F. CLONING, SEQUENCING AND CHARACTERIZATION OF OPD GENES AND THEIR BROAD-SPECTRUM ORGANOPHOSPHATE HYDROLASES FROM SOIL BACTERIA

J.R. Wild*, L.L. Harper, C.S. McDaniel and F.M. Raushel
Departments of Biochemistry & Biophysics and Chemistry
Texas A&M University, College Station, Texas 77843

Two homologous organophosphate degrading genes (opd) have been identified and subcloned from nonhomologous plasmids isolated from divergent soil bacteria. These genes produce broad-spectrum organophosphate hydrolases which appear to have a membrane-associated somanase activity (see Albizo and White, this symposium). During late stages of growth, the enzyme activity is released into the growth media as a membrane-independent complex of 60Kd or associate. Characterization of the substrate specificity of the enzymes indicate that the nature of the triester linkage is critical to hydrolytic cleavage. While the substituent at the para-position has no effect on binding affinity, it does have a dramatic effect on catalytic activity. The genes have been subcloned and expressed in various bacterial backgrounds. The nucleotide sequences of the open reading frames of the two genes are identical even though neighboring regions show restriction site polymorphism and sequence divergence.

Synthetic organophosphorus compounds have been used as xenobiotic pesticides, insecticides, fungicides, herbicides and as other biological control agents. Although some of the modern organophosphates are thought to be among the safest of all chemical pesticides, compounds of dangerously high mammalian toxicity are also found in this group. Some of the more toxic organophosphates are potent agonists for mammalian acetylcholinesterase (AChE) and pose important detoxification challenges. A variety of organophosphate hydrolyzing enzymes have been identified by their ability to detoxify the powerful cholinesterase (Che) inhibitor and neurotic agent DFP (diisopropyl-phosphonofluoridate) (1). The "squid type" DFPase has been extensively purified (2) and it hydrolyzes DFP more rapidly than Soman (1,2,2-trimethyl propylmethylphosphonofluoridate). The enzyme has a molecular weight of 26 Kd and is narrowly distributed in specific organs of cephalopods (3). The "Mazur-type" DFPase has a larger molecular weight (45 - 65 Kd) and hydrolyzes Soman many times faster than DFP. Recent studies on the inhibition of DFPases by the insecticide Mipafox have suggested that Escherichia coli may contain a third species of DFPase (4). The "Mazur-type" DFPase is an ubiquitous class of enzymes which have been identified in various organisms from bacteria to the mammalian kidney; however, the enzymes are not identical. Studies with the bacterial DFPase or DFPases compared DFP and paraoxon specificity and concluded that the enzyme was not an A-esterase (5).

It has been observed that a variety of microbial systems may be involved in the degradation of organophosphate insecticides in the soil and associated water systems. Parathion has been reported to be readily degraded in the soil by a variety of bacteria, fungi and algae. Furthermore, a broad spectrum of other organophosphates (including phorate, dialkyl phenyl phosphates, carbophenothion, dichlorovos, and fenthion) appear to be substrates for microbial degradation (6). A variety of parathion-degrading microorganisms possess phosphotriesterases which can hydrolyze a broad spectrum of organophosphates including parathion and amino-parathion. The esterase hydrolyzes parathion to diethylthiophosphate and p-nitrophenol. Several of these parathion hydrolases have been partially characterized (7,8). In two cases, these enzymes have been demonstrated to be carried on specific bacterial plasmids (Pseudomonas diminuta, 9; and Flavobacterium, 10). The genes have been designated opd (organophosphate degrading) and have were transferred to plasmid or phage cloning vehicles and expressed in various bacterial hosts (10-12). The enzymes were observed to be membrane-associated and the Fl-enzyme was reported to have a molecular weight of approximately 60,000 d (7,8).

