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

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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 Rₚ-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 Rₚ-enantiomer of the sarin analog. The k_{cat}/K_m of the Rₚ-enantiomer is 250 M⁻¹ s⁻¹, while that of the Sₚ-enantiomer is 110 M⁻¹ s⁻¹. The order of preference for the stereoisomers of the soman analog is RₚSₐ > RₛRₜ > SₛRₜ > SₚSₐ. 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 RₚSₐ-isomer of the soman analog is therefore preferred by a factor of 7000 over the SₚSₐ-isomer.

Key Words: organophosphorus 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).

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 $1.4 \times 10^7$ M$^{-1}$ min$^{-1}$, while the rate constant for the $R_P$-enantiomer is $3 \times 10^3$ 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 $\mu$g/kg for mice while the two $R_P$-stereoisomers have values in excess of 2000 $\mu$g/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.
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 stereoselectivity 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 $R_P$-$S_C$- and $S_P$-$S_C$-stereoisomers was prepared by chemical resolution of racemic pinacolyl alcohol.
prior to coupling with bis(p-nitrophenyl)methylphosphonate (6,14). The individual isomers were enzymatically resolved from the diastereomeric mixture using PTE (12). The \( R_P R_C / S_P R_C \) 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 \( R_P R_C \)-, \( R_P S_C \)-, \( S_P R_C \)-, and \( S_P S_C \)-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 \((\text{NH}_4)_2\text{SO}_4\) 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 \( \sim 350 \) mM NaCl. Fractions containing the enzyme were
pooled and concentrated with (NH₄)₂SO₄ 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 Pipette (Rainin, Woburn, MA) fitted with a multichannel adapter. Hydrolysis of substrate was monitored at 400 nm (p-nitrophenolate, ε = 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 MnCl₂. 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], A₀ is the initial substrate concentration and k₀ is the first-order rate constant. In Eq. [2], A₁ and A₂ are the initial concentrations of the two enantiomers of the racemic mixture and k₁ and k₂ are the respective first-order rate constants. The values of Kₘ, kₜₐₐ/ₚ, and kₜₐₐ/Kₘ were determined by fitting Eq. [3] to

\[
y = A_0(1 - e^{-k_0t})
\]

\[
y = A_1(1 - e^{-k_1t}) + A_2(1 - e^{-k_2t})
\]

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

\[
v/E_t = k_{cat}A/K_m
\]

**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 kₜₐₐ/Kₘ for the racemic sarin analog, determined from a substrate saturation curve, is 230 M⁻¹ s⁻¹. The values for the individual Sₚ- and Rₚ-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) $R_P S_C/S_P S_C$-7, (D) $R_P R_C/S_P R_C$-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>
<thead>
<tr>
<th>Substrate</th>
<th>OPAA (mg/ml)</th>
<th>$k_1$ (min$^{-1}$)</th>
<th>$k_2$ (min$^{-1}$)</th>
<th>$k_3$ (min$^{-1}$)</th>
<th>$k_4$ (min$^{-1}$)</th>
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<tbody>
<tr>
<td>6</td>
<td>Both isomers</td>
<td>0.11</td>
<td>0.048</td>
<td>0.011</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0037</td>
<td>0.195</td>
<td>0.0083</td>
<td>0.0023</td>
</tr>
<tr>
<td>7</td>
<td>All four isomers</td>
<td>0.0073</td>
<td>0.352</td>
<td>0.0067</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.073</td>
<td>—</td>
<td>0.0474</td>
<td>0.0023</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.22</td>
<td>—</td>
<td>0.1409</td>
<td>0.0093</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.73</td>
<td>—</td>
<td>0.415</td>
<td>0.0325</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.73</td>
<td>—</td>
<td>—</td>
<td>0.0624</td>
</tr>
<tr>
<td>7</td>
<td>$R_P S_C/S_P S_C$</td>
<td>0.0073</td>
<td>0.393</td>
<td>—</td>
<td>0.0028</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.73</td>
<td>—</td>
<td>—</td>
<td>0.0019</td>
</tr>
<tr>
<td>7</td>
<td>$R_P R_C/S_P R_C$</td>
<td>0.0073</td>
<td>—</td>
<td>0.0073</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.073</td>
<td>—</td>
<td>0.0673</td>
<td>0.0049</td>
</tr>
</tbody>
</table>
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 $R_P S_C / S_P S_C$- or the $R_P R_C / S_P R_C$-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 $R_P S_C / S_P S_C$- or the $R_P R_C / S_P R_C$-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 $R_P S_C$-configuration of 7 would be preferred to the $S_P S_C$-configuration.

