

72. KINETICS OF NERVE AGENT HYDROLYSIS BY A HUMAN PLASMA ENZYME

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ABSTRACT

In the search for new nerve agent scavengers the organophosphorus acid anhydride hydrolase (OPAA hydrolase) enzymes from human plasma (paraoxonase 1, PON1) have emerged as very promising candidates. Although the turnover rates of these enzymes are not believed to be high enough to confer a great amount of protection against nerve agents, it is probable that those rates can be improved by protein engineering once high-resolution crystal structures are available. It was previously shown that both known isozymes of PON1 are able to hydrolyze the toxic isomers of soman, sarin and, to a lesser extent, tabun. We now have shown that in spite of the rather high K_m values of these enzymes with these agents, sarin and soman are hydrolyzed quite rapidly even at submicromolar concentrations. We have also demonstrated that the enzyme is able to catalyze the hydrolysis of VX. In contrast to the reactions with soman and sarin, both of which produced good Lineweaver-Burk plots from which K_m and V_{max} could be calculated, this reaction appears not to follow Michaelis-Menten kinetics; the rate of reaction continues to increase with substrate concentration at least up to 3.2 mM VX at which point the hydrolysis rate is 0.1 $\mu\text{mole per minute per mg enzyme}$, a value that is comparable to the value of 0.16 $\mu\text{mole per minute per mg enzyme}$ reported for the hydrolysis of VX by the Co^{++} form of the OPAA Hydrolase from *Pseudomonas diminuta*, the most efficient VX-hydrolyzing enzyme reported previously.

INTRODUCTION

In the search for a suitable *in vivo* scavenger to protect personnel against nerve agent intoxication, we have considered many enzymes. In general, those that are available in nature are not suitable catalytic scavengers because they bind the nerve agents poorly and their hydrolytic turnover rates are too slow. We have attempted to remedy this situation by engineering enzymes that are inhibited by the organophosphorus anticholinesterases in a way that they behave as organophosphorus acid anhydride hydrolases (OPAH), that is, they catalyze the hydrolysis of nerve agents. We began with that strategy because the crystal structure and DNA sequence were available for acetylcholinesterase (AChE) [1] and from that crystal structure a model could be constructed of butyrylcholinesterase (BuChE) [2] and carboxylesterase (CaE) [3], whereas the folded structures of OPAH enzymes were (and remain) unknown. Unfortunately, enzyme-engineering experiments have been only partially successful to this point in time and though the desired activity was produced the required turnover rates have not been approached [4, 5, 6]. This leads us to pursue other avenues for the discovery of a catalytic scavenger. One promising candidate is the in human plasma OPAH, PON1, a member of a family of esterases associated with high-density lipoprotein. Their natural function has been shown to include the hydrolysis of homocysteine thiolactone, a protective mechanism against protein N-homocysteinylation [7]. Mice in which this gene has been knocked out are highly sensitive to organophosphorus insecticides and are susceptible to atherosclerosis [8]. It has a molecular weight of about 35 kD, similar to that of OPAH from *Ps. diminuta*, the crystal structure of which has been solved [9]. However, there appears to be no homology between the two classes of enzymes. The enzymes differ in metal ion requirements (PON1 requires Ca^{++} , the *Pseudomonas* enzyme requires Zn^{++} or Co^{++}). Although there are three cysteine residues in each protein their positions in the sequence are completely different.

Several years ago we studied the hydrolysis of soman, sarin and tabun by human PON1 [10]. The results are shown in Table 1.

In humans PON1 exists as two phenotypes with either Gln (type Q) or Arg (type R) at position 192 in the amino acid sequence. As can be seen in the Table, both soman and sarin are hydrolyzed by either form, but with a high K_M , though the Q variant seems to be significantly faster. Tabun was also hydrolyzed measurably, but at a much slower rate (data not shown). It was of interest to determine whether VX hydrolysis would also be catalyzed by this enzyme and whether this enzyme activity is of practical significance at the low concentrations attained in an exposure of a few times the LD50.

MATERIALS AND METHODS

PON1: Drs. Masson and Josse have been involved in an intense study of the PON enzymes recently and have produced highly purified preparations, which they provided to us at Edgewood for study of the kinetics of VX hydrolysis. They have also produced several mutants in cell culture media that they provided for some of the low concentration studies.

VX Hydrolysis: The hydrolysis of VX is conveniently measured by following colorimetrically the liberation of the thiocholine analog using 5,5'-dithiobis-(2-nitrobenzoic acid).

By utilizing a microtiter plate reader with a 405nm filter, a series of up to 12 VX concentrations were measured simultaneously in triplicate; thus all reactions in any given run proceed under identical conditions, an ideal arrangement for kinetics measurements. Beginning with the highest concentration of VX that may be handled in our laboratory under surety regulations, 3.44 mM, a series of dilutions was made using a mixture of 90% 1.1M NaCl/2.2mM CaCl₂ and 10% 0.2M

Tris buffer (either pH 7.4 or pH 8.5) as a dilutant. Generally the dilutions were serially 4:5. To each concentration of VX (1 mL) was added 20 uL of 0.02M DTNB, the solutions were mixed thoroughly and 100 uL of each concentration was admitted to a row of wells in a microtiter plate. The final VX concentrations ranged from 3.21 down to 0.28 mM. Three microliters of the appropriate enzyme solution was added to each well and the plate was read for 20 min. One row was kept without enzyme to measure background hydrolysis, if any. The hydrolysis rate in each well was calculated from the slope of the line measured between 0 and 20 minutes, a total of 41 points. Each enzyme sample was run in triplicate and at two pH values.

