Stereochemical Specificity of Organophosphorus Acid Anhydrolase toward *p*-Nitrophenyl Analogs of Soman and Sarin

Craig M. Hill,* Wen-Shan Li,* Tu-Chen Cheng,† Joseph J. DeFrank,†
and Frank M. Raushel*,[1]

**Department of Chemistry, Texas A&M University, P.O. Box 30021, College Station,
Texas 77842-3021; and †Environmental Technology Team, U.S. Army Edgewood Research,
Development and Engineering Center, Aberdeen, Maryland 21010*

Organophosphorus acid anhydrolase (OPAA) catalyzes the hydrolysis of *p*-nitrophenyl analogs of the organophosphonate nerve agents, sarin and soman. The enzyme is stereoselective toward the chiral phosphorus center by displaying a preference for the R_P-configuration of these analogs. OPAA also exhibits an additional preference for the stereochemical configuration at the chiral carbon center of the soman analog. The preferred configuration of the chiral carbon center is dependent upon the configuration at the phosphorus center. The enzyme displays a two- to four-fold preference for the R_P-enantiomer of the sarin analog. The k_{cat}/K_m of the R_P-enantiomer is 250 M^{-1} s^{-1}, while that of the S_P-enantiomer is 110 M^{-1} s^{-1}. The order of preference for the stereoisomers of the soman analog is $R_P S_C > R_P R_C > S_P R_C > S_P S_C$. The k_{cat}/K_m values are 36,300 M^{-1} s^{-1}, 1250 M^{-1} s^{-1}, 80 M^{-1} s^{-1} and 5 M^{-1} s^{-1}, respectively. The $R_P S_C$-isomer of the soman analog is therefore preferred by a factor of 7000 over the $S_P S_C$-isomer.   © 2001 Academic Press

Key Words: organophosphate hydrolysis; stereochemical preferences; organophosphorus acid anhydrolase.

INTRODUCTION

Organophosphorus acid anhydrolase (OPAA) from *Alteromonas* sp. JD6.5 is one member of an expanding class of enzymes that is capable of catalytically hydrolyzing a wide variety of organophosphorus compounds. DNA sequence and biochemical evidence have indicated that OPAA belongs to a proline dipeptidase family of enzymes (EC 3.4.13.9) *(1,2)*. Enzymes of this type catalyze the cleavage of the peptide bond within a dipeptide containing a prolyl residue at the carboxy terminus (Xaa-Pro). Organophosphorus substrates of OPAA include the insecticide paraoxon (*O, O*-diethyl
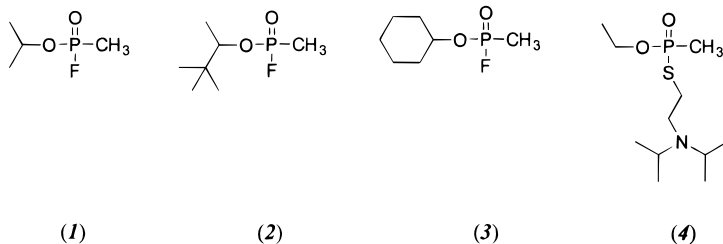

SCHEME 1. The phosphonofluoridates sarin (**1**), soman (**2**), GF (**3**), and the phosphonothiolate VX (**4**).

p-nitrophenyl phosphate) and the phosphonofluoridate chemical warfare (CW) agents sarin (**1**), soman (**2**) and GF (**3**) (*3,4*). However, the V-class agent VX (**4**) is not a substrate for this enzyme. All of these organophosphorus compounds have a chiral phosphorus center while soman has an additional stereogenic carbon center within the *O*-pinacolyl substituent (Scheme 1). The reaction catalyzed by this enzyme with the insecticide paraoxon (**5**) is shown in Scheme 2.

