J. Enzyme Inhibition, 1999, Vol. 14, pp. 125–149
Reprints available directly from the publisher
Photocopying permitted by license only

STRUCTURE-FUNCTIONAL EFFECTS OF ETHANOL ON *DROSOPHILA MELANOGASTER* ACETYLCHOLINESTERASE PROBED BY KINETIC STUDIES WITH SUBSTRATE AND INHIBITORS

FLORENCE ORTÉGA*, DELPHINE GARCIA
and JEAN-LOUIS MARTY

*Centre de Phytopharmacie, CNRS UA 461, Université de Perpignan,
52 avenue de Villeneuve, 66860 Perpignan cedex, France*

(Received 30 April 1998; In final form 20 June 1998)

Ethanol is commonly used to extract and dissolve insecticides acting as inhibitors of acetylcholinesterase (EC 3.1.1.7). Here, experiments were undertaken to investigate the influence of solvent on the reaction and inhibition of the enzyme from *Drosophila melanogaster*. Ethanol (up to 20% by volume) is shown to induce a dramatic reduction of the affinity of acetylcholinesterase for the acetylthiocholine iodide substrate and all the edrophonium chloride, paraoxon ethyl and propidium diiodide inhibitors, with little influence on the rate constants. Taken together, these results point to a main perturbation of active-center related components involved in the formation and/or stability of Michaelis complexes. Inactivation and ligand-stabilization studies of acetylcholinesterase activity further indicate the occurrence of specific "conformational scrambling" at catalytic and regulatory sites. It is proposed that ethanol affects the enzyme reactivity by modifying the conformation of the aromatic gorge containing the active centre and hence, interactions involved in the molecular recognition of substrates and ligands.

Keywords: Acetylcholinesterase; Ethanol; Acetylthiocholine iodide; Paraoxon ethyl; Edrophonium chloride; Propidium diiodide; Michaelis complexes

* Corresponding author. 1 avenue Général Leclerc, B3D, 91190 Gif sur Yvette, France. Tel.: (33) 1 64 46 02 43. E-mail: rimai@easynet.fr.

INTRODUCTION

Acetylcholinesterase (AChE, acetylcholine hydrolase, EC 3.1.1.7) plays a key role at cholinergic synapses, where it rapidly terminates the influx transmission by catalyzing the hydrolysis of the neurotransmitter acetylcholine (ACh). AChE is a serine hydrolase that possesses a bipartite active center composed of the catalytic site and the anionic site, though misnamed, involved in binding of the choline moiety.[1-5] Solution of the three-dimensional structure of AChE revealed that its active site is buried 20 Å from the enzyme surface at the bottom of a deep and narrow cavity. This cavity was named 'the aromatic gorge', since its surface is lined predominantly by rings of 14 conserved aromatic residues.[1,6] Due to asymmetric distribution, AChE displays a large dipole moment aligned along the aromatic gorge, which would provide guidance of positively charged ion into the gorge towards the active site.[7] In addition, AChE has been found to be composed of a peripheral anionic site (PAS), which is located at the entrance of the active-site gorge, 9 Å remote from the active center. PAS has been implicated in the transfer of the substrate to the active site,[8,5] in ionic strength monitoring[9] and in substrate inhibition.[10] The AChE reaction involves three main forms with respect to the active centre: the free, the reversible Michaelian complex, and the acetylated enzyme whose deacylation regenerates the free enzyme. As a key enzyme in neuromuscular transmission, AChE has been a primary target for the blockage of synaptic events, which has led to the development of nerve agents and insecticides.

Two main classes of AChE inhibitors have been used as insecticides: carbamates, and organophosphates which are both competitive analogues of the substrate.[11,12] The insecticides react rapidly and irreversibly with AChE by phosphorylating or carbamoylating the active site Ser-203(276)[13] within the esteratic center. Phosphorylation or carbamoylation of the active-site serine blocks the hydrolysis of the neurotransmitter acetylcholine and hence, causes death. Intensive use of organophosphate and carbamate insecticides during the past thirty years have lead to many ecotoxicological problems, resulting in the exposure of wildlife to hazardous chemicals. Indeed, since cholinergic transmission is well conserved, these compounds are also potentially toxic for all animals. In response to the need to monitor the levels of these insecticides in waters and agricultural run-offs, an advantageous alternative for rapid, sensitive and specific detection of organophosphates and carbamates emerged based on the determination of these insecticides using free or immobilized AChE.[14-18] Yet, many organophosphates and carbamates used as anticholinesterases are insoluble in water

and polar solvents are usually required to dissolve them and to study their inhibition of AChE *in vitro*. The presence of solvents for the extraction and the concentration of the insecticides is likely to alter the enzyme properties and this remains a main drawback in such a biological approach.

In order to facilitate a better insight into the solvent-induced modifications of AChE together with the design of operating conditions for the increased efficiency of AChE, the purpose of this work was to determine the effect of a water-soluble solvent, ethanol, on *Drosophila* AChE. This solvent/enzyme system appeared the most suitable due to the fact that, (1) ethanol is commonly used to dissolve the highly hydrophobic organophosphates and carbamates and, (2) *Drosophila* AChE has been shown to provide a good model for studying adaptation of eucaryotes to agricultural and medical pests[19] in addition to showing the best sensitivity to the insecticides. The systematic evaluation of the effects of alcohol on AChE is not an easy task, due to the occurrence of enzyme instability, solvent-induced dielectric effects and nucleophilic competition of alcohol with water for enzyme deacylation during reaction.[20,21] So far, the investigation of ethanol action on cholinesterase reactivity has been restricted to either activity studies of AChE-low alcohol concentration systems i.e. $< 1\%$ by volume[22–24] or to alcoholysis competition studies.[25,26] Only a few attempts have been made to analyze the reaction of AChE with substrates or ligands upon exposure to appreciable amounts of ethanol. In previous studies, Ronzani[27,28] showed an apparent competitive effect between the substrate or the active-centre selective inhibitor, neostigmine and the water-soluble organic solvent for binding to the enzyme. However, a definite conclusion has not yet been drawn, leaving several questions unanswered. Is the competition observed between ethanol and substrate to be ascribed to the protic nature of the solvent *i.e.* nucleophilic competition or to the exclusion of substrates and ligands from active sites by the solvent molecule, or both? Does the alteration of the enzyme reaction also result from a solvent effect at sites distinct from the active-center such as PAS involved in the enzyme allosteric regulation? What is the relevance of specific ethanol antagonism of the AChE reactivity? Due to the complex nature of AChE catalysis, the change in its kinetic parameters upon exposure to water-miscible solvents allows no simple interpretation in terms of the structure of the enzyme-substrate complex. Still, the chemical diversity of ligands binding to AChE can be used advantageously to delineate distinct steps in the catalytic pathway and hence, the specificity of the component steps as dependent variables in the ethanol effect. Apart from the esteratic site, the various sites related to the AChE activity thus can be distinguished biochemically: edrophonium

chloride exclusively binds to the so-called anionic subsite in the active-centre of AChE, whereas propidium diiodide binds preferentially to PAS.[29] Both are aromatic cationic reversible inhibitors of the enzyme activity.

In an attempt to further assess the effect of ethanol on the functional characteristics of AChE, we investigated the enzyme reaction with acetylthiocholine iodide as substrate, its corresponding inhibition by the inhibitors, edrophonium chloride and propidium diiodide, and that by the organophosphate paraoxon ethyl. The specificity of the effect of ethanol on the functional stability of AChE is also discussed.

MATERIALS AND METHODS

Materials

AChE from *Drosophila melanogaster* was kindly provided by D. Fournier (Université Paul Sabatier-Toulouse, France). Acetylthiocholine iodide (ATC), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), propidium diiodide, and anhydrous dibasic potassium phosphate (ACS reagent) (KH_2PO_4) were all purchased from Sigma® Chemical Company. Edrophonium chloride was purchased from ICN. Paraoxon ethyl (*o*,*o*-diethyl *o*-4-nitrophenyl phosphate) was obtained from Cluzeau Info Labo, France. Disodium hydrogenophosphate (Rectapur™) (Na_2HPO_4) was from Prolabo (France). The analytical grade ethanol was kept dry over sodium.

