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Structure-Based Rational Design of a Phosphotriesterase▽†

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In silico substrate docking of both stereoisomers of the pesticide chlorfenvinphos (CVP) in the phosphotriesterase from *Agrobacterium radiobacter* identified two residues (F131 and W132) that prevent productive substrate binding and cause stereospecificity. A variant (W131H/F132A) was designed that exhibited ca. 480-fold and 8-fold increases in the rate of *Z*-CVP and *E*-CVP hydrolysis, respectively, eliminating stereospecificity.

Synthetic organophosphate pesticides (OPs) can cause acute neurotoxicity in insects and humans as a result of their inhibition of acetylcholinesterase at the nerve synapse (15). The >90% identical bacterial phosphotriesterases (PTEs) from *Pseudomonas diminuta* (*oph*; PTE_{Pd}) (5) and *Agrobacterium radiobacter* (*opdA*; PTE_{Ar}) (9) efficiently catalyze the hydrolysis of a broad range of OPs, effectively detoxifying them. This has led to the commercialization of PTE_{Ar} as a free-enzyme bioremediant (14) and its use in treating OP poisoning in animal studies (1). However, not all OPs are efficiently turned over by the PTEs. For instance, despite having a reasonably reactive leaving group (Fig. 1), the turnover of chlorfenvinphos (CVP) by PTE_{Ar} was not detected in a previous study (9).

Kinetic analysis. For a detailed analysis of CVP turnover by PTE_{Ar} (expressed and purified as previously described [10]), the *E* and *Z* isomers were separated by liquid chromatography, and hydrolysis was measured by liquid chromatography/mass spectrometry (see the supplemental material). These data demonstrate that both isomers of CVP are substrates of PTE_{Ar}, although the catalytic efficiencies, particularly for the *Z* isomer, are very low (Table 1). Additionally, PTE_{Ar} exhibits clear isomer specificity, with the k_{cat}/K_m of the *E* isomer exceeding that of the *Z* isomer by 87-fold. This finding has particular relevance to the application of PTE_{Ar} in bioremediation, since the *E*/*Z* isomer ratio is approximately 1:9 in commercially available CVP.

Substrate docking. Structures of *E*- and *Z*-CVP (obtained from Spartan06; Wavefunction, Inc.) were manually docked at the active site of PTE_{Ar} using COOT (6). These poses were then used as starting points for a substrate docking experiment using CDOCKER (16) as previously described (11). As a control, diethyl-4-methoxyphenylphosphate (EPO) was docked, and the resulting pose was found to be effectively identical to the conformation obtained experimentally (Fig. 2b) (11). Of the three unique poses obtained from docking *E*-CVP (Fig. 2c), only one was productive in the sense that it was aligned such that nucleophilic attack was possible. The hydrogen and chloride atoms of the vinyl moiety straddle the active-site cleft

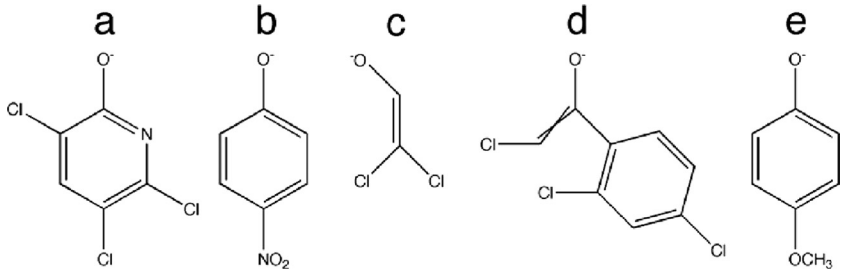

FIG. 1. Structures of the leaving groups of all the substrates discussed in this work. (a) 3,5,6-Trichloro-2-pyridinol for methyl chlorpyrifos oxon; (b) 4-nitrophenol for methyl paraoxon and methyl parathion; (c) 2,2-dichloroethenol for dichlorvos; (d) *Z*/*E*-2-chloro-1-(2,4-dichlorophenyl)ethanol for *E*/*Z*-CVP; (e) 4-methoxyphenol for EPO.

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TABLE 1. k_{cat}/K_m (s^{-1} M^{-1}) values for PTE_{Ar} and variants with different OPs[a]

Substrate	k_{cat}/K_m (s^{-1} M^{-1}) for PTE_{Ar}:			
	WT	F132A	W131H	W131H/F132A
MPS	2.7×10^7	1.8×10^6	1.3×10^6	8.7×10^5
DCV	8.1×10^5	NA	NA	NA
Z-CVP	9.6×10^0	3.9×10^3	2.4×10^3	4.6×10^3
E-CVP	8.4×10^2	4.3×10^3	3.1×10^3	6.0×10^3

[a] MPS, methyl parathion (ChemService); DCV, dichlorvos (ChemService); CVP, chlorfenvinphos (Bayer Crop Science, Australia); WT, wild type; NA, not available. Error is within 10%.

in this pose, with the larger chloride group on the outside of the cleft. No productive poses were obtained with *Z*-CVP (Fig. 2d), possibly because the analogous pose to the productive *E*-CVP pose would result in the chloride group (which is positioned on the opposite side of the vinyl bond in the Z-isomer) clashing with the protein. These docking results suggest that the low catalytic efficiency of PTE_{Ar} with both isomers is primarily a result of steric hindrance and that the stereospecificity is a result of more severe steric hindrance to *Z*-CVP binding.

