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Accommodation of physostigmine and its analogs by acetylcholinesterase is dominated by hydrophobic interactions

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Abstract

The role of the functional architecture of the human acetylcholinesterase (HuAChE) in reactivity toward the carbamates pyridostigmine, rivastigmine and several analogs of physostigmine, that are currently used or considered for use as drugs for Alzheimer's disease, was analysed using over 20 mutants of residues that constitute the interaction subsites in the active center. Both steps of the HuAChE carbamylation reaction, formation of the Michaelis complex as well as the nucleophilic process, are sensitive to accommodation of the ligand by the enzyme. For certain carbamate - HuAChE combinations, the mode of inhibition shifted from a covalent to a noncovalent type, according to the balance between dissociation and covalent reaction rates. Whereas the charged moieties of pyridostigmine and rivastigmine contribute significantly to the stability of the corresponding HuAChE complexes, no such effect was observed for physostigmine and its analogs, phenserine and cymserine. Moreover, physostigmine-like ligands carrying oxygen instead of nitrogen at position - 1 of the tricyclic moiety (physovenine and tetrahydrofurobenzofuran analogs) displayed comparable structure - function characteristics toward the various HuAChE enzymes. The essential role of the HuAChE hydrophobic pocket, comprising mostly of residues Trp86 and Tyr337, in accommodating (–)-physostigmine and in conferring ~300-fold stereoselectivity toward physostigmynes, was elucidated through examination of the reactivity of selected HuAChE mutations toward enantiomeric pairs of different physostigmine analogs. The present study demonstrates that certain charged and uncharged ligands, like analogs of physostigmine and physovenine, seem to be accommodated by the enzyme mostly through hydrophobic interactions.

1. INTRODUCTION

The enzyme acetylcholinesterase (AChE) is presently the most important molecular target for therapeutic intervention in symptomatic treatment of senile dementia of the Alzheimer's type (AD) [1]. The ongoing effort to develop more therapeutically efficacious AChE inhibitors is currently driven by the remarkable progress, made during the last 15 years, in elucidating the structural and functional properties of the enzyme through x-ray crystallography [2,3] and site directed mutagenesis [4-8]. Combination of these two powerful techniques allowed for the detailed mapping of the human acetylcholinesterase (HuAChE) active center, delineating the functional subsites involved in reactivity toward substrates and other covalent modifiers as well as toward noncovalent ligands specific for the active center. These include the catalytic triad (Ser203, His447, Glu334), the "oxyanion hole" consisting of residues Gly120, Gly121

and Ala204 as well as different combinations of the 14 aromatic amino acids which line about 40% of the HuAChE active-center gorge surface, e.g. the acyl pocket (Phe295 and Phe297); the 'hydrophobic subsite' (Trp86, Tyr133, Tyr337 and Phe338); the cation - π interaction locus for charged moieties of substrates and other ligands at the active center (Trp86) and the peripheral anionic site (PAS, Tyr72; Tyr124 and Trp286).

Further examination of the functional architecture of the HuAChE active center revealed that reactivity of the enzyme toward substrates and other ligands can be also affected through perturbation of functional domains which may include multiple subsites in the active center. Thus, enhanced conformational mobility of the catalytic histidine was recently implicated in the activity differences between human butyrylcholinesterase (HuBChE) and the hexamutant HuAChE carrying aliphatic replacements of all the active site gorge aromatic residues (Tyr72, Tyr124, Trp286, Phe295, Phe297, Tyr337) distinguishing between the two enzymes [9,10]. Modulation of ligand interactions with the enzyme can be also effected through disruption of polar networks in the active center. One of these may include residues Ser229 and the catalytic triad residue Glu334 [11].

Most of the AChE inhibitors approved for clinical use as AD drugs (Cognex, Aricept, Nivalin) are noncovalent inhibitors and hence their AChE complexes are amenable for crystallographic analysis [12-14]. For the recently approved covalent AChE modifier, the carbamate rivastigmine (Exelon), such analysis can be carried out only on the carbamylated enzyme and is therefore relevant primarily to the enzyme regeneration step [15]. Yet, the overall inhibition process by carbamates is determined by properties of both the carbamylated enzyme and the transient Michaelis complex. These two species determine the rates of decarbamylation and carbamylation, respectively, and hence both contribute to the efficacy of the carbamate as a drug. Therefore, dissection of the affinity characteristics toward carbamates, through functional analysis of the carbamate-HuAChE Michaelis complexes, should provide information relevant to the design of more efficacious carbamate AD therapeutics [16]. In the past, we have shown that functional analysis of such Michaelis complexes can be carried out much in the same manner as for the noncovalent ligands [8].

In the present study, we examined the reactivity of HuAChE enzymes modified at relevant binding subsites, toward the carbamates rivastigmine and analogs of physostigmine, that are currently used [17,18] or considered for use as AD drugs [19-22]. Elements of the binding environment that determine the fate (carbamylation or dissociation; see scheme 1) of the particular Michaelis complexes have been identified. We conclude that interactions of HuAChE enzymes with analogs of physostigmine are dominated by hydrophobic interactions of the tricyclic eseroline moiety, and therefore the properties of the corresponding Michaelis complexes are quite different from those of rivastigmine and pyridostigmine. Thus, functional analysis appears to be a tool of choice for analysis of molecular complexes, which are too unstable for structural studies and yet are important as templates for drug design.

