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Substrate and Stereochemical Specificity of the Organophosphorus Acid Anhydrolase from *Alteromonas* sp. JD6.5 toward *p*-Nitrophenyl Phosphotriesters

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Abstract—The enzyme OPAA hydrolyzes *p*-nitrophenyl phosphotriesters bearing substituents at the phosphorus center ranging in size from methyl to phenyl. The enzyme exhibits stereoselectivity toward the hydrolysis of chiral substrates with a preference for the S_P enantiomer.

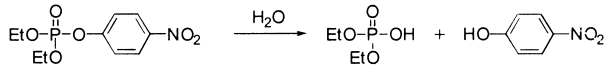
Introduction

The enzymatic detoxification of chemical warfare (CW) agents has received considerable experimental attention for over 50 years. Although the currently fielded decontamination solutions are effective in the hydrolysis of CW agents, they are corrosive in nature and result in enormous amounts of hazardous waste. In contrast, the enzymatic detoxification of this class of compounds can be conducted at neutral pH under very mild reaction conditions while the associated logistical burden is significantly reduced.

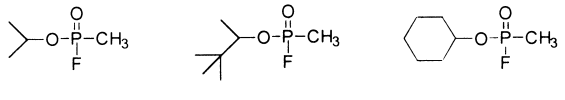
Orgonophosphorus acid anhydrolase (OPAA; E.C. 3.1.8.2) is one member of a growing class of enzymes that is capable of catalytically hydrolyzing a wide variety of organophosphorus compounds including the phosphotriester paraoxon (*O, O*-diethyl *p*-nitrophenyl phosphate) and the phosphonofluoridates soman (*O*-pinacolyl methyl phosphonofluoridate), sarin (*O*-isopropyl methyl phosphonofluoridate) and GF (*O*-cyclohexyl methyl phosphonofluoridate).[1,2] The reaction products for the hydrolysis of the insecticide paraoxon are shown in Scheme 1.

Many of the OPAA substrates have a stereogenic phosphorus center while soman has an additional stereogenic carbon center as illustrated in Scheme 2. High levels of OPAA activity have been found in a number of *Alteromonas* species.[1–3] Recently Cheng et al. reported the nucleotide sequence of the gene encoding OPAA from *Alteromonas* sp. JD6.5.[4] DNA sequence and biochemical evidence for the cloned enzyme have established this OPAA to be a proline dipeptidase (EC 3.4.13.9). This type of enzyme cleaves a dipeptide bond with a prolyl residue at the carboxy terminus (Xaa-Pro).[4,5] The activity of the proline dipeptidase with organophosphorus compounds has been proposed to be due to the similarity of the physical and chemical properties shared by these compounds and the dipeptide substrates.

Numerous other enzymes hydrolyze organophosphorus compounds and among them the phosphotriesterase (PTE) from *Pseudomonas diminuta* is the best characterized.[6,7] This enzyme also has a rather broad substrate specificity and exhibits a stereoselectivity that is highly dependent on the absolute configuration at the



Scheme 1. Paraoxon hydrolysis.



Scheme 2. The phosphonofluoridates (A) sarin, (B) soman and (C) GF.

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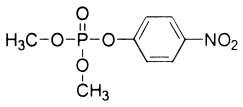
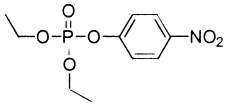
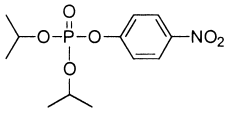
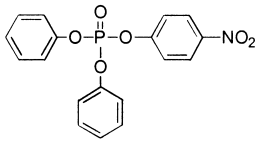
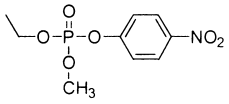
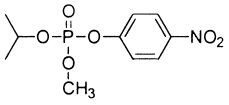
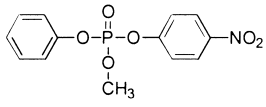
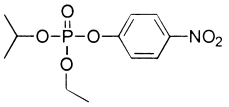
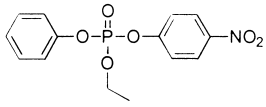
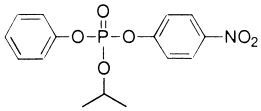
phosphorus center.[8,9] It has recently been demonstrated using a library of chiral and racemic paraoxon analogues that the wild type PTE favors the hydrolysis of the S_P enantiomer over the R_P enantiomer by nearly two orders of magnitude.[9] This report describes the utilization of a series of 16 paraoxon analogues (Table 1) to investigate the substrate and stereochemical specificity of the OPAA from *Alteromonas* sp. JD6.5 toward *p*-nitrophenyl phosphotriesters.

