Exploration of the Drosophila Acetylcholinesterase Substrate Activation Site Using a Reversible Inhibitor (Triton X-100) and Mutated Enzymes*

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Cholinesterases are activated at low substrate concentration, and this is followed by inhibition as the level of substrate increases. However, one of these two components is sometimes lacking. In Drosophila acetylcholinesterase, the two phases are present, allowing both phenomena to be studied. Several kinetic schemes can explain this complex kinetic behavior. Among them, one model assumes that activation results from the binding of a substrate molecule to a non-productive site affecting the entrance of a substrate molecule into the active site. To test this hypothesis, we looked for an inhibitor competitive for activation and we found Triton X-100. Using organophosphates or carbamates as hemisubstrates, we showed that Triton X-100 inhibits or increases phosphorylation or carbamoylation of the enzyme. In vitro mutagenesis of the residues lining the active site gorg allowed us to locate the Triton X-100 binding site at the rim of the gorge with glutamate 107 playing the major role. These results led to the hypothesis that substrate binding at this site affects the entrance of another substrate molecule into the active site cleft.

Cholinesterases belong to the family of serine hydrolases. In vertebrates, they are present in two forms: acetylcholinesterase (AChE, EC 3.1.1.7), which hydrolyzes the neurotransmitter acetylcholine in order to stop nervous impulse transmission at the cholinergic synapses, and butyrylcholinesterase (EC 3.1.1.8), for which the physiological role is not well known. Two substrate binding sites are present on these enzymes: the catalytic site, located at the bottom of a gorge (the so-called “catalytic gorge”; Ref. 1), and a peripheral site that can bind various ligands bearing a quaternary ammonium (2, 3). Hydrolysis of the substrate occurs in three steps (1, 4–6): (i) formation of an enzyme-substrate complex, (ii) acylation of the enzyme, and (iii) deacylation by hydrolysis. To analyze the first two steps, we can use a hemisubstrate: an organophosphate. Phosphorylation of the active serine by this compound can be considered as irreversible since dephosphorylation is a very slow step, the half-life for hydrolysis of the phosphoryl enzyme being measured in days (7).

In insects, only one form of the enzyme, called acetylcholinesterase, is present in the central nervous system. While in vertebrates, AChE is inhibited by excess substrate and butyrylcholinesterase is activated by low substrate concentrations (8), insect AChE combines both types of kinetic behavior, suggesting that the two phenomena are independent and result from different bindings, and not from different manifestations, acceleration or inhibition of catalysis, of the same binding (9). In a recent paper (10), we proposed a kinetic scheme to describe this complex kinetic behavior. The scheme suggests the existence of a high affinity non-productive site for the substrate. The binding of a substrate molecule to this site has the consequence of slowing down the positioning of a second substrate molecule at the active site, resulting in an apparent activation of the enzyme.

In order to precisely analyze the activation phenomenon observed, we searched for a reversible inhibitor that binds only to the activation site. Among several AChE inhibitors tested: choline, tetramethylammonium, edrophonium (11), propidium, and Triton X-100, only the latter affected activation by the substrate. Triton X-100 is a non-ionic detergent that is widely used to solubilize membrane-bound proteins, but it has also been described to inhibit cholinesterase activity (12–14). We used this molecule in inhibition experiments and found that it is in competition with the substrate for binding at the activation site.

Comparing the phosphorylation rate constants of several mutated enzymes, we saw that Triton X-100 binds at the rim of the gorge and, as a consequence, inhibits the enzyme activity by hindering access to the entrance of the active site, thus decreasing the acylation step.

EXPERIMENTAL PROCEDURES

Chemicals and Source of Enzyme—Triton X-100, polyethylene glycol, reduced Triton X-100, edrophonium, and propidium were from Sigma. As Triton X-100 is a mixture of two chain lengths (n = 8 and 10), its concentration will be expressed in g/liter.

