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DECREASED REACTIVITY OF ORGANOPHOSPHORUS INHIBITORS TOWARDS A MODIFIED CHYMOTRYPSIN

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SUMMARY

Alkylation of the methionine-192 residue of chymotrypsin with *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate (W. B. LAWSON AND H. J. SCHRAMM, *Biochemistry*, 2 (1965) 252, decreases reactivity to organophosphorus compounds. The ratios of rates of inhibition, native to methionine modified, are 3:2 for isopropyl methylphosphonofluoridate (sarin), 2:1 for diisopropylphosphofluoridate (soman) and 7:1:1 for cyclohexyl methylphosphonofluoridate, 6.7:1 for pinacolyl methylphosphonofluoridate. The bulkier the alkoxy group and generally the more potent the inhibitor of native chymotrypsin, the slower the relative rate of inhibition of the modified enzyme. The pH optimum for sarin inhibition was also studied and found to be essentially the same value for native and modified preparations.

Native chymotrypsin inhibited by sarin or soman could be reactivated by pyridine-2-aldoxime methochloride more rapidly than the corresponding inhibited modified enzyme. The ratios of rates of reactivation when comparing native to methionine-modified chymotrypsins were 2:1 for sarin and 7.5:1 for soman inhibitions. Native chymotrypsin inhibited by soman can be reactivated twice as fast as when inhibited by sarin, whereas the reverse occurs with the modified enzyme. The ability to be reactivated was preserved for 24 h, indicating that "aging" does not occur with the modified as well as native enzymes.

INTRODUCTION

Specific chemical alkylation of enzymes offers a theoretical approach to the prophylaxis and therapy of organophosphorus poisoning. A modified enzyme would have to retain activity against its substrate yet be less reactive to the inhibitors.

LAWSON AND SCHRAMM[1,2] elegantly demonstrated that α-chymotrypsin alkylated on Met-192 by *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate gave an altered enzyme which possessed activity although K_m was increased eleven fold (v_{max} was little changed). They used [^{32}P]DFP to demonstrate stoichiometry of binding—*i.e.* each active center serine of the alkylated α-chymotrypsin was capable

Abbreviations: ATEE, acetyl-L-tyrosine ethyl ester; sarin, isopropyl methylphosphonofluoridate; soman, pinacolyl methylphosphonofluoridate.

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of being phosphorylated—but they did not distinguish between rates of inhibition with DFP.

An investigation of the kinetics of inhibition of LAWSON AND SCHRAMM's[1,2] methionine-modified α-chymotrypsin by selected organophosphorus inhibitors provides an opportunity to establish the feasibility of chemical modification as an approach to the management of poisoning by these compounds, using a model enzyme system. Rates of reactivation of the inhibited modified and native enzymes are also of interest.

EXPERIMENTAL PROCEDURE

Materials

α-Chymotrypsin (EC 3.4.4.5) was purchased from Worthington Biochemical Corp. as 3-times-crystallized protein. *p*-Nitrophenyl bromoacetyl-α-aminoisobutyrate, melting point 149.5–150.0°, was generously provided by Dr. W. B. Lawson (State Department of Health, Albany, N.Y.). Acetyl-L-tyrosine ethyl ester (ATEE) was purchased from Aldrich Chemical Co. Solutions of pyridine-2-aldoxime methochloride (Ayerst Labaratories) were made up fresh each day, adjusted to pH 7.5, and stored at 2° until use.

Isopropyl methylphosphonofluoridate (sarin, GB) assayed greater than 98% pure by elemental carbon and hydrogen analysis and by gas chromatography. Pinacolyl methylphosphonofluoridate (soman, GD) was found to be 91% pure, cyclohexyl methylphosphonofluoridate 92% pure, and DFP 78% pure by hydrolytic titration analysis[3]. Concentrations reported were not corrected for purity. To avoid significant hydrolysis, fresh vials of these inhibitors were opened for each experiment, diluted in glass-distilled water, and used immediately (except with sarin where control inhibition experiments revealed no significant loss of inhibitory potency for 2 h at 25°. Other chemicals used were reagent grade.

Modification of chymotrypsin

Chymotrypsin was reacted with *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate at pH 5.0, using the procedure of LAWSON AND SCHRAMM[2]. After 5 h the enzyme was dialyzed overnight against 1 mM HCl. Native chymotrypsin was treated identically, except for the absence of the modifying reagent in the ethanol. Concentrations of the dialyzed enzymes were measured by absorption at 280 nm following a 1:10 dilution in 10 mM acetate buffer at pH 5.0. Using a molar absorptivity of 0.495 (ref. 4) and assuming a mol. wt. of 25 000 (ref. 5), each stock enzyme concentration was found to be 52 μM. Both native and modified preparations were stored at 4°, and activity was unchanged during a 1-month period. Binding constants of the enzymes, determined by several concentrations of ATEE, were in excellent agreement with reported values[2].