Table 1. OPAA substrates

Compound	Structure
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

Results and Discussion

OPAA hydrolyzes *p*-nitrophenyl phosphotriesters bearing substituents at the phosphorus center ranging in size from methyl to phenyl.[10–12] The K_m values for substrates containing a phenyl group are all below 2.0 mM except for (S_P)-methyl phenyl *p*-nitrophenyl phosphate (**7**) where a value of 4 mM was observed. The remaining substrates all have higher K_m values. The R_P-isomer of methyl isopropyl *p*-nitrophenyl phosphate (**6**) displays the highest K_m of 19 mM (Table 2). The K_m values for the diethyl (**2**), and ethyl isopropyl (**8**) substrates could not be determined precisely, due to their relatively high values and limited solubility in the assay solution.

The k_{cat} values for the substrates containing a phenyl group are less than 1 s^{-1} and are lower than the values obtained for the other substrates. The single exception is (S_P)-methyl phenyl *p*-nitrophenyl phosphate (**7**) where a k_{cat} of 40 s^{-1} was observed. (S_P)-Methyl ethyl *p*-nitrophenyl phosphate (**5**) has a k_{cat} of 47 s^{-1} while the (S_P)-methyl isopropyl *p*-nitrophenyl phosphate (**6**) has the highest k_{cat} of 54 s^{-1}. These results clearly indicate that a relatively small substituent, such as a methyl group, is required to be present in a substrate with the S_P configuration in order for appreciable substrate turnover to be obtained (Table 2). OPAA displays the highest value of k_{cat}/K_m with (S_P)-methyl phenyl *p*-nitrophenyl phosphate (**7**) with a value of k_{cat}/K_m of 1×10^4 M^{-1} s^{-1}. All other substrates except (S_P)-methyl ethyl *p*-nitrophenyl phosphate (**5**), (S_P)-methyl isopropyl *p*-nitrophenyl phosphate (**6**) and (R_P)-methyl phenyl *p*-nitrophenyl phosphate (**7**) have values of $k_{cat}/K_m < 1\times10^3$ M^{-1} s^{-1}.

OPAA exhibits stereoselectivity toward phosphotriester substrates with a clear preference for the S_P enantiomer over the R_P enantiomer. The selectivity is most apparent for the methyl ethyl (**5**) and methyl isopropyl (**6**) substrates where chiral preferences of 112-fold and 100-fold were observed, respectively. Figure 1 graphically illustrates that upon the addition of OPAA ~50% of the racemic methyl isopropyl *p*-nitrophenyl phosphate (**6**) in the reaction mixture is rapidly hydrolyzed. When KOH is added to the reaction mixture the (R_P)-isomer is subsequently hydrolyzed. The chiral selectivity is considerably diminished for the methyl phenyl substrate (**7**) (14-fold preference) and the stereoselectivity is essentially eliminated when the methyl substituent is replaced with other substituents. The loss in stereoselectivity displayed with these other pairs of racemic mixtures is also accompanied by poorer overall kinetic parameters by these same substrates. This latter result may indicate that neither enantiomer is optimally positioned within the active site with these compounds.

The demonstrated stereoselectivity toward this series of phosphotriesters suggests that OPAA will also display a similar discrimination against the various stereoisomers

Table 2. Kinetic constants determined from initial velocity assays

Substrate	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	$(k_{cat}/K_m)_{S_P}/(k_{cat}/K_m)_{R_P}$	k_1/k_2[a]
1	140 ± 10	1.4 ± 0.2	9.5 ± 2.0		
2	280 ± 10[b]	1.8[c]	>7.6		
3	480 ± 20	5.7 ± 1.3	12 ± 3		
4	530 ± 80	0.1 ± 0.02	0.2 ± 0.1		
5-(R_P)	90 ± 10	0.8 ± 0.1	8.3 ± 1.6	36	112
5-(S_P)	3250 ± 120	47 ± 10	14 ± 4		
6-(R_P)	190 ± 10	3.6 ± 0.2	19 ± 1	23	100
6-(S_P)	4510 ± 70	54 ± 3	12 ± 1		
7-(R_P)	2340 ± 60	1.8 ± 0.2	0.8 ± 0.1	4	14.5
7-(S_P)	9990 ± 250	40 ± 15	4.0 ± 1.6		
8-(R_P)	560 ± 10[b]	1.2[d]	>2.3	≤3	2.5
8-(S_P)	440 ± 10[b]	1.4[e]	>2.4		
9-(R_P)	300 ± 20	0.2 ± 0.03	0.6 ± 0.1	≤3	2.8
9-(S_P)	360 ± 10	0.7 ± 0.1	2.0 ± 0.4		
10-(R_P)	240 ± 10	0.1 ± 0.001	0.30 ± 0.04	≤3	3.5
10-(S_P)	150 ± 10	0.2 ± 0.05	1.3 ± 0.4		

[a]Determined from progress curves.
[b]Determined by a linear fit to data.
[c]Determined at a substrate concentration of 7.6 mM.
[d]Determined at a substrate concentration of 2.3 mM.
[e]Determined at a substrate concentration of 2.4 mM.

