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Probing the acyl binding site of acetylcholinesterase by protein engineering [1]

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Abstract

Recombinant acetylcholinesterase from rat brain and two mutants were studied for their hydrolytic activity toward acetyl- and butyrylthiocholine substrates and for their sensitivity toward organophosphate and carbamate inhibitors. Both mutants, a point mutant where F295 was replaced by leucine, and a second mutant where loop PQES was replaced by SG, were designed for increased size of the acyl binding pocket. Wild type and mutant enzymes were expressed in baculovirus-infected insect cells and biochemically characterized. As expected, wild type rat brain acetylcholinesterase hydrolyzed acetylthiocholine, but not butyrylthiocholine. Sensitivity toward small- and medium-sized organophosphate inhibitors like paraoxon-methyl and paraoxon-ethyl was comparable, but bulky organophosphates like ethoprophos were less efficient inhibitors. This tendency applied to carbamates as well, since small carbamoyl moieties like carbofuran and aldicarb were stronger inhibitors than furathiocarb which features a bulky carbamoyl moiety. In contrast to wild type enzyme, both mutants were capable of hydrolyzing butyrylthiocholine. However, k_{cat}/K_m toward acetylthiocholine of the F295L mutant was reduced if compared to the wild type enzyme. All five organophosphate and three carbamate inhibitors inhibited mutant F295L more efficiently than the wild type enzyme. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Protein engineering; Mutation; Acetylcholinesterase; Inhibitors

1. Introduction

Acetylcholinesterases (AChE; EC 3.1.1.7) are widely distributed in the nervous system, where they catalyze hydrolysis of the neurotransmitter acetylcholine (ACh) [1,2]. They have been investigated as amino acid protecting groups in peptide synthesis [3], and they have found application as reporter enzymes for synthetic organophosphorus and carbamate compounds by which they are inhibited [4]. Residual activity in AChE based inhibition tests can be determined through colorimetric [5], radiochemical [6] or electrochemical indicator reactions [7]. A spectrophotometrical method based on the hydrolysis of acetylthiocholine (ASCh) has become an industry standard in Germany for the determination of insecticides in drinking water [8].

Abbreviations: AChE = acetylcholinesterase; rbAChE = rat brain acetylcholinesterase; dAChE = *Drosophila* acetylcholinesterase; w.t. = wild type; ACh = acetylcholine; BuCh = butyrylcholine; ASCh = acetylthiocholine; BuSCh = butyrylthiocholine

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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Studies on the mechanism of AChE inhibition by organophosphates and carbamates have suggested that the enzyme is acylated by organophosphates and carbamates at a nucleophilic serine [9]. The inhibition constant depends on the chemical structure of the substituents and the leaving group [10]. This model was validated and refined when structures of AChE from *Torpedo californica* [11], mouse [12], and of a transition state analog complex [13] became available. AChEs belong to the class of α/β hydrolases [14] with a catalytic triad Ser–His–Glu or Asp on top of a central beta sheet. They are thus closely related to the lipases and serine proteases.

The mechanism of AChE inhibition by organophosphates and carbamates could not yet be studied on a molecular level, since no structures of complexes with organophosphates or carbamates are yet available. However, for other serine hydrolases, which have a similar enzymatic mechanism, such complexes were studied by X-ray analysis: for lipases from *Rhizomucor miehei* [15], *Candida rugosa* [16], *Candida antarctica* [17] and human pancreas [18], structures of complexes with substrate analogous inhibitors demonstrated that phosphates and phosphonates mimic the substrate intermediate. In serine proteases inhibited by hydroxylamine, the inhibitor covalently binds to the nucleophilic serine in a carbamate structure, but not in a substrate-analogous orientation [19].

In order to understand the molecular basis of substrate specificity and sensitivity toward substrate analogous inhibitors, the recognition sites have been probed by analyzing naturally occurring resistant *Drosophila* strains to identify mutation sites with changed susceptibility [20], and by site directed mutagenesis [21–27]. From these studies, it was concluded that there are distinct domains which confer specificity to substrate or inhibitor (for an overview see Ref. [28]). The acyl binding pocket (residues 288–293 in *Torpedo* AChE) determines specificity for the substrates ACh and butyrylcholine (BuCh), whereas resistance toward organophosphate inhibitors is mediated by positions 78, 119, 129, 227, 288, 290, 331, 442, and organophosphate specificity is mediated by position 288 (*Torpedo* AChE numbering). To our knowledge, unique sites responsible for carbamate specificity have not yet been identified.

