Involvement of Deacylation in Activation of Substrate Hydrolysis by Drosophila Acetylcholinesterase*

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The cholinesterases (ChE), including acetylcholinesterases (AChE) and butyrylcholinesterases (BuChE), are serine hydrolases that catalyze the hydrolysis of choline esters in two steps, enzyme acylation followed by deacylation involving a water molecule (1). This very efficient catalysis shows an acetylcholinesterase (AChE); BuChE, butyrylcholinesterase; ACh, acetylcholine; ATCh, acetylthiocholine; ChAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; BSA, bovine serum albumin.

The process takes place at the acylation site located at the bottom of a long narrow active site gorge. This site includes a tryptophan (Trp-84 using the numbering of the Torpedo enzyme) that interacts with the trimethylammonium group of acetylcholine and a serine (Ser-200) that is acylated and deacetylated (hydrolyzed) during substrate turnover (3). Another substrate binding site, located at the mouth of the active site gorge, has been discovered. This site is non-productive and is thought to be the initial binding site for substrate on the ChE catalytic pathway (4, 5). This binding decreases association and dissociation substrate rate constants at the acylation site and blocks product release (5, 6).

Analysis of substrate hydrolysis at different concentrations reveals that insect cholinesterases display activation at low substrate concentrations similar to vertebrate BuChE and inhibition at high substrate concentrations similar to vertebrate AChE (7). Analysis of product-substrate curves and the effects of a competitive inhibitor of activation led to the hypothesis that substrate binding at the peripheral site accounts for apparent activation by affecting the entrance rate constant of a second molecule to the catalytic site (8, 9). Although apparent activation may be explained by steric hindrance at the mouth of the gorge, it cannot be excluded that another mechanism participates in the activation. Here, we consider a second hypothesis proposed by Eriksson and Augustinsson in 1979 (10): the modulation of the deacylation rate constant follows the binding of an additional substrate molecule to the acyl-enzyme intermediate.

MATERIALS AND METHODS

Enzyme Sources—Truncated cDNA encoding soluble AChEs (wild-type and mutated) from Drosophila melanogaster was expressed with the baculovirus system (11). Secreted AChEs were purified and stabilized with 1 mg ml⁻¹ BSA as previously reported (12). Residue numbering was according to the sequence of the mature Drosophila AChE (13), and the numbering in parentheses corresponds to the Torpedo AChE sequence.

Chemicals—Acetylcholine (ACh), acetylthiocholine (ATCh), Triton X-100, edrophonium and propidium were purchased from Sigma (St Louis, MO). Carbaryl, i.e. 1-naphthyl methylcarbamate, was purchased from Cil Cluzeau Info Labo (Sainte-Foi-La-Grande, France). The substrate analog i.e. 4-oxo-N,N,N-trimethylpentanaminium iodide (14) was synthesized according to Thanei-Wyss and Waser (15). 7-(methylthioethoxyphosphoryloxy)-1-methyl-quinolinium iodide was synthesized as described by Levy and Ashani (Ref. 16; Fig. 1).

Kinetics of Substrate Hydrolysis—Kinetics were studied at 25 °C in 25 mM phosphate buffer, pH 7, 1 mg ml⁻¹ BSA. Hydrolysis of acetylthiocholine iodide was followed spectrophotometrically at 412 nm using the method of Ellman et al. (17) at substrate concentrations from 2 μM to 300 mM. Active site titration was carried out using 7-(methylthioethoxy-phosphoryloxy)-1-methyl-quinolinium iodide.

Determination of the Decarbamoylation Rate Constant of AChE—Enzyme was incubated with carbaryl in 25 mM phosphate buffer, pH 7, 1 mg ml⁻¹ BSA until more than 95% of the enzyme was inhibited. The mixture was loaded onto a gel filtration column (P10, Amersham Pharmacia Biotech).
macia Biotech) and eluted with 25 mM phosphate buffer, pH 7, 1 mg ml \(^{-1}\) BSA. Enzyme fractions were collected. The decarbamoylation rate was followed with time at 25 °C for 9 h by sampling aliquots of the reaction mixture and by estimating free enzyme concentration spectrophotometrically through its activity with 1 mM ATCh. The reaction can be described by a simple first-order rate equation. The decarbamoylation rate constant observed (\(k_{\text{obs}}\)) was calculated by non-linear regression analysis using the equation,

\[
\frac{\text{d}[E]}{\text{dt}} = k_{\text{R}}[E_0] - k_{\text{a}}[S] + K_d \frac{[S] + [E]}{[S]}
\]

(Eq. 1)

where \([E]\) represents the free enzyme concentration at time \(t\), \([E_0]\) the initial concentration of free enzyme, and \([E_0]\) the initial concentration of monomethylcarbamoylated enzyme.

