydroxide on target substrates. The architecture for the metal center has remained remarkably intact, but the individual active sites have been tailored through molecular evolution to



Scheme 2 Catalytic reaction mechanism for dihydroorotase.

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recognize a specific set of substrates and associated functional groups for binding and chemical cleavage. Therefore, the amidohydrolase superfamily of enzymes offered a rather attractive target with which to test the limits for a rational reconstruction of an active site. Modulation of the substrate and stereoselectivity of PTE through site-directed mutagenesis were utilized as a stringent test of this proposition.

3 PHOSPHOTRIESTERASE

A bacterial version of phosphotriesterase (aka organophosphate hydrolase or OPH) has been discovered in strains of *Pseudomonas* and *Flavobacterium* (7,8). The *Flavobacterium* isolate was originally identified from a rice patty in the Philippines where bacterial soil samples had been tested for their ability to hydrolyze specific organophosphate insecticides (7). The gene responsible for the coding of the enzyme was identified, cloned, and overexpressed in *Escherichia coli*, and the protein was purified to homogeneity (9). The three-dimensional x-ray structure of PTE has been determined by the Holden laboratory to very high resolution (10).

4 REACTION MECHANISM

Bacterial PTE hydrolyzes a variety of organophosphate triesters of the type shown in Sch. 1 using the insecticide, paraoxon, as an example. The substrate specificity is such that the substituent that functions as the leaving group is very much dependent on the pK_a and, thus, with paraoxon, only the *p*-nitrophenol group is cleaved from the phosphorus center (11). The enzyme does not hydrolyze diesters at an appreciable rate and thus only a single substituent is subjected to cleavage (12). The substrate specificity of the native enzyme is reasonably broad in that the phosphoryl oxygen can be substituted with sulfur and the other three substituents can be replaced with a variety of other groups (13).

The native bacterial phosphotriesterase served as an ideal candidate for the directed reconstruction of a substrate-binding site. The breadth of substrates recognized by the amidohydrolase superfamily of enzymes convinced us that the structural fold of the $(\beta\alpha)_8$ barrel could accommodate a variety of perturbations to the specific interactions between proteins and substrates. Moreover, the binuclear metal center within these proteins demonstrated that hydroxide could be delivered to a variety of trigonal and tetrahedral reaction centers. The construction of mutant variants of PTE would be quite useful in the detoxification and detection of chemical warfare agents and agricultural insecticides. Our initial objective was to first identify the structural determinants of substrate specificity for wild-type protein and then to

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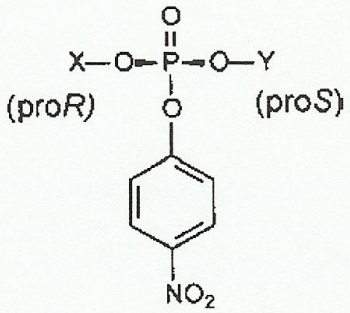

Scheme 3 Generic substrate for phosphotriesterase.

utilize this information to construct mutant forms of PTE where substrate specificity would be enhanced for specific substrates.

The initial substrate library was a series of organophosphate triesters bearing a *p*-nitrophenol leaving group of the type presented in Sch. 3. The *p*-nitrophenyl group was chosen because of the ease with which the kinetics of hydrolysis could be monitored spectrophotometrically. The substituents **X** and **Y** could be varied with large and small alkyl groups through straightfor-