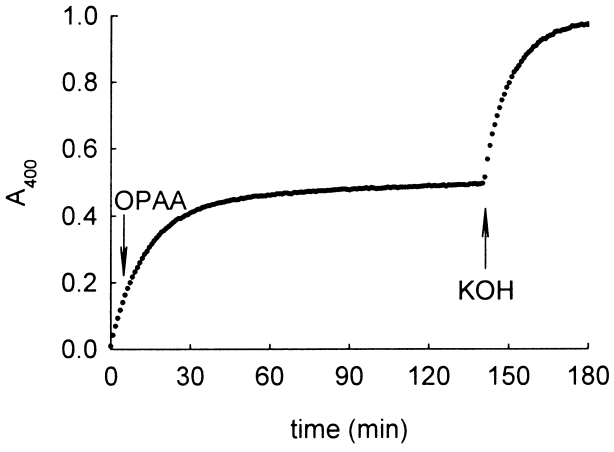
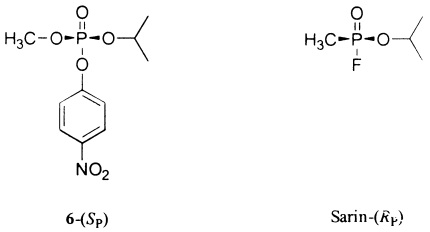


Figure 1. Time course for OPAA hydrolysis of racemic methyl isopropyl *p*-nitrophenyl phosphate.

of the toxic nerve agents, sarin and soman. We predict that the time courses for the OPAA catalyzed hydrolysis of sarin should proceed in two phases that reflect the configuration at the phosphorus center and that the R_P enantiomer of sarin will be the preferred substrate. This prediction is based on the relative structural similarities for the preferred isomers as illustrated in Scheme 3. Soman has four stereoisomers, each of which may be hydrolyzed at a different rate by the enzyme. The time course for the OPAA catalyzed hydrolysis of soman is expected to display at least two phases that are dependent upon the stereochemistry at the phosphorus center. An additional two phases may be observed that reflect the preference of the enzyme for the stereochemical configuration of the *O*-pinacolyl substituent. It has been reported that the S_P-isomer is more toxic than is the R_P-isomer.[13]



6-(S_P) Sarin-(R_P)

Scheme 3. The relative stereochemistry of the phosphotriester **6**-(S_P) and the phosphonofluoridate, sarin-(R_P).

Acknowledgements

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10. An *Escherichia coli* XL1 culture containing the plasmid pTCJS4 was grown at 37 °C in 5 L of LB containing 0.1 mM $MnCl_2$. Protein expression was induced by the addition of 0.6 mM IPTG to the 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 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 $MnCl_2$ (buffer A) and dialysed 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.
11. Continuous assays were conducted at 25 °C and carried out on a SPECTRAmax-340 microplate spectrophotometer (Molecular Devices Inc., Sunnyvale, CA, USA). Enzyme (50 μL, 1 μg) was dispensed into the wells of a multiwell plate. The assay was started by the addition of 200 μL of assay buffer to the enzyme using an *edp plus* Motorized Microliter Pippette (Rainin, Woburn, MA, USA) fitted with a multi-channel adapter. Hydrolysis of substrate was monitored at 400 nm (*p*-nitrophenol $\epsilon=17{,}000$ M^{-1} cm^{-1}). The assay buffer contained 50 mM bis-Tris propane (pH 8.5), 100 mM NaCl, 0.1 mM

$MnCl_2$. Diisopropyl-*p*-nitrophenyl phosphate (**3**) and substrates containing a single phenyl substituent were assayed in the presence of 5% methanol. Diphenyl-*p*-nitrophenyl phosphate (**4**) was assayed in the presence of 20% methanol. Time course assays were conducted at 25 °C on a Gilford 260 spectrophotometer. The assay was 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 exponential and double exponential time courses were fitted to eqs 1 and 2, respectively.

$$y = A_0(1 - e^{-k_0 t}) \quad (1)$$

In eq 1 A_0 is the initial substrate concentration and k_0 is the first order rate constant.

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

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.

$$v = k_{cat}EA/(K_m + A) \quad (3)$$

where E is the concentration of active sites and A is the substrate concentration. The differences in enantiomeric specificity ratios obtained by progress curves or by substrate saturation experiments are likely to arise from a small amount of the S_P enantiomer contaminating the R_P enantiomer so that the R_P enantiomer k_{cat} and hence the k_{cat}/K_m is over-estimated. In addition the high K_m values makes their precise measurement difficult. The more reliable estimate of the specificity ratio is obtained from the progress curves.

12. The substrates were synthesized as described in 9. The enantiomeric purity for each isomer was greater than 95%. The toxic properties of these compounds have not been determined.
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