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Flexibility versus “rigidity” of the functional architecture of AChE active center

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Abstract

Functional architecture of the AChE active center appears to be characterized by both structural “rigidity”, necessary to stabilize the catalytic triad as well as by flexibility in accommodating the different, high affinity AChE ligands. These seemingly conflicting structural properties of the active center are demonstrated through combination of structural methods with kinetic studies of the enzyme and its mutant derivatives with plethora of structurally diverse ligands and in particular with series of stereoselective covalent and noncovalent AChE ligands. Thus, steric perturbation of the acyl pocket precipitates in a pronounced stereoselectivity toward methylphosphonates by disrupting the stabilizing environment of the catalytic histidine rather than through steric exclusion demonstrating the functional importance of the “rigid” environment of the catalytic machinery. The acyl pocket, the cation-binding subsite (Trp86) and the peripheral anionic subsite were also found to be directly involved in HuAChE stereoselectivity toward charged chiral phosphonates, operating through differential positioning of the ligand cationic moiety within the active center. Residue Trp86 is also a part of the “hydrophobic patch” which seems flexible enough to accommodate the structurally diverse ligands like tacrine, galanthamine and the two diastereomers of huperzine A. Also, we have recently discovered further aspects of the role of both the unique structure and the flexibility of the “hydrophobic patch” in determining the reactivity and stereoselectivity of HuAChE toward certain carbamates including analogs of physostigmine. In these cases the ligands are accommodated mostly through hydrophobic interactions and their stereoselectivity delineates precisely the steric limits of the pocket. Hence, the HuAChE stereoselectivity provides a sensitive tool in the in depth exploration of the functional architecture of the active center. These studies suggest that the combination of “rigidity” and flexibility within the HuAChE gorge are an essential element of its molecular design.

Keywords

AChE; Stereoselectivity; Mutagenesis

1. Introduction

The ability to use conformational flexibility is a hallmark of enzyme function [1,2]. Multiple conformational changes are thought to be involved in optimization of structures of the

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consecutive steps in the substrate-enzyme reaction sequence. These changes add up to maximize substrate binding and to orient it precisely with respect to the enzyme catalytic machinery, as exemplified by the “induced fit model”. On the other hand, the catalytic process of bond breaking or formation seems to require a structurally fine tuned array of functional groups juxtaposed for optimal electrostatic and steric stabilization of the transition state [3,4]. Yet most of the structural data of AChE complexes with various ligands did not reflect such combination of flexibility and rigidity in the active center. In the TcAChE complexes with tacrine [5], edrophonium [5], galanthamine [6] and huperzine A [7], the respective structures of the active center are nearly equivalent to that of the free enzyme, despite the obvious differences in the shape and volume of the inhibitors. Due to the restrictive dimensions of the AChE active center “gorge” it seems quite evident that formation of these complexes required pronounced side chain mobility of residues comprising the walls of the “gorge” [8,9]. Indeed, modification of the acyl pocket subsite could be observed in the crystallographic structure of TcAChE adduct with DFP [10]. Also, positional shifts of the catalytic histidine were noticed in the adducts of AChEs with VX [11], tabun [12] and rivastigmine [13]. Yet, in most cases these motions cannot be observed in the crystal structures of AChE adducts and this may indicate that the mobile residues reassume their equilibrium positions once the formation of the Michaelis complex is complete. Similarly, formation of the Michaelis-Menten complex with the natural substrate has to include widening of the “gorge” bottle neck due to the side chains of Tyr121 and Phe330 [14].

To further explore the contributions of “rigidity” and flexibility to the reactivity properties of AChE we examined structural perturbations of three of the major subsites of the HuAChE active center “gorge” [5,15,16]—the aromatic trap; the hydrophobic pocket and the peripheral anionic site (PAS). Through combination of structural methods with kinetic studies of the enzyme and its mutant derivatives with plethora of structurally diverse ligands and in particular with series of diastereomeric covalent and noncovalent AChE ligands, we demonstrate that AChE stereoselectivity may be the consequence of either differential flexibility or perturbation of the rigid environment of the active center.