### TABLE 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 $S_P / R_P$</td>
<td>$230 \pm 6^c$</td>
<td>$\geq 2.3^b$</td>
<td>$&gt; 10$</td>
</tr>
<tr>
<td>6 $S_P$</td>
<td>$110 \pm 2^a$</td>
<td>$0.9^c$</td>
<td>$&gt; 8.4$</td>
</tr>
<tr>
<td>6 $R_P$</td>
<td>$250 \pm 12^a$</td>
<td>$2.2^c$</td>
<td>$&gt; 8.4$</td>
</tr>
<tr>
<td>7 4 isomers</td>
<td>$6,030 \pm 360$</td>
<td>$11^c$</td>
<td>$&gt; 2$</td>
</tr>
<tr>
<td>7 $R_P S_C / S_P S_C$</td>
<td>$15,400 \pm 900$</td>
<td>$40 \pm 4$</td>
<td>$2.7 \pm 0.4$</td>
</tr>
<tr>
<td>7 $R_P R_C / S_P R_C$</td>
<td>$1,250 \pm 70$</td>
<td>$9 \pm 2$</td>
<td>$7.2 \pm 2.2$</td>
</tr>
<tr>
<td>7 $S_P S_C$</td>
<td>$5 \pm 0.2^a$</td>
<td>$0.004^c$</td>
<td>$&gt; 1$</td>
</tr>
<tr>
<td>7 $S_P R_C$</td>
<td>$80 \pm 2^a$</td>
<td>$0.07^c$</td>
<td>$&gt; 1$</td>
</tr>
<tr>
<td>7 $R_P R_C$</td>
<td>$1,250 \pm 16^a$</td>
<td>$1.7^c$</td>
<td>$&gt; 1$</td>
</tr>
<tr>
<td>7 $R_P S_C$</td>
<td>$36,300 \pm 1,900$</td>
<td>$270 \pm 90$</td>
<td>$7.5 \pm 2.8$</td>
</tr>
</tbody>
</table>

*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 \( R_P R_C \)-configuration is preferred to the \( S_P R_C \)-configuration (Table 1).

Thus, the progress curve data indicate the following order for the OPAA stereochemical preference with 7: \( R_P S_C > R_P R_C > S_P R_C > S_P S_C \). This order of preference was confirmed by substrate saturation experiments using the four individual stereoisomers of 7. The \( R_P S_C \)-stereoisomer has the highest \( k_{cat}/K_m \) of \( 3.6 \times 10^4 \) M\(^{-1}\) s\(^{-1}\) followed by \( R_P R_C \) (1.3 \( \times 10^3 \) M\(^{-1}\) s\(^{-1}\)), \( S_P R_C \) (80 M\(^{-1}\) s\(^{-1}\)), and \( S_P S_C \) (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 \( R_P S_C \)-isomer is six-fold higher than that of the totally racemic mixture where the \( R_P S_C \)-isomer concentration is four-fold lower. Similarly \( k_{cat}/K_m \) for the pure \( R_P S_C \)-isomer is twice that of the \( S_C \)-pair racemate where the \( R_P S_C \)-isomer concentration is halved.

The value of \( k_{cat}/K_m \) for the \( R_P R_C \)-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 \( R_P S_C \)-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 \( R_P S_C \)-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

\[ \text{SCHEME 5. The relative stereochemistry of sarin (1), } p \text{-nitrophenyl sarin analog (6), and methyl isopropyl } p \text{-nitrophenyl phosphate (8).} \]
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 $8.2 \times 10^6$ and $4.1 \times 10^5$ 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 $1.6 \times 10^5$, $1.2 \times 10^4$, $3.8 \times 10^2$, and $1.6 \times 10^1$ 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.

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