The organophosphorus compounds are potent neurotoxins and they exert their effect by inactivation of acetylcholinesterase (AChE), an enzyme essential for repolarization of the postsynaptic neuronal membrane in the central nervous system (*5*). The sensitivity of AChE to inactivation by individual stereoisomers of sarin and soman can vary over a wide range (*6*). It has been demonstrated that the S_P-stereoisomers of both sarin and soman are significantly more toxic than the R_P-stereoisomers of these compounds (*6*). For example, the S_P-enantiomer of sarin inactivates bovine erythrocyte AChE with a second-order rate constant of 1.4×10^7 M^{-1} min^{-1}, while the rate constant for the R_P-enantiomer is 3×10^3 M^{-1} min^{-1}. Similarly, inactivation of electric eel AChE by the two S_P-stereoisomers of soman, is characterized by rate constants of $1.8–2.8 \times 10^8$ M^{-1} min^{-1}, while the rate constants for the R_P-stereoisomers are $< 3 \times 10^5$ M^{-1} min^{-1} (*6*). Thus, the S_P-stereoisomers of sarin and soman inactivate AChE 10^3- to 10^4-fold faster than those of the R_P-configuration. The preference of AChE for soman of the S_P-configuration is reflected in the relative lethality of the individual stereoisomers. The two S_P-stereoisomers have LD$_{50}$ values of 38–99 μg/kg for mice while the two R_P-stereoisomers have values in excess of 2000 μg/kg (*6*). Scheme 3 illustrates the structures of the six stereoisomers of sarin and soman.

OPAA has reported k_{cat} values of 2500 s^{-1} and 380 s^{-1} for the racemic mixtures
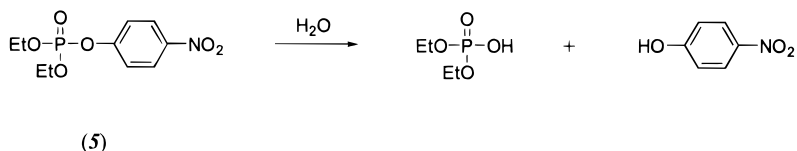
SCHEME 2. Hydrolysis of paraoxon (**5**) by OPAA.
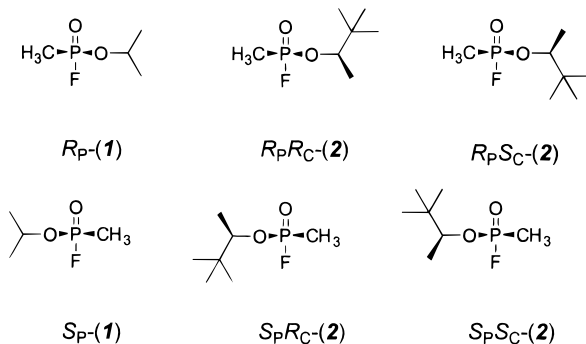
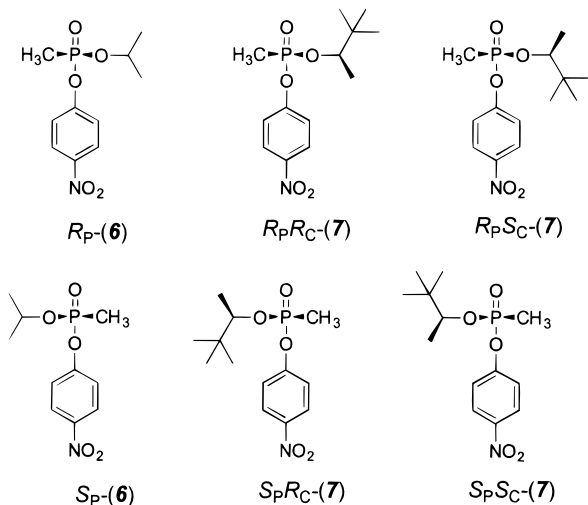**SCHEME 3.** The individual stereoisomers of sarin (**1**) and soman (**2**).

of soman and sarin, respectively (*7*). The enzyme has been proposed as an enzymatic alternative for the catalytic detoxification of G-agent neurotoxins (*7*). Therefore, it is of critical importance to determine the stereospecificity of this enzyme toward such neurotoxins. The substrate and stereochemical specificity of OPAA toward *p*-nitrophenyl phosphotriesters has been previously characterized with 16 paraoxon analogs (*8*). The enzyme exhibits stereoselectivity toward these substrates with a clear preference for the S_P-enantiomer over the R_P-enantiomer. OPAA displays the greatest discrimination toward the methyl ethyl and methyl isopropyl *p*-nitrophenyl phosphate substrates, where 112- and 100-fold preferences for the S_P-enantiomers were observed, respectively. The chiral selectivity was reduced to a 14-fold preference for the S_P-enantiomer of methyl phenyl *p*-nitrophenyl phosphate and the stereoselectivity is almost eliminated when the methyl group is replaced with other substituents.

