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SUBSTRATE PREFERENCES OF WILD AND A MUTANT HOUSE FLY ACETYLCHOLINESTERASE AND A COMPARISON WITH THE BOVINE ERYTHROCYTE ENZYME

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Summary

Comparisons were made of purified acetylcholinesterase from the heads of wild type house flies with a mutant form (which bound organophosphates and carbamates less tightly). Using 12 substrates and 6 quaternary inhibitors, the only substantial difference was that the K_m for butyrylcholine was 25 times greater for the mutant enzyme, suggesting that butyrylcholine and the organophosphates and carbamates shared a common binding site.

The pure enzyme from the wild type house fly was also compared with bovine erythrocyte acetylcholinesterase. The major difference was again with butyrylcholine as substrate: the ability to acylate or deacylate was 30 times greater in the fly enzyme (the K_m values differed by a factor of 4).

Introduction

We reported earlier [1] that a mutant house fly strain (Cornell-resistant), which was resistant to the organophosphate, tetrachlorvinphos, had an acetylcholinesterase in the head which was about 200-fold less sensitive compared to the enzyme from the wild type fly (Cornell-susceptible) to inhibition by tetrachlorvinphos in vitro. The reduced sensitivity was caused by a 573-fold decrease in affinity of tetrachlorvinphos for the enzyme. There was little change in the reactivity to its substrate, acetylcholine. Subsequently, Tripathi [2] showed that there were lesser differences (7- to 117-fold) in the reactivity to 4 other organophosphates and 2 carbamates. The differences were again exclusively due to substantial reductions in affinity of the enzyme for the inhibitors.

The gene which produces mutant acetylcholinesterase was introduced into the genome of a susceptible wild strain (sbo). Genetic studies showed that mutant acetylcholinesterase was controlled by a gene on chromosome II,

approx. 5 crossover units from the marker, stubby wing, and in the resistant sbo flies the crude enzyme was found to be 30 times less susceptible to tetrachlorvinphos than was the wild sbo form [3]. Purified acetylcholinesterase from the Cornell-resistant strain also showed decreased sensitivity to tetrachlorvinphos (39-fold) compared to the pure enzyme from the wild sbo strain. 94% purification of enzymes from mutant (Cornell-resistant) and wild sbo types has now been achieved [4] and there are no gross structural differences between the two forms.

In the present study, we report the substrate preferences of the enzymes from the mutant (Cornell-resistant) strain and wild (sbo) strain. We also compare the substrate profile for the highly purified enzyme from wild type (sbo) flies with that for bovine erythrocyte acetylcholinesterase.

Materials and Methods

Acetylcholinesterases were derived from heads of house flies (*Musca domestica*), a wild strain (sbo) [3] and a tetrachlorvinphos-resistant strain [1]. In addition, bovine erythrocyte acetylcholinesterase (Sterwin Laboratories, New York) was used. 5 substrates were chromogenic and were assayed spectrophotometrically: acetylthiocholine iodide, propionyl-β-methylthiocholine iodide, acetyl-β-methylthiocholine iodide, indophenyl acetate, and *p*-nitrophenyl acetate. The other substrates were assayed in a pH-stat: acetylcholine iodide, butyrylcholine iodide, phenyl acetate, indoxyl acetate, 1-naphthyl acetate, 2-naphthyl acetate and triacetin.

The spectrophotometric method utilized a Varian Techtron double-beam recording spectrophotometer with a temperature-controlled compartment at 25°C. Because of different enzymic activities and quantities available, the volumes of enzyme varied. The following protocol applies to the mutant house fly enzyme with acetylthiocholine; variations were necessary for other substrates. Thiocholine substrates and 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) were prepared separately in 50 mM sodium phosphate buffer (pH 8.0) and 0.2% NaCl. The reaction mixture contained 0.15 ml substrate and 0.15 ml 5,5′-dithiobis-(2-nitrobenzoic acid), then 4 μl enzyme in 50 mM sodium phosphate buffer (pH 7.4) was injected with a micro-syringe and the reaction followed at 412 nm. For indophenyl acetate and *p*-nitrophenyl acetate, stock solutions in 95% ethanol were diluted into the above salt/phosphate buffer to be 1% in ethanol, and 0.15 ml was mixed with 0.05 ml salt/buffer and 0.1 ml enzyme. The spectrophotometer was read at 625 nm for indophenyl acetate and 402 nm for *p*-nitrophenyl acetate.

In the pH-stat method, the reaction was performed in a Radiometer pH-stat with a 10 ml or 5 ml jacketed vessel at 25°C, and using 2.5 mM NaOH as titrant under N_2. The protocol for the mutant enzyme and acetylcholine was as follows (minor variants were made for other substrates and enzymes): to the 10 ml vessel was added 2 ml 5 mM sodium phosphate buffer (pH 8.0) 1% NaCl, and then 1 ml substrate in water plus 6.93 ml water. The pH was then adjusted to 8 and 0.07 ml enzyme in 50 mM phosphate buffer (pH 7.4) was injected, the pH adjusted to 8 and the titration followed with time. The results for house fly enzymes were expressed in nmol · min^{-1} · mg^{-1}; because the turnover number

of these enzymes is not known, the rate per mol cannot be calculated.