Buffer

The buffer employed was 0.06 M Na_2HPO_4/KH_2PO_4 equilibrated at the appropriate pH. The presence of ethanol increased all pH values by around 0.01 per percentage increment. The pH of the starting buffer was accordingly decreased with 0.06 M KH_2PO_4 prior to addition of ethanol and controlled for its final value.

Determination of the Activity of AChE

AChE activity was evaluated at 30°C by monitoring the hydrolysis of ATC in the presence of 0.33 mM DTNB in 1 ml of sodium-phosphate buffer, pH 8.0, according to the Ellman procedure.[30] The ATC hydrolysis reaction was initiated by addition of the enzyme solution to the sample cuvette. The increase in the reaction product was recorded for 30 s at $\lambda = 412$ nm by

measuring the absorbance of 5-thio-2-nitrobenzoate formed in the reaction ($\varepsilon_{412\,nm} = 13{,}600\,M^{-1}\,cm^{-1}$), using a Hewlett-Packard 8452A spectrophotometer. The AChE concentration is expressed as the concentration of the active monomer (molecular weight 71,000 Da[31]). The enzyme control activity during assays was 0.1 μmol/min/ml at 30°C for hydrolysis of 0.7 mM ATC, using 3 nM AChE. The measured rate was found to be proportional to the enzyme concentration over a wide range of enzyme concentrations. Experiments were carried out in either duplicate or triplicate.

Inhibition of the Activity of AChE by Ethanol

The activity of AChE was assayed in the absence (control activity) or presence of various concentrations of ethanol (5–20%), as described above. Using a dilution method, we verified that the absorbance at $\lambda = 412$ nm of 5-thio-2-nitrobenzoate formed in the reaction was not modified by the presence of the alcohol under the conditions of assays. Unless stated otherwise, the range used for ATC concentration (0.015–0.8 mM) allowed data to be analyzed according to the reciprocal form of the simplified Michaelis–Menten velocity equation [Eq. (1)] for up to 20%[32] ethanol:

$$(1/v) = (K_m/V_{max}) \times (1/[S]) + 1/V_{max} \quad (1)$$

where K_m and V_{max} refer to the substrate S overall affinity constant and the apparent maximal enzymatic rate, respectively.

Reversible Inhibition of AChE Activity by Cationic Ligands

Reaction mixtures contained ATC (0.02–0.8 mM) and edrophonium chloride [0 (control) to 50 μM] in buffer solutions were prepared at pH 8.0 in the absence or presence of 5–17.5% ethanol. Similar experiments were performed with propidium diiodide [0 (control) to 50 μM] in the absence or presence of 15% ethanol. Activity was initiated by addition of the enzyme solution and the product formed was determined as outlined above. Equilibrium reversible inhibitions were analyzed according to the reciprocal form of the velocity equation for a linear mixed-type model [Eq. (2)] that degenerates to a competitive-type model [Eq. (3)] in the case of edrophonium chloride:

$$(1/v) = (K_m/V_{max})(1 + [I]/K_i) \times 1/[S] + (1/V_{max})(1 + [I]/\alpha K_i) \quad (2)$$

$$(1/v) = (K_m/V_{max})(1 + [I]/K_i) \times 1/[S] + 1/V_{max} \quad (3)$$

where K_i refers to the dissociation constant of the complex formed between inhibitor (I) and free enzyme (E). $\alpha(\geq 1)$ is the coefficient that affects the K_i value for the formation of an unfavored ESI ternary complex.

The K_i (propidium diiodide), in the absence and presence of ethanol, was determined from the replots of primary slopes obtained according to Eq. (2) *versus* the concentration of propidium diiodide. In order to determine the K_i values and to standardize the graphic representation, data with edrophonium chloride were further analyzed according to Eq. (4):

$$\text{slope}(1/v \textit{ versus } 1/[S])_{(I)}/\text{slope}(1/v \textit{ versus } 1/[S])_{(0)} = 1 + [I]/K_i \quad (4)$$

where the value of the primary slopes from Eq. (3) in the presence of edrophonium chloride (slope($1/v$ *versus* $1/[ATC]$)$_{(I)}$) were divided by that of the control (slope($1/v$ *versus* $1/[ATC]$)$_{(0)}$) and reported as a function of the inhibitor concentration at each ethanol content.

Irreversible Inhibition of AChE by Paraoxon Ethyl

Experimental protocol Into disposable glass test tubes was added 2 ml of an aqueous solution of ethanol at the desired concentration (none for regular irreversible inhibition or 5%–15% in the sodium-phosphate buffer adjusted to pH 8.0 as described above) and in the absence or presence of cationic ligands at the appropriate concentration (see figure legends). The mixture was preheated to 30°C. Irreversible inhibition of AChE by paraoxon ethyl (0.03–20 μM) was then started by the addition of the enzyme solution so as to reach an enzyme concentration of 25–50 nM during the subsequent period of incubation. This was taken as zero-time for the inhibition. Controls for correction of the solvent-induced inactivation of AChE contained no organophosphate. Each sample was then immersed into a thermostatically controlled bath maintained at 30°C and regular time intervals, homogeneous aliquots (100 μl) were withdrawn from the incubation mixture and the enzyme activity was recorded for 30 s in 1 ml of sodium-phosphate buffer, pH 8.0 at 30°C containing 0.7 mM ATC and 0.33 mM DTNB as described above. Similar experiments were carried out in the presence of 100 μM edrophonium chloride or propidium diiodide, where the zero-time inhibited activity was taken as the reference value for either inactivation or covalent inhibition. Experiments were performed in duplicate

using twelve data points for each condition. The inactivation rate of AChE by ethanol was found to be independent of protein concentration within a concentration range of 10–200 nM. HPLC analysis also indicated that the presence of ethanol in the assays does not modify the chemical structure of paraoxon ethyl under our conditions (unpublished).

Data analysis Data for ethanol inactivation of the enzyme were analyzed by a general two-exponential factor model where it was assumed that each exponential term represents an independent, potentially active form of the enzyme following first-order inactivation kinetics. The residual enzyme activity was expressed according to Eq. (5a):

$$E/E_0 = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \tag{5a}$$

where E_0 is the initial enzyme activity at time-zero, E is the enzyme activity at any time t in the inactivation process, and k_1 and k_2 are the inactivation rate constants of the native E_0 form. A_1 and A_2 represent the corresponding labile and stable fractions of the initial enzyme activity. A non-linear regression program (DeltaGraph® Pro3) was used to estimate the kinetic parameters.

Except for the experiment at 15% ethanol in the absence of cationic ligands, all covalent-inhibition kinetics followed a pseudo-first order model i.e. $E/E_0 = e^{-k_{obs}t}$, with k_{obs} corresponding to the observed overall inactivation rate constant for the native enzyme (E_0). The corresponding k_{obs} at 15% ethanol without reversible ligands was taken as the k_{obs_1} inactivation rate constant for the labile fraction in Eq. (5b) equivalent to Eq. (5a):

$$E/E_0 = A_1 e^{-k_{obs_1} t} + A_2 e^{-k_{obs_2} t} \tag{5b}$$

All data were then analyzed according to the generally accepted mechanism of inhibition of AChE by organophosphate insecticides, as described by Aldridge,[33] taking the corresponding control rate (k_1) into account to determine the k_i bimolecular rate constant for paraoxon ethyl [Eq. (6)]:

$$1/(k_{obs} - k_1) = [\text{Paraoxon ethyl}]/k_i + 1/k_2 \tag{6}$$

All the plots fitted by Eq. (6) satisfied a linear relationship ($0.973 < R^2 < 0.996$), suggesting that the restricted Aldridge model *i.e.* the covalent inhibition of AChE by paraoxon ethyl is quasi-irreversible, holds true under all experimental conditions.