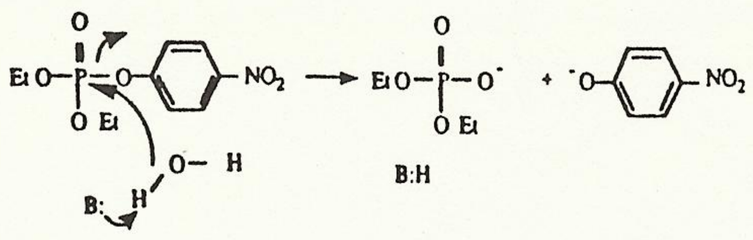
Phosphotriesterase substrate characterization. The substrate specificity of an acetone-extracted, cell-free extracts of the OP phosphotriesterase from P. diminuta MG (obtained from C. Serdar) was evaluated by gas chromatography analysis. The catalysis of 31 dialkoxy, organophosphates obtained from the Environmental Protection Agency was evaluated under gentle shaking at 37°C, pH 8.5 for 30'. The reaction was stopped with 100 microliters of 2-mercaptoethanol, extracted with hexane, dried and concentrated (13). Five microliters was analyzed in a Tracor gas chromatograph at various temperatures on a 6' x 2mm glass column with 3% OV101 and monitored in a nitrogen-phosphorus detector. The results summarized in Table 1 indicate that the nature of the triester linkage is critical to its susceptibility to hydrolytic cleavage. Alterations to the acid leaving moiety appears to stabilize the ester linkage (compare the effect of the addition of an electrophilic halogen substituent such as parathion vs bromophos ethyl). Thiol esters seems to be much less susceptible although there may be slight cleavage of some of these (e.g. malathion). Dimethyl substitution of the phosphorate and thiophosphorate moieties dramatically reduces hydrolysis. In addition, there may be small differences attributable to the shift between sulfones and oxones.

TABLE 1
Degradation of Organophosphates by Pd-phosphotriesterase

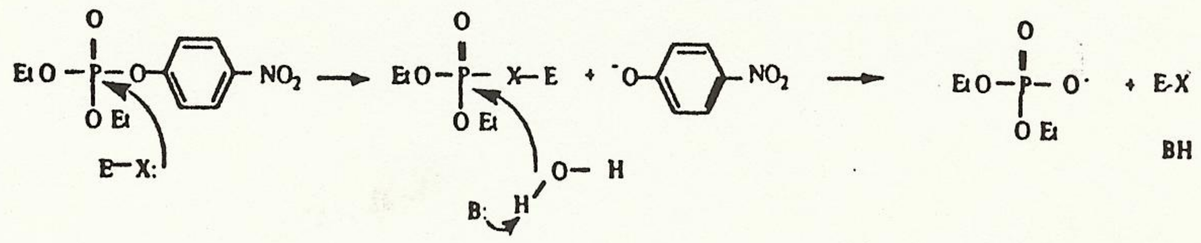
GROUP I		GROUP II		GROUP III
Bromophos-ethyl	53.2	Chlorfenvinphos	7.4	Chlorpyrifos-methyl
Coumaphos	100.0	Chlorothiophos	11.4	Ethoprop
Diazinon	72.1	Chlopyrifos	37.2	Etrimifos
Fensulfothion	100.0	Crufomate	0.9	Famphur
Leptophos-oxon	100.0	Dichlofenthion	32.6	Fenthion
Parathion	98.4	EPN	29.3	Isofenphos
Paraoxon	100.0	Fenitrothion	7.9	Jodfenphos
Pirimiphos-ethyl	94.3	Malathion	16.4	Ronnel
Pyrazophos	89.1	Parathion-methyl	31.3	Sulfopros
Quinalphos	99.3	Pirimiphos-methyl	17.1	Sulfotepp
		Trichloronate	19.8	

More detailed studies have been performed in order to describe the mechanism of catalysis and describe the nature of substrate specificity. At least three mechanisms can be written for hydrolytic cleavage of paraoxon (chosen as the model substrate for better aqueous solubility).

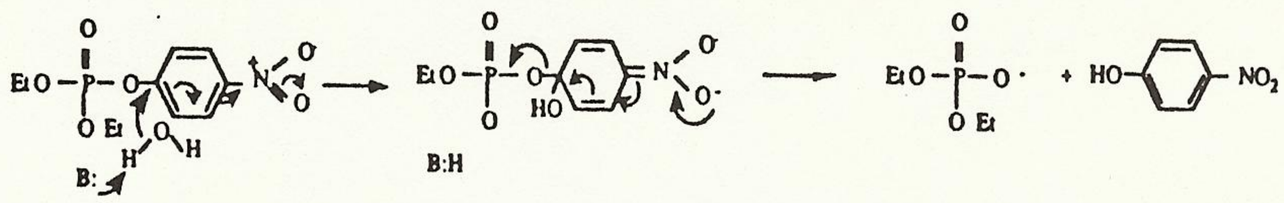
A.



B.



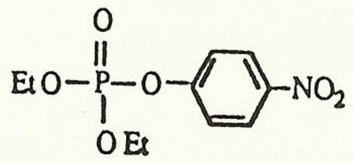
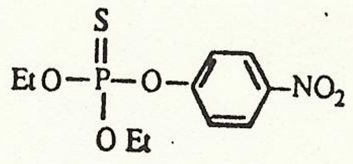
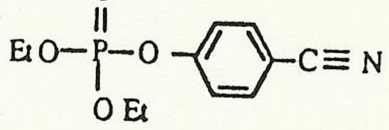
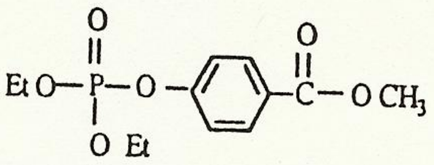
C.



