Cooperative Binding of the Organophosphate Paraoxon to the (Na⁺ + K⁺)-ATPase

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Paraoxon, the main active metabolite of the organophosphorus insecticide parathion, exerted a dose-dependent inhibitory effect on the activity of pig kidney (Na⁺ + K⁺)-ATPase contained in microsomal fraction and purified from it. Substrate kinetics studies revealed the existence of two active sites with high and low affinity to ATP. The Dixon analysis of the mode of the inhibition indicated its noncompetitive character. The purified enzyme was more affected than enzyme contained in the microsomal fraction. The inhibition constant K_i ranged from 73 to 246 μM depending on the type of preparation. The Hill coefficient (n) fulfilled the relationship $1 < n < 3$. These properties of the interaction suggest the cooperative binding of paraoxon to the enzyme. An indirect mechanism of the interaction was proposed: paraoxon could inhibit the activity of the (Na⁺ + K⁺)-ATPase by excluding the enzyme protein from its normal lipid milieu.

Introduction

Parathion is a *O*-phenyl derivative of phosphoric acid (Fig. 1a) that has belonged to the most heavily used organophosphorus insecticides (Ware, 1978; Qiao *et al.*, 1994). The insecticide, in its original form, has extremely low acute toxicity (Gage, 1953). It is activated to its oxygen analogue, paraoxon (Fig. 1b) trough the cytochrome P450-dependent oxidative desulfuration of parathion (Kulkarni and Hodgson, 1980; Neal, 1972; Whitehouse and Ecobichon, 1975). Parathion and its metabolite can be extruded from the organism by the kidney through the secretory pathway of the organic anions (Reyes *et al.*, 1983). This secretion is active and depends on the activity of the tubular ATPases, mainly (Na⁺ + K⁺)-ATPase (EC 3.6.1.3) (Jaramillo-Juarez *et al.*, 1989). Some organophosphorus compounds are reported to inhibit the activity of this enzyme (Dierkes-Tizek *et al.*, 1984; Robineau *et al.*, 1991; Imamura and Hasegawa, 1984), thus (Na⁺ + K⁺)-ATPase can be involved both in toxic action of insecticides and in the processes of their detoxification.

(Na⁺ + K⁺)-ATPase is the enzyme which is present in biological membranes (Jörgensen, 1982). On the other hand organophosphorus insecticides,
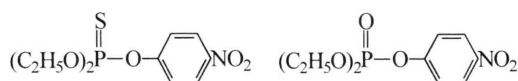
Fig. 1. Chemical structures of parathion (*O,O*-diethyl *O*-4-nitrophenyl phosphorothioate) (a) and paraoxon (*O,O*-diethyl *O*-4-nitrophenyl phosphate) (b).

due to their lipophilic character, may interact with membranes as structures rich in lipids (Antunes-Madeira and Madeira, 1982), thus the interaction of the insecticides with membrane enzymes may be taken into account as their possible biological side effect. Although the inhibition of the active site of acetylcholinesterase is the main mode of the toxic action of organophosphorus insecticides, there are some organophosphorus esters that cause an insidious neuropathic anomaly which is not related to acute cholinergic effects (Ohkawa, 1982). It is also important to explore the effects of insecticides in nontarget tissues to evaluate their environmental safety.

In the present work the activity of the pig kidney (Na⁺ + K⁺)-ATPase in the presence of paraoxon was investigated. Two kinds of enzyme preparations were used: the enzyme located in the microsomal fraction and the enzyme purified from it. These two preparations have different phospholipid and cholesterol contents as related to protein (Jörgensen, 1974). Organophosphorus insecticides

have a strong affinity to phospholipids (Antunes-Madeira and Madeira, 1984, 1987) and their action can be modulated by cholesterol (Blasiak, 1993a, 1993b, 1995; Blasiak and Walter, 1992), so the possible action of paraoxon can differ for these two enzyme preparations.

Materials and Methods

The procedure of Jörgensen (1988) was used to prepare (Na⁺ + K⁺)-ATPase from pig kidney.

Protein was estimated by Peterson's (1977) modification of Lowry *et al.* (1951) procedure. The quantitative determination of phospholipids was done according to the method of Bartlett (1959). Cholesterol was determined by the method of Babson *et al.* (1962).

The activity of (Na⁺ + K⁺)-ATPase was determined spectrophotometrically using an assay coupling ADP production to NADH oxidation (Josephson and Cantley, 1978). The molar extinction coefficient at 340 nm was taken as 6.22×10^3 M^{-1} cm^{-1} (Moczydlowski and Fortes, 1981). The activity of (Na⁺ + K⁺)-ATPase was expressed as the difference between amounts of µmoles oxidized NADH without and with oubain. This quantity corresponds to the amount of µmoles ADP or inorganic phosphate released during the enzymatic hydrolysis of ATP.