RESULTS

Effect of Ethanol on the AChE Activity

The hydrolysis of ATC by AChE was determined at fixed concentrations of ethanol (from 0 up to 20%) as a function of ATC concentration ranging from 0.015 to 0.8 mM at pH 8.0 where the enzyme reaction can be described by the general Michaelis–Menten scheme. In agreement with previous observations,[27,28] substantial amounts of ethanol causes a dramatic decrease in the activity of AChE. As shown in Figure 1A, the double-reciprocal plots of the reaction rate *versus* ATC concentration were linear up to 20% ethanol. Analysis of the straight-line plots shows a common intercept on the $(1/v)$-axis, indicating that the apparent V_{max} is not affected by ethanol. The values of the apparent K_m (ATC) affinity constant were calculated from the slopes according to Eq. (1) (Table I). The AChE-catalysis of ATC hydrolysis is well-known to involve allosteric interactions between PAS and the enzyme active-center. One of the characteristics of allosteric modulation is substrate inhibition due to additional binding at PAS. Therefore, we also investigated the enzyme behavior over a wide range of substrate concentration (0.015–75 mM ATC), where ATC inhibition can be detected. Under

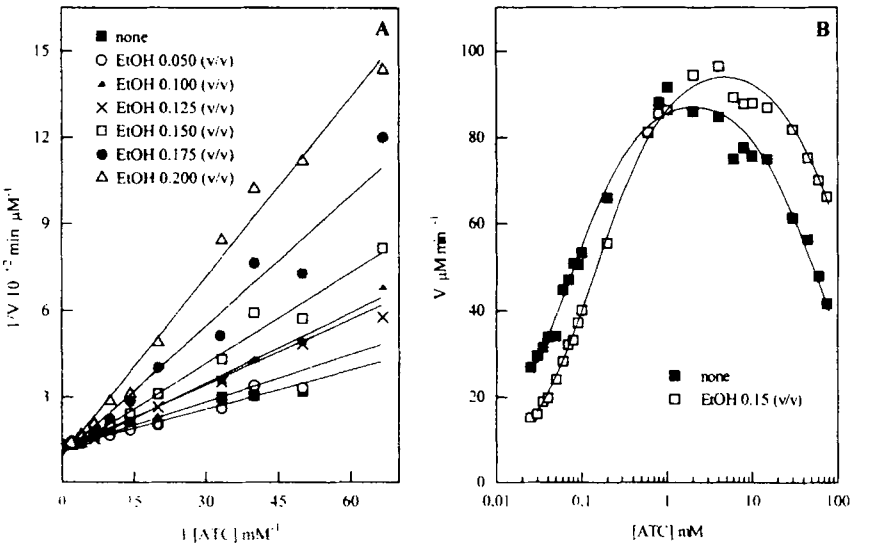

FIGURE 1 (A) Lineweaver–Burk plots of the AChE-catalyzed hydrolysis of ATC (0.015–0.8 mM) at various concentrations of ethanol, pH 8.0 and 30°C, [AChE] = 3 nM. (B) Concentration dependency of ATC hydrolysis by AChE over a wide range of substrate (0.015–75 mM) in the absence and presence of 15% (by volume) ethanol, [AChE] = 3 nM.

TABLE I Calculated affinity constants of *Drosophila* AChE for hydrolysis of ATC and binding of edrophonium chloride in the presence of various concentrations of ethanol. The corresponding K_i (propidium diiodide) values were 0.05 and 5.9 μM in the absence and presence of 15% ethanol, respectively

EtOH%	K_m (ATC) (μM)	K_i (edrophonium chloride) (μM)
0.0	40.7	2.7
5.0	41.4	5.7
10.0	53.3	5.6
12.5	60.8	10.5
15.0	111.8	19.5
17.5	121.1	29.3
20.0	170.7	

these conditions, *Drosophila* AChE clearly displays a non-Michaelian component (Figure 1B), in agreement with previous findings. In the presence of 15% ethanol, the observed substrate inhibition at concentrations up to and above 1 mM ATC is reduced, indicating that the organic solvent additionally affects the binding of the substrate molecule to PAS on the enzyme. The fact that 15% ethanol also antagonizes the binding of the PAS-selective inhibitor, propidium diiodide to AChE, as seen by the decrease of the K_i (propidium diiodide) dissociation constant value (cf. Table I), confirms the occurrence of modulations at PAS due to solvent effect.

Effect of Ethanol on the Inhibition of the Activity of AChE by Edrophonium Chloride

If K_m values mostly reflect the association of the enzyme with ATC, the observed decrease in the overall affinity of AChE upon exposure to ethanol would similarly result in a decrease of the affinity for substrate analogues. In order to better establish a fundamental distinction between the binding of substrates and the kinetic properties of AChE, we investigated the effect of ethanol on the inhibition of the activity of AChE (measured in the Michaelis region) by fixed concentrations of edrophonium chloride. In pure water medium, edrophonium chloride exhibits a competitive pattern of inhibition (Figure 2A) in agreement with the facts that, (1) inhibition mainly corresponds to binding of the ligand to the free enzyme and, (2) inhibitor association with the acetylenzyme, if it were to occur, is kinetically silent. In the presence of up to 17.5% ethanol, data still fit well to a common intercept on the $(1/v)$-axis (Figure 2B) indicating that the basic enzyme kinetics of edrophonium chloride as a competitive inhibitor are not affected by the solvent within the range tested for the AChE reaction. The values of the K_i

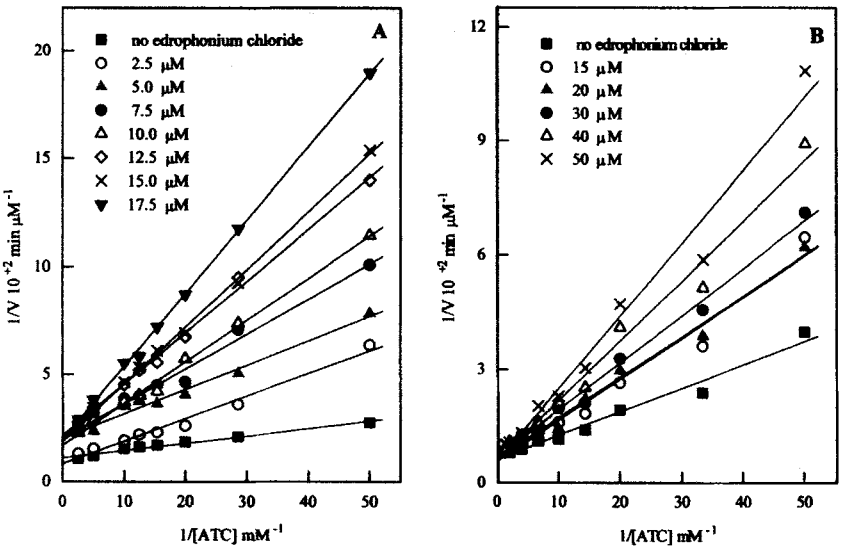

FIGURE 2 Lineweaver–Burk plots of the AChE-catalyzed hydrolysis of ATC (0.02–0.8 mM) at several fixed concentrations of edrophonium chloride, pH 8.0 and 30°C: (A) in pure water medium, [AChE] = 3 nM, (B) in the presence of 17.5% (by volume) ethanol, [AChE] = 7.5 nM.

(edrophonium chloride) dissociation constant as a dependent variable of solvent effect were determined from the replots of primary Lineweaver–Burk slopes *versus* the concentration of edrophonium chloride according to Eq. (4) which allows direct analysis of the net influence of ethanol on the association between inhibitor and free AChE (Figure 3). Table I shows the decrease in the inhibition efficiency of the competitive edrophonium chloride.