MATERIALS AND METHODS

Materials and enzymes

Acetylthiocholine iodide (ATC), 5,5'-dithiobis-(2-nitrobenzoic acid), pyridostigmine bromide and (-)-physostigmine salicylate were purchased from Sigma, and rivastigmine was obtained from Teva Ltd. (+)-Physostigmine, enantiomers of physovenine, phenserine as well as the enantiomeric pairs of cymserine, cymyl carbamate of physovenol and cymyl carbamate of tetrahydrofurobenzofurof were synthesized according to published procedures [23-26]. The chemical structures of the AChE inhibitors are shown in Figure 1, and full chemical characterization was performed to insure chemical and chiral purity.

Expression of recombinant enzymes, as well as the construction of all the HuAChE mutants, was described previously [4,6,7,9,27-31]. Construction of the double mutant W86A/Y337A was carried out by replacement of the appropriate DNA fragments of the AChE-w7 variant [4] with the respective fragments of the W86A and Y337A variants. Stable recombinant cell clones expressing high levels of each of the mutants were established according to the procedure described previously [27]. Enzymes were purified (over 90% purity) as described previously [27,32].

Determination of HuAChE activity and analysis of kinetic data

HuAChE activity was assayed according to the method of Ellman et al [33] in the presence of 0.1 mg/ml BSA, 0.3 mM DTNB, 50 mM sodium phosphate buffer (pH 8.0) and various concentrations of ATC at 27 °C and monitored with a Thermomax microplate reader (Molecular Devices).

The rate constants of progression of the carbamylation reactions (see Scheme 1) were estimated for at least four different concentrations (and at least a 10-fold in ligand concentration, around the estimated value of K_d) of carbamate (CR), by adding substrate at various time intervals and measuring the enzyme residual activity (E) (enzyme concentration was ca. 1.0nM). To avoid interference from regeneration of enzyme activity due to dissociation of enzyme carbamate conjugates, the initial velocity was used to determine k_{obs} ($V=k_{obs} [E]$) at each carbamate concentration. Thus, values of k_{obs} were calculated from the slope of the straight lines obtained from the plots of $1/\ln(E)$ vs. time of incubation prior to addition of substrate (cf. Figure 2 middle panels A and B). Double reciprocal plot of $1/k_{obs}$ vs. $1/[CR]$ were used to compute k_2 from the intercept, k_i from the slope and K_d from the ratio between the slope and the intercept according to scheme 1 and equation 1 [8] (cf. Figure 2 right panels A and B)

$$1/k_{obs} = 1/k_2 + (1/k_i) \times (1/[CR]) = 1/k_2 + (K_d/k_2) \times (1/[CR]) \quad \text{Equation 1}$$

In cases where steady state with respect to E was formed rapidly (within few minutes, cf. panels C,D in Figure 2), and immediate recovery of full enzymatic activity was observed upon dilution (cf. Figure 3), the inhibition was treated kinetically as reversible. Thus, Lineweaver –Burk plots in the absence and in the presence of different carbamate concentrations (cf. Figure 2 middle panels C and D) yielded values of relative slopes R_s (note that the $R_s = (1 + (1/K_d)[CR])$ according to ref 6 and 31). The R_s values were plotted against the carbamate concentration and the reciprocal of the slope provided the K_d values (cf. Fig. 2 right panels C and D).

Molecular modeling

Building and optimization of three-dimensional models of the HuAChE adducts with the various carbamates were performed on a Silicon Graphics workstation Octane2, using the SYBYL modeling software (Tripos Inc.). The initial models were constructed by manual docking of the ligands into the HuAChE active centre guided by interactions with residue Trp86, the active-site nucleophile (Ser203) and residues of the oxyanion hole. The initial models were optimized by molecular mechanics using the MAXMIN force field (and AMBER charge parameters for the enzyme) and zone-refined, including 122 amino acids [1.5 nm (15 Å) substructure sphere around $^o\text{O-Ser203}$]. Initial optimization included restriction of the distances between the carbonyl oxygen and the amide nitrogen atoms of residues Gly121 and Gly122, that between the carbonyl carbon and $^o\text{O-Ser203}$ as well as positions of residues Cys69 and Cys96, the ends of the omega loop. Those constraints were relieved in the subsequent refinement [8].

RESULTS AND DISCUSSION

Reactivity of rivastigmine toward HuAChE enzymes modified at various binding subsites in the active center

In a previous study, comparison of the reactivity characteristics of rivastigmine and pyridostigmine toward HuAChE enzymes suggested that accommodation of these carbamates in the active center is analogous to that of noncovalent inhibitors like edrophonium [16]. Results presented here indicate that although pyridostigmine and rivastigmine share the same binding subsites in the HuAChE active center, their distinct orientations with respect to the active site seem to influence the outcome of the carbamylation process. These results are summarized in Table 1 that includes HuAChE enzymes that carry replacements at the hydrophobic pocket, H-bond network, oxyanion hole, acyl pocket and the peripheral anionic site [6-8,10,11,30, 31]. In addition, we report on reactivities of both carbamates toward HuAChEs, which were engineered to resemble HuBChE active center [9,11].