In this report we investigated the stereoselectivity of OPAA toward *p*-nitrophenyl phosphonate analogs of sarin and soman. The stereoselective properties of this enzyme were characterized with racemic mixtures and preparations of individual enantiomers. The results clearly indicate that OPAA will have a stereoselective preference for the R_P-enantiomers of sarin and soman.

MATERIALS AND METHODS

Chemical Syntheses

Racemic mixtures of *O*-isopropyl *p*-nitrophenyl methylphosphonate (**6**) and *O*-pinacolyl *p*-nitrophenyl methylphosphonate (**7**) were synthesized by modification of standard procedures (*9–11*) (Scheme 4). Individual enantiomers of **6** were isolated through a kinetic resolution of the racemic mixture using an enzymatic method employing wild-type and mutant variants of the phosphotriesterase (PTE) from *Pseudomonas diminuta* (*12*). The enantiomeric purity of the two sarin analogs was verified by chiral capillary electrophoresis (*13*). For the preparation of S_P-**6,** the ratio of S_P-**6** to R_P-**6** was found to be 99:1. For the preparation of R_P-**6,** the ratio of R_P-**6** to S_P-**6** was found to be 93:7. The diastereomeric mixture of **7** containing the R_PS_C- and S_PS_C-stereoisomers was prepared by chemical resolution of racemic pinacolyl alcohol

SCHEME 4.   The individual *p*-nitrophenyl analogs of sarin (**6**) and soman (**7**).

prior to coupling with bis(*p*-nitrophenyl)methylphosphonate (*6,14*). The individual isomers were enzymatically resolved from the diastereomeric mixture using PTE (*12*). The R_PR_C/S_PR_C diastereomeric mixture of **7** and the individual isomers were prepared in a similar manner. The purity of the single stereoisomer preparations were verified by chromatography using a chiral HPLC column ((*R, R*)-Whelk-O,1) from Regis Technologies. The stereochemical purity for the individual preparations of the R_PR_C-, R_PS_C-, S_PR_C-, and S_PS_C-stereoisomers of the soman analog **7** was 90, 92, 92, and 98%, respectively. In addition, a totally racemic mixture of **7** was prepared. The synthetic and enzymatic procedures yield three different preparations of **6** and seven different preparations of **7.** The toxicity of these compounds has not been determined and thus they should be used with caution.

Purification of OPAA

An *Escherichia coli* XL1 culture containing the plasmid pTCJS4 was grown at 37°C in 5 liters of LB containing 0.1 mM MnCl₂. Protein expression was induced by the addition of 0.6 mM IPTG to the cell culture at A_{600} = 0.5. Incubation was continued at 37°C for another 5 h. The cells were harvested by centrifugation and disrupted by two passages through a French pressure cell. Cell debris was removed by centrifugation and the supernatant solution fractionated with $(NH_4)_2SO_4$ at 40–65% saturation. The pellet was dissolved in 10 mM Bis-Tris Propane (pH 7.2) containing 0.1 mM MnCl₂ (buffer A) and dialyzed against the same buffer. The protein solution was applied to a Q-Sepharose column (3 × 14.5 cm) and loosely bound material was removed by washing the column with buffer A containing 0.2 M NaCl. The enzyme was eluted from the column with a linear gradient of buffer A containing 0.2–0.6 M NaCl. The enzyme eluted at ~350 mM NaCl. Fractions containing the enzyme were

pooled and concentrated with $(NH_4)_2SO_4$ at 65% saturation and then dialyzed against buffer A.