Effect of Ethanol on the Irreversible Inhibition of AChE by Paraoxon Ethyl

The organophosphate insecticides permanently block enzyme catalysis by phosphorylating the esteratic serine. Since this reaction does not require concomitant AChE-hydrolysis of ATC, the use of such anticholinesterase agents is convenient as a complementary approach to further explore the influence of ethanol at the AChE active-centre. Paraoxon ethyl represents well this family of compounds and has therefore been chosen to investigate the effect of ethanol on the covalent inhibition of AChE. In all cases, AChE was incubated in the absence of ATC. The residual activity of the enzyme as

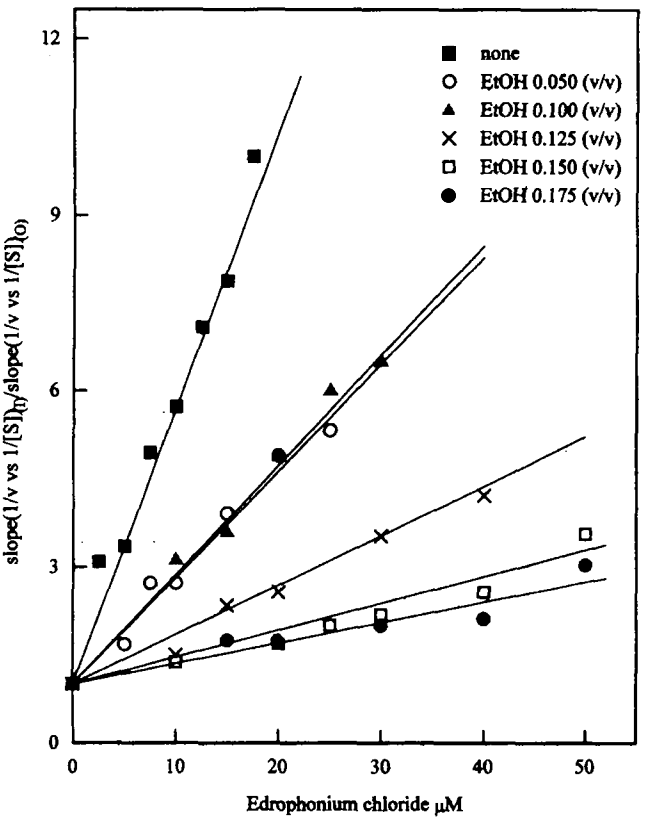

FIGURE 3 Secondary plots of slopes of reciprocal velocity as a function of 1/[ATC] at various concentrations of ethanol (e.g. none and 17.5% in Figure 2) *versus* edrophonium chloride concentration, pH 8.0 and 30°C, as defined by Eq. (4).

a function of the incubation time was then monitored following the addition of the substrate and the measurement of the initial hydrolytic rates.

Operational stability of AChE in the presence of ethanol Prior to inhibition studies, we examined the operational stability of AChE in the presence of ethanol. The enzyme displays fairly stable activity at pH 8.0 in the absence or the presence of ethanol concentrations up to 10%, as shown by the analysis of residual activity (Figure 4). However beyond this level, inactivation of AChE took place in a biphasic inactivation process followed by rapid (apparent single-step kinetic model) and dramatic inactivation at ethanol concentrations greater than 15% (Figure 4). The loss of the activity of AChE in response to the increase in ethanol content was analyzed according to Eq. (5a). The dramatic increase of the value of both the k_1 first-order

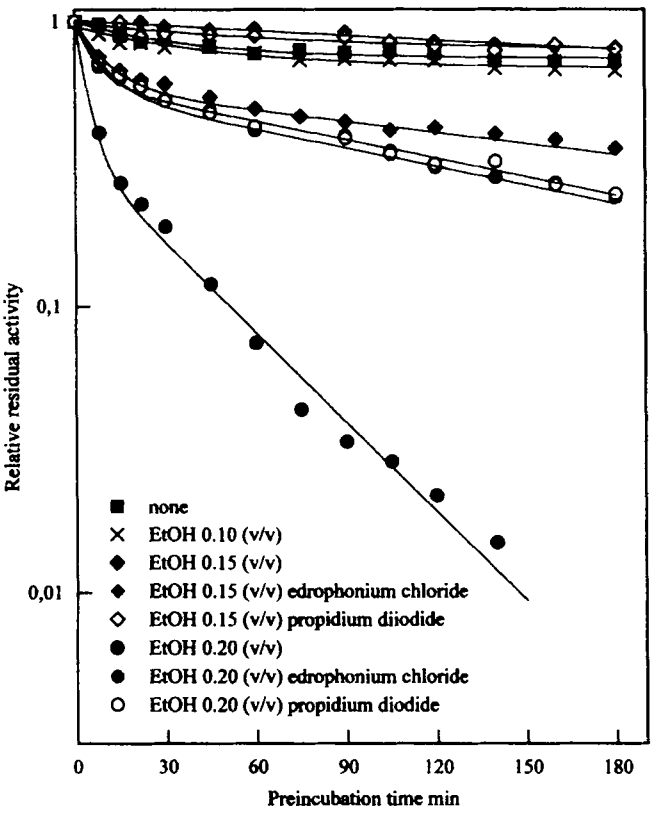

FIGURE 4 Kinetics of AChE inactivation *versus* ethanol concentration and ligand stabilization. AChE (25–50 nM) was incubated at pH 8.0 and at 30°C in the absence (control) or presence of 5–20% ethanol. Experiments were reproduced in the presence of 100 μM edrophonium chloride or propidium diiodide. Activities have been normalized as a fraction of the activity measured at time zero.

inactivation rate constant and the A_1 labile-activity fraction parameter well represent the extent of AChE inactivation at different fixed concentrations of ethanol (Table II).

Stabilization of AChE in the presence of ethanol Both active-site and peripheral-site cationic ligands which modulate catalytic activity have been previously reported to retard the inactivation rate of AChE with respect to thermal and urea-dependent inactivation.[34,35] We also found cationic ligands to slow down the ethanol-induced inactivation of AChE. In the presence of 15% ethanol, the stability of AChE was optimal using 100 μM edrophonium chloride or propidium diiodide (Figure 4). As a comparison, the addition of glycerol, polyethylene glycol or sodium chloride (up to

TABLE II Kinetic inactivation parameters for *Drosophila* AChE as a function of ethanol concentration. Values were determined according to Eq. (5a), where A_1 is the relative activity of the labile over the native form of AChE

EtOH%	$k_1 10^2$ (min^{-1})	A_1 (%)
0.0	2.7	26
5.0	1.6	37
10.0	3.0	30
15.0	9.5	41
17.5	14.9	42
20.0	22.9	66

molar concentrations) provided poor protection to the enzyme or none against the inactivation induced by ethanol (data not shown).

Cation antagonism of the paraoxon ethyl reaction in pure water system The irreversible inhibition of AChE by paraoxon ethyl was examined in the presence and the absence of 100 μM edrophonium chloride and propidium diiodide, in accordance with our previous experiments (see above). Different ranges of paraoxon ethyl concentrations were used to analyze the covalent inhibition of AChE under each condition. High concentrations of the organophosphate (0.15–15 μM) *i.e.* up to 50-times higher than in the absence of ligands (0.05–0.3 μM), were actually required in the presence of edrophonium chloride to reach significant inhibition of the enzyme activity, indicating that the active-center ligand strongly antagonizes the irreversible reaction. On the other hand, inhibition of AChE by paraoxon ethyl showed no dependence on the presence of propidium diiodide (cf. Figure 5). In all cases, inhibition displayed a pseudo-first order kinetic behaviour regardless of the concentration of paraoxon ethyl (data not shown). From the slopes of semilog plots of residual activity *versus* time, double-reciprocal plots of pseudo-first order rate constants (k_{obs}) against inhibitor concentration were drawn to obtain the bimolecular rate constants. The values of the k_i bimolecular inhibition constant are shown in Table III, where the active center-selective edrophonium chloride (100 μM) cation is seen to decrease the basic k_i value of $\sim 4 \times 10^6\,M^{-1}min^{-1}$ for the covalent reaction by $\sim$60-fold. These findings are compatible with previous observations.[9] Propidium diiodide thus appears as the best candidate both to stabilize the activity of AChE in the presence of non-negligible amounts of ethanol and to study the irreversible inhibition of the enzyme under such conditions.

