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An OPAA enzyme mutant with increased catalytic efficiency on the nerve agents sarin, soman, and GP

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ABSTRACT

The wild-type OPAA enzyme has relatively high levels of catalytic activity against several organophosphate G-type nerve agents. A series of mutants containing replacement amino acids at the OPAA Y212, V342, and I215 sites showed several fold enhanced catalytic efficiency on sarin, soman, and GP. One mutant, Y212F/V342L, showed enhanced stereospecificity on sarin and that enzyme along with a phosphotriesterase mutant, GWT, which had the opposite stereospecificity, were used to generate enriched preparations of each sarin enantiomer. Inhibition of acetylcholinesterase by the respective enantioenriched sarin solutions subsequently provided identification of the sarin enantiomers as separated by normal phase enantioselective liquid chromatography coupled with atmospheric pressure chemical ionization–mass spectrometry.

1. Introduction

Sarin, soman (GD and, GP (Fig. 1) are all G-type chemical nerve agents; members of a class of organophosphates that exert their toxic effect by phosphorylating the catalytically active serine in the active site of acetylcholinesterase and preventing it from catalyzing the breakdown of acetylcholine in the neuromuscular junction. These, and other compounds are included in the Chemical Weapons Convention which prohibits their development, production, stockpiling, and use in the 192 (as of 17 Oct, 2015) signatory nations [1]. However, not all countries have signed the Convention and reports of the use of chemical weapons have continued to appear [2–4]. Two websites provide regularly updated lists of reports of chemical weapons use, many of which have been verified by the Organization for the Prohibition of Chemical Weapons [5,6].

Catalytic enzymes are currently being investigated for their potential as *in vivo* medical countermeasures for nerve agent poisoning [7]. Current therapies comprise symptomatic treatment such as atropine (a muscarinic antagonist) and benzodiazepines (to control seizures), in addition to etiologic treatment of the poisoned acetylcholinesterase with 2-PAM (an oxime reactivator). Given the high toxicity of nerve agents and the fact that existing treatments typically confer protection against only a few lethal doses, treatments can easily be overwhelmed by agent exposure [8,9]. This concern has led to the effort to develop catalytic antidotes which might confer greater protection than would be possible with stoichiometric approaches. The particular advantage of circulating enzymes is that they can detoxify organophosphates in the blood before they enter the tissue where they bind to acetylcholinesterase [10]. For that reason, they have the potential to function either alone or synergistically with existing treatments [7].

Organophosphorus Acid Anhydrolase (OPAA; EC 3.1.8.2) is one of the enzymes described for the catalytic defluorination of G-agents. The wild-type (WT) OPAA enzyme has relatively high levels of activity against soman and cyclosarin [11,12]. WT OPAA has slight activity against Russian VX; some mutants have greater activity and, in the case of one mutant, enhanced stereospecificity such that both enantiomers of Russian VX are catalyzed at similar rates [13]. WT OPAA activity against sarin is significantly less than seen with soman and cyclosarin [15]. In this study we initially used site-directed mutagenesis in an effort to increase the catalytic efficiency of OPAA against sarin. One of those mutants, FL, proved particularly promising. Therefore, we studied its kinetics against soman and a more recently described nerve agent, GP [16]. Finally, enzymatic reactions were used to generate enantioenriched preparations of sarin which were used along with

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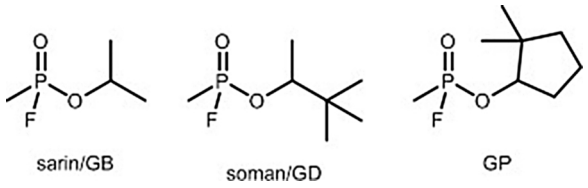

Fig. 1. Structures of sarin/GB (propan-2-yl methylphosphonofluoridate), soman/GD (3-methylbutan-2-yl methylphosphonofluoridate), and GP (2, 2-dimethylcyclopentyl methylphosphonofluoridate).

Table 1
Enzyme genotypes.

Source	Genotype	Abbreviation
OPAA	Y212F/V342L	FL
OPAA	Y212F/V342I	FI
OPAA	Y212F/V342Y	FY
Phosphotriesterase	H254G/H259W/L303T	GWT

acetylcholinesterase inhibition assays to assign the respective sarin enantiomers separated and identified by chiral liquid chromatography coupled with atmospheric pressure chemical ionization–mass spectrometry (LC-APCI-MS).

harvested and the enzyme was purified by ammonium sulfate fractionation and the 45–65% pellet obtained was redissolved, passed through a size exclusion column and the active fractions were pooled and loaded on a Q Sepharose column followed by a 0.2–0.6 M NaCl gradient elution.

The resulting sample was apparently homogeneous by polyacrylamide gel electrophoresis.