In order to probe the acyl binding site of rat brain AChE (rbAChE), the acyl binding was re-engineered by site-directed mutagenesis, and the biochemical properties of the mutated enzymes were studied.

2. Materials and methods

2.1. Materials

The rbAChE cDNA was obtained from Jean Massoulié (ENS, Paris) and the dAChE cDNA from Didier Fournier (University of Toulouse, Toulouse). The baculovirus transfer vector pVL1393 and linearized baculoviral DNA (bacoluGold) were obtained from Pharmingen (San Diego, USA). *Spodoptera frugiperda* Sf9 insect cells were a gift from D. Moosmayer (Institute of Cell Biology and Immunology, University of Stuttgart). TNM-FH insect cells medium was purchased from Pharmingen. All inhibitors were obtained from Riedel de Haën (Seelze, Germany).

2.2. Preparation of recombinant enzymes

Cloning and expression of AChEs using a baculovirus transfer vector system were performed as described before [29]. Two mutated AChE genes, the F295L point mutant and the 'loop mutant' (replacement of PQES in SG), were prepared by PCR technology. The point mutation F295L was generated by two successive rounds of PCR and using four primers according to Mikaelian and Sergeant [30]. The loop mutant was obtained by synthesis of a long PCR fragment (the complete transfer vector and AChE gene) containing the change of bases in one primer. The mutated recombinant transfer

vector was then analyzed by restriction enzyme mapping and DNA sequencing. The recombinant transfer vector containing only the expected mutation and correct position in respect to the polyhedrin promoter was then purified and used for co-transfection. The co-transfection was realized by calcium precipitation using recombinant transfer. Sf9 cells were cultured and supernatant was recovered for further biochemical characterization as described before [29].

2.3. Mutants

In the first mutant, F295 was exchanged for leucine while maintaining the orientation of the side chain. This mutation removes three carbon atoms from the binding site and thus should allow binding of the substrate or inhibitor molecules with three additional CH_2 groups. In the second mutant, the loop 290–293 (PQES) was replaced by SG as in dAChE. This loop is not in contact with the substrate but makes the binding site more flexible.

2.4. Determination of AChE activity

Supernatant fractions of 10 μl were assayed directly in 300 μl final reaction medium volume by the spectrophotometric Ellman method [31] and read for 1 to 10 min at 412 nm. The activities were normalized for total protein concentration and expressed as ΔOD min^{-1} μl^{-1} enzyme. All further experiments were done with an enzyme activity normalized at 0.2 mOD min^{-1} μl^{-1}. For the determination of biochemical constants, the Haldane equation was used in order to account for excess of substrate inhibition (Eq. (1)), where K_m is the substrate affinity constant and K_{ss} the substrate inhibition constant representing the dissociation of a second substrate molecule.

$$\nu = \frac{V_{max}}{1 + \frac{[S]}{K_{ss}} + \frac{K_m}{[S]}} \quad (1)$$

The active-site concentration in the various preparations of enzyme studied were titrated with MTP (*O*-ethyl-*S*2-diisopropylaminoethyl methylphosphothionate) using a concentration range from 0.05 to 0.5 nM of inhibitor and either acetylthiocholine or butyrylthiocholine (BuSCh) as substrate. After 18 h incubation at 4°C, the kinetic of the reaction was measured. The enzyme reaction followed a second-order kinetics. The slopes of the linear plots representing residual activity as a function of inhibitor were used to determine the turnover numbers of the different recombinant enzymes.