Enzyme Assays

Continuous assays were conducted at 25°C and carried out on a SPECTRAmax-340 microplate spectrophotometer (Molecular Devices Inc, Sunnyvale, CA). Enzyme (10–50 μl) was dispensed into the wells of a multiwell plate. The assays were started by the addition of 200–250 μl of assay buffer to the enzyme using an *edp plus* Motorized Microliter Pippette (Rainin, Woburn, MA) fitted with a multichannel adapter. Hydrolysis of substrate was monitored at 400 nm (*p*-nitrophenolate, $\varepsilon = 17,000 \ M^{-1} \ cm^{-1}$). The assay buffer contained 50 mM Bis-Tris Propane (pH 8.5), 100 mM NaCl, and 0.1 mM $MnCl_2$. The soman analogs were assayed in the presence of 10% methanol. Progress curves were conducted at 25°C on a Gilford 260 spectrophotometer. The assays were started by the addition of enzyme to 3.0 ml of assay buffer. The composition of the assay buffer was as above except that methanol was omitted. Single and double exponential time courses were fitted to Eqs. [1] and [2], respectively. In Eq. [1], A_o is the initial substrate concentration and k_o is the first-order rate constant. In Eq. [2], A_1 and A_2 are the initial concentrations of the two enantiomers of the racemic mixture and k_1 and k_2 are the respective first-order rate constants. The values of K_m, k_{cat}, and k_{cat}/K_m were determined by fitting Eq. [3] to the initial velocity data, where E_t is the concentration of active sites and A is the substrate concentration. When the enzyme could not be saturated with the substrate due to the limited solubility, k_{cat}/K_m was obtained by fitting the data to Eq. [4].

$$y = A_0(1 - e^{-k_0 t}) \qquad [1]$$

$$y = A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t}) \qquad [2]$$

$$v/E_t = k_{cat}A/(K_m + A) \qquad [3]$$

$$v/E_t = k_{cat}A \, /K_m \qquad [4]$$

RESULTS AND DISCUSSION

OPAA catalyzes the hydrolysis of the *p*-nitrophenyl analogs of sarin (**6**) and soman (**7**). A racemic mixture of **6** and the individual enantiomers were synthesized and tested as substrates for OPAA. A biphasic progress curve was observed for the enzymatic hydrolysis of the racemic mixture of **6** (Fig. 1A), indicating stereoselectivity toward the chiral phosphorus center. The ratio of the first-order rate constants for the two phases of the progress curve is 4:1 (Table 1). The value of k_{cat}/K_m for the racemic sarin analog, determined from a substrate saturation curve, is 230 $M^{-1} \ s^{-1}$. The values for the individual S_P- and R_P-enantiomers are 110 and 250 $M^{-1} \ s^{-1}$, respectively
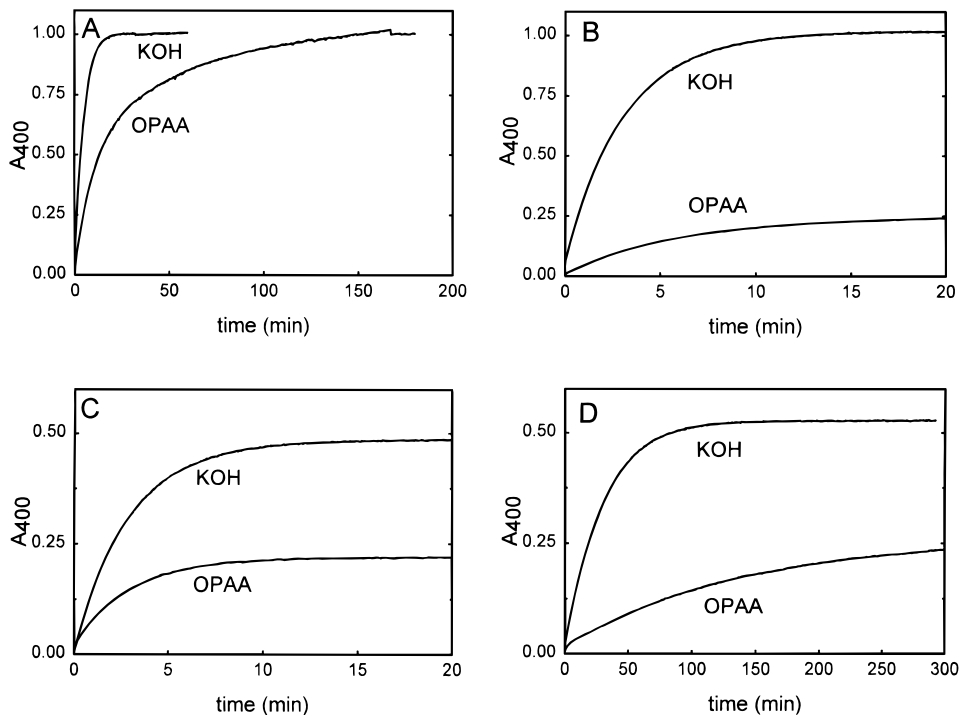
FIG. 1. Progress curves for KOH and OPAA catalyzed hydrolysis of **6** and **7**. (A) racemic **6,** (B) totally racemic **7,** (C) R_PS_C/S_PS_C-**7,** (D) R_PR_C/S_PR_C-**7**. Assays were performed at 25°C, pH 8.5, and contained 60 μM of **6** or **7**. KOH was added to a final concentration of 0.6 M. OPAA was added at various concentrations: (A) 1860, (B) 62, (C) 124, and (D) 124 nM.

