The ‘aromatic patch’ of three proximal residues in the human acetylcholinesterase active centre allows for versatile interaction modes with inhibitors

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The role of the functional architecture of the human acetylcholinesterase (HuAChE) active centre in accommodating the non-covalent inhibitors tacrine and huperzine A, or the carbamates pyridostigmine and physostigmine, was analysed using 16 mutants of residues lining the active-centre gorge. Despite the structural diversity of the ligands, certain common properties of the complexes could be observed: (a) replacement of aromatic residues Tyr¹³³, Tyr³³⁷ and especially Trp⁸⁶, resulted in pronounced changes in stability of all the complexes examined; (b) effects due to replacements of the five other aromatic residues along the active-centre gorge, such as the acyl pocket (Phe²⁹⁵, Phe²⁹⁷) or at the peripheral anionic site (Tyr¹²⁴, Trp²⁸⁶, Tyr³⁴¹) were relatively small; (c) effects due to substitution of the carboxylic residues in the gorge (Glu²⁰², Glu⁴⁵⁰) were moderate.

These results and molecular modelling indicate that the aromatic side chains of residues Trp⁸⁶, Tyr¹³³ and Tyr³³⁷ form together a continuous ‘aromatic patch’ lining the wall of the active-centre gorge, allowing for the accommodation of the different ligands via multiple modes of interaction. Studies with HuAChE mutants carrying replacements at positions 86, 133 and 337 indicate that the orientations of huperzine A and tacrine in the HuAChE complexes in solution are significantly different from those observed in X-ray structures of the corresponding complexes with *Torpedo californica* AChE (TcAChE). These discrepancies may be explained in terms of structural differences between the complexes of HuAChE and TcAChE or, more likely, by the enhanced flexibility of the AChE active-centre gorge in solution as compared with the crystalline state.

INTRODUCTION

The catalytic efficiency of acetylcholinesterase (AChE, EC 3.1.1.7) and its high reactivity towards various covalent and non-covalent inhibitors are believed to be determined by the unique architecture of the AChE active centre, currently investigated by X-ray crystallography [2–5] and site-directed mutagenesis [6–10]. The crystallographic structure of AChE is characterized by a deep and narrow ‘gorge’, which penetrates halfway into the enzyme and contains the catalytic site at about 0.4 nm (4 Å) from its base [2]. About 40 % of the inner gorge inner surface is lined by 14 aromatic residues which are presumably involved in facilitating the two-dimensional diffusion of acetylcholine (ACh) to the active site [2], in accommodation of the substrate in the Michaelis–Menten complex and in allosteric modulation of catalysis [6,7,11,12]. Investigation of the specific roles for most of these aromatic amino acids, as well as for many other residues in the active-centre gorge, permitted identification of several functional subsites in the active-centre gorge, including the catalytic triad [Ser²⁰³⁽²⁰⁰⁾, His⁴⁴⁷⁽⁴⁴⁰⁾, Glu³³⁴⁽³²⁷⁾] [2,11]; the acyl pocket [Phe²⁹⁵⁽²⁸⁸⁾ and Phe²⁹⁷⁽²⁹⁰⁾] [6,8,9] and the ‘hydrophobic subsite’. The latter accommodates the alcoholic portion of the covalent adduct (tetrahedral intermediate) and may include residues Trp⁸⁶⁽⁸⁴⁾, Tyr¹³³⁽¹³⁰⁾, Tyr³³⁷⁽³³⁰⁾ and Phe³³⁸⁽³³¹⁾, which operate through non-polar and/or stacking interactions, depending on the substrate [6,10,13]. Stabilization of the charged moieties of substrates and other ligands at the active centre is mediated by cation– π interactions with the residue at position 86 rather than through true ionic interactions [2,3,6,13]. In addition, residues

Tyr⁷²⁽⁷⁰⁾, Tyr¹²⁴⁽¹²¹⁾, Trp²⁸⁶⁽²⁷⁹⁾ and Tyr³⁴¹⁽³³⁴⁾, which are localized at or near the rim of the active-centre gorge, together with Asp⁷⁴⁽⁷²⁾ constitute the peripheral anionic subsite of AChE [3,11,12,14].

The recent developments in our understanding of the AChE structure–function characteristics were used to guide the design of novel AChE inhibitors as potential therapeutic agents for senile dementia of the Alzheimer’s type [15]. The underlying assumption in the modelling experiments was that the solution structures of AChE–inhibitor complexes closely resemble those in the crystal. Indeed, the crystal structure of the *Torpedo californica* acetylcholinesterase (TcAChE) complex with the prototypical active-centre inhibitor edrophonium [3] is fully consistent with the findings from mutagenesis studies of the corresponding human AChE (HuAChE) complexes [6,13]. These studies demonstrated the involvement of the hydrophobic subsite residues (Trp⁸⁶, Tyr¹³³ and Tyr³³⁷), as well as of residue Glu²⁰², which is a part of the hydrogen-bond network in the active centre [10], in accommodation of the ligand and suggested a structural template for examination of HuAChE interactions with other active-centre inhibitors. On the other hand, the effects of replacement of residue Tyr³³⁷ in the mouse AChE on reactivity toward tacrine [14] appear to be incompatible with the crystallographic structure of the TcAChE–tacrine complex [3]. Furthermore, the recently published X-ray structure of the TcAChE–(–)-huperzine A complex [5] does not seem to account for the previously observed role of Tyr³³⁷ in accommodating huperzine A in complexes with mammalian cholinesterases [16–18].

