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Protection against tabun toxicity in mice by prophylaxis with an enzyme hydrolyzing organophosphate esters

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Abstract—We demonstrate here the correlation between protection afforded by pretreatment alone with parathion hydrolase purified from *Pseudomonas* sp. against tabun toxicity in mice and the kinetic parameters which are assumed to determine the *in vivo* detoxification of tabun by the same enzyme. Results show that 15 and 22 μg of parathion hydrolase per animal conferred a protective ratio of 3.94 and 5.65 respectively, against tabun toxicity, without post-exposure treatment.

Pretreatment with as little as 7.5 and 26 μg of parathion hydrolase purified from *Pseudomonas* sp. has been demonstrated in mice to confer, without additional treatment, protection against multiple median lethal doses (LD_{50}) of diethyl *p*-nitrophenyl phosphate (paraoxon) and its P-F-containing analogue, diethyl fluorophosphate

(DEFP*), respectively (see Scheme 1) [1]. The ability of organophosphorus acid anhydride (OPA) hydrolases to protect against these anti-cholinesterase organophosphates (OP) suggests that they may be considered as suitable prophylactic drugs against toxic nerve agents [1–3].

$(C_2H_5O)(R)P(O)X$

	R	X
Paraoxon:	C_2H_5O	*p*-nitrophenoxy
DEFP:	C_2H_5O	F
Tabun:	$(CH_3)_2N$	CN

Scheme 1

Ethyl *N,N*-dimethylamidophosphorocyanidate (tabun) is considered a hazardous anti-cholinesterase nerve agent. The combination of a P—CN moiety and a phosphoroamidate function (i.e. P—N bond) appears to constitute a unique electrophilic character of the phosphorus atom in tabun. Indeed, recent studies with OPA hydrolases isolated from human serum [4], a mouse neuroblastoma–rat glioma hybrid cell line [5] or rat liver [6] have indicated that the hydrolysis of tabun by these enzymes is significantly slower than their catalytic hydrolysis of OP esters which do not contain a P—N bond. Furthermore, mipafox (*N,N'*-diisopropyldiamidophosphorofluoridate), which also contains a P—N bond, was hydrolyzed slowly by an OPA hydrolase from *Pseudomonas diminuta*, compared to various dialkyl and methylphosphonofluoridates [7]. Since parathion hydrolase from *Pseudomonas* sp. has been demonstrated to confer excellent protection against paraoxon and DEFP, it seemed important to determine the kinetic constants of the enzyme-catalyzed hydrolysis of tabun and to test the ability of this particular parathion hydrolase to protect against poisoning with this OP.

Materials and Methods

Materials. Tabun was prepared according to Holmstedt [8] and purified to homogeneity by silica gel column chromatography (TLC one spot, >98% purity by gas chromatography, and ^{1}H- and ^{31}P-NMR). *N*-[(2-Acetamido)iminodiacetic acid] disodium salt (ADA) was purchased from Sigma (St. Louis, MO, U.S.A.). Parathion hydrolase, purified from *Pseudomonas* sp. [9] and stored at −20° in 50 mM Tris–50% glycerol buffer, pH 8.0, was dialyzed against 5 mM Tris, pH 7.4, before being used.

Animals. Adult male ICR mice weighing 23–26 g were cared for in accordance with the principles enunciated in the "Guide for Care and Use of Laboratory Animals" (NIH Publication No. 85-23, 1985 revision).

Enzymatic hydrolysis of tabun. The kinetic constants of the enzymatic hydrolysis of tabun were determined by monitoring the release of cyanide ion with a cyanide selective electrode (Corning 476127, Medfield, MA, U.S.A.) attached to a pH-meter (Radiometer PHM 82, Copenhagen, Denmark), equipped with a double-junction reference electrode. The concentration of cyanide was determined from a calibration curve constructed by describing relative mV against the logarithm of known concentrations of KCN in the same buffer.

