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# Structural modifications of the Ω loop in human acetylcholinesterase

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**Abstract Conformational mobility of the surface Ω loop (Cys-69–Cys-96) in human acetylcholinesterase (HuAChE) was recently implicated in substrate accessibility to the active center and in the mechanism of allosteric modulation of enzymatic activity. We therefore generated and kinetically evaluated the following modifications or replacements in HuAChE: (a) residues at the loop ends, (b) residues involved in putative hydrogen-bond interactions within the loop and between the loop and the protein core, (c) ChEs conserved proline residues within the loop and (d) a deletion of a conserved segment of 5 residues. All the residue replacements, including those of the prolines, had either limited or no effect on enzyme reactivity. These results suggest that unlike the case of lipase, the Ω loop in the HuAChE is not involved in large lid-like displacements. In cases where modifications of the loop sequence had some effect on reactivity, the effects could be attributed to an altered position of residue Trp-86 supporting the proposed coupling between the structure of the Ω loop and the positioning of the Trp-86 indole moiety, in catalytic activity and in allosterism.**

*Key words:* Acetylcholinesterase; Ω loop; Mutagenesis; Deletion; Allosteric effect

## 1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is among the most efficient enzymes, with a turnover number of over $10^4$ $s^{-1}$ [1]. Its remarkable catalytic power is presumably determined by the unique architecture of the AChE active center, which has been recently elucidated by 3D structure analysis of *Torpedo* AChE [2], site-directed mutagenesis and molecular modeling together with kinetic studies of the AChE muteins with substrates and reversible inhibitors [3–17]. However, the location of the active center at the bottom of a deep and narrow 'gorge' and the crystallographic dimensions of this gorge pose an intriguing question regarding the substrate and ligand access to the catalytic site [18]. In addition, the structure reveals an uneven overall distribution of negative charge giving rise to a large electrostatic dipole, aligned along the active site gorge [19]. Such a dipole would draw the positively charged substrate down the gorge, however, it could also interfere with the release of the reaction product – choline. In order to reconcile the apparent steric and electrostatic impediments in the trafficking of substrate and products with the high turnover number of the enzyme, conformational adjustments of the gorge dimensions during ligand approach [18] or a possible back door path for product release have been suggested [20]. Subsequently, it was shown, by septuple replacement of negatively charged amino acids, that electrostatic attraction does not contribute to the catalytic rate of the enzyme [21]. In addition, mutagenesis of key residues located along the putative back door channel does not support its proposed role in AChE activity [15,22].

Another intriguing feature of AChE reactivity is the allosteric modulation of its catalytic activity [23], following ligand binding to a peripheral site (PAS) at the enzyme surface [24], which is located 20 Å away from the active center and has been recently characterized by site-directed mutagenesis [7,13,14]. Although binding of PAS ligands was shown to affect the conformation of the active center over 15 years ago [17,25], the actual mechanism of this modulation was only recently proposed, suggesting the involvement of a conformational transition of residue Trp-86 [14]. Residue Trp-86 which in its active conformation is the main element of the classical 'anionic subsite' [6,10,11,26,27] essential for substrate accommodation, can occupy an alternative conformational state in which it occludes the active center [11]. It has been further suggested that such conformational mobility of Trp-86 is governed by the dynamic behavior of the cysteine loop (Cys-69–Cys-96) on the protein surface and that modulation of this behavior, through occupation of the PAS, provides the means of transmission of the allosteric signal [28]. The HuAChE disulfide surface loop (Cys-69–Cys-96), which is a typical Ω loop [29], is a structural element conserved throughout the esterase/lipase family of hydrolytic enzymes sharing the α/β hydrolase fold [30] (classified as the $L_{b3,2}$ variable length loop [31]). In the case of lipases, X-ray crystallographic studies of free versus complexed structures show that the $L_{b3,2}$ loop is one of the most mobile structural elements [18,22,32]. Simulated annealing experiments of HuAChE suggest that, also for this enzyme, a large segment of the $L_{b3,2}$ loop is the most mobile part of the molecule [33]. Furthermore, this mobility was shown to induce the conformational transitions of residue Trp-86 and to modify the dimensions of the active site gorge rendering it more accessible to inbound ligands [28,33].