TABLE 1

First-Order Rate Constants Determined from Progress Curves

	Substrate	OPAA (mg/ml)	k_1 (min^{-1})	k_2 (min^{-1})	k_3 (min^{-1})	k_4 (min^{-1})
6	Both isomers	0.11	0.048	0.011	—	—
7		0.0037	0.195	0.0083	—	—
7	All four isomers	0.0073	0.352	0.0067	—	—
7		0.073	—	0.0474	0.0023	—
7		0.22	—	0.1409	0.0093	—
7		0.73	—	0.415	0.0325	—
7		0.73	—	—	0.0624	0.0059
7	R_PS_C/S_PS_C	0.0073	0.393	—	—	0.0028
7		0.73	—	—	—	0.0019
7	R_PR_C/S_PR_C	0.0073	—	0.0073	—	—
7		0.073	—	0.0673	0.0049	—

(Table 2). Thus, OPAA displays a two- to fourfold preference for the R_P-enantiomer of this substrate.

The stereospecificity of OPAA was further investigated with three different sets of racemic mixtures and the four individual stereoisomers of **7**. Two diastereomeric mixtures of **7** containing the R_PS_C/S_PS_C- or the R_PR_C/S_PR_C-isomers were prepared. A totally racemic mixture containing all four stereoisomers of **7** was also synthesized. The four individual stereoisomers were obtained through kinetic resolutions of the two diastereomeric mixtures (*12*). OPAA exhibits stereoselectivity toward the two chiral centers of **7**. Addition of OPAA to a racemic mixture containing the four stereoisomers results in the initial hydrolysis of only 25% of the total substrate present, reflecting stereoselectivity toward the phosphorus center, and an additional preference for the stereochemical configuration at the chiral carbon of the *O*-pinacolyl group (Fig. 1B). The addition of OPAA to the diastereomeric mixtures containing either the R_PS_C/S_PS_C- or the R_PR_C/S_PR_C-stereoisomer pairs results in the rapid initial hydrolysis of 50% of the substrate (Figs. 1C and 1D). This result indicates a clear preference of OPAA for a single stereochemical configuration at the phosphorus center

Four phases were observed for the OPAA catalyzed hydrolysis of the totally racemic mixture of **7** by incrementing the amount of enzyme in a series of assays (Table 1). The ratios of the first-order rate constants obtained from the progress curves, after correction for the amount of added enzyme, are 20,000:315:21:1. Correlation of the first-order rate constants, obtained from the totally racemic mixture, with those from the S_C-pair racemate indicates that the S_C-pair contains the fastest and the slowest stereoisomers (Table 1). The preference of OPAA for the R_P-configuration of **6** suggests that the R_PS_C-configuration of **7** would be preferred to the S_PS_C-configuration.

TABLE 2

Kinetics of OPAA Catalyzed Hydrolysis of Soman and Sarin Analogs

Substrate		k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)
6	S_P/R_P	230 ± 6^a	$\geq 2.3^b$	> 10
6	S_P	110 ± 2^a	$\geq 0.9^c$	> 8.4
6	R_P	250 ± 12^a	$\geq 2.2^c$	> 8.4
7	4 isomers	$6{,}030 \pm 360$	$\geq 11^d$	> 2
7	R_PS_C/S_PS_C	$15{,}400 \pm 900$	40 ± 4	2.7 ± 0.4
7	R_PR_C/S_PR_C	$1{,}250 \pm 70$	9 ± 2	7.2 ± 2.2
7	S_PS_C	5 ± 0.2^a	$\geq 0.004^e$	> 1
7	S_PR_C	80 ± 2^a	$\geq 0.07^f$	> 1
7	R_PR_C	$1{,}250 \pm 16^a$	$\geq 1.7^g$	> 1
7	R_PS_C	$36{,}300 \pm 1{,}900$	270 ± 90	7.5 ± 2.8

Note. Assays were performed at 25°C and pH 8.5. Analog **7** was assayed in the presence of 10% methanol.
[a] Determined by fitting of the data to equation 4.
[b] Determined at a concentration of 10 mM.
[c] Determined at a concentration of 8.4 mM.
[d] Determined at a concentration of 1.9 mM.
[e] Determined at a concentration of 0.9 mM.
[f] Determined at a concentration of 1.0 mM.
[g] Determined at a concentration of 1.5 mM.