Truncated cDNA encoding soluble AChE of Drosophila melanogaster was expressed with the baculovirus system (15). Secreted AChE were purified and stabilized with 1 mg/ml bovine serum albumin according to Estrada-Mondaca and Fournier (16). The concentration of the enzymes was determined by active site titration using 7-(methylthio)phosphinyl)oxy)-1-methylquinolinium iodide, which was synthesized as described by Levy and Ashani (17). Residue numbering follows that of the precursor (18); correspondence with the Torpedo AChE sequence was done according to Stojan (19) and is given in parentheses in Table I.

Kinetics of Substrate Hydrolysis—Kinetics were followed at 25 °C in 25 mM phosphate buffer, pH 7. Hydrolysis of acetylthiocholine iodide was studied spectrophotometrically at 412 nm using the method of Ellman et al. (20), at substrate concentrations from 2 μM to 200 μM in 1-cm path length cuvettes using a Sadas DES spectrophotometer. Activity was measured for 1 min, from 10 to 70 s after addition of the enzyme to the mixture. kobs is the number of micromoles of product

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from 0 to 10 gliter\(^{-1}\) of Triton X-100 to Equation 2, corresponding to Scheme 2.

The rate constant of enzyme phosphorylation or carbamoylation was determined by the dilution method of Aldridge (21); at time 0, the organophosphorus compound or the carbamoyl was added to enzyme solutions (~10 nm) in 25 mm phosphate buffer, pH 7, with 1 mgml\(^{-1}\) bovine serum albumin. Inhibition of the enzyme with time was followed by measuring the free enzyme concentration after dilution in the presence of substrate. The inhibition followed pseudo-first order kinetics since the concentration of the irreversible inhibitor was always at least 10-fold higher than the enzyme concentration.

\[ k_i = -\ln \left( \frac{[E]}{[E]_0} \right) = \ln \left( \frac{[PX_i]}{[PX]} \right) \]

\([E]\) and \([E]_0\) are the enzyme concentrations at times \(t\) and 0, respectively, and \([PX_i]\), the organophosphate (or carbamate) concentration. The experimental phosphorylation (or carbamoylation) rate constants (\(k_{i\text{(obs)}}\)) were estimated in the presence of different concentrations of Triton X-100, reduced Triton X-100, or polyethylene glycol (0–10 gliter\(^{-1}\)).

**Molecular Modeling**—Building and optimization of three-dimensional models of Triton X-100/Drosophila AChE were performed on a Silicon Graphics IRIS workstation using Biosym modeling software with a Drosophila AChE scheme built by homology with the Torpedo enzyme (19) with five layers of surrounding water molecules. Docking was performed using a distance constraint between the iso-octyl part of Triton X-100 and the amino acid at position 107. Molecular dynamics calculations were carried out as follows; the structure was equilibrated at 300 K for 20 ps, followed by subsequent 10-ps dynamic runs at 300 K. Second, after minimization, the same sequence was applied without any constraints on the whole system.

**RESULTS**

**Inhibition of Substrate Hydrolysis by Triton X-100**—Acetylthiocholine hydrolysis by Drosophila AChE was studied in the presence of different concentrations of Triton X-100 (0–10 gliter\(^{-1}\)). As opposed to electric eel AChE (12), the activity of the enzyme in the presence of Triton X-100 was found to be linear with time. Thus, Triton X-100 was considered to be a reversible inhibitor. Several kinetic models can be used to describe substrate hydrolysis by the Drosophila enzyme (9). The one proposed by Cauet et al. (23) and reduced to Scheme 1 describes activation and inhibition by substrate and leads to the determination of kinetic parameters (10).

\[ E + S \rightarrow EA \rightarrow E + P \]

\[ SE + S \rightarrow SEA \rightarrow SE + P \]

**SCHEME 1**

\[ E = \text{the free enzyme, } KA \text{ the acylated enzyme, and } SE \text{ represents the binding of a substrate molecule onto the activation site. } k_{a} \text{ represents the bimolecular rate constant for acylation and } k_{cat} \text{ the rate constant for deacylation. } a \text{ and } b \text{ coefficients represent the effect of the activation site occupation by a substrate molecule on deacylation and acylation respectively. } K_s \text{ and } K_r \text{ represent the affinity of the activation site for a substrate molecule. This scheme was chosen because it can be further reduced when the deacylation step is ignored, i.e. when organophosphorous compounds are used as substrates instead of acetylcholine (see below).} \]