Effect of ethanol on the paraoxon ethyl reaction Although the stability of AChE is affected by ethanol, it was still feasible to investigate the effect of the organic solvent on the kinetics of covalent inhibition by paraoxon ethyl.

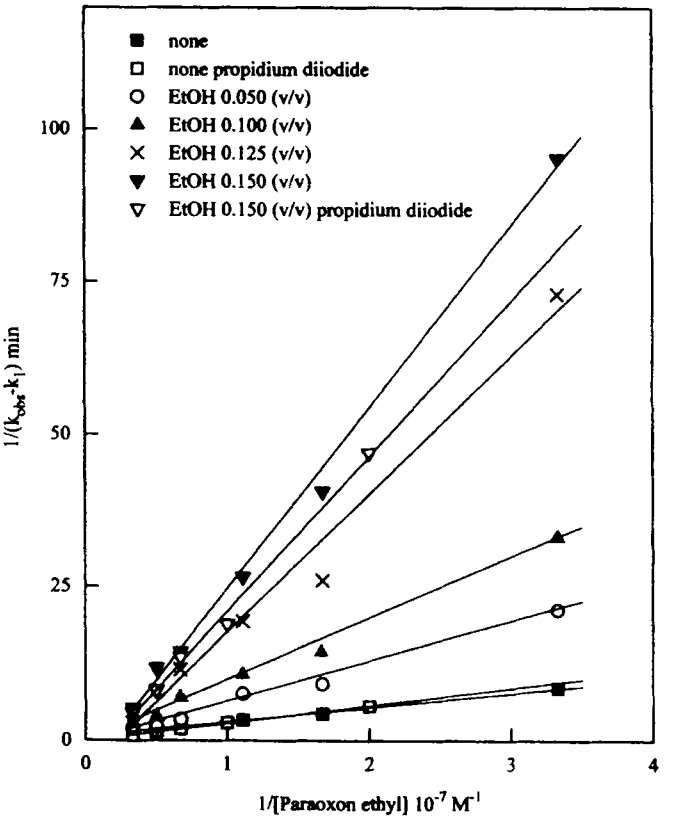

FIGURE 5 Reciprocal secondary plots of $k_{obs}-k_1$ values calculated from (residual activity *versus* time) plots at various ethanol concentrations, as a function of paraoxon ethyl concentration. k_{obs} and k_1 are the inactivation rate constants determined in the presence and the absence of paraoxon ethyl, respectively. Conditions for inhibition were pH 8.0 and 30°C, [AChE] = 25–50 nM.

Indeed, the values of the k_1 first-order rate inactivation constants are at least ten-times lower (cf. Table II) than that of k_{obs} overall inactivation rate constants usually observed in the presence of active concentrations of organophosphates. Furthermore, propidium diiodide can be used in an additional experiment to compare the values obtained for covalent inhibition when restoring the best functional stability of AChE. Based upon our previous results, the net inhibition reactions were determined by measuring the residual activity of AChE in the presence of up to 15% ethanol and by taking the corresponding control rate into account. All the observed covalent-inhibition kinetics followed a pseudo-first order model except for the experiment at the highest concentration of organic solvent where the k_{obs}

TABLE III k_i bimolecular velocity constant for phosphorylation of *Drosophila* AChE by paraoxon ethyl at different ethanol concentrations, in the absence and the presence of 100 μM cationic ligands. The values were determined from Figure 5 (data not shown for edrophonium chloride) according to Eq. (6)

EtOH%	$k_i 10^{-5}$ ($M^{-1} min^{-1}$)
0.0	43.1
5.0	14.6
10.0	10.0
12.5	4.4
15.0	3.4
+ propidium diiodide	
0.0	35.1
15.0	5.2
+ edrophonium chloride	
0.0	0.6

values were then determined from the corresponding initial rates of AChE inhibition [cf. Eq. (5b)]. The progressive phosphorylation of AChE was also investigated in the presence of propidium diiodide (100 μM) in the reaction medium containing 15% ethanol. In the presence of propidium diiodide kinetics again obeyed a pseudo-first order model. The k_{obs} pseudo-first order rate constants were plotted against the concentration values of paraoxon ethyl according to Eq. (6) (Figure 5) allowing the determination of the k_i bimolecular rate constants. Clearly, ethanol induces a major slope effect that appears to account for the decrease in the k_i values (Table III). No remarkable change is seen to occur at the $1/(k_{obs}-k_1)$ axis *i.e.* in the k_2 phosphorylation rate of the enzyme, suggesting that the increase in the k_i value mainly derives from an increase in the K_d constant value for association between AChE and paraoxon ethyl. Similar results were obtained in the presence of propidium diiodide and ethanol (cf. Figure 5 and Table III), which lends further support to our approach.

Quantitative Effects Induced by Ethanol on the Reactivity of AChE

In analyzing the effect of ethanol on the reactivity of AChE, it was noted that an exponential increase in all the K_m (ATC), K_i (edrophonium chloride) and k_i (paraoxon ethyl) values occurred with gradual increase of the organic solvent. Such a behavior is usually ascribed to general dielectric effects by the surrounding solvent. Therefore, the data of Table I for K_m (ATC) and K_i (edrophonium chloride) and that of Table III for k_i (paraoxon ethyl) were plotted as dependent variables in χ according to Eq. (7) (Figure 6), that

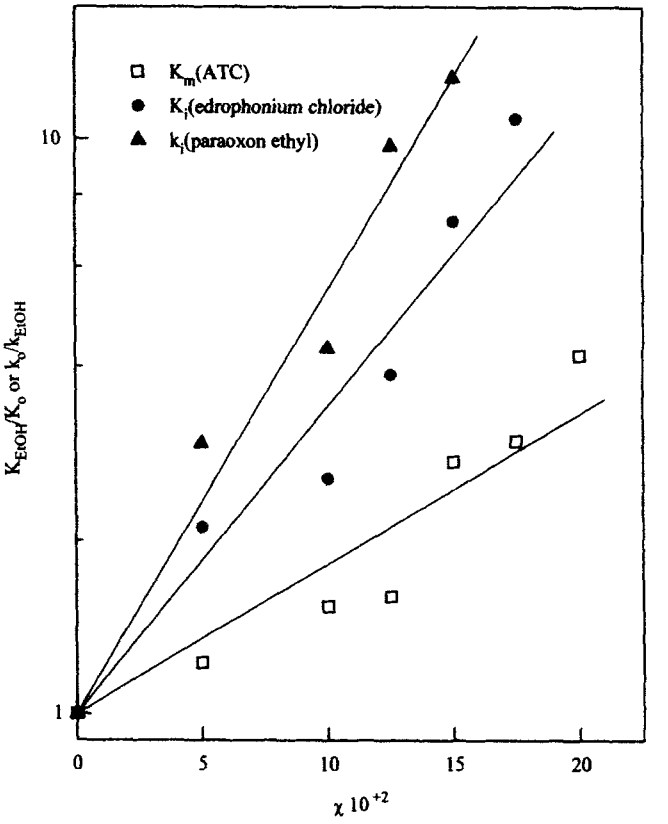

FIGURE 6 Exponential dependence of the affinity, dissociation, and bimolecular reaction constants of AChE with ATC, edrophonium chloride and paraoxon ethyl, respectively on χ [cf. Eq. (7)], where χ is related to the dielectric constant of the medium and hence, to the ethanol concentration. Experiments were performed at pH 8.0 and 30°C.

allows the influence of the dielectric effect to be approximated:[36]

$$K_{\text{ethanol}}/K_0 \text{ (or } k_0/k_{\text{ethanol}}) = e^{A\chi} \tag{7}$$

where the values of the K_0 and K_{ethanol} equilibrium constants and that of the k_0 and k_{ethanol} rate constants represent the absence and presence of ethanol, respectively, A is a constant which measures the electrostatic repulsion between AChE and ligands, χ is the difference between the reciprocal dielectric constant of the organic solvent–water mixture and that of the pure water.