2.5. Determination of inhibition constants

Reaction of insecticide inhibitors such as irreversible organophosphate compounds and reversible carbamate insecticides were analyzed using the German standard method DIN 38 415-TI [8]. After 30 min pre-incubation of the different enzyme preparations and an inhibitor, residual activities were measured using the Ellman colorimetric method [31]. The approach to inhibition was first order and no deacylation or decarbamylation occurred. Thus, the $t_{1/2}$ value was calculated according to Eq. (2), where $t_{1/2}$ is the half value time for each sample or standard, t_i the incubation time (30 min) and A_r the residual activity after inhibition (in %).

$$t_{1/2} = \frac{\ln 2\, t_i}{\ln \frac{1}{A_r}} \text{min} \quad (2)$$

The inhibition constant k_i (in M^{-1} min^{-1}) for each inhibitor was calculated according to Eq. (3), where $t_{1/2}$ is the half value time, and C the concentration of the inhibitor in the inhibition mixture (in M)

$$k_i = \frac{\ln 2}{t_{1/2} C}\ \text{M}^{-1}\ \text{min}^{-1} \quad (3)$$

2.6. Modeling of the acyl binding site

Sequence information of rbAChE [32] and *Drosophila* AChE (dAChE) [33] were retrieved from the sequence database SwissProt [34] (entries ACES_RAT and ACES_DROME, respectively), sequence and structure of *T. californica* [11,13] and mouse AChE [12] and of *C. rugosa* lipase (CRL) [16] from the Protein Data Bank [35]. The sequences were aligned using Clustal W [36] and Match-Box [37]. The secondary structure was determined from X-ray data using the program DSSP [38]. Visualization and protein modeling were done using the modeling programs ProExplore (Oxford Molecular Group) and InsightII (Molecular Simulations).

As the sequence of rbAChE is 98% homologous to mouse AChE, the structure of the acyl binding pocket of rbAChE was modeled using mouse AChE by exchanging the differing side chains while maintaining side chain torsion angles, whenever possible.

2.7. Docking of substrate and inhibitors

The structure of paraoxon-methyl in the binding site of rbAChE was derived from the X-ray structure of the homologous CRL complexed with the inhibitor by menthyl-hexyl-phosphonate. The sequences of rbAChE and CRL were aligned and the most homologous 107 residues superimposed (C_α root mean square (r.m.s.) deviation 0.57 Å, data not shown). Paraoxon-methyl was placed in the binding site of rbAChE according to the respective phosphonate atoms in the CRL complex. The complex of rbAChE with paraoxon-ethyl was created by adding two CH_3 groups in *all trans*. Complexes of substrates in the tetrahedral intermediate state were created from the organophosphate inhibitors by replacing the phosphorus atom by a tetravalent carbon and replacing one CH_3–O– group of paraoxon-methyl by CH_3– to yield the acetyl moiety of ACh, and replacing CH_3–CH_2–O– of paraoxon-ethyl by CH_3–CH_2–CH_2– to yield the butyryl moiety of BuCh, respectively.

