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A second site of action of soman on acetylcholinesterase

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Acetylcholinesterase (AChE, acetylcholine acetylhydrolase, EC 3.1.1.7) is involved in terminating the actions of acetylcholine at cholinergic synapses [1]. AChE is also located in a number of noncholinergic tissues and has been shown to possess a peptidase activity in addition to its ability to hydrolyse acetylcholine [2–5]. In addition to the esteratic and tryptic sites, AChE possesses peripheral sites which modify the esteratic activity. The modulation of AChE activity is carried out at these sites by a broad range of compounds [6–10]. AChE is inhibited by a number of irreversible or slowly reversible inhibitors. Many of these compounds are used as therapeutic agents [11] or insecticides [12]. One group of such compounds, referred to as nerve agents, has been produced for use as chemical weapons [13].

The mechanism by which these compounds inhibit AChE

can be summarised as [14]:

$$EH + IX \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} EH \cdot IX \xrightarrow{k_{+2}} EI + HX \xrightarrow[H_2O]{k_{+3}} EH + IOH + HX \qquad (1)$$

where EH is the enzyme, IX the inhibitor and EH·IX the Michaelis complex. For the nerve agents, k_{+3} is much smaller than k_{+2} and no reactivation is observed over the time course of most experimental studies.

The theoretical treatment of this kinetic system has been carried out thoroughly [15–17], and the values of the dissociation constant K_d and the unimolecular rate constant k_{+2} have been measured for a number of compounds, including the nerve agents [18, 19]. Nishioka *et al.* [20] observed a concentration dependence of these values for some of the carbamates. The results suggest the presence of an allosteric site to which the carbamates may bind [20, 21]. In addition, Friboulet *et al.* [22, 23] have also proposed the existence of a peripheral site to which organophosphorus compounds may bind. Soman (pinacolylmethylphosphonofluoridate) is an organophosphorus nerve agent and a potent inhibitor of acetylcholinesterase [18]. We report here that an allosteric site may exist to which soman binds. DFP (diisopropyl fluorophosphate) has been shown to bind to two separate sites on AChE [5] and was used here for comparison.

Materials and Methods

Stopped-flow studies were carried out as described by Gray and Dawson [19]. DFP, eel acetylcholinesterase (type V1-S, 340–350 units/mg), acetylthiocholine iodide (substrate) and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from the Sigma Chemical Co. (St. Louis, MO). Soman was greater than 95% pure. The other experimental details are as described in Gray and Dawson [19].

Gray [24] has shown that calculation of k_{+2} and K_d from single progress curves is very sensitive to systematic errors in the parameters V_{max} and K_m. However, the first-order rate constant, k', may be calculated quite accurately [25], and is a function of both inhibitor and substrate concentrations [15, 16]:

$$k' = \frac{k_{+2}[IX]}{K_d\left(1 + \frac{[S]}{K_m} + \frac{[IX]}{K_d}\right)} \qquad (2)$$

where K_m is the Michaelis constant and [S] the substrate concentration.

A plot of $[IX]/k'$ against [IX] or [S] is therefore linear [12]. Furthermore, varying [S] at constant [IX] or vice versa should give rise to a series of parallel lines. Whether or not the plots are linear may also allow the detection of additional binding sites for the inhibitor or substrate [12, 26].

The parameter k' was calculated from progress curves using the non-linear regression program of Duggleby [27] as described by Gray and Duggleby [25]. Each estimate of k' was calculated from the numerical average of at least five progressive inhibition curves. Each of these estimates was repeated an average of eight times.

Results and Discussion

In Fig. 1 is shown a plot of $[DFP]/k'$ vs [DFP] at three different substrate concentrations. The DFP concentrations were in the range 0.5 K_d to 2.5 K_d [18]. The data are fitted very well by a first-order polynomial. However, the lines are not parallel and intersect to the left of the ordinate. A replot of these data as $[DFP]/k'$ vs [substrate] at varying DFP concentrations also produced straight but nonparallel lines (not shown). The corresponding data for soman are shown in Figs. 2 and 3. For Fig. 2, soman concentrations

