Two invertebrate acetylcholinesterases show activation followed by inhibition with substrate concentration

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In vertebrates there are two cholinesterases, with differences in catalytic behaviour with respect to substrate concentration: butyrylcholinesterase displays an increased activity at low substrate concentrations, whereas acetylcholinesterase displays inhibition by excess substrate. In two invertebrates, *Drosophila melanogaster* and *Caenorhabditis elegans*, we found cholin-

esterases that showed both kinetic complexities: substrate activation at low substrate concentrations followed by inhibition at higher concentrations. These triphasic kinetics can be explained by the presence of two enzymes with different kinetic behaviours or more probably by the existence of a single enzyme regulated by the substrate concentration.

INTRODUCTION

Cholinesterases are serine hydrolase enzymes that belong to the α/β -fold protein family [1]. They are classified according to their catalytic properties, inhibition specificities and tissue distributions: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BuChE, EC 3.1.1.8). AChE has a key role in the nervous system in terminating the neurotransmitter action of acetylcholine. No physiological function has yet been ascribed to BuChE. In vertebrates both types of enzyme are found and several catalytic parameters allow them to be distinguished. Among them, the most discriminating feature is the profile of activity against substrate concentration. AChE undergoes inhibition by an excess of substrate, whereas BuChE displays activation at low concentrations of aliphatic carboxylic acid monocholine esters [2].

In *Drosophila melanogaster* there is only one cholinesterase, an amphiphilic dimer bound to the membrane via a glycolipid anchor. In the nematode *Caenorhabditis elegans* three different

genes encode three AChEs [3] differing in their substrate specificities [4,5]. One of these genes, coding for an amphiphilic tetramer, has recently been cloned and expressed *in vitro* [6]. These two enzymes, from *Drosophila* and the nematode, can be considered as AChEs because they are present in the central nervous system where they metabolize the neurotransmitter acetylcholine. Unlike vertebrate AChE, these enzymes efficiently hydrolyse butyrylthiocholine [4,7].

AChE is the functional target of the carbamates and organophosphates used as insecticides, nematicides and chemical warfare nerve agents. These compounds are quasi-irreversible inhibitors. They act as pseudo-substrates and inactivate cholinesterases by carbamylating or phosphorylating the active serine [8]. Insect AChE resistance to these compounds originates from point mutations in the enzyme that modify either the inhibitor affinity and/or the inhibition rate constants [9,10]. Knowledge of wild-type AChE kinetics is a prerequisite for understanding the effects of mutations in resistant pests if anti-resistant compounds are to be designed. The purpose of this work was to understand the regulation of substrate hydrolysis by invertebrate cholinesterases that are present in insects and can be called AChEs because they are present in the central nervous system where they metabolize the neurotransmitter, acetylcholine. We therefore chose to study, in depth, the catalytic behaviour of the *D. melanogaster* and one of the *C. elegans* AChEs with different thiocholine esters (acetylthiocholine iodide, butyrylthiocholine iodide, propionylthiocholine iodide, acetyl- β -methylthiocholine iodide) and a neutral substrate, *o*-nitrophenyl acetate (Table 1). All the substrates we assayed were efficiently metabolized by the two enzymes from *Drosophila* or the nematode. This finding cannot be generalized because some insect AChEs do not hydrolyse *p*-nitrophenyl acetate [11], whereas others do not hydrolyse butyrylthiocholine iodide [12].

MATERIALS AND METHODS

Purification of *Drosophila* AChE

The enzyme was purified to homogeneity as previously described but with a procainamine affinity column instead of trimethylammonium-6-hexylamine [13].

Table 1 Substrates used for kinetic studies

Name	Structure
Acetylthiocholine	$\text{CH}_3\text{C}(\text{O})\text{-SCH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3$
Butyrylthiocholine	$\text{CH}_3\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{-SCH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3$
Propionylthiocholine	$\text{CH}_3\text{CH}_2\text{C}(\text{O})\text{-SCH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3$
Acetyl- β -methylthiocholine	$\text{CH}_3\text{C}(\text{O})\text{-SCH}(\text{CH}_3)\text{CH}_2\text{N}^+(\text{CH}_3)_3$
<i>o</i> -Nitrophenyl acetate

Abbreviations used: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase.

