Communications to the Editor

Rationally Engineered Mutants of Phosphotriesterase for Preparative Scale Isolation of Chiral Organophosphates

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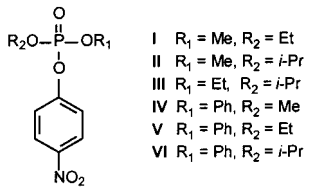
There are important national security issues and considerable commercial interests associated with organophosphorus compounds because of their demonstrated value as agricultural insecticides and their perceived threat as chemical warfare nerve agents. The toxicological properties of this class of compounds are due specifically to the inactivation of the enzyme acetylcholinesterase and the subsequent loss of nerve function.[1] The biological profiles for some organophosphates are very much dependent on the chirality of the tetrahedral phosphorus center.[2] Therefore, there is particular interest in the efficient preparation of pure enantiomeric organophosphorus compounds that can be utilized as potential enzyme substrates, potent irreversible inhibitors, and organometallic catalysts. However, the lack of chiral organophosphorus compounds found as natural products has hampered the development of general chemical methods for the stereospecific syntheses of these compounds. Nevertheless, the preparation of chiral organophosphate triesters has been documented.[3] However, these methods require the stereospecific alcoholysis of an optically active derivative or the employment of a chiral resolving ligand. Moreover, these protocols incorporate lengthy chemical procedures and/or the very tedious chiral separation of diastereomeric intermediates.

Herein we report the first stereoselective preparation of chiral organophosphate triesters through the kinetic resolution of racemic mixtures via the hydrolysis of a single enantiomer by the bacterial phosphotriesterase (PTE). The phosphotriesterase from *Pseudomonas diminuta* is a zinc-containing enzyme that catalyzes the hydrolysis of a variety of organophosphorus compounds, which includes an array of insecticides and chemical nerve agents.[4] The wild type PTE is catalytically efficient and is reasonably stereoselective during the hydrolysis of racemic organophosphate triesters. For example, the value of k_{cat} for the hydrolysis of the insecticide paraoxon approaches 10^4 s^{-1} while the k_{cat}/K_m is nearly 10^8 M^{-1} s^{-1}.[5] Moreover, the reported k_{cat}/K_m values for the series of organophosphates shown in Scheme 1 are 1- to 90-fold greater for the S_P-isomers than for the corresponding R_P-isomers.[6] PTE

Scheme 1



will catalyze the hydrolysis of the most acidic phenolic substituent from an organophosphate triester. The overall rate of hydrolysis is very much dependent on the pK_a of the leaving group phenol.[4b]

To *enhance* the substrate stereoselectivity exhibited by the wild-type enzyme, we designed and characterized several site-directed mutants of PTE. Three regions within the active site of PTE (*small*, *large*, and *leaving group*) have been identified that interact with the three substituents attached to the phosphorus center of typical substrates.[7] These subsites were graphically localized using the X-ray crystal structure of PTE in the presence of a bound substrate analogue. The relative sizes of these binding subsites appear to play the dominant roles in establishing the stereoselective properties of the wild-type PTE.[8] Thus, when the *small* subsite is reduced in size by mutating Gly60 to an alanine residue, the observed stereoselectivity of the mutant enzyme is dramatically *enhanced* toward the hydrolysis of the S_P-isomer. For the racemic pairs of substrates **I**, **II**, and **III**, the k_{cat}/K_m values are 10- to 400-fold greater for the S_P-isomers than for the corresponding R_P-isomers and 10 000- to 15 000-fold greater for the substrates **IV**, **V**, and **VI**.

Similarly, a *reversal* in the stereoselectivity is achieved upon the expansion of the *small* subsite coupled with a simultaneous contraction within the *large* subsite. The *large* subsite was reduced in size by mutation of His257 to tyrosine or tryptophan while the *small* subsite was enlarged by the double substitution of Phe132 and Ile106 with either glycine or alanine.[9] The mutant I106G/F132G/H257Y hydrolyzes the R_P-isomers of **IV**, **V**, and **VI** 50- to 100-fold faster than the corresponding S_P-isomers. Furthermore, the mutant I106A/F132A/H257W hydrolyzes the R_P-isomer of **II** significantly faster than the S_P-isomer.

A graphical illustration of the opposing stereoselectivity exhibited by the mutants G60A and I106A/F132A/H257W for the hydrolysis of a racemic mixture of compound **II** is presented in Figure 1. When G60A is added to the racemic mixture, only one-half of the total organophosphate present in solution is hydrolyzed enzymatically. The remaining isomer is rapidly hydrolyzed only after the addition of I106A/F132A/H257W.

The individual R_P- and S_P-isomers of the organophosphate triesters **I**−**VI** were synthesized and isolated on a preparative scale through an enzymatic kinetic resolution of the corresponding racemic mixtures. The racemic organophosphate triesters[10] **I**−**VI** were dissolved in 100 mM CHES buffer (pH 9.0) containing

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(4) For notable examples, see the following. (a) Lewis, V. E.; Donarski, W. J.; Wild, J. R.; Raushel, F. M. *Biochemistry* **1988**, *27*, 1591. (b) Hong, H.-B.; Raushel, F. M. *Biochemistry* **1996**, *35*, 10904. (c) Shim, H.; Hong, S.-B.; Raushel, F. M. *J. Biol. Chem.* **1998**, *273*, 17445. (d) Raushel, F. M.; Holden, H. M. *Adv. Enzymol. Relat. Areas Mol. Biol.* **2000**, *74*, 51.