3. Results

3.1. Structure modeling of the acyl binding site

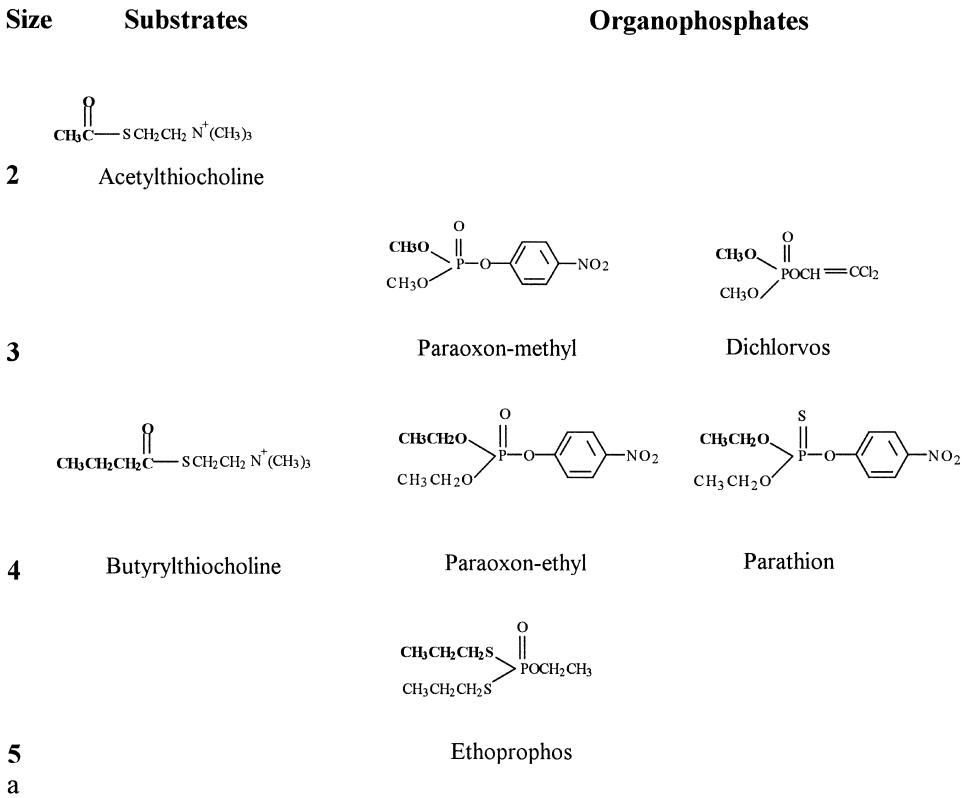
rbAChE was compared to dAChE, which had been shown to hydrolyze butyrylcholine, a substrate which is not accepted by rbAChE [20,33]. Sequence alignment of the acyl binding site region (Fig. 1) identified two major differences between the two proteins: (1) Residue 295 of rbAChE, which is phenylalanine in rbAChE but leucine in dAChE. This side chain is in direct contact with the substrate or organophosphate inhibitors. (2) The loop 290–293 (PQES) of rbAChE, which in dAChE is shortened to SG. The side chains of this loop are not in contact with the substrate.

3.2. Interaction with substrates and inhibitors

The acyl binding site has been probed by protein engineering and by measuring substrates and inhibitors of different size. W.t. rbAChE and the two mutants were compared. The F295L mutant has an increased acyl binding pocket. In the loop mutant, the shorter loop was expected to render the acyl binding pocket more flexible

rbAChE	274	RTRPAQDLVDHEWHVL**PQES**I**F**RFSFVPVVDG
***Torpedo* AChE**	267	REKKPQELIDVEWNVL**PFDS**I**F**RFSFVPVIDG
dAChE	347	RSVDAKTISVQQWNSY**SG..**I**L**SFPSAPTIDA
AChE secondary structure		HH HHHHHHHHHH SS

Fig. 1. Sequence alignment of rbAChE, *Torpedo* AChE and dAChE (highly homologous residues in boxes); the secondary structure of *Torpedo* is given in line 4 (H: α helix, S: β strand). The sites of mutation are in bold.