To probe further the interactions of representative AChE

Abbreviations used: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; HuAChE, human acetylcholinesterase; TcAChE, *Torpedo californica* acetylcholinesterase; ACh, acetylcholine; ATC, acetylthiocholine; SAR, structure–activity relationship; Y337A etc., Tyr³³⁷ → Ala mutant etc.; amino acids and numbers refer to HuAChE (the numbers in parentheses refer to the positions of analogous residues in TcAChE according to the recommended nomenclature [1]; residues in TcAChE are shown in italics).

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Figure 1 Chemical formulae of the anti-cholinesterase agents used in the present study

At physiological pH the neutral molecules are protonated (tacrine at the endocyclic nitrogen; huperzine A at the amine substituent; physostigmine at N¹).

agents like tacrine, huperzine A or the carbamates pyridostigmine and physostigmine (see Figure 1) with HuAChE, we examined their inhibitory activity toward a defined series of enzyme mutants representing specific perturbations at the various subsites of the active-centre-hydrophobic site, acyl pocket, H-bond network and the peripheral anionic site. We show that the functional architecture of the AChE active centre, characterized in the past through studies with both covalent and non-covalent ligands, can be applied to the construction of plausible molecular models of the enzyme-inhibitor complexes. A continuum of spatially adjacent aryl moieties of the hydrophobic subsite residues Trp⁸⁶, Tyr¹³³ and Tyr³³⁷, forms an aromatic patch which appears to play a dominant role in accommodation of the structurally diverse ligands. In certain cases these models suggest also that the structure-function characteristics of AChE-inhibitor complexes may not fully correspond to the structural information from X-ray crystallography of the corresponding TcAChE complexes.

MATERIALS AND METHODS

Materials and enzymes

Acetylthiocholine iodide (ATC), 5,5'-dithiobis-(2-nitrobenzoic acid), ethyl-(*m*-hydroxyphenyl)dimethylammonium chloride (edrophonium), 3-hydroxy-1-methylpyridinium bromide dimethylcarbamate (pyridostigmine) and physostigmine salicylate were purchased from Sigma, and synthetic (±)-huperzine A was obtained from Calbiochem. The chemical structures of the AChE inhibitors are shown in Figure 1.

Expression of recombinant enzymes, as well as the construction of all the HuAChE mutants, was described previously [6,11,13,19]. Stable recombinant cell clones expressing high levels of each of the mutants were established according to the procedure described previously [19]. Enzymes were purified (over 90% purity) as described previously [12,19].

Determination of HuAChE activity and analysis of kinetic data

Assays of activity of HuAChE enzymes were carried out as described previously [6,7,13]. All the HuAChE enzymes

Scheme 1

examined, formed rapid equilibria with tacrine and huperzine A, allowing for an immediate addition of increasing amounts of ATC to the enzyme/inhibitor mixture (preincubation of the enzymes with huperzine A for 10 min before addition of the substrate or simultaneous mixing yielded the same results). Values of K_i and K_{si} (the competitive and uncompetitive inhibition constants) were determined from weighted non-linear regression analyses of eqn. (1), which is a general kinetic description of the AChE catalysis inhibition scheme [6,13,16], assuming that v has a constant percentage error (experimental values were weighted by $1/v^2$; Prism V2.0, GraphPad Software Inc.):

$$v = V_{\max}([S]/K_{\text{app}})\{1 + ([S]/K_{\text{app}}) + ([I]/K_i) + ([S][I]/K_{\text{app}}K_{si})\}^{-1} \quad (1)$$

The rate constants of progression of the carbamylation reactions (see Scheme 1) were estimated for at least four different concentrations (and at least 10-fold in ligand concentration, around the estimated value of K_d) of carbamate (CX), by adding substrate at various time intervals and measuring the enzyme residual activity (E). The apparent bimolecular carbamylation rate constants (k_{obs}), at different carbamate concentrations, were computed from the plot of slopes of $\ln(E)$ versus time. Only the initial slopes were considered in order to minimize the errors due to re-activation of the carbamylated enzymes. Double-reciprocal plots of k_{obs} versus [CX] were used to compute k_2 and K_d from the intercept and from the ratio of the slope and the intercept respectively, according to the equations [10]:

$$1/k = 1/k_2 + 1/k_i[\text{CX}]$$

$$K_d = k_2/k_i$$

Note that when $k_{-1} \gg k_2$, K_d approaches the value of dissociation constant for the corresponding Michaelis complex.

Molecular modelling

Building and optimization of three-dimensional models of the HuAChE complexes with the various anti-cholinesterase agents were performed on a Silicon Graphics workstation Indigo II, using the SYBYL modelling software (Tripos Inc.). The initial models were constructed by manual docking of the ligands into the HuAChE active centre [13,20] following the findings from mutagenesis studies. Positioning of the ligands was also guided by interactions with residue Trp⁸⁶ and, in the case of carbamates, by interactions with the active-site nucleophile (Ser²⁰³) and residues of the oxyanion hole. In the case of huperzine A, an additional model was constructed in which the HuAChE was modified with respect to orientation of the peptide bond Gly¹²⁰⁽¹¹⁷⁾–Gly¹²¹⁽¹¹⁹⁾. Such conformational change was reported in the past [5] and also observed during simulated annealing experiments with the ligand-free model of HuAChE [12]. Comparison of the huperzine A models, with the regular and the modified structures of HuAChE, shows the latter to be more stable.

