Augmented Hydrolysis of Diisopropyl Fluorophosphate in Engineered Mutants of Phosphotriesterase*

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The phosphotriesterase from *Pseudomonas diminuta* hydrolyzes a wide variety of organophosphate insecticides and acetylcholinesterase inhibitors. The rate of hydrolysis depends on the substrate and can range from 6000 s^{-1} for paraoxon to 0.03 s^{-1} for the slower substrates such as diethylphenylphosphate. Increases in the reactivity of phosphotriesterase toward the slower substrates were attempted by the placement of a potential proton donor group at the active site. Distances from active site residues in the wild type protein to a bound substrate analog were measured, and Trp[131], Phe[132], and Phe[306] were found to be located within 5.0 Å of the oxygen atom of the leaving group. Eleven mutants were created using site-directed mutagenesis and purified to homogeneity. Phe[132] and Phe[306] were replaced by tyrosine and/or histidine to generate all combinations of single and double mutants at these two sites. The single mutants W131K, F306K, and F306E were also constructed. Kinetic constants were measured for all of the mutants with the substrates paraoxon, diethylphenylphosphate, acephate, and diisopropylfluorophosphate. V_{max} values for the mutant enzymes with the substrate paraoxon varied from near wild type values to a 4-order of magnitude decrease for the W131K mutant. There were significant increases in the K_m for paraoxon for all mutants except F132H. V_{max} values measured using diethylphenylphosphate decreased for all mutants except for F132H and F132Y, whereas K_m values ranged from near wild type levels to increases of 25-fold. V_{max} values for acephate hydrolysis ranged from near wild type values to a 10^3-fold decrease for W131K. K_m values for acephate ranged from near wild type to a 5-fold increase. V_{max} values for the mutants tested with the substrate diisopropylfluorophosphate showed an increase in all cases except for the W131K, F306K, and F306E mutants. The V_{max} value for the F132H/F306H mutant was increased to 3100 s^{-1}. These studies demonstrated for the first time that it is possible to significantly enhance the ability of the native phosphotriesterase to hydrolyze phosphorus-fluorine bonds at rates that rival the hydrolysis of paraoxon.

The phosphotriesterase (PTE)[1] from *Pseudomonas diminuta* has a rather broad substrate specificity. This protein has been

shown to catalyze the hydrolysis of the insecticides parathion, paraoxon, coumaphos, methylparathion, and diazonin, among others (1). The enzyme also detoxifies the potent acetylcholinesterase nerve agents sarin, soman, and diisopropylfluorophosphate (2). The enzymatic reaction mechanism has been shown to proceed via an S_N2-like process whereby an activated water molecule attacks the phosphoryl center resulting in the displacement of the leaving group with an inversion of stereochemical configuration (3). The active site of this enzyme is comprised primarily of a binuclear metal cluster that contains Zn^{2+} but can also accommodate Co^{2+}, Mn^{2+}, Cd^{2+}, or Ni^{2+} with full retention of catalytic activity (1, 4). High resolution x-ray crystallography of the Cd^{2+}-substituted enzyme has established the identity of the metal binding ligands (5). The crystal structure of the Zn^{2+} enzyme, with a bound nonhydrolyzable substrate analog, has illustrated the probable orientation of the substrate relative to the binuclear metal center (6). The more buried zinc interacts with His[55], His[57], Asp[301] and two bridging ligands in a distorted trigonal bipyramidal geometry. The more solvent exposed zinc interacts directly with His[201], His[230], and the two bridging ligands in a distorted tetrahedral geometry. The two bridging ligands are a carbamylated lysine residue (Lys[169]) and a solvent water molecule.

