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RELIEVING MIPAFox INHIBITION IN ORGANOPHOSPHORUS ACID ANHYDROLASE BY RATIONAL DESIGN

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14. ABSTRACT: Organophosphate acid anhydrolase (OPAA) is a bimetalloenzyme that hydrolyzes acetylcholinesterase-inhibiting organophosphorus compounds, including fluorine-containing nerve agents such as soman [pinacolyl methylphosphonofluoridate]. The insecticide mipafox (<i>N,N'</i> -diisopropyldiamidofluorophosphate), which is a close analog of the nerve agent substrate diisopropyl fluorophosphonate, is a special case for OPAA because it reversibly inhibits the enzyme's hydrolysis activity. It is believed that OPAA hydrolyzes mipafox to DDP (<i>N,N'</i> -diisopropyldiamidophosphate), which becomes bound to the OPAA active site through a hydrogen bond network. Evaluation of the crystal structure of the OPAA-DDP complex suggests that disruption of two of these bonds, one involving Asp244 and the other involving Glu381, could potentially release DDP from the OPAA active site to give the enzyme catalytic mipafox hydrolysis activity. To test this hypothesis, we produced three mutant OPAA enzymes including one enzyme with an Asp244-to-alanine mutation, a second with a Glu381-to-alanine mutation, and a third with both mutations, to remove the corresponding hydrogen bonds between these amino acids and the bound DDP. In all cases, the mutant enzymes did not have mipafox hydrolysis activity but retained their high hydrolysis rates against soman, which indicated that the remaining OPAA-DDP interactions were adequate to stabilize their complex and keep DPP bound to the enzyme.					
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PREFACE

The work described in this report was authorized under the ECBC Research and Technology Directorate SEED Program.

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RELIEVING MIPAFOX INHIBITION IN ORGANOPHOSPHORUS ACID ANHYDROLASE BY RATIONAL DESIGN

1. INTRODUCTION

In 1984, Hoskins and his coworkers¹ identified two enzymes that could be used to detoxify fluorine-containing nerve agents such as diisopropyl fluorophosphonate (DFP, Figure 1) and soman (GD, Figure 1). These enzymes are generally referred to as organophosphate/organophosphorus hydrolyases. Seven years later, an organophosphate acid anhydrolase (OPAA, EC 3.1.8.2) was purified from halophilic *Alteromonas* sp. bacteria. OPAA displayed hydrolysis activity against several highly toxic organophosphorus compounds, including the fluorine-containing nerve agents tabun (GA), sarin (GB), and GD.³ OPAA has high hydrolysis activity against phosphorus–fluorine bonds in organophosphorus compounds but very minimal activity for phosphorus–oxygen and phosphorus–carbon bonds in the compounds. However, mipafox, which is an organophosphorus insecticide containing a phosphorus–fluorine bond, is not an OPAA substrate under normal assay conditions but a competitive inhibitor with an inhibition constant (K_i) of 0.49 mM for DFP substrate hydrolysis.^{3,4} The OPAA enzyme is active at pH ~8.5 and requires divalent cations.^{3–5} Evaluation of a recent crystal structure of OPAA that was co-crystallized with mipafox² suggested that the enzyme hydrolyzed the insecticide to *N,N'*-diisopropyldiamidophosphate (DDP, Figure 1), and it was this hydrolysis product, rather than mipafox, that became bound to the OPAA active site to inhibit DFP substrate hydrolysis. The DDP is reversibly held in the enzyme's active site by an intricate network of hydrogen bonds involving both divalent cations and three amino acids. There was no evidence in the crystal structure for a covalent bond between the enzyme and DDP.

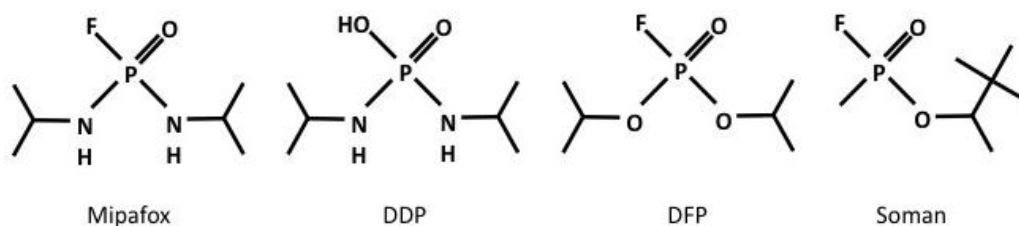


Figure 1. OPAA substrates mipafox, DFP, and GD, along with the DDP reaction product.