These three mechanisms can be distinguished by appropriate labelling experiments in oxygen-18 H_2O. If mechanism C is operating, hydrolysis in the presence of water will result in the O-18 exclusively associated with p-nitrophenol, whereas mechanism A or B will leave the label associated with diethylphosphate. The 31P-NMR spectra of diethylphosphate was determined after enzyme-catalyzed hydrolysis of paraoxon in the presence of 70% oxygen-16 and 30% oxygen-18 water. The diethylphosphate clearly showed the incorporation of oxygen-18 water as indicated by the 0.03 ppm upfield chemical shift relative to standards containing only oxygen-16. Mechanism C can thus be eliminated. It will be possible to differentiate between mechanisms A and B through the analysis of a chiral phosphotriester (studies in progress, F.M. Raushel). A detailed examination of the pH profile of the Pd-enzyme reveals that there is a single break at pH 6.2 relative to both Vmax and Vmax/Km. This would appear to represent the titration of the basic residue responsible for activation of the water molecule implicated in the reaction mechanism. There is no indication of an acidic group at high pH that could be used to protonate the phenol (data presented, not published here).

A series of phenol substituted phosphotriesters have been recently synthesized and the relative Km and Vmax values are indicated in Table 2.

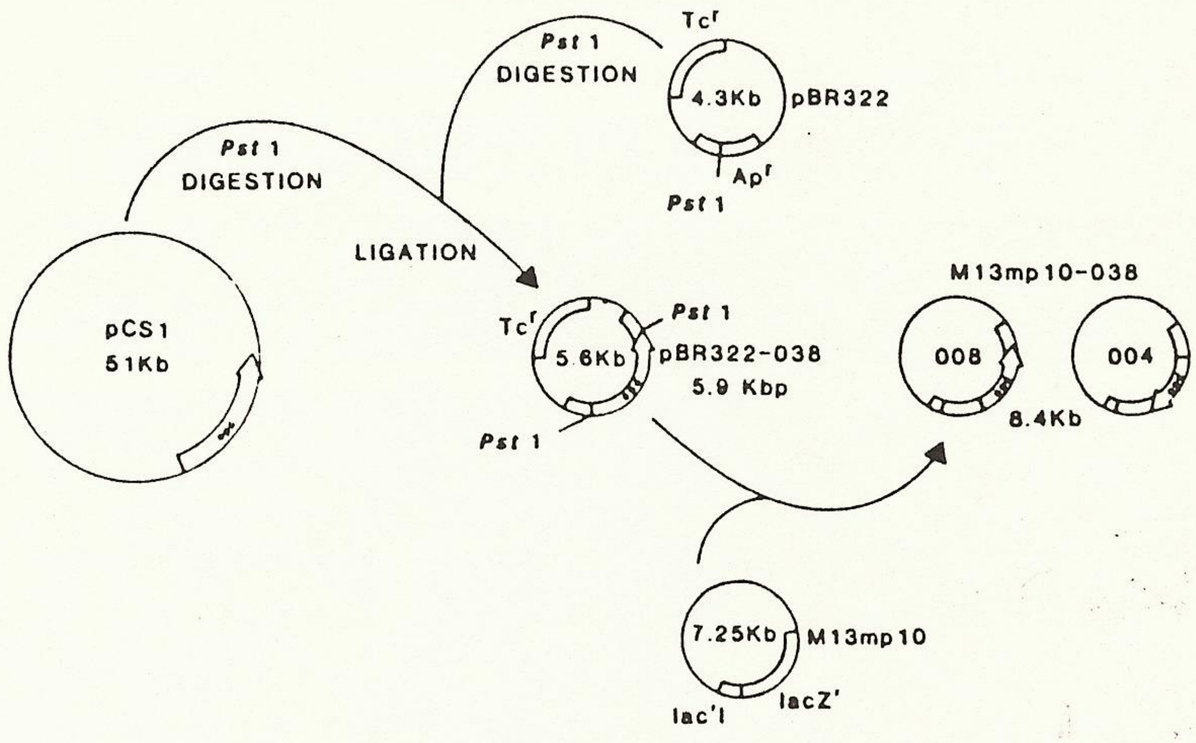
TABLE 2
Comparison of Phenol Substituted Phosphotriesters.