Based on the generally-accepted assumption that, (1) the organic solvent–AChE interaction is much less susceptible to a dielectric effect than is the ligand-enzyme reaction and (2) a more positive A value corresponds to an increase in electrostatic repulsion, these results quantitatively account for the dielectric effect of ethanol on the binding of ligands to AChE according to the following order: paraoxon ethyl ($A \approx 0.17$) > edrophonium chloride ($A \approx 0.12$) > ATC ($A \approx 0.06$).

DISCUSSION

Effects of Ethanol as Determined in the Presence of ATC

Undoubtedly, the most sensitive tests for detecting effects on an enzyme are those involving its catalytic properties. It is well known that minor structural changes can produce drastic effects on enzyme catalysis. In a preliminary approach we investigated the Michaelis region of the AChE-hydrolysis of the ATC substrate to describe the interaction of ethanol with the enzyme. In agreement with previous observations,[27,28] it was found that ethanol undergoes an apparent competition with ATC as determined from the variation of the overall K_m value along with the invariance of the V_{max} value in response to the alcoholic solvent (cf. Figure 1A). The K_m value theoretically reflects all the kinetic components of ATC hydrolysis and is related to the equilibrium binding dissociation constant K_s according to Eq. (8):

$$K_m = K_s/(1 + k_2/k_3) \tag{8}$$

where k_2 is the rate constant for acylation of AChE and k_3 is the rate constant for deacylation of the acyl-intermediate.

Accordingly, K_m represents the reversible association of AChE and substrate only if acylation is rate-limiting *i.e.* $k_2/k_3 < 1$. This has actually been reported for the hydrolysis of ATC by insect AChE.[25] It would therefore appear that the apparent K_m, determined in the pure water system, well-approximates the K_s for the ATC/AChE complex under the conditions here. However, in the presence of ethanol, other components of the enzyme reaction can participate in the observed solvent effect, thereby hindering a straightforward interpretation from the estimated K_m and V_{max} values. Indeed, alcoholic solvents can undergo nucleophilic competition with water for deacylation of the acyl–AChE intermediate[20] and hence, modify the relative rates between acylation and deacylation together with the significance of K_m. The investigation of ATC hydrolysis by AChE from *Drosophila melanogaster* rather than from other sources (e.g. electric eel)

appears advantageous from this point of view since it would preclude problems arising from changes in the rate limiting step. Increased nucleophilicity of ethanol leading to an increase in the deacylation rate should better tend to enhance the original tendency of the insect AChE to keep the acyl–enzyme intermediate kinetically silent. The fact that both the apparent V_{max} value and the basic kinetic mechanism of edrophonium chloride as a competitive inhibitor (cf. Figures 1A and 2B, respectively) are not significantly affected by the organic solvent supports this idea. Further examination the effect of ethanol on the inhibition of AChE by edrophonium chloride also showed a substantial increase in the value of the K_i inhibition constant with the organic solvent (cf. Figure 3 and Table I). The values of K_i (edrophonium chloride) *versus* ethanol concentration were determined through the inhibition of the AChE-catalysis of ATC rather than by direct measurements of the ligand binding. Yet, edrophonium chloride is exclusive for the anionic active-center subsite, suggesting that there should be no artefact in the current analysis due to modulation at other sites by the surrounding medium. Therefore, under conditions where the Michaelis–Menten scheme apparently prevails, ethanol appears to antagonize AChE catalysis essentially by altering the binding properties of the active-center of the protein. Still, the analysis of the system with ATC requires further arguments to understand the effect of ethanol on the enzyme reaction.

Another aspect, when analyzing the AChE-catalysis of ATC, concerns the complex interplay between PAS and the active-center to bind ATC and its consequence on the kinetic properties of the enzyme. Ethanol was shown to alter the inhibition of AChE both by high concentrations of ATC (cf. Figure 1B) and by propidium diiodide (cf. Table I). Reciprocally, propidium diiodide appears to specifically protect the functional integrity of AChE against inactivation by ethanol (cf. Figure 4). Obviously, ethanol brings about modifications at PAS. Ligand binding at PAS has long been known to induce conformational changes at the substrate binding site.[37–39] Ordentlich *et al.*[3,5] and Barak *et al.*[40] also suggested a possible effect of PAS occupation in positioning the substrate at the catalytic center. In addition, evidence has been provided that occupation of PAS alters the hydrolysis of ATC by slowing the rate of deacylation so that the acetyl enzyme is no longer kinetically silent.[9] Owing to the fact that ethanol relieves inhibitions originating at PAS, the observed solvent effect at very high concentrations of ATC can be interpreted as ethanol lowering the amount of the substrate molecules bound to PAS, thereby restoring the best deacylation rate for the acetylated enzyme. Alternatively, the decrease in the occupation of PAS by ATC could originate from the nucleophilic activity of ethanol.

This would lead to a decrease in the accumulation of the acyl-intermediate. In any case, the occurrence of ethanol antagonism of ligand binding at PAS points to the fact that under non-Michaelis conditions, alteration at PAS either participates to or accounts for the observed solvent effect. It seems that, the increase in the value of K_i (propidium diiodide) together with that of K_i (edrophonium chloride) and K_m (ATC) with ethanol suggests a common underlying mechanism interfering with ligand binding at PAS and at the AChE active-center.

Effect of Ethanol as Determined by Residual Activity Analysis

Earlier studies with insect AChE resistant to organophosphate inhibition[41] indicated that modifications of the active site which render the phosphorylation of the active serine less efficient, should also impair the catalysis of the substrate. Organophosphates bring about irreversible anticholinesterase inhibition in essentially the same manner as choline esters up to the point where a phosphorylated enzyme is formed. Since the process of de-phosphorylation, similar to deacylation does not occur (or it takes place at a very slow rate), the estimated k_i bimolecular velocity constant of the AChE–organophosphate complex possibly reflects the effect of ethanol devoid of the nucleophilic-competition component. Therefore, the competitive effect of ethanol can be belier demonstrated by investigating the reaction of AChE with organophosphates rather than with substrates, provided that no interaction occurs at PAS. Accordingly, the inhibition was processed with the uncharged organophosphate paraoxon ethyl in the absence of ATC to further explore and to compare ligand exclusion at the AChE catalytic center by ethanol. The pattern of covalent inhibition with increasing concentrations of ethanol clearly shows a dramatic decrease in the k_i bimolecular rate constant value due to an essential increase of the K_d dissociation constant value (cf. Figure 5). Indeed, the absence of variation near the maximal rate of covalent inhibition indicates a negligible ethanol effect on the phosphorylation step of the nucleophilic serine at the AChE active-center. Ordentlich *et al.*[42] explained the relative invariance of the k_2 phosphorylation constant by the lack of sensitivity of the nucleophilic reaction at the phosphorus to the alteration in the architecture of the active center. Furthermore, the authors showed that irrespective of the enzyme source or the structure of the organophosphate inhibitor, the unique organization of the AChE active-center contributes predominantly to the formation of the enzyme-organophosphate Michaelis complexes. Therefore, the stabilization of these complexes appears as a major determinant of AChE reactivity with