The initial models were optimised by molecular mechanics using the MAXMIN force field (and AMBER charge parameters

for the enzyme) and zone-refined, including 127 amino acids [1.5 nm (15 Å) substructure sphere around O^γ-Ser²⁰³]. In the cases of carbamates, initial optimization included restriction of the distances between the carbonyl oxygen and the amide nitrogen atoms of residues Gly¹²¹ and Gly¹²², as well as that between the carbonyl carbon and O^γ-Ser²⁰³, which were relieved in the subsequent refinement. Initial models of HuAChE complexes with tacrine and (–)-huperzine A, where the ligand orientation mimics that observed in the corresponding X-ray structure of TcAChE complexes, were constructed using the crystallographic ligand co-ordinates [3,5]. In the huperzine A complex, the conformation of the peptide bond Gly¹²⁰⁽¹¹⁷⁾–Gly¹²¹⁽¹¹⁹⁾ was also adjusted (see above).

RESULTS

Binding activity of various HuAChE mutants towards non-covalent active-centre ligands

Interactions with tacrine

Similar to the case of the prototypic active-centre inhibitor edrophonium, the inhibitory activity of tacrine toward HuAChE enzymes is affected predominantly by replacement of the hydrophobic-pocket residues Trp⁸⁶, Tyr¹³³ and Tyr³³⁷ (see Table 1). In particular, substitution of the 'cation- π interaction subsite' Trp⁸⁶ by Ala brings about a 20000-fold increase of the corresponding value of K_i relative to the wild-type HuAChE (see Table 1), whereas mutation of this residue to another aromatic amino acid, Phe, has a much smaller effect (70-fold). This pattern

is consistent with stacking of the positively charged aromatic ligand (protonated tetrahydroacridine) against the π electron system of residue at position 86, as suggested by the crystal structure of the TcAChE–tacrine complex [3]. Replacement of residue Tyr¹³³ by Ala or by Phe results in nearly equal reduction in affinity toward tacrine (Table 1), indicating that the hydroxy group rather than the aromatic moiety of this residue participates in ligand accommodation. Replacement of the aromatic residue at position 337 by Ala *increases* the affinity of the resulting enzyme toward tacrine [the K_i value for the Y337A (Tyr³³⁷ → Ala) mutant is 11-fold lower than that for the wild-type enzyme; see Table 1]. This result is consistent with that reported for an analogous replacement in mouse AChE [14] and can partially account for the higher affinity of tacrine toward butyrylcholinesterase (BuChE) than toward AChE [14,21].

Replacement of residue Glu²⁰² by Gln as well as mutation of Glu⁴⁵⁰ to Ala result in a moderate increase of the corresponding values of K_i relative to wild-type HuAChE (6- and 7-fold respectively), demonstrating that disruption of the hydrogen-bond network [22] in the active centre has only a minor effect on the affinity toward tacrine. Therefore the 68-fold increase in the corresponding value of K_i , caused by replacement of the third constituent of this hydrogen-bond network (residue Tyr¹³³) to Phe, indicates a direct participation of this residue in accommodation of tacrine. Replacement of Glu²⁰² by Ala results in a much larger effect than the corresponding replacement by glutamine, suggesting that, in addition to the hydrogen-bond network, this residue is also involved in a direct interaction with the ligand.

Table 1 Inhibition constants of HuAChE and its mutants with active-centre ligands

HuAChE	10 ⁷ × Inhibition constant (M)		10 ⁸ × Inhibition constant (M)			
	Edrophonium*		Tacrine		Huperzine A‡	
	K_i	K_{si}	K_i	K_{si}	K_i	K_{si}
WT	7 ± 1.3	50 ± 20	8.5 ± 1.2	9 ± 2	1.6 ± 0.1	1.2 ± 0.4
Hydrophobic pocket						
W86F	380 ± 150	1300 ± 100	610 ± 100	2700 ± 500	200 ± 16	210 ± 60
W86A	(> 450 000)†	–	150 000 ± 40 000	–§	(> 100 000)†	–
Y133A	7800 ± 500	1800 ± 670	920 ± 100	1000 ± 200	120 ± 10	75 ± 7
Y337A	57 ± 1.3	210 ± 55	0.8 ± 0.2	0.8 ± 0.2	490	–
Y337F	7 ± 0.1	50 ± 3.5	21 ± 2	25 ± 5	11.3	–
F338A	8 ± 0.4	80 ± 6	4.0 ± 0.5	5 ± 1	6.5 ± 0.4	4.4 ± 0.4
H-bond network						
Y133F	180 ± 10	1100 ± 60	500 ± 80	950 ± 180	3.2 ± 0.4	7.6 ± 0.8
E202A	1860 ± 70	5000 ± 1000	330 ± 15	1600 ± 300	160 ± 30	–§
E202Q	128 ± 5	830 ± 100	45 ± 2	73 ± 8	15	–
E450A	29 ± 4	–§	53 ± 5	87 ± 7	0.6 ± 0.1	–§
Acyl pocket						
F295A	4 ± 0.7	26 ± 2	6.0 ± 0.8	3 ± 1	20.5	–
F297A	7 ± 1	120 ± 10	23 ± 1.2	72 ± 7	6.2 ± 0.7	8 ± 2
Peripheral site						
D74N	35 ± 0.8	280 ± 70	460 ± 70	6500 ± 1500	1.7 ± 0.2	2.5 ± 0.5
Y124A	15 ± 0.6	90 ± 6	3 ± 0.4	2.5 ± 0.4	1.8 ± 0.3	6 ± 1.6
W286A	6 ± 0.3	27 ± 0.3	8 ± 1	15 ± 2	7.4	–
Y341A	21 ± 1.3	150 ± 2	12.0 ± 1.5	28 ± 4	3.4 ± 0.1	2.2 ± 0.4

* Values of K_i compiled from our previous studies [8,18] are presented here for comparison.

† Values in parentheses refer to the highest concentration used (45 mM for edrophonium; 1 mM for huperzine A); yet these concentrations were still insufficient to cause detectable inhibition of activity.

‡ Racemic mixture.

§ Values of standard error were too large to permit accurate determination of K_{si} .

|| Values reported previously [24].

Figure 2 Stereo view of models of HuAChE complexes with tacrine and (–)-huperzine A

(A) HuAChE–tacrine complex (model according to mutagenesis). The ligand exocyclic amine is within H-bond distance from *O*⁴-Tyr¹³³ and the tetrahydroacridinium nitrogen is within interaction (cation– π) distance from the indole moiety of residue Trp⁸⁶. (B) HuAChE–tacrine complex (model according to the X-ray structure of the TcAChE complex). The optimized HuAChE model is similar to the X-ray structure with respect to the relative orientation of the ligand [3]. (C) HuAChE–(–)-huperzine A complex (model according to mutagenesis). The ammonium nitrogen of the ligand is closer to the aromatic moiety of Trp⁸⁶ than to that of Tyr³³⁷ and shows no indication of H-bonding between the 2-pyridone oxygen and *O*⁴-Tyr¹³³. (D) HuAChE–(–)-huperzine A complex (according to the X-ray structure of the TcAChE complex [5]). The ammonium nitrogen of the ligand points toward the gorge entrance and is within similar distances from the aromatic moieties of Trp⁸⁶ and Tyr³³⁷. The 2-pyridone oxygen is within H-bond distance from *O*⁴-Tyr¹³³.

Mutation of residues of the acyl pocket and of the peripheral anionic site have either minor effects or no effect on the inhibitory activity of tacrine. The only outstanding case is that of the D74N enzyme, where the corresponding value of K_i was 60-fold higher than that for the wild-type HuAChE (Table 1). It appears that, as already proposed in the past [11,12], replacement of Asp⁷⁴ affects the binding properties of the active centre, since, according to the results from mutagenesis of other peripheral anionic site residues (Table 1) and the X-ray structure of its complex with TcAChE [3], tacrine is primarily an active-centre ligand. A similar reduction in affinity towards tacrine (150-fold increase in the value of K_i relative to the wild-type enzyme) was reported for the ‘atypical’ BuChE variant [21], where Asp⁷⁰ (corresponding to Asp⁷⁴ in HuAChE) is replaced by glycine.

Modelling of the HuAChE–tacrine complex was guided by (a) stacking interaction of the protonated tetrahydroacridinium moiety with the indole ring of residue Trp⁸⁶, and (b) interaction of the hydroxy group of Tyr¹³³ with the amine substituent of tacrine (Figure 1). According to this model, the orientation of tacrine in which the aliphatic portion is directed toward residue Tyr³³⁷ (Figure 2A) is energetically favourable by over 42 kJ/mol (10 kcal/mol) compared with that in which residue Tyr³³⁷ is juxtaposed with an aromatic ring of the ligand (not shown). Such preference is also consistent with the findings from a structure–activity relationship (SAR) study of tacrine analogues [23], which suggest that interaction with the aromatic moiety of Tyr³³⁷ is not involved in stabilizing interactions but rather seems to

hinder sterically the accommodation of tacrine in the active centre (Table 1).

Interactions with huperzine A

In a previous study with racemic huperzine A we showed that 2 mol of inhibitor were required to inhibit completely the activity of 1 site-equivalent of HuAChE [16]. This stoichiometry is consistent with the reported 38-fold stereoselectivity of mammalian AChE toward (–)-huperzine A [17]. As in the cases of edrophonium and tacrine, the single most important interaction of huperzine A is with the cation– π interaction site residue Trp⁸⁶ (Table 1). Replacement of this residue by Ala lowers the inhibitory activity of huperzine A by over 55 000-fold relative to the wild-type enzyme, while its substitution by Phe results in a 180-fold increase of the corresponding value of K_i , underscoring the role of the aromatic system at position 86 in accommodating the inhibitor. While substitution of Tyr¹³³ by Ala resulted in a 50-fold increase of the corresponding value of K_i , replacement of this residue by Phe has no effect on the affinity toward huperzine A. This observation demonstrates that residue Tyr¹³³ participates indirectly in the accommodation of huperzine A through interactions of the aromatic system rather than through the hydroxy group.