The bacterial phosphotriesterase catalyzes the hydrolysis of paraoxon at the diffusion controlled limit. The Co^{2+}-substituted enzyme has a reported k_{cat} and k_{cat}/K_m for paraoxon hydrolysis of nearly 10^4 s^{-1} and 10^8 M^{-1} s^{-1}, respectively (4). However, an extensive structure reactivity analysis for the hydrolysis of paraoxon analogues has clearly demonstrated that the magnitude of these kinetic constants is very much dependent on the pK_a of the leaving group phenol (7). Moreover, the rate-limiting step changes from diffusion of the enzyme-substrate or enzyme-product complex to hydrolytic cleavage of the P-O bond when the pK_a of the phenol exceeds ~7. The Brønsted value (β_{lg}) for this series of compounds with pK_a values exceeding 7 is approximately −2. Therefore, the rate for the enzymatic hydrolysis of paraoxon with a *p*-nitrophenolate leaving group ($pK_a = \sim 7$) is nearly a million times faster than is diethylphenylphosphate with a phenolate leaving group ($pK_a \sim 10$). The very substantial Brønsted value for this reaction indicates two important factors about the enzymatic machinery at the active site of this protein. First, the transition state for the hydrolysis of paraoxon analogues is very late, suggesting that the P-O bond is nearly fully broken. Second, the enzyme does not appear to be contributing to the activation of the leaving group through Lewis acid catalysis, via complexation with the binuclear metal center, or by general acid catalysis through protonation of the phenolic oxygen from a nearby acidic residue. In addition, it would appear that the environment of the leaving group binding site is quite hydrophobic.
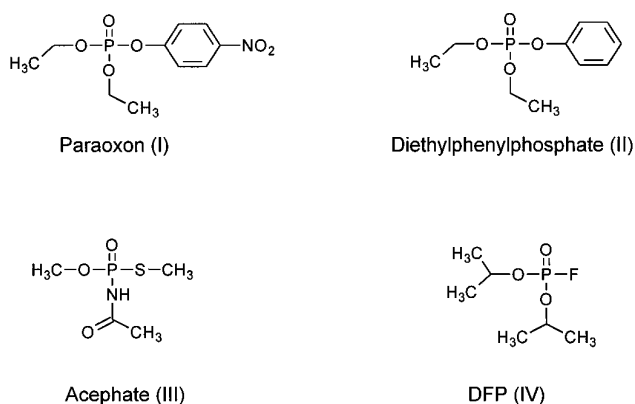
[1] The abbreviations used are: PTE, phosphotriesterase; DEPP, dieth-

ylphenylphosphate; DFP, diisopropyl fluorophosphate; PCR, polymerase chain reaction.

SCHEME 1

Indeed, the crystal structure of phosphotriesterase shows that the binding site for the putative leaving group is lined with a series of very hydrophobic residues (6). These amino acids include Trp[131], Phe[132], Leu[271], Phe[306], and Tyr[309]. The side chains from Trp[131], Phe[132], and Phe[306] are oriented toward the oxygen of the leaving group, but there are no acidic residues in the immediate vicinity that could potentially donate a proton to the leaving group during P-O bond cleavage. Therefore, mutants of phosphotriesterase were created with the goal of transforming this protein into an enzyme that would be capable of hydrolyzing the poorer substrates, containing leaving groups with high pK_a values, at a faster rate. An additional objective was to enhance the rate of phosphofluoridate hydrolysis. It was anticipated that the introduction of potential hydrogen bond donors to the leaving group pocket would effectively lower the pK_a of the leaving group and increase the hydrophilic character of this site. These changes would potentially increase the rate of the reaction with some substrates through alteration of the Brønsted β value. The side chains of the closest amino acids (Trp[131], Phe[132], and Phe[306]) were mutated to residues capable of hydrogen bond formation and proton donation to the leaving group. The catalytic effects of these mutations were determined for the hydrolysis of paraoxon (I), diethylphenylphosphate (II), acephate (III), and diisopropylfluorophosphate (IV) (Scheme 1).

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma, Aldrich, Fisher, or U. S. Biochemical Corp. Bacto-tryptone and Bacto-Yeast extract were acquired from Difco Laboratories. PCR amplification was conducted using Vent$_R$ DNA Polymerase (New England Biolabs), and dNTPs were obtained from Life Technologies, Inc. T4 DNA ligase, restriction enzymes, Magic Miniprep DNA purification kits, and Magic PCR Prep DNA purification kits were purchased from Promega. Gene-Clean DNA purification kits were obtained from Bio 101. The synthesis of all oligonucleotides and DNA sequencing reactions were carried out by the Gene Technology Laboratory at Texas A & M University.

Bacterial Strains and Plasmids—The *Escherichia coli* strains used for this study were XL1-Blue (8) and BL21 (9). The pBS$^+$ phagemid (Stratagene) was used as the vector for all genetic manipulations. The reconstructed *opd* gene encoding the mature phosphotriesterase (pJK01) was used as the initial template in the mutagenesis experiments (10).