G Agent Hydrolysis, millimolar range: In order to compare the VX hydrolysis rates with those of G agents the hydrolysis of soman was measured under comparable conditions. Soman was diluted serially beginning at 11mM and then each of those dilutions was brought to the same ionic strength by the addition of an equal volume of 1M NaCl/2 mM CaCl₂, creating a concentration range from 5.45 to 0.16 mM. Detection of hydrolysis rate was by pH stat. All data were normalized to nmol agent hydrolyzed per minute per μ L enzyme used so that they could be compared.

Agent hydrolysis, micromolar range: First, a solution of cholinesterase (either acetyl or butyryl) was prepared such that when 10 microliters of 1 micromolar agent was added to a 50 microliter aliquot the activity was just shy of being completely inhibited. Then, to 100 microliters of a solution of the enzyme to be tested, equilibrated to 30 deg., was added a solution of the agent to be tested such that the final concentration was exactly one micromolar and mixed on a vortex mixer. As quickly as possible (usually 10-12 seconds) a 10 microliter sample of the mixture was transferred to 50 microliters of the cholinesterase solution, mixed and held at room temperature for 10 minutes to allow reaction between the cholinesterase and the residual agent. Similar samples were taken at predetermined times after being mixed and treated in the same manner. After the ten-minute incubation period the cholinesterase activity of the sample was determined and the concentration of residual agent calculated. The linear portion of plots of residual agent concentration versus time provided initial rates of agent hydrolysis. Minor variations of this procedure permitted determination of agent hydrolysis rates between 0.25 and 10 micromolar initial concentrations.

RESULTS

The VX hydrolysis results at millimolar concentrations are shown in Figure. 2. The black circle is a determination at a single substrate concentration (1 millimolar) with the OPAH from *Pseudomonas diminuta* for comparison; this enzyme is reported to have the highest turnover rate for VX of any enzyme tested so far. It is noted that VX hydrolysis by the human PON is faster at pH 8.5 than at 7.4. This is not unexpected; with the G agents and insecticides rates of hydrolysis are faster at higher pH. However, it is surprising that the human enzyme is faster at both pH values than the rabbit enzyme is at the higher pH. It should be pointed out that the enzyme solutions used for the VX data are the same as those for the soman data in Figure. 3. The rabbit enzyme hydrolyzes soman about 10 times as fast as the human enzyme while VX is hydrolyzed much more slowly. It is also interesting to note that the *Ps. Diminuta* OPAH is much slower with GD than the rabbit PON, but much faster with VX than either PON.

The results of studies at low micromolar substrate concentrations are not yet as quantitative as those at the higher concentrations, though they are, perhaps, more pertinent. It is clear that both human PON variants are able rapidly to hydrolyze the G agents tested, with very little difference at these concentrations. The Q variant (the most abundant one) is also able to catalyze the hydrolysis of VX. The ability of the R variant to hydrolyze VX has not yet been conclusively demonstrated; pure R type PON is difficult to obtain from natural sources. Recombinant Q192R has been made by Dr. Josse and will be available for future testing.

CONCLUSION

We have shown that PON1, the OPAH from human plasma, is able to catalyze the hydrolysis of VX at a rate comparable to the OPAH from *Pseudomonas diminuta*, the best enzyme for that purpose yet described. Additionally, we have shown that nerve agents are destroyed by PON1 even at the low concentrations attained in blood at lower levels of exposure. Furthermore, since this enzyme obviously was not designed to catalyze the hydrolysis of nerve agents, it should be amenable to enzyme-engineering techniques to improve that activity. Because it is a natural plasma enzyme it should be compatible with the human organism and expected to be tolerated well if exogenously

augmented. Efforts are in progress to obtain diffracting crystals of PON1 so that a crystal structure can be obtained that we hope will enable us, and others, to design possible mutants with improved nerve agent binding and faster hydrolysis rates that can be used as practical nerve agent scavengers.

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FIGURES AND TABLES

Figure 1. The hydrolysis of VX is conveniently measured by following colorimetrically the liberation of the thiocholine analog using 5,5'-dithiobis-(2-nitrobenzoic acid).