Each experiment was performed five times. One-way analysis of variance was used. The differences between means were compared using Scheffe's multiple comparison test (Zar, 1974).

Results and Discussion

The relative content of phospholipids and cholesterol in microsomal fraction was 564 and 158 µg/mg protein, respectively; in purified enzyme these values equalled 626 and 270 µg/mg protein, respectively.

The activity of (Na⁺ + K⁺)-ATPase was measured as the function of ATP concentration in the range 25 µM – 10 mM for both kinds of enzyme preparations, i.e. the enzyme contained in microsomal fraction and the purified enzyme. Results, presented in double reciprocal Lineweaver-Burk form (Fig. 2), show that the response of the enzyme to ATP concentration is biphasic. The curves in Fig. 2 can therefore be represented by the sum of two Michaelis-like equations: one with high af-
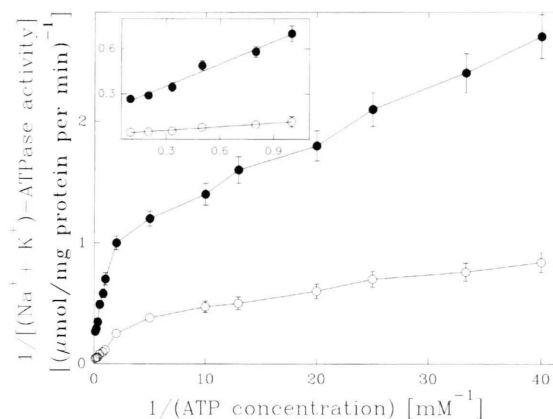

Fig. 2. Double-reciprocal plot of the substrate kinetics of the pig kidney (Na⁺ + K⁺)-ATPase contained in microsomal fraction (○) and purified from it with SDS (●). Error bars denote SE, each experimental point is the mean of three replications. In the inset the data with higher concentrations of ATP are replotted; regression lines were calculated by means of the least squares method, the R values were 0.989 for upper line and 0.990 for lower one. In some cases error bars were smaller than the symbol radius.

finity and low maximum rate and the other with low affinity and high maximum rate. These equations describe two functional active sites of the enzyme. The influence of the insecticides on the activity of (Na⁺ + K⁺)-ATPase was studied separately for low and high affinity active sites.

Paraoxon inhibited the activity of the (Na⁺ + K⁺)-ATPase. The effect was dose-dependent. The lowest paraoxon concentration at which the effect was significant was 25 µM. The purified enzyme was more affected than the enzyme contained in microsomal fraction. Results displayed as the

Table I. The values of inhibition constant (K_i) for paraoxon inhibition of the activity of pig kidney (Na⁺ + K⁺)-ATPase contained in the microsomal fraction and purified from it for high and low affinity sites for ATP[a].

Preparation	Affinity sites for ATP	K_i [µM]
Purified enzyme	low	73 ± 9
	high	91 ± 11
Enzyme contained in	low	138 ± 20
microsomal fraction	high	246 ± 22

[a] The values of K_i were calculated using the least square method on the basis of the results displayed in Fig. 3. The results are given as mean ± SE.

Dixon plots (Fig. 3) indicate that the enzyme was inhibited noncompetitively. The values of the inhibition constant (K_i) are displayed in Table I. The Hill analysis of inhibition (Fig. 4) always yielded a value of the Hill coefficient (n) greater than 1, which suggests cooperative binding of paraoxon to both forms of the enzyme. More precisely, the n value fulfilled the relationship $1 < n < 3$. Based on the Hill coefficient one can draw a conclusion that there is cooperative interaction between a minimum of three paraoxon binding sites on the enzyme.