Replacements of aromatic residues comprising the HuAChE active center hydrophobic pocket had a similar effect on the rates of carbamylation by rivastigmine and by pyridostigmine, implying that in both cases the positively charged moiety interacts with the cation binding subsite - Trp86. The pronounced increase of the respective dissociation constants, due to replacement of Trp86 (4400- and 6150-fold for pyridostigmine and rivastigmine respectively), resembles that for all the charged active center inhibitors [8,34,35]. Like for pyridostigmine [8], replacement of Tyr133 by alanine but not by phenylalanine had a pronounced effect on the affinity of Y133A HuAChE toward rivastigmine. As noted previously, replacement of Tyr337 by alanine had little effect on interactions of cationic ligands, although corresponding crystal structures of AChE complexes [12-14] and molecular model of HuAChE-pyridostigmine Michaelis complex [8] indicate close proximity of this residue to the ligand charged moiety.

Perturbations of the H-bond network, through replacements of residues Tyr133, Glu202 and Glu450 [10], had a relatively uniform effect on the corresponding rates of carbamylation by pyridostigmine (see Table 1). Yet, for two of those enzymes, E202Q and E450A, interaction with rivastigmine did not lead to carbamylation but rather to a regular, albeit low affinity (for corresponding values of the dissociation constants K_d see Table 1), *noncovalent* inhibition. This observation suggests that the balance between the rates of carbamylation and of dissociation of the corresponding Michaelis complexes can be easily tipped away from the covalent reaction. This facet of carbamate reactivity toward HuAChEs will become even more evident for certain analogs of physostigmine.

Structural modification of the oxyanion hole through replacement of residue Gly121 by alanine [31] alters the reactivity characteristics of both carbamates, converting them into noncovalent inhibitors. We have already shown that interactions of the acyl oxygen (acetyl, carbamyl or phosphoryl) with the oxyanion hole are important for both stabilization of the Michaelis complex and activation of the acyl moiety for nucleophilic attack by the catalytic Ser203 [31]. Thus, dissociation of the G121A HuAChE- rivastigmine complex is probably much faster than that of the corresponding complex of the wild type enzyme (the pronounced increase of the value of K_d is mostly due to increase of the dissociation rate constant k_{-1}), while its conversion to carbamylated enzyme is slower.

Replacements at the peripheral anionic site had only a limited effect on the carbamylation rate constants by rivastigmine. In particular, the corresponding value of k_i for carbamylation of D74N HuAChE was 3-fold lower than that of the wild type enzyme. On the other hand, carbamylation of this enzyme by pyridostigmine was nearly 150-fold slower, with the corresponding value of K_d being 200-fold higher than that of the wild type HuAChE. It is

already reported that this replacement resulted in a 50-fold increase of the dissociation constant for tacrine, while having only a small effect on the corresponding constant for edrophonium (5-fold increase) and no effect on huperzine A [8]. The reason for these uneven effects on affinities of the D74N enzyme toward the various charged (at the experimental pH) active center ligands still remains elusive.

While replacement of acyl pocket residue Phe295 by alanine, had little effect on the carbamylation rate by pyridostigmine, the corresponding rate for rivastigmine was 30-fold higher. This observation seems consistent with the size of the substituents on the carbamyl nitrogen. Namely, while interaction of rivastigmine with residue Phe295 of the WT HuAChE may perturb the “aromatic trap” and affect the carbamylation step, such perturbation is avoided in binding to the F295A enzyme. Accordingly, analogous substitution of the second acyl pocket residue Phe297 had a similar effect on the carbamylation rates by both carbamates. Reactivity of rivastigmine toward HuBChE, in which the corresponding acyl pocket is lined by aliphatic residues, has been found to be 15-fold higher than toward HuAChE. Most of this difference was due to the 9-fold higher value of the carbamylated enzyme formation step rate constant k_2 (see Table 1). Such reactivity enhancement was not observed for HuAChE enzymes in which the active center was engineered to resemble that of HuBChE. For the “butyryl – like” enzyme carrying replacements of aromatic residues vicinal to the active site (F295L/F297V/Y337A HuAChE) as well as for that substituted at both the active center and the peripheral anionic site (Y72N/Y124Q/W286A /F295L/F297V/Y337A HuAChE) [9], carbamylation rates by rivastigmine resembled those of the wild type enzyme. On the other hand, the corresponding rates of carbamylation by pyridostigmine were 15-fold and 73-fold lower, respectively.

Analogues of physostigmine display distinct inhibition profiles toward HuAChE enzymes

Unlike the pronounced effects (over 4000-fold) of replacing Trp86 by alanine on the inhibitory activities of pyridostigmine and rivastigmine, the inhibition rate constant of physostigmine toward the W86A HuAChE was only less than 50-fold lower than toward the wild type enzyme (see Table 2). Replacement of Tyr133 by alanine had larger effect (700-fold), implying that steric congestion rather than cation- π interaction is the dominant factor in the accommodation of physostigmine in the hydrophobic pocket [28]. Perturbations of the H-bond network affect reactivities of the corresponding enzymes toward physostigmine to a similar extent as for pyridostigmine and rivastigmine. Modifications of the acyl pocket had lower effect on reactivities toward physostigmine than toward rivastigmine (see Tables 1,2 and reference 8)

To further explore the reactivity characteristics of HuAChE toward physostigmine, we now examine physostigmine analogues differing in substitution at the carbamyl nitrogen as well as analogs with a modified tricyclic moiety. Thus, physostigmine, phenserine and cymserine (see Figure 1) were expected to display similar accommodation in the HuAChE hydrophobic pocket while differing with respect to the acyl pocket and the peripheral anionic site. From the results exemplified in Figure 2 and the regeneration of HuAChE activity from the respective enzyme-inhibitor conjugate (Figure 3), it appears that while the inhibition characteristics of phenserine toward the HuAChE enzymes resembles that of physostigmine, the reactivity of cymserine is that of a *noncovalent inhibitor*

Notwithstanding the difference in the inhibitory activity of physostigmine and phenserine as compared to cymserine, it seems that the three compounds are similarly accommodated in the hydrophobic pocket. In all cases affinities were affected by replacements at positions 86 and 133 by alanine, while not being sensitive to substitutions of Tyr337. Substitution of position 338 by alanine had some effect on the values of K_d for phenserine and cymserine (30-fold and 6-fold, respectively) but not on the corresponding value for physostigmine (see Table 2). Thus it appears that the different reactivity characteristics of cymserine, as compared to physostigmine and phenserine is not due to interactions of the respective eseroline moieties

with the HuAChE hydrophobic pocket. Therefore, it seems reasonable to assume that in the HuAChE- cymserine Michaelis complexes the ligand is sub-optimally oriented with respect to the catalytic machinery of the enzyme.

Substitutions at the H-bond network had similar effects on affinities toward phenserine and cymserine, as was the case for most of the replacements at the peripheral anionic site. The outstanding case was the failure of phenserine to carbamylate W286A HuAChE. Similar noncovalent inhibition was observed for interactions of phenserine with F295A/F338A and with the "butyryl like" HuAChEs (see Table 2).

Perturbations of the acyl pocket had comparable effects on the affinities toward phenserine and cymserine, as well as minor effects on the carbamylation rate constant (k_2) for phenserine. Thus, it does not appear that the failure of cymserine to carbamylate HuAChE enzymes results from delocalization of the catalytic His447 due to perturbations of the acyl pocket [10]. Moreover, while the prototypical perturbation of the His447 positioning (F295A/F338A HuAChE see [29]), abolished carbamylation by phenserine, the reactivity was restored by additional replacement at position 407. Such a reactivity profile [29] could not be observed in the case of cymserine (Table 2).

In order to gain further insight into the unique inhibitory characteristics of cymserine toward all the HuAChE enzymes, molecular modelling experiments of the Michaelis complexes of wild type HuAChE with physostigmine, phenserine and cymserine have been constructed (Figure 4). While in the model of phenserine, interactions of the N-aryl moiety with residues of the acyl pocket and with Phe338 could be observed, the disposition of the carbamyl moiety with respect to the active site residues resembled that of physostigmine. On the other hand, interactions of the 4-isopropyl aromatic substituent of cymserine with aromatic residues lining the mouth of the active center gorge forced an alternative conformation of the N-aryl moiety and consequently the removal of the carbamyl oxygen from the oxyanion hole. This finding seems consistent with the reversible noncovalent nature of cymserine interaction with wild type HuAChE and any of its mutant derivatives.

The notion that cymserine fails to carbamylate HuAChEs (see Figures 2 and 3) due to impaired polarization of the carbamyl oxygen, is consistent with the carbamylation profile of the G121A enzyme with physostigmine [31] as well as with pyridostigmine and rivastigmine.

Accommodation of the tricyclic moiety in the hydrophobic pocket

Another manifestation of the tight accommodation of the tricyclic moiety in the hydrophobic pocket is the marked stereoselectivity toward the (–)-physostigmine (3aS-diastereomer) enantiomeric form [20]. Since the main difference between the diastereomers is in the eseroline moiety, stereoselectivity should originate from the asymmetric interactions in the hydrophobic pocket. It was therefore reasonable to assume that through identifying the specific interactions leading to stereoselectivity toward (–)-physostigmine, a better understanding of its accommodation in the hydrophobic pocket would be achieved.

