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Stereochemical preferences for chiral substrates by the bacterial phosphotriesterase

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Abstract

The bacterial phosphotriesterase from *Pseudomonas diminuta* catalyzes the hydrolysis of organophosphate nerve agents such as paraoxon (diethyl *p*-nitrophenyl phosphate) with a turnover number of $\sim 10^4 \text{ s}^{-1}$. The active site of the enzyme has been shown to be composed of a binuclear Zn^{2+} complex with a bridging hydroxide. The utilization of chiral phosphotriesters has demonstrated that the overall hydrolytic reaction occurs with net inversion of stereochemistry at the phosphorus center. The stereochemical constraints of the active site have been probed by the synthesis and characterization of paraoxon analogs. One or both of the two ethoxy substituents of paraoxon have been replaced with various combinations of methyl, isopropyl, or phenyl groups. Racemic mixtures and individual enantiomers were tested as substrates for the phosphotriesterase. In general, the kinetic constants (k_{cat} and $k_{\text{cat}}/K_{\text{m}}$) for the (–)-enantiomers were one to two orders of magnitude greater than the (+)-enantiomer. Conversely, acetylcholinesterase was more rapidly inactivated by the (+)-enantiomers than the (–)-enantiomers. These results were examined in the context of the three-dimensional structure of the bacterial phosphotriesterase. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Phosphotriesterase; Organophosphorus substrates; Acetylcholinesterase; Detoxification

Abbreviations: AChE, acetylcholinesterase; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; PTE, phosphotriesterase.

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1. Introduction

The phosphotriesterase (PTE) from *Pseudomonas diminuta* catalyzes the hydrolysis of a variety of organophosphorus nerve agents. The enzyme has exhibited a high catalytic efficiency and potential utility in degrading organophosphorus compounds [1,2]. The k_{cat} and V/K_m values for the hydrolysis of paraoxon by the enzyme have been determined to be nearly 10^4 s^{-1} and $10^8 \text{ M}^{-1} \text{ s}^{-1}$, respectively [3]. The active site of the native enzyme consists of a binuclear metal center and the two native zinc ions can be substituted with either Co^{2+} , Cd^{2+} , Ni^{2+} , or Mn^{2+} to yield catalytically active enzyme [3]. A three-dimensional structure of the Cd^{2+} -substituted holoenzyme by X-ray crystallography has demonstrated that the binuclear metal center is embedded within a cluster of histidine residues [4]. Many of the details of the roles of the two metal ions and active site residues involved in binding and catalysis remain unknown.

The chemical mechanism for the hydrolysis reaction catalyzed by PTE is initiated by an activated water molecule that directly attacks the phosphorus center, resulting in inversion of configuration [5]. It has been shown that the hydrolysis of asymmetric organophosphorus compounds catalyzed by phosphotriesterase is stereoselective [5,6]. For example, PTE hydrolyzes only the S_p enantiomer of *O*-ethyl *O*-(4-nitrophenyl) phenylphosphonothioate (EPN). The R_p isomer of EPN is not hydrolyzed by the enzyme at a significant rate. In addition, the S_p isomers of *O,S*-dimethyl *N*-acetylphosphoramidothioate (acephate) and *O,S*-dimethyl phosphoramidothioate (methamidophos) are preferentially hydrolyzed by the enzyme.

Organophosphorus nerve agents react with acetylcholinesterase (AChE), resulting in an inactive enzyme in which the nucleophilic serine residue is phosphorylated. The (–)-(S_p) enantiomer of sarin inactivates acetylcholinesterase 4200 times faster than does the (+)-(R_p) enantiomer [7]. This implies that the chiral enantiomers of organophosphorus compounds differ greatly in their acute toxicity. Thus, the relative stereospecificities of phosphotriesterase and acetylcholinesterase would be important not only to fully understand the chiral nature of the reaction of organophosphorus nerve agents with PTE and AChE but also in the design of more effective enzymes for detoxification of organophosphates. In this paper, we have examined the stereoselective hydrolysis of chiral phosphotriesters with phosphotriesterase and the stereoselective inhibition of human recombinant acetylcholinesterase by these same chiral phosphotriesters.