**Results**

**Acceleration of the Decarbamoylation Rate by the Substrate Analog**—The study of the effect of potential deacetylation accelerators on acetyl-enzyme was not possible because the deacetylation rate was too rapid. To slow down this step, we used an analog of acetyl-enzyme, the carbamoyl-enzyme. The decarbamoylation rate constant of the wild-type enzyme (0.79 \(\pm\) 10\(^{-2}\) s\(^{-1}\)) is about 10\(^6\)-fold slower than that of deacetylation.

To test if the substrate molecule accelerates decarbamoylation of the enzyme, we first used acetylcholine and acetylthiocholine. However, because some free enzyme always remains in solution, substrate hydrolysis occurs releasing choline or thiocholine molecules into the solution. These latter molecules accelerate decarbamoylation (18) because AChE is able to catalyze transesterification (19, 20).

With the *Drosophila* enzyme, acceleration of decarbamoylation by choline and thiocholine at 1 mM was 4.7 and 140-fold, respectively. Consequently, it was impossible to separate the potential acceleration caused by the substrate molecule from that caused by choline or thiocholine.

Thus to analyze acceleration of decarbamoylation by a substrate molecule, we synthesized a substrate analog, 4-oxo-N,N,N-trimethylpentanaminium iodide, which cannot be hydrolyzed (Fig. 1). We checked that this molecule is indeed a competitive inhibitor of the substrate ATCh (data not shown). The decarbamoylation rate was determined in the presence of various concentrations of substrate analog from 10–500 \(\mu\text{M}\) (Fig. 2). Considering only the decarbamoylation step and one non-productive binding site, the data were analyzed according to Scheme 1, where \(E_c\) represents the monomethylcarbamoylated AChE; \(E\), the decarbamoylated AChE; \(S\), the substrate analog; \(S_E\), the substrate analog molecule bound on the peripheral site; \(k_r\), the decarbamoylation rate constant; \(a\), the coefficient of acceleration or deceleration; and \(K_D\), the dissociation constant of the substrate analog for the peripheral site.

\[
k_{\text{obs}} = k_r \left( \frac{K_D + a[S]}{K_D + [S]} \right)
\]

(Eq. 2)

The fit of data using Equation 2, which is derived from Scheme 1, gave the following results: the decarbamoylation rate constant \(k_r\) was estimated to be 79 \(\pm\) 4 \(\times\) 10\(^{-6}\) s\(^{-1}\); the substrate analog activates decarbamoylation with a coefficient of acceleration \(a\) estimated at 2.2 \(\pm\) 0.1; and the dissociation constant \(K_D\) was estimated to be 130 \(\pm\) 47 \(\mu\text{M}\).

**Effect of Ligands on the Decarbamoylation Rate**—We investigated the effect of several ligands on the decarbamoylation rate of monomethylcarbamoylated *Drosophila* AChE: Triton X-100, propidium, edrophonium, CHAPS, tetramethylammonium, and decamethonium. The results are presented in Table I. The fit of data was achieved with Equation 2, previously used for the substrate analog. All ligands tested showed a significant effect on the decarbamoylation rate of the enzyme, where a decrease or increase was observed. This effect has already been
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reported by Roufogalis and Thomas (21) who described the acceleration of decarbamylation by tetramethylammonium using bovine erythrocyte AChE. However, the effect of ligand binding varies depending on the enzyme species, because a deceleration by tetramethylammonium using Drosophila AChE was observed (Table I). Effects were small for monovalent cations; the strongest activation having a factor of 3 and the greatest inhibition a factor of 1.6. This suggests that ligand binding to the rim or the bottom of the gorge has a limited effect on deacylation.