Replacement of the tricyclic eseroline group in physostigmine by physovenyl moiety (see Figure 1) resulted in an analogous physovenine [24], with similar chirality due to asymmetric carbon at position - 3a. Yet, the overall inhibitory activities, and in particular the affinities of both diastereomers of physovenine toward HuAChE were similar to that of (–)-physostigmine (see Table 3). It has been already proposed that the low affinity of AChE toward (+)-physostigmine is due to its N¹-methyl group interfering with Trp84 (86 in HuAChE) [36]. However this residue is usually thought to interact with N-methyl groups, as in the case for its endogenous substrate – acetylcholine [4,6].

As already reported [8,11], replacement of Trp86 by alanine had a moderate effect (~25-fold) on the affinity toward the natural (–)-physostigmine. Here we find that this modification has practically no effect on the affinity toward the (+)-diastereomer (see Table 3). Thus, the diminished stereoselectivity exhibited by W86A HuAChE toward physostigmine diastereomers, as compared to the wild type enzyme (20-fold, see Table 3), was solely due to a decrease in affinity toward (–)-physostigmine. This suggests that residue Trp86 does not participate at all in the interactions of HuAChE hydrophobic pocket with (+)-physostigmine (see Figure 5). On the other hand, W86A HuAChE displayed a similar decrease in affinity toward both diastereomers of physovenine and therefore, both the wild type and the W86A HuAChEs show nearly no stereoselectivity toward the physovenines.

Substitution of Tyr337 by alanine maintained the stereoselectivity toward physostigmines while inducing limited stereoselectivity toward the physovenines (18-fold, see Table 3). For both cases, stereoselectivity is completely abolished in the double mutant W86A/Y337A HuAChE. We note that while replacement of the aromatic residues Trp86 and Tyr337 led to a moderate decrease in affinity toward (–)-physostigmine (28- and 3-fold respectively), the corresponding effect for the double mutant is quite dramatic (4100-fold). In contrast, only a 10-fold decline in affinity of the W86/Y337 HuAChE toward the (+)-enantiomer has been observed, demonstrating that interactions with the hydrophobic pocket determine stereoselectivity toward physostigmines (see Table 3). For both physovenine enantiomers, affinities of the double mutant are comparable to those of the W86A enzyme. Thus, physostigmines and physovenines seem to be somewhat differently oriented with respect to the hydrophobic patch in the active center [8]. While residue Trp86 is essential in accommodation of (–)-physovenine, residues Trp86 and Tyr337 seem to compensate for one another in the case of (–)-physostigmine. Such compensation seems to account for the intriguing observation that removal of the aromatic moiety from position 86 had a larger effect on affinity toward the uncharged physovenines than toward the charged (–)-physostigmine.

The results described above seem consistent with the idea that steric congestion of Trp86 and the N1-methyl of (+)-physostigmine, indeed, interferes with accommodation of this diastereomer in the hydrophobic pocket. To further examine this hypothesis, affinities of HuAChE enzymes modified at the hydrophobic pocket, toward cymserine and cymyl carbamates of physovenol and of tetrahydrofurobenzofuran [26], have been compared. As for cymserine, all the cymyl analogs are noncovalent inhibitors. While HuAChE displayed ~50-fold stereoselectivity toward (–)-cymserine, practically no stereoselectivity was observed toward the diastereomers of analogs bearing the physovenol and tetrahydrofurobenzofuran moieties. Replacement of Trp86 by alanine practically abolished stereoselectivity toward diastereomers of cymserine yet had only a limited effect on their binding affinities. Other cymyl carbamates were also little affected by residue replacements at the hydrophobic pocket (see Table 4).

The notion that AChE stereoselectivity toward physostigmine is mainly due to interactions of the alkyl substituent at position -1 is supported also by previous studies on physostigmine analogues [36-38]. In particular, it was interesting to examine the low AChE stereoselectivity toward analogs of 8-carbaphysostigmine, since these structures do contain the N(1)- alkyl substituent [39]. Examination of molecular models of the corresponding Michaelis complexes indicates that due to bending of the tricyclic moiety at the sp^3 -C⁸, both enantiomers could be accommodated in the hydrophobic pocket without steric occlusion of Trp86. Thus the structural features of the eseroline moiety that contribute to AChE stereoselectivity toward physostigmine, are the alkyl substituent at position -1 combined with the planar disposition of the tricyclic ring system.

Accommodation of carbamates in the HuAChE active center

Carbamates are unique HuAChE inhibitors, binding both as covalent and noncovalent ligands to the different HuAChE enzymes. It appears that this property of carbamates originates from the particular dependence of the carbamyl moiety reactivity on its juxtaposition with the elements of the enzyme catalytic machinery. Carbamylation of AChEs involves nucleophilic attack on a relatively nonreactive carbonyl group, and therefore its rate depends critically upon the stability of the corresponding Michaelis complex, which manifests itself predominantly by variation in the values of the dissociation rate constant k_{-1} (under equilibrium conditions $K_d = k_{-1}/k_1$). Thus, the efficiency of the carbamylation process depends mainly on the relative values of the rate constants k_{-1} and k_2 (see scheme 1), with the latter displaying rather limited variability [40]. Accommodation of the carbamylating agent in the AChE active center is hence the most significant molecular event in the carbamylation process. The finding that the affinity of HuAChE toward the charged physostigmine is remarkably similar to that toward the structurally similar yet uncharged physostigmine, indicates that both inhibitors are accommodated mainly through hydrophobic interactions.

