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INHIBITION OF A SOMAN- AND DIISOPROPYL PHOSPHOROFLUORIDATE (DFP)-DETOXIFYING ENZYME BY MIPAFOX

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Abstract—Mipafox, *N,N'*-diisopropylphosphordiamidofluoridate, has been found to be a reversible competitive inhibitor of a diisopropyl phosphorofluoridate hydrolyzing enzyme (DFPase) isolated from hog kidney and *Escherichia coli*. Heretofore, this DFPase was characterized by its more rapid hydrolysis of Soman (1,2,2-trimethylpropyl methylphosphonofluoridate), its stimulation by Mn^{2+}, and its wide distribution. In sharp contrast, Mipafox did not inhibit the DFPase found only in cephalopod nerve, hepatopancreas, and saliva, and further characterized by its more rapid hydrolysis of DFP than of Soman, and its indifference to Mn^{2+}. Neither of these two DFPases hydrolyzed Mipafox.

The present status of the enzymatic detoxication of DFP* and some related ChE inhibitors has recently been presented, together with a brief entrée to the history of this subject [1]. While certain practical implications arise from this work [2], more fundamental progress continues to be impeded by a lack of knowledge of a natural substrate or physiological role for DFPase. In addition, there are at least two enzymes, and perhaps more,† to which this name applies. Squid type DFPase, narrowly distributed in nerve, hepatopancreas, and saliva of the cephalopods hydrolyzes DFP several times faster than Soman, has a molecular weight of about 26,000, and is indifferent to Mn^{2+}; the more widely distributed enzyme—we have introduced the term "Mazur type DFPase" [1] in recognition of the discoverer [3]—hydrolyzes Soman many times faster than DFP, has a much higher molecular weight (reports differ, but between 45,000 and 65,000) and is stimulated several-fold by Mn^{2+}.

In a continuing search for substrates and inhibitors of DFPase, many organophosphorus compounds have been considered [4], especially those displaying properties in addition to the inhibition of the ChEs. One such, Mipafox, had shown considerable promise as an insecticide, inhibiting AChE at about 10^{-4} M, and ChE at about 10^{-7} M, each to a comparable degree for comparable times and conditions [5]. The notable additional property is that, from its earliest (and hence limited) use, Mipafox was implicated in a more slowly developing muscle paralysis [6], the cause of which appears to be the inhibition of another "inessential" enzyme [7, 8] abbreviated as NTE and the subsequent degeneration of nerve fiber.

Evidence is now presented that Mipafox is a potent, reversible, competitive inhibitor of Mazur type DFPase, but not of squid type DFPase, whether DFP or Soman is used as substrate. Nevertheless, Mazur type DFPase is not NTE. These results may offer a new approach to the study of the active site and physiological role of one of the DFPases, but at the same time suggest additional hazards in the use of Mipafox and perhaps similar compounds.

MATERIALS AND METHODS

Squid type DFPase is routinely prepared in large quantity in this laboratory [9], now by much improved methods, for the eventual production of antibodies. For reasons of economy, the enzyme used here was not of the highest purity, but it was free of any of the Mazur type enzyme by virtue of the method of purification [1]. Mazur type DFPase was partially purified from hog kidney by published methods [10]. It was immobilized (see Table 2 of Ref. 1) by the method published for squid type DFPase [2]. *Escherichia coli* (ATCC25922) was grown, harvested, and sonicated as described previously [1]. Newly hatched squids, *Loligo pealei* [11], were homogenized in 20% (w/v) buffer [12]. *Escherichia coli* sonicate and whole squid homogenate were used without further purification.

DFP was purchased from the Sigma Chemical Co., St. Louis, MO, and Mipafox from Dr. R. J. Richardson, University of Michigan, Ann Arbor, MI. Soman was synthesized as previously described [13].

In a typical DFPase determination, 3.4 ml buffer [12] with or without Mn^{2+} and 1.5 ml of 0.01 M Soman in buffer were combined and fluoride release was followed for 5–10 min by means of a fluoride-sensitive electrode and a model 901 Orion meter (Fig. 1A). With DFP there was no detectable fluoride

* Abbreviations: DFP, diisopropyl phosphorofluoridate; ChE, cholinesterases, especially the non-specific esterases; Soman, 1,2,2-trimethylpropyl methylphosphonofluoridate; Mipafox, *N,N'*-diisopropylphosphordiamidofluoridate; AChE, the specific esterase, acetylcholinesterase; NTE, neurotoxic esterase; and Tabun, ethyl *N,N*-dimethylphosphoramidocyanidate.