Inhibition by Triton X-100 (Fig. 1) was analyzed assuming that two molecules of Triton X-100 can bind at two sites, a catalytic site and a peripheral site, whether the enzyme is free or acetylated, as tetramethylammonium does (11). We derived a complete scheme, and the resulting kinetic equation was used to simultaneously analyze 11-pS curves obtained with Triton X-100 concentrations varying from 1 mgml\(^{-1}\) to 100 gliter\(^{-1}\). In a second step, we eliminated inoperative constants and the scheme became reduced to Scheme 2 without any decrease of goodness of fit.
In this scheme, the ternary complex (two inhibitor molecules bound to the enzyme) is not taken into account. Triton X-100 binds competitively with the substrate at the non-productive, or activation, site. TE represents the binding of a Triton X-100 molecule onto the activation site; $c$ and $d$ represent the effects of activation site occupation by Triton X-100 on acylation and deacetylation, respectively.

$$k_{obs} = \frac{k_{cat}[S]}{K_S} \left(1 + \frac{[S]}{K_S} + \frac{[T]}{K_T}ight) \left(1 + \frac{[S]}{K_S} + \frac{[T]}{K_T}ight)$$

( Eq. 2 )

Non-linear fit allowed the estimation of some constants: affinity of Triton X-100 for the free enzyme, $K_t = 0.033 \pm 0.003$ g liter$^{-1}$; effect of Triton X-100 binding on acylation, $c = 0.136 \pm 0.04$. However, the affinity of Triton X-100 for acetylated enzyme, $K'_t$, was above 10 g liter$^{-1}$ and therefore could not be estimated and the effect of Triton X-100 on deacetylation, $d$, correlated with $K'_t$, could not be determined either.

Thus, Triton X-100 is a competitive inhibitor of activation by the substrate. According to Scheme 2, Triton X-100 decreases acetylation of the enzyme to 13.6%. To test this scheme, we used hemisubstrates, substrates for which the second step of the reaction (dealkylation or dephosphorylation) does not take place during the time of the experiment.

**Effect of Triton X-100 on Phosphorylation by Malaoxon—** We observed an inhibition of phosphorylation by malaoxon with increasing concentrations of Triton X-100, up to a plateau (Fig. 2A). Initially, we thought that malaoxon was trapped in the hydrophobic core of Triton X-100 micelles and, consequently, the concentration of the organophosphate available for the enzyme would be overevaluated. However, two observations led us to reject this hypothesis. First, phosphorylation was already diminished at a concentration below the CMC, 0.28 g liter$^{-1}$ in the experimental conditions used. Second, no effect of Triton X-100 on phosphorylation was detected with the *Torpedo* AChE (Fig. 2B). Thus, both results suggest that inhibition of the phosphorylation constant by Triton X-100 originated from an interaction of Triton X-100 with the *Drosophila* AChE and not from an artifact. Phosphorylation can be analyzed with Scheme 3, which is identical to Scheme 2 when deacetylation of the enzyme is negligible.

As the dilution method was used to phosphorylate the active site, it was not possible to test a large range of organophosphate concentrations. Indeed, above 3 $\mu$M, inhibition was too fast and, below 0.1 $\mu$M, the kinetics were no longer pseudo first-order since the free inhibitor concentration cannot be considered as constant. As a consequence, it was not possible to estimate the affinity of malaoxon for the non-productive site ($K_{m'}$) or the effect of occupation of the non-productive site on phosphorylation ($b$). Then, Scheme 3 was reduced to Scheme 4, where $k_i$ corresponds to the overall inhibition constant.

