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# HOMOLOGY BUILT MODEL OF ACETYLCHOLINESTERASE FROM *DROSOPHILA MELANOGASTER*

JURE STOJAN*

*Institute of Biochemistry, Medical faculty, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia*

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Acetylcholinesterases from *Drosophila melanogaster* and *Torpedo marmorata* possess 35% identical residues. We built a homology model of the *Drosophila* enzyme on the basis of the known three-dimensional structure of *Torpedo* acetylcholinesterase, which revealed an oval rim of the active site gorge with an additional hollow which could accept small charged ligands more firmly than the corresponding surface in the *Torpedo* enzyme. This difference at the peripheral site, together with the kinetics of W121A and W359L mutants, suggests coordinate action of important hydrophobic residues that form the active site gorge during the catalytic process. It may also account for the activation–inhibition kinetic pattern which is characteristic for the insect enzyme.

*Keywords:* Homology built model; Acetylcholinesterase; *Drosophila melanogaster*; Mutants

## INTRODUCTION

Two cholinesterases are responsible for acetylcholine hydrolysis in vertebrates: acetylcholinesterase (EC 3.1.1.7., AChE) which terminates the impulse transmission at cholinergic synapses, and butyrylcholinesterase (EC 3.1.1.8.) whose physiological role is still unclear.[1] The latter enzyme is less specific and also hydrolyzes other choline esters, among which butyrylcholine appears to be the best substrate.[2]

* Tel.: +386-61-1320019. Fax: +386-61-1320016. E-mail: stojan@ibmi.mf.uni-lj.si.

Invertebrates and insects posses only one enzyme which converts butyrylcholine with only slightly lesser activity than acetylcholine.[3] Recently, the biphasic activation–inhibition kinetics, also seen with human butyrylcholinesterase,[4] was reported for the enzyme from *Drosophila Melanogaster* (DM).[3] This phenomenon could only be explained by a strong influence on the binding of the second substrate molecule to the allosteric site by the acylation of the active site.[5]

Cloning and sequencing reveal 35% identical amino acids in the *Torpedo* and *Drosophila* AChE, with the conserved three intrachain disulfides, and an additional 7% of similar residues (i.e. A and G; T and S; D and E; K and R; F and Y; N and Q; L, V, I and M).[6] Although there are several deletions and insertions in the first 534 amino acids, it seems that only deletion of two residues between positions 361 and 366 (281 and 288 in *Torpedo* numbering) could influence catalytic activity in the *Drosophila* enzyme.

In order to reach an understanding of how the unusual kinetic behavior of DM-AChE could be explained in terms of structural differences from vertebrate enzymes, a homology built model was worked out. An attempt to explain its validity with comparative kinetics on the wild type and the two mutant enzymes with an important substituted amino acid was also carried out.

## METHODS AND MATERIALS

### Model Building

Modeling was performed with WHATIF[7] on an IBM PC compatible computer with two pentium-pro processors running LINUX. Further refinement and the molecular dynamics of the DM-AChE structure were carried out using macromolecular simulation program CHARMM.[8]

### Kinetic Experiments

The hydrolysis of acetylthiocholine catalyzed by the wild type, W359L and W121A mutant DM-AChE was recorded on a stopped-flow apparatus. The product formation was followed photometrically[9] after mixing together the aliquots of two buffer solutions, one containing the enzyme and the other the substrate and Ellman's reagent, in the mixing chamber of the stopped-flow apparatus. The time course of product formation was followed at various substrate concentrations. At low concentrations the reaction was followed to its completion while at high concentrations only the initial

$$\begin{array}{ccc} S + SE & \xrightarrow{bk_i} & SEA \xrightarrow{ak_3} SE + P_2 \\ \updownarrow K_3 & & \updownarrow K_4 \\ S + E & \xrightarrow{k_i} & EA \xrightarrow{k_3} E + P_2 \end{array}$$

SCHEME 1 Kinetic model for AChE.

TABLE I Characteristic constants for the hydrolysis of acetylthiocholine by wild type and two mutant *Drosophila* acetylcholinesterases according to Scheme 1. The values were obtained by direct fