A putative kinetic model for substrate metabolisation by *Drosophila* acetylcholinesterase

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Abstract Insect acetylcholinesterase, an enzyme whose catalytic site is located at the bottom of a gorge, can metabolise its substrate in a wide range of concentrations (from 1 μM to 200 mM) since it is activated at low substrate concentrations. It also presents inhibition at high substrate concentrations. Among the various rival kinetic models tested to analyse the kinetic behaviour of the enzyme, the simplest able to explain all the experimental data suggests that there are two sites for substrate molecules on the protein. Binding on the catalytic site located at the bottom of the gorge seems to be irreversible, suggesting that each molecule of substrate which enters the active site gorge is metabolised. Reversible binding at the peripheral site of the free enzyme has high affinity (2 μM), suggesting that this binding increases the probability of the substrate entering the active site gorge. Peripheral site occupation decreases the entrance rate constant of the second substrate molecule to the catalytic site and strongly affects the catalytic activity of the enzyme. On the other hand, catalytic site occupation lowers the affinity of the peripheral site for the substrate (34 mM). These effects between the two sites result both in apparent activation at low substrate concentration and in general inhibition at high substrate concentration.

Key words: Acetylcholinesterase; Substrate metabolization; *Drosophila*

1. Introduction

Cholinesterases are serine hydrolases which have been divided into two classes, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8) according to their catalytic properties, inhibition specificities and tissue distribution (for a review of cholinesterases, see Massoulié et al. [1]). Two substrate binding sites are present on each monomer: a catalytic site which lies at the bottom of a 20 Å deep gorge, and a peripheral anionic site represented by some of the numerous aromatic residues at the rim of the active site gorge. The catalytic site is composed of two subsites: a catalytic 'anionic' site which recognises the choline moiety of the substrate, and an 'esteratic' site responsible for the hydrolysis of the substrate. The hydrolysis is described as occurring in three steps: (a) reversible Michaelis complex formation, (b) acylation of the enzyme and (c) deacylation induced by a water molecule [2–5].

Most cholinesterases, from different sources, do not follow Michaelis-Menten kinetics: vertebrate butyrylcholinesterase displays activation at low substrate concentration and vertebrate acetylcholinesterase displays an inhibition by an excess of substrate. Two main hypotheses have been proposed to explain these kinetics. The first assumes a modulation of the deacylation rate constant by the binding of an additional substrate molecule on the acyl-enzyme intermediate [6] and the second hypothesis involves the non-competitive binding of the substrate to a peripheral site, increasing or decreasing the catalytic ability of the enzyme [7]. Insect cholinesterase kinetics which combine activation and inhibition [8] cannot be analysed by such models. Thus, we propose a putative kinetic model, with a reasonable number of parameters, able to describe the complex kinetics of insect AChE.

2. Materials and methods

2.1. Source of enzyme

A truncated cDNA encoding a soluble AChE of *Drosophila melanogaster* was expressed with the baculovirus system [9]. Secreted AChE was purified and stabilised with 1 mg/ml BSA according to Estrada-Mondaca and Fournier [10].

2.2. Kinetics of substrate hydrolysis

Kinetics were studied at 25°C in 25 mM phosphate buffer pH 7. Hydrolysis of thiocholine iodide esters was followed spectrophotometrically at 412 nm using the method of Ellman et al. [11] at substrate concentrations from 1 μM to 200 mM. Active site titration was carried out using 7-(methylethoxy phosphinyloxy)-1-methylquinolinium iodide which was synthesised as described by Levy and Ashani [12].

2.3. Data analysis

The initial rate constants were analysed according to mathematical models which result from different kinetic mechanisms, using a non-linear regression program [13]. Discriminations between the different models tested were made according to general criteria for goodness of fit [14].

3. Results and discussion

3.1. Choice of the kinetic model

From the complex kinetic behaviour of *Drosophila* AChE, three main hypotheses can be suggested: (1) two kinetically distinct enzymatic forms or two enzymatic forms in equilibrium are present; (2) one enzymatic form with three substrate binding sites; (3) one enzymatic form with two substrate binding sites. For each hypothesis, we deduced different plausible kinetic schemes. In order to have a reason-

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Abbreviation: AChE, acetylcholinesterase

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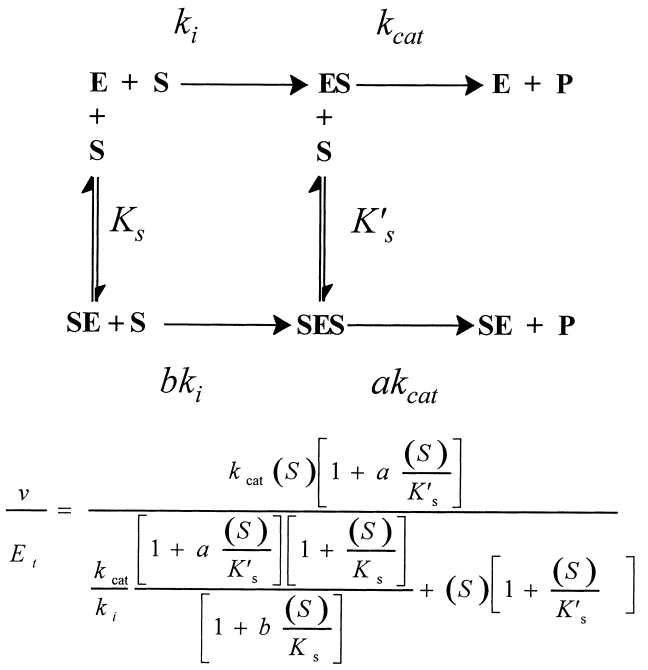

Scheme 1. General model for activation and inhibition of *Drosophila* AChE depending on substrate concentration. E is the free enzyme, ES is a Michaelian complex representing the binding of a substrate molecule onto the catalytic site and SE represents the binding of a substrate molecule onto the peripheral site.

able number of parameters, different simplifications were made, for example, inclusion of formation of the Michaelian complex and acylation in the same step, or inclusion of acylation and deacylation in the same step. From these schemes, kinetic equations were derived and used to analyse initial rate constants by non-linear regression.

It appeared evident that models with three binding sites or with two independent enzyme species were appropriate to resolve data sets. But since (i) docking assays of three substrate molecules on the active site failed due to the narrowness of the active site gorge, (ii) we were unable to characterise two enzymatic forms and (iii) all known post-translational modifications of the enzyme did not change the kinetic behaviour [15], we excluded such schemes and we considered only models with one enzymatic form and two substrate binding sites.

From this assumption, the model presented in Scheme 1 appeared to be appropriate. It was functional for all sets of data obtained in several conditions (pH, temperature), with different mutated enzymes on amino acids lining the active site gorge (Fig. 1), with different substrates (Fig. 2) or in the presence of reversible inhibitors (in this latter case, parameters describing the binding of inhibitor molecules on each enzymatic complex were added).

3.2. Significance of the model

Although other models may also be adequate and although it is speculative to describe the biological functioning of an enzyme from pS curves only, we tried to evaluate the significance of this model.

The main point is that the formation of the complex ES is an irreversible step. This irreversibility, as implied by the model, seems to corroborate structural information. Indeed, asymmetric charge distribution of the enzyme suggests that every substrate molecule that encounters the entrance of the active site gorge proceeds to reaction. An electrostatic gradient attracts the substrate inside the gorge, where it is blocked and most probably metabolised [16,17]. To analyse this hypothesis, we performed a dynamic simulation with the *Drosophila* active site gorge deduced from the *Torpedo* enzyme coordinates [4]. The tetrahedral intermediate was minimised in the active site to mimic the substrate positioning in the Michaelian complex. It seems that the choline part is enclosed in a box composed of four aromatic residues at positions 330, 334, 331 and 84 (*Torpedo* numbering) which form an aromatic cage [18]. This arrangement could hinder substrate exit from the gorge and explain the apparent irreversibility of ES complex formation.

The second point is the presence of a non-productive site. Such a site has already been described in vertebrate enzymes, is usually called a peripheral anionic site and is located at the rim of the active site gorge [19]. The model also assumes the presence of this site in insect AChE. But, in contrast to previous models [7,20], the affinity of this site for the substrate is very high (2 μM). We can hypothesise that this high affinity allows substrate molecules to be trapped at the entrance of the active site gorge, locally increasing the substrate concentration and hence the probability of the substrate entering and reaching the active site. The affinity of the peripheral site changes according to the enzyme form: the high substrate affinity for the peripheral anionic site on the free enzyme is strongly reduced (~4 orders of magnitude) by the presence of substrate at the catalytic site (34 mM). To explain this variation of affinity of the peripheral site when the catalytic site is occupied, one hypothesis can be expressed: the arrangement of the aromatic residues at the catalytic site around the choline moiety of the substrate changes the arrangement of aromatic residues located at the rim of the gorge, and thus the putative peripheral site.

The third point is the effect of peripheral site occupation on the entrance of a new molecule inside the gorge. This effect is represented by the *b* factor, which, with K_s, is responsible for the apparent activation observed in the insect AChE (Fig. 3). The *b* factor is less than 1 (see Table 1) which indicates that the entering of a substrate molecule into the gorge and/or the positioning at the catalytic site are slowed down by a substrate molecule bound to the rim of the gorge, most probably

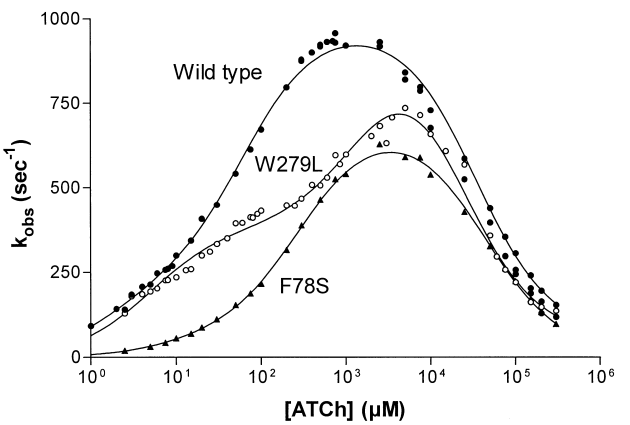

Fig. 1. pS activity curves for acetylthiocholine hydrolysis by wild type *Drosophila* acetylcholinesterase, W279L and F78S mutants (*Torpedo* numbering). The curves were obtained by fitting to the equation in Scheme 1. Parameter values are presented in Table 1.

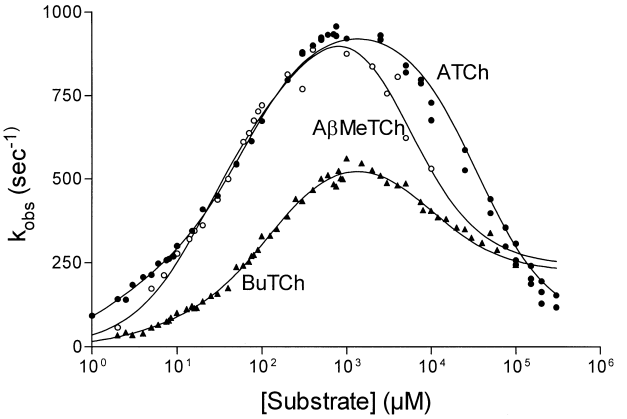

Fig. 2. pS activity curves for acetylthiocholine (ATCh), acetyl-β-methylthiocholine (AβMeTCh) and butyrylthiocholine (BuTCh) hydrolysis by wild type *Drosophila* AChE. The curves were obtained by fitting to the equation in Scheme 1. Parameters values are presented in Table 1.