$$TE + PX \xrightarrow{ch_i} TEP$$

$||K_t$

$T$ $\xrightarrow{k_i}$

$E + PX \xrightarrow{c[T]} Ep$

$+$ $PX$

$||K_{m'}$ $bk_i$

$PXE + PX \xrightarrow{c[T]} PXEp$

**Scheme 3**

The phosphorylation rates observed ($k_{obs}$) were determined at different concentrations of Triton X-100 (0–10 g liter$^{-1}$) allowing the estimation of Triton X-100 affinity ($K_t$) and the effect of Triton X-100 on phosphorylation ($c$): $K_t = 0.06 \pm 0.02$ g liter$^{-1}$ and $c = 0.18 \pm 0.04$. These estimations are in agreement with $c$ and $K_t$ values obtained by measuring acetylthiocholine hydrolysis inhibition according to Scheme 3.

Triton X-100 inhibits phosphorylation of the active site by malaoxon, suggesting that activation of substrate hydrolysis results from inhibition of active site acetylation. Thus, Triton X-100 binds at the activation site.

**Effect of Triton X-100 on Phosphorylation or Carbamylation by Various Molecules—** Scheme 4 was used to study the effect of Triton X-100 on other hemisubstrates. The results are presented in Table I. It appears that Triton X-100 affects phosphorylation or carbamylation of the serine; for some compounds such as paraoxon, it decreases the phosphorylation rate but for other compounds, such as aldicarbe or metamidophos, Triton X-100 significantly increases the phosphorylation or...
carbamoylation rate. Thus, binding of a substrate molecule at the activation site may either increase or decrease the acylation of the enzyme.

**Competition of Triton X-100 Inhibition by Inhibitors Specific for Anionic and Peripheral Sites**—Inhibition of phosphorylation by Triton X-100 was studied in the presence of two inhibitors specific for anionic and peripheral sites, edrophonium or propidium. The data were analyzed with Scheme 5, which is an extension of Scheme 4.

![Scheme 5](image)

\[
k_{da} = \frac{k_0}{1 + \frac{a_1[T]}{K_a} + \frac{a_2[I]}{K_i} + \frac{b[I][T]}{K_{ai}K_{iT}}} \quad \text{(Eq. 4)}
\]

\(I\) represents edrophonium or propidium, and \(T\) indicates the Triton X-100. Both inhibitors decreased the phosphorylation rate; results of the fit are presented in Fig. 3. With propidium, including the ternary complex (ITE) did not improve the fit, suggesting that the two inhibitors, Triton X-100 and propidium, are competitive, i.e. that they cannot bind simultaneously to the enzyme; their respective binding sites are overlapping. By contrast, simultaneous binding of edrophonium and Triton X-100 to the enzyme was revealed because \(K_a'\) was operative for the fit, suggesting that the two binding sites are different. Taking in account that propidium binds at the rim of the active site gorge while edrophonium binds at the bottom of the gorge, this suggests that Triton X-100 binds at the rim of the gorge.

**Triton X-100 Inhibition of Mutated Enzymes**—In order to obtain information on the Triton X-100 binding site, we analyzed the phosphorylation of several mutated enzymes in the presence of Triton X-100 (Fig. 4). \(K_a'\), \(K_i'\), and \(c\) were determined (Table II). The mutations E107Y and E107W strongly increased the affinity of Triton X-100; mutants W359L and D413G moderately increased the affinity while mutants Y412A, E107K, and Y109K slightly decreased the affinity of Triton X-100. On the other hand, some mutations affected the \(c\) factor. When classified following their effects, mutations E107W, E107Y, and D413G increased inhibition by Triton X-100 while mutations W359L, Y362A, Y111Q, and Y412A slightly decreased the effect of Triton X-100.

**Effect of Triton X-100 Analogues**—Triton X-100 includes a hydrophobic part, the aromatic ring, coupled with a hydrocarbon chain and a hydrophilic part, the polyethylene glycol chain. To evaluate the involvement of each part of the molecule in its recognition by the activation site, we studied the inhibition of the wild type enzyme by malaoxon in the presence of polyethylene glycol or reduced Triton X-100. It was ground that reduced Triton X-100 presents the same affinity as Triton X-100, while the affinity of polyethylene glycol was much lower (\(K_{PEG} = 2.64 \pm 1.76 \text{ g liter}^{-1}\)).