To further test the notion that the dynamic behavior of the $L_{b3,2}$ loop is a major functional characteristic of AChE activity, governing the mobility of Trp-86 as well as ligand accessibility to the active center, we generated and characterized HuAChE mutants carrying replacement of residues which presumably contribute to the loop structure. Our results suggest that unlike the case of lipases, the $L_{b3,2}$ loop in HuAChE is not involved in large lid-like displacements but more likely in specific low amplitude motions of selected loop residues. Such limited motions could be sufficient for providing access to the active center and for controlling the positioning and conformation of residue Trp-86.

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## 2. Materials and methods

*2.1. Mutagenesis of recombinant HuAChE and preparation of enzymes*

Mutagenesis of HuAChE [34] was performed by DNA cassette replacement and involved substitution by synthetic DNA duplexes [6]. For generation of the Y77A, P78A, P85A and N87A mutants the *Acc*I-*Nru*I fragment of pACHEw4 was replaced by a synthetic fragment carrying the corresponding substitution. For generation of Y449A HuAChE, the *Bst*BI-*Bam*HI fragment of pACHEw3 was substituted. The Ala codon used in all mutations was GCC. The sequences of all new clones were verified by the dideoxy sequencing method (US Biochemical Corp. sequenase kit). HuAChE mutants Y72A, D74N, E84Q, D95N and Y341A were described previously [6,7,14]. All recombinant HuAChE cDNA mutants were expressed in bipartite vectors which allow expression of the *cat* reporter gene. Human embryonal kidney 293 cells were transfected with various purified plasmids as described previously [35,36]. The various AChE polypeptides secreted into the medium and were quantified by AChE-protein determination relying on specific ELISA [6,37].

*2.2. Substrates, inhibitors and kinetic studies*

Acetylthiocholine iodide (ATC), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) and ethyl(*m*-hydroxyphenyl)dimethylammonium chloride (edrophonium), 3,8-diamino-5,3′-(trimethylammonium)propyl-6-phenyl phenanthridinium iodide (propidium), di(*p*-allyl-*N*-dimethylaminophenyl)pentane-3-one (BW284C51) and 1,10-bis(trimethylammonium)decane (decamethonium) were all purchased from Sigma.

Catalytic activity of the recombinant HuAChE and its mutant derivatives collected from transient transfections was assayed according to Ellman et al. [38] as described previously [6]. Assays were performed with $\sim 10^{-10}$ M enzymes (total volume 0.1 ml) in growth medium in the presence of 0.1 mg/ml bovine serum albumin, 0.3 mM (5,5-dithiobis(2-nitrobenzoic acid) in 50 mM sodium phosphate buffer pH 8.0 and varying ATC (0.01–25 mM) concentrations. The assays were carried out at 27°C and monitored by a Thermomax microplate reader (Molecular Devices) and corrected for background readings using medium collected from mock-transfected cells. Data was analyzed according to the kinetic treatment described previously by Ordentlich et al. [10].

*2.3. Structure analysis and molecular graphics*

Building and analysis of the 3D models was performed on a Silicon Graphics workstation Indigo2 using SYBYL 6.0 modeling software (Tripos Inc.). Construction of models for the HuAChE and the mutated enzymes was based upon the model structure of the enzyme obtained by comparative modeling [39] from the X-ray structure of TcAChE [2]. Optimization of the resulting structures was carried out as described before [10,11,14] using the TRIPOS force field and Kollman all-atom charges for the enzyme [40].

## 3. Results and discussion

*3.1. Potential structural modifications of the $L_{b3.2}$ loop: generation and analysis*

The HuAChE $L_{b3,2}$ surface loop constitutes a relatively long stretch of residues (28 residues, Fig. 1) folded on itself and exhibiting a limited ordered structure (Fig. 2A,B). It has an elongated shape and lies flat on the protein surface forming the thin wall of the active site gorge. The notion that loop mobility may be important to AChE reactivity is suggested by the involvement of an equivalent loop in lipase, another member of the $\alpha/\beta$ hydrolase fold, in interfacial activation and substrate accommodation [32]. However, while the loop motion in lipases resembles a lid opening, controlled by *cis-trans* isomerization of proline (Pro-92 in *Candida rugosa* lipase) [32], molecular dynamics simulations indicate that the corresponding motion in HuAChE can be best characterized as gradual unwinding [33].