These results are consistent with the general inhibitory effects of different organophosphorus compounds on various ATPases (Robineau *et al.*, 1991). The differences in the degree of inhibition for the two kinds of enzyme preparation can be explained by different accessibility of the insecticide than by different phospholipid and cholesterol content.
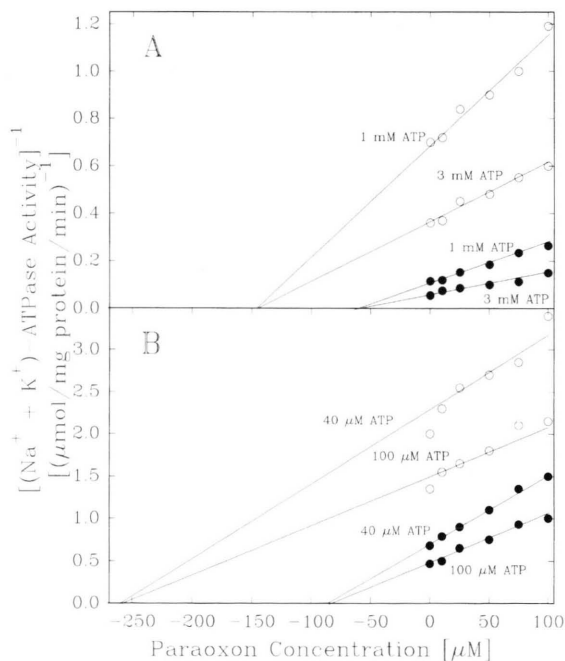

Fig. 4. The Hill plots of paraoxon inhibition of the pig kidney (Na$^+$ + K$^+$)-ATPase at low (A, B) and high (C, D) affinity sites for ATP for the enzyme contained in microsomal fraction (B, D) and purified enzyme (A, C). V$_0$ and v denote the rate of enzymatic reaction in the absence and in the presence of the insecticide at indicated concentrations. Low affinity sites were stimulated at 3 mM ATP, high affinity at 0.1 mM. Regression lines were calculated by means of the least square method. The R values belong to the range 0.924–0.989. The slope of each line is indicated in the figure. Error bars were omitted.

Fig. 3. The Dixon plots of paraoxon inhibition of the pig kidney (Na$^+$ + K$^+$)-ATPase purified (●) and contained in microsomal fraction (○) at low (A) and high (B) affinity sites for ATP. Low affinity sites were stimulated at 1 and 3 mM ATP, high affinity at 0.04 and 0.1 mM. Regression lines were calculated by means of the least square method. The R values belong to the range 0.965–0.998. Error bars were omitted to increase the clarity of the figures.
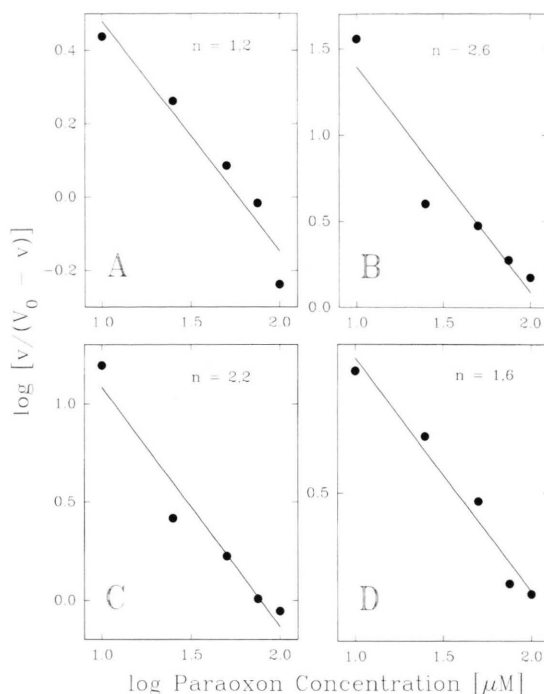
Although paraoxon and its parent compound parathion are primarily neurotoxic, their neurotoxicity is not connected with the inhibition of (Na$^+$ + K$^+$)-ATPase. Our findings can therefore be taken as evidence of paraoxon action on a nonspecific tissue. Further studies are needed to evaluate whether the observed effect can be related to chronic toxicity reported of this compound (Okhawa, 1984).

The values of the Hill coefficient suggest multiple binding sites for paraoxon. It has been shown that a methylated homologue of parathion has multiple binding sites in the photosynthetic membranes of *Chlorella* (Saraja-Subbaraj and Bose, 1983).

The values of the Hill coefficient and the non-competitive character of the inhibition suggest that paraoxon could be an allosteric inhibitor of the (Na$^+$ + K$^+$)-ATPase. Because the lipid milieu of the enzyme may be considered as a large allosteric site and the phospholipid-water partition coefficient of parathion is very large (Antunes-Madeira and Madeira, 1984), ensuring practically quantitative uptake of the compound by the membrane phase of the ATPase assay medium, para-oxon could interact with (Na$^+$ + K$^+$)-ATPase by excluding the enzyme protein from its normal lipid milieu. The fact that the inhibition of the purified enzyme and the enzyme contained in microsomal fraction was qualitatively the same, also support this hypothesis.

Acknowledgements

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