2. Materials and methods

2.1. General

^1H and ^{13}C NMR spectra were recorded on a Varian XL-200E spectrometer. ^{31}P NMR spectra were obtained on a Varian Unity 300 spectrometer. Polarimetry was conducted on a Jasco DIP-360 digital polarimeter. A Gilford Model 260 spectrophotometer was used to determine reaction rates.

2.2. Chemicals

Dimethyl *p*-nitrophenyl phosphate (**I**) was obtained from Chem Service. Paraoxon (diethyl *p*-nitrophenyl phosphate) (**II**), acetylthiocholine chloride, and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma. Paraoxon analogs in which the two ethyl group were replaced with isopropyl (**III**) or phenyl groups (**IV**) were synthesized as described previously [2]. Racemic analogs (**IV–VIII**) in which one or both of the two ethyl substituents of paraoxon differ were synthesized as previously described from *p*-nitrophenyl phosphorodichloridate and the corresponding alcohols [2]. Optically active phosphotriesters (**V–VII**) were synthesized by a modification of the previously reported procedure for similar compounds [8]. The (+)-enantiomer of isopropyl phenyl *p*-nitrophenyl phosphate (**VIII**) was obtained from the enzymatic resolution of the racemic phosphotriester by means of stereospecific hydrolysis by phosphotriesterase.

2.3. Enzymes

The purification of wild-type phosphotriesterase and reconstitution of the isolated enzyme with Zn^{2+} , Co^{2+} , and Cd^{2+} have been described previously [3]. Human recombinant acetylcholinesterase was purchased from Sigma and used without further purification.

2.4. Kinetic studies

The kinetic parameters, K_m and V_{\max} , for the hydrolysis of phosphotriesters catalyzed by PTE were determined in 100 mM CHES (pH 9.0) at 25°C. Owing to limited solubility, compounds **IV**, and **VII–VIII** were tested as substrates in 20 and 5% methanol/water solution, respectively. The kinetic constants, K_m and V_{\max} , were calculated from a fit of the data to Eq. (1), where v is the initial velocity, V_m is the maximum velocity, K_m is the Michaelis constant, and A is the substrate concentration.

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

Human recombinant acetylcholinesterase was incubated with 500 nM phosphotriester in 0.1 M phosphate buffer (pH 7.0) containing 1 mg/ml BSA at 25°C. Aliquots were withdrawn and residual activity was measured with 1.0 mM acetylthiocholine and 0.3 mM DTNB in 0.1 M phosphate buffer, pH 7.0 at 25°C according to Ellman et al. [9]. The pseudo-first-order rate constants were obtained by a fit of the data to Eq. (2) where A is the concentration of the substrate, k is the pseudo-first-order constant, and t is time.

$$A = A_0 e^{-kt} \quad (2)$$

2.5. Absolute configuration

Nakayama and Thompson have demonstrated that the alcoholysis of phosphotriesters proceeds stereospecifically with inversion of configuration [10]. The (+)-isopropyl methyl *p*-nitrophenyl phosphate (**VI**) was converted into ethyl isopropyl methyl phosphate by treatment with a sodium ethoxide. Likewise, (+)-ethyl *p*-nitrophenyl phenyl phosphate (**VII**) and (+)-isopropyl *p*-nitrophenyl phenyl phosphate (**VIII**) were treated with sodium methoxide to yield ethyl methyl phenyl phosphate and isopropyl methyl phenyl phosphate, respectively. Nitration of isopropyl methyl phenyl phosphate with ammonium nitrate gave isopropyl methyl *p*-nitrophenyl phosphate.