Inhibitory potencies against cationic inhibitors were determined in 0.05 M sodium phosphate buffer (pH 7.4), 1 mM acetylthiocholine, 0.5 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 75 mM NaCl and 40 mM $MgCl_2$ at 25°C on a Beckman Acta III spectrophotometer. Dissociation constants (K_d) of inhibitors were calculated according to the following equation [5]:

$$K_d = \frac{[I]\ (1 - i)}{i\ (1 + \frac{[S]}{K_m})} \tag{1}$$

where K_m is the Michaelis constant for acetylthiocholine, [I] is the concentration of inhibitor, [S] is the concentration of substrate, and i the fractional inhibition.

Results and Discussion

In the following discussion, we will utilize the terminology:

$$AX + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AXE \overset{k_2}{\rightarrow} AE + X \overset{k_3}{\rightarrow} A + E \tag{2}$$

where AX is the substrate, E is the enzyme, and k_2 and k_3 are the acylation and deacylation steps respectively. Gutfreund and Sturtevant [6] have shown for the analogous case of chymotrypsin that:

$$K_m = \frac{k_{-1} + k_2}{k_1} \cdot \frac{k_3}{k_2 + k_3} \tag{3}$$

$V = k_2\,[E]$ or $k_3\,[E]$, depending on whether k_2 or k_3 is smaller. In the following discussion, the key issue will be the extent to which observed differences in K_m and V can be used to make deductions about differences in affinity (k_1/k_{-1}) and acylating ability (k_2) and deacylating ability (k_3).

The K_m values are of course independent of the absolute amount of enzyme used in the assays. But the V values are linearly dependent on the amount. Because we did not know the absolute amounts present, we calculated the relative V values of Table II on a normalized basis, setting the V ratio for acetylcholine as 1. To the extent that reality departs from this approximation, the whole set of V ratios will be off-set accordingly, but because we were looking for differences between substrates, such an off-set would not affect our conclusions.

Comparison of house fly wild and bovine erythrocyte enzyme

It is well known that crude house fly enzyme differs profoundly from the other acetylcholinesterase such as that of bovine erythrocyte, lobster axon or *Torpedo* or eel electroplax in hydrolyzing butyrylcholine very well, better than acetylcholine at substrate concentrations above 10^{-2} M [7—9]. This difference is particularly notable in that one major criterion for distinguishing acetylcholinesterase from the related enzyme, butyrylcholinesterase, is inability to hydrolyze butyrylcholine effectively [9]. We are now able to affirm (Tables I,

TABLE I

REACTIVITY OF ACETYLCHOLINESTERASES

Parenthetic values are standard errors as percent. Data was analyzed by a computerized version of the Wilkinson weighted regression technique [12].

	Bovine		Wild house fly		Mutant house fly	
	$10^4 \times K_m$ (M)	V *	$10^4 \times K_m$ (M)	V **	$10^4 \times K_m$ (M)	V **
Spectrophotometric assay						
Acetylthiocholine	0.623	18.8	0.098	0.752	0.304	0.752
	(23)	(5)	(31)	(8)	(14)	(4)
Propionyl-β-methylthiocholine	1.99	6.63	0.129	0.677	0.114	0.591
	(10)	(4)	(13)	(5)	(13)	(4)
Acetyl-β-methylthiocholine	1.76	15.9	0.458	1.15	0.393	1.26
	(6)	(3)	(7)	(3)	(18)	(10)
Indophenyl acetate	13.1	0.392	1.21	0.0782	5.51	0.0405
	(58)	(64)	(8)	(3)	(14)	(9)
p-Nitrophenyl acetate	3.19	0.0852	19.1	0.0183	5.94	0.00584
	(26)	(15)	(17)	(17)	(19)	(12)
pH-stat assay						
Acetylcholine	0.568	10.7	0.767	1.05	0.289	0.983
	(10)	(3)	(18)	(9)	(28)	(12)
Butyrylcholine	3.04	0.152	0.804	0.447	19.83	0.788
	(13)	(6)	(27)	(7)	(35)	(16)
Phenyl acetate	35.0	13.7	12.3	0.667	53.1	1.93
	(37)	(20)	(23)	(10)	(41)	(32)
Indoxyl acetate	129.0	21.7	2.85	3.51	19.2	0.895
	(63)	(55)	(16)	(6)	(24)	(14)
1-Naphthyl acetate	16.1	4.23	1.55	0.341	4.19	0.822
	(15)	(11)	(18)	(4)	(19)	(6)
2-Naphthyl acetate	35.0	2.49	5.29	0.642	49.7	0.528
	(37)	(37)	(14)	(9)	(70)	(65)
Triacetin	138.0	0.602	183.0	0.307	53.9	0.0572
	(70)	(63)	(65)	(88)	(49)	(37)

* V in nmol · min^{-1} · U^{-1}.