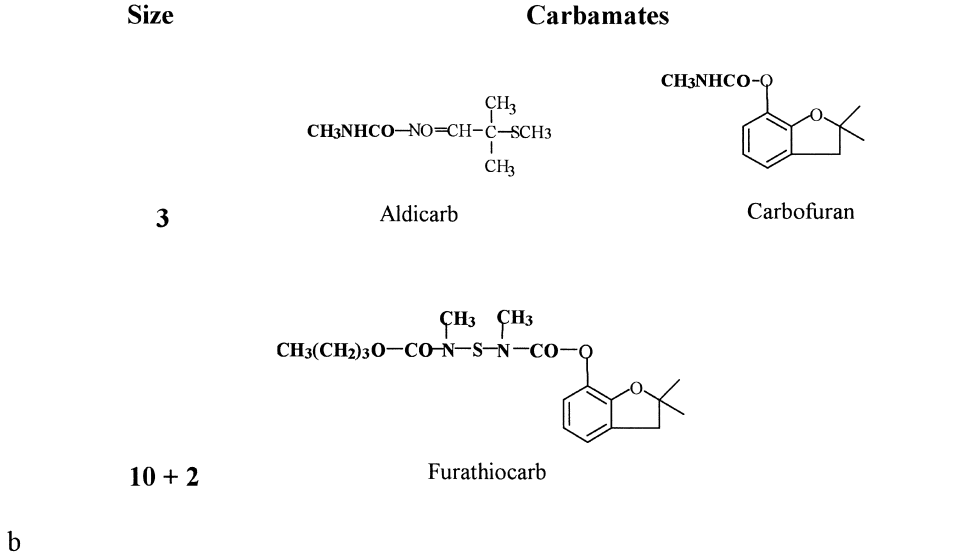


Fig. 2. (a) Substrates and organophosphate inhibitors grouped by size of moiety (in bold) assumed to bind to the acyl binding pocket. For a comparison of substrates and organophosphate inhibitors, the phosphorus atom is included in the ‘size’ value. (b) Carbamate inhibitors (carbamoyl moiety in bold).

compared to the w.t. enzyme. This mutation was expected to mediate the binding of bulky substrates or inhibitors by induced fit effects. Two substrates (ASCh and BuSCh), five organophosphates (paraoxon-methyl, paraoxon-ethyl, ethoprophos, parathion and dichlorvos) and three carbamates (aldicarb, carbofuran and furathiocarb) were tested (Fig. 2). These substrates and inhibitors were chosen since they differ in size of their acyl, alcohol or carbamoyl moieties, respectively. To determine the influence of the leaving groups, pairs of inhibitors of identical alcohol or carbamoyl moieties but different leaving groups, were compared.

Substrates and organophosphate inhibitors were expected to bind in a similar mode to the acyl binding pocket of AChE, therefore, the acyl moiety of substrates corresponds to the alcohol moiety of organophosphates. We have grouped substrates and organophosphate inhibitors by size of the moieties which interact with the acyl binding pocket (Fig. 2a). In this model, paraoxon-ethyl and butyrylcholine occupy the same space in the acyl binding pocket and contact the nearest F295 atom at a distance of only 3.1 Å (Fig. 3). ACh fitted better into the pocket, with a distance of 4.2 Å to the nearest F295 atom. Replacing F295 with leucine increased the distance from butyryl and acetyl CH_3 to the nearest L295 atom to 3.6 Å and 5.1 Å, respectively. Since L295 can reorientate more easily than F295, due to its smaller size, the distance between L295 and substrate or inhibitor may even increase. The influence of the leaving group was studied comparing paraoxon-methyl and dichlorvos. A possible interference of the phosphate group was probed by comparing paraoxon-methyl to parathion containing a phosphorothioate group. The carbamate inhibitors were selected as follows: aldicarb and carbofuran have an identical carbamoyl moiety (CH_3–NH–CO) but a different leaving group. Furathiocarb has the same leaving group as carbofuran, but a much longer carbamoyl moiety.

3.3. Expression

Actively expressed proteins were obtained using the baculovirus-insect cells system. The biochemical characterization of w.t. rbAChE was studied and compared to tissue derived rbAChE [29]. In all cases, i.e., activity measurement or inhibition determination, both enzymes were identical and reacted in the same manner. The expression of active dAChE confirmed results already obtained and the calculation of a biochemical constant or inhibition rate were quite similar [39]. The amount of produced enzymes, about 1.5 μg of pure AChE from a standard cultivation of 15 ml, was sufficient for direct titration of active sites with the highly affine MTP alkyl phosphate and a high concentration of enzyme were employed for the monitoring of excess substrate inhibition. All four recombinant AChEs were inhibited by an excess of substrate (data not shown).

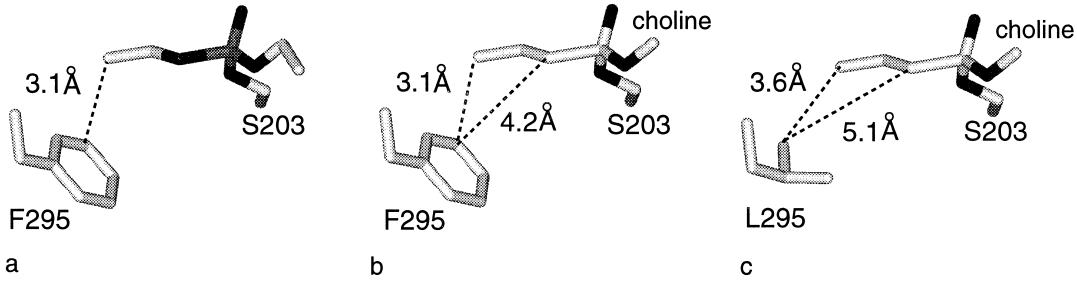


Fig. 3. Interaction of w.t. rbAChE with (a) paraoxon-ethyl and (b) the butyryl moiety of BuCh, and (c) of the F295L mutant with the butyryl moiety of BuCh. The side chains of F295, L295 and the catalytic serine S203 are displayed (carbon: light grey, phosphorus: dark grey, oxygen: black).