3. Results

3.1. Enzymatic hydrolysis of phosphotriester enantiomers

Phosphotriesterase enzyme can efficiently hydrolyze a variety of phosphotriesters. Paraoxon (**II**) is the best substrate known to date. The two ethyl groups in paraoxon can be replaced with methyl (**I**) isopropyl (**III**), and phenyl (**IV**) groups but kinetic values of k_{cat} and K_m decrease substantially. The kinetic constants for the hydrolysis of optically active phosphotriesters by PTE are listed in Table 1. Reaction of phosphotriesterase with racemic compounds **VI**–**VIII** resulted in the faster degradation of one enantiomer over the other enantiomer (Fig. 1). When the reaction was half-complete, the residual unhydrolyzed isomer was extracted with chloroform and the (+)-enantiomer was isolated in every instance. This result demonstrates that phosphotriesterase predominantly hydrolyzes the (–)-enantiomer of the racemic mixture for compounds **VI**–**VIII**. The enzymatic hydrolysis of the (–)-isomers is one to two orders of magnitude faster than the (+)-isomers. It should be noted that there is no significant difference in enzymatic activity between both isomers of ethyl methyl *p*-nitrophenyl phosphate (**V**).

3.2. Inhibition of acetylcholinesterase with phosphotriester enantiomers

The phosphotriester stereoisomers were used to inhibit human recombinant acetylcholinesterase. Table 2 lists the pseudo-first-order rate constants calculated from Eq. (2). The stereoselectivity of the inhibition of AChE with the chiral phosphates is opposite to that of hydrolysis reaction of PTE since the (+)-enantiomers are more potent inhibitors of AChE than the (–)-enantiomers (Fig. 2).

3.3. Absolute configuration

(–)-Ethyl methyl *p*-nitrophenyl phosphate (**V**) was prepared by nitration of (–)-ethyl methyl phenyl phosphate. It is reasonable to assume that this reaction occurs with retention of configuration. The configuration of (–)-ethyl methyl

phenyl phosphate is known to have the S_P configuration [11], and thus the (–)-isomer of compound **V** must also have S_P configuration. Treatment of (+)-ethyl *p*-nitrophenyl phenyl phosphate (**VII**) with sodium methoxide afforded (S_P)-(–)-ethyl methyl phenyl phosphate. This reaction takes place with inversion of configuration [10]. Thus, (+)-**VII** was assigned as the R_P configuration. The (+)-enantiomer of isopropyl phenyl *p*-nitrophenyl phosphate (**VIII**) was treated with sodium methoxide to yield (–)-isopropyl methyl phenyl phosphate. The (–)-isopropyl methyl phenyl phosphate was converted into (–)-isopropyl methyl *p*-nitrophenyl phosphate (**VI**) by nitration.

Table 1
Kinetic constants for the hydrolysis of phosphotriesters catalyzed by the Zn^{2+}/Zn^{2+} -PTE^a

	Compound	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
I		1050	7500	7.2×10^6
II		35	2200	6.2×10^7
III		47	170	3.5×10^6
IV ^a		230	3700	1.6×10^6
(+)- V		160	4000	2.5×10^7
(–)- V		160	5800	3.7×10^6
(+)- VI		490	180	3.6×10^5
(–)- VI		43	2000	4.7×10^7
(+)- VII ^b		170	310	1.8×10^6
(–)- VII ^b		18	3100	1.8×10^8
(+)- VIII ^b		110	100	9.5×10^5

^a Determined in 20% MeOH solution.

^b Determined in 5% MeOH solution.

4. Discussion

The bacterial phosphotriesterase is a remarkably efficient enzyme for the hydrolysis of a wide variety of organophosphorus nerve agents. The X-ray crystal structure of the Zn^{2+} -substituted PTE with a bound substrate analog, diethyl *p*-methylbenzylphosphonate, has shown that the active site of the enzyme consists mainly of hydrophobic residues [12]. The observed interactions between the enzyme and the substrate analog have provided valuable information on how the substrate binds to the active site and the potential role of other residues in binding and catalysis. Since the enzyme exhibits a stereospecific preference in favor of the hydrolysis of the S_p enantiomer of EPN, the results require the proper binding orientation of the substrate in the active center of the enzyme. Based on modeling of EPN into the active site, the regions of the active site interacting with the leaving group and the two other substituents to the phosphorus center have been identified.