The most apparent element of secondary structure in the $L_{b3.2}$ loop of HuAChE is a helical turn (Glu-81–Asn-87) containing the anionic subsite residue Trp-86 (Fig. 2B). Structural modifications of this element as well as other perturbations of the loop shape may be expected to affect the catalytic properties of the enzyme through relocation of the choline binding residue Trp-86. Examination of molecular models suggests that such modifications could include replacement of polar residues contributing to the loop structure through salt bridges or H-bond interactions, replacement of the loop proline residues or deletion of part of the loop. Such types of modified enzymes were generated and kinetically monitored for effects on the catalytic activity as well as on reactivity toward reversible inhibitors. For the latter, three different types of reversible inhibitors were used. Affinity toward the cationic active center ligand edrophonium was determined to probe the extent of interaction with Trp-86 since loop modifications were not expected to affect other elements of the active center. The inhibitory activity of a specific PAS ligand propidium was tested as it is expected to depend both upon the location of Trp-86 and upon the manner by which loop mobility affects Trp-86 conformation [27]. Inhibition by the specific bisquaternary ligands (decamethonium and/or BW284C51) which span the distance between the active center and the PAS was examined to evaluate possible changes at both binding loci.

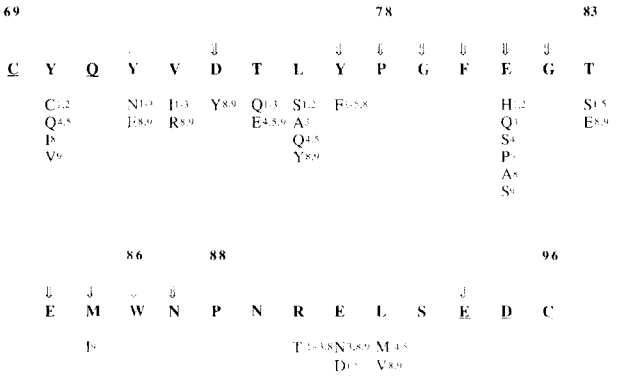


Fig. 1. Amino acid sequence of the surface Ω loop (Cys-69–Cys-96) in HuAChE as compared to other ChEs. Source of ChE is marked by numbers, as follows: 1, human BuChE; 2, rabbit BuChE; 3, mouse BuChE; 4, *Torpedo californica* AChE; 5, *Torpedo marmorata* AChE; 6, fetal bovine AChE; 7, mouse AChE; 8, *Anopheles stephensi* AChE; 9, *Drosophila* AChE. Amino acid squences are according to [45]. Residues conserved in nearly all the proteins maintaining the α/ß hydrolase fold are underlined. Positions at which replacements or deletions have been made by site-directed mutagenesis are denoted by arrows.

*3.2. Structural modifications of the loop ends*

The $L_{b3.2}$ loop appears to be tightly fixed at its base by the Cys-69–Cys-96 disulfide bridge. In fact, molecular dynamics simulations indicate that the mobile segments of the $L_{b3.2}$ loops, in both lipases and cholinestrases, do not involve movement of residues at their N- and C-termini (in HuAChE Cys-69–Tyr-72 and Glu-91–Cys-96, respectively [32,33]). Therefore, replacement of residues located near the loop termini should have only a limited effect on its shape and dynamic properties. On the other hand, the C-terminus residues Ser-93 to Cys-96 are strictly conserved in the lipase/esterase family and therefore may play a structural role in maintaining the loop position. In particular, residue Glu-94 and the conserved