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Abbreviations

ATC	acetylthiocholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
HuAChE	human acetylcholinesterase
HuBChE	human butyrylcholinesterase

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Figure 1.

Chemical structures of the carbamates used in this study. Only the (–)-3aS-enantiomers of the various physostigmine analogues are shown. The numbering shown for eseroline is applicable to all the other physostigmine analogues.

Fig. 2.

Derivation of inhibition rate and equilibrium constants of wild type HuAChE and its mutant F295A/F338A by the carbamates physostigmine, phenserine and cymserine. Left panels: Inhibition time curve at indicated concentrations of the various carbamates (the actual curve was not calculated and serves as illustration). Middle panels (A, B) - The plots of $\ln(E)$ vs time, allow for derivation of the observed first-order carbamylation rate constants (k_{obs}), at indicated carbamate concentrations, Middle panels (C, D) - Lineweaver-Burk plots for the wild type HuAChE and the F295A/F338A enzymes with or without the indicated carbamates. Right panels (A, B) - Double reciprocal plots of k_{obs} (derived from their respective middle panels) versus the respect carbamate concentrations, from which k_2 , k_i and K_d were derive (as described

in the materials and methods section). Right panels (C, D) – R_s (relative slope) values as determined from the corresponding middle panels were plotted against the concentration of the carbamates. The value of K_d constant for cymserine and phenserine were obtained from the slope of these plots (see materials and methods section).

Fig. 3. Regeneration of enzymatic activity, following 50-fold dilution, from HuAChE phenserine and cymserine conjugates.

Figure 4.

Relative orientations of (-)-physostigmine and (-)-cymserine in models of their respective Michaelis complexes with HuAChE. Superposition of the carbamyl moieties is emphasized by the shaded area. Please notice that the carbamyl oxygen of physostigmine (cyan) is 2.997 Å and 2.387 Å from the peptidic NH groups of Gly121 and Gly122 respectively (shown in broken lines). Similar orientation of the carbamyl oxygen was observed also for phenserine (not shown for the sake of clarity). The corresponding distances for cymserine (grey) are 3.255 Å and 3.980 Å.

Figure 5.

Gradual displacement of the cation binding residue Trp86 from the hydrophobic pocket. Models of HuAChE Michaelis complexes with pyridostigmine and physostigmine enantiomers are shown in panels A-C. A, The positioning of residue Trp86 in the Michaelis complex of pyridostigmine is typical for complexes with charged noncovalent ligands and is shown here as a reference. B, Docking of the (-)-physostigmine in the HuAChE hydrophobic pocket results in vertical displacement of the indole moiety of Trp86. Yet, the conformation of the indole moiety relative to the ligand remains unchanged. C, The modelled displacement of residue Trp86 (1.310 Å between the centroids of the indole moieties), in complex depicted in panel A (cyan) as compared to that shown in panel B (magenta), is facilitated by flexibility of the Cys69-Cys93 omega loop. D, Steric interference due to the N-1 methyl substituent of (+)-physostigmine results in further displacement of residue Trp86 and to additional deformation of the Cys69-Cys93 omega loop. In this case, the indole moiety is too far removed from Tyr133 or from other residues stabilizing the overall structure of the omega loop [46] and therefore residue Trp86 is conformationally labile. E, Superposition of the omega loop segments Phe80-Pro88 in the complexes of (-)-physostigmine (cyan) and (+)-physostigmine (red) shown in panels B and D, respectively. The conformational change of Trp86 implies its practical removal from the hydrophobic pocket

Scheme 1.

Under conditions where $k_{-1} \gg k_2$: $k_d = k_{-1}/k_1$ (dissociation constant of the Michaelis complex);
 $k_i = k_2/k_d$ (bimolecular rate constant of carbamylation)

Table 1
Effects of mutations at the various subsites of the HuAChE active center on reactivity toward pyridostigmine and rivastigmine^a