Analysis of the structural data derived from OPAA co-crystallized with the mipafox substrate² revealed that in addition to coordinating the two metal cations, the DDP product interacted with the enzyme's amino acids via electrostatic interactions through its two nitrogen atoms and one of its carboxyl oxygen atoms. This is illustrated in Figure 2, where one DDP nitrogen atom donated a hydrogen bond to a carboxyl oxygen atom of Asp244, and the other DDP nitrogen atom donated a hydrogen bond to a Glu381 carboxyl oxygen atom. Additionally, a DDP oxygen atom accepted a hydrogen bond from the His343 nitrogen atom, and the two DDP isopropyl groups participated in hydrophobic

interactions with several OPAA amino acids. In an effort to investigate the forces stabilizing the OPAA–DDP complex, we chose to systematically eliminate hydrogen bonds between the enzyme and DDP for its release from the OPAA active site, giving rise to catalytic mipafox hydrolysis activity. Our initial effort focused on the DDP–Asp224 and DDP–Glu381 hydrogen bonds. We produced three mutant OPAA enzymes: one enzyme with an Asp244-to-alanine mutation, a second with a Glu381-to-alanine mutation, and a third with both mutations. This was done to remove their corresponding hydrogen bonds in the OPAA–DPP complex and create an OPAA variant with catalytic mipafox activity. This report contains our preliminary results for these three mutants.

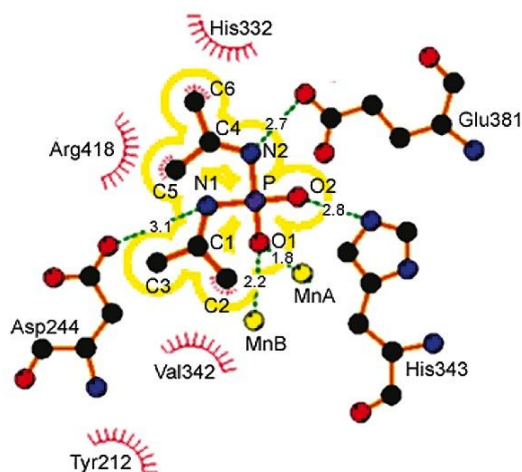


Figure 2. Ligplot⁶ schematic representation of the interactions between DDP (circumscribed by a yellow boundary), the two Mn²⁺ cations in the OPAA active site (MnA and MnB), and the OPAA amino acids via hydrogen bonds (dashed green lines) and nonpolar interactions (residues demarcated by a spoked arc).

2. EXPERIMENTAL PROCEDURES

Coding regions for each of three OPAA variants, an Asp244-to-alanine mutant (D244A), a Glu381-to-alanine mutant (E381A), and a variant with both (D244A–E381A), were separately ligated into the promotor region of pSE420 plasmids between their *RcaI* and *EcoRI* restriction sites for expressing the variant proteins. For each, an *Escherichia coli* DH5 α culture containing one of the plasmids was grown at 37 °C in 1 L of Luria-Bertani (LB) broth containing 0.1 mg/mL ampicillin and 0.1 mM manganese (II) chloride. Cells were grown to mid-log phase ($A_{600} = 0.5$) before the addition 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for cell induction. After 4 h, the cells were harvested by centrifugation, and the pellets were resuspended in 0.1 mM manganese (II) chloride and 10 mM bis-tris-propane (pH 7.2) before disruption by three passes through a French pressure cell. After cell debris was removed by centrifugation, the crude lysates were precipitated with ammonium sulfate at 40–65% saturation. The precipitated protein was resuspended and dialyzed extensively against 0.1 mM of manganese (II) chloride and 10 mM bis-tris-propane (pH 7.2) to create enriched variant OPAA preparations. Aliquots of the crude cell lysates and the enriched OPAA preparations were taken for analysis by

sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). OPAA activity against GD and mipafox was determined by monitoring the release of free fluoride with a fluoride-specific probe at 25 °C. For each determination, 10 μ L of an enriched variant OPAA preparation was added to 990 μ L of 50 mM bis-tris-propane buffer (pH 7.2) containing either 3 mM GD or mipafox as substrates.