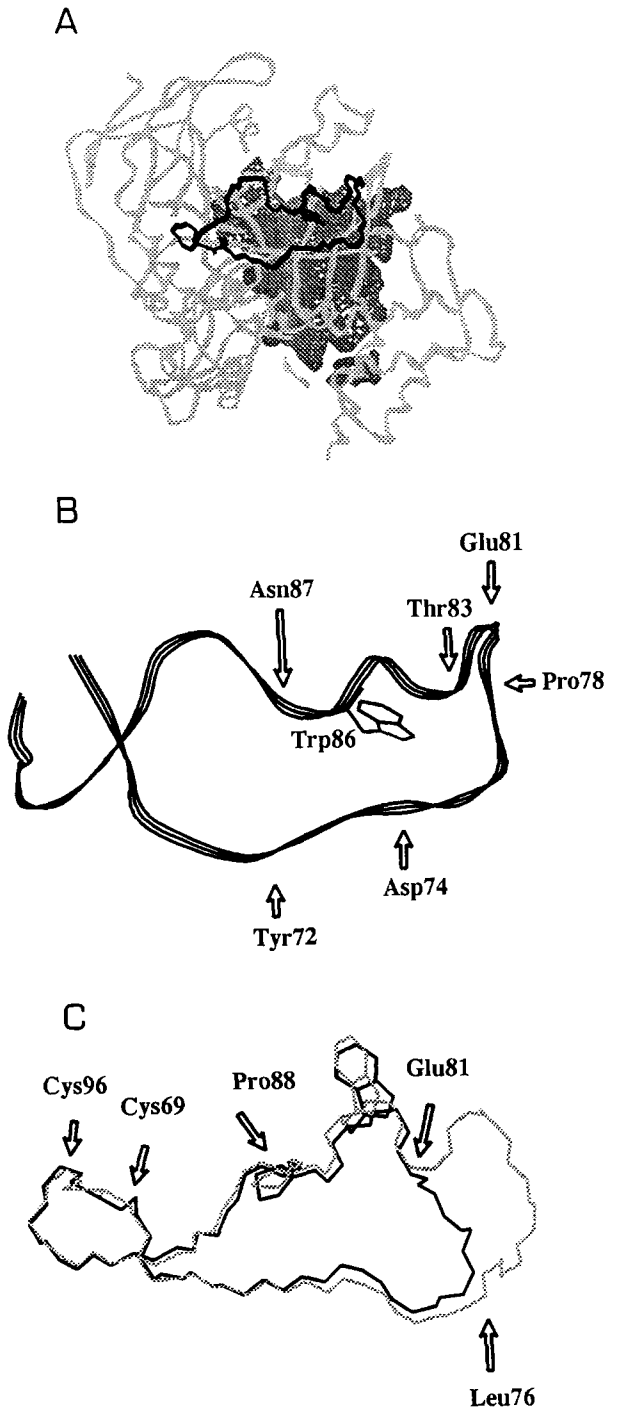


Fig. 2. Surface Ω loop (Cys-69–Cys-96) structure in HuAChE. (A) Illustration of the Ω loop (heavy line) disposition relative to the protein core, with residues of the active site gorge shown in grid surface and the rest of the protein in line ribbon. Only the loop Cα backbone and the indole side chain of residue Trp-86 are presented. (B) Ribbon representation of the Ω loop main chain illustrating the α-helical turn (Glu-81–Asn-87), the loop tip (Pro-78–Thr-83) and position of the choline binding Trp-86 side chain. (C) Superposition of the Ω loop trace of the wild-type HuAChE (light line) and that of the modeled loop structure of the Del-78–82 HuAChE mutant (heavy line). The two traces diverge mainly in the vicinity of the deleted segment (between Leu-76 and Glu-84 of the original loop sequence), while the Cα position of residue Trp-86 is nearly unchanged. Note also the slight difference in orientation of the catalytically important indole moiety in the loops of the wild-type and Del-78–82 HuAChE mutant enzymes.

residue Arg-46 form a salt bridge which was shown as critical for maintenance of the 3D fold of the enzyme [41]. In addition, ligand binding to the PAS array, including residue Tyr-72 at the N-terminus of the loop, has been suggested to precipitate allosteric effects through modulation of the loop motion [28,33]. Thus, examination of the reactivity properties of enzymes carrying replacements of the PAS residue Tyr-72 and of the conserved residue Glu-95, at the N- and C-termini, respectively, may allow for a better understanding of coupling between the relatively stationary and the mobile portions of the $L_{b3,2}$ loop.

Examination of the kinetic parameters for Y72A HuAChE (Table 1) demonstrates that replacement of residue Tyr-72 does not affect catalytic activity, suggesting that the positioning of residue Trp-86 has not changed. The anionic subsite in the Y72A enzyme is fully functional, as indicated also by its wild-type-like affinity toward the active center inhibitor edrophonium. The 4-fold decrease in the inhibition constant of propidium is probably due to the decrease in affinity toward the modified PAS of Y72A HuAChE [14], rather than due to the effect on the conformational behavior of the loop. The affinity toward the bisquaternary ligand BW284C51 is similarly affected (2-fold). Replacement of the C-terminal residue Asp-95, which is conserved in all the cholinesterases, affects neither the catalytic activity nor the reactivity of the D95N enzyme toward inhibitors (Table 1).