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M N K L A V L Y A E   H I A T L Q K R T R   E I I E R E N L D G   V V F H S G Q A K R   40
Q F L D D M Y Y P F   K V N P Q F K A W L   P V I D N P H C W I   V A N G T D K P K L   80
I F Y R P V D F W H   K V P D E P N E Y W   A D Y F D I E L L V   K P D Q V E K L L P   120
Y D K A R F A Y I G   E Y L E V A Q A L G   F E L M N P E P V M   N F Y H Y H R A Y K   160
T Q Y E L A C M R E   A N K I A V Q G H K   A A R D A F F Q G K   S E F E I Q Q A Y L   200
L A T Q H S E N D T   P Y G N I V A L N E   N C A I L H Y T H F   D R V A P A T H R S   240
F L I D A G A N F N   G Y A A D I T R T Y   D F T G E G E F A E   L V A T M K Q H Q I   280
A L C N Q L A P G K   L Y G E L H L D C H   Q R V A Q T L S D F   N I V N L S A D E I   320
V A K G I T S T F F   P H G L G H H I G L   Q V H D V G G F M A   D E Q G A H Q E P P   360
E G H P F L R C T R   K I E A N Q V F T I   E P G L Y F I D S L   L G D L A A T D N N   400
Q H I N W D K V A E   L K P F G G I R I E   D N I I V H E D S L   E N M T R E L E L D   440
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2. Material and methods

Sarin and soman were chemical agent standard analytical reference material from our stocks. Sarin was 97.4% weight percent pure by acid base titration (traceable to the National Institute of Standards and Technology, or NIST through potassium phthalate). Purity by gas chromatography/thermal conductivity detection (GC/TCD) was 97.9%. Purity by ^{31}P nuclear magnetic resonance (NMR) was 97.7 wt%. Soman was 95.3 wt% pure by ^{31}P NMR relative to a triethylphosphate internal standard and traceable to NIST through the internal standard with purity of 99.87 wt% relative to a NIST traceable standard of dimethylsulfone via quantitative ^{1}H NMR measurements. The purity of the soman by GC/FPD was 98.95%.

GP was synthesized in a two-step procedure from 2,2-dimethylcyclopentanone. First, a reduction of the ketone by $LiAlH_4$ in Et_2O yielded racemic 2,2-dimethylcyclopentanol after acid workup. Then reaction of 2,2-dimethylcyclopentanol with methylphosphonyl difluoride in the presence of trimethylamine in Et_2O yielded the desired 2,2-dimethylcyclopentyl methylphosphonofluoridate after filtration to remove triethylammonium hydrofluoride, removal of the solvent, and distillation under reduced pressure.

Note: GP is extremely toxic and its synthesis is regulated by the Chemical Weapons Convention.

All reagents and solvents were HPLC grade. Hexane and isopropyl alcohol were purchased from Fisher Scientific (Waltham, MA). For the LC–MS analytical analysis, the MS system was operated in total ion chromatogram (TIC) mode at *m/z* 50–300. The analytical separations of the enantiomers were characterized using an Agilent 1200 LC with atmospheric pressure chemical ionization–mass spectrometry (LC-APCI-MS) performed on a Phenomenex Lux Cellulose-1 column, 250 × 4.6 mm, 5 μm with a mobile phase consisting of *n*-hexane (A) and isopropyl alcohol (B) and a sample volume of 20 μL. The enantiomers were baseline resolved within 15 min with a mobile phase of 95/5 A/B (v/v%) with a flow rate 0.6 mL/min. Samples for analytical separation were prepared at 0.1 mg/mL.

The OPAA enzyme was prepared as described previously [12]. Briefly, the *Escherichia coli* host cell containing the cloned OPAA gene was grown to late log phase in 1 L of Luria Broth in a flask. Cells were

The wild-type opaa amino acid sequence is as follows:

The opaa gene was cloned into the *Nco*I and the *Eco*RI sites of the pSE420 expression vector. The cloned gene lacks the last 77 carboxyl-terminus amino acid residues of the OPAA enzyme; these residues were removed in a previous investigation and found to have no discernible effect on enzymatic activity [15]. The OPAA enzyme with the FL mutations was constructed by DNA 2.0 (Menlo Park, CA) by site-directed mutagenesis [17]. The mutants have the Y212F mutation in combination with L, I, or Y substitutions at the V342 site (Table 1). The GWT mutant of the PTE gene was made by directed mutagenesis performed using the Quick Change (Agilent Technologies) protocol [18].

Enzyme activity was determined with a fluoride electrode connected to an Accumet XL250 ion selective meter (Thermo Fisher Scientific, Inc.) calibrated against authentic standards. Assays were conducted in 2.0 mL of 50 mM bis-tris-propane buffer, pH 8.0, containing 0.1 mM $MnCl_2$ which was added just prior to the assay. Data were logged every 30 s. Sarin was used at a concentration of only 0.5 mM for stereochemistry assays and for enantioenriched preparations since the rate of racemization is at least partially a function of the concentration of fluoride in the sample [19].

3. Theory/calculation

Kinetic parameters were calculated using Biosoft EnzFitter© software (Biosoft.com). Activity data were generally collected at substrate concentrations ranging from 1/3 to three times the K_m under conditions that consumed less than 10% of the substrate. At least six, more typically ten, different substrate concentrations were used for each curve. The percent uncertainties of the Vmax and K_m values determined from the software were added together to determine the percent uncertainties of the k_{cat}/K_m values.

Acetylcholinesterase inhibition was determined on a plate reader (BioTek Synergy4) using a modified Ellman Assay, as described in Results

The rationale for selecting the respective OPAA sites for mutation was described previously [13]. Briefly, the OPAA enzyme active site comprises a small pocket, a large pocket and a leaving group pocket.