The disappearance of tabun and the concomitant appearance of hydrolysis products were monitored by ^{31}P-NMR spectroscopy at 121.6 MHz using a GN 300WB (General Electric) spectrometer. The temperature during data acquisition was 18–20°.

Protection experiments. Since the blood–enzyme concentration was maintained at a constant level only during the first 10–15 min following an i.v. injection of parathion hydrolase [1], mice were challenged with an i.v. bolus injection of tabun in saline 5 min after the administration of the enzyme. No additional supporting therapy was given to tabun-challenged mice. The median lethal dose was calculated (N = 16) on the basis of 24-hr mortality [1].

Results and Discussion

Kinetic constants of hydrolysis of tabun by parathion hydrolase. Tabun released 1 ± 0.07 equivalents of cyanide either in alkaline solution (0.1 N NaOH) or in the presence of parathion hydrolase at pH 7.5 (10 mM ADA buffer). The spontaneous hydrolysis of tabun at pH 7.5 was negligible. ^{31}P-NMR spectroscopy revealed (Fig. 1, inset) that the enzymatic hydrolysis of tabun occurred by breaking the P—CN bond, to form the corresponding ethyl *N,N*-dimethylamidophosphoric acid. The enzymatic enhancement of the hydrolysis of tabun followed Michaelis–Menten kinetics throughout the entire range of substrate concentrations used (7–140 μM). Figure 1 shows the goodness-of-fit ($r^2 = 0.992$) of the curve obtained by non-linear regression analysis of the data in accordance with the standard hyperbola equation. The Michaelis–Menten constant (K_m) and the maximum velocity (V_{max}) were found to be 0.101 ± 0.016 mM and 128 ± 11 $\mu mol \cdot mg^{-1} \cdot min^{-1}$, respectively (Table 1). Assuming a molecular weight of 36 kDa for parathion hydrolase [9], the turnover (k_{cat}) and the bimolecular rate constant (k_{cat}/K_m) values for the reaction of parathion hydrolase with tabun were calculated to be $4.6 \times 10^3\ min^{-1}$ and $4.56 \times 10^7\ M^{-1}\ min^{-1}$, respectively. Although it is difficult to compare the kinetic constants for the hydrolysis of tabun at 25° with constants determined at 38° for the hydrolysis of paraoxon and DEFP by the same enzyme (Table 1) [1], it seems that parathion hydrolase catalyzes the hydrolysis of tabun and DEFP at a similar rate. Further, the enhancement of the hydrolysis of paraoxon by parathion hydrolase was only 10- and 30-fold (k_{cat} and k_{cat}/K_m, respectively) higher than that of tabun. Thus, the kinetic constants for the hydrolysis of tabun, DEFP, parathion and paraoxon illustrate, in

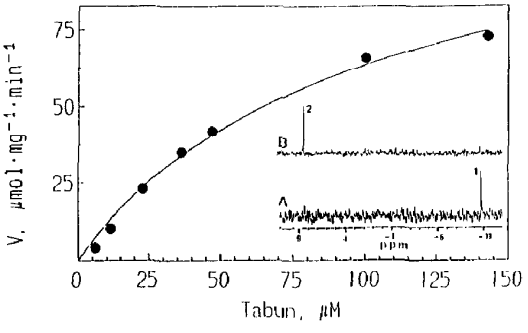

Fig. 1. Michaelis–Menten plot for the hydrolysis of tabun by parathion hydrolase from *Pseudomonas* sp. (10 mM ADA buffer, pH 7.5, 25°). Curve fitting was carried out by the method of non-linear regression analysis of data points (average of three determinations; SEM < 15%) in accordance with a hyperbola equation. Inset: ^{31}P-NMR spectra of tabun (7 mM) recorded after 5 min in 0.1 M ADA buffer (pH 7.4) (A) and 8 min following the addition of *ca.* 1 μg parathion hydrolase to the same NMR tube (B). Signals 1 and 2 were assigned to tabun and $(CH_3)_2N(C_2H_5O)P(O)OH$, respectively. Chemical shifts are relative to external trimethylphosphate.