† F. C. G. Hoskin, International Conference on Environmental Hazards of Agrochemicals in Developing Countries, p. 50 (abstract). University of Alexandria, Egypt (1983).

release over this time period (Fig. 1B). Then 0.1 ml of enzyme solution or source was added and fluoride measurement was continued. With a potential inhibitor, e.g. Mipafox, the components were combined in a different order. Enzyme and inhibitor were mixed, fluoride release was measured (almost negligible) and then substrate, i.e. DFP or Soman, was added. The immediate jump in fluoride was ignored, and the subsequent steady rate of fluoride release was corrected by substraction of the very low (if any) fluoride release from Mipafox. When Soman was used as substrate, the non-enzymatic release found in the first type of experiment without Mipafox [(o) Fig. 1A] was also subtracted from the second type of experiment [(x) Fig. 1A].

RESULTS

Two typical experiments, one with Mazur type DFPase and Soman as substrate, and the other with squid type DFPase and DFP as substrate, are shown in Fig. 1. The Soman solution, initially about 2% decomposed, showed about 1% non-enzymatic hydrolysis over the next 10 min, and thereafter a high rate of enzymatic hydrolysis. Mipafox showed little or no evidence of enzymatic, nor of non-enzymatic, hydrolysis. Thereafter, on addition of Soman, there was an immediate jump to the level of non-enzymatic decomposition, and little or no evidence of additional enzymatic hydrolysis. The DFP solution, initially about 1% decomposed, showed no detectable non-enzymatic hydrolysis over the next 5 min and thereafter a high rate of enzymatic hydrolysis which was not inhibited by Mipafox. The same kinds of results were seen when substrates were switched. In view of the complete lack of inhibition of squid type DFPase by Mipafox, suitably confirmed, this phase of the work was discontinued.

Table 1. Inhibition of Mazur type DFPase by Mipafox with DFP or Soman as substrate

Mipafox (μM)	Inhibition (%) when substrate was DFP	Soman
3000	100	
1000	98	
300	93	87
100	79	68
88	78	
30		33
10		16

Table 1 and Fig. 2 present, respectively, the percentage of inhibition of Mazur type DFPase caused by various concentrations of Mipafox with both DFP and Soman as substrates, and the degree of inhibition (expressed as a reciprocal plot) caused by Mipafox as a function of the concentration of substrate, i.e. DFP. Because of the extreme toxicity of the compounds involved, a minimum number of experiments were performed. Even so, these were sufficient to permit a judgement that Mipafox is a competitive inhibitor of Mazur type DFPase, and an estimation, with certain assumptions implicit, that the binding of Mipafox to Mazur type DFPase is about 100 times stronger than that of DFP to the enzyme ($K_M \approx 1.7 \times 10^{-3}$ M; $K_i \approx 1.7 \times 10^{-5}$ M).

An aliquot of a suspension of agarose-immobilized Mazur type DFPase was tested for its ability to hydrolyze DFP; the suspension was centrifuged, aspirated, resuspended in Mipafox solution and similarly tested; the suspension was then washed by five centrifugations, aspirations and resuspensions, and an aliquot was again tested. The results, presented in Fig. 3, show that the inhibition of Mazur type DFPase by Mipafox was reversible. As expected for a competitive reversible inhibitor, the degree of