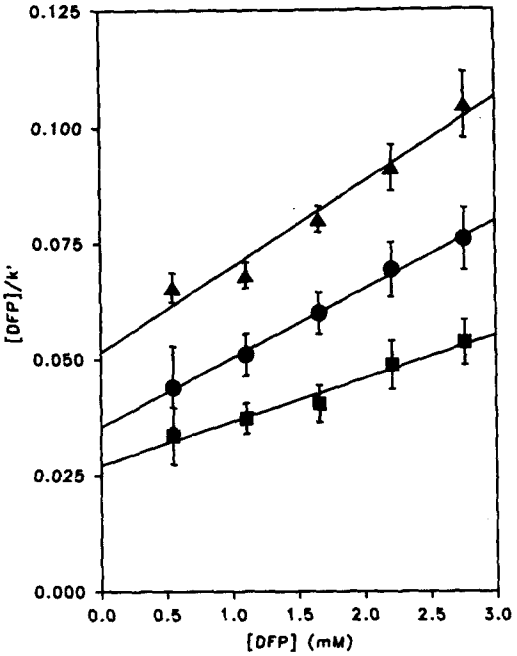

Fig. 1. Plot of $[IX]/k'$ vs [IX] for DFP. The data are shown as the mean ± SD of between six and nine replicate experiments. The fitted lines were calculated by linear regression. The substrate concentrations used were: (▲–▲) 1×10^{-3} M, (●–●) 7.5×10^{-4} M, and (■–■) 5.0×10^{-4} M.

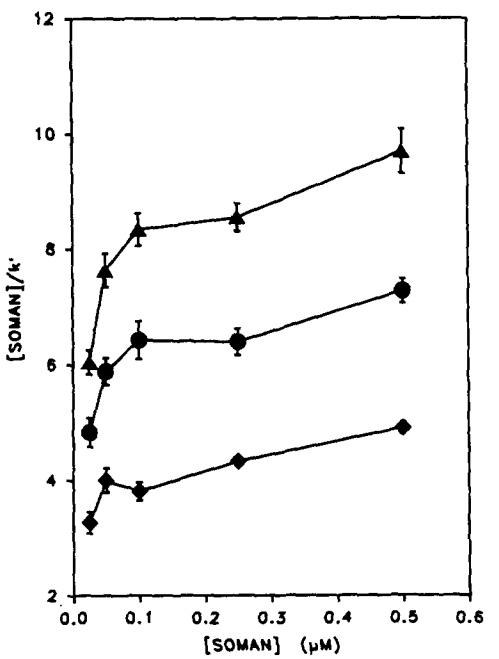

Fig. 2. Plot of $[IX]/k'$ vs [IX] for soman. The data are plotted as the mean ± SD of eight replicate experiments. The substrate concentrations were: (▲–▲) 1×10^{-3} M, (●–●) 7.5×10^{-4} M, and (◆–◆) 5.0×10^{-4} M.

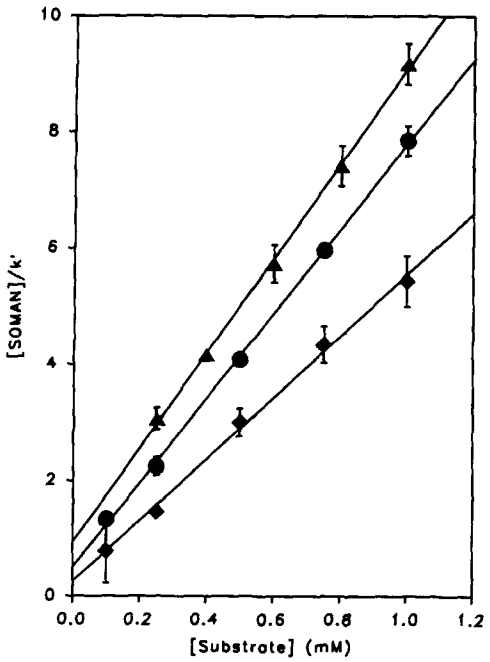

Fig. 3. Plot of $[IX]/k'$ vs [S] for soman. The data were plotted as the mean ± SD of eight replicate experiments. The soman concentrations were: (▲–▲) 1.0×10^{-6} M, (●–●) 3.0×10^{-7} M, and (◆–◆) 4.1×10^{-8} M.

were varied from $0.04\,K_d$ to $0.82\,K_d$. The substrate concentrations were the same as those used for DFP (Fig. 1). At each substrate concentration the plots were nonlinear, with the slope decreasing as the soman concentration increased. The curvature also increased with increasing substrate concentration. The effect of varying substrate concentration is shown in Fig. 3. The soman concentration ranged from $0.067\,K_d$ to $1.64\,K_d$. As for DFP, a series of straight but nonparallel lines resulted.