**DISCUSSION**

**Inhibition by Triton X-100**—Triton X-100 is a detergent widely used to solubilize membrane-anchored proteins such as insect cholinesterase, which is linked to the membrane via a glycolipid (24, 25). Devonshire (26) noted that Triton X-100 increased the apparent \(K_m\) for the substrate, but activation was not evidenced because the presence of the glycolipid anchor did not allow complete elimination of the detergent from the solution. In *vitro* expression of a soluble cholinesterase, devoid of any lipid anchor, allowed kinetic studies to be performed in the absence of detergent, activation to be demonstrated at low substrate concentration (9), and the effect of Triton X-100 to be studied. It appears that this molecule is a competitive inhibitor of activation with a dissociation constant of 0.033 g liter\(^{-1}\).

Since kinetic models resulting from analysis of pS curves predicted that apparent activation may result from an increase or a decrease of the acylation rate constant depending on the scheme chosen (9, 10), we looked for a hemisubstrate, for which the second step, deacylation, is negligible. As phosphorylation by organophosphate is considered to be equivalent to acylation by acetylcholine, organophosphates and carbamates were used to evaluate the effect of Triton X-100 on the “acylation” step. As previously observed by Devonshire (26), we found a decrease of the phosphorylation rate constant with malaoxon in the presence of Triton X-100; however, using other hemisubstrates, we found that Triton X-100 can either increase or decrease the acylation rate.

**Localization of the Triton X-100 Binding Site**—Triton X-100 increases or only partially inhibits phosphorylation and thus does not compete with irreversible inhibitors at the productive site. This is the first evidence that Triton X-100 most probably binds to a peripheral site. As a reference, edrophonium, which binds at the productive site, totally suppresses phosphorylation by malaoxon (\(a_1\) is not significantly different from zero). However, this result does not give information about the location of this peripheral site. Binding of Triton X-100 and propidium were competitive, suggesting that the ternary complex, Triton X-100-propidium-enzyme, does not exist. So the binding sites for Triton X-100 and propidium are identical or overlapping.

**In vitro mutagenesis** was used to locate the Triton X-100 binding site independently. Triton X-100 inhibition of phosphorylation by malaoxon was observed with the *Drosophila* enzyme, but Triton X-100 did not significantly affect phosphorylation of *Torpedo* enzyme. We first hypothesized that phosphorylation enzyme inhibition came from one of the residues that differs between the two enzymes. Several mutations were chosen on residues lining the active site gorge in order to mimic the *Torpedo* enzyme inhibition: E107Y (Y70); R108V (V71); Y109D (D72); Y111Q (Q74); V356D (D276); L366F (F288); Y408F (F330); V356D (D276) and D413G (G335). However, these single mutations did not abolish the effect of Triton X-100, suggesting that either another residue is effective for Triton X-100 inhibition or that the differential behavior of the two enzymes originates from a combination of several mutations. Mutations inside the gorge (G303A, E275G, W309G, W121A, and F368L)
did not significantly change the affinity of Triton X-100 ($K_t$) compared with the wild type, confirming that Triton X-100 does not enter the active site gorge (Table I). Among the mutations located at the rim of the gorge, E107Y and E107W increased the affinity for Triton X-100 10-fold. This suggests that Triton X-100 binds at the entrance of the gorge and that residue Glu-107 forms a major part of this site.

Glu-107 has been shown to belong to the peripheral anionic site but is not conserved in the ChE family; at this position, we can find a methionine in Bungarus fasciatus AChE, a tyrosine in Torpedo and human AChEs, or an asparagine in human butyrylcholinesterase. In vitro mutagenesis of AChE from vertebrates has shown that this residue affects the binding of peripheral site ligands propidium, gallanine, and tubocurarine, as well as the binding of bis-quaternary ligands such as decamethonium and BW284C51, which bind at both the active and peripheral sites (6, 27–29).