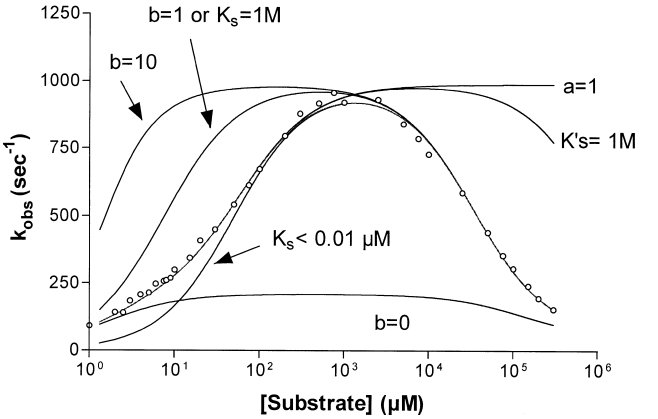

Fig. 3. Curves generated using the equation in Scheme 1 and the parameters of Table 1 (wild type/ATCh). Some parameters were modified to show their influence on the *Drosophila* AChE kinetics.

by steric interactions. This effect has recently been described for some ligands [21].

Finally, binding of a substrate molecule at the peripheral site decreases the metabolisation of the substrate at the bottom of the gorge ($a < 1$), resulting in the inhibition of the enzyme (Fig. 3). At very high substrate concentrations, the molecule binds at the peripheral anionic site even if another substrate molecule is present at the active site, but with very low affinity. This binding greatly hinders the metabolisation of a new substrate molecule and so results in a general inhibition of the enzyme. This inhibition of the catalytic step may reflect either jam in the traffic between substrate molecules and products circulating in the gorge or a direct effect on the positioning of the substrate molecule at the catalytic site as previously suggested by the change of peripheral site affinity when the catalytic site is occupied.

This model was deduced from pS curves obtained with the *Drosophila* enzyme. If it is applied to vertebrate enzymes, the lack of one phase, i.e. activation in AChE or inhibition in butyrylcholinesterase, may be explained either by the fact that these phases do not exist in these enzymes or because one phase is masked. In vitro mutagenesis sometimes produces enzymes which reveal both phases: activation and inhibition [22], so the second possibility appears plausible. In vertebrate enzymes or in mutant enzymes in which the activation phase is lacking, this could be due to a lower affinity for the peripheral site or to lack of effect of peripheral site occupation on the entrance to the catalytic site. Absence of inhibition at high substrate concentrations may only indicate that the value of K_s is higher than the substrate solubility in aqueous solvent and so was undetectable (Fig. 3).

3.3. Has the complex behaviour of cholinesterase any physiological significance?

The activation of the enzyme depending on substrate concentration may serve to adjust the catalytic activity to fluctuations in the concentration of the neurotransmitter, following its release in the synaptic cleft. The peripheral site would act as a sensor allowing substrate molecules to be caught but only when present at low concentrations and may serve to control the neurotransmitter concentration in the synaptic cleft. It is believed that acetylcholine is released in the synapse in a concentration range of 0.1–1 mM [23] which corresponds to the maximal activity of the enzyme. After its release, the neurotransmitter binds to the receptor and is simultaneously metabolised by AChE. It should be noted that the receptor exists in two states (resting and desensitised) and has two binding sites for acetylcholine resulting in different affinities for acetylcholine, from 10^{-3} to 10^{-9} M. Thus, ligand concentration between two pulses determines the relative concentration of resting and desensitised receptors and consequently, the response to the subsequent pulse [24]. Thus, allostery in AChE seems to be correlated to receptor function; the combination of the two kinetics allows the response to successive nerve impulses. Inhibition at high substrate concentration is puzzling, but these concentrations are not likely to occur under physiological conditions except following organophosphorus compound intoxication. Nevertheless, we suggest here that activation and inhibition originate from the same interaction between the catalytic site and the peripheral site and that the inhibition

Table 1
Kinetic parameters obtained by data analysis with the equation in Scheme 1, for the wild type *Drosophila* enzyme and mutant enzymes W279L and Y78F (*Torpedo* numbering)

Enzyme Substrate	Wild type ATCh	Wild type AβMeTCh	Wild type BuTCh	W279L ATCh	F78S ATCh
K_s (μM)	2	55	6	8	12
K'_s (mM)	34	4	9	22	43
k_{cat} (s^{-1})	986	1166	611	991	696
k_2 (μM^{-1} s^{-1})	133	36	18	77	8.5
a	0.06	0.20	0.36	0.06	0.018
b	0.15	0.19	0.28	0.01	0.31

by excess substrate is a consequence of the peripheral site saturation.

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