In previous studies with various dialkyl paraoxon analogs, it was shown that the K_m and V_m values decline with increasing alkyl chain length and branching [2]. These results suggest that the active site can accommodate a wide variety of hydrophobic organophosphate substrates. To further characterize the stereochemical constraints of the active site, one or both of the two ethoxy substituents of paraoxon have been replaced with various combinations of methyl, isopropyl, or phenyl groups and then tested as substrates of the phosphotriesterase. The enzyme is quite tolerant of alterations to this structure. The negligible chiral preference seen for the hydrolysis of enantiomeric pairs of ethyl methyl *p*-nitrophenyl phosphate by the enzyme is attributable to the small difference in chain length of the two alkoxy groups. When the chiral groups differ considerably in bulkiness, the enzyme hydrolyzes the (–)-enantiomer one to two orders of magnitude faster than the (+)-enantiomer. These results suggest that binding and reactivity of (–)- and (+)-enantiomers is governed by the sizes of the subsites at the active site accommodating the alkyl or aryl substituents. According to the three-dimensional structure

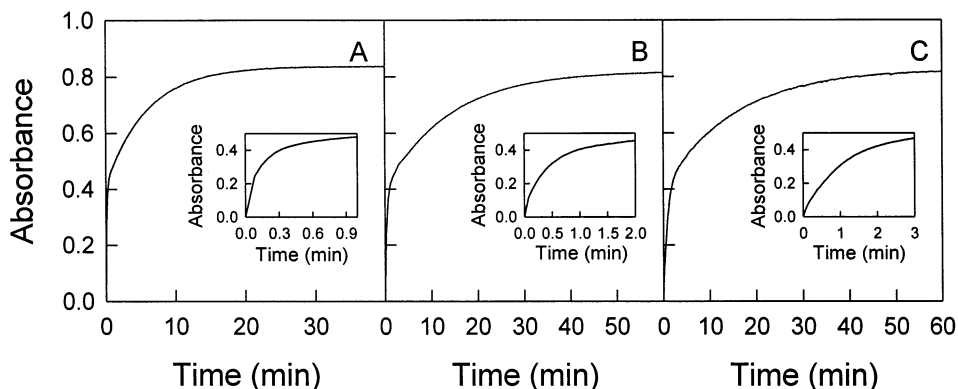
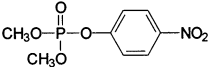
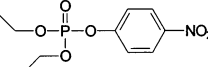
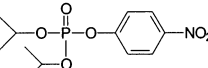
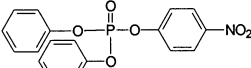
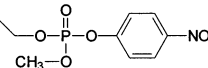
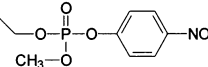
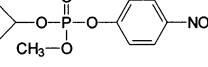
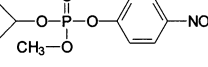
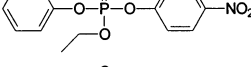
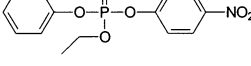
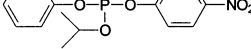


Fig. 1. Time course for the hydrolysis of racemic phosphotriesters (50 μ M) by phosphotriesterase. (A) Isopropyl methyl *p*-nitrophenyl phosphate. (B) Ethyl *p*-nitrophenyl phenyl phosphate. (C) Isopropyl *p*-nitrophenyl phenyl phosphate.

Table 2
Inhibition of acetylcholinesterase by phosphotriesters (500 nM)

	Compound	k (min^{-1})
I		0.15
II		0.80
III		0.87
IV		0.19
(+)-V		1.6
(-)-V		0.12
(+)-VI		0.39
(-)-VI		0.10
(+)-VII		0.44
(-)-VII		0.16
(+)-VIII		0.16