Some mutations located in the proximity of Glu-107 (W359L, D413G, and R108V; see Fig. 4) also affected the affinity for Triton X-100, but to a lesser extent (Table I). This suggests that other residues in the vicinity of Glu-107 participate in Triton X-100 binding. Among them are Trp-359, Asp-413, and Arg-108. The first, Trp-359, has been shown to be one of the most important residues for the binding of several ligands such as propidium in vertebrate AChE (27, 30, 31). Similarly, this residue is important for propidium binding on Drosophila AChE; the dissociation constant of propidium (8 nM for the wild type AChE) increases to 12 μM for the W359L mutant. As mutation W359L only slightly affects the binding of Triton X-100, as binding of Triton X-100 and propidium are competitive, and as the two residues, Glu-107 and Trp-359, are close, we can conclude that the two peripheral sites partially overlap.

### Table II

| Enzymes       | $k_i$ | $K_i$ | $c$  |
|---------------|------|------|-----|
| Wild type     | 37 ± 1 | 0.060 ± 0.015 | 0.180 ± 0.040 |
| E107Y(Y70)    | 330 ± 4.1 | 0.005 ± 0.0004 | 0.013 ± 0.008 |
| E107W(Y70)    | 179 ± 9  | 0.008 ± 0.0020 | 0.011 ± 0.004 |
| E107K(Y70)    | 8 ± 6   | 0.140 ± 0.0800 | 0.250 ± 0.090 |
| R108V(V71)    | 54 ± 4  | 0.012 ± 0.0030 | 0.054 ± 0.025 |
| Y109D(D72)    | 41 ± 6  | 0.059 ± 0.0120 | 0.210 ± 0.030 |
| Y109K(D72)    | 17 ± 6  | 0.140 ± 0.0800 | 0.250 ± 0.090 |
| Y111Q(Q74)    | 71 ± 2.2 | 0.075 ± 0.0300 | 0.550 ± 0.040 |
| F11SS(F78)    | 9 ± 1   | 0.040 ± 0.0300 | 0.320 ± 0.090 |
| W121A/W84     | 0.9 ± 0.03 | 0.054 ± 0.0110 | 0.150 ± 0.040 |
| E275G/E199    | 1.1 ± 0.03 | 0.080 ± 0.0400 | 0.340 ± 0.050 |
| G303A(G227)   | 12.3 ± 0.8 | 0.040 ± 0.0200 | 0.260 ± 0.060 |
| W309G(W233)   | 2.3 ± 0.04 | 0.000 ± 0.0070 | 0.110 ± 0.020 |
| V356DD(D276)  | 56 ± 5  | 0.020 ± 0.0200 | 0.130 ± 0.080 |
| W359L(W279)   | 60 ± 2  | 0.015 ± 0.0080 | 0.650 ± 0.030 |
| Y362A(F284)   | 63 ± 2  | 0.090 ± 0.0400 | 0.600 ± 0.040 |
| L366FF(W288)  | 37 ± 0.6 | 0.068 ± 0.0110 | 0.150 ± 0.020 |
| F368LI(F290)  | 58 ± 4  | 0.076 ± 0.0460 | 0.320 ± 0.080 |
| Y408F(F331)   | 166 ± 4 | 0.190 ± 0.0600 | 0.480 ± 0.040 |
| D413G(G335)   | 77.8 ± 3.1 | 0.019 ± 0.0031 | 0.059 ± 0.021 |

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Interaction of Triton X-100 with the Activation Binding Site—Reduced Triton X-100 had the same affinity as Triton X-100 for the activation site; π interactions between the aromatic moiety of Triton X-100 and an aromatic residue of the active site can thus be ruled out. Mutagenesis of residue Glu-107 suggests that the interaction is mainly hydrophobic according to the hydrophobicity indices of Hopp and Woods (32). Indeed, the replacement of glutamate by a more hydrophobic residue (tyrosine, tryptophan, or leucine) increases the Triton X-100 affinity for the protein, whereas a residue that presents approximately the same hydrophobicity as glutamate, a lysine, does not modify the affinity. This result is tentatively illustrated in Fig. 5. Triton X-100 has been made to dock on E107Y mutant because it displayed the highest affinity for Triton X-100 (Table I), and we mimicked the hydrophobic interaction by imposing a distance constraint between the iso-octyl moiety and the aromatic ring for the first minimization; this constraint was removed for the following steps.