Inhibition of AChE by irreversible inhibitors which obey the mechanism shown in the introduction should produce straight, parallel lines when $[IX]/k'$ is plotted against inhibitor or substrate concentration for varying concentrations of substrate or inhibitor, respectively. This was not observed for either DFP or soman.

For DFP (Fig. 1), the lines are described adequately by a first-order polynomial and intersect to the left of the ordinate. The observation of the linear relationship between $[IX]/k'$ and [IX] for DFP confirms the work of Liu and Tsou [14]. These workers observed the linear relationship for both DFP and paraoxon, but did not extend their studies to multiple substrate concentrations [14]. The linear relationship is consistent with irreversible inhibition of the competitive complexing type [14]; however, the lack of parallelism indicates the involvement of additional factors. Small and Chubb [5] have demonstrated that DFP binds to both the esteratic site and a tryptic site. The lack of parallelism may reflect binding to these two different sites.

The lack of parallelism in the plot of $[soman]/k'$ vs [substrate] (Fig. 3) suggests the interaction of soman with a site other than the esteratic site. Increasing the inhibitor concentration, at constant substrate concentration (Fig. 2), resulted in inhibition greater than that predicted by the model. This is manifested as an increased first-order rate constant (k') and consequent smaller values of $[soman]/k'$. Friboulet *et al.* [23] have observed a peripheral site for the organophosphorus compound *O*-ethyl *S*-[2-(diisopropylamino)ethyl]methylphosphonothioate (MPT). However, in that case, interaction with the peripheral site diminished the inhibition [23]. The shape of the plots in Fig. 2 suggests a complex interaction between soman and acetylcholinesterase. The complexity may arise from the binding of soman to peripheral sites which are linked or overlap. Such an interaction of peripheral sites has been suggested to occur between MPT and propidium [23].

The increase in the effect with increasing substrate concentration (Fig. 2) may be explained by competition between substrate and soman for the free active sites. As the increasing substrate concentration inhibits soman binding to the active site, the amount of soman available to bind to the peripheral site would increase. Soman binding to the peripheral site may then either inhibit substrate hydrolysis or increase the inhibition.

There are several experimental factors which may be invoked to explain the results observed with soman. In the first instance, soman exists as a mixture of four stereoisomers [28]. However, two of these isomers have such high bimolecular inhibition rate constants ($\sim 10^8$) while the other two are so low, $< 5 \times 10^3$ [28], that such an explanation is unlikely. Second, substrate depletion is not a likely cause of the curvature as this would also be expected to cause the same effect in the DFP results. Finally, an effect of substrate alone is unlikely because the DFP data were collected over the same range of substrate concentrations.

In summary, we have shown that the kinetics of inhibition of acetylcholinesterase by soman are not consistent with the action of this inhibitor at only one site on the enzyme. This study provides an addition to the growing body of evidence which supports the existence of a second site on the acetylcholinesterase surface, other than the esteratic site, to which organophosphorus inhibitors may bind. The existence of such a site requires modification of the currently accepted model for inhibition of acetylcholinesterase by these compounds.

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Enhancement of cross-linking of presynaptic plasma membrane proteins by phospholipase A_2 neurotoxins

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β-Bungarotoxin (β-BuTX*) and notexin are members of a group of snake venom toxins which have phospholipase A_2 (PLA_2) enzymatic activity and whose neurotoxicity is due to a presynaptic action. These neurotoxins alter the release of acetylcholine in the peripheral nervous system and the release of many neurotransmitters (i.e. acetylcholine, γ-aminobutyric acid, norepinephrine, and serotonin) in the central nervous system (for reviews, see Refs. 1–4). The mechanism(s) by which β-BuTX and notexin alter neurotransmitter release, however, is not completely understood. PLA_2 activity alone cannot account for neurotoxicity since there is no direct relationship between the enzymatic activities and neurotoxicities of these neurotoxins and chemically related, non-neurotoxic PLA_2 enzymes (e.g.

* Abbreviations: β-BuTX, β-bungarotoxin; BS^3, bis-(sulfosuccinimidyl) suberimidate; FFA, free fatty acids; PLA_2, phospholipase A_2; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and SPM, synaptic plasma membrane(s).