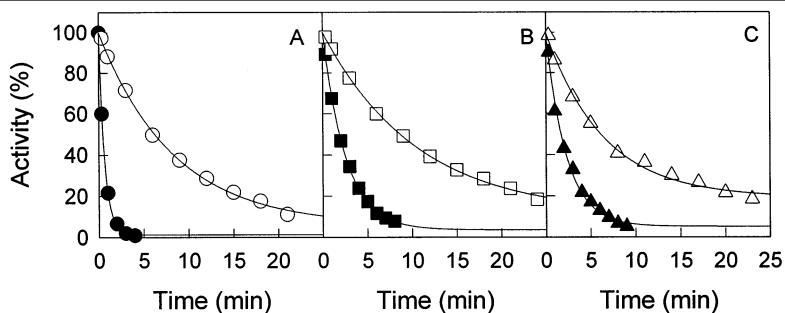
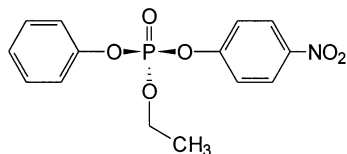
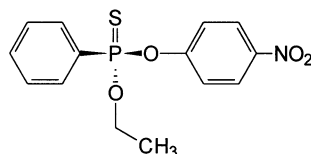
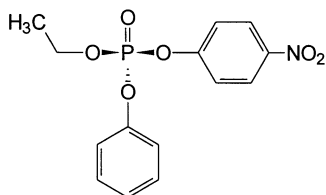
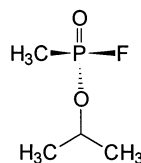


Fig. 2. Time course for the inactivation of acetylcholinesterase with both enantiomers of phosphotriesters (500 nM). In panels A–C, the open symbols refer to the (–)-enantiomer, and the close symbols to the (+)-enantiomer. (A) Ethyl methyl *p*-nitrophenyl phosphate. (B) Isopropyl methyl *p*-nitrophenyl phosphate. (C) Ethyl *p*-nitrophenyl phenyl phosphate.

of PTE, the leaving group is embedded in a subsite directed towards the solvent. Since (–)-ethyl *p*-nitrophenyl phenyl phosphate (**VII**) was determined to have the S_P configuration, it is possible to correlate the configuration of (S_P)-(–)-**VII** with S_P enantiomer of EPN. Based on this correlation and the known chiral preferences of the enzyme, the phenoxy group of (S_P)-(–)-**VII** and the phenyl group of S_P -EPN would occupy the same binding region.

 (S_P) - (–) - **VII** (S_P) - (–) - EPN

Organophosphorus nerve agents also react with acetylcholinesterase by phosphorylating the serine residue in the active site. Earlier studies have shown that chiral reactions with AChE are dependent on the absolute configuration at the phosphorus atom [13–15]. Distinct subsites could serve to optimize orientation of one enantiomer more preferably than the other enantiomer. The (+)-enantiomers of phosphotriesters are more potent inhibitors of AChE than the (–)-enantiomers. However, the rate of inactivation of AChE by the (–)-isomer of sarin are three orders of magnitude faster than the (+)-isomer [7]. Since the absolute configuration of (–)-sarin was determined to be S_P [16], it is possible to correlate the configuration of (R_P)-(+)-**VII** with (S_P)-(–)-enantiomer of sarin. By fixing the relative location of the *p*-nitrophenol leaving group of (R_P)-(+)-**VII** and fluoride of (S_P)-(–)-sarin, then the phenoxy group of (S_P)-(–)-**VII** and isopropyl group of R_P enantiomer of sarin would occupy the same position in the active site of phosphotriesterase. This implies that phosphotriesterase would hydrolyze (R_P)-(+)-sarin faster than (S_P)-(–)-sarin. These results clearly show that PTE has the chiral preference for the hydrolysis of enantiomeric organophosphorus nerve agents.

 (R_P) - (+) - **VII** (S_P) - (–) - sarin

5. Conclusion

Various enantiomeric paraoxon analogs containing combinations of methyl, isopropyl, and phenyl substituents were synthesized and tested as substrates of the bacterial phosphotriesterase and inhibitors of human recombinant acetylcholinesterase. PTE hydrolyzed the (–)-enantiomers of these phosphotriesters one to two orders of magnitude faster than the (+)-enantiomers. These results suggest that the binding orientation of the substrate is important for the hydrolytic reaction of PTE. In contrast, AChE exhibits a preference in favor of the (+)-enantiomers of these phosphotriesters over the (–)-enantiomers. The redesign of catalytic activity of phosphotriesterase would be needed to increase the rate of hydrolysis of chiral nerve gases.

