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KINETIC INVESTIGATIONS INTO THE INTERACTIONS OF APROPHEN WITH CHOLINESTERASES AND A CARBOXYLESTERASE

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Abstract—Acetylcholinesterases, butyrylcholinesterases, and carboxylesterases appear to form kinetically a homologous enzyme series with respect to many substrates and inhibitors. The present paper evaluates the interaction of aprophen with acetylcholinesterases, butyrylcholinesterases, and carboxylesterases with respect to (1) protecting the enzyme from organophosphate and carbamate inhibition, (2) accelerating pralidoxime iodide (2-PAM) regeneration of the diisopropylphospho-enzyme, and (3) comparing the inhibition and regeneration kinetics of a soluble mammalian acetylcholinesterase with that of bovine erythrocyte acetylcholinesterase. The irreversible inhibition kinetics of diisopropyl fluorophosphate (DFP) and eserine inhibition of fetal bovine serum acetylcholinesterase were typical of other acetylcholinesterases as indicated by the bimolecular inhibition rate constants, k_i, of $7.7 \pm 1.3 \times 10^4\ M^{-1}\ min^{-1}$ and $2.9 \pm 1.7 \times 10^6\ M^{-1}\ min^{-1}$, respectively. Similarly, the bimolecular regeneration rate constant, k_r, for 2-PAM regeneration of the diisopropylphospho-acetylcholinesterase was $14.7\ M^{-1}\ min^{-1}$. The bimolecular rate constants, k_i and k_r, were not statistically perturbed when the reaction was monitored in the presence of aprophen with the fetal bovine serum acetylcholinesterase. Human serum butyrylcholinesterase was partially protected from DFP inhibition by aprophen with no detectable change in the bimolecular inhibition rate constant, k_i. The regeneration of the diisopropylphospho-butyrylcholinesterase by 2-PAM was accelerated in the presence of aprophen by a factor of 2.7 over that of 2-PAM alone ($8.4 \pm 2.2\ M^{-1}\ min^{-1}$ to $23.1 \pm 2.6\ M^{-1}\ min^{-1}$ respectively). Neither the inhibition (DFP) nor the regeneration (2-PAM) kinetics observed for the carboxylesterase was perturbed by the presence of aprophen.

It has been shown recently that aprophen is a good reversible inhibitor of the human serum butyrylcholinesterases (BuChE†; EC 3.1.1.8); it is also a substrate for both the BuChE and rabbit liver oligomeric carboxylesterases (CE; EC 3.1.1.1) [1]. Standard therapy in cases involving organophosphate intoxication employ the administration of atropine and 2-PAM to regenerate the inhibited serine hydrolase(s). Various other drugs have been tested as adjuncts to this therapy: these include benactyzine, chlorpromazine, and more recently aprophen [1–3]. The present paper reports on an investigation to evaluate the interaction of aprophen with acetylcholinesterases (AChE; EC 3.1.1.7), butyrylcholinesterases and carboxylesterases with respect to (1) protecting the enzyme from binding an organophosphate or carbamate inhibitor, (2) accelerating 2-PAM regeneration of the diisopropylphospho-serine hydrolase, and (3) comparing the inhibition and regeneration kinetics of a soluble mammalian G_4 AChE [4] with that of bovine erythrocyte AChE.

The bimolecular rate constant was evaluated in the presence or absence of aprophen for both the inhibition and regeneration reactions. These constants are kinetically a composite constant reflecting equilibrium binding and elementary rate terms [5, 6]. A change in the bimolecular rate constant would be expected if either of the composite terms was altered by the binding of aprophen. The focus of much of this work has been directed toward the AChE because of its physiological role in neural transmission; however, the BuChEs and the CEs are also present in neural, as well as, other tissues [7–9].

MATERIALS AND METHODS

Aprophen, α-methyl-α-phenylbenzeneacetic acid-2-(diethylamino) ethyl ester (WR-3563-01-7), was synthesized as previously described [10]. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), diisopropyl fluorophosphate (DFP), and (3a-*S*-*cis*)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo-[2,3-b]indol-5-ol methylcarbamate (ester) (eserine) were from the Sigma Chemical Co., St. Louis, MO. Acetylthiocholine iodide (ATC) and butyrylthiocholine iodide (BTC) were from Eastman Kodak, Rochester, NY. Isopropanol was obtained from Burdick & Jackson Laboratories, Muskegon, MI, and phenylthiobutyrate was custom synthesized by Steroids Ltd., Chicago, IL. Pralidoxime iodide (2-PAM) was obtained from the Aldrich Chemical Co., Milwaukee, WI. All other reagents were purchased

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† Abbreviations: BuChE, butyrylcholinesterase; 2-PAM, pralidoxime iodide; DFP, diisopropyl fluorophosphate; CE, carboxylesterase; AChE, acetylcholinesterase; ATC, acetylthiocholine; BTC, *n*-butyrylthiocholine; and aprophen, α-methyl-α-phenyl-benzeneacetic acid-2-(diethylamino) ethyl ester.

from commercial sources and were of the highest purity available.