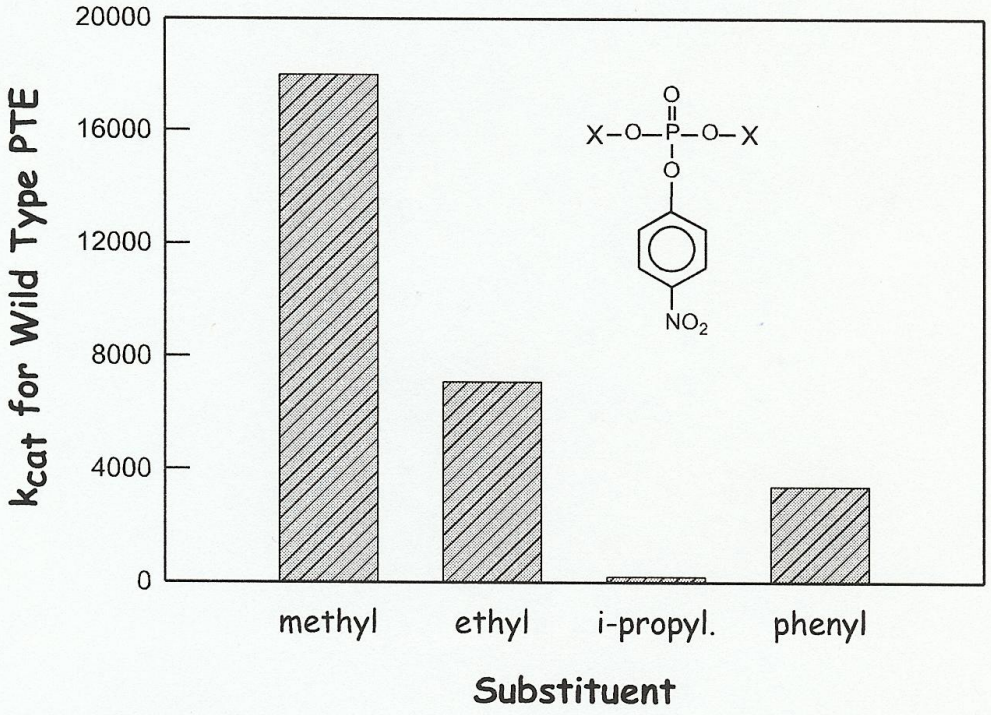

Figure 2 Relative values for k_{cat} for the wild-type phosphotriesterase with achiral substrate analogs. (From Ref. 11.)

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ward synthetic procedures, and chiral substrates could be constructed of either stereochemistry. Altogether, 16 such substrates were prepared using all possible combinations of *methyl*, *ethyl*, *isopropyl*, and *phenyl* groups. Shown in Fig. 2 are the relative k_{cat} values for the four possible achiral variants of the target substrate (**Y** and **X** are the same substituents). These studies demonstrated that the wild-type protein accepted any of the four substituents in either the pro*S* of pro*R* position, but that not all of these substituents were accommodated in the same way by the protein (10).

Kinetic assays of the six pairs of racemic mixtures demonstrated that the wild-type enzyme exhibited a distinct preference for one stereoisomer over the other, as shown in Fig. 3. In every case, except for the pair of *methyl* and *ethyl*, there was a > 20-fold preference for one isomer over the other and this catalytic preference rose to about 100:1 for the methyl phenyl substrate. Kinetic assays with the individual enantiomers demonstrated that the S_P-stereoisomer, in every case examined, was preferred over the R_P-enantiomer for this series of chiral substrates. If this preference is defined in terms of steric