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References

- [1] D.P. Dumas, S.R. Caldwell, J.R. Wild, F.M. Raushel, Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*, *J. Biol. Chem.* 264 (1989) 19659–19665.
- [2] W.J. Donarski, D.P. Dumas, D.P. Heitmeyer, V.E. Lewis, F.M. Raushel, Structure-activity relationships in the hydrolysis of substrates by the phosphotriesterase from *Pseudomonas diminuta*, *Biochemistry* 28 (1989) 4650–4655.
- [3] G.A. Omburo, J.M. Kuo, L.S. Mullins, F.M. Raushel, Characterization of the zinc binding site of bacterial phosphotriesterase, *J. Biol. Chem.* 267 (1992) 13278–13283.
- [4] M.M. Benning, J.M. Kuo, F.M. Raushel, H.M. Holden, Three-dimensional structure of the binuclear metal center of phosphotriesterase, *Biochemistry* 34 (1995) 7973–7978.
- [5] V.E. Lewis, W.J. Donarski, J.R. Wild, F.M. Raushel, Mechanism and stereochemical course at phosphorus of the reaction catalyzed by a bacterial phosphotriesterase, *Biochemistry* 27 (1988) 1591–1597.
- [6] M.Y. Chae, J.F. Postula, F.M. Raushel, Stereospecific enzymatic hydrolysis of phosphorus-sulfur bonds in chiral organophosphate triesters, *Bioorg. Med. Chem. Lett.* 4 (1994) 1473–1478.
- [7] H.L. Boter, A.J.J. Ooms, G.R. van der Berg, C. van Dijk, The synthesis of optically active isopropyl methylphosphonofluoridate (sarin), *Recl. Trav. Chim. Pays-Bas* 85 (1966) 147–150.
- [8] T. Koizumi, Y. Kobayashi, H. Amitani, E. Yoshii, A practical method of preparing optically active dialkyl phenyl phosphates, *J. Org. Chem.* 42 (1977) 3459–3460.
- [9] G.C. Ellman, K.D. Courtney, V. Andres Jr, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, *Biochem. Pharmacol.* 7 (1961) 88–95.
- [10] K. Nakayama, W.J. Thompson, A highly enantioselective synthesis of phosphate triesters, *J. Am. Chem. Soc.* 112 (1990) 6936–6942.
- [11] C.R. Hall, T.D. Inch, Preparation of some chiral alkyl phosphorothioates and stereochemical studies of their conversion into other chiral organophosphates, *J. Chem. Soc. Perkin Trans. 1* (1979) 1104–1111.
- [12] J.L. Vanhooke, M.M. Benning, F.M. Raushel, H.M. Holden, Three dimensional structure of the zinc-containing phosphotriesterase with the bound substrate analog diethyl 4-methylbenzylphosphonate, *Biochemistry* 35 (1996) 6020–6025.

- [13] N.P.B. Dudman, B. Zerner, Enzymatic preparation of an optically active phosphotriester, asymmetric only at phosphorus, *J. Am. Chem. Soc.* 95 (1973) 3019–3021.
- [14] N.P.B. Dudman, J.D. Jersey, B. Zerner, The stereospecificity of hydrolysis of methyl *n*-butyl *p*-nitrophenyl phosphate by serum phosphotriesterases and by acetylcholinesterase, *Biochim. Biophys. Acta* 481 (1977) 127–139.
- [15] H.A. Berman, K. Leonard, Chiral reaction of acetylcholinesterase probed with enantiomeric methylphosphonothioates, *J. Biol. Chem.* 264 (1989) 3942–3950.
- [16] H.P. Benschop, The absolute configuration of chiral organophosphorus anticholinesterases, *Pestic. Biochem. Physiol.* 4 (1974) 348–349.