organophosphate inhibitors. This is in accordance with the fact that ethanol seems to affect mainly the K_d value of the AChE-paraoxon ethyl complex. However, the high efficiency of paraoxon ethyl determined by measurements of residual activity rather than that of product formation, did not allow us to determine a precise value of the k_2 phosphorylation constant. Following this aim, we attempted to investigate further the covalent inhibition in the presence of edrophonium chloride which can compete with paraoxon ethyl for binding to the AChE active-center. This approach was comparable to that generally used with ATC[43,44] except for monitoring residual activity rather than product formation. In agreement with previous observations,[9] we showed that the occupation of the AChE active-center by edrophonium chloride effectively antagonizes the irreversible inhibition by paraoxon ethyl (cf. Table III). However, in the presence of ethanol, linearity was lost in the plots of $1/k_{obs}$ *versus* edrophonium chloride concentration at several fixed concentrations of paraoxon ethyl. These results emphasize a differential and/or combined effect of the alcoholic solvent on the interactions involved in binding of either edrophonium chloride or paraoxon ethyl to the AChE active-center. Thus, despite our efforts, it was not possible to compare further the system in the absence and in the presence of ethanol. Contrary to edrophonium chloride, the PAS-selective propidium diiodide affects the inhibition potency of the paraoxon ethyl organophosphate neither in the absence nor in the presence of ethanol (cf. Figure 5) indicating no significant interaction between the covalent inhibitor and PAS as required for analysis of solvent effect at the active center. The "kinetic neutrality" of propidium diiodide towards covalent inhibition by paraoxon ethyl and its ability to stabilize the activity of AChE (cf. Figure 4) were used advantageously to verily the permanency of a pseudo-first order kinetic behaviour for the inhibition mechanism in the presence of ethanol. Our results indicate that, (1) the biexponential behavior that eventually occurs at high ethanol concentration arises from a solvent effect on enzyme stability and, (2) the restricted Aldridge model i.e. the covalent inhibition of AChE by paraoxon ethyl is quasi-irreversible, as expected, holds true in the presence of ethanol. In agreement with previous findings,[42] the picture emerging from these results is that ethanol contributes predominantly to inhibit the formation of the enzyme-paraoxon ethyl Michaelis complex in the AChE active-center.

Dielectric Effects of Ethanol and Specific Antagonism of Ligand Binding

All of our observations are consistent with specific modulation of the enzyme binding properties by ethanol. The solvent effect may well arise

from changes in the enzyme structure thereby modifying the hydration state of AChE and the local dielectric constant around the active center and the regulatory PAS site. A decrease in the dielectric constant of the medium leads to an increase in the electrostatic repulsion between ligands and their sites and hence, yields lower binding. Previous studies with other enzymes e.g. chymotrypsin and trypsin[45-47] and papain,[48] also showed a main change in the dissociation constants due to the presence of water-soluble organic solvents, with little influence on the reaction rates. This was ascribed to general dielectric effects by the surrounding solvent in addition to competitive interactions with the enzyme molecule. Such an interpretation would equally apply to our results. The adverse effect of ethanol on the AChE reaction was shown to increase exponentially with its gradual addition for all the tested substrate analogues (cf. Figure 6). It is quite remarkable that analyzing the data as a function of dielectric effects leads to a similar extent of ethanol antagonism (within a factor three), indicating the occurrence of a common underlying mechanism as the concentration of the alcoholic solvent becomes significant. Yet, the observed order for increasing dielectric effect i.e. paraoxon ethyl > edrophonium chloride > ATC, suggests a more pronounced solvent effect on AChE interacting with aromatic molecules than with aliphatic molecules. However, the complex organization of the functional architecture of AChE still calls for caution. Specific "conformational scrambling" by ethanol at the AChE catalytic and regulatory sites additionally was shown in inactivation and stabilization studies on AChE. Both the propidium diiodide and edrophonium chloride aromatic cationic inhibitors were found to stabilize the activity of the enzyme upon exposure to ethanol, in agreement with previous observations that AChE stability involves maintenance of the functional integrity of the active center.[34,35] Furthermore, our findings with propidium diiodide provide the evidence for specific functional stabilization of AChE due to the prevention of conformational changes by binding at the sole PAS. For comparison, protection of AChE by non-specific additives e.g. sodium chloride, PEG or glycerol was marginal further demonstrating the occurrence of catalytic instability on the addition of ethanol. It would thus appear that the effect of ethanol specifically originates from conformational changes of the amino acid residues involved in the molecular recognition of substrates and ligands. In earlier studies, inactivation of AChE from *Torpedo californica* by acid, alkali, heat or urea was also shown to result from partial unfolding of Trp amino acid residue(s) at or near the active center.[49] A role of the orientation and/or bonding of an aromatic amino acid residue within the active center of AChE was proposed to explain the maintenance of the functional

integrity of the enzyme by ligands bearing specifically-oriented quaternary alkyl ammonium substituents.[34] Apart from the esteratic subsite containing the active serine, the AChE active center consists of several major domains: (1) the "anionic subsite", Trp-84(121), that through cation-aromatic interactions accommodates the quaternary ammonium moiety of choline as well as the active center inhibitors such as edrophonium chloride,[1-3,50 53] (2) the hydrophobic site for the alcoholic portion of the covalent adduct (tetrahedral intermediate) including residues Trp-86(121), Tyr-337(Tyr-334), and Phe-338(Tyr-408) which operate through nonpolar and/or stacking interactions in binding aryl substrates, uncharged ligands or the alkyl portion of the acyl moiety,[1] and (3) the acyl pocket outlined by the side chains of Phe-295(Phe-368) and Phe-297 that through steric limitations governs selectivity for the acyl moiety of the substrate[1,2,54,55] and for phosphorylating agents.[42,56] In addition to cholinesterases active-center domains affecting ligand specificity, PAS comprising Trp-286(Trp-359) and Asp-74(Tyr-109) close to the entrance of the active site gorge is a primary determinant in dictating specificity to cationic substrates and ligands[1,52] and cationic organophosphates.[57] Therefore, different aromatic amino acids within the aromatic gorge are critical to the overall binding efficiency of individual ligands depending on their nature and/or charge. Since we have shown a common yet specific dielectric effect of ethanol occurring at both PAS and the AChE active-center, it seems reasonable to infer that the solvent affects the AChE reactivity by overall "scrambling" the conformation of the aromatic gorge. Ethanol thus would either modify the cation-α and/or aromatic interactions between aromatic residues and charged or neutral ligands, respectively and/or impose new steric limitations to the acyl pocket. According to this, differential patterns for the ethanol effect would lie in the original bonding/orientation of aromatic amino acids within the aromatic gorge governing the specificity of interaction between AChE and substrate or ligands.

Perspectives

The results of the present studies have both practical and theoretical consequences. The analysis of the effect of ethanol on the reaction and the inhibition of *Drosophila* AChE facilitates a clearer insight into the process of solvent action and affords basic information on the operational stability of AChE that will be required in order to further investigate the behavior of the enzyme in the presence of polar solvents. Additional evidence is provided that the major determinant of the enzyme reactivity towards

substrates and inhibitors is the formation and/or the stability of Michaelis complexes rather than the nucleophilic activity of the AChE catalytic machinery. Our findings also suggest that ethanol predominantly modulates the functional conformation of the aromatic cavity and hence, that of the AChE active-center and PAS. Therefore, the pattern of the ethanol effect with other substrate analogues will depend on the occupation of particular sites and its consequence on the enzyme reaction. For example, anti-cholinesterase agents interacting with PAS in addition to the active center may show a covalent inhibition of AChE enhanced by ethanol unlike that observed with paraoxon ethyl. This dual solvent effect is well represented by our results for substrate activation and inhibition of the AChE reaction (cf. Figure 1).

The development of an analytical method based on the insecticide inhibition of AChE activity thus requires to minimize the action of ethanol on the functional characteristics of the enzyme. One solution would be to perform *in vitro* mutagenesis of residues lining the aromatic gorge so as to obtain a higher stability of inhibitors at the enzyme active-site. Such mutant AChE is also predicted to be more susceptible to insecticide compounds than the wild type. Still, the best sensitivity of *Drosophila* AChE towards the organophosphate insecticides allows efficient inhibition by paraoxon ethyl in the presence of ethanol at up to 15% with bimolecular reaction constant k_i values similar to that reported for inhibition of anthor AChE (e.g. electric eel) in a pure water system.