Site-directed Mutagenesis—The individual mutations (F132H, F132Y, F306H, F306Y, F306E, F306K, and W131K) were generated using the method of overlap extension PCR as described previously (11). After amplification, the mutagenic PCR fragments were purified from an agarose gel, cut with *Bam*HI, concentrated with *Bam*HI-digested pBS$^+$, ligated with T4 DNA ligase, and transformed into XL1-Blue cells. The double mutants (F132H/F306H, F132H/F306Y, F132Y/F306H, and F132Y/F306Y) were prepared by taking advantage of the unique *Sty*I and *Eco*RV restrictions sites on each side of the codon for the phenylalanine at position 132. The mutant plasmids containing either the single tyrosine or histidine mutation at position 132 was digested with *Sty*I and *Eco*RV to obtain a fragment containing the mutation at this site. This oligonucleotide fragment was then ligated into an identically cut plasmid containing only the single mutation at position 306. The *opd* gene in each of the mutants generated from PCR were completely sequenced to ensure that only the desired base changes were present.

Expression and Purification of Mutant Enzymes—Overnight cultures of the transformed BL21 cells grown in Luria-Bertani (LB) broth (12) were used to inoculate Terrific broth (TB) containing 50 µg/ml ampicillin and 1 mM CoCl$_2$. The cultures were incubated at 30 °C and induced with IPTG in early log phase, and the cells harvested in stationary phase. Mutant enzymes were purified from BL21 cells according to previously reported procedures (4, 10). SDS-polyacrylamide gel electrophoresis indicated that the isolated proteins were greater than 95% pure.

Preparation and Reconstitution of Apo-enzyme—Apo-enzyme was prepared by incubating each enzyme with 2 mM *o*-phenanthroline at 4 °C overnight or until there was less than 1% residual activity. The *o*-phenanthroline was removed by diafiltration with metal-free 50 mM HEPES (pH 8.5) buffer using an Amicon PM10 membrane or by passing the solution through a Pharmacia PD-10 desalting column. Apo-enzyme was reconstituted with the desired metal by adding 5 mol equivalents of either ZnSO$_4$, CdSO$_4$, or CoCl$_2$ in the presence of 10 mM KHCO$_3$ at 4 °C for at least 2 h.

Enzyme Assays and Data Analysis—The rate of paraoxon hydrolysis by each of the mutants was measured by monitoring the appearance of *p*-nitrophenol at 400 nm (ϵ = 17,000 M^{-1} cm^{-1}) at pH 9.0 (10). The rate of hydrolysis of diethylphenylphosphate (DEPP) was measured by monitoring the appearance of phenol at 280 nm (ϵ = 1,130 M^{-1} cm^{-1}) as previously reported (7). The rate of hydrolysis of acephate (*O*,*S*-dimethyl *N*-acetylphosphoramidothioate) was measured by monitoring the appearance of product at 412 nm (ϵ = 13,600 M^{-1} cm^{-1}) in the presence of 5,5′-dithiobis (2-nitrobenzoic acid) (13) as previously reported (14). The rate of hydrolysis of diisopropylfluorophosphate (DFP) was determined by monitoring the appearance of fluoride ion with an Orion perpHect 570 pH Meter equipped with an Orion combination fluoride ion electrode as described previously (15). The kinetic constants were obtained by a fit of the data to Equation 1 using the software supplied by Savanna Shell Software, where v = velocity, V = maximum velocity, A = substrate concentration, and K_a is the Michaelis constant.

$$v = VA/(K_a + A) \tag{Eq. 1}$$

RESULTS

Construction, Expression, and Purification of Mutant Enzymes—The W131K, F132H, F132Y, F306E, F306H, F306K, and F306Y phosphotriesterase mutants were made using the method of overlap extension PCR. The F132H/F306H, F132H/F306Y, F132Y/F306H, and F132Y/F306Y double mutants were constructed directly from the single site mutants by taking advantage of the unique *Sty*I and *Eco*RV restriction sites within the PTE gene. The isolated *Sty*I/*Eco*RV restriction fragments from the F132H and F132Y mutants were substituted for the corresponding fragments within the already altered genes for the F306H and F306Y mutants. The genes for the mutant proteins were fully sequenced and shown to contain only the desired modifications. The mutant proteins were expressed in BL21 cells and purified to homogeneity.