3. RESULTS AND DISCUSSION

The electrophoretogram of the crude cell lysates and enriched variant OPAA preparations is shown in Figure 3, along with molecular weight standards and a wild-type OPAA preparation for reference. In all of the cell lysates and enriched preparations, the single, most intense band appeared at ~50 kDa, which was expected for OPAA under the electrophoresis conditions used. This was consistent with our previous results, where over-expressed, wild-type OPAA constituted ~50% of the soluble protein in *E. coli* cell extracts. The ~50 kDa band also appeared in the wild-type OPAA preparation; however, the preparation also contained four high molecular weight protein contaminants (between 64 and 97 kDa) as well as lower molecular weight components, which were most likely from OPAA degradation. Considered collectively, these results indicated that the three OPAA variants were over-expressed in the *E. coli* cultures, and although they were not homogeneous, the protein preparations were enriched for their respective OPAA variants.

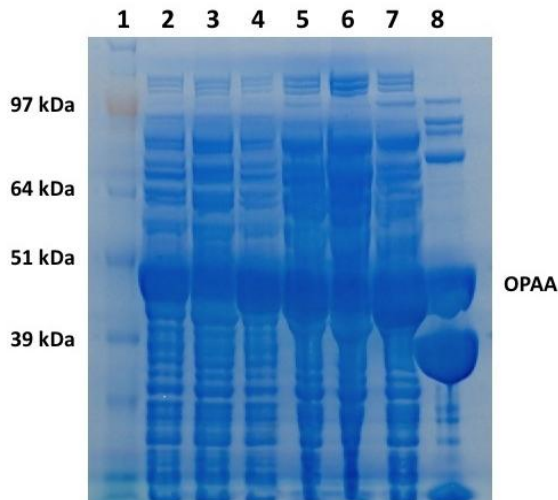


Figure 3. Electrophoretogram from the SDS-PAGE analysis of the OPAA variants. Molecular weight standards are shown (lane 1) with labels for alcohol dehydrogenase (39 kDa), glutamic dehydrogenase (51 kDa), bovine serum albumen (64 kDa), and phosphorylase (97 kDa). Cell lysates of D224A (lane 2), E381A (lane 3), and D224A-E381A (lane 4) are shown, as well as the enriched protein preparations for these three variants (lanes 5, 6, and 7, respectively). A wild-type OPAA preparation (lane 8) is included for reference.

As illustrated in Figure 4, each of the three OPAA variants had catalytic hydrolysis activity against GD that was substantially above the rate for GD alone in aqueous solution. This confirmed the conclusion that the variants were over-expressed in the *E. coli* cultures. Because the mutant OPAA concentrations in the enriched preparations were not known, the activity of each variant relative to the others could not be established. It was likely that many of the relative differences observed in GD hydrolysis between the three variants represented different variant concentrations in the enriched variant preparations rather than differences in specific activities. Finally, the figure shows that all three OPAA variants did not have mipafox hydrolysis activity significantly different from the spontaneous rate for the insecticide alone in aqueous solution. Data for all three variants and spontaneous mipafox hydrolysis are superimposed over one another in Figure 4. Clearly, neither the D244A nor the E381A mutations or both used together were able to release the DDP from the enzyme active site and create mipafox hydrolysis activity.

