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Received for review September 12, 1985. Accepted February 3, 1986. Supported by Grant DE01850 from the National Institutes of Health, Bethesda, MD.

Hydrolysis of 4-Nitrophenyl Organophosphinates by Arylester Hydrolase from Rabbit Serum

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The substrate specificity of arylester hydrolase partially purified from rabbit serum was studied. It was found that 10 of 13 4-nitrophenyl organophosphinate compounds tested were substrates for the enzyme. Michaelis constants were determined and ranged from 0.021 mM for 4-nitrophenyl methyl(2-furyl)phosphinate to 0.49 mM for 4-nitrophenyl bis(chloromethyl)phosphinate as compared to 0.61 mM for ethyl paraoxon. Specific activities for a number of other substrates were also determined for this enzyme.

Arylester hydrolase (arylesterase EC 3.1.1.2) is an enzyme that can detoxify the oxon metabolites of various organophosphorothioate insecticides such as paraoxon. The enzyme was first studied by Mazur (1946) and Aldridge (1953). Mazur found that rabbit serum was capable of hydrolyzing paraoxon and DFP. Aldridge demonstrated that the paraoxon hydrolyzing activity was enzymatic and that it was heat labile, pH dependent, and substrate concentration dependent. This enzyme has been referred to as paraoxonase, since paraoxon hydrolysis has been used to measure its activity, and also as phosphotriesterase because it does not hydrolyze monoesters of orthophosphoric acid (Aldridge and Reiner, 1972). Paraoxonase has been purified from sheep serum by Main (1960), but much of the work on paraoxonase specificity has been done with crude serum or tissue (Lenz et al., 1973; Zech and Zurcher, 1974; Chemnitius et al., 1983). Since there have been few purifications of arylesterase, its substrate specificity is unclear. A few organophosphonates were not hydrolyzed by rabbit serum (Lenz et al., 1973).

We report hydrolysis of certain 4-nitrophenyl organophosphinates by arylesterase partially purified from rabbit serum. The study of the metabolism of phosphinates is very important because they are a class of organophosphorus compounds that do not undergo the "aging" reaction and as such should not be capable of producing organophosphate-induced delayed neuropathy. These compounds provide transient protection against organophosphorus compounds that do cause delayed neuropathy (Johnson, 1982). Phosphinates could also provide prophylaxis against acute poisoning by phosphates and phosphonates.

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MATERIALS AND METHODS

Chemicals. Organophosphinates I–XIII were provided by the U.S. Army Medical Research Institute of Chemcal Defense (Aberdeen Proving Ground, MD) following synthesis by Ash-Stevens, Inc. (Detroit, MI) (Lieske et al., 1982, 1984). Paraoxon, MOPS [3-(3-morpholino)-propanesulfonic acid], 1- and 2-naphthyl acetate, 4-nitrophenyl acetate, and 4-nitrophenyl butyrate were from Sigma Chemical Co. (St. Louis, MO), methyl paraoxon was from the U.S. Environmental Protection Agency, and parathion and methyl parathion were from Monsanto Agricultural Products Co. Phenyl thioacetate, phenyl thiopropionate, and phenyl thiobutyrate were generously provided by Dr. A. R. Main, North Carolina State University. Ethanethiol and butyryl chloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ethyl thioacetate and ethyl thiopropionate were from Fairfield Chemical Co. (Blythewood, SC) and were distilled before use. Bio-Rad protein assay reagent was purchased from Bio-Rad Laboratories (Richmond, CA).

Handling of Organophosphinates. The organophosphinates have mammalian toxicities comparable to paraoxon and were handled accordingly. The LD_{50} values for some of the phosphinates in rats are published (Lieske et al., 1984).

Synthesis of Ethyl Thiobutyrate. Ethyl thiobutyrate was synthesized by an adaption of procedures described by Booth and Metcalf (1970). Butyryl chloride and ethanethiol were added in a 1.5:1 molar ratio and refluxed for 2 h. The reaction mixture was cleaned up by distillation.

0021-8561/86/1434-0513$01.50/0

Material that distilled at 115 °C and lower was discarded. The material that distilled between about 135 and 143 °C was collected and then run through a 1 × 4.5 cm activated silicic acid column with methylene chloride as the solvent. The methylene chloride was removed from the sample by nitrogen stream. Purity was checked by thin-layer chromatography on silica gel using hexane-ethyl acetate (60:40) as the developing solvent. The silica gel plates had a fluorescent dye (silica gel 60 F254; E. Merck, Darmstadt Germany) that enabled visualization of the spots under ultraviolet light. A sample of the ethyl thiobutyrate was checked for reaction with DTNB, prior to and after being treated with 1 M NaOH, to confirm that the sample was in fact a thioester.