Rational design. Based on the substrate-docking results, the W131A, W131H, F132A, F132L, and F132R mutants were constructed (see the supplemental material) and screened for activity with CVP using a previously described plate-clearing assay (3) at a concentration of 10 mM racemic CVP. The most active variants (W131H and F132A) were identified on the basis of their clearing rates and were combined to produce a W131H/F132A double mutant. Kinetic analysis of these variants was performed (Table 1). The F132A mutation had the largest effect on the rate of CVP hydrolysis, improving k_{cat}/K_m by 5.1-fold for *E*-CVP and 400-fold for *Z*-CVP, while the W131H mutation improved the rates by 3.7-fold and 250-fold, respectively. Combining the two mutations improved the k_{cat}/K_m for *E*-CVP by 7.1-fold and *Z*-CVP by 480-fold. The observation that the improvements were nonadditive suggests that the two mutations have similar or overlapping effects. These residues also appear to be responsible for the stereoselectivity of PTE_{Ar}, which was relaxed 67-fold from 87:1 in the wild-type enzyme to 1.3:1 in the W131H/F132A variant. Interestingly, the activities of the mutants with the "ideal" substrate methyl parathion were reduced ca. 30-fold, most probably through a loss of stabilizing π-π interactions between W131/F132 and the leaving group in the transition state (reduced k_{cat}; see Table S1 in the supplemental material) and less hydrophobic interaction with the substrate (increased K_m; see Table S1 in the supplemental material). The structural effects

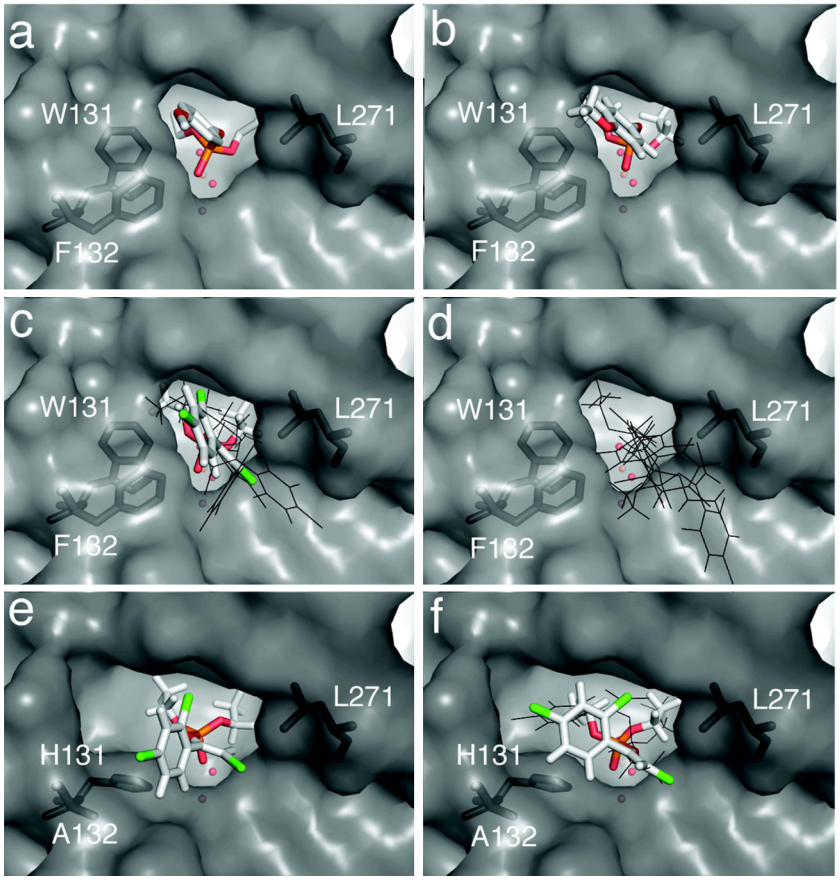

FIG. 2. Substrate docking using CDOCKER. Unique productive docking poses are shown as colored sticks, whereas unique unproductive docking poses are shown as black lines. The crystal structure of EPO-PTE_{Ar} is shown in panel a. Hydrogen atoms are not included since they are not observed in the crystal structure. In silico "docked" poses are also shown as follows: EPO-PTE_{Ar} (b), *E*-CVP–PTE_{Ar} (c), Z-CVP–PTE_{Ar} (d), *E*-CVP–PTE_{Ar} W131H/F132A (e), and Z-CVP–PTE_{Ar} W131H/F132A (f).