2. Results and discussion

2.1 Stereoselectivity due to perturbation of the “aromatic trap”

“Aromatic trap” is a functional subsite in the HuAChE active center involved in maintaining the optimal positioning of the catalytic His447 and include elements of the acyl pocket (Phe295, Phe297) as well as residue Phe337 [17]. Early hypotheses and modeling experiments related the pronounced stereoselectivity of AChE toward methylphosphonofluoridates such as sarin or soman to the structure of the acyl pocket [15,18]. The main reason for this assessment was that the homologous enzyme butyrylcholinesterase (BChE), in which residues at positions 295 and 297 are isoleucine and valine, respectively [19], was found to be more reactive than AChE toward bulky substrates such as butyrylcholine (BCh) and organophosphorus inhibitors such as diisopropyl phosphorofluoridate (DFP) while lacking stereoselectivity toward sarin [15, 18]. Indeed, studies of HuAChE reactivity using site-directed mutagenesis and enzyme kinetics suggested that replacement of acyl pocket residues Phe295 and to a lesser extent Phe297, by leucine and valine respectively, alter the specificity toward acylating (substrates) and phosphorylating agents [15,18,20]. Yet, compared to the wild type enzyme, the F295L/F297V HuAChE displayed a major decrease in catalytic activity toward ATC and only a minor increase toward BTC [15,21]. Since the pronounced activity of BChE toward ATC indicates that the restrictive dimensions of the acyl pocket are not required for efficient catalysis, the corresponding drop in catalytic activity of the F295L/F297V enzyme must be related to the role of residues F295 and F297 in maintaining the functional positioning of the catalytic His447 [17,21]. This notion is consistent also with the catalytic activities of the wild type and the

F295L/F297V HuAChEs toward BTC since accommodation of its bulky acyl moiety in the wild type enzyme disrupts the stabilizing interactions of the acyl pocket residues with His447, much like the structural perturbation effected by modification of the acyl pocket in the F295L/F297V enzyme. Thus, the mechanism of substrate selectivity in HuAChE, which besides the size of the acyl moiety was believed to consist of steric interaction with the acyl pocket, involves mainly perturbation of the “aromatic trap”.

Similar reasoning can be applied to the examination of stereoselectivity in reactions of HuAChE active center mutants with diastereomers of soman [22]. While the wild type HuAChE displays a 6×10^4 -fold stereoselectivity toward the P^S -diastereomers of soman, the corresponding value for the F295L/F297V enzyme is 80 (Table 1). The main reason for this decrease, the 50-fold drop in reactivity toward the P^S -diastereomers, can be related to perturbation in the positioning of His447. The F295L/F297V enzyme is also 15-fold more reactive than the wild type HuAChE toward the P^R -diastereomers of soman suggesting that steric effects may also contribute to the overall stereoselectivity originating from interactions with the “aromatic trap”.

The notion that the specific architecture of the acyl pocket in HuAChE is required for enzyme covalent reactivity rather than presenting a rigid hindrance to ligand accommodation suggests that it should not affect noncovalent binding of active center ligands. Indeed, recently published structures of AChE complexes with C₇ and C₅ bis-tacrines show a major deformation of the acyl pocket only for the C₅ complex while the corresponding value of IC₅₀ is merely 14-fold higher than that for the C₇ complex [23].

2.2. Stereoselectivity precipitated by charge interactions with the PAS

While the over 10^4 -fold stereoselectivity of AChE toward the P^S -diastereomers of soman originates mainly from perturbation of the “aromatic trap”, a more complex array of interactions seems to underlay the stereoselectivity toward methylphosphonates bearing positively charged leaving groups. Indeed, AChE inhibition studies with various charged and noncharged *O*-ethyl methyl-phosphonothioates demonstrated the contribution of polar interactions to the reactivities of the respective inhibitors [24]. Also, AChE stereoselectivity toward certain *O*-alkyl *S*-[(trimethylamino)ethyl] methylphosphonothioates was found to be much lower than that found for analogous phosphonofluoridates [25,26]. These, as well as modeling studies, indicated that the thiocholine leaving group of the inhibitors is oriented toward the gorge entrance and that the PAS residue Asp74 is a primary determinant in AChE specificity for cationic organophosphorus agents [25,27]. This conclusion is quite surprising in view of the X-ray structures of AChE in complex with noncovalent inhibitors like edrophonium [5] or conjugated with covalent modifiers like TMTFA [28] that show invariably that the ammonium cationic head points toward the anionic subsite residue Trp86 and being quite remote from Asp74.