References

[1] Sussman, J.L., Harel, M., Frolow, F., Oefner, C., Goldman, A. and Silman, I. (1991) *Science*, **253**, 872–879.
[2] Harel, M., Schalk, I., Ehret-Sabatier, L., Bouet, F., Goeldner, M., Hirth, C., Axelsen, P., Silman, I. and Sussman, J.L. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 9031–9035.
[3] Ordentlich, A., Barak, D., Kronman, C., Flashner, C., Leitner, M., Segall, Y., Ariel, N., Cohen, S., Velan, B. and Shafferman, A. (1993) *J. Biol. Chem.*, **268**, 17083–17095.
[4] Radic, Z., Pickering, N.A., Vellom, D.C., Camp, S. and Taylor, P. (1993) *Biochemistry*, **32**, 12074–12084.
[5] Ordentlich, A., Barak, D., Kronman, C., Ariel, N., Segall, Y., Velan, B. and Shafferman, A. (1995) *J. Biol. Chem.*, **270**, 2082–2091.
[6] Axelsen, P.H., Harel, M., Silman, I. and Sussman, J.L. (1994) *Prot. Sci.*, **3**, 188–197.
[7] Ripoll, D.R., Faerman, C.H., Axelsen, P.H., Silman, I. and Sussman, J.L. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 5128–5132.
[8] Haas, R., Adams, E.W., Rosenberry, M.A. and Rosenberry, T.L. (1992) In *Multidisciplinary Approaches to Cholinesterase Functions* (A. Schafferman and B. Velan (eds.)), pp. 131–139. New York: Plenum Press.
[9] Berman, H.A. and Leonard, K. (1990) *Biochemistry*, **29**, 10640–10649.
[10] Radic, Z., Reiner, E. and Taylor, P. (1991) *Mol. Pharmacol.*, **39**, 98–104.
[11] Main, A.R. (1969) *Can. Med. Assoc. J.*, **100**, 161–167.

[12] Aldridge, W.N. and Reiner, E. (1972) *Frontiers Biology*, **26**, 1–328.
[13] Amino acids and numbers refer to human AChE, and the numbers in parentheses refer to the position of analogous residues in *Drosophila melanogaster* AChE.
[14] Battacharya, S., Alsen, C., Kruse, H. and Valentin, P. (1981) *Envir. Sci. Technology*, **15**, 1352–1355.
[15] Razumas, V.J., Kulis, J.J. and Malinauskas, A.A. (1981) *Envir. Sci. Technology*, **15**, 360–361.
[16] Morelis, R.M. and Coulet, P.R. (1990) *Anal. Chim. Acta*, **231**, 27–32.
[17] Salamoun, J. and Remien, J. (1993) *J. Pharmacol. Biomed. Analy*, **10**, 931–936.
[18] Marty, J.L., Sode, K. and Karube, I. (1992) *Electroanalysis*, **4**, 249–252.
[19] Fournier, D., Karch, F., Bride, J.M., Hall, L.M., Berge, J.B. and Spiere, P. (1989) *J. Mol. Biol.*, **210**, 15–22.
[20] Seydoux, F. and Yon, J. (1967) *Eur. J. Biochem.*, **3**, 42–56.
[21] Hinberg, I. and Laidler, K.J. (1972) *Can. J. Biochem.*, **50**, 1334–1359.
[22] Shin, S., Wu, P. and Chen, C.H. (1991a) *Int. J. Biochem.*, **23**, 169–174.
[23] Shin, S., Roth, L.G. and Chen, C.H. (1991b) *Int. J. Biochem.*, **23**, 1131–1136.
[24] Lasner, M., Roth, G.L. and Chen, C.-H. (1995) *Arch. Biochem. Biophys.*, **317**, 391–396.
[25] Hellenbrand, K. and Krupka, R.M. (1970) *Biochemistry*, **9**, 4665–4672.
[26] Dawson, R.M. and Crone, H.D. (1974) *J. Neurochem.*, **24**, 411–414.
[27] Ronzani, N. (1993) *Tetrahedron Lett.*, **34**, 3867–3870.
[28] Ronzani, N. (1994) *Analysis*, **22**, 249–256.
[29] Taylor, P. and Lappi, S. (1975) *Biochemistry*, **14**, 1989–1997.
[30] Ellman, G.L., Courtney, K.D., Andres, K.D. and Featherstone, R.M. (1961) *Biochem. Pharmacol.*, **7**, 88–95.
[31] Fournier, D., Mutero, A. and Rungger, D. (1992) *Eur. J. Biochem.*, **203**, 513–519.
[32] All the ethanol concentrations hereafter are volumetric.
[33] Aldridge, W.N. (1950) *Biochem. J.*, **46**, 451–460.
[34] Payne, C.S., Saeed, M. and Wolfe, A.D. (1989) *Biochim. Biophys. Acta*, **999**, 46–51.
[35] Wilson, E.J., Massoulié, J., Bon, S. and Rosenberry, T.L. (1996) *FEBS Letters*, **379**, 161–164.
[36] Clement, G.E. and Bender, M.L. (1963) *Biochemistry*, **2**, 836–843.
[37] Changeux, J.P. (1966) *Mol. Pharmacol.*, **2**, 369–392.
[38] Kitz, R.J., Braswell, L.M. and Ginsburg, S. (1970) *Mol. Pharmacol.*, **6**, 108–121.
[39] Berman, H.A., Becktel, W. and Taylor, P. (1981) *Biochemistry*, **20**, 4803–4810.
[40] Barak, D., Ordentlich, A., Bromberg, A., Kronman, C., Marcus, D., Lazar, A., Ariel, N., Velan, B. and Shafferman, A. (1995) *Biochemistry*, **34**, 15444–15452.
[41] Fournier, D. and Mutero, A. (1994) *Comp. Biochem. and Phys.*, **108**, 19–31.
[42] Ordentlich, A., Barak, D., Kronman, C., Ariel, N., Segall, Y., Velan, B. and Shafferman, A. (1996) *J. Biol. Chem.*, **271**, 11953–11962.
[43] Hart, G.J. and O'Brien, R.D. (1973) *Biochemistry*, **12**, 2940–2945.
[44] Liu, W. and Tsou, C.L. (1986) *Biochim. Biophys. Acta*, **870**, 185–190.
[45] Fink, A.L. (1973) *Biochemistry*, **9**, 1736–1742.
[46] Fink, A.L. (1974) *Biochemistry*, **13**, 277–280.
[47] Mares-Guia, M. and Figueiredo, A.F.S. (1972) *Biochemistry*, **11**, 2091–2097.
[48] Fink, A.L. and Angelides, K.J. (1976) *Biochemistry*, **15**, 5287–5293.
[49] Wu, C.-S.C., Gan, L. and Yang, J.T. (1987) *Biochim. Biophys. Acta*, **911**, 25–36.
[50] Weise, C., Kreiekamp, H.J., Raba, R., Pedak, A., Aaviksaar, A. and Hucho, F. (1990) *EMBO J.*, **9**, 3885–3888.
[51] Shafferman, A., Kronman, C., Flashner, Y., Leitner, M., Grosfeld, H., Ordentlich, A., Gozes, Y., Cohen, S., Arid, N., Barak, D., Harel, M., Silman, I., Sussman, J.L. and Velan, B. (1992) *J. Biol. Chem.*, **267**, 17640–17648.
[52] Shafferman, A., Velan, B., Ordentlich, A., Kronman, C., Grosfeld, H., Leitner, M., Flashner, Y., Cohen, S., Barak, D. and Ariel, N. (1992) *EMBO J.*, **11**, 3561–3568.
[53] Ordentlich, A., Kronman, C., Barak, D., Stein, D., Ariel, N., Marcus, D., Velan, B. and Shafferman, A. (1993) *FEBS Lett.*, **334**, 215–220.
[54] Vellom, D.C., Radic, Z., Li, Y., Pickering, N.A., Camp, S. and Taylor, P. (1993) *Biochemistry*, **32**, 12–17.

[55] Hosea, N.A., Harvey, A., Berman, H.A. and Taylor, P. (1995) *Biochemistry*, **34**, 11528–11536.
[56] Fournier, D., Mutero, A., Pravalario, M. and Bride, J.-M. (1993) *Chem. Biol. Interact.*, **87**, 233–238.
[57] Hosea, N.A., Radic, Z., Tsigelny, I., Berman, H.A., Quinn, D.M. and Taylor, P. (1996) *Biochemistry*, **35**, 10995–11004.