Preparation of Arylester Hydrolase. The arylester hydrolase used for this study was purified 25-fold from rabbit serum. The purification involves two PEG 4000 fractionations and two DEAE-Sepharose column procedures. The 14–25% PEG 4000 fraction of the serum is resuspended in 10 mM MOPS with 2.5 mM $CaCl_2$ (pH 7.0) and applied to a DEAE-Sepharose column. A linear gradient, 0–400 mM NaCl in MOPS buffer, was run, and active fractions were pooled and dialyzed before being applied to a second DEAE column. The active fractions from the second DEAE column were pooled and frozen dropwise in liquid nitrogen and stored at −60 °C. Details of the complete purification procedrue are available (Zimmerman and Brown, 1986).

Determination of Enzyme Activity. Organophosphinates were assessed as substrates for paraoxonase in 1.2 mL of 0.1 M MOPS buffer (pH 7.5 with 2.5 mM calcium chloride) containing 10 μL of the organophosphinate in acetonitrile. Absorbance at 405 nm was measured at 22–24 °C on a Bausch and Lomb Spectronic 2000 UV/vis spectrophotometer against a blank lacking only the phosphinate and recorded on an X-Y recorder to obtain a rate of spontaneous hydrolysis. Then 25-μL enzyme solution was added, and the hydrolysis was determined. Replicate determinations were performed on separate days, each with five substrate concentrations. Replicates were done with at least two different enzyme preparations. Nonenzymatic hydrolysis was determined also by adding 25 μL of the same enzyme solution from an aliquot that had been denatured by holding in a sealed plastic microcentrifuge tube in a steam bath for 10 min.

Velocities of hydrolysis at the various substrate concentrations were corrected for nonenzymatic hydrolysis at each concentration in which the nonenzymatic rate exceeded 10% of total hydrolysis. Michaelis constants were obtained from computer analysis of Woolf–Augustinsson–Hofstee plots of v vs. $v/[s]$ (Segal, 1975).

Specific activities were measured with high substrate concentrations and diluted enzyme samples. To ensure saturation kinetics, assays were done at half the substrate concentration as that used for the V_{max} assay. If no difference in rate was observed, then it was assumed that the higher substrate concentration was saturating. The values were corrected for nonenzymatic hydrolysis as described above. Protein concentration was measured by the Bio-Rad Protein Assay with γ-globulin as the standard.

Enzyme assays for ethyl and methyl parathion and for 4-nitrophenyl acetate and 4-nitrophenyl butyrate were done the same as the saturating substrate assays for the phosphinates. The liberation of 4-nitrophenol in each case was followed continuously at 405 nm at 22–24 °C.

Assays for activity against ethylthio and phenylthio esters were done with Ellman's reagent (Ellman, 1959). The assay mixture consisted of 1 mL of MOPS buffer (pH 7.5), 1 mL of DTNB [5,5′dithiobis(2-nitrobenzoic acid)] in MOPS buffer (15.9 mg/100 mL), 20 μL of substrate in acetonitrile or acetone, and 20 μL of enzyme solution. The reactions were followed at 405 nm at 22–24 °C.

Table I. Michaelis Constants and Specific Activities of 4-Nitrophenyl Organophosphinates, Paraoxon, and Methyl Paraoxon with Arylester Hydrolase from Rabbit Serum

	substituents	R_t[a]	K_M, mM ± SE (n)	sp act., μmol/min per mg
		Phenyl Series		
I	methyl phenyl	3.85	0.0750 ± 0.047 (4)	2.9
II	ethyl phenyl	5.05	0.285 ± 0.048 (4)	0.64
III	isopropyl phenyl	6.58	[b] (2)	<0.0025
IV	diphenyl	9.68	[b] (2)	<0.0025
		Methyl Series		
V	methyl 2-furyl	3.12	0.0210 ± 0.014[c] (2)	1.4
VI	methyl 2-thienyl	3.68	0.0309 ± 0.0054 (4)	2.5
(I)	methyl phenyl	3.85	0.0750 ± 0.047 (4)	2.9
VII	methyl 2-naphthyl	7.31	0.124 ± 0.0621 (4)	0.19
		Heterocycle Series		
(V)	methyl 2-furyl	3.12	0.0210 ± 0.014[c] (2)	1.4
(VI)	methyl 2-thienyl	3.68	0.0309 ± 0.0054 (4)	2.5
VIII	di-2-thienyl	7.67	**0.0744 ± 0.0134 (4)**	**0.039**
		Halogen Series		
IX	bis(chloromethyl)	3.83	0.434 ± 0.104 (4)	nd
X	chloromethyl phenyl	5.74	0.0567 ± 0.0082 (4)	1.6
XI	dichloromethyl phenyl	8.89	0.167 ± 0.011 (4)	0.33
XII	trichloromethyl phenyl	15.87	[b] (2)	<0.0025
XIII	methyl (trifluoromethyl)phenyl	nd	0.123 ± 0.039 (3)	1.5
XIV	ethyl paraoxon	5.1	0.609 ± 0.023 (17)	0.056
XV	methyl paraoxon	nd	nd	0.036

[a] Retention times on octasilyl-bonded column from Brown and Grothusen (1984) corrected for dead volume. [b] Enzymatic hydrolysis was below detection. [c] Corrected for spontaneous hydrolysis.

HPLC of Organophosphinates. The retention times listed in Table I are from Brown and Grothusen (1984) for HPLC on octasilyl-bonded silica. The times have been corrected for dead volume.