HuAChE Type	Pyridostigmine			Rivastigmine		
	k_i $\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$	k_2 min^{-1}	K_d $\times 10^7 \text{ M}$	k_i $\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$	k_2 min^{-1}	K_d $\times 10^7 \text{ M}$
Wild Type	43.5±1.6	0.77±0.3	18±5	1.50±0.1	0.10±0.005	70±3
Hydrophobic pocket						
W86A	0.01±0.001	0.68±0.2	79000±2400	0.0007±0.0001	0.30±0.06	430000±80000
W86F	0.3±0.05	0.65±0.2	2300±500	0.60±0.02	0.70±0.01	1100±30
Y133A	0.02±0.001	1.80±0.3	94400±20000	0.0006±0.0002	0.60±0.2	1000000±300000
Y337A	7.6±1.0	0.80±0.1	105±27	0.50±0.05	0.10±0.01	160±10
Y337F	31.4±1.1	0.68±0.2	20±6	3.10±0.1	0.10±0.01	32±1
F338A	25.0±1.3	1.20±0.4	48±15	0.07±0.01	0.10±0.01	1430±70
H-bond network						
Y133F	1.4±0.1	0.20±0.04	120±30	0.07±0.01	0.10±0.01	1430±100
E202A	4.2±0.5	0.20±0.05	50±15	0.20±0.02	0.10±0.01	510±50
E202Q	2.5±0.1	1.00±0.2	410±80	-	-	10000±100
E450A	1.3±0.2	0.15±0.02	120±34	-	-	3700±40
Oxycation hole						
G121A	-	-	2500±30	-	-	160000±1000
G122A	0.76±0.2	0.12±0.02	154±2	0.09±0.01	0.10±0.01	1100±20
Acyl pocket						
F295A	62.0±3.4	0.17±0.02	2.7±0.5	44±2	0.50±0.05	11±1
F297A	5.1±1	0.64±0.1	125.0±40	0.55±0.03	0.10±0.01	190±10
Peripheral anionic site						
D74N	0.3±0.03	1.0±0.1	3600±730	0.60±0.03	0.60±0.1	1000±50
Y124A	13.8±0.4	0.1±0.01	6±0.8	1.30±0.03	0.08±0.01	63±2
W286A	14.0±0.6	1.8±0.5	124±35	3.30±0.1	0.70±0.1	200±15
Y341A	16.6±0.5	1.0±0.1	61±8	0.30±0.02	0.30±0.05	1000±70
His447 'trapping'						
F295A/F338A	0.8±0.5	0.5±0.4	625±500	-	-	12±2
F295A/F338A/V407F	16.5±2.0	0.5±0.15	30.0±12.6	1.00±0.1	0.10±0.01	90 ±5

HuAChE Type	Pyridostigmine			Rivastigmine		
	k_i $\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$	k_2 min^{-1}	K_d $\times 10^7 \text{ M}$	k_i $\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$	k_2 min^{-1}	K_d $\times 10^7 \text{ M}$
Butyryl-like						
Y72N/Y124Q/W286A	9.3±0.8	0.80±0.07	88±7	1.40±0.1	0.10±0.01	70±3
F295L/F297V/Y337A	2.8±0.1	0.55±0.09	195±30	3.70±0.1	0.30±0.06	80±8
Y72N/Y124Q/W286A/ F295L/F297V/Y337A	0.6±0.1	0.40±0.1	665±150	1.10±0.05	0.10±0.01	91±7
HuBChE-Wild Type	12.7±0.5	0.30±0.03	24±2	22±1.0	0.90±0.03	38±1

^aValues are means ± S.D. for at least three independent measurements.

Table 2

Effects of mutations at the various subsites of the HuAChE active center on reactivity toward physostigmine phenserine and cymserine^a

HuAChE Type	(-)-Physostigmine				(-)-Phenserine				(-)-Cymserine			
	k_i $\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$	k_2 min^{-1}	K_d $\times 10^7 \text{ M}$	k_i $\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$	k_2 min^{-1}	K_d $\times 10^7 \text{ M}$	k_i $\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$	k_2 min^{-1}	K_d $\times 10^7 \text{ M}$	k_i $\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$	k_2 min^{-1}	K_d $\times 10^7 \text{ M}$
Wild Type	350±20	1.2±0.5	3.4±1.0	89±30	0.9±0.5	13.9±5.5						6.3±0.7
Hydrophobic pocket												
W86F	190±2	0.90±0.20	4.6±1.0	150±20	1.3±0.9	8.7±3.4						1.5±0.5
W86A	8.2±0.7	0.80±0.30	95±40	5±0.9	1.3±1.0	200±90						54±12
Y133A	0.5±0.1	0.40±0.02	820±150	0.5±0.09	0.4±0.06	760±150						220±100
Y337A	120 ± 5	1.10±0.50	9.5±3.5	28±10	0.8±0.4	27.5±2.8						3.8±0.9
Y337F	360 ±50	1.90±0.40	5.2±1.5	171±49	0.9±0.07	2.8±0.3						3.0±0.3
W86A/Y337A	866 ±130	1.2 ±0.18	14000 ±2100	ND	ND	ND						470±127
F338A	100±3	0.40±0.20	4.2±1.0	1.6±0.5	0.7±0.1	413±95						40±18
H-bond network												
Y133F	19±0.2	0.13±0.01	6.9±1.0	4.2±0.8	0.3±0.09	76±19						37±13
E202A	7.7±1.0	0.24±0.05	32±4	2.2±0.6	0.2±0.1	91±36						120±50
E202Q	43±1.0	0.80±0.20	18±5	15±1.6	3.2±1	174±34						23±6
E450A	26±0.3	0.15±0.01	4.6±0.5	2.7±0.8	0.2±0.04	74±19						135±8
Peripheral anionic site												
D74N	140±4	2.40±0.50	17±4	21±5	0.7±0.01	34±6.1						8.7±3.5
Y72N	320 ±48	0.9 ±0.14	3.0±0.2	229±70	1.7±0.2	7.4±1.9						2.9±1.1
Y124A	150±3	6.0±1.8	0.9±0.3	ND	ND	ND						ND
W286A	365±40	2.70±0.80	1.0±0.2	-	-	1.6±0.3						7.2±2
Y341A	425±15	1.90±0.30	0.8±0.1	101±18	0.7±0.3	6.9±2.1						1.9±0.5
Acyl pocket												
F295A	1770±60	1.60±0.50	0.9±0.3	2300±480	2.1±1.2	0.9±0.4						0.4±0.1
F297A	160±15	0.60±0.10	3.8±1.0	35±6	1.0±0.5	2.9±1.0						7.5±1.4
F295L/F297V	90±9	2.0±0.4	23±5	377±170	3.0±1.8	8.0±3.6						1.0±0.05
F295A/F297A	55±17	0.6±0.2	11±3	305±90	8.5±2.5	28±8						1.6±0.3