Chymotrypsin assay

Enzyme activity was measured with ATEE as substrate by a modification of the titrimetric method of H. O. MICHEL (personal communication). A Radiometer TTT 1-C titrator with SBR2/SBU1/TTA3 titration assembly was used with the temperature controlled at 25°. Standard base, 0.01 M NaOH, was made fresh each

day. 2 ml of a buffered gelatin solution, consisting of 1.0 mM Tris, 1.0 M KCl, and 3.0 mg of gelatin per ml, were pipetted into the reaction vessel, and 2–5 μl of the dialyzed enzyme were added. The mixture was quickly equilibrated by magnetic stirring and the reaction was begun by the addition of 1.0 ml of 11 mM ATEE. Final chymotrypsin concentrations ranged from 0.034 to 0.085 μM, and the final ATEE concentration was 3.7 mM. In control studies nonenzymatic hydrolysis was negligible under these conditions. The addition of standard base was continuously recorded, and activity was expressed as μmoles of base per min. The substrate concentration was checked daily by measuring the total amount of base titrated to neutralize 0.20 ml of 11 mM ATEE with complete hydrolysis by 20 μl of native enzyme.

Inhibition conditions

To measure rates of inhibition, 0.1 ml of inhibitor solution, or of distilled water for control, was added to the enzyme in the gelatin buffer in the pH stat reaction vessel at 25° and pH 8.0, unless otherwise specified. The substrate was subsequently added after separate 30-, 60-, 90-, and 120-sec incubations. The inhibition rate constant, k_i, was then calculated from the slopes of plots of log A_0/A_t *versus* inhibition time where a bimolecular rate relationship was seen to hold, *i.e.*

$$k_i = \log (A_0/A_t) \times 2.3/[I]t$$

where [I] is the inhibitor concentration in M, t is time in min, A_0 is the activity of the uninhibited control, and A_t is the remaining activity at time t. Concentrations of the inhibitors were selected to demonstrate a wide range of percent inhibition.

Reactivation conditions

The enzymes were rapidly inactivated by final concentrations of 1.2 mM sarin or 0.66 mM soman by adding 0.03 ml inhibitor to 0.20 ml enzyme (52 μM) in 0.02 ml of 10 mM Tris (pH 7.5, 25°). At varying intervals, 0.1 ml pyridine-2-aldoxime methochloride (final concentration 0.40 M and in great excess over sarin) was added after the activities of appropriate controls were measured. Aliquots of 2–5 μl were removed for assay in the pH stat at 0.5, 1.5, 3, 6, 12, and 24 h to establish the percentage reactivation. Under these conditions, spontaneous reactivation was insignificant. Uninhibited controls containing pyridine-2-aldoxime methochloride showed progressive inactivation: 8% loss at 6 h, 20% at 24 h. Consequently, pyridine-2-aldoxime methochloride was included in the controls. Rates of reactivation, k_r, were calculated from the relationship:

$$k_r = \log (I_0/I_t) \times 2.3/[R]t$$

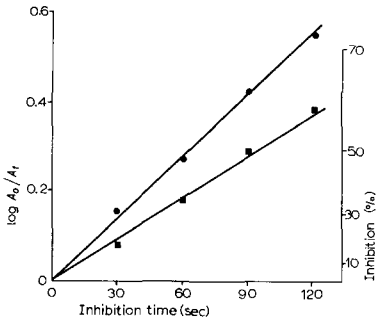
where $[R]$ is concentration of the reactivator in M, t is time in h, I_t/I_0 is the fraction of inhibited enzyme remaining at time t. Concentrations of pyridine-2-aldoxime methochloride in μg/ml were found by multiplying the absorbance values at 335 nm at pH 10 by the molar absorptivity, 1.06 (ref. 6).

RESULTS

Inhibition studies

Initial observations indicated that 41 μM sarin inhibits native chymotrypsin more rapidly than it inhibits the preparation modified by alkylation with *p*-nitro-

phenyl bromoacetyl-α-aminoisobutyrate. While a final concentration of 3.6 mM ATEE blocked inhibition of the native enzyme by sarin, a similar concentration of the substrate did not prevent additional sarin interaction with the methionine-modified enzyme, since inhibition of the modified enzyme continued at a decreased rate. When a lower concentration of substrate (0.74 mM ATEE following 41 μM sarin) was used, inhibition of the native enzyme could also be detected in the presence of substrate. This result was attributed to the increased binding constant and decreased



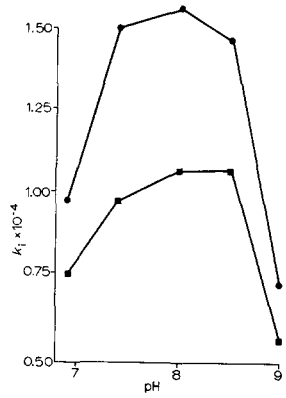


Fig. 1. Inhibition of native chymotrypsin (●) and modified chymotrypsin (■) by sarin. Conditions: 41 μM sarin, pH 8.0, 25°. Abscissa represents time of preincubation with inhibitor.