### 3.3. *Modification of interactions of the potentially mobile part of the $L_{b3,2}$loop with protein core*

As could be expected, the interactions of the $L_{b3,2}$ loop with the protein core are predominantly hydrophobic [31,42,43], but several polar interactions with the core can be identified. In the wild-type HuAChE residue Asp-74 interacts with the protein core residue Tyr-341. Disruption of this interaction has already been shown to affect HuAChE reactivity [10,14] (see also Table 1). Two other residues participating in the loop-protein core polar interactions are Tyr-77 (with residue Tyr-341) and Glu-84 (with Lys-348). Both residues are located near the center of the loop, flanking its highly mobile tip (Tyr-77–Thr-83) [33]. In fact, dissociation of this tip from the protein core has been suggested, by high temperature molecular dynamics simulations, as one of the characteristics of the $L_{b3,2}$ loop motions in HuAChE [33]. Another interaction of this type may involve the indole moiety of Trp-86 and the adjacent side chain of residue Tyr-449. However, due to the poor H-bond donating properties of the indole nitrogen, the contribution of this interaction to either the conformational stability of residue Trp-86 or to the overall shape of the loop has been difficult to predict.

Examination of kinetic parameters for the Y77A, E84Q, Y341A and Y449A enzymes (Table 1) shows that neither one of these structural modifications had a significant effect on HuAChE reactivity. The fact that perturbations of polar interactions between the loop and the protein core had only a marginal effect on enzymatic activity may suggest that the coupling between loop mobility and conformation of Trp-86 does not involve large loop displacements separating it from the core. The recently reported activity of TcAChE mutant, in which a disulfide bridge between the loop tip and the core has been introduced (*G80C/V431C*) [22], and our results with the similar double mutant E81C/S438C (unpublished results) appear to support such a conclusion.

Table 1
Kinetic constants of catalysis and inhibition constants $K_i$ for HuAChE and its mutants

| AChE | $K_m$ (mM) | $k_{cat}$ ($\times 10^{-5}$) ($min^{-1}$) | $k_{cat}/K_m$ ($\times 10^{-8}$) ($M^{-1}$ $min^{-1}$) | Edrophonium (μM) | Propidium (μM) | BW284C51 (nM) |
|---|---|---|---|---|---|---|
| WT | 0.14 | 4.0 | 29.0 | 0.75 | 1.4 | 10 |
| Y72A[a] | 0.14 | 4.7 | 32.0 | 0.3 | 5.3 | 22 |
| D74N[a] | 0.60 | 2.5 | 4.2 | 3.5 | 7.8 | 2450 |
| Y77A | 0.12 | 2.9 | 24.2 | 0.83 | 1.7 | 3.9 |
| P78A | 0.10 | 3.0 | 30.0 | 0.8 | 0.7 | 7.5 |
| E84Q[b] | 0.27 | 4.0 | 15.0 | 1.2 | 2.4 | 78.5 |
| M85A | 0.14 | 4.6 | 33.0 | 2 | 1.3 | 5.2 |
| N87A | 0.23 | 6.1 | 27.0 | 3.4 | 6.5 | 105 |
| P88A | 0.14 | 5.0 | 37.5 | 1.6 | 0.7 | 14 |
| D95N | 0.13 | 4.4 | 34.0 | 0.43 | 2.2 | 3.2 |
| Y341A[a] | 0.30 | 2.5 | 8.3 | 2.0 | 3.4 | 322 |
| Y449A | 0.13 | 1.5 | 11.5 | 5.5 | 2.9 | 24 |

[a]Values for Y72A, D74N and Y341A HuAChE are cited from Barak et al. [28].
[b]Some of the kinetic parameters for E84Q were determined previously [21].

### 3.4. Modifications of interactions within the potentially mobile part of the $L_{b3,2}$ loop

Most of the loop polar residues including Asp-74, Thr-75, Glu-81, Thr-83, Glu-84, Asn-87, Asn-89 and Arg-90 are involved in H-bond contacts within the loop. These contacts may be instrumental in maintaining the local kinks and turns characteristic to the $L_{b3,2}$ loop structure (see Fig. 2B). Among these interactions, particularly interesting were the two in the immediate vicinity of Trp-86 (Met-85–Glu-81 and Asn-87–Thr-83) since they seem to participate in maintaining the helical turn structure ($\alpha_{b3,2}$, see [31]), providing a flexible main chain linkage for residue Trp-86. Such linkages at the tip of small loops or turns are characteristic for residues of the catalytic machineries (e.g. those of the catalytic triad in serine hydrolases [31]), allowing for minute conformational adjustments in order to achieve optimal interaction geometry. In addition, interaction of the Met-85 side chain with the indole moiety has been proposed to contribute to the stabilization of Trp-86 active conformation [44].