Furthermore, this experiment allowed us to determine the affinity of each ligand for the carboxamoylated enzyme (Table I), which we compared with its affinity for the free enzyme that we had previously estimated (9). The affinity of edrophonium for the free enzyme (K_{d} = 0.84 \mu M) was higher than for the carboxamoyl form (K_{d} = 4.7 \mu M). This could be explained by the fact that acetylation should change the edrophonium binding site located at the bottom of the active site gorge, at the catalytic site (22). By contrast, the affinity of propidium was not significantly different for the free enzyme (K_{d} = 119 nM) and for the carboxamoyl form (K_{d} = 92 nM). This is in accordance with the observation of Taylor and Lappi (23) who reported that the affinity of propidium is not affected by mephenesulfonylaion of Torpedo californica AChE. The affinity of Triton X-100 for the free enzyme and for the carboxamoyl form was also not significantly different: K_{d} = 0.052 g/liter and K_{d} = 0.047 g/liter, respectively. Thus, binding of a ligand at the rim of the active site gorge does not seem to be affected by acetylation.

Kinetics of Substrate Hydrolysis—We previously used a simple model to describe the kinetic pathway of Drosophila AChE activity as a function of substrate concentrations (8), AChE being activated at low substrate concentrations and inhibited at high substrate concentrations. This model indicated that activation of substrate hydrolysis at low substrate concentrations could be explained by the binding of a substrate molecule to the peripheral non-productive site, located at the rim of the active site gorge, which affects the entrance of another substrate molecule into the active site gorge (9). However, in this model, an increase of the deacylation rate as a possible additional explanation of the activation phenomenon was not considered. Because activation of Drosophila AChE results in part from an acceleration of the deacylation (Fig. 2), we had to change our previous kinetic model. Moreover, a previous model hypothesized that the affinity of the activation site for the substrate molecule decreases when the enzyme is acetylated. As carbamylation did not affect the affinity of ligands that bind at the rim of the gorge such as propidium and Triton X-100 (Table I and Ref. 9), we could hypothesize that acylation does not affect the binding of a substrate molecule at the rim of the gorge. Consequently, the same parameter K_{S1} (the affinity of the substrate molecule for the peripheral binding site involved in activation) could be applied to the free enzyme and the acetylated form. Taking into account that inhibition of substrate hydrolysis occurs for high substrate concentrations (above 1 mM), we propose here a new model, which considers the existence of two different non-productive sites, where substrate molecules (S1 and S2) bind but are not hydrolyzed (Scheme 2). In this way, both activation of the enzyme activity at low substrate concentrations and its inhibition at higher substrate concentrations could be explained. Binding of S1 would result in substrate hydrolysis activation and binding of S2 would result in substrate hydrolysis inhibition. In Scheme 2, E represents the AChE; EA, the acetyl enzyme; S1, the substrate molecule responsible for activation; S2, the substrate molecule responsible for inhibition; a, the coefficient of acceleration; b, the coefficient of inhibition of the deacylation rate constant; c, the effect of peripheral activation site occupation on the entrance of a new molecule inside the gorge; and d, the effect of peripheral inhibition site occupation on the entrance of a new molecule inside the gorge. k_{r} represents the bimolecular rate constant for acylation and k_{cat} the rate constant for deacylation. K_{S1} and K_{S2} represent the dissociation constants of S1 and S2 for the peripheral activation site and the peripheral inhibition site, respectively. Free substrate molecules and reaction products are not represented for clarity of the scheme.