Paraoxon Hydrolysis—The kinetic parameters for paraoxon hydrolysis were determined for the mutant enzymes reconstituted with Co^{2+}, Cd^{2+}, and Zn^{2+}. These kinetic constants are presented in Table I. In general, the single site mutants were more active than the mutants constructed at two sites simultaneously. With the cobalt-substituted enzymes, the single site mutations made at Phe[132] and Phe[306] with either tyrosine or histidine replacements had approximately the same V_{max} as the wild type enzyme. However, the mutants made at Phe[306] all had Michaelis constants that were elevated by an order of magnitude or more, whereas the two mutants made at Phe[132] had essentially no changes in the K_m values. With the double mutants, the K_m values were further elevated, whereas the V_{max} values were reduced relative to the single site mutations. The K_m value for the F132H/F306Y double mutant was significantly greater than the solubility of paraoxon, and thus an accurate value could not be obtained for either K_m or V_{max}, but

DFPase Activity in Mutants of PTE

TABLE I
Hydrolysis of paraoxon by phosphotriesterase

PTE enzyme	Co^{2+}-reconstituted enzyme		Cd^{2+}-reconstituted enzyme		Zn^{2+}-reconstituted enzyme	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
	mM	*s^{-1}*	*mM*	*s^{-1}*	*mM*	*s^{-1}*
Wild type	0.3	5100	0.67	2500	0.052	780
W131K	0.58	0.85	0.95	2.1	ND	ND
F132Y	0.71	1900	1.2	740	0.30	1700
F132H	0.35	6600	1	2800	0.16	2500
F306Y	8.6	7000	6.6	360	1.9	870
F306H	2.4	2300	4	370	1.8	320
F306K	17	1500	2.2	31	14	440
F306E	4	38	0.34	1.2	2.3	5.1
F132H/F306H	16	2000	5	67	14	230
F132Y/F306Y	9.5	590	2.9	27	3.2	230
F132Y/F306H	4.4	860	4.4	85	3.4	140
F132H/F306Y	ND[a]	ND	2.8	86	6	750

[a] ND, not detected.

the V/K_m value was determined to be 280 M^{-1} s^{-1}. The F306E mutant was the least active catalyst of any of the mutants made at this position, whereas the W131K mutant was reduced in activity by over 3 orders of magnitude.

Similar trends were observed with these proteins for the hydrolysis of paraoxon when the mutants were substituted with either Cd^{2+} or Zn^{2+}. The single site mutants were more active than the double mutants, and the mutations made at Phe[306] were less active than those made at Phe-132. In nearly every case, the zinc-substituted mutants were more active than the cadmium-substituted enzyme, even though the wild type enzyme is more active with cadmium than with zinc. The W131K and F306E mutants were the least active mutants with either Zn^{2+} or Cd^{2+} as the divalent cationic activator.

Hydrolysis of Diethylphenylphosphate, Acephate, and Diisopropyl Fluorophosphate—The mutant enzymes were also tested for their ability to hydrolyze the alternate substrates diethylphenylphosphate, acephate, and diisopropyl fluorophosphate. The kinetic constants obtained with these substrates are presented in Table II for the cobalt-substituted enzyme. Except for the two mutants made at position Phe[132], all of the mutants were less active than the wild type enzyme for the hydrolysis of the slow substrate, diethylphenylphosphate. In every case the K_m values were elevated except for the F306E mutation. The W131K mutant possessed catalytic activity barely detectable above background (<0.0006 s^{-1}) at a substrate concentration of 2.3 mM.

For the wild type enzyme, the hydrolysis of acephate is relatively slow, whereas the Michaelis constant is quite high. Similar parameters were observed with all of the mutants constructed for this investigation. The V_{max} values for the F132Y, F132H, F132Y/F306Y, and F132H/F306Y mutants were essentially the same as the wild type enzyme, whereas the F306Y mutant doubled in activity. The rest of the mutants were reduced in activity about an order of magnitude with the exception of W131K, where no activity could be detected above background. The K_m values for all mutants were elevated by factors ranging from 1 to 5.