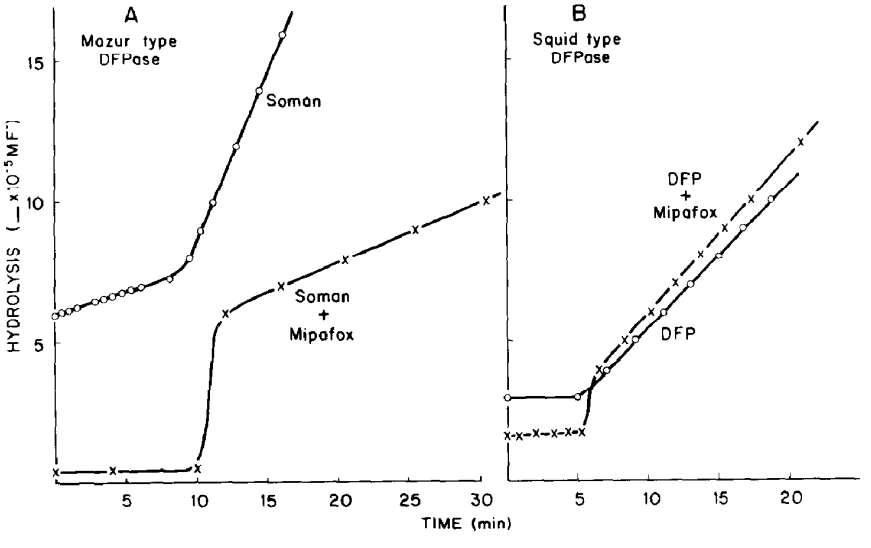

Fig. 1. Effect of Mipafox on the enzymatic hydrolysis of DFP and Soman. (A) Soman, 0.003 M; Mipafox, 0.001 M; 0.1 ml enzyme purified about 15-fold from hog kidney. Order of addition: (○), Soman, enzyme; (×), Mipafox, enzyme, Soman. (B) DFP, 0.003 M; Mipafox, 0.003 M: 0.1 ml enzyme purified about 500-fold from squid optic ganglion. Order of addition: (○), DFP, enzyme; (×), Mipafox, enzyme, DFP. Final volumes throughout, 5.0 ml; temperature, 23°. Note that activities in (A) or in (B) are comparable, but not between (A) and (B).

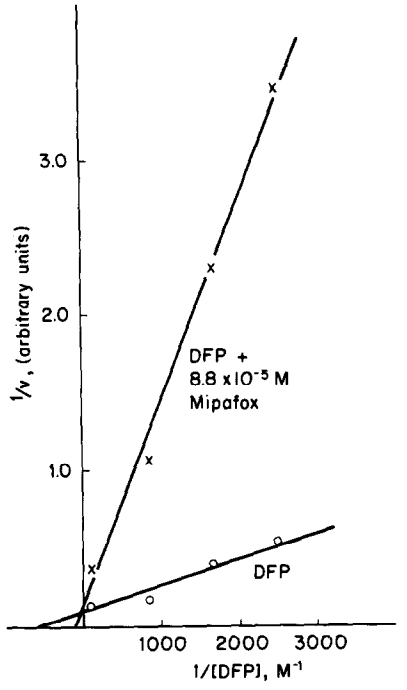

Fig. 2. Inhibition of Mazur type DFPase by Mipafox as a function of substrate (DFP) concentration. Order of additions was as in Fig. 1.

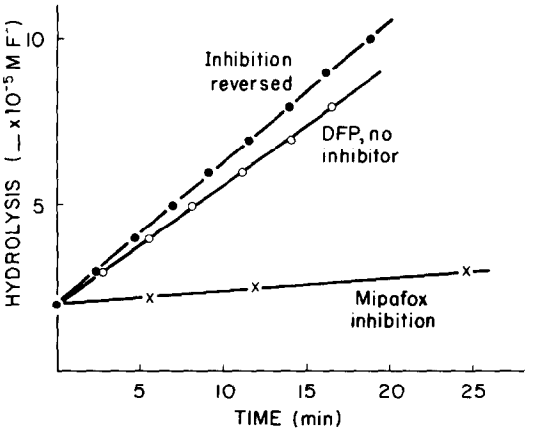

Fig. 3. Reversibility of Mipafox inhibition of Mazur type DFPase. Hydrolysis of DFP by an aliquot of a suspension of agarose-immobilized Mazur type DFPase purified from hog kidney (○). Suspension centrifuged, aspirated, and resuspended in 0.001 M Mipafox; aliquot tested in 0.001 M Mipafox for its ability to hydrolyze DFP (×). Same suspension centrifuged, aspirated, and resuspended in buffer five times; aliquot then tested without Mipafox for its ability to hydrolyze DFP (●). In all determinations, DFP was 0.003 M; other conditions were as in Fig. 1.

inhibition was found to be the same whether enzyme was added to a solution of DFP and Mipafox, or enzyme was incubated with Mipafox for 30 min before addition of DFP.