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Figure 1 Activation and inhibition of *D. melanogaster* AChE by substrate

(A) k_{obs} (Ellman method) plotted against acetylthiocholine iodide (ASCh) concentration (1–100 μM); □, electric eel AChE; ●, *Drosophila* AChE. The two curves were fitted to the Michaelis–Menten equation, showing that the *Drosophila* enzyme deviates from this equation. (B) Eadie–Hofstee plots of k_{obs} of the *Drosophila* enzyme (Ellman method) over range of substrate concentrations from 1 μM to 1 mM compared with the curve generated with parameters (K_{m1} , K_{m2} , V_{max1} and V_{max2}) determined from the fit with eqn. (2). (C) Lineweaver–Burk plots of initial rate obtained on a pH-stat over a range of substrate concentrations from 10 μM to 1 mM for the *Drosophila* enzyme. Activation is present and so cannot be attributed to the Ellman method used. (D) k_{obs} plotted against substrate concentration (1 μM to 200 mM), for the *Drosophila* enzyme (Ellman method), analysed in accordance with eqn. (2). (A), (B) and (C) describe the activation phase because in (D) the two phenomena, activation and inhibition, are represented.

Transient expression of recombinant *Drosophila* AChE in *Xenopus* oocytes

We used the pX construction as previously described. This clone contains the entire coding sequence for AChE except for the C-terminal region, which is the signal inserting the glycolipid anchor. This construct directs the expression of a soluble protein secreted into the oocyte incubation medium, which remains essentially free of other proteins [9].

AChE synthesis in baculovirus-infected cells

cDNA fragments encoding AChE of *D. melanogaster* or AChE1 of *C. elegans* were cloned in the transfer vector pVL941 or pBacPAK9 for expression with the baculovirus system [6,14]. Transfer vector was co-transfected in SF9 cells with previously linearized baculovirus DNA [15]. After culture for 4 days in protein-free medium [16], the virus was purified and titrated. Enzyme production was monitored in the same medium but without serum to avoid further co-purification of bovine AChE. The cell culture medium containing secreted AChE was centrifuged and dialysed against 50 mM phosphate buffer, pH 7.0;

recombinant AChE was purified by affinity chromatography essentially as for the natural insect AChE.

Kinetics of thiocholine substrate hydrolysis

Ellman method

Kinetic analysis was performed at 25 °C in 25 mM phosphate buffer, pH 7.0 or 7.25 depending on the substrate (see Table 2), with 10–100 ng of enzyme. Hydrolysis of thiocholine iodide esters was followed at 412 nm by using the method of Ellman et al. [17], with 48 different substrate concentrations from 1 μM to 200 mM. For low concentrations of substrate (less than 20 μM), two different methods were used to measure the enzyme activity. One consisted of measuring the initial velocity with a small amount of enzyme, which allowed us to assume that we were working at the stationary state. This could be checked by the absorbance obtained at the end of the measurement. Another less conventional method was also used: the enzyme was mixed with an initial concentration of substrate ($c_i < 50$ μM) and the change in absorbance was followed with time. At time t the

concentration of substrate was $c_t = c_i - A/0.0136$. The corresponding part of the curve at the substrate concentration studied was selected and checked to be linear over the time range chosen. Then a linear regression was made on this part of the curve to give the initial velocity. At such low substrate concentrations, thiocholine had no effect on the rate of the reaction because its K_i is two orders of magnitude above the concentration used. Furthermore this method allowed the verification of the initial concentration of substrate corresponding to the absorbance when the reaction was complete.

pH-stat

The acetic acid produced by the enzyme during acetylcholine hydrolysis was neutralized directly by a solution of 0.001 M NaOH. The experiment was performed at 25 °C in 0.155 M NaCl at pH 7.0.

Kinetics of *o*-nitrophenyl acetate hydrolysis

o-Nitrophenyl acetate solutions were prepared in methanol. Kinetic analysis was performed with 10–100 ng of enzyme in 25 mM phosphate buffer, pH 7.0, at 25 °C in the presence of 5 % (v/v) methanol. This methanol concentration did not significantly affect the enzymic hydrolysis. The appearance of *o*-nitrophenol was followed at 410 nm (ϵ_{410} 2400 M⁻¹·cm⁻¹ at pH 7.0 in the presence of 5 % methanol) and spontaneous substrate hydrolysis was subtracted.