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(7) Vanhooke, J. L.; Benning, M. M.; Raushel, F. M.; Holden, H. M. *Biochemistry* **1996**, *35*, 6020. The PTE binding pockets have been designated as the *small*, *large*, and *leaving group* subsites. The *small* subsite is defined by the side chains of Gly60, Ile106, Leu303, and Ser308. The pocket for the *large* subsite is lined with residue His254, His257, Leu271, and Met317. The residues that are located around the *leaving group* subsite are Trp131, Phe132, Phe306, and Try309.

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(9) Chen-Goodspeed, M.; Sogorb, M. A.; Wu, F.; Raushel, F. M. *Biochemistry.* Submitted for publication.

(10) Steurbaut, W.; DeKimpe, N.; Schreyen, L.; Dejonckheere, W. *Bull. Soc. Chim. Belg.* **1975**, 84, 791.

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Table 1. Enzymatic Resolution of Organophosphate Triesters by Mutants of Phosphotriesterase

compd	phosphotriesterase mutant	concn (mM)	enzyme (nM)	solvent (mL)	CH_3CN (%)	time (min)	yield (%)	config	ee[a,b] (%)	turnovers/ enzyme
I	G60A	10.2	0.8	400	5	15	71	R_P	99[a]	6.4×10^6
II	G60A	11.6	1.6	400	10	15	91	R_P	95[a]	3.6×10^6
II	I106A/F132A/H257W	15.8	7.7	300	15	15	84	S_P	99[a]	1.0×10^6
III	G60A	10.7	1.6	400	15	30	92	R_P	98[a]	3.3×10^6
IV	G60A	7.1	3.1	400	20	45	95	R_P	98[b]	1.1×10^6
IV	I106G/F132G/H257Y	6.5	8.9	400	20	30	62	S_P	98[b]	3.6×10^5
V	G60A	8.7	2.3	400	20	70	93	R_P	98[b]	1.9×10^6
V	I106G/F132G/H257Y	9.0	5.9	400	20	35	67	S_P	96[b]	7.6×10^5
VI	G60A	8.0	2.6	600	20	40	99	R_P	98[b]	1.5×10^6
VI	I106G/F132G/H257Y	10.8	10.4	400	20	40	97	S_P	96[b]	5.2×10^5

[a] Enantiopurity was determined by capillary electrophoresis. [b] Determined by HPLC analysis with a chiral column, (*R*,*R*)-Welk-01. For compounds **IV** and **V** the eluent was hexane:dichloromethane:95% ethanol = 104:8:1 and for compound **VI** the eluent was hexane:dichloromethane:95% ethanol = 173:12:1. The flow rate was 1.0 mL/min and the elution profile was monitored at 280 nm. The structures of all compounds were confirmed by ^1H, ^{13}C, and ^{31}P NMR spectroscopy.

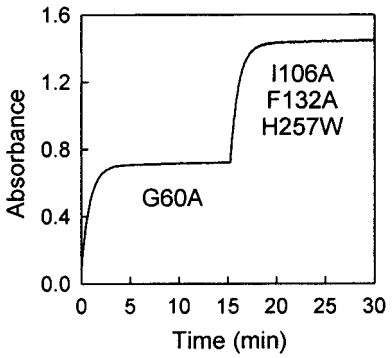

Figure 1. Time course for the consecutive hydrolysis of racemic **II** by G60A and I106A/F132A/H257W at pH 9.0 and 25 °C. The reaction was monitored at 400 nm.

acetonitrile (5−20%) and 100 μM Co^{2+}. The reactions were initiated by the addition of the appropriate mutant enzyme (G60A, I106G/F132G/H257Y, or I106A/F132A/H257W). The solution was stirred at room temperature while the reaction was monitored spectrophotometrically for the release of *p*-nitrophenol. When the reaction was half-complete the reaction was quenched by extraction of the solution with chloroform. The combined organic layers were washed with dilute $NaHCO_3$ solution until the aqueous layer was colorless, and then washed with brine. The organic solution was dried over anhydrous $MgSO_4$, filtered, and concentrated in a vacuum to provide the unhydrolyzed enantiomer in excellent yield (62−99%) with a high enantiomeric excess (95−99% ee).[11]

(11) Zhu, W.; Wu, F.; Raushel, F. M.; Vigh, G. *J. Chromatog.* **2000**, in press.

The general scope of the resolving power of the engineered mutants of PTE for the preparation of enantiomerically pure organophosphates is summarized in Table 1. For compounds **II**, **IV**, **V**, and **VI** either enantiomer can be prepared with the simple selection of the appropriate mutant form of PTE. However, we have thus far been unable to create a mutant that is able to more rapidly hydrolyze the R_P-enantiomer of compounds **I** or **III** faster than the corresponding S_P-enantiomer.

In conclusion, we have documented a new strategy for the efficient enantioselective synthesis of organophosphate triesters by using engineered mutants of phosphotriesterase to kinetically resolve racemic mixtures. Rational manipulation of the active site structure has allowed us to construct mutants where the stereoselectively of the native enzyme has been either *enhanced* or *reversed* through a very small number of amino acid changes to PTE. The yields, enantiomeric purity, and turnover numbers are superior to the previously reported chemical approaches for the synthesis of this class of chiral compounds. The reaction times are inversely proportional to the amount of enzyme added. For the studies reported here the minimum reaction time desired was approximately 15 min. This time interval corresponded to about a million turnovers for each enzyme molecule during the hydrolysis reaction. The ease with which we have altered the substrate and stereochemical specificity of PTE suggests that further refinements to the catalytic activity are quite likely to be discovered.

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