Reactivity of recombinant wild type HuAChE toward VX^S was 115-fold higher than toward the VX^R (Table 2), with the respective values of the bimolecular rate constants being $1.4 \times 10^8 \text{ min}^{-1} \text{ M}^{-1}$ and $1.2 \times 10^6 \text{ min}^{-1} \text{ M}^{-1}$. While interactions with the charged leaving group

seem to make up the major contribution to the HuAChE affinity toward the two VX enantiomers, the question regarding the specific interactions contributing to stereoselectivity toward VX^S remains open. The lower reactivity of HuAChE toward VX^R may still be a result of inferior accommodation of the ethoxy substituent in the acyl pocket. On the other hand, AChE displayed no stereoselectivity toward enantiomers of MEPQ [29], indicating that the facility with which AChE accommodates methyl or ethoxy groups in the acyl pocket may depend on the nature of the charged leaving group and its interactions within the active center. In order to explore these interactions we examined the stereoselectivity toward VX of HuAChE enzymes carrying mutations at the peripheral anionic site (PAS) and the choline-binding site.

Residues Tyr72, Tyr124, Trp286 and Tyr341 are localized at or near the rim of the active center gorge, and together with Asp74 constitute the peripheral anion site [16]. Replacement of Asp74 by asparagine practically abolished stereoselectivity toward the VX^S enantiomer. The D74N HuAChE was 130-fold less reactive toward VX^S while its reactivity toward VX^R resembled that of the wild type enzyme (see Table 2). In contrast, substitution of the aspartate at position-74 by another acidic residue glutamate yielded an enzyme with nearly the same affinities toward both VX enantiomers as the wild type HuAChE. These results show that VX^S interacts with the acidic residue at position-74 of HuAChE, suggesting that in the case of VX enantiomers, charged interactions of the leaving group are a major determinant of stereoselectivity. Replacement of other components of the PAS Tyr341 and Trp286 had only limited effect on reactivity toward VX enantiomers.

Replacement of residue Trp86, which is the main component of the choline binding site, by alanine, had a dramatic effect on the reactivity toward both VX enantiomers, yet only a minor effect on stereoselectivity toward the VX^S (Table 2). The decrease of the W86A HuAChE reactivity toward the VX^S and VX^R enantiomers as compared to the wild type enzyme (the values of bimolecular rate constants decreased by 4500-fold and almost 1000-fold respectively, see Table 2) was compatible with what was observed in the past for other covalent and noncovalent charged ligands [15,24,30]. The corresponding effects of the replacement of Trp86 by phenylalanine were much smaller and consistent with the well-established role of the aromatic residue at position-86 as the main interaction locus of the charged moieties of AChE ligands [30,31].

Thus, for both the VX^S and the VX^R enantiomers, interactions of the charged leaving group appear to be the main determinant of HuAChE stereoselectivity. The discovery that the D74N HuAChE was practically devoid of stereoselectivity toward VX^S and yet that the reactivity of this mutant toward VX^R was nearly equivalent to that of the wild type enzyme indicated that the difference in the HuAChE accommodation of the two VX enantiomers can be reduced to a single interaction of the charged phosphyl substituent with residue Asp 74. Thus, while the diisopropylammonium moiety in Michaelis complexes of both VX^S and VX^R enantiomers interacts mainly with the anionic subsite residue Trp86, as demonstrated by the respective 980-fold and 4500-fold decreases in reactivity toward the W86A enzyme (Table 2), in the HuAChE- VX^S complex additional stabilization is provided by its interaction with the carboxylate of residue Asp74.