The rate of ATP hydrolysis was measured versus substrate concentration, and data were fitted using Equation 3, which was derived from Scheme 2 (Fig. 3, Equation 3). But the fitting yielded a poor estimation of the numerous kinetic parameters. To be able to fit data accurately, we fixed two parameters, K_{S1} and a, previously estimated by the effect of the substrate analog on the carbamylation rate. Parameter d was not neces-

![Image 308x188 to 555x291](image)

**SCHEME 2.**

**TABLE I**

| Ligand          | K_{d} (M)       | a          |
|----------------|----------------|------------|
| Propidium      | 92 \times 10^{-6} | 2.5 (0.26) |
| Triton X-100   | 47 \times 10^{-3} | 2.28 (0.29) |
| Edrophonium     | 4.7 \times 10^{-6} | 3 (0.3)    |
| CHAPS          | 53 \times 10^{-6} | 0.63 (0.03) |
| Tetramethylammonium | 2.8 \times 10^{-6} | 0.62 (0.03) |
| Decamethonium  | 1.23 \times 10^{-6} | 7 (1.3)    |
| Substrate analog| 130 \times 10^{-6} | 2.2 (0.1)  |
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The data of the rate of ATCh hydrolysis versus substrate concentration were fitted using Equation 4, which was derived from Scheme 3 (Fig. 4, Equation 4), after having fixed two parameters obtained by studying the effect of the substrate analog on the decarbamoylation rate of AChE. The activation coefficient of the decarbamoylation rate constant ($a$) was fixed at 2.2 and the dissociation constant of S1 ($K_s$) for the peripheral activation site was fixed at 130 μM (Table I). The fit was obtained with 0.996 as the global correlation coefficient and 5.2% as the average relative deviation per point (Fig. 5).

Acceleration of the Decarbamoylation Rate of Mutated Enzymes—To locate the amino acids involved in the binding of substrate analogs and in signal transmission, several enzymes with a mutation on residues lining the active site gorge were used (Table II, Fig. 6). The effect of the substrate analog on the decarbamoylation rate of mutated enzymes was studied from a concentration of 1 μM to 1 mM substrate analog. Several mutations induced a decrease in acceleration, which was in some cases no longer detectable. This could be interpreted either by a decrease in the affinity of the activation site for the substrate analog ($K_d > 1$ μM) or by the absence of the effect of substrate analog binding on the activation site on decarbamoylation rate ($a$ is not significantly different from 1). For only one mutant, Y374A, the binding of the substrate analog induced a decrease in the decarbamoylation rate ($a < 1$).

**DISCUSSION**

**Substrate Binding Sites in Drosophila AChE**—Data from ATCh hydrolysis versus substrate concentration can be analyzed considering the existence of three sites: a productive site where binding of substrate molecule triggers its hydrolysis, and two distinct non-productive sites, one responsible for the activation and the other for the inhibition of ATCh hydrolysis by Drosophila AChE. The productive site, also called the catalytic site, is located at the bottom of the gorge with Trp-83(84) as a key component interacting with the quaternary ammonium moiety of choline (24, 25, 26). Some ligands such as edrophonium and N-methylacridinium are specific for this site (22, 27). A non-productive site, also called a peripheral binding site, was first proposed from steady-state kinetic inhibition with various ligands (28). It has been implicated by equilibrium dialysis experiments (29), by direct fluorescence (23, 27, 30), and by NMR (31). Site-directed labeling (24), site-directed mutagenesis (32), and x-ray crystallography (22, 33) have shown that this peripheral site is located at the rim of the active site gorge and that Trp-321(279) was a key component. A multiplicity of subsites, designated as P1 through P4 by Rosenberry (34), has been implicated on the basis of inhibition kinetics (35, 36).

The existence of these subsites was confirmed by site-directed mutagenesis, which showed that each inhibitor binds in a distinct manner (32). The presence of several ligand peripheral sites is consistent with the presence of two non-productive substrate binding sites, one responsible for the activation and the other for the inhibition of ATCh hydrolysis by Drosophila AChE.