** V in nmol · min^{-1} · ng^{-1} of purified enzyme.

II) that the ability to hydrolyze butyrylcholine well is an intrinsic property of the purified house fly enzyme, and is not caused by a contaminating butyrylcholinesterase.

Considering the details of the butyrylcholine difference, the difference in K_m (ratio = 0.26) for house fly and bovine enzyme is close to the average for all substrates (0.84) and thus one can virtually exclude the possibility that the enzyme differ greatly in affinity for this substrate. This view is confirmed by observing the data on all the substrates for the bovine enzyme (Table I): the K_m of butyrylcholine is quite good (better than seven other substrates); it is the V which is extremely poor. In the house fly set, the K_m is also good (again better than seven other substrates) but in this case the V is about average. We conclude that the primary difference between the enzymes with butyrylcholine is in the unusually slow acylating or deacylating step in the bovine enzyme. This conclusion differs from the proposal of Hopff et al. [10], who interpret the inability of *Torpedo* enzyme to hydrolyze butyrylcholine on the basis of an "esteratic crevice" of limited size.

TABLE II

RATIOS OF ACTIVITIES

The ratios are calculated from the data given in Table I.

	K_m ratio		V ratio (normalized to acetylcholine) *	
	Mutant/wild	Wild/bovine	Wild/mutant	Bovine/wild
Acetylthiocholine	3.10	0.16	0.94	2.45
Propionyl-β-methylthiocholine	0.88	0.065	1.07	0.96
Acetyl-β-methylthiocholine	0.86	0.260	0.86	1.36
Indophenyl acetate	4.55	0.093	1.81	0.49
p-Nitrophenyl acetate	0.31	5.97	2.95	0.46
Acetylcholine	0.38	1.35	1	1
Butyrylcholine	24.7	0.26	0.53	0.033
Phenyl acetate	4.32	0.35	0.32	2.02
Indoxyl acetate	6.74	0.022	3.67	0.61
1-Naphthyl acetate	2.70	0.096	0.39	1.22
2-Naphthyl acetate	9.39	0.15	1.14	0.38
Triacetin	0.29	1.33	5.03	0.19

* Obtained by dividing the ratio of the V values for any substrate by the ratio of the V values for acetylcholine.

Comparison of mutant and wild house fly enzyme

The K_m ratios in Table II show that 11 of the 12 lie within a reasonably narrow range of 0.3—6.7. But butyrylcholine is a striking exception, with its K_m being 25 times greater for the mutant than for the wild enzyme. This contrasts with the moderate V ratio of 0.5 which probably implies that the mutant enzyme enjoys a small, 2-fold, advantage in the rate of deacylation. We cannot distinguish between two possible explanations: the acylation step may differ profoundly, or else the affinity for butyrylcholine of the mutant enzyme is much less than that of the wild.

The general similarity of most binding characteristics of the wild and mutant enzyme was also established by a different approach. We examined the ability of six reversible inhibitors to block acetylthiocholine hydrolysis by the mutant

TABLE III

DISSOCIATION CONSTANTS OF CATIONIC REVERSIBLE INHIBITORS FOR WILD AND MUTANT UNPURIFIED HOUSE FLY HEAD ACETYLCHOLINESTERASE

Values are means of triplicate measurements.

	K_i (μM)		Ratio wild/mutant
	Wild	Mutant	
Tetramethylammonium	4600	2840	1.61
Decamethonium	46	29.7	1.54
Phenyltrimethylammonium	119	172	0.69
Edrophonium	25.2	64.6	0.39
Gallamine	110	135	0.81
BW 284 C 51 *	0.066	0.097	0.68

* 1,5-bis(4-allyl dimethyl ammoniumphenyl)-pentan-3-one dibromide.

and wild enzyme. Since there is only one acetylthiocholine-hydrolyzing enzyme in the house fly head [11], we were able to utilize unpurified material for this part of the study. The results (Table III) show no substantial differences in binding.

What information does all this add to our understanding of the nature of the mutant as compared with the wild enzyme? Studies with organophosphates and carbamates [2] showed that all the loss in reactivity of the mutant enzyme was caused by a reduction in binding, not to reactivity. If the loss in K_m for butyrylcholine is also due to a reduction in binding, then the butyrylcholine binding site may be identical with the organophosphate binding site, and the 25-fold increase in K_m is comparable with the 39-fold decrease in the reactivity with tetrachlorvinphos. The simplest conclusion is that the mutation involves a residue or set of residues which is crucial to binding of organophosphates, carbamates and butyrylcholine, but unimportant for other substrates and inhibitors.

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