If a stabilizing interaction of the diisopropylammonium moiety with Asp74 is possible in the VX^S Michaelis complex, why is it absent in the corresponding VX^R complex? The reason is

suggested by the molecular simulation experiments, which show the cationic moiety of VX^S to be located nearly 2 Å closer to the Asp74 carboxylate than the corresponding moiety of VX^R . The respective locations of the VX cationic moieties seem to be determined by interactions with the phosphonyl ethoxy substituent. In the HuAChE- VX^S complex, the ethoxy group, positioned in the hydrophobic pocket, allows for an apparently optimal juxtaposition of the diisopropylammonium group with respect to residues Trp86 and Asp74. The carboxylate moiety of the latter is also displaced toward the VX^S ammonium moiety (Fig. 1). On the other hand, in the HuAChE- VX^R complex, the ethoxy group emerging from the acyl pocket induces an alternative conformation of the leaving group, in which interaction with residue Asp74 is practically precluded (Fig. 1).

Thus, stereoselectivity of HuAChE toward VX enantiomers is determined primarily by interactions of the charged leaving group with the rigid choline-binding site (Trp86) and the flexible PAS (Asp74). For a different charged leaving group, like the methylquinolinium moiety of MEPQ, tight interactions of both enantiomers with Trp86 may preclude any involvement of the PAS in accommodation of the corresponding Michaelis complexes. Consequently AChE displays no stereoselectivity toward MEPQ.

2.3. Stereoselectivity due to steric interactions with the hydrophobic site/cation-binding subsite

The unique characteristic of the AChE cation-binding subsite, Trp86, is its capacity for nonpolar interactions and therefore participation, together with residues Tyr337 and Phe338, in accommodation of hydrophobic moieties of active center ligands [31]. Although nonpolar interactions with the hydrophobic site contribute little to the binding of prototypical AChE active center inhibitors [31], the asymmetry of this site can still give rise to stereoselectivity toward certain chiral inhibitors. Thus, molecular models of HuAChE complexes with huperzine A diastereomers (Fig. 2) indicated that while the main interaction for both isomers was that with Trp86, the 50-fold stereoselectivity toward (-)-huperzine A [32] was mostly due to the differential interaction with Tyr337.

(-) Huperzine-A

In this respect, reactivity of HuAChE toward the physostigmine series is quite interesting since the enzyme displays marked stereoselectivity toward (-)-physostigmine (350-fold), which seems to originate from interactions with the hydrophobic site (Table 3). Yet, replacements of either Trp86 or Tyr337 by alanine had only a limited effect on reactivity toward physostigmine

[31]. To further explore the source of HuAChE stereoselectivity toward the physostigmine series, we compared the affinities of enzymes mutated at the hydrophobic site for enantiomers of physostigmine and physovenine [33]. Since both physostigmine and physovenine are AChE carbamylating agents, their overall reactivities should depend predominantly upon the stability of the respective Michaelis complexes [34].

Wild type HuAChE displays similar affinity toward (–)-physostigmine and (–)-physovenine despite the fact that the latter does not introduce a positively charged nitrogen (at experimental pH) proximal to Trp86. Furthermore, the decline in affinity of the W86A enzyme for (–)-physovenine is larger than that for (–)-physostigmine, indicating that Trp86 interacts with the physostigmine series as part of the hydrophobic site rather than as the cation-binding site. Finally, while HuAChE stereoselectivity toward (–)-physostigmine declines gradually with modifications of the hydrophobic pocket (see Table 3), none of these enzymes display significant stereoselectivity toward (–)-physovenine. Thus, it appears that the origin of HuAChE stereoselectivity toward physostigmine is indeed in the asymmetry of the hydrophobic pocket. However, the specific interactions resulting in the superior accommodation of (–)-physostigmine do not involve directly the chiral center of the molecule.