Fig. 2. pH curves for rates of inhibition of native chymotrypsin (●) and modified chymotrypsin (■) by sarin at 25°; k_i is the bimolecular rate constant.

specificity of the substrate to modified as compared with native enzyme. In instances where inhibition continued, the rate of addition of base was calculated from the tangent drawn to the initial rate (usually taken as the rate in the first 30 sec after addition of the substrate). A typical plot showing sarin inhibition of the enzyme at pH 8.0 is shown in Fig. 1.

The pH profiles for sarin inhibiton (expressed as the inhibiton rate constants, k_i) of native and modified chymotrypsins are shown in Fig. 2. The optima are broad at pH values of 7.5–8.5. (Fig. 1 represents sarin inhibition at pH 8.0.) Rates of

TABLE I

RATES OF INHIBITION OF NATIVE AND MODIFIED CHYMOTRYPSINS WITH ORGANOPHOSPHORUS COMPOUNDS

k_i's are bimolecular rate constants for native and modified chymotrypsins; inhibitions were performed at 25°, pH 8.0.

Inhibitor	*Inhibitor concn. (μM)*	$k_i \times 10^{-4}$ ($l \cdot mole^{-1} \cdot min^{-1}$)		*k_i ratio native: modified*
		Native	*Modified*	
Sarin	41.0	1.55	1.06	3:2
DFP	37.4	2.4	1.24	2:1
Soman	2.7	16.8	2.5	6.7:1
Cyclohexyl methylphosphonofluoridate	0.11	360.0	51.0	7.1:1

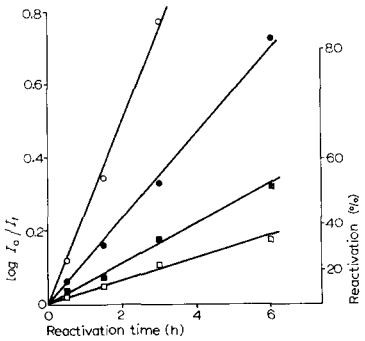


Fig. 3. Reactivation of chymotrypsins by pyridine-2-aldoxime methochloride: reactivation of native chymotrypsin inhibited by sarin (●) and soman (○) and of modified chymotrypsin inhibited by sarin (■) and soman (□). Conditions: 0.4 M pyridine-2-aldoxime methochloride, pH 7.5, 25°. Abscissa represents time after addition of pyridine-2-aldoxime methochloride.

inhibition of the other organophosphorus compounds (at pH 8.0) were calculated from similar plots. The results are summarized in Table I. In every instance the inhibitor reacted more slowly with the modified enzyme, the greatest difference being approximately seven fold with cyclohexyl methylphosphonofluoridate.

Reactivation studies

Reactivation results are presented in Fig. 3. The modified enzyme, inhibited by either sarin or soman, was reactivated more slowly by pyridine-2-aldoxime methochloride than the native chymotrypsin. Reactivation data are further summarized in Table II. Inhibited enzymes could be completely reactivated when the oxime was added 24 h after inhibition indicating that the phenomenon of "aging" (ref. 7) does not occur. Of additional interest, native chymotrypsin inhibited by soman was reactivated more rapidly than native enzyme inhibited by sarin; while the reverse was found for the methionine-modified enzyme—*i.e.* the modified enzyme was reactivated faster with sarin than soman (Table II).

TABLE II

RATES OF REACTIVATION OF INHIBITED NATIVE AND MODIFIED CHYMOTRYPSINS WITH PYRIDINE-2-ALDOXIME METHOCHLORIDE

Conditions: 0.4 M pyridine-2-aldoxime methochloride, 25°, pH 7.5; $t_{\frac{1}{2}}$ is the half time of reactivation; k_r is the reactivation rate constant. Final activity was taken as percent uninhibited control at 24 h.