The effects of substitution of residue Tyr³³⁷ (Phe³³⁰) by Phe and by Ala in AChEs, as well as replacements of Ala³²⁸ (Phe³³⁰) in human BuChE by Phe and Tyr, have already been reported

Table 2 Kinetic constants for carbamylation reactions* of HuAChE and its selected active-centre-gorge mutants

HuAChE	Pyridostigmine			Physostigmine		
	$10^7 \times K_d$ (M)	$10^{-4} \times k_1$ ($M^{-1} \cdot \text{min}^{-1}$)	k_2 (min^{-1})	$10^7 \times K_d$ (M)	$10^{-4} \times k_1$ ($M^{-1} \cdot \text{min}^{-1}$)	k_2 (min^{-1})
WT	$18 \pm 5^\dagger$	$43.5 \pm 1.6^\dagger$	$0.77 \pm 0.3^\dagger$	$3.4 \pm 1^\ddagger$	350 ± 20	$1.2 \pm 0.5^\ddagger$
Hydrophobic pocket						
W86F	2300 ± 500	0.3 ± 0.05	0.65 ± 0.2	4.6 ± 1	190 ± 2	0.88 ± 0.15
W86A	$79\,000 \pm 2400$	0.009 ± 0.001	0.68 ± 0.2	94.5 ± 40	8.2 ± 0.7	0.78 ± 0.3
Y133A	$94\,400 \pm 20\,000$	0.02 ± 0.001	1.8 ± 0.3	820 ± 150	0.5 ± 0.1	0.44 ± 0.02
Y337A	105 ± 27	7.6 ± 1	0.8 ± 0.1	9.5 ± 3.5	120 ± 5	1.1 ± 0.5
Y337F	22 ± 6	31.4 ± 1.1	0.68 ± 0.16	5.2 ± 1.5	360 ± 50	1.9 ± 0.4
F338A	48 ± 15	25 ± 1.3	1.2 ± 0.4	4.2 ± 1	100 ± 3	0.43 ± 0.2
H-bond network						
Y133F	120 ± 30	1.4 ± 0.06	0.2 ± 0.04	6.9 ± 1	19 ± 0.2	0.13 ± 0.01
E202Q	410 ± 80	$2.5 \pm 0.03^\S$	1.0 ± 0.2	17.5 ± 4.6	43 ± 1	0.76 ± 0.18
E202A	50 ± 15	4.2 ± 0.5	0.2 ± 0.05	31.5 ± 4	7.7 ± 1	0.24 ± 0.05
E450A	120 ± 34	1.3 ± 0.2	0.15 ± 0.02	4.6 ± 0.5	26.8 ± 0.3	0.15 ± 0.01
Acyl pocket						
F295A	2.7 ± 0.5	62 ± 3.4	0.17 ± 0.02	0.9 ± 0.3	1770 ± 60	1.6 ± 0.5
F297A	125 ± 40	5.1 ± 1	0.64 ± 0.1	3.8 ± 1	160 ± 15	0.6 ± 0.1
Peripheral site						
D74N	3600 ± 730	0.3 ± 0.03	0.98 ± 0.1	17 ± 4	140 ± 4	2.4 ± 0.5
Y124A	6 ± 0.8	13.8 ± 0.44	0.1 ± 0.01	6 ± 1.8	150 ± 3	0.91 ± 0.26
W286A	124 ± 35	14 ± 0.6	1.8 ± 0.5	2.7 ± 0.8	365 ± 40	1.0 ± 0.2
Y341A	61 ± 8	16.6 ± 0.5	1.0 ± 0.1	1.9 ± 0.3	425 ± 15	0.8 ± 0.1

* Rate and dissociation constants are those depicted in Scheme 1 in the Materials and methods section.

† The corresponding values computed for mouse AChE were: $k_1 = 49 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$; $k_2 = 4.6 \text{ min}^{-1}$; $K_d = 95 \times 10^{-7} \text{ M}$ [8].

‡ The values reported from direct stop-flow measurements for electric-eel AChE were: $K_d = 4.3 \times 10^{-7} \text{ M}$; $k_2 = 2.5 \text{ min}^{-1}$ [25].

§ The ratio of k_1 values of wild-type TcAChE to the E199Q mutant was reported as 21 [26] compared with the ratio of 17 in the corresponding HuAChE enzymes.

[16–18] to demonstrate that this residue participates in accommodation of huperzine A predominantly through interactions of its aromatic moiety. In addition, it was shown [17] that, unlike fetal-bovine serum AChE and TcAChE, which were stereoselective toward (–)-huperzine A versus the corresponding (+) stereoisomer (35-fold and 95-fold respectively), mammalian BuChEs displayed no such stereoselectivity. In the latter, the position corresponding to 337(330) is replaced by Ala. All these findings are consistent with the notion that the stereoselectivity of AChEs toward huperzine A stereoisomers is determined mainly by the substituent at position 337, as suggested previously [17].