Table 1
Biochemical characterization of w.t. rbAChE, loop and F295L mutant, and dAChE using the substrates ASCh and BuSCh

	Acetylthiocholine			Butyrylthiocholine		
	k_{cat} (10^5 min^{-1})	K_m (10^{-5} M)	k_{cat}/K_m (10^6 min^{-1} M^{-1})	k_{cat} (10^5 min^{-1})	K_m (10^{-5} M)	k_{cat}/K_m (10^6 min^{-1} M^{-1})
W.t. rbAChE	0.26 ± 0.01	12.7 ± 0.6	205	no hydrolysis		
Loop mutant	0.25 ± 0.01	12.5 ± 1.2	200	0.03 ± 0.01	204 ± 4	1.5
F295L mutant	0.06 ± 0.01	169 ± 7	3.6	0.034 ± 0.002	36 ± 0.5	9.4
dAChE	0.28 ± 0.06	7.1 ± 0.6	39	0.18 ± 0.01	43 ± 0.4	41

K_m and k_{cat} as calculated from Eq. (1). K_{ss} for hydrolysis of ASCh by w.t. rbAChE was 16.9 ± 3.6 mM, K_{ss} values of the mutants and for hydrolysis of BuSCh are not shown.

3.4. Substrates

Hydrolysis of two substrates of different size, ASCh and BuSCh, by w.t. rbAChE, the loop mutant and the F295L mutant were measured (Table 1). ASCh was an equally good substrate to both w.t. rbAChE and the loop mutant, but was much less efficiently hydrolyzed by the F295L mutant. The decrease of selectivity k_{cat}/K_m by a factor 57 was mainly due to a 13-fold increase of the Michaelis constant K_m, while the turnover number k_{cat} decreased only 4-fold. The more bulky substrate BuSCh was hydrolyzed by both mutants, whereas w.t. rbAChE did not show any hydrolytic activity. The F295L mutant was even 2.6-fold more active toward BuCh than toward ASCh.

3.5. Organophosphate inhibitors

Inhibition of ASCh hydrolysis by five organophosphate inhibitors were measured (Table 2). The absolute value of inhibition constant k_i varied by a factor of 1000 for the w.t. enzyme and 300 for the F295L mutant, depending on the structure of the organophosphate inhibitor. This considerable difference in inhibitory potential is due to the size of the alcohol moiety, the chemical structure of the leaving group and the presence of a phosphorothioate group instead of phosphate. Mutation of the acyl binding pocket increased the sensitivity toward all five organophosphate inhibitors. The increase in sensitivity was most pronounced for the bulky ethoprophos, which was the weakest inhibitor of w.t. rbAChE. The loop mutant was more sensitive toward this inhibitor (factor 2.6), while the F295L mutant showed the largest increase in sensitivity (factor 80), compared to the other inhibitors. For paraoxon-ethyl, the effect of the mutations was less pronounced. W.t. rbAChE was efficiently inhibited, and mutation of the loop did hardly change the sensitivity of the enzyme, whereas the F295L mutation increased sensitivity 7-fold. Compared to paraoxon-ethyl, paraoxon-methyl had a slightly reduced inhibition efficiency toward the w.t. rbAChE. The increase of sensitivity by the F295L mutation was only 2-fold. As for paraoxon-ethyl, the loop mutant behaved as the w.t. rbAChE. Inhibition of w.t. rbAChE was decreased nearly 300-fold, if the phosphate

Table 2
Inhibition constants of w.t. rbAChE, loop and F295L mutant, and dAChE for five organophosphate inhibitors