Determination of kinetic parameters

Enzymes studied were either Michaelian or non-Michaelian, depending on the nature of the substrate and the concentration range used. For Michaelian behaviour, K_m and V_{max} were determined with Eadie–Hofstee plots. For non-Michaelian behaviour with activation at low substrate concentration, the two K_m values and V_{max} values were estimated by non-linear regression from the graph of k_{obs} against substrate concentration by using [18]:

$$v = V_{max1}[S]/(K_{m1} + [S]) + V_{max2}[S]/(K_{m2} + [S]) \quad (1)$$

When the substrate inhibition constant exists and leads to $k_{obs} = 0$, inhibition constants K_{ss} were calculated in accordance with:

$$v = V_{max1}/(1 + K_{m1}/[S] + [S]/K_{ss1}) + V_{max2}/(1 + K_{m2}/[S] + [S]/K_{ss2}) \quad (2)$$

assuming that inhibition by excess substrate is due to the binding of a second substrate molecule to the peripheral site and that the ternary complex is non-productive.

If the inhibition leads to a constant k_{obs} , the following equation is used:

$$v = V_{max1}[S]/(K_{m1} + [S]) + V_{max2}/(1 + K_{m2}/[S] + [S]/K_{ss2}) \quad (3)$$

To determine k_{obs} (number of molecules of substrate hydrolysed per second per catalytic centre), active-site titration was performed with 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide, which was synthesized as described by Levy and Ashani [19]. Enzyme titration was performed by two methods: by the residual activity method of Levy and Ashani [19] and by the 'burst' method of monitoring the fluorescence of the leaving group, 1-methyl-7-hydroxyquinolinium iodide. This compound was synthesized from 7-hydroxyquinoline and methyl iodide by the method of Rosenberry and Bernhard [20].

RESULTS AND DISCUSSION

Kinetics of substrate hydrolysis

To study the kinetic behaviour of *Drosophila* and nematode AChE, we used a very wide substrate concentration range: from 1 μ M to 200 mM thioesters. Surprisingly, hydrolysis of substrates by *Drosophila* and nematode AChEs between 1 and 100 μ M deviated from the kinetic model established for vertebrate AChEs such as that of the electric eel (Figure 1A) [21]. This deviation was observed for the *Drosophila* enzyme whether the enzyme was from fly heads or was a recombinant enzyme produced in *Xenopus* oocytes or baculovirus. *Drosophila* and nematode AChEs showed triphasic kinetics for the hydrolysis of thiocholinesters (including activation at low substrate concentrations and inhibition at high concentrations). In Figures 1 and 2 the results shown were obtained with recombinant enzymes produced in baculovirus. Similar findings were obtained with acetylcholine chloride as substrate and by pH-stat monitoring of hydrolysis with higher substrate concentrations, indicating that the catalytic behaviour observed was not dependent on the assay method, i.e. the Ellman reaction (Figure 1C). The same experiment was also performed in the presence of 1 mM NaCl to avoid any changes in the ionic strength of the medium with increasing substrate

Figure 2 Activation and inhibition of *C. elegans* AChE by the substrate

(A) Eadie–Hofstee plot for substrate concentrations between 6 μ M and 7.5 mM compared with the curve generated with parameters (K_{m1} , K_{m2} , V_{max1} and V_{max2}) obtained from the fit with eqn. (2). (B) k_{obs} plotted against substrate concentration (6 μ M to 200 mM) analysed with eqn. (2). Abbreviation: ASCh, acetylthiocholine iodide.

Figure 3 Hydrolysis of *o*-nitrophenyl acetate (concentrations from 75 μ M to 5 mM) by various cholinesterases

Eadie-Hofstee plots of substrate hydrolysis by (A) *D. melanogaster* AChE, (B) electric eel AChE, (C) human BuChE and (D) *C. elegans* AChE.

concentration. Under these conditions the same triphasic kinetic pattern was observed. The kinetic behaviour of *Drosophila* AChE towards *o*-nitrophenyl acetate from 75 μ M to 5 mM showed biphasic kinetics (Figure 3), with an activation phase. Inhibition by excess of substrate, if it existed, would not be seen because the limit of solubility of *o*-nitrophenyl acetate is 5 mM. In contrast, for nematode AChE, human BuChE and electric eel AChE, we did not observe any significant deviation from a linear Eadie-Hofstee plot for the *o*-nitrophenyl acetate substrate. From the total substrate concentration range we determined K_{m1} , K_{m2} , V_{max1} and V_{max2} from eqn. (1). When substrate inhibition occurred, the equilibrium constants K_{ss} were also calculated from eqns. 2 or 3 (Figures 1D and 2B). Results are reported in Table 2.