HuAChE Type	(-)-Physostigmine			(-)-Phenserine			(-)-Cymserine		
	k_i $\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$	k_2 min^{-1}	K_d $\times 10^7 \text{ M}$	k_i $\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$	k_2 min^{-1}	K_d $\times 10^7 \text{ M}$	k_i $\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$	k_2 min^{-1}	K_d $\times 10^7 \text{ M}$
His447 'trapping'									
F295A/F338A	78±19	4.5±0.9	58±14	-	-	0.3±0.08	1.3±0.3		
F295A/F338A/V407F	197±30	0.2±0.03	92±14	1.1±0.1	0.3±0.1	272±55	22±5		
Butyryl-like									
F295L/F297V/Y337A	58±1	0.75±0.20	13±2	303±80	0.9±0.2	3±0.06	2.7±0.8		
Y72N/Y124Q/W286A/ F295L/F297V/Y337A	34±1.2	0.20±0.05	6.1±1.5	-	-	1.2±0.3	2.3±0.5		
Y72N/Y124Q/W286A/ F295L/F297V/Y337A/ V407F	18±3	1.25±0.25	71±17	-	-	203±61	20±4		
HuBChE-Wild Type	1030±37	0.55±0.05	0.5±0.04	6.6±2.2	0.6±0.2	90±30	$k_i=20 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$		

^aValues are means ± S.D. for at least three independent measurements.

Table 3

Stereoselectivity of HuAChE enzymes mutated at the hydrophobic pocket and at the acyl pocket toward (+)- and (–)-enantiomers of physostigmine and physovenine^a

HuAChE type	K_d ×10⁷M^b			
	Physostigmine		Physovenine	
	(–)	(+)	(–)	(+)
Wild Type	3.4±1.0 (1) ^c	1200±310 (1)	4.3±0.9 (1)	14±1.3 (1)
Hydrophobic pocket				
W86A	95±40 (28)	2000±460 (1.7)	1200±240 (279)	2800±285 (200)
Y133A	820±150 (241)	NI ^d	NI	NI
Y337A	9.5±3.5 (2.8)	482±120(0.4)	8.5±2 (2)	150±16 (10.7)
F338A	14±1.0 (4.1)	2300±810 (1.9)	25.6±6 (6)	7±1 (0.5)
W86A/Y337A ^e	14000±2100 (4100)	12000±2760 (10)	4130±850 (960)	2810±450 (201)
Acyl pocket				
F295A	0.9±0.3 (0.3)	90±26 (0.1)	1±0.27 (0.2)	0.9±0.25 (0.1)
F297A	3.8±1.0 (1.1)	3850±1150 (3.2)	6.3±0.4 (1.5)	10±3.5 (0.7)
F295A/F297A	11.4±5 (3.4)	7700±1850 (6.4)	2.3±0.7 (0.5)	2.8±0.8 (0.2)

^aValues are means ± S.D. for at least three independent measurements.

^bValues of K_d were determined using linear regression of Eq. 1 and plots like those presented in the middle and right panels of Fig.2A, 2B

^cValues are presented with SD; Numbers in brackets represent relative K_d (mutant/wt)

^dNo inhibition between 7.5×10^{–6}- 2.5×10^{–4}M

^eActivity measured with TB

Table 4

Stereoselectivity of HuAChE enzymes mutated at the hydrophobic pocket toward (+)- and (–)-enantiomers of cymserine and its analogues^a

HuAChE type	K _d × 10 ⁷ M ^b					
	Cymserine		Cymyl carbamate of physovenol		Cymyl carbamate of tetrahydrofurobenzofuran	
	(–)	(+)	(–)	(+)	(–)	(+)
Wild Type	6.3±1.7	300±76	7.1±1.2	10±2	17±3.5	22±9
W86A	54±16	130±58	25±2	50±13	71±17	66±25
Y133A	220±57	4800±1900	ND	ND	ND	ND
Y337A	3.8±1	50±13	27±10	22±7	200±45	123±50
F338A	40±11	135±14	400±120	571±150	1607±480	2900±870

^aValues are means ± S.D. for at least three independent measurements.

^bValues of K were determined from analysis of competition with ATC using plots like these presented in the middle and right panels of Fig. 2C, 2D.