Substantial improvements in substrate turnover were observed for most of the mutants relative to the wild type enzyme when DFP was utilized as the test substrate. The V_{max} values increased by over an order of magnitude for the double mutant F132H/F306H, whereas slightly smaller enhancement factors were observed for either of the phenylalanine or histidine substitutions at Phe[132] or Phe[306]. The glutamate substitution for Phe[306] was approximately half as active as the wild type enzyme, whereas the W131K mutant was reduced in activity by 3 orders of magnitude. With all of the mutants, the K_m values for

DFP were elevated by factors ranging from 2 to 100. The k_{cat} and k_{cat}/K_m values for the various mutants with DFP as a substrate are graphically presented in Fig. 1.

DISCUSSION

Most of the reported biochemical studies employing site-directed mutagenesis as an investigative tool in enzyme chemistry have been directed toward the elucidation of the specific roles of individual amino acids in catalysis, substrate binding, and maintenance of the overall protein structure. However, successful perturbations to the native substrate specificity and associated enhancements in the magnitude of the residual catalytic activity have been achieved with a more limited but growing repertoire of enzymes. To be marginally successful, a rational redesign of an enzyme active site to one with modified properties requires a comprehensive and detailed understanding of the catalytic mechanism of the protein under study, coupled with the availability of high resolution structural data. For example, the catalytic properties of papain have been transformed from a cysteine protease to a nitrile hydratase with a single point mutation (16). The substrate specificity for subtilisin has been reconfigured to accommodate substrates with large hydrophobic side chains by making multiple mutations within the ligand binding pocket (17). The coenzyme specificity for isocitrate dehydrogenase has been changed from NADP to NAD by the introduction of mutations that removed favorable interactions for the binding of NADP (18). In addition, a novel hydratase activity has been introduced into D-alanyl-D-alanine ligase by mutation of three amino acids within the active site (19). Other successful examples have also been described (20).

The catalytic and structural properties of the phosphotriesterase from *P. diminuta* have been extensively studied, making it an ideal target for programmed alterations in substrate specificity and catalytic activity. The chemical mechanism has been elucidated using structure-reactivity relationships, heavy atom isotope effects, and changes in solvent viscosity (7, 21, 22). Several mutagenic studies have been successfully employed for the identification of active site ligands and their probable role in catalysis and binding (10, 23, 24). High resolution crystal structures have been solved for the apo-enzyme and the Cd^{2+}-substituted protein in addition to the structure for Zn^{2+}-PTE complexed with a nonhydrolyzable substrate analogue bound at the active site (5, 6, 25). Although the bacterial phosphotriesterase has a rather broad substrate specificity, the actual rate of hydrolysis varies over many orders of magnitude, depending on the particular alteration in the structure of the target compound. The successful redesign of the substrate spec-

TABLE II
Hydrolysis of DEPP, acephate, and DFP with Co^{2+}-phosphotriesterase

PTE enzyme	DEPP		Acephate		DFP	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
	mM	*s^{-1}*	*mM*	*s^{-1}*	*mM*	*s^{-1}*
Wild type	0.33	0.029	86	1.5	0.57	290
W131K	ND[a]	<0.0006	ND	<0.002	2.48	0.33
F132Y	1.4	0.047	120	1.3	2.4	2300
F132H	0.99	0.038	440	1.3	1.2	1400
F306Y	4.4	0.022	150	3.3	1.5	990
F306H	2.5	0.011	53	0.19	16	1400
F306K	1.3	0.0043	160	0.23	51	120
F306E	0.25	0.0024	120	0.085	ND	ND
F132H/F306H	2.4	0.013	69	0.13	10	3100
F132Y/F306Y	2.1	0.0028	210	1.49	4.8	1300
F132Y/F306H	1.3	0.0049	77	0.14	13	2400
F132H/F306Y	8.2	0.018	420	1.5	3.1	1400

[a] ND, not detected.