Activation is not dependent on substrate charge

Biphasic kinetics, including activation at low substrate concentration, has already been observed for BuChE but only with charged substrates [22–25]. Here we also observed activation with *o*-nitrophenyl acetate (Figure 3A) between 1 and 5 mM, which is a concentration range never explored before [26,27]. This suggests that the charge of the substrate is not the most important factor in the observed phenomenon. To check whether this activation was not an artifact, we performed the same experiment under the same conditions with electric eel AChE (Figure 3B). This enzyme yielded a linear Eadie-Hofstee plot.

Results obtained for human BuChE and nematode AChE with *o*-nitrophenyl acetate were more ambiguous: it seems that there is substrate activation but it was too weak to be measured under the experimental conditions used (Figures 3C and 3D).

Activation and inhibition

There is a body of evidence that vertebrate AChEs possess a secondary substrate-binding site called the peripheral anionic site [28,29], which regulates catalysis in an allosteric fashion [30,31]. More recently it has been argued that this peripheral site is involved in substrate inhibition [32–36]. This site is located at the entrance of the active-site gorge [37]. A general model has been proposed to describe the behaviour of vertebrate cholinesterases [21]. The model assumes that substrate can combine at two distinct sites, the active site and a peripheral anionic site, forming two different enzyme-substrate complexes, a binary ES and a ternary SES complex (see Scheme 1). Both ES and SES lead to substrate hydrolysis but with different efficiencies; k_{cat} for the binary and $b k_{cat}$ for the ternary complex, where b indicates the efficiency of hydrolysis. This scheme is interesting because it is simple but it can only account for inhibition or activation depending on the value of b , and thus cannot be applied to invertebrate AChEs.

One possibility to afford both inhibition and activation would be the occurrence of two enzymes with different kinetic behaviours: two enzymes with different K_m values displaying an

Table 2 k_{cat} , K_m and K_{ss} values for different cholinesterases for the hydrolysis of various substratesAbbreviations: acetyl- β -methylSCh, acetyl- β -methylthiocholine iodide; ASCh, acetylthiocholine iodide; BuSCh, butyrylthiocholine iodide; PrSCh, propionylthiocholine iodide.

	K_{m1} (μM)	$k_{\text{cat}1}$ (s^{-1})	K_{ss1} (mM)	K_{m2} (μM)	$k_{\text{cat}2}$ (s^{-1})	K_{ss2} (mM)
<i>Drosophila</i> AChE						
ASCh (pH = 7.25)	2.95	334	6.2	84	720	46
BuSCh (pH = 7.25)	26	251		273	460	3.6
PrSCh (pH = 7.0)	6.8	449		441	216	
Acetyl- β -methylSCh (pH 7.25)	8.5	319	11	66	685	11
<i>o</i> -Nitrophenyl acetate (pH 7.0)	61	228		3485	1494	
Nematode AChE						
ASCh (pH = 7.25)	26	432	13.7	1873	540	286
<i>o</i> -Nitrophenyl acetate (pH 7.0)	451	585				
Electric eel AChE						
<i>o</i> -Nitrophenyl acetate (pH 7.0)	354	3606				
Human BuChE:						
<i>o</i> -Nitrophenyl acetate (pH 7.0)	966	129

Scheme 1 General model of behaviour of vertebrate cholinesterases with two substrate-binding sites

The substrate can bind to two sites, the anionic site (ES) and the peripheral anionic site (SE). Binding to the peripheral anionic site changes the k_{cat} of the enzyme. This scheme can explain either activation or inhibition.

inhibition by excess of substrate. However, this hypothesis seems improbable because the two enzymes would have to be very similar, because they are not distinguishable by physical separation methods, and equally represented whatever the enzyme source, *Drosophila* head or production *in vitro*.