The relative affinities of huperzine A toward the Y133F, E202Q and E450A mutant enzymes demonstrate that disruption of the hydrogen-bond network does not, in itself, affect binding. The moderate effect due to replacement of Glu²⁰² by Gln and the more pronounced effect due to its replacement by Ala (relative values of K_d were 8- and 100-fold respectively compared to wild-type HuAChE) indicate that Glu²⁰² interacts directly with the ligand, probably through electrostatic attraction with the ammonium moiety of huperzine A, which can be partially maintained by Gln, but not by Ala, at this position.

Modelling of a structure of HuAChE(–)-huperzine A complex that is consistent with the mutagenesis data (Figure 2C), was guided mainly by substantial interaction of the protonated ammonium group with Trp⁸⁶ and of the pyridone oxygen of the ligand (Figure 1) with active-site residues. The model (Figure 2C) provides an explanation for the contribution of the aromatic moiety of residue Tyr³³⁷ to the binding of huperzine A. It is also consistent with the marginal effects on inhibition due to perturbations of the hydrogen-bond network (i.e. minimal in-

teraction of the pyridone ring with Glu²⁰²). The relative position of the ligand in our model of HuAChE(–)-huperzine A complex is somewhat similar to that proposed earlier [17]. In particular, the ammonium group points away from residue Asp⁷⁴ at the gorge entrance and is much closer to the aromatic moiety of Trp⁸⁶ than to that of Tyr³³⁷. In this model the pyridone oxygen does not interact with the hydroxy group of residue Tyr¹³³ (in accordance with mutagenesis data).

Reactivity of HuAChE mutants towards carbamates

Although carbamates react with AChE to yield covalent adducts, their anti-cholinesterase potency seems also to be critically dependent upon their complementarity with the architecture of the active centre. Structure–activity relationship studies with various carbamates suggest that structural variability affects the affinity towards the enzyme (K_d), rather than the rate of carbamyl-AChE adduct formation (k_2 ; see Scheme 1) [24,25]. Thus reactivities of carbamates with the HuAChE enzymes modified at the active-centre gorge should reflect mainly the relative stabilities of the corresponding Michaelis complexes, and be comparable with those HuAChE complexes with noncovalent inhibitors like tacrine, huperzine A or edrophonium. To examine this issue kinetics of inhibition by pyridostigmine and physostigmine was studied under conditions which allowed for derivation of values for K_d and k_2 (see the Materials and methods section and Scheme 1). As predicted, the kinetic constants of these reactions (see Table 2) demonstrate that, for both agents, most of the kinetic variability, brought about by the structural modifications of the enzymes, is in the values of the Michaelis-complex dissociation constants (K_d) (see Scheme 1).

Figure 3 Stereo view of the HuAChE-carbamate Michaelis complexes

In both structures the carbamyl oxygen is positioned proximal to the amide nitrogen atoms of Gly¹²¹ and Gly¹²² (oxyanion hole). **(A)** HuAChE-pyridostigmine complex; the pyridinium methyl substituent is within interaction distance from the aromatic moieties of Trp⁸⁶ and Tyr¹³³. **(B)** HuAChE-physostigmine complex; the conformations of the Trp⁸⁶ and Tyr¹³³ side chains are quite different from those in the free enzyme.

Effect of substitution of HuAChE active-centre gorge residues on the non-covalent interactions with physostigmine and pyridostigmine

Unlike the dramatic effects of replacing Trp⁸⁶ by Ala on the inhibitory activities of the non-covalent inhibitors studied, the corresponding effects for both carbamates are more moderate and comparable with those resulting from replacing Tyr¹³³ with Ala (Table 2). In addition, whereas the inhibition constants of the W86A and Y133A enzymes by pyridostigmine decreased 4400- and 5250-fold respectively, relative to the wild-type HuAChE, the corresponding constants of physostigmine decrease by merely 20- and 240-fold respectively. Replacement of Tyr³³⁷ by Ala resulted in a 6- and 3-fold effect for pyridostigmine and physostigmine respectively, while replacement of this residue by Phe is of no consequence. A minor effect on the K_i value of pyridostigmine was observed, also attributable to a mutation of residue Phe³³⁸ to Ala.

Replacement of residue Tyr¹³³ by Phe and of Glu⁴⁵⁰ by Ala had equivalent effect on the affinity of the resulting enzymes toward pyridostigmine, whereas the corresponding effects for physostigmine were negligible (Table 2). Replacement of Glu²⁰², the third residue of the hydrogen-bond network, by Gln had somewhat larger effects on the affinity toward both inhibitors. For pyridostigmine this reactivity pattern is consistent with the involvement of this network in facilitating the interaction of Glu²⁰² carboxylate with the aromatic ring of the inhibitor, as already proposed for edrophonium. On the other hand, the observation that affinity of E202A toward pyridostigmine was higher than that of E202Q, is puzzling, since the reactivity order is reversed in these mutants for all the other inhibitors tested (see Tables 1 and 2). The importance of Glu^(199/202) in interaction of AChEs with physostigmine was reported in the past by Radic et al. [26]. It is also noteworthy that, of all the residue substitutions at the HuAChE active-centre gorge, only those related to the hydrogen-bond network had somewhat more significant effect on the values of the carbamylation rate constants (k_2). Similar characteristics of these mutant HuAChEs have already been reported for reactions with organophosphates [10].