FIG. 1. **Kinetic constants for the hydrolysis of diisopropylfluorophosphate by phosphotriesterase mutants.** *A*, bar graph of k_{cat} for each mutant. The k_{cat} for W131K was 0.33 s^{-1}, and the k_{cat} for F306E was not determined. *B*, bar graph for *V/K* with diisopropylfluorophosphate for each mutant. The *V/K* for W131K was 0.13 mM^{-1} s^{-1}, and the *V/K* for F306E was 2.4 mM^{-1} s^{-1}.
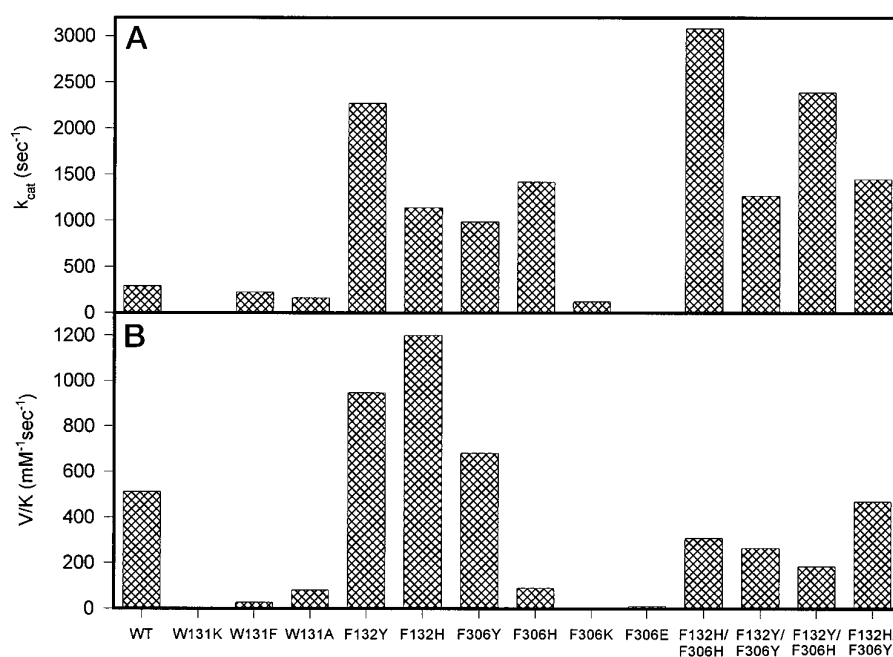
ificity for the bacterial phosphotriesterase would have significant implications for the practical destruction of chemical warfare agents and bioremediation of pesticide contaminated soils. However, for phosphotriesterase to become a particularly effective tool for these endeavors, mutant enzymes must be constructed with enhanced catalytic activity for these slower substrates.

A partial explanation for the broad substrate specificity of the bacterial phosphotriesterase can be found by examination of the three-dimensional structure of the enzyme (6). There are relatively few direct interactions of the bound inhibitor with the side chains of the amino acids found in the active site. However, electrostatic interactions apparently occur between the phosphoryl oxygen of the substrate and the side chains for two amino acids in the active site of phosphotriesterase, Trp[131] and His[201]. The environment of the binding site for the remainder of the substrate has been identified and consists primarily of hydrophobic residues. The pocket that holds the leaving group of the substrate is lined with Trp[131], Phe[132], Leu[271], Phe[306], and Tyr[309]. Three of the side chains from these amino acids are oriented in a way to potentially act as hydrogen bond donors upon mutation to other residues as illustrated in Fig. 2*A*. A model for the structure of the F132Y/F306Y mutant is presented in Fig. 2*B*. The overall effectiveness of adding hydrogen bond donors to the active site to facilitate leaving group cleavage was tested by creating several mutations at these sites. Trp[131] was mutated to lysine, and Phe[132] and Phe[306] were mutated to histidine and/or tyrosine.