Another possibility is illustrated in Scheme 2 taking into

Scheme 3 Model of cholinesterase behaviour with three substrate-binding sites

There are three sites of binding for the substrate to the enzyme. One is the anionic site, another is responsible for activation and the last is responsible for inhibition. The three sites have different affinities for the substrate.

account that there exists a reactive intermediate, the acetyl enzyme. In this model, binding of the substrate molecule to the peripheral site occurs with different affinities (K_2 , K'_2 and K''_2) depending on whether the enzyme is free (E), involved in a Michaelian complex (ES) or acetylated (EA).

Scheme 2 Model of cholinesterase behaviour with two substrate-binding sites on different enzyme forms

The substrate can bind to two sites on all enzyme forms (free enzyme, Michaelis complex and the acetylated enzyme). This scheme explains both the activation and the inhibition of the enzyme depending on the substrate concentration.

The last possibility considered involves a third binding site for the substrate (Scheme 3). Accordingly, three binding sites for the substrate might exist on the enzyme (denoted OOEO), the choline-binding site and two peripheral sites. Several simplifications have been made in this cubic model, by assuming for example that the third substrate molecule binds only to binary or ternary complexes [27].

It would be of interest to know whether there is a feature common to all cholinesterases. Substrate activation is not visible in vertebrate AChE and substrate inhibition is not visible in vertebrate BuChE, although recently Masson et al. [38] showed that the activation phase is followed by an inhibition phase in human BuChE. The percentage similarity in the amino acid sequences between insect and vertebrate AChEs indicates that these enzymes derive from a common ancestral gene [39]. Thus invertebrate AChEs are useful models to understand the regulation of catalysis in vertebrate cholinesterases.

Does the complex behaviour of cholinesterase have a physiological significance? Substrate concentration-dependent activation of the enzyme function might serve to adjust the catalytic activity of AChE to fluctuations in the concentration of the neurotransmitter after its release in the synaptic cleft. Such a hypothesis has already been suggested for the modulation of the catalytic activity of AChE by cations on binding to the peripheral site [40] and suggests that the peripheral anionic site is a sensor that exerts remote control on catalysis.

Insecticide resistance involving AChE is usually correlated with modifications in acetylcholine hydrolysis. Most often, the catalytic efficiency (k_{cat}/K_m) decreases because of a decrease in k_{cat} or an increase in K_m . However, more intriguing results have been reported. For example a decrease in K_m , or a loss of inhibition at high substrate concentrations, were correlated with resistance to insecticides (reviewed in [41]). These characteristics could be used as markers to follow the dynamics of resistant alleles in field populations if the relationships between the kinetic parameters and the mutations that confer insecticide resistance can be established.

We thank Michèle Willson for her help in the synthesis of 7-(methylethoxy phosphinyloxy)-1-methylquinolinium iodide, and Frédéric Magné for his technical assistance. This research was supported by grants from DRET (94-084), IFREMER and CNRS (GDR 1105 and ACS-SV3).