Both pyridostigmine and physostigmine seem to interact with the acyl pocket residues Phe²⁹⁵ and Phe²⁹⁷. Replacement of Phe²⁹⁵ by Ala results in a small, but significant, increase in affinity for both inhibitors. This may indicate that the methyl substituents in both the dimethylamino (pyridostigmine) and the monomethylamino (physostigmine) carbamates are oriented

toward this residue. Analogous substitution of residue Phe²⁹⁷ affects only the affinity towards the dimethylamino carbamate, pyridostigmine.

Substitution of the aromatic elements of the peripheral anionic site had only minor effects on the affinities of the resulting enzymes toward carbamates, as observed also in the cases of edrophonium, huperzine A or tacrine (see Tables 1 and 2). On the other hand, the dissociation constant of the D74N enzyme by pyridostigmine is 200-fold higher compared with the wild-type enzyme, whereas the corresponding effect on physostigmine was only a 5-fold increase. Such outstanding influence of residue Asp⁷⁴ could be attributed to the formal positive charge of the pyridostigmine molecule, yet an analogous effect is also observed in the case of the formally neutral tacrine.

Modelling of the HuAChE-pyridostigmine and HuAChE-physostigmine Michaelis complexes

The model of the HuAChE-pyridostigmine Michaelis complex (Figure 3A) is consistent with the large and nearly equivalent contributions of the aromatic rings of residues Trp⁸⁶ and Tyr¹³³ to the accommodation of pyridostigmine (the corresponding values of K_d are 4400- and 5250-fold higher than those for the wild-type enzyme), as well as with the less pronounced participation of residue Tyr³³⁷. In addition, the involvement of both residues of the acyl pocket (Phe²⁹⁵, Phe²⁹⁷) is compatible with the location of the dimethylamino carbamyl moiety. The model is also in agreement with the effects of perturbations of the hydrogen-bond network, through replacements of residues Tyr¹³³ and Glu⁴⁵⁰. The model does not suggest a straightforward explanation for the changes in the apparent dissociation constant K_d due to replacements of residue Glu²⁰² or of the peripheral anionic site residues Asp⁷⁴ and Trp²⁸⁶.

As in case of pyridostigmine, model building of the HuAChE-physostigmine Michaelis complex was guided by the requirement for a properly juxtaposed carbamyl moiety relative to the elements of HuAChE catalytic machinery and the oxyanion hole. Accommodation of the ligand induced considerable conformational changes in the side chains of residues Trp⁸⁶ and Tyr¹³³ (see Figure 3B). These conformational changes separate the aromatic moieties of Trp⁸⁶ and Tyr¹³³, eliminating the aromatic-aromatic interaction between the two residues, which has been implicated in maintaining the active conformation of the anionic subsite, Trp⁸⁶ [13]. The relatively strained side-chain confor-

mations of residues Trp⁸⁶ and Tyr¹³³ around the charged region of the physostigmine may account for the relatively small decrease in affinity, due to replacements of these residues by Ala, since the loss of cation- π interaction energy is partially offset by removal of steric strain. The model seems to be compatible with the anti-cholinesterase activities of physostigmine analogues bearing replacements of the neutral (N⁸) and the protonated (N¹) nitrogen atoms. Replacement of N⁸ by carbon has little effect on the inhibitory activity [27], supporting the conclusion that this nitrogen atom is not involved in specific interactions of physostigmine with the enzyme. The AChE inhibitory activity of physostigmine analogues bearing oxygen at position 1 (physovenins) [28] is also consistent with the proposed model, since the O¹ oxygen can readily form a hydrogen bond with the hydroxy moiety of Tyr¹³³.

DISCUSSION

The aromatic patch, consisting of residues Trp⁸⁶, Tyr¹³³ and Tyr³³⁷, allows for accommodation of the different covalent and non-covalent HuAChE ligands

In the present study we have examined the contribution of 12 HuAChE active-centre residues, including nine out of the 14 aromatic amino acids lining the active-site gorge [3], to the accommodation of certain active-centre ligands which are likely to serve as lead compounds for the design of new HuAChE inhibitors. From the six aromatic residues adjacent to the active site, Trp⁸⁶ and Tyr¹³³, and to a lesser extent Tyr³³⁷, appear to be the major participants in the stabilization of HuAChE complexes, *irrespective* of the ligand structure. Such an ability of these elements of the HuAChE hydrophobic subsite to accommodate structurally diverse ligands originates from the different modes of interaction exhibited by these residues, including polar (cation- π , hydrogen-bond) and non-polar (aromatic-aromatic, hydrophobic), as well as from the spatial organization of their aromatic moieties which form an aromatic patch directly above the catalytic Ser²⁰³. The extent of contact with this elongated patch and the contribution of the different interaction modes depend on the structure of the ligand and partially determine the binding specificity. Participation of residue Phe³³⁸, also associated with the hydrophobic subsite, and of the acyl pocket residues Phe²⁹⁵ and Phe²⁹⁷ is less significant and is characterized mostly by non-specific interactions. The three aromatic residues of the peripheral anionic site, Tyr¹²⁴, Trp²⁸⁶ and Tyr³⁴¹, do not contribute to the stabilization of HuAChE complexes with the active-centre ligands examined here, as shown in the past for other active-centre ligands such as substrates or organophosphate inhibitors [3,7,10,12,14].