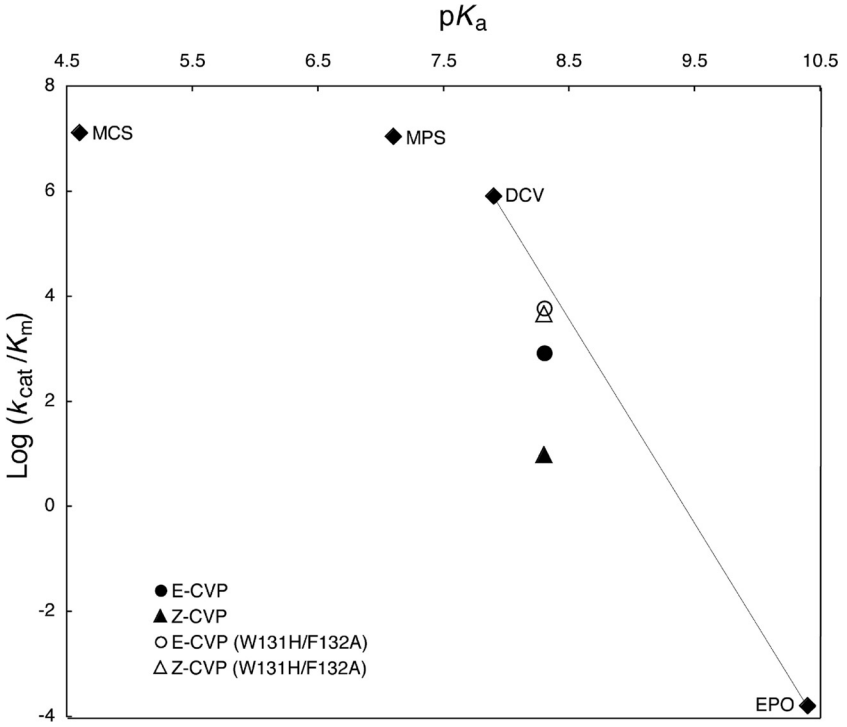

FIG. 3. Bronsted plots of log(k_{cat}/K_m) versus pKa of the leaving group. The pK_a values of the dichlorvos (7.9) and CVP (8.3) leaving groups were calculated using the SPARC online pK_a calculator (http://ibmlc2.chem.uga.edu/sparc/) (7), while values for MPO (7.1), MCO (4.6), and EPO were taken from published sources (8, 11, 13). Values of k_{cat}/K_m for MCO, MPO, and EPO were taken from previous work (11, 12). The biphasic dependence of the enzyme on pK_a is shown, with the curve flattening below a pK_a of ~8.0. A linear dependence on pK_a is observed at pK_a values below ca. 8.0. The catalysis of *Z*- and *E*-CVP hydrolysis by wild-type PTE$_{Ar}$ is shown to deviate from the predicted values, while for PTE$_{Ar}$ (W131H/F132A), the catalytic rates are close to the expected values.

of the W131H and F132A mutations are shown in Fig. 2. It is clear that both mutations widen the active-site cleft, allowing productive docking of both isomers, and that this improvement is most significant for *Z*-CVP.

Previous work has shown that a rational redesign of W131 could affect the stereospecificity of PTE$_{Pd}$ (2). In that work, W131 was considered part of the small side chain pocket and changes at this position had large effects on the stereospecificity of enantiomers with different-sized alkyl side chains. Here, it is characterized as forming part of the active-site cleft of the enzyme and clearly affects the stereospecificity of the enzyme for substrates with enantiomeric leaving groups. Thus, it appears W131 forms parts of both the small side-chain pocket and the active-site cleft.

Steric and chemical factors in catalysis. The PTEs exhibit a linear relationship between the pK_a value of the leaving group and k_{cat}/K_m below a pK_a value of ca. 8.0; above this value, other factors, such as diffusion or conformational change, become rate limiting (8). Despite the 480-fold increase in the catalytic efficiency of PTE$_{Ar}$ for *Z*-CVP in the double mutant, its k_{cat}/K_m is still ~10^3-fold lower than that for methyl paraoxon hydrolysis catalyzed by wild-type PTE$_{Ar}$, and it does not exceed the value that would be expected on the basis of the pK_a of the leaving group (Fig. 3). Large catalytic improvements are frequently reported when the substrate specificities of enzymes change as a result of changes in the substrate-binding pocket (3, 4, 17). However, as shown here, the catalytic efficiencies of enzymes are ultimately limited by the underlying reaction chemistry. Improving this facet of catalysis is a looming and markedly more difficult challenge for protein engineers and will most likely require more sophisticated approaches than the relatively simple randomization-based strategies described here and elsewhere.

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