	Dichlorvos	Paraoxon-methyl	Parathion	Paraoxon-ethyl	Ethoprophos
			k_i (10^3 M^{-1} min^{-1})		
W.t. rbAChE	36 ± 1.0	520 ± 20	2.9 ± 0.8	800 ± 25	0.69 ± 0.1
Loop mutant	41 ± 1.3	550 ± 50	1.8 ± 0.1	750 ± 40	1.8 ± 0.4
F295L mutant	57 ± 0.9	1200 ± 90	17 ± 0.6	5500 ± 80	56 ± 9.4

Table 3
Inhibition constants of w.t. rbAChE, loop and F295L mutant, and dAChE for three carbamate inhibitors

	Aldicarb	Carbofuran	Furathiocarb
	k_i (10^3 M^{-1} min^{-1})		
W.t. rbAChE	5.0 ± 0.7	290 ± 16	2.4 ± 0.4
Loop mutant	3.9 ± 0.9	250 ± 18	2.8 ± 0.5
F295L mutant	12.0 ± 1.0	710 ± 15	12.0 ± 2.0

group in paraoxon-ethyl was exchanged for a phosphorothioate group in parathion. However, the effect of the F295L mutation was similar for both inhibitors. Dichlorvos differs from paraoxon-methyl by its leaving group, which reduced its inhibition efficiency by more than an order of magnitude. However, the relative inhibition of w.t. and mutant rbAChEs was similar as for paraoxon-methyl.

3.6. Carbamate inhibitors

The most efficient carbamate inhibitor for rbAChE was carbofuran (Table 3). Aldicarb and carbofuran both have an identical, small carbamoyl moiety, but a different chemical structure of the leaving group, which accounts for a factor of 60 in inhibition efficiency. Increasing the size of the carbamoyl moiety decreased sensitivity by an even larger factor of 120 by comparing carbofuran to furathiocarb. The loop mutant had about the same sensitivity as the w.t. rbAChE for all three carbamates, while the F295L mutant had a 2-fold and 5-fold sensitivity toward aldicarb and carbofuran, and furathiocarb, respectively.

4. Discussion

4.1. Acyl binding site

rbAChE was compared to dAChE which hydrolyzes BuSCh [39]. Sequence alignment of rbAChE and dAChE pointed to two major differences. First, position 295 is phenylalanine in rbAChE instead of leucine in dAChE. Second, loop 290–293 in rbAChE is by two residues longer than the respective loop 363–364 in dAChE. Although these amino acids are not directly in contact with the substrate or inhibitor, shortening by two amino acids may lead to an increased flexibility of the acyl binding pocket. To investigate the contribution of F295 and loop length to substrate specificity and sensitivity toward organophosphate and carbamate inhibitors, two independent mutations of F295 and of loop length were performed, and the mutant enzymes characterized with substrates and inhibitors of different size.

4.2. Hydrolysis of substrates

Increasing the acyl binding pocket by replacing F295 with leucine had two effects: (1) the mutant hydrolyzes BuSCh, and (2) its catalytic activity toward ASCh decreased. Transfer of BuCh activity to rbAChE was in accordance with previous experiments on human AChE, where F295 was replaced with alanine [22,25] and leucine [22], and with mouse AChE, where F295 was replaced with leucine [23,24] and tyrosine [24]. Notably, optimal fit of substrate and binding site were prerequisite to efficient catalysis, since catalytic activity decreased if the binding site was too small (w.t. enzyme hydrolyzing BuSCh) and if it was too large (F295L mutant hydrolyzing ASCh).

The similarity of K_m and k_{cat} of w.t. AChE and loop mutant toward the substrate ASCh indicated that the structure of the acyl binding site was not substantially changed by this mutation. However, it increased the flexibility of the acyl binding pocket, since the larger substrate BuSCh was hydrolyzed by the loop mutant, but not by the w.t. enzyme.