Fig. 5. Proposed binding orientation of Triton X-100 at the rim of the active site gorge, in hydrophobic interaction with tyrosine 107; water molecules are not shown. A, side view; B, upside view.

Effect of Triton X-100 Binding at the Activation Site—Triton X-100 binding at the rim of the active site affects the acylation rate constant. Three, not mutually exclusive, interpretations can be proposed.

First, Triton X-100 binding at the rim of the gorge modifies the active site conformation as do peripheral site ligands. Bermann et al. (34) showed conformational changes of AChE following binding of peripheral site ligands by methylphosphonofluoridate. Studies with mutated proteins confirmed this hypothesis since binding of ligands to the peripheral site, affects the Trp-84 orientation (6, 35). The hypothesis of a conformation change induced by Triton X-100, affecting acylation of the substrate, cannot be rejected or confirmed with the data presented here.

In the second hypothesis, Triton X-100 affects enzyme phosphorylation by steric hindrance at the entrance of the active site gorge. This hypothesis appears to have the greatest weight considering the position of the Triton X-100 binding site at the rim of the gorge, and the bulkiness of the Triton X-100 molecule. This explanation has been proposed by Szegletes et al. (37) for binding of another inhibitor to the peripheral site. Analysis of the effects of mutations on the $c$ value, which expresses the Triton X-100 effect, corroborates this steric effect hypothesis. Indeed, the E107W mutation narrows the active site gorge and thus increases the effect of Triton X-100 on phosphorylation by malaoxon ($c = 0.01$). On the other hand, W359L, Y362A, Y111Q, and Y412A enlarge the active site gorge and thus decrease the effect of Triton X-100 on the phosphorylation rate ($c > 0.4$).

A third interpretation involves the role of the peripheral site for substrate hydrolysis, taking into account that Triton X-100 and substrate binding at the peripheral site are competitive. Szegletes et al. (37) assumed that the primary role of the AChE peripheral site is to accelerate the hydrolysis of acetylcholine at low substrate concentrations, so accordingly we can hypothesize that the function of the activation binding site would be to attract the substrate molecule in solution, which would be suitably positioned to reach the catalytic site at the bottom of the gorge. The peripheral site helps direct the substrate to the active site. Inhibition of Triton X-100 at the rim of the gorge would compete with the initial binding of substrate and result in inhibition at low substrate concentration (see Fig. 1). Anal-
ysis of the effect of mutations at position 107 on malaoxon phosphorylation is in agreement with this interpretation. Actually, with the four amino acids modified at this position, a correlation exists between Triton X-100 affinity and phosphorylation (Fig. 6). As Triton X-100 affinity represents substrate affinity at the non-productive site and as phosphorylation represents acylation of the substrate, the correlation between substrate affinity and acylation suggests that an increase of affinity for the substrate at the rim of the gorge increases acylation.