Inhibitor	*Inhibitor concn. (mM)*	*Enzyme*	*Inhibition time (min)*	*Initial activity (% of control)*	*Final activity (% of control)*	$t_{\frac{1}{2}}$ *(h)*	k_r *(l · mole^{-1} · h^{-1})*	*k_r ratio native: modified*
Sarin	1.20	Native	10	1	98	3	0.70	2:1
		Modified	10	2	95	6	0.33	
Soman	0.66	Native	40	1	99	1.5	1.48	7.8:1
		Modified	90	4	90	12	0.19	

DISCUSSION

Active site-directed enzyme modification (affinity labeling) has proved to be an extremely valuable tool for studying protein structure[8], and has also been investigated as an approach to cancer chemotherapy[9]. We believed that prophylaxis and therapy for management of organophosphorus poisoning could also be approached by enzyme modification where the altered enzyme would still hydrolyze a substrate ester, yet be less reactive to inhibitors. The feasibility of this general approach has now been demonstrated with an alkylated Met-192 derivative of chymotrypsin.

Chymotrypsin alkylated on histidine-57 by tosyl phenylalanine chloromethyl ketone loses its ability to react with DFP[10]; and it fails to react with the small organophosphonate, sarin[11]. Alkylation of Met-192 by *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate was found to alter the kinetics of substrate hydrolysis, but complete reactivity towards DFP was retained[1,2]. KEZDY *et al.*[12] investigated the reactivity of alkylated chymotrypsin with a large number of substrates and found the K_m of all specific substrates increased, whereas binding of some nonspecific substances was unaltered. Their data support a hypothesis that the increase in binding constants is caused by intramolecular interactions between the substrate and the alkyl group on the methionine. ROYER AND CANADY[13] noting the increased polarity of the modified methionine residue, demonstrated decreased affinity for simple hydrocarbon inhibitors in modified chymotrypsin. They attributed the decreased inhibitor affinity to decreased hydrophobic bonding, although benzamide, a hydrophobic substrate, had been found to have the same K_m for each α-chymotrypsin[12]. Benzyl bromide also alkylates the Met-192 residue of chymotrypsin specifically[14]; and MARSHALL[15] found that this alkylation caused a broadening of the chlorine nuclear magnetic resonance line after subsequent binding of an alkyl mercurihalide to the active center serine. It would appear that the alkyl group restricts the rotational motion of the bound inhibitor largely through direct interaction rather than alteration of the secondary or tertiary structure around the active site since he further found that 8 M urea abolished the difference between the native and alkylated enzymes.

Decreased rates of inactivation of methionine-modified chymotrypsin with various organophosphorus compounds used in the present investigation can be attributed to decreased binding due to intramolecular interactions between the organophosphorus inhibitors and the alkyl-methionine group. This interaction could represent decreased nonpolar affinity due to the increased polarity of the modified residue. Of note, the increase in size of the hydrocarbon portion of the alkoxy group from isopropyl to cyclohexyl markedly decreased the reactivity of the inhibitor with the modified chymotrypsin relative to the native (despite the general increase, to over 200-fold, in the corresponding rates of inhibition of the native enzyme). In contrast, current modes of treatment of organophosphorus poisoning become less effective against the inhibitors with larger alkoxy groups.

DFP was found to be an intermediate-type inhibitor, with a slightly higher k_i than sarin in both chymotrypsin preparations. The pH curves for sarin inhibition of the native enzyme show a broad optimal range. The native curve is in fair agreement with the 8–8.5 optimum for sarin inhibition of chymotrypsin found by H. O. MICHEL (personal communication).

The reactivation rates of inhibited native chymotrypsins are of the same order as the rates found by GREEN AND NICHOLLS[16] for sarin-inhibited chymotrypsin using oximes other than pyridine-2-aldoxime methochloride, and hydroxamic acids, as reactivators. It is of interest in the present study that the reactivation rate constant for soman-inhibited native chymotrypsin was twice that of the sarin-inhibited native preparation, especially since cholinesterase inhibited by many of the more potent inhibitors rapidly loses its ability to be reactivated[7]. As with other studies on reactivation of inhibited native chymotrypsin[16,17], our preparations, both native and modified, could be completely reactivated, and this ability was not subsequently lost.

Chemical modification of cholinesterase to decrease reactivity towards organophosphorus compounds while preserving reactivity against acetylcholine could best be approached by designing a specific substrate-like, active site-directed reagent which would alkylate a reactive residue near the active center. Design and proof of such is facilitated by knowledge of the amino acid sequence and tertiary structure, which in turn must await purification of the enzyme. Recent studies with an impure red cell cholinesterase preparation inhibited by certain aziridinium ions[18] suggest that under certain conditions, alkylation is occurring, presumably at the "anionic site" as the inhibition becomes irreversible[19]. Although changes in reactivity to certain charged organophosphorus compounds were noted, no effects upon organophosphonate inhibition were observed[20], and O'BRIEN[21] only found decreased inhibition of the modified cholinesterase using the neutral indophenylacetate (rather than acetylcholine) as substrate. Alkylation at or near the anionic site of cholinesterase by these aziridinium compounds also explains why charged substrates and charged inhibitors are both affected, whereas inhibition by uncharged compounds is unaltered.

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