Similarly, the R_C-pair racemate contains the second and third fastest isomers and it is expected that the R_PR_C-configuration is preferred to the S_PR_C-configuration (Table 1). Thus, the progress curve data indicate the following order for the OPAA stereochemical preference with **7**: $R_PS_C > R_PR_C > S_PR_C > S_PS_C$. This order of preference was confirmed by substrate saturation experiments using the four individual stereoisomers of **7**. The R_PS_C-stereoisomer has the highest k_{cat}/K_m of 3.6×10^4 M^{-1} s^{-1} followed by R_PR_C- (1.3×10^3 M^{-1} s^{-1}), S_PR_C- (80 M^{-1} s^{-1}), and S_PS_C- (5 M^{-1} s^{-1}) stereoisomers (Table 2). The ratios of the k_{cat}/K_m values obtained from substrate saturation curves are 7250:250:16:1. These ratios are close to those obtained from an analysis of the progress curves at variable enzyme concentration. The k_{cat}/K_m for the pure R_PS_C-isomer is six-fold higher than that of the totally racemic mixture where the R_PS_C-isomer concentration is four-fold lower. Similarly k_{cat}/K_m for the pure R_PS_C-isomer is twice that of the S_C-pair racemate where the R_PS_C-isomer concentration is halved. The value of k_{cat}/K_m for the R_PR_C-isomer is similar to that for the R_C-pair racemate, where the value might be expected to be halved.

The K_m values of most substrates could not be determined due to their relatively high values and limited solubility in the assay solution. In such cases, where the enzyme could not be saturated, the k_{cat} is reported for the highest concentration of substrate assayed (Table 2). However, K_m values could be determined for the R_PS_C-stereoisomer and the R_C- and S_C-stereoisomer pairs. A k_{cat} of 270 s^{-1} and a K_m of 7.5 mM were obtained for the R_PS_C-stereoisomer. Smaller k_{cat} values of 40 s^{-1} and 9 s^{-1} were obtained for the S_C- and R_C-pairs, respectively, while K_m values of 2.7 and 7.2 mM were observed.

OPAA hydrolyzes the *p*-nitrophenyl analogs of sarin and soman. The enzyme exhibits a stereoselectivity toward these substrates with a clear preference for the R_P-configuration at the phosphorus center. This result is consistent with the stereoselectivity displayed by OPAA toward the phosphotriester substrates where the S_P-enantiomer is preferred (8). The relative stereochemistry of **1, 6,** and methyl isopropyl *p*-nitrophenyl phosphate (**8**) is illustrated in Scheme 5. The enzyme also displays a stereoselectivity toward the chiral carbon center of the *O*-pinacolyl substituent of **7**. The preference for a particular configuration at the carbon center is dependent upon the configuration at the phosphorus center. The S_C-configuration is preferred with the R_P-configuration while the R_C-configuration is preferred with the S_P-configuration. The R_P-enantiomer
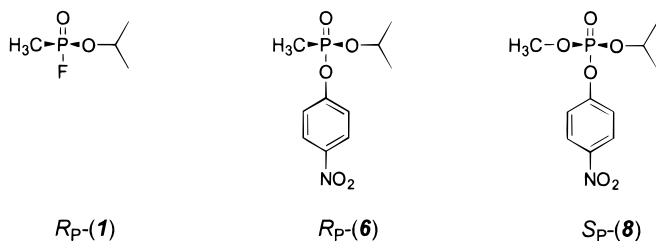
SCHEME 5. The relative stereochemistry of sarin (**1**), *p*-nitrophenyl sarin analog (**6**), and methyl isopropyl *p*-nitrophenyl phosphate (**8**).

of **6** is preferred two- to fourfold over the S_P-enantiomer. The fastest stereoisomer of **7** is preferred about 7000-fold over the slowest stereoisomer.