4.3. Inhibition by organophosphates

Since no structure of a complex of AChE with any organophosphate has been published yet, we modeled the complex based on X-ray

structures of the homologous lipase from *C. rugosa* inhibited by menthyl-hexyl-phosphonate [16]. For BuChE and AChE, it has been demonstrated that the size of the acyl binding pocket mediates sensitivity and stereospecificity toward optically active phosphonates of different size [40]. We have shown that increasing the size of the acyl binding pocket increased sensitivity toward organophosphate inhibitors. These changes depended on the chemical structure of the organophosphate. By modeling the interaction of rbAChE with organophosphate inhibitors, one of the two alcohol moieties of the bound organophosphate was predicted to bind to the acyl binding pocket. This model was confirmed by the experimental observation that the relative increase of sensitivity by mutating this pocket depended on the size of the alcohol moiety: the larger the alcohol moiety the larger the effect, whereas towards inhibitors with different leaving groups of phosphorothioate instead of phosphate, the effect of the mutation was similar, as concluded from the similarity of the ratio R_{F295L} of k_i between the F295L mutant and w.t. rbAChE for two pairs of inhibitors (ratio $R_{F295L} = k_i^{F295L}/k_i^{w.t.}$): paraoxon-methyl ($R_{F295L} = 2.3$)/dichlorvos ($R_{F295L} = 1.6$), which differ only in their leaving group, and paraoxon-ethyl ($R_{F295L} = 7$)/parathion ($R_{F295L} = 6$) with phosphorothioate instead of phosphate. Thus, the size of the alcohol moiety was the major factor which dominated the interaction with the acyl binding pocket. Mutating F295 to leucine increased the sensitivity of rbAChE toward organophosphates with increasing size of the alcohol moiety: $R_{F295L} = 2.3$, 7 and 80 for paraoxon-methyl, paraoxon-ethyl and ethoprophos, respectively. In addition, increasing the flexibility of the acyl binding pocket in the loop mutant increased its sensitivity for bulky inhibitors. The ratio of k_i between loop mutant and w.t. rbAChE was 2.6 for ethoprophos, while there was no difference in sensitivity for the smaller organophosphates. The effect of this mutation could be explained by an increased flexibility of the acyl binding pocket, as it was already observed for substrate specificity.

4.4. Inhibition by carbamates

The factors which govern the binding of carbamates are different from organophosphates. The most prominent result for carbamates was that even carbamates with very bulky carbamoyl moieties still bind well to rbAChE. k_i of furathiocarb was more than three times the k_i of ethoprophos, while the size of its carbamoyl moiety is two times the size of the alcohol moiety of ethoprophos. We concluded from this observation that carbamates were likely to bind differently from organophosphates. For the binding of carbamate inhibitors, no structure data for any α/β hydrolase is available. The only indication how a carbamate could bind to AChE comes from X-ray studies on the inhibition of serine proteases by *N*-peptidyl-*O*-aroylhydroxylamine [19]. The inhibitor binds to the protease in a carbamate-like structure. Although the inhibitor is peptide-like, it did not bind in a substrate-analogous orientation, but protrudes out of the active site. To probe the interaction of carbamate and rbAChE, we compared the effect of the F295L mutation on sensitivity toward aldicarb, furathiocarb and carbofuran. The latter two differ considerably in the length of their carbamoyl moiety, while aldicarb and carbofuran differ in their leaving group. The ratio of k_i for aldicarb and carbofuran was identical ($R_{F295L} = 2.4$) and similar to R_{F295L} of the organophosphates paraoxon-methyl and dichlorvos. All four inhibitors have the same size of carbamoyl or alcohol moiety, but differ in their leaving group. Thus, we concluded that small carbamates may bind with their carbamoyl moiety to the acyl binding pocket like organophosphate inhibitors. Increasing drastically the carbamoyl moiety, as furathiocarb relative to carbofuran, decreased the inhibition constant only 120-fold and doubled relative inhibition efficiency for the F295L mutant ($R_{F295L} = 5$). Since furathiocarb is too bulky to fit into the

acyl binding pocket of either w.t. or mutant rbAChE, we concluded that there must be an alternative binding mode of large carbamates, which allow them to inhibit rbAChE, in contrast to organophosphates.

Our results demonstrated that protein engineering of recombinant rbAChE can be applied to change the specificity profile of this enzyme, and to increase its activity for non-natural substrates and its sensitivity toward organophosphate and carbamate inhibitors.

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