REFERENCES

- Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, J., Silman, I., Schrag, J. et al. (1992) *Prot. Engin.* **5**, 197–211
- Nachmansohn, D. and Wilson, I. B. (1951) *Acta Enzymol.* **12**, 259–339
- Johnson, C. D., Duckett, J. G., Culotti, J. G., Herman, R. K., Meneely, P. M. and Russell, R. L. (1981) *Genetics* **97**, 261–279
- Johnson, C. D. and Russell, R. L. (1983) *J. Neurochem.* **41**, 30–46
- Johnson, C. D., Rand, J. R., Herman, R. K., Stern, B. D. and Russell, R. L. (1988) *Neuron* **1**, 165–173
- Arpagaus, M., Fedon, Y., Cousin, X., Chatonnet, A., Bergé, J.-B., Fournier, D. and Toutant, J.-P. (1994) *J. Biol. Chem.* **269**, 9957–9965
- Gnagey, A. L., Forte, M. and Rosenberry, T. L. (1987) *J. Biol. Chem.* **262**, 13290–13298
- Ballantyne, B. and Marrs, T. C. (1992) *Clinical and Experimental Toxicology of Organophosphates and Carbamates*, Butterworth-Heinemann, Oxford
- Fournier, D., Bride, J. M., Hoffmann, F. and Karch, F. (1992) *J. Biol. Chem.* **267**, 14270–14274
- Mutero, A., Pralavorio, M., Bride, J. M. and Fournier, D. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 5922–5926
- Brestkin, A. P., Maizel, E. B., Moralev, S. N., Novozhilov, K. V. and Sazonova, I. N. (1985) *Insect Biochem.* **15**, 309–314
- Zhu, K. Y. and Clark, J. M. (1994) *Insect Biochem. Molec. Biol.* **24**, 453–461
- Fournier, D., Cuany, A., Bride, J. M. and Bergé, J. B. (1987) *J. Neurochem.* **49**, 1455–1461
- Chaabihi, H., Fournier, D., Fedon, Y., Bossy, J. P., Ravallec, M., Devauchelle, G. and Cérutti, M. (1994) *Biochem. Biophys. Res. Commun.* **203**, 734–742
- Kitts, P. A., Ayres, M. D. and Possee, R. D. (1990) *Nucleic Acids Res.* **18**, 5667–5672
- Inlow, D., Shaugar, A. and Maiorella, B. (1988) *J. Tiss. Cult. Methods* **12**, 13–16
- Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7**, 88–95
- Spears, G., Sneyd, J. G. T. and Loten, E. G. (1971) *Biochem. J.* **125**, 1149–1151
- Levy, D. and Ashani, Y. (1986) *Biochem. Pharmacol.* **35**, 1079–1085
- Rosenberry, T. L. and Bernhard, S. A. (1971) *Biochemistry* **10**, 4114–4120
- Radic, Z., Pickering, N. A., Vellom, D. C., Camp, S. and Taylor, P. (1993) *Biochemistry* **32**, 12074–12084
- Christian, S. T. and Beasley, J. G. (1968) *J. Pharmacol. Sci.* **57**, 1025–1027
- Eriksson, H. and Augustinsson, K.-B. (1979) *Biochim. Biophys. Acta* **567**, 161–173
- Cauet, G., Friboulet, A. and Thomas, D. (1987) *Biochem. Cell Biol.* **65**, 529–535
- Masson, P., Adkins, S., Gouet, P. and Lockridge, O. (1993) *J. Biol. Chem.* **19**, 14329–14341
- Ferro, A. and Masson, P. (1987) *Biochim. Biophys. Acta* **916**, 193–199
- Cléry, C., Bec, N., Balny, C., Mozhaev, V. V. and Masson, P. (1995) *Biochim. Biophys. Acta* **1253**, 85–93
- Changeux, J. P. (1966) *Mol. Pharmacol.* **2**, 369–392
- Aldridge, W. N. and Reiner, E. (1969) *Biochem. J.* **115**, 147–162
- Rosenberry, T. L. (1975) *Adv. Enzymol.* **43**, 103–218
- Epstein, D. J., Berman, H. A. and Taylor, P. (1979) *Biochemistry* **18**, 4749–4754
- Gibney, G., Camp, S., Dionne, M., MacPhee-Quigley, K. and Taylor, P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7546–7550
- Radic, Z., Reiner, E. and Taylor, P. (1990) *Mol. Pharmacol.* **39**, 98–104
- 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
- Ordentlich, A., Barak, D., Kronman, C., Segall, Y., Velan, B. and Shafferman, A. (1995) *J. Biol. Chem.* **270**, 2082–2091
- Barak, D., Ordentlich, A., Bromberg, A., Kronman, C., Marcus, D., Lazar, A., Ariel, N., Velan, B. and Shafferman, A. (1995) *Biochemistry* **34**, 15444–15452
- Weise, C., Kreienkamp, H. J., Raba, R., Aaviksaar, A. and Hucho, F. (1990) *EMBO J.* **9**, 53–57
- Masson, P., Froment, M. T., Bartels, C. and Lockridge, O. (1996) *Eur. J. Biochem.* **235**, 36–48
- Meyers, M., Richmond, R. C. and Oakeshott, J. G. (1988) *Mol. Biol. Evol.* **5**, 113–119
- Berman, H. A. and Leonard, K. (1990) *Biochemistry* **29**, 10640–10649
- Fournier, D. and Mutero, A. (1994) *Comp. Biochem. Physiol.* **108C**, 19–31