**Significance of the Model**—The rate of ATCh hydrolysis was measured versus substrate concentration. Fig. 4 shows the goodness of the fit (0.996 as global correlation coefficient and 5.2% as average relative deviation per point), allowing us to consider Scheme 3 as a good model for the kinetic pathway of Drosophila AChE activity. Thus we could interpret the regulation of AChE activity as the binding of substrate molecules at two non-productive sites, one being responsible for activation and one for inhibition. However, it remains to be elucidated if these two sites are independent or overlap, i.e., binding of the substrate responsible for inhibition depends on the occupation of the activation site or not. If they are dependent there are two possibilities: either the substrate molecule responsible for inhibition binds only to acetylated enzyme and the ternary complex (S1S2E) does not form, or the substrate binds only to the S1EA complex and the S2EA complex does not occur. These two possibilities were tested. Product-substrate data did not fit the equations derived from these two simplified models, suggesting that the two sites are independent. The decarbamoylation rate constant was estimated to be 464 s$^{-1}$. By taking into account the acceleration of the decarbamoylation rate constant of 2.2 (Table I), we calculated a turnover number of 1020 s$^{-1}$, as illustrated in Fig. 2 and which is similar to another estimation made for Drosophila AChE turnover, 1783 s$^{-1}$ (37). In Scheme 3, the affinity of the substrate molecule for the peripheral binding site involved in the activation is considered to be the same for the free enzyme (E) and the acetylated form (EA). This is consistent with the observation of inhibitors binding at a peripheral site that is not modified by the carbamoylation of the enzyme. In contrast, this is contradictory to an hypothesis of our previous model, where catalytic site occupation lowers the affinity of...
the substrate for the peripheral site.

Residues Affecting Decarboxylation Rate—To locate potential sites involved in the acceleration of the decarboxylation rate, we studied the effect of the binding of different ligands and the effect of a single mutation on the decarboxylation rate. Binding of all the ligands tested affected the decarboxylation rate. Edrophonium, which binds to the catalytic site (22), increased the decarboxylation rate. Propidium also increased the decarboxylation rate, binding to the rim of the gorge on Trp-321(279) in vertebrates (25, 26, 38), similar to Drosophila, because the dissociation constant of propidium (8 nM for the wild-type AChE) increases to 12 µM for the W321L mutant.³ Triton X-100 increased the decarboxylation rate, binding at the rim of the gorge at a site distinct from the propidium binding site with Glu-69(70) as the main residue involved in its binding (9). CHAPS, a non-competitive inhibitor (39), decreased the decarboxylation rate. Taking into account its size, it probably also binds to the rim of the gorge. Decamethonium accelerated decarboxylation; this bis-quaternary ammonium ligand bridges catalytic and peripheral sites (22). Tetramethylammonium decreased the decarboxylation rate, and this small quaternary ligand binds to peripheral and catalytic sites (40). Thus, the binding of ligands to the catalytic site or to a peripheral site affects the decarboxylation rate, where acceleration or deceleration was observed.

Studies using mutated enzymes led to the same result: several mutations affect the decarboxylation rate. Mutated residues that affect the decarboxylation rate can be located anywhere in the gorge from the rim to the bottom, i.e. the decarboxylation rate can be modified by mutations anywhere in the active site gorge. For example, F330S, a mutation located in the acyl pocket, E237Q located at the bottom of the gorge, F371G located at the middle of the gorge, and V318D located at the rim of the gorge decreased the decarboxylation rate constant (kₜ, Table II).

Localization of the Substrate Activation Site—The acceleration in decarboxylation rate by substrate analog was consistent with the existence of only one substrate activation site. This substrate activation site has been recently located at the rim of the gorge with Glu-69(70) as the main component, using an inhibitor competitive with activation (Triton X-100; Ref. 9). This location is consistent with mutational effects because the three mutations located at position 69 completely abolished activation of the decarboxylation rate by the substrate analog. However, other mutations also suppressed activation by the substrate analog. We can assume that they are involved in signal transmission because they are located between the rim and the bottom of the gorge.

This result consolidates two hypotheses that have been proposed to explain the activation of hydrolysis by substrate molecules in human BuChE. In the first hypothesis, a substrate molecule binds to the acyl-enzyme and accelerates decarboxylation (10, 41). In the second, binding of a substrate molecule at the rim of the gorge allosterically regulates the catalytic activity through a conformational change (42, 43). Results obtained for activation in insect AChE show that these two hypotheses are not exclusive; binding of a substrate molecule at the rim of the gorge accelerates decarboxylation (Table I).