The crystal structure of PTE shows that Trp[131] resides between the leaving group pocket and the pocket modeled for the *pro-R* ethoxy group of paraoxon (6). This residue also interacts with the phosphoryl oxygen of the substrate. The mutation of Trp[131] to an alanine or phenylalanine has been characterized previously (24). W131F displayed essentially wild type activity, whereas the W131A mutant exhibited a decreased affinity for paraoxon. The mutation of Trp[131] to lysine has a much more dramatic consequence. Depending on the particular metal bound at the active site, paraoxon hydrolysis decreased by 3 or 4 orders of magnitude. The K_m values for paraoxon did not increase substantially for the Co^{2+}- or Cd^{2+}-reconstituted enzyme, but the K_m for the Zn^{2+}-reconstituted enzyme increased over 100-fold. Catalytic activity of W131K with either DEPP or acephate was not detectable over background. The k_{cat} for DFP hydrolysis decreased by 10^3 and the K_m for DFP increased 4-fold. The DFPase activity of W131F and W131A were near wild type levels.
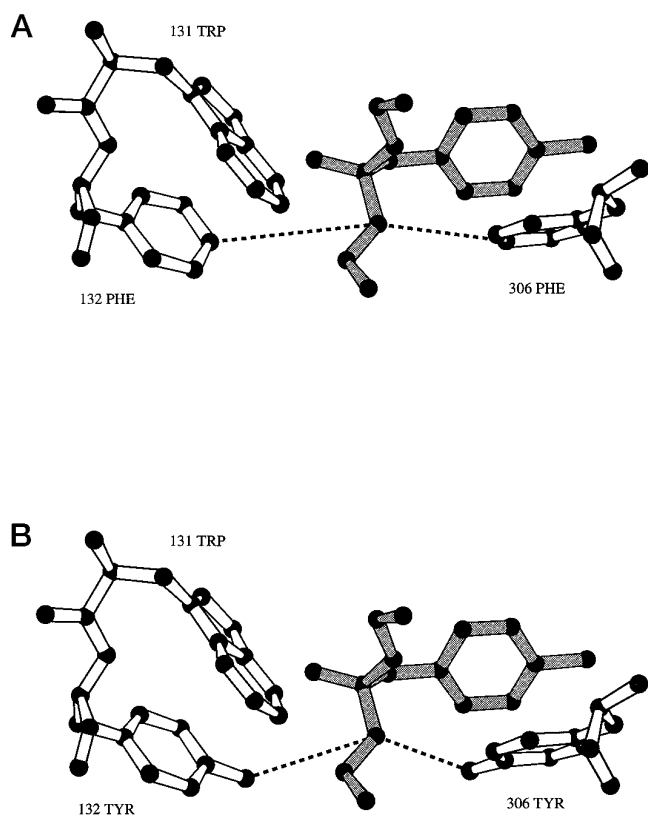
A

B

FIG. 2. **Active site structure for the zinc-substituted phosphotriesterase.** The bound substrate analogue is shown in *gray*. *A*, the area depicted shows the region of the protein near the leaving group of the bound substrate analogue, diethyl 4-methylbenzylphosphonate. The residues Trp[131], Phe[132], and Phe[306] are highlighted. The distances from the oxygen of the ethoxy group of the bound analogue to the aromatic ring of Phe[132] and Phe[306] are 5.0 and 3.9 Å, respectively. *B*, a model for the same region of the protein when Phe[132] and Phe[306] have each been mutated to a tyrosine. The distances in the model from the oxygen of the ethoxy group of the substrate analog to the phenolic oxygen of the Tyr[132] and Tyr[306] are 3.8 and 2.7 Å, respectively. The coordinates for the zinc-substituted wild type enzyme in the presence of the substrate analogue were taken from Vanhooke *et al.* (6).

The large decrease in k_{cat} upon introduction of a positive charge at position 131 suggests that dramatic changes have occurred within the active site. The previously reported mutations of Trp[131] to either alanine or phenylalanine were electrostatically more conservative and resulted in mutant enzymes with elevated K_m values for paraoxon. Those studies supported the role of Trp[131] in substrate and/or product interactions. Because the alanine substitution had a more significant effect than the phenylalanine mutation, it was proposed that a hydrophobic interaction with the aromatic ring of Trp[131] plays an important role in paraoxon binding. The W131K mutation may have caused significant alterations in the organization about the binuclear metal center. Mutational studies of the Zn^{2+} site in carbonic anhydrase indicate that second sphere residues around the metal center play a crucial role in the proper orientation of the active site ligands. These alterations were found to affect the reactivity of Zn^{2+}-bound water, the stabilization of the transition state, and protein-metal affinity (26, 27). Similar effects may also be modulating the active site of phosphotriesterase. When a known second sphere ligand of PTE, Asp[253], was mutated to alanine, a substantial decrease in catalytic activity was observed that could be attributed to the loss of a hydrogen bond interaction with His[230], a direct amino acid ligand to the binuclear metal center (24).