The absolute configuration of R_P-sarin (**1**) can be correlated with the absolute configuration of R_P-**6** (Scheme 5). Thus, the stereospecificities of OPAA and AChE, which is more rapidly inactivated by the S_P-isomers of sarin and soman, are opposite and thus OPAA more efficiently hydrolyzes analogs of the less toxic stereoisomers of sarin and soman.

The stereochemical preference of OPAA for the two stereoisomers of the sarin analog **6** is similar to that of PTE. The k_{cat}/K_m values obtained for PTE with R_P-**6** and S_P-**6** are 8.2×10^6 and 4.1×10^5 M^{-1} s^{-1}, respectively (K. Lum, W-S. Li, and F. M. Raushel, unpublished results). However, PTE is able to hydrolyze each of these analogs at least 1000 times faster than does OPAA. The stereochemical preference of OPAA for the four stereoisomers of **7** is similar to that of PTE, except that the relative order of the $R_P S_C$- and $R_P R_C$-isomers is reversed. The k_{cat}/K_m values obtained for PTE with the $R_P R_C$-, $R_P S_C$-, $S_P R_C$-, and $S_P S_C$-stereoisomers of the soman analog **7** are 1.6×10^5, 1.2×10^4, 3.8×10^2, and 1.6×10^1 M^{-1} s^{-1}, respectively (K. Lum, W-S. Li, and F. M. Raushel, unpublished results). OPAA and PTE thus catalyze the hydrolysis of the stereoisomers of the soman analog **7** at comparable rates. However, the analogs of the most toxic isomers ($S_P R_C$, and $S_P S_C$) are hydrolyzed the slowest by these two enzymes. If OPAA and PTE are to achieve their full potential for the catalytic detoxification of sarin and soman, then these enzymes will have to be structurally modified to enhance the inherent catalytic activity toward the most toxic stereoisomeric forms of these organophosphorus nerve agents.

ACKNOWLEDGMENTS

REFERENCES

1. Cheng, T.-C., Harvey, S. P., and Chen, G. L. (1996) *Appl. Environ. Microbiol.* **62,** 1636–1641.
2. Cheng, T.-C., Liu, L., Wang, B., Wu, J., DeFrank, J. J., Anderson, D. M., Rastogi, V. K., and Hamilton, A. B. (1997) *J. Ind. Microbiol.* **18,** 49–55.
3. DeFrank, J. J., and Cheng, T.-C. (1991) *J. Bacteriol.* **173,** 1938–1943.
4. Cheng, J. J., Beaudry, W. T., Cheng, T-C., Harvey, S. P., Stroup, A. N., and Szafraniec, L. L. (1993) *Chem-Biol. Interact.* **87,** 141–148.
5. Ecobichon, D. J. (1995) *in* Casarett and Doull's Toxicology: The Basic Science of Poisons (Klaassen, C. D., Ed.), pp. 655–666, McGraw Hill, New York, NY.
6. Benschop, H. P., Konings, C. A. G., Van Genderen, J., and De Jong, L. P. A. (1984) *Toxicol. Appl. Pharmacol.* **72,** 61–74.
7. Cheng, T.-C., DeFrank, J. J., and Rastogi, V. P. (1999) *Chem.-Biol. Interact.* **119–120,** 455–462.
8. Hill, C. M., Wu, F., Cheng, T.-C., DeFrank, J. J., and Raushel, F. M. (2000) *Bioorg. Med. Chem. Lett.* (2000) **10,** 1285–1288.
9. Herriott, A. W. (1971) *J. Am. Chem. Soc.* **93,** 3304–3305.
10. Boter, H. L., and Platenburg, D. H. J. M. (1967) *Rec. Trav. Chim.* **86,** 399–404.
11. Hoffmann, F. W., Wadsworth, D. H., and Weiss, H. D. (1958) *J. Am. Chem. Soc.* **80,** 3945–3948.
12. Wu, F., Li, W.-S., Chen-Goodspeed, M., Sogorb, M., and Raushel, F. M. (2000) *J. Am Chem. Soc.* **122,** 10206–10207.
13. Zhu, W. Wu, F., Raushel, F. M., and Vigh, G. (2000) *J. Chrom.* **895,** 247–254.
14. Pickard, R. H., and Kenyon, J. (1914) *J. Chem. Soc.* 1115–1131.