Molecular modeling of the HuAChE Michaelis complexes with the enantiomers of physostigmine and physovenine suggested an explanation for the difference between the two with respect to interaction with the hydrophobic site. While both physovenine enantiomers seem comparably well accommodated in the hydrophobic site (Fig. 3), binding of the enantiomers of physostigmine involves “pushing up” of the Trp86 indole moiety (Fig. 4). In the (–)-physostigmine complex this distortion of the hydrophobic site appears to be within its flexibility limits and therefore binding affinity is comparable to that of the corresponding complex with (–)-physovenine. However, in the (+)-physostigmine complex the steric congestion introduced by the *N*-methyl substituent apparently leads to a total displacement of the Trp86 side chain. Thus, flexibility of the hydrophobic pocket, and in particular of residue Trp86, determines HuAChE stereoselectivity toward physostigmine and the lack thereof toward physovenine.

2.4. Flexibility versus “rigidity” of the AChE active center

HuAChE stereoselectivity provided a sensitive tool for the in depth exploration of the functional architecture of the active center and, in particular, for demonstrating its seemingly conflicting requirements for structural flexibility and rigidity. Yet, combination of “rigidity” and flexibility within the HuAChE gorge seems to be an essential element of its molecular design. In fact, recent structural insights into substrate traffic and inhibition in acetylcholinesterase reveal conformationally distinct transient enzyme-substrate complexes [14,35]. Thus, the static picture of the AChE active center gorge, that started a new era in the cholinesterase research over 15 years ago [8], becomes gradually a dynamic one where the balance of conformational flexibility and “rigidity” has been optimized for catalytic perfection.

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Fig. 1.

Average structures from molecular dynamics simulations of Michaelis complexes of the VX^S and VX^R enantiomers with HuAChE. Note that the ammonio group of the VX^S complex (violet) is proximal to both Asp74 carboxylate and the indole moiety of Trp86, while that of the VX^R complex (red) points exclusively in the direction of residue Trp86. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 2. Superposition of molecular volumes of the docked ligands in complexes of (-)-huperzine A (red) and (+)-huperzine A (violet) with HuAChE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 3. Superposition of the ligand molecular volumes in the models of HuAChE Michaelis complexes with (-)-physostigmine (green) and (+)-physostigmine (violet). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 4. Superposition of Trp86 in models of free HuAChE and its Michaelis complex with (-)-physostigmine.

Table 1
Stereoselectivity of HuAChE enzymes carrying mutations at the acyl pocket toward P-soman diastereomers

HuAChE	P ^S -soman		P ^R -soman		WT/mutant	VX ^S /VX ^R
	<i>k</i> _i (×10 ⁻⁴ M ⁻¹ min ⁻¹)		<i>k</i> _i (×10 ⁻⁴ M ⁻¹ min ⁻¹)			
Wild type	12,000	1	0.2	1	1	60,000
F295A	1,200	10	1.0	0.2	0.2	2
F297A	3,300	4	0.5	0.4	0.4	6,600
F295L/F297V	240	50	3.0	0.07	0.07	80

Table 2
Stereoselectivity of HuAChE enzymes carrying mutations at the cation-binding site and peripheral anionic site (PAS) toward enantiomers of VX

HuAChE	VX ^S		VX ^R		WT/mutant	VX ^S /VX ^R
	$k_i(\times 10^{-4} \text{M}^{-1} \text{min}^{-1})$	WT/mutant	$k_i(\times 10^{-4} \text{M}^{-1} \text{min}^{-1})$	WT/mutant		
Wild type	13,700	1	118	1	115	
W86A	3	45,00	0.1	980	30	
D74N	107	130	48	2.5	2	
D74E	59,00	2	31	4	190	
W286A	13,000	1	85	1	170	
Y341A	5,100	3	13	9	390	

Table 3

Stereoselectivity of HuAChE enzymes carrying mutations at the hydrophobic site toward enantiomers of physostigmine and physovenine

HuAChE	Physostigmine		Physovenine	
	Relative k_i^a		Relative k_i^b	
	(-)	(+)	(-)	(+)
Wild type	1	350	1	3
W86A	28	590	280	650
Y337A	3	480	2	4
W86A/Y337A	4120	3530	960	650

^a Values relative to the dissociation constant of the WT-(-)-physostigmine complex.

^b Values relative to the dissociation constant of the WT-(-)-physovenine complex.