	Vmax	Km
	100	0.0514
	34.3	0.177
	30.0	0.0177
	6.9	0.0232

Several analogues were completely inactive as substrates ($-CH_3$ and -F) and it appears that the determinative characteristic may be the pKa generated by the phenol complex. In addition, various para-substituted phosphotriesters have been evaluated as competitive inhibitors for paraoxon hydrolysis (data presented, not published here). The Ki values ranged from 0.039 for the cyano-substituted phenol to 0.222 for the non-substituted phenol. The relatively constant values indicate that the nature of the substituent at the para positon has no effect on binding affinity but it does have a dramatic effect on hydrolytic cleavage.

Cloning and sequencing of the opd genes. The plasmid CS1 carrying the opd gene from P. diminuta MG was isolated by a mild lysis procedure adapted from Berns and Thomas (14). Isolated plasmid DNA was restricted with a variety of restriction endonucleases and the resulting fragments were sized by agarose electrophoresis. The plasmid was approximately 50 Kb based on independent digests with 4 restriction endonucleases (PstI, BamHI, SmaI, and HindIII). This size differs from that reported earlier by electron microscope estimation (9) but similar EM-analysis in our laboratory (12) provided that same estimation. The entire degradative plasmid was subcloned into the PstI site of the bla gene of pBR322 and parathion-degradating transformants of E. coli HB101-4442 (15) were identified. Each of the active transformants were identical (pBR322-038) and carried a 1300 bp insert. This fragment was isolated and transferred into the polyclonal region of M13mp10 where it became obvious that external promotion was necessary for expression. In one orientation (008) (Figure 1) opd was expressed and in the other (004) it was not. Subsequent studies have revealed that expression can be enhanced by removing approximately 250bp of the 5'-flanking sequence of the cloned fragment and introducing it into a tac expression vector (16). Upon induction with IPTG (isopropyl-thiogalactoside) the specific activities of the op-phosphotriesterase in E. coli are similar to those in the native host. The same procedure has been utilized to enhance the expression of the initially cloned Flavobacterium gene (10).

FIGURE 1
Cloning of opd from P. diminuta MG.



The DNA sequences for both of the opd genes are virtually identical through their 846 bp open reading frame and should produce an amino acid with 282 amino acids (data presented, not published here). An interesting feature of the homologous structural gene regions is that they are contained in plasmids of different size and possessing neighboring restriction site polymorphism (10). CS1 from P. diminuta is estimated to be 50Kb by restriction fragment analysis (60-70 Kb by other analyses) and pPDL2 from Flavobacterium is approximately 40Kb. The DNA sequence homology begins to diverge in the 5'-flanking region of the genes and there is no evidence of homology outside of the common 1300 bp regions.

Characterization of the OP-phosphotriesterases. The phosphotriesterases from both native bacterial hosts and their tac cloned opd genes expressed in E. coli are membrane-associated during active growth and expression is constitutive, unrelated to growth phase. However, at later growth phases, activity begins to appear in the supernatant. When chromatographed by molecular weight sieves, the activity was distributed between a large, heterogenous membrane associated fraction (Mr > 300,000 d) and a discrete molecular weight species of 60,000 d. It was possible to use detergents to chase OPD activity from its membrane-association into the free aggregate. This form of the enzymes appears to be a dimer of approximately 30,000 d (data not shown). Triton X-100 and Tween 20 (.1 - 1%) were effective for releasing the enzyme, while SDS, CETAB and laurylsarcosine disrupted activity. It is possible to release greater than 90% of the enzymatic activity associated with whole cells by a hypertonic wash (0.25 - 1.0M NaCl). Most of the activity is associated with a membrane component which is indistinguishable from that released into the supernatant. The enzyme, in its various forms, is relatively stable at a variety of temperatures if the pH is maintained between 8.5 and 9.0. In addition, the enzyme is active in various organic solvents (MeOH, Dioxane and DMF) up to 20-25% but then becomes irreversibly inactivated. Studies are underway to detail the stability of the free form of the enzyme. The membrane-associated form has been frozen and kept at room temperature for months without appreciable loss of activity.

CONCLUSIONS

Two genes which produce a broad-spectrum organophosphate hydrolase have been cloned from different plasmids isolated from soil bacteria. The DNA sequences of the two genes have been determined and the nature of the substrate specificity is being examined. The enzyme appears to have an extremely broad binding affinity for organophosphate but significantly more limited hydrolytic cleavage. The combination of these studies will permit the genetic manipulation of the opd genes in order to design specific catalysis. In studies that are more important for the nearer time frame, it has been possible to construct expression systems so that enzyme can be produced in high concentration in E. coli and other bacterial hosts. This will result in greater availability of the enzyme from bacterial systems that can grow more quickly and in more limited environments.

ACKNOWLEDGMENTS

This research was supported by the U.S. Army Research Office, contract DAAG29-84-0075 and the Texas Agricultural Experiment Station, H6458.

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