Replacement of residue Met-85 had no effect on the catalytic activity of the enzyme and only a minor ($>3$-fold) effect on affinity toward edrophonium. Although an interaction Ala-85–Glu-81 is still possible in the M85A enzyme, that of the methionine side chain with the indole moiety of Trp-86 is certainly lost. Thus, the proximity of Met-85 to residue Trp-86 does not influence in a substantial way the conformation of the latter.

In the case of N87A enzyme, a small effect on the $K_m$ value (2-fold increase relative to the wild type) has been observed. This slight impairment in accommodating active center ligands is also reflected in the 5-fold decrease in affinity toward edrophonium. Interestingly, the inhibitory activity of the PAS specific ligand propidium is also about 5-fold lower. Such reactivity phenotype is shared by enzymes carrying replacements of residue Asp-74 or Y133, in which the position or conformational properties of residue Trp-86 have been affected, [10,11,14]. Like in these cases, the somewhat altered properties of residue Trp-86 due to the replacement of Asn-87 affect both the binding characteristics of the active center and the efficiency of transmitting the allosteric signal from the PAS. This conclusion is further supported by a 10-fold increase in the inhibition constant ($K_i$) of BW284C51 toward N87A HuAChE, as compared to the wild-type enzyme (Table 1).

### 3.5. Structural modifications of the $L_{b3,2}$ loop involving its proline residues

The significance of proline residues in the loop mobility of lipase (from *Candida rugosa*; CRL), was recently demonstrated by comparison of the X-ray structures of the closed and open forms [32]. Transition between the two forms involves a nearly 90° rotation of the loop around hinge residues Pro-92 and Glu-66 (numbering according to the CRL sequence) with residue Pro-65 probably providing additional rigidity to the adjacent hinge region. The corresponding $L_{b3,2}$ loops in cholinesterases also contain two conserved proline residues (Fig. 1) which may fulfil a similar function in controlling the loop mobility. However, according to the results from molecular dynamics simulations, isomerization of residues Pro-78 and Pro-88 does not take place in HuAChE [28,33]. Furthermore, while the hinge residue of CRL Pro-92 is located near the C terminus of the loop sequence, the analogous HuAChE residue Pro-78 (according to comparison of the lipase and cholinesterase loop sequences [45]) is located near the center of the HuAChE loop sequence and therefore may be unsuitable to function as a *cis-trans* conformational switch of the loop.

Even without being involved in *cis-trans* isomerizations, proline residues should locally restrict the conformational mobility of the adjacent residues [46]. In particular, this may be

Table 2
Kinetic constants of catalysis and inhibition constants $K_i$ for HuAChE and its mutants

| AChE type | $K_m$ (mM) | $k_{cat}$ ($\times 10^{-5}$) ($min^{-1}$) | $K_{cat}/K_m$ ($\times 10^{-8}$) ($M^{-1}$ $min^{-1}$) | Edrophonium (μM) | Propidium (μM) | BW284C51 (nM) | Decamethonium (μM) |
|---|---|---|---|---|---|---|---|
| WT | 0.14 | 4.0 | 29.0 | 0.75 | 1.4 | 10 | 6 |
| W86A[a] | 93 | 0.8 | 0.009 | >45000 | 870 | 40000 | 90000 |
| W86F[a] | 0.8 | 2.1 | 2.6 | 38 | 3.2 | 1200 | 500 |
| Del 78–82 | 1.0 | 1.5 | 1.5 | 84 | 6.6 | 2300 | 880 |

[a]Values for W86A and some of those reported for W86F were determined previously [11,28].