Enzymes utilized in this study were obtained from fetal bovine serum, human serum, bovine erythrocytes and rabbit liver. The fetal bovine serum AChE and the rabbit liver oligomeric CE were electrophoretically pure and were purified as described [4, 11]. Human serum BuChE was obtained by the method of Ralston *et al.* [12]. Bovine erythrocyte AChE was a commercial product (Worthington Biochemicals) and was used without further purification.

Determination of enzymatic activities. Enzymatic activities were determined by the method of Ellman *et al.* [13] as described [1]. Enzymatic activities were measured with 1.0×10^{-3} M ATC, BTC, and phenylthiobutyrate for the AChEs, BuChEs and CE respectively.

Reaction conditions for DFP and eserine inhibition. The following stock enzyme solutions were used: fetal bovine serum AChE contained 65 ATC units/ml, 2.32×10^{-10} moles active sites/ml (sp. act. 3300 units/mg, subunit weight 85,000 daltons) [4]; bovine erythrocyte AChE contained 24 ATC units/ml; human serum BuChE contained 81 BTC units/ml, 1.85×10^{-9} moles active sites/ml (sp. act. 546 units/mg, subunit weight 80,000 daltons) [12], and the oligomeric CE contained 59.8 phenylthiobutyrate units/ml, 5.01×10^{-9} moles active sites/ml (sp. act. 168 units/mg, subunit weight 71,000 daltons) [11]. Stock DFP solutions were in isopropanol. Eserine stock solutions were in distilled water, pH 3.0. The inhibition reactions were initiated by adding 5 μl of the inhibitor to the temperature-equilibrated enzyme solution. At various times, 5 μl of the preincubation mixture was assayed at the designated temperature using the appropriate substrate; the inhibitors were diluted by a factor of 601 in the assay mixture.

Reaction conditions for 2-PAM regeneration of enzymatic activity. Oxime regeneration experiments were conducted similarly to the inhibition experiments. The enzyme (50 μl) was preincubated at 37° with a fixed concentration of DFP or eserine for a designated time, and then 5 μl of stock 2-PAM (1.0×10^{-2} M) was added. When aprophen was used in conjunction with 2-PAM the appropriate quantity of aprophen was dissolved in the stock 2-PAM. The recovery of enzymatic activity was monitored with the appropriate substrate at 37°. Just prior to the addition of the 2-PAM, a zero time point was obtained.

RESULTS

Inhibition of the AChEs. DFP inhibition of fetal bovine serum AChE followed linear first-order kinetics. Five concentrations of DFP were used to evaluate the bimolecular inhibition rate constant. The range of DFP concentration was 25, from 4.55×10^{-6} M to 1.82×10^{-7} M. The bimolecular inhibition rate constant, k_i, was calculated from established kinetic treatments. The average inhibition rate constant is given in Table 1.

Aprophen did not alter the inhibition of fetal bovine serum AChE either in terms of the bimolecular rate constant (Table 1) nor did it shift the final equilibrium position attained at a given DFP concentration (data not shown). The ratio of aprophen to DFP was 1000:1, 2.74×10^{-4} M aprophen and 2.74×10^{-7} M DFP.

Eserine inhibition of fetal bovine serum AChE followed second-order kinetics. Five concentrations of eserine were employed to evaluate the bimolecular inhibition rate constant reported in Table 1. The range of inhibitor was varied from 5.0×10^{-7} M to 3.3×10^{-8} M, i.e. fifteen times. Appropriate replots of this inhibition data were linear, confirming second-order kinetics. Aprophen did not perturb the inhibition kinetics or the final equilibrium position attained at 1.23×10^{-7} M eserine. The ratio of the concentration of aprophen to eserine was 545:1.