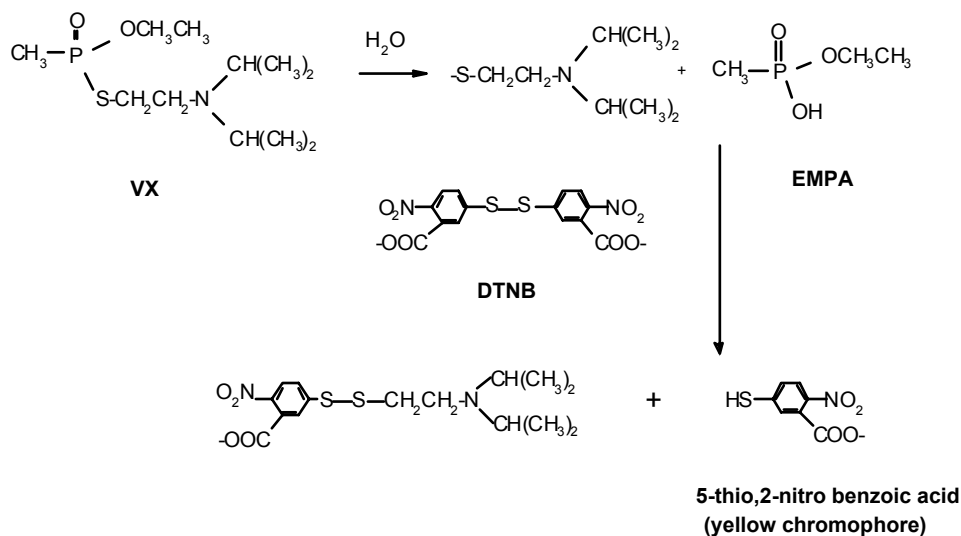


Figure 2. The VX hydrolysis results at millimolar concentrations. The black circle is a determination at a single substrate concentration (1 millimolar) with the OPAH from *Pseudomonas diminuta* for comparison

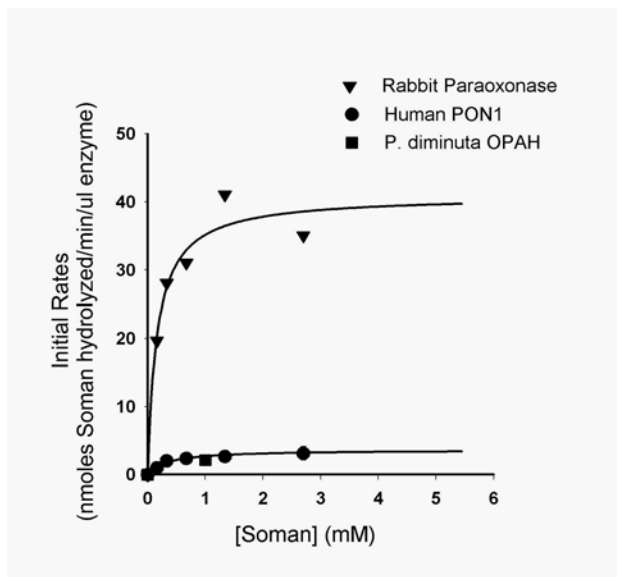


Figure 3. Hydrolysis of VX.

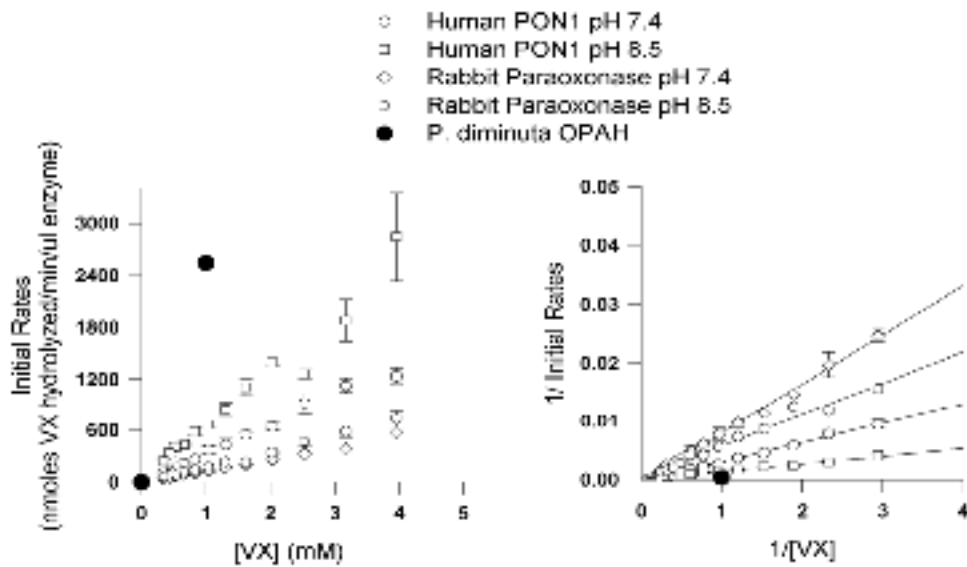


Table 1. Hydrolysis of Sarin and Soman by Plasma OPAH

Enzyme	Sarin		Soman	
	Km	Vmax	Km	Vmax
Hu 192Q	0.21	69	0.41	82
Hu 192R	0.31	21	0.25	31
Heterozygous	0.15	24	0.39	36

Units for Vmax = micromoles/minute/milligram protein