The crystal structure of PTE indicates that Phe[132] resides in the hydrophobic pocket that engulfs the leaving group of the

substrate during catalysis. Phe[306] resides at the interface of the three pockets that contain the ethoxy and phenoxy substituents attached to phosphorus center of the paraoxon substrate but is oriented more toward the leaving group pocket. Mutation of Phe[306] to either of the charged residues, lysine or glutamic acid, results in significant decreases in k_{cat} and substrate affinity for paraoxon. This result indicates that the hydrophobicity of the amino acid side chain is important in substrate binding and in the organization of the active site. Mutation of Phe[132] to histidine or tyrosine resulted in enzymes with wild type levels of activity when tested with paraoxon, DEPP, or acephate. The K_m values for these substrates ranged from near wild type to a modest 6-fold increase. Mutation of Phe[306] to histidine or tyrosine resulted in a variety of different effects on k_{cat} and K_m, but the general trend was a slightly lower catalytic activity and significantly elevated K_m values. These results support the role of Phe[132] and Phe[306] as previously speculated (6). These hydrophobic residues facilitate the binding of substrates that contain hydrophobic leaving groups. Introduction of more polar and bulkier substituents decreases substrate affinity, especially for the Phe[306] site. Smaller but usually detrimental effects are also observed on catalytic hydrolysis of these substrates. The kinetic parameters of the double mutants of Phe[132] and Phe[306] confirmed these roles. The placement of two polar groups in the leaving group pocket resulted in even higher K_m values and slower rates of hydrolysis.

The tyrosine and histidine mutants of Phe[132] and Phe[306] were able to hydrolyze DFP up to an order of magnitude faster than that of the wild type enzyme. The double mutants, F132H/F306H and F132H/F306Y, had higher catalytic activity than the individual mutations, whereas the catalytic activity of F132Y/F306Y and F132Y/F306H was intermediate in value. The turnover number for the F132H/F306H mutant of 3100 s^{-1} is nearly as high as the k_{cat} for the wild type enzyme with paraoxon. These results clearly indicate that the likelihood of creating even more complex mutants of PTE that are fully optimized for the catalytic hydrolysis of sarin and soman is quite high. The K_m values remained high for all double mutants. The alterations in kinetic parameters can be attributed to a direct interaction of the fluoride leaving group with the leaving group pocket. However, the elevated K_m values for some of the mutants may also indicate an unfavorable interaction between the modified sidechains and one of the isopropyl substituents of DFP. The fluoride substituent, in contrast with the paraoxon or DEPP leaving groups, is smaller and more hydrophilic.

The active sites that are known to bind halogens have been characterized in several enzymes. In haloalkane dehalogenase, two tryptophan residues form hydrogen bonds to the cleaved halogen and apparently stabilize the transition state during carbon-halogen bond cleavage (28, 29). A threonine is involved in bromide binding in carbonic anhydrase II (30) and a lysine and a histidine bind to a chloride ion in human color vision pigments (31). The interaction of the fluoride with the more polar residues of the active site could facilitate phosphorus-fluoride bond cleavage. Also, the structural changes introduced into the active site pocket by the addition of the polar substituents could orient the substrate such that there is better alignment for attack by the active site water. Understanding the chemistry of the phosphorus-fluoride bond cleavage event will be essential in the development of rationally designed mutants of phosphotriesterase that are capable of efficient hydrolysis of phosphofluoridate compounds such as sarin and soman.

The rational design of mutant enzymes with novel activities is dependent on a thorough understanding of the chemical mechanism and the three-dimensional structure of the enzyme

under investigation. Despite the lack of a complete understanding of the specific mechanism of phosphorus-fluorine bond cleavage, the information known about phosphotriesterase was used to create a series of phosphotriesterase mutants with an enhanced ability to facilitate the cleavage of the phosphorus-fluorine bond. Mutants of PTE were created with DFPase activity that was increased up to an order of magnitude. However, the redesign effort failed in increasing the rate of hydrolysis of phosphotriesters with poorer leaving groups. The improved DFPase activity is a critical first step in the design of phosphotriesterase mutants with increased activity toward other phosphofluoridate compounds such as the neurotoxins sarin and soman. Although protein redesign studies of this sort are important in the understanding of protein structure-function relationships, this effort also shows the feasibility of an enzymatic solution for the destruction and protection against the lethal effects of chemical warfare agents. Furthermore, this study clearly affirms the role of the phosphotriesterase from *P. diminuta* as the leading candidate for the design of mutants to be used for the biological degradation of these compounds.

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ENZYMOLOGY:

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