Inhibition of the human serum BuChE. DFP inhibition of human serum BuChE was extremely rapid and did not follow first-order kinetics. The rate was not measurable above 5° under our experimental

Table 1. Bimolecular inhibition rate constants in the absence or presence of aprophen for selected serine hydrolases

Enzyme	Inhibitor	Aprophen	k_i (M^{-1} min^{-1})	T (°C)	pH	Reference
AChE*	DFP	Absent	$7.70 \pm 1.33 \times 10^4$(7)	37	8.0	
		Present	4.67×10^4	37	8.0	
	Eserine	Absent	$2.94 \pm 1.66 \times 10^6$(4)	37	8.0	
		Present	3.20×10^6	37	8.0	
AChE†	DFP	Absent	$4.64 \pm 0.53 \times 10^4$(4)	37	8.0	
	DFP	Absent	3.4×10^4	25		[5]
	Eserine	Absent	3.3×10^6			[5]
BuChE‡	DFP	Absent	$7.02 \pm 1.18 \times 10^5$(6)	5	8.0	
		Present	$4.57 \pm 0.78 \times 10^5$(4)	5	8.0	
BuChE§	DFP	Absent	1.10×10^6	5		[5]
CE‖	Eserine	Absent	5.3×10^6	37	7.4	[6]

Values for k_i are means ±SE; numbers in parentheses are the number of experiments.
* Fetal bovine serum.
† Bovine erythrocyte.
‡ Human serum.
§ Horse serum.
‖ Rat intestinal mucosa.

Table 2. Bimolecular regeneration rate constants in the absence or presence of aprophen for selected diisopropylphospho-serine hydrolases

Enzyme	Aprophen	k_r (M^{-1} min^{-1})	T (°C)	pH	Reference
AChE*	Absent	14.72 ± 1.25 (4)	37	8.0	
	Present	14.15 ± 1.92 (4)	37	8.0	
AChE†	Absent	17	25	7.4	[5]
AChE‡	Absent	140	25	7.4	[5]
BuChE§	Absent	8.36 ± 2.17 (5)	37	8.0	
	Present	23.08 ± 2.55 (5)	37	8.0	
CE‖	Absent	2.74 ± 0.54 (3)	37	8.0	
	Present	3.62 ± 0.73 (3)	37	8.0	

Values for k_r are means ±SE; numbers in parentheses are the number of experiments.
* Fetal bovine serum.
† Human erythrocyte.
‡ Eel.
§ Human serum.
‖ Rabbit liver.

conditions. The k_i for DFP inhibition is given in Table 1. Aprophen did not statistically alter the k_i; the final equilibrium position, at a concentration of 4.55×10^{-7} M DFP, was dramatically raised from 53.4% of the uninhibited velocity to 68.4% in the presence of 4.55×10^{-4} M and 4.55×10^{-7} M DFP.

Inhibition of the rabbit liver oligomeric CE. DFP inhibition of rabbit liver oligomeric CE was extremely rapid and could not be followed under our experimental conditions even at 5°. The final equilibrium position could be measured accurately, and it was not perturbed by the presence of aprophen at a ratio of 1000:1 (aprophen to DFP). The concentration of DFP was 4.55×10^{-7} M.

2-PAM Regeneration of DFP-inhibited serine hydrolases. The bimolecular regeneration rate constant, k_r, was calculated according to established kinetic procedures [5, 6]. Regeneration of DFP inhibited fetal bovine serum AChE, and rabbit liver oligomeric CE was not affected by the presence or absence of aprophen. The concentration of 2-PAM and aprophen was 9.1×10^{-4} M and 2.8×10^{-4} M and 9.1×10^{-4} and 9.2×10^{-4} M, respectively, for the fetal calf serum AChE and the rabbit liver oligomeric CE. The regeneration rate constants are summarized in Table 2. The regeneration of DFP-inhibited human serum BuChE exhibited a marked difference in k_r as a function of the presence or absence of aprophen. In the presence of 9.1×10^{-4} M 2-PAM and 2.8×10^{-4} M aprophen, the regeneration rate constant was increased from 8.36 M^{-1} min^{-1} to 23.08 M^{-1} min^{-1} (Table 2) or by a factor of 2.76.

DISCUSSION

Fetal bovine serum AChE inhibited by either DFP or eserine showed typical first-order and second-order kinetics similar to those observed for other acetylcholinesterases [5, 14]. The bimolecular inhibition rate constant, k_i, was 7.7×10^4 M^{-1} min^{-1} and 2.9×10^6 M^{-1} min^{-1}, respectively, for DFP and eserine inhibition of fetal calf serum AChE. The k_i values also were not statistically different from those reported for the bovine erythrocyte AChE, 4.64×10^4 M^{-1} min^{-1} and 3.3×10^6 M^{-1} min^{-1} for DFP and eserine respectively (see Table 1). It was concluded that the fetal bovine serum AChE was similar to other AChEs with respect to inhibition by organophosphate and carbamate inhibitors. The values were not statistically perturbed by the presence of aprophen.

The bimolecular inhibition rate constant, k_i, for human serum BuChE was determined to be 7.0×10^5 M^{-1} min^{-1} at 5°, pH 8.0. In the presence of aprophen, the rate constant was lowered to 4.6×10^5 M^{-1} min^{-1}. There was a marked elevation of the final steady-state level when aprophen was present with the DFP compared to the DFP control. The final steady-state level for the DFP control was 50 ± 2.2% of the initial uninhibited activity. In the presence of aprophen, the final steady-state level was 68 ± 0.9%.