Transmission of Information from the Rim to the Bottom of the Gorge—Several reports suggest that peripheral sites are allosteric and affect the reactivity of the esterase site. Specific inhibitors for some peripheral sites enhance the decarboxylation rate of the carbamoylated enzyme or dephosphorylation of the phosphorylated enzyme (18, 44, 45). Here, we confirm these results because Triton X-100 and propidium that bind to the rim of the gorge increased the rate of decarboxylation, which takes place at the bottom of the gorge. This allosterism was also implicated by the effect of mutations at the rim of the gorge. Mutations at the peripheral site, Asp-70(72) in human BuChE, have been shown to be involved in activation by the substrate (4, 46) and in dealkylation of the phosphoryl enzyme (47). Results obtained with the Drosophila enzyme are in accordance because mutations located at the entrance of the gorge (at positions 69(70), 71(72), 321(279), or 375(335)) cancelled the activation of decarboxylation, showing allosterism between the rim of the gorge and its bottom.

The question arising now is how the information is transmitted from the rim to the bottom of the gorge. Several non-exclusive hypotheses can be put forward: (i) The information is transmitted via the backbone; the binding of the substrate to the rim of the gorge changes or stabilizes a loop conformation more favorable for decarboxylation. (ii) The information is transmitted by the substrate molecule; the binding at the rim of the gorge orients the molecule favorably, which then slides down and increases the decarboxylation rate at the bottom, and (iii)

³ D. Fournier, unpublished results.
the information is transmitted via motions of the side chains of amino acids lining the gorge and of water molecules.

The first hypothesis involves the movement of a flexible loop (Ω-loop) from Cys-66(65) to Cys-93(94) (47). This hypothesis is consistent with the observation that mutation of several residues located on the Ω-loop affects the acceleration of decarbamylation by substrate binding at the peripheral site (Table II). However, mutations of some residues, such as Tyr-370 or Tyr-374, also have an important effect. As these residues are not located on the loop, according to this hypothesis, their mutation would affect the conformation of the Ω-loop.

The second hypothesis involves a shift of the substrate molecule that first binds at the rim, slides down the gorge, and then increases deacetylation. This hypothesis agrees with the normal path of the substrate molecule. However, inhibitor effects do not support this because all inhibitors have a significant effect on decarbamylation rate. Some of them such as propidium or CHAPS are too cumbersome to enter the gorge and product molecules that exit the gorge. These inhibitors affect the entrance of another substrate molecule inside the site (9); and it may increase deacetylation of the serine (this study) and therefore clean up the active site gorge before the entrance of a new substrate molecule. Thus, the activation site seems to be a regulator of the traffic between substrate molecules that enter the gorge and product molecules that exit the gorge.

The third hypothesis involves side chains of the amino acids paving the gorge. Sussman et al. (3) showed that the active site gorge is lined by connected aromatic residues. Information could be transmitted by changes in stacking arrangement or by gear effect. Certain interactions between aromatic residues have been hypothesized and some demonstrated. Shafferman et al. (25) assumed that tyrosine (334) interacts with phenylalanine (330), which may reduce the deacylation efficiency of the acyl-enzyme complex. Tyrosine 374(334) has been shown to interact with tyrosine 71(72) in the Torpedo enzyme (3) and in the Drosophila enzyme (48).

The efficiency of deacetylation is dependent on the position of a water molecule. The location of water molecules inside the site depends on the arrangement of the amino acid side chains lining the active site gorge and on the position of the loops structure forming the gorge wall. When a molecule binds somewhere in the active site gorge, the arrangement of water molecules is modified (49) resulting in a change of deacetylation rate. In this way, the binding of a substrate molecule at the peripheral site would accelerate or decelerate deacetylation if the water molecule involved in the hydrolysis is closer or further from the acetyl-serine in the new arrangement. The fact that a large number of mutations affects the acceleration of decarbamylation by the substrate supports this last hypothesis and suggests that the information is transmitted through the motions of the network of side chains lining the gorge and by water molecules.

Roles of the Activation Site—The binding of a substrate molecule at the rim of the gorge seems to have several effects. It changes the probability of a substrate molecule entering the active site gorge (9); it may correctly orient positively charged substrates to slide down to the bottom of the gorge (4); it may affect the entrance of another substrate molecule inside the site (9); and it may increase decylation of the serine (this study) and therefore clean up the active site gorge before the entrance of a new substrate molecule. Thus, the activation site seems to be a regulator of the traffic between substrate molecules that enter the gorge and product molecules that exit the gorge.

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