REFERENCES
1. Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., and Silman, I. (1991) Science 253, 872–878
2. Changeux, J. P. (1966) Mol. Pharmacol. 2, 369–392
3. Roufogalis, B. D., and Quist, E. E. (1972) Mol. Pharmacol. 8, 41–49
4. Wilson, I. B., and Cabib, E. (1956) J. Am. Chem. Soc. 78, 202–207
5. Quinn, D. (1987) Chem. Rev. 87, 955–979
6. Barak, D., Kronman, C., Ordentlich, A., Ariel, N., Bromberg, A., Marcus, D., Lazar, A., Velan, B., and Shafferman, A. (1994) J. Biol. Chem. 269, 6296–6305
7. Rosenberry, T. L., and Bernhard, S. A. (1971) Biochemistry 10, 4114–4120
8. Nachmanson, D., and Wilson, I. B. (1951) Acta Enzymol. 12, 259–339
9. Marcel, V., Gagnon-Palacios, L., Pertuy, C., Masson, P., and Fournier, D. (1998) Biochem. J. 329, 329–334
10. Stojan, J., Marcel, V., Estrada-Mondaca, S., Klaebe, A., Masson, P., and Fournier, D. (1998) FEBS Lett. 440, 85–88
11. Stojan, J., Marcel, M., and Fournier, D. (1999) Chem. Biol. Interact. 119–120, 137–146
12. Millar, D. B., Christopher, J. P., and Bishop, W. H. (1979) Biophys. Chem. 10, 147–151
13. Kamaric, L. (1982) Period. Biol. 84, 143–146
14. Johnson, C. D., and Russell, R. L. (1983) J. Neurochem. 41, 30–46
15. Chaashi, H., Fournier, D., Fedon, Y., Bossy, J. P., Ravalier, M., Devauchelle, G., and Céritti, M. (1994) Biochem. Biophys. Res. Commun. 203, 734–742
16. Estrada-Mondaca, S., and Fournier, D. (1998) Protein Exp. Purif. 12, 166–172
17. Levy, D., and Ashani, Y. (1986) Biochem. Pharmacol. 35, 1079–1085
18. Hall, L. M. C., and Spierez (1986) EMBO J. 5, 2949–2954
19. Stojan, J. (1999) J. Enzyme Inhib. 14, 193–201
20. Ellman, G. L., Courtney, K. D., Andres, V., and Feathersone, R. M. (1961) Biochem. Pharmacol. 7, 88–95
21. Aldridge, W. N. (1950) Biochem. J. 46, 451–456
22. Ross, S., and Olivier, P. (1959) J. Phys. Chem. 63, 1671–1674
23. Cauet, G., Friboulet, A., and Thomas, D. (1987) Biochem. Cell Biol. 65, 529–535
24. Fournier, D., Bergé, J. B., Cardoso de Almeida, M. L., and Bordier, C. (1988) J. Neurochem. 50, 1158–1163
25. Haas, R., Marshall, T. C., and Rosenberry, T. L. (1988) Biochemistry 27, 6453–6457
26. Devonshire, A. (1975) Biochem. J. 149, 463–469
27. Radic, Z., Pickering, N. A., Vellom, D. C., Camp, S., and Taylor, P. (1993) Biochemistry 32, 12074–12084
28. Cousin, X., Ben, S., Duval, N., Massoulié, J., and Ben, C. (1996) J. Biol. Chem. 271, 15099–15108
29. Loewenstein-Lichtenstein, Y., Click, D., Gluzman, N., Sternfeld, M., Zakut, H., and Soreq, H. (1996) Mol. Pharmacol. 56, 1423–1431
30. Shafferman, A., Velan, B., Ordentlich, A., Kronman, C., Grosfol, H., Leitner, M., Flashner, Y., Cohen, S., Barak, D., and Ariel, N. (1992) EMBO J. 11, 3561–3568
31. Harel, M., Sussman, J. L., Krejci, E., Ben, S., Chanal, P., Massoulié, J., and Silman, I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10827–10831
32. Hopp, T. P., and Woods, K. R. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3824–3828
33. Fraenkel, Y., Gershoni, J. M., and Navon, G. (1994) Biochemistry 33, 644–650
34. Berman, H. A., Becktel, W., and Taylor, P. (1981) Biochemistry 20, 4803–4810
35. Ordentlich, A., Barak, D., Kronman, C., Ariel, N., Segall, Y., Velan, B., and Shafferman, A. (1995) J. Biol. Chem. 270, 2082–2091
36. Deleted in proof
37. Szegletes, T., Mallender, W. D., and Rosenberry, T. (1998) Biochemistry 37, 4206–4216
38. Szegletes, T., Mallender, W. D., Thomas, P. J., and Rosenberry, T. (1999) Biochemistry 38, 122–133
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