Polyacrylamide Gel Electrophoresis. Native slab gel electrophoresis was run on 5% acrylamide gels, 1.5 mm thick, made with 0.37 M Tris-HCl (pH 8.8) with a 3% stacking gel made with 0.12 M Tris-HCl (pH 6.8). The gels were run at 10 °C in a 5 mM Tris, 38 mM glycine buffer (pH 9.0) with a constant current of 35 mA per slab gel. The gels were stained with 1- or 2-naphthyl acetate by using a modification of the procedure of Saul et al. (1976), in which MOPS buffer, 0.1 M MOPS, and 2.5 mM $CaCl_2$, pH 7.5, were used.

RESULTS AND DISCUSSION

The results of K_M determinations for the organophosphinates are presented in Table I. The values span a wide range but all are lower than that for ethyl paraoxon. In general the K_M increases with increasing size of the second substituent within a group and with the retention times of these phosphinates on octasilyl-bonded silica. The retention times listed are from Brown and Grothusen (1984) and are in excellent agreement with octanol–water partition values for these phosphinates (Lieske, C. N., personal communication). The only phosphinates tested that did not show measurable enzymatic or spontaneous hydrolysis were III, IV, and XII. Although the K_M is often used as a measure of substrate suitability, or of substrate affinity, it does not necessarily give information on how good a substrate is. A substrate with a low K_M could also have a low V_{max} and as such could be considered a competitive inhibitor. The results of saturating substrate

Table II. Specific Activities for Ester Compounds with Arylester Hydrolase from Rabbit Serum

	sp act., μmol/min per mg of protein
ethyl thioacetate	0.012
ethyl thioproprionate	0.010
ethyl thiobutyrate	0.0039
phenyl thioacetate	6.1
phenyl thioproprionate	0.71
phenyl thiobutyrate	0.22
4-nitrophenyl acetate	1.2
4-nitrophenyl butyrate	0.062
ethyl parathion	<0.002
methyl parathion	<0.002

assays for a number of the compounds are presented in Table I. These values for specific activity (μmol/min per mg protein) indicate that these organophosphinates are good substrates for this enzyme preparation, since the Michaelis constants are lower and the specific activities are higher than paraoxon.

Additional proof that the enzyme preparation is an A esterase is given in Table II. The specific activities for a number of other compounds studied are presented. Aldridge (1972) states that, for A esterases, acetate esters are hydrolyzed faster than butyrate esters. In all three groups of esters, the specific activity against the acetate compound is greater than that of the butyrate. It should also be noted that the phenyl compounds have higher specific activities than the ethyl compounds.

Incubation of the enzyme for 40 min at 25 °C in the presence of 0.18 mM *p*-chloromercuribenzoic acid inhibited more than 90% of the paraoxonase activity and more than 92% of the 4-nitrophenyl ethyl(phenyl)phosphinate hydrolysis. This is further evidence that the enzyme preparation is arylester hydrolase since Aldridge (1972) states that A esterases unlike B esterases are –SH enzymes and as such are inhibited by mercurials, or other reagents capable of forming mercaptides. The inhibition of phosphinate hydrolysis by the *p*-chloromercuribenzoic acid is further evidence that phosphinates are hydrolyzed by paraoxonase.

Native polyacrylamide gel electrophoresis was run on the enzyme preparations. Gels stained with 2-naphthyl acetate showed one red-staining esterase band only, and gels stained with 1-naphthyl acetate showed no esterase band.

This is the first known report of hydrolysis of phosphinates by arylester hydrolase. A goal of the work presented here is the development of structure–function relationships for this important detoxicating enzyme. We are currently extending this study to examine stereoselectivity of hydrolysis by using Pirkle chiral stationary phase HPLC columns (Grothusen and Brown, 1986).

ACKNOWLEDGMENT

This was Technical Contribution No. 2511 of the South Carolina Agricultural Experiment Station, Clemson University. This work was performed under Contract DAMD17-82-C-2193 from the U.S. Army Medical Research and Development Command, Fort Detrick, MD.

Registry No. I, 35691-25-9; II, 80751-40-2; III, 80751-39-9; IV, 10259-20-8; V, 81425-59-4; VI, 91308-90-6; VII, 91308-91-7; VIII, 81425-58-3; IX, 33714-89-5; X, 88144-99-4; XI, 81344-27-6; XII, 81344-26-5; XIII, 81542-84-9; XIV, 311-45-5; XV, 950-35-6; AcSEt, 625-60-5; EtC(O)SEt, 2432-42-0; PrC(O)SEt, 20807-99-2; AcSPh, 934-87-2; EtC(O)SPh, 18245-72-2; PrC(O)SPh, 18245-73-3; *p*-$AcOC_6H_4NO_2$, 830-03-5; *p*-$PrC(O)OC_6H_4NO_2$, 2635-84-9; ethyl parathion, 56-38-2; methyl parathion, 298-00-0; arylester hydrolase, 9032-73-9.

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Received for review June 20, 1985. Accepted January 27, 1986.