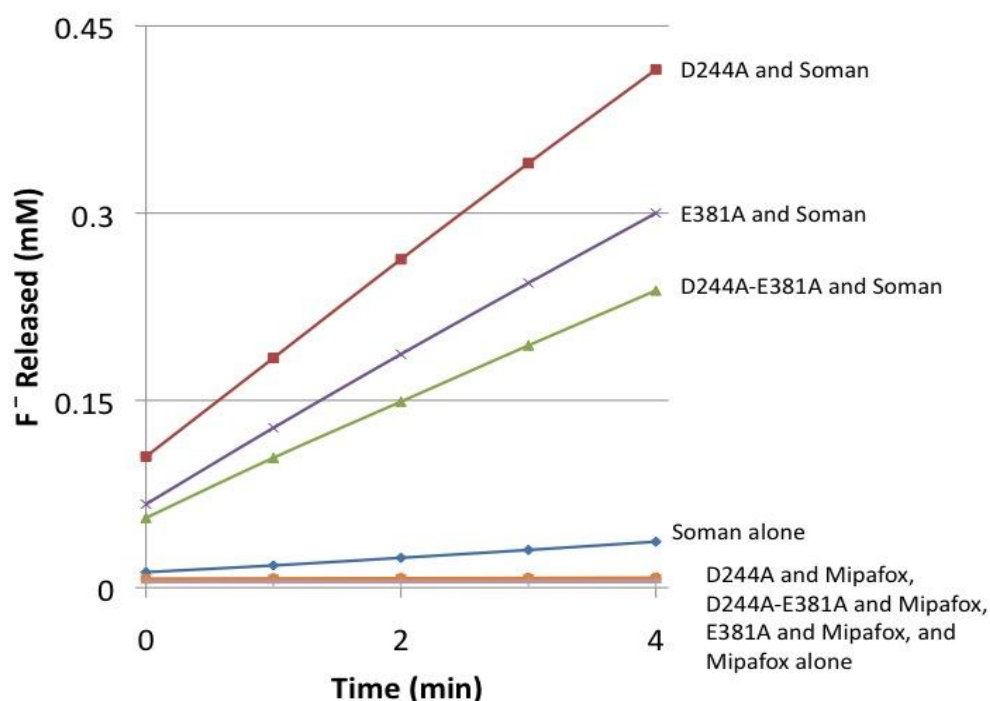


Figure 4. Kinetic results of the OPAA variants D244A, D244A-E381A, and E381A against GD and mipafox. Results for the spontaneous hydrolysis of GD (GD alone) and mipafox (mipafox alone) are also included.

We investigated two hydrogen bonds in the OPAA-DDP complex and determined their contributions toward stabilizing this complex. For both bonds, DDP nitrogen atoms (N1 and N2 in Figure 2) donated hydrogen bonds to the carboxyl group oxygen atoms of the native Asp244 and Glu381 amino acids.² The removal of these hydrogen bonds, either individually with an Asp244-to-alanine mutation or a Glu381-to-alanine mutation or both mutations together, did not create catalytic hydrolysis activity against the mipafox insecticide. Because the creation of catalytic hydrolysis activity requires the release of DDP from the enzyme, and because it is known that the OPAA–

DPP complex is a consequence of numerous stabilizing forces between the enzyme and DDP hydrolysis product, it was reasonable to assume that removal of these hydrogen bonds would not release DPP from the enzyme. The reasons for this could not be determined without further investigation; however, the removal of these two hydrogen bonds leaves three more in place (one involving His343 and two involving each of the Mn^{2+} cations [Figure 2]) as well as at least two hydrophobic interactions between DDP and OPAA amino acids (involving Tyr212, His332, Val342, and Arg418) to stabilize the OPAA-DDP complex.² Therefore, it was reasonable to assume that these remaining hydrogen bonds and hydrophobic interactions would provide adequate stabilization of the enzyme-hydrolysis product complex to keep it undisrupted. It was possible that the two point mutations to alanine residues contributed to the existing hydrophobic interactions between OPAA and DDP, created new hydrophobic interactions, or obliterated the enzyme's hydrolysis activity. The latter case was believed to be unlikely, given that all three OPAA variants retained high hydrolysis rates against GD.

4. CONCLUSIONS

We demonstrated conclusively that removal of the Asp244-DDP and Glu331-DPP hydrogen bonds in the OPAA-DDP complex did not create catalytic mipafox hydrolysis activity. We attributed this to the fact that the removal of these hydrogen bonds left three more in place between OPAA and DDP, and at least two hydrophobic interactions, which provided sufficient stabilizing forces to the complex, prohibited the release of DPP from the enzyme active site.

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ACRONYMS AND ABBREVIATIONS

DDP	<i>N,N'</i> -diisopropylidiamidophosphate
DFP	diisopropyl fluorophosphonate
GA	ethyl dimethylphosphoramidocyanidate (tabun)
GB	isopropyl methylphosphonofluoridate (sarin)
GD	pinacolyl methylphosphonofluoridate (soman)
IPTG	isopropyl β -D-1-thiogalactopyranoside
K_i	inhibition constant
LB	Luria-Bertani (broth)
OPPA	organophosphorus acid anhydrolase
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis