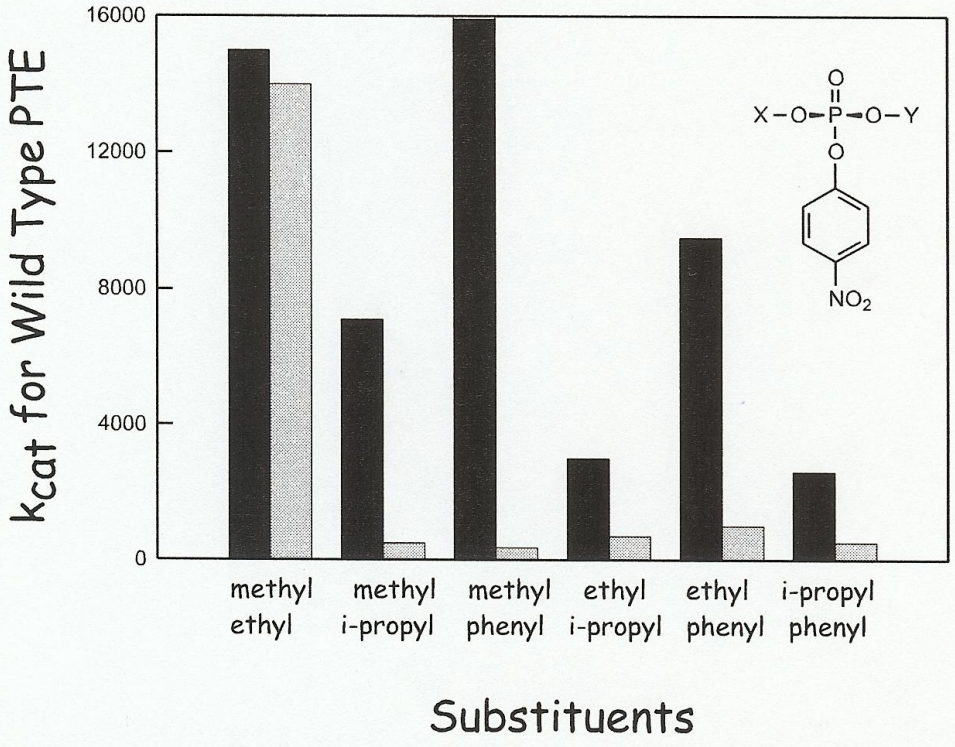

Figure 3 Relative values for k_{cat} for the wild-type phosphotriesterase with chiral substrate analogs. The values for S_P enantiomers are shown in black whereas the R_P enantiomers are shown in gray. (From Refs. 16,17.)

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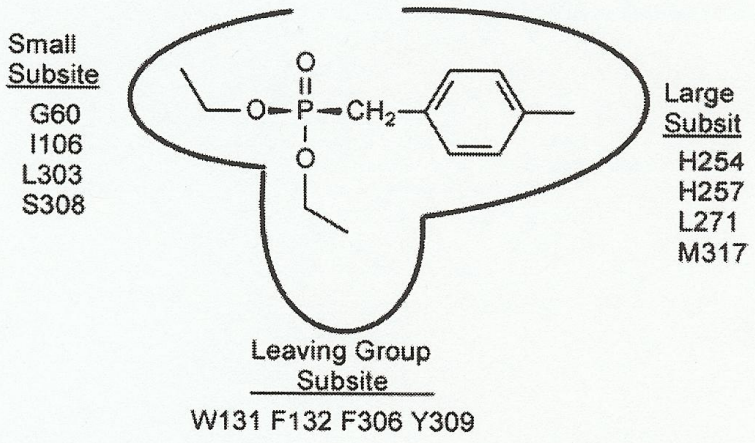
bulk, then the "large" substituent is preferred in the pro*S* position whereas the "smaller" substituent is preferred in the pro*R* position, as illustrated in Sch. 3.

5 KINETIC ENGINEERING OBJECTIVES

Our immediate goals for the mutagenesis of PTE were focused on a rational rearrangement of the active site binding cavity such that the inherent stereoselectivity using the aforementioned library of 16 model substrates could be manipulated. Thus, we were interested in *enhancing* the stereoselectivity possessed by the wild-type enzyme such that the catalytic preference for the S_P isomer would be even more pronounced. The construction of mutants of this type would be quite useful, in a practical sense, for the kinetic resolution of racemic mixtures through the hydrolysis of a single stereoisomer while leaving the other enantiomer intact (14). Second, we were interested in *relaxing* the stereoselectivity of the wild-type enzyme. The goal here was to make the initially slower R_P isomer as fast as the S_P isomer (rather than making the S_P isomer as slow as the R_P isomer). Such mutants would be appropriate for the detoxification of racemic mixtures of organophosphate triesters where both isomers are toxic. Mutants of this type would also be useful as an initial stepping stone for the final objective, which was to create mutants where the stereoselective preference was *reversed.* With such mutants, the R_P isomer would be hydrolyzed in preference to the S_P isomer. In order to accomplish this goal, the S_P isomers must be made poorer substrates while, simultaneously, the R_P isomers must be made much better.



Scheme 4 Cartoon of the substrate binding pocket for phosphotriesterase.

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To accomplish these objectives, we set out to retool the active site in a semirational manner. Single-site mutants were constructed sequentially and then specific mutations were combined with one another to achieve the desired effect. Our starting premise for this endeavor was based on the assumption that alterations to substrate specificity could be accommodated by the expansion and contraction of the individual subsites for each of the three substituents attached to the phosphorus core. A cartoon showing these three subsites is illustrated in Sch. 4. An additional assumption for this endeavor was that only one of these three subsites would be properly oriented for the expulsion of the leaving group. Therefore, the remaining two subsites would define the substrate and sterospecificity for PTE. The most obvious problem here is that substrate binding can occur in any one of the three possible orientations and thus there is the potential for nonproductive binding.

6 IDENTIFICATION OF SUBSITES

In order to identify those amino acid side chains that came together in three-dimensional space to form the individual subsites for the substrate, Vanhooke et al. (15) (University of Wisconsin) solved the structure of PTE bound to the nonhydrolizable substrate analog shown in Sch. 4. From the x-ray structure, it was concluded that the pro*S* ethoxy group was oriented in what we defined as the *leaving group* pocket. The remaining two substituents (*methylbenzyl*) and the pro*R* ethoxy group were oriented within the *large* and *small* pockets, respectively. The designation for the *large* and *small* pockets was defined to acknowledge the stereoselective preference exhibited by the wild-type enzyme for the initial library of organophosphate esters (13). The residues that surrounded the leaving group pocket included W131, F132, F306, and Y309, whereas those that comprised the large pocket included H254, H257, L271, and M317. The small pocket was defined by the side chains of G60, I106, L303, and S308. However, it should be noted that many of these residues are actually localized between these subsites, and thus the assignments are in some way rather arbitrary (15).

7 CONTRACTION OF SMALL SUBSITES

In order to construct mutants of PTE that were more stereoselective than the wild-type enzyme, we anticipated that the *small* subsite would have to be reduced in size. This reduction in the cavity size for the *small* subsite would likely obstruct or impair the positioning of substrates with bulky groups that would be required to bind within this region of the active site. Of the four residues that were probed in this manner, the mutation of Gly60 to an alanine proved to be the most effective. Shown in Fig. 4 is a direct comparison of

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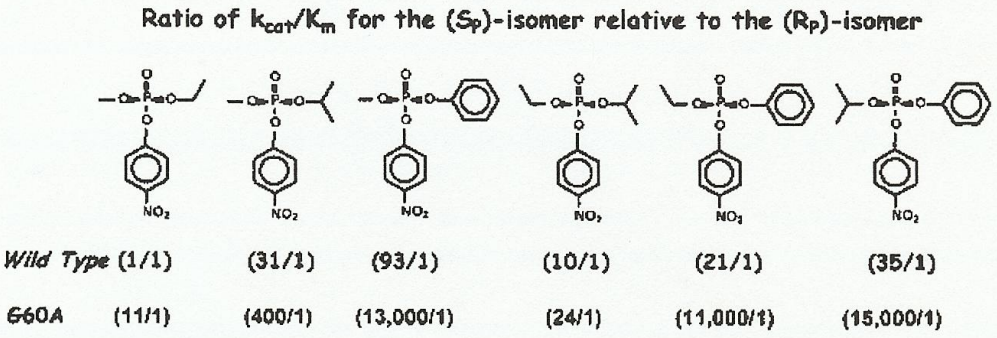

Figure 4 Ratios of k_{cat}/K_m for chiral substrate analogs with the wild-type and *G60A* mutant of phosphotriesterase. The ratios are given for S_P/R_P. (From Ref. 16.)

the stereoselectivity (ratio of k_{cat}/K_m values for the S_P and R_P isomers) for the wild-type and *G60A* mutant (16). The results are extraordinary considering that only a single $-CH_2-$ group has been added to a sea of nearly 2000 carbon atoms. In every case, the *G60A* mutant is considerably more stereoselective than the wild-type enzyme. For example, the S_P isomer of methyl ethyl *p*-nitrophenyl phosphate is hydrolyzed 10 times faster than the R_P isomer, where no difference in the rate of hydrolysis for these enantiomers was observed with the wild-type enzyme. Moreover, the ratio of k_{cat}/K_m values for the two enantiomers of methyl phenyl *p*-nitrophenyl phosphate increased from 20:1 to 10,000:1 with the mutant *G60A*. This mutant has proven to be quite effective in the kinetic resolution of organophosphate triesters (14). Gram quantities of single R_P isomers with *ee* values $>98\%$ have been obtained in a few minutes with this enzyme.

8 RELAXATION OF STEREOSELECTIVITY

In order to relax the stereoselectivity of the wild-type PTE, our approach was to enlarge the cavity space of the small subsite by mutation of residues within this site to either alanine or glycine. A simple alanine scan of residues C59, G60, S61, I106, W131, F132, H254, H257, L271, L303, F306, S308, Y309, and M317 showed that a significant increase in the overall rate of hydrolysis of the initially slower R_P isomers could be realized when some of these residues are changed to alanine (16). In general, the initially slower R_P isomer has gotten faster in every case. Those residues that had the greatest overall impact in the improvement of the rate for the initially slower R_P isomers were found to be I106, F132, and S308 (16). Further improvements in the relaxation of stereoselectivity were achieved by the construction of glycine mutants at the critical residue positions and through the combination of multiple alanine or glycine mutants at selected residue positions (17).

9 REVERSAL IN SPECIFICITY

In order to *reverse* the stereoselectivity inherent within the wild-type PTE, two adjustments to the active site needed to be accomplished simultaneously. The *small* subsite must be expanded whereas the *large* subsite must be shrunk in size. The constriction of the large subsite was initiated in an attempt to make it more difficult for the larger and bulkier groups to properly fit within this portion of the active site. If effective, this would reduce the rate of hydrolysis of the S_P stereoisomers, relative to the values exhibited by the wild-type enzyme. The overall dimensional space of the large subsite was reduced by replacing H254, H257, L271, and M317 with the larger aromatic residues tyrosine, phenylalanine, or tryptophan. Shown in Fig. 5 are the effects of these mutations on the k_{cat} values for the hydrolysis of methyl phenyl *p*-nitrophenyl phosphate. The k_{cat} value has been reduced from a value that exceeds 40,000 s^{-1} for the wild-type enzyme to a value that is about 200 s^{-1} for the H254F mutant. Overall, the most interesting mutant within this series of modified enzymes was *H257Y* (17). The kinetic constants for the S_P isomers for the six chiral organophosphates were all reduced, relative to those of the wild-type enzyme. The largest reductions were observed for those compounds containing a single phenyl substituent. Therefore, the *H254Y* mutation at the *large* subsite was combined with the mutations previously

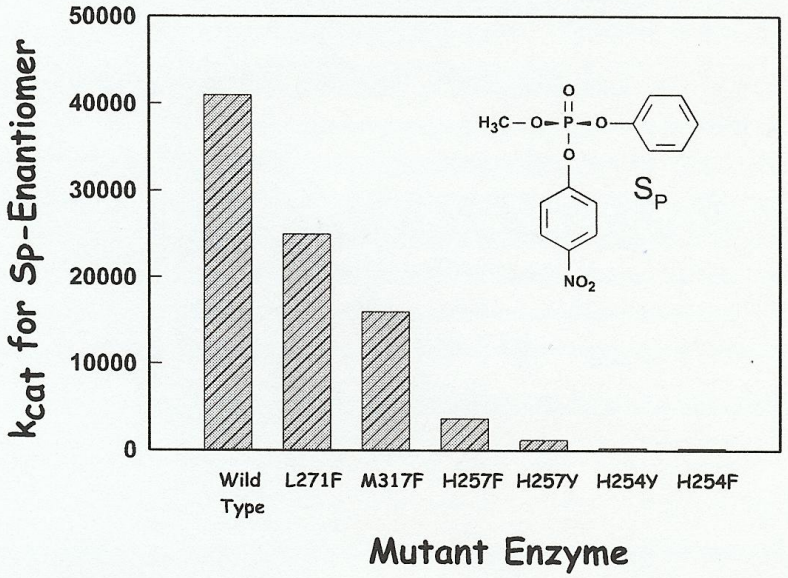

Figure 5 Diminution in the value of k_{cat} for the S_P enantiomer of methyl phenyl *p*-nitrophenyl phosphate when the indicated residues within the large subsite of phosphotriesterase are mutated. (From Ref. 17.)

made within the *small* subsite in the rational search for novel proteins where the stereoselectivity was the opposite to that of the wild-type enzyme.

We had demonstrated that enlargement of the *small* subsite with the substitution of glycine and/or alanine residues for I106, F132, and S308 resulted in significant improvements in the rates of hydrolysis for most of the initially slower R_P enantiomers of the substrate library. However, the mutations made to the *small* subsite had much smaller effects on the rates of hydrolysis for the initially faster S_P enantiomers. In contrast, reduction in the size of the *large* subsite with the mutant *H254Y* 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 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 (17). Mutant enzymes were identified for the reversal of each pair of stereoisomers, with the single exception of ethyl isopropyl *p*-nitrophenyl phosphate. The most dramatic example is the case for the two stereoisomers of the substrate, isopropyl phenyl *p*-nitrophenyl phosphate. The wild-type enzyme prefers the S_P isomer by a factor of 35 whereas the mutant *I106G/H254Y/S308G* prefers the R_P stereoisomer by a factor of 460. The enhancements in the rates of hydrolysis for the R_P isomers caused by these mutants were very similar to those observed with the glycine and alanine mutants of I106, F132, and S308 that only enlarged the *small* subsite.

10 SUMMARY

The investigation of the enantiomeric selectivity of PTE is of considerable practical significance. A variety of toxic pesticides and chemical warfare agents are phosphorus compounds that contain a chiral phosphorus center (18). Previous studies have shown that the more toxic isomers of these acetyl cholinesterase inactivators are the poorer substrates of the wild-type PTE (19). This study has clearly demonstrated that the reactivity and stereoselectivity of PTE can be *enhanced*, *relaxed*, or *reversed* by the rational evolution of specific active site residues. The *enhancement* and *reversal* of stereoselectivity have made it possible to utilize variants of PTE for the kinetic resolution of racemic mixtures of chiral organophosphates and to obtain either isomer with substantial enantiomeric excess (14). The relaxation of stereoselectivty is desired for bioremediation when catalysts are needed to efficiently detoxify hazardous pesticides and chemical warfare agents. The

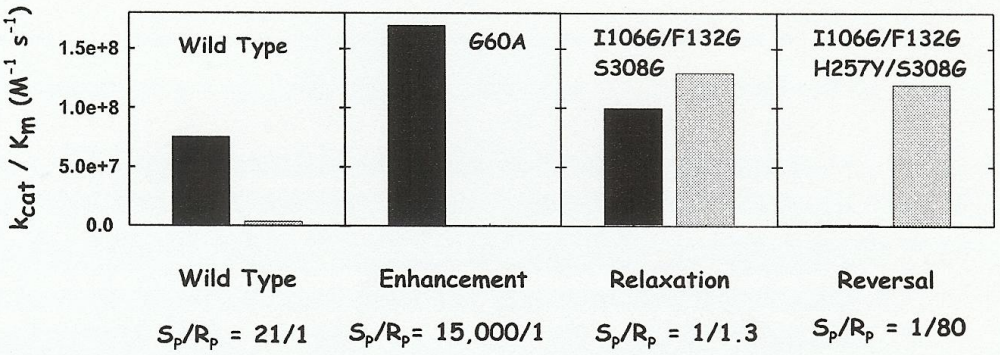

Figure 6 Manipulation of the stereoselectivity of phosphotriesterase for the chiral forms of ethyl phenyl *p*-nitrophenyl phosphate. The ratios of k_{cat}/K_m for the wild-type and selected mutants where the stereoselectivity has been *enhanced* (*G60A*), *relaxed* (*I106G/F132G/S308G*), and *reversed* (*I106G/F132G/H257Y/S308G*) are presented.

overall success of this effort, directed at the modulation of the kinetic properties of the wild-type enzyme, is graphically presented in Fig. 6. The relative kinetic parameters for the kinetic parameters for ethyl phenyl *p*-nitrophenylphosphate with the wild-type enzyme and the best mutant enzyme, where the relative kinetic parameters have been enhanced, relaxed, or reversed, are provided. Enhancements in stereoselectivity for the preferred S_P enantiomer up to three orders of magnitude have been achieved by the mutant *G60A* for all substrates tested. Multiple mutations within the active site have led to a complete reversal of the original chiral selectivity. These results suggest that further mutations within the active site could be engineered to accommodate nearly any organophosphate.

ACKNOWLEDGMENTS

Financial support for this project has been obtained from the National Institutes of Health, Office of Naval Research, and the Advanced Technology Program from the state of Texas.

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