In a recent presentation,* it was suggested that the DPFase from *Escherichia coli* [1] might contain a significant amount of an enzyme preferentially hydrolyzing DFP (thus like the squid type DFPase) along with the predominant Mazur type DFPase. It has also been consistently found in this laboratory that cephalopod nerve, hepatopancrease and saliva are the only rich sources of squid type DFPase, whereas other squid parts—blood, gills, heart, mantle muscle—contain predominantly the Mazur type enzyme, and that in low levels.* On the assumption that both *Escherichia coli* and whole squid contain mixtures of two, or perhaps more, kinds of DFPase, sonicated *Escherichia coli* without further purification, and a squid hatchling [11] homogenate were tested for their DFPase response to Mipafox. The results are presented in Table 2. In contrast to the results in Table 1, some activity remained in the *Escherichia coli* sonicate even at high Mipafox concentration and, conversely, Mipafox caused some inhibition of the DFPase activity in squid homogenate.

* F. C. G. Hoskin, International Conference on Environmental Hazards of Agrochemicals in Developing Countries, p. 50 (abstract). University of Alexandria, Egypt (1983).

DISCUSSION

Earlier publications from this laboratory [1] have shown that a phosphor*mono*amide, Tabun, is more rapidly hydrolyzed than DFP by Mazur type DFPase. Indeed, that was originally the basis for its differentiation from squid type DFPase. In the present experiments, however, the phosphor*di*amide, Mipafox, was barely hydrolyzed by either enzyme (about 1% the rate of DFP), and was a moderately strong competitive inhibitor of only the Mazur type DFPase. Thus, for the first time these superficially similar enzymes are distinguishable on the basis of what must be a difference in the nature of their active sites or the immediate environs.

Table 2. Effect of Mipafox on hydrolysis of DFP and Soman by mixed sources of DFPase

Enzyme source	Mipafox (mM)	Enzymatic hydrolysis of DFP (μmoles/min/g tissue)	Soman	Inhibition by Mipafox (%, approx.)	Ratio Soman/DFP
Escherichia coli	0	1.27	9.25	0	7.3
	1	0.20	2.02	80	10.1
	3	0.083	0.88	90	10.6
Squid hatchling	0	0.87	0.78	0	0.89
	3	0.82	0.62	10–20	0.76

While an inhibitor of DFPase might be expected to lead to a fruitful exploration of the physiological role of such an enzyme, three factors probably offset this. First, Mipafox is not as powerful and as irreversible an inhibitor of DFPase as, for example, Soman is of AChE; second, Mipafox is already an inhibitor of two other enzymes, AChE and NTE, the inhibition of one of which might be expected to have precipitous effects of its own; and third, Mipafox is an inhibitor of the ubiquitous Mazur type DFPase rather than the narrowly distributed squid type DFPase.

The other enzyme that Mipafox inhibits, NTE, may raise the question as to whether Mazur type DFPase and NTE are synonymous. The manner of purification of the DFPase [10], the association of NTE with synaptosomal membranes [8], and above all the role of DFP as a substrate rather than an inhibitor of DFPase, whereas DFP is a 10-fold more potent inhibitor of NTE than Mipafox [14], seem to dismiss this possibility.

The low level of DFPase inhibition by Mipafox in whole squid (hatchling) homogenate is probably to be expected in an organism already described here and elsewhere* as containing both kinds of DFPase. The small but significant amount of DFPase activity remaining in *Escherichia coli* sonicate after Mipafox, and the Soman/DFP ratio (see Table 1 of Ref. 1) either remaining unchanged or even increasing (see Table 2) seem to suggest yet a third kind of DFPase. Several preliminary chromatographic separations presented from this laboratory* but not yet in print tend to support this.

A practical utilization of the DFPase from *Escherichia coli* for the detoxication of hazardous organophosphorus compounds, alluded to elsewhere [1], may be negated by Mipafox and compounds of a similar structure. Such compounds may even present hitherto unsuspected hazards. A solution to one of these problems, if such is desirable, would be to obtain the gene for the squid type DFPase and to determine whether it can be expressed in a suitable host, possibly even in *Escherichia coli*.

* F. C. G. Hoskin, International Conference on Environmental Hazards of Agrochemicals in Developing Countries, p. 50 (abstract). University of Alexandria, Egypt (1983).

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