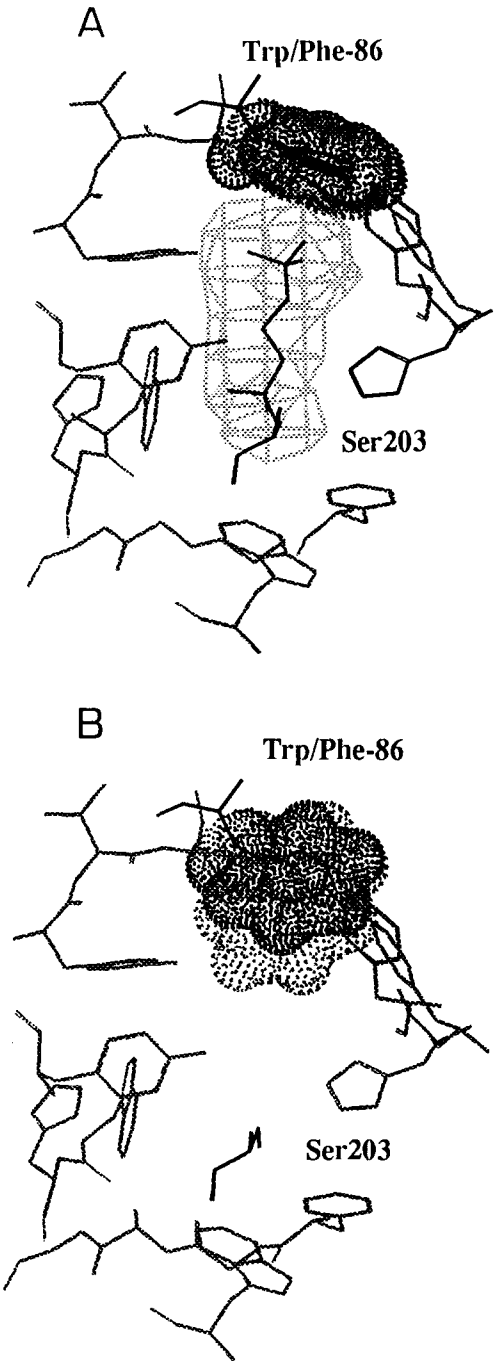


Fig. 3. Conformational transition of aromatic residue (tryptophan and phenylalanine) at position 86. (A) Superposition of the catalytically functional conformations of residues Trp-86 (lighter volume) and Phe-86 (darker volume) in wild-type and W86F HuAChE, respectively, with the aromatic moiety shown to accommodate the tetramethylammonium group of ACh (presented as a grid). (B) Non-active conformation of the extended aromatic side chain occluding the active center interfering with complexation of substrates and active center inhibitors. Note the greater hindrance by the indole moiety as compared to that of the phenyl ring.

the case for Pro-88 located at the C terminus of the $\alpha_{b3,2}$ helical turn (Glu-81–Asn-87). Nevertheless, replacements of both proline residues did not affect catalytic activity and had only marginal (~2-fold) effect on the affinity toward edrophonium and BW284C51 (see Table 1). These results suggest that although HuAChE and CRL share the same 3-dimensional topology, both conforming to the $\alpha/\beta$ hydrolase fold, the functional significance of analogous structural details can be very different. Namely, while proline residues in the CRL $L_{b3,2}$ loop are essential for normal enzymatic activity, the analogous residues in HuAChE appear to be devoid of any functional or even structural role. Such apparent lack of structural significance of the loop proline residues is very surprising since they are conserved in all the cholinesterases and since proline residues are recognized as major determinants of secondary structure in proteins [42,43].

### 3.6. *Structural modification of the $L_{b3,2}$ loop through residue deletion*

Since replacement of single residues within the loop or in its vicinity failed to produce sizable effects on HuAChE reactivity, we turned to a more drastic way to modify the $L_{b3,2}$ loop structure, through deletions of selected loop segments. Such deletions are bound to alter the loop span and consequently the relative positions of its individual residues. Yet, a total loss of activity due to a drastic deletion would be difficult to interpret in terms of specific structural modifications.

In a preliminary experiment, designed to test whether the proper positioning of Trp-86 in the active center is the only catalytically significant function of the $L_{b3,2}$ loop, deletion of most of its mobile part was attempted. However, attempts to express a protein, carrying excision of the sequence Asp-74–Asn-87, were not successful implying that in this case the folding process was probably affected (data not shown). To overcome this limitation we have generated and examined molecular models of truncated loop structures, looking for those in which positions of Trp-86 and adjacent residues are the least affected. In construction of the truncated loop models we adjusted the geometry of residues adjacent to the excised sequence, only as necessary to re-close the loop. Several of these initial loop structures were subsequently optimized to obtain acceptable model alternatives. One such model structure has been obtained by deletion of the sequence Pro-78–Gly-82, resulting in a loop in which the loop tip is missing (Fig. 2C). Despite such drastic alteration of the loop sequence, the model indicated only a minor displacement of the Trp-86 indole moiety relative to its position in the wild type enzyme (Fig. 2C). This is especially interesting in view of the fact that the sequence indicated for the deletion (Pro-Gly-Phe-X-Gly) is conserved in all the cholinesterases (Fig. 1).