In the detailed analysis presented above, we assume that residue replacements result only in a local perturbation of the active-centre functional architecture. That such structural integrity of the active centre is likely to be maintained is suggested by the pronounced reactivity of *all* the mutant enzymes examined here toward substrates and covalent inhibitors. Even replacement of the cation- π subsite residue Trp⁸⁶ by Ala, which may presumably create a void in the active centre, results in an HuAChE mutant enzyme exhibiting a nearly wild-type reactivity toward non-charged substrates and organophosphorus inhibitors [6,10].

The AChE active centre may accommodate the same ligand in more than one orientation

In the crystallographic structure of the TcAChE-tacrine complex, residues Trp⁸⁴ and Phe³³⁰ seem to participate in a similar manner

in ligand accommodation, through aromatic-aromatic interactions with the protonated acridinium moiety [3]. The protonated ring nitrogen forms a hydrogen bond with the carbonyl group of the catalytic His⁴⁴⁰ and the exocyclic amine group projects toward the gorge opening (see Figure 2B for analogous model of the HuAChE-tacrine complex). However, such orientation of the ligand appears to be at odds with previous observations from SAR studies of tacrine analogues [23,29] as well as with the mutagenesis data reported here. According to the X-ray structure, the aromatic ring C of tacrine (Figure 1) is the ligand moiety participating in stacking interaction with the phenyl group of Phe³³⁰. Yet, comparison of the AChE inhibitory activities of tacrine and its octahydroacridine analogue demonstrated [23] that the aromaticity of ring C (see Figure 1) is of no consequence to the inhibitory activity (the respective values of K_i are within factor 2; see [22]). As already mentioned, replacement of Tyr³³⁷(Phe³³⁰) by Ala increased the affinity of the resulting HuAChE enzyme toward tacrine, implying that the aromatic moiety at position 337 may present a steric obstruction to ligand binding rather than participating in stabilizing interactions. Also, from the X-ray structure of the complex one could predict that replacement of residue Phe³³⁰ by tyrosine should allow for an additional hydrogen bond with the amine substituent of tacrine (see Figure 2B). In HuAChE, tyrosine is actually present at position 337(330), yet its replacement by Phe had only a marginal effect on enzyme affinity toward tacrine. Furthermore, while removal of the hydroxy group from position 133 of HuAChE results in a 68-fold decrease of the tacrine inhibitory activity toward the Y133F enzyme, the X-ray structure of the complex does not show any direct involvement of residue Tyr¹³⁰ in the accommodation of tacrine. In conclusion, both kinetic studies with tacrine derivatives and inhibition patterns with the Y337F, Y337A, Y133F and Y133A HuAChE enzymes suggest that the crystallographic orientation of the ligand may not be unique in the different AChE complexes.

Similarly, in the recently described X-ray structure of TcAChE-(–)-huperzine A complex [5], two principal protein-ligand interactions were pointed out: (a) hydrogen-bond of the pyridone oxygen to the hydroxy group of Tyr¹³⁰, (b) cation- π interaction of the protonated amino group of the ligand with the aromatic moieties of residues Trp⁸⁴ and Phe³³⁰ [distances between the nitrogen and the centroids of the rings 0.48 and 0.47 nm (4.8 and 4.7 Å) respectively] [5]. In contrast with these observations, we find that: (a) replacement of residue Tyr¹³³(130) by Phe has practically no effect on the affinity toward huperzine A, suggesting that, in the HuAChE-huperzine A complex, there is no hydrogen bond between residue Tyr¹³³ and the ligand; (b) the inhibitory activity of huperzine A toward W86(84)A is over 55000-fold lower than that toward the wild-type enzyme, whereas the corresponding ratio for the Y337F and Y337A mutant enzymes is only 43-fold. Such large difference in the roles of the aromatic residues at positions 86 and 337 is difficult to reconcile with the crystallographic position of the ligand ammonium group relative to these residues in the TcAChE-(–)-huperzine A complex.

The apparent inconsistencies between the X-ray data for the TcAChE complexes with tacrine and huperzine A and the mutagenesis data for the corresponding HuAChE complexes may simply point to the actual differences between the complex structures of HuAChE and TcAChE with the same ligand, owing to differences in their respective active-centre regions. Such an explanation does not seem to account for the following observations: (a) the only difference in the composition of the amino acids lining the AChE active-centre gorge (over 30 residues), is Phe at position 330(337) of TcAChE instead of Tyr in the

mammalian AChEs; (b) the recently reported crystallographic analyses of the fasciculin complexes with TcAChE and with HuAChE demonstrate the nearly equivalent molecular architectures of the active-centre regions for the two AChEs [4,30]. It is therefore not unlikely that the differences between the X-ray data and the mutagenesis results reflect a somewhat different accommodation of the same ligand when the AChE binding environment is modified by the protein transition from the crystal to solution. We note that the flexibility of the active-centre gorge, in response to external signals, is currently regarded as essential for the AChE enzymic activity and for its modulation by peripheral anionic site ligands [12,31,32], presumably involving conformational changes of the active-centre binding environment [7,12].

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