It is known that aprophen is a substrate for the BuChE [1]. Since the k_i values in the absence of presence of aprophen are the same, it follows that neither the equilibrium binding constant, K_a, nor the phosphorylation rate constant, k_2, were perturbed by binding aprophen ($k_i = k_2/K_a$) to human serum BuChE; otherwise a change in k_i should have been observed [5, 6]. It appears that the protection of the active site from DFP inhibition is due primarily to blocking the active site with this ligand and not to altering the inhibition kinetic mechanism. This explanation is supported by comparing the equilibrium binding constant, K_d, for DFP inhibition of the horse serum BuChE ($K_d = 2.6 \times 10^{-5}$ M) [5] with the competitive inhibition constant, K_i, for aprophen and the human serum BuChE ($K_i = 3.7 \times 10^{-7}$ M)[1]. Aprophen binds to the active site 100 times better than DFP.

The final steady-state level observed with the carboxylesterase from rabbit liver was not affected by the presence or absence of aprophen. The initial rate of inhibition was extremely fast [15], and could not be monitored, even at 5°, under the experimental conditions employed. The fact that no shift in the

final equilibrium position was observed implies that the binding constant K_m for aprophen to the CE is higher relative to the K_d for DFP binding. It is known that CEs can hydrolyze aprophen [1] but the kinetics of aprophen carboxylesterase interaction have not been investigated thoroughly.

Regeneration of the diisopropylphospho-serine hydrolase by 2-PAM appears to follow the rank order AChE > BuChE > CE. This ordering probably reflects the efficiency of binding of the 2-PAM, within a Michaelis complex, to the respective phosphoenzymes [6]. The accelerated regeneration of human serum BuChE in the presence of both 2-PAM and aprophen is an unexpected result. It is possible that the acceleration may be due to tighter binding of aprophen to the BuChE active site relative to 2-PAM. If the tertiary N of aprophen could act as a nucleophile, it might attack the diisopropylphosphoryl moiety in a manner similar to the oxime functionality of 2-PAM. Alternatively, aprophen may bind to an allosteric site (it has been shown to be hydrolyzed by BuChE sigmoidally) [1] modulating the conformation of the active site in such a manner as to increase the binding of 2-PAM, thus indirectly accelerating the oxime-mediated regeneration of the active enzyme. Both arguments have been invoked as possible explanations for chlorpromazine acceleration of diethylphosphoryl-bovine erythrocyte AChE [2].

REFERENCES

1. R. S. Rush, J. S. Ralston and A. D. Wolfe, *Biochem. Pharmac.* **34**, 2063 (1985).
2. R. M. Dawson and M. P. Bladen, *Biochem. Pharmac.* **28**, 2211 (1979).
3. R. S. Rush, A. R. Main, B. F. Kilpatrick and G. D. Faulkner, *J. Pharmac. exp. Ther.* **216**, 586 (1981).
4. J. S. Ralston, R. S. Rush, B. P. Doctor and A. D. Wolfe, *J. biol. Chem.* **260**, 4312 (1985).
5. A. R. Main, *Pharmac. Ther.* **6**, 579 (1979).
6. W. N. Aldridge and E. Rainer, *Enzyme Inhibitors as Substrates, Interaction of Esterases with Esters of Organophosphorus and Carbamic Acids*, p. 37. North-Holland Publishing, Amsterdam (1975).
7. A. M. Graybiel and C. W. Ragsdale, Jr., *Nature, Lond.* **299**, 439 (1982).
8. J. G. Clement, *Biochem. Pharmac.* **33**, 3807 (1984).
9. J. M. Chemnitius and R. Zech, *Molec. Pharmac.* **23**, 717 (1983).
10. N. D. Brown, H. K. Sleeman, B. P. Doctor and J. P. Scovill, *J. Chromat.* **195**, 146 (1980).
11. S. B. Miller, A. R. Main and R. S. Rush, *J. biol. Chem.* **255**, 7161 (1980).
12. J. S. Ralston, A. R. Main, B. F. Kilpatrick and A. L. Chasson, *Biochem. J.* **211**, 243 (1983).
13. G. L. Ellman, K. D. Courtney, V. Anders, Jr. and R. M. Featherstone, *Biochem. Pharmac.* **7**, 88 (1961).
14. H. A. Berman, J. Yguerabide and P. Taylor, *Biochemistry* **19**, 226 (1980).
15. W. Junge and K. Krisch, *CRC Crit. Rev. Toxic.* **3**, 371 (1975).