Kinetic characterization of the Del-78–82 HuAChE reveals that its reactivity toward the substrate ATC is nearly 20-fold lower compared to the wild type enzyme. Most of this decrease is due to a 7-fold increase in the respective value of $K_m$, indicating deficient substrate accommodation in the Michaelis-Menten complex. Such destabilization is probably the outcome of an impaired interaction with residue Trp-86, since structural modifications of the loop should not affect other elements of the active center. However, the Michaelis Menten constants ($K_m$) only approximate the true dissociation constants of the non-covalent complexes. Therefore, it was important to examine the effects of loop deletion on the affinities toward inhibitors interacting with the active center. Indeed, the affinities of Del-78–82 HuAChE toward edrophonium, BW284C51 and decamethonium were 110-, 230- and 140-fold lower, respectively (see Table 2). According to the molecular models, these suboptimal interactions are the outcome of

the improper positioning of residue Trp-86 indole moiety. Interestingly, a very similar reactivity phenotype was observed for the W86F enzyme where the relative 11-fold decrease in the reactivity toward ATC also originates mostly from the increase in $K_m$ (6-fold) and the affinities toward edrophonium BW284C51 and decamethonium are 50-, 120- and 80-fold lower, respectively. In this case, the impaired interaction with the anionic subsite is probably effected through modification of the π-electron system rather than by repositioning of the interaction locus for the ligands charged groups. On the other hand, Del-78–82 HuAChE is still a highly efficient enzyme suggesting that the small relocation of Trp-86 is indeed the only structural consequence of the loop deletion. This conclusion seems to be consistent with the nearly equal effects on the affinities toward the inhibitors edrophonium BW284C51 and decamethonium. For the latter, we have already shown that changes in affinity represent a combination of effects on the active center and the PAS [14]. Thus, loop deletion appears to have no structural effect on the PAS.

The inhibition of Del-78–82 HuAChE catalytic activity by the PAS ligand propidium is about 9-fold less effective than that of the wild-type enzyme. This result indicates that truncation of the loop has not abolished the mechanism of the allosteric modulation of AChE activity [14] and that the lower inhibitory efficiency is due to the diminished capacity of Trp-86 to block the access to the active center (Fig. 3). Such interpretation is consistent with the conclusion that Trp-86 is somewhat relocated in the truncated loop structure. Furthermore, similar characteristics of inhibition by propidium were also observed for the W86F enzyme, where the smaller phenyl ring could be expected to be somewhat less efficient, than the indole moiety, in blocking the active center (Fig. 3B). Thus, the reactivity characteristics of Del-78–82 HuAChE appear to be consistent with the proposed involvement of the conformational mobility of Trp-86 in the mechanism of allosteric modulation of AChE activity.

### 3.7. Conclusions

Previous investigations of the structure-function characteristics of HuAChE demonstrated that the precise positioning of residue Trp-86 in the active center is essential for the AChE catalytic activity [10,11,27]. According to the X-ray structure of AChE [2], such positioning is achieved through a specific structure of the $L_{b3,2}$ loop and its juxtaposition against the protein core. Although this structural assembly is held together mainly by hydrophobic interactions, the revealed resistance of the loop 3D shape to modifications through selected replacements of its polar residues is rather unexpected. Particularly surprising is the insensitivity of the loop structure to replacements of the conserved proline residues. Nevertheless, in cases in which enzyme reactivity was somewhat affected by modification of the loop sequence, like that of Del-78–82 HuAChE, the effects were consistent with small relocations of residue Trp-86. Such a conclusion is also supported by the resemblance of the reactivity phenotypes of the Del-78–82 and the W86F enzymes. The interdependence between the location of Trp-86 and the loop structure is also consistent with the previously proposed mechanism of allosteric modulation of AChE activity [14]. However, the relative rigidity of the loop, indicated by the present results, precludes large amplitude loop motions like those observed in the lipases. It appears that the conformational transition of residue Trp-86, implicated in the allosteric mechanism, takes place in response to minor and specific conformational changes of the loop. In fact, such sensitivity of the Trp-86 conformation has been suggested by the high temperature dynamic simulations [33], even though the minimal loop motion precipitating Trp-86 conformational transition could not be derived from these simulations. Minor motions of the central loop portion (particularly of the main chain around position 74) may also allow for relatively unrestricted ligand access to the active site. Such motion may again be a result of specific conformational changes along the loop, presenting a far more complex dynamic behavior than the flap motions in lipases which initially served as a model for the $L_{b3,2}$ loop mobility in cholinesterases.

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