* Abbreviations: ADA, *N*-[(2-acetamido)iminodiacetic acid] disodium salt; DEFP, diethyl fluorophosphate; OP, organophosphate(s); and OPA, organophosphorus acid anhydride.

Table 1. Summary of kinetic constants of the hydrolysis of $(C_2H_5O)RP(O)X$, catalyzed by parathion hydrolase

OP (name/structure)	V_{max} ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)	K_m (mM)	k_{cat}* (min^{-1})	k_{cat}/K_m ($M^{-1} min^{-1}$)
Tabun† ($R = (CH_3)_2N$; X = CN)	128 ± 11‡	0.101 ± 0.016‡	4.6×10^3	4.56×10^7
Paraoxon§ ($R = C_2H_5O$; X = pNP)‖	1387 ± 46	0.035 ± 0.003	5.0×10^4	1.4×10^9
DEFP§ ($R = C_2H_5O$; X = F)	350 ± 75	0.25 ± 0.10	1.3×10^4	5.2×10^7

* Calculated assuming a molecular weight of 36 kDa [9].
† Determined in 10 mM ADA buffer, pH 7.5, 25°.
‡ Means ± SEM, N = 7.
§ Taken from Ref. 1 (determined at pH 7.4, 38°).
‖ pNP = *p*-nitrophenol.

Table 2. Protection of mice against tabun toxicity by pretreatment with parathion hydrolase

Parathion hydrolase* (μg protein/mouse)	LD_{50}† (μg/kg)	Protective ratio‡	Time§ (sec)
Non-treated	223 (204–244)	1	
15	878 (742–1041)	3.94	11
22	1259 (1199–1320)	5.65	11

* Both parathion hydrolase and tabun were administered by an i.v. bolus injection.
† Figures in parentheses are 95% confidence limits (N = 16, each group).
‡ LD_{50} in protected animals divided by LD_{50} in naive mice.
§ Time required to decrease the initial concentration of tabun to a level of $1 \times LD_{50}$ in non-protected mice was calculated by using the integrated form of the Michaelis–Menten equation [1].

absolute terms, the ability of parathion hydrolase from *Pseudomonas* sp. to hydrolyze both OP esters and an OP amidate extremely rapidly. Based on the concentration–time curve for the release of cyanide and ^{31}P-NMR spectroscopy, both stereoisomers of tabun appeared to be hydrolyzed at a similar rate. Lack of stereospecificity in the hydrolysis of certain OPs has also been demonstrated for other OPA hydrolases [4, 6, 7].

Prophylaxis against tabun toxicity. Results show that 15 and 22 μg of parathion hydrolase per animal conferred protective ratios of 3.94 and 5.65 against tabun toxicity, respectively (Table 2). The time required to decrease the initial concentration of either 3.94 or $5.65 \times LD_{50}$ dose of tabun to a concentration level of $1 \times LD_{50}$ dose of tabun in naive mice was estimated to be 11 sec at 25° (see Table 2). Since the blood circulation time in mice has been estimated to be 8 sec [10], we predict that at 38° the rate of *in vivo* detoxification should be higher and sufficient to decrease tabun to a level below $1 \times LD_{50}$ dose in less than one circulation time.

To the best of our knowledge, parathion hydrolase purified from *Pseudomonas* sp. is the first enzyme which has been reported to exhibit substantial rate enhancement towards a phosphoroamidate, and to protect against multiple lethal doses of tabun, without the need of post-exposure treatment. The kinetic constants of the hydrolysis of three different OPs (see Scheme 1), taken together with results from protection experiments with the same enzyme, suggest that future research should be directed at the exploration of OPA hydrolases capable of hydrolyzing methylphosphonate nerve agents (e.g. sarin, soman). Low doses of these enzymes should have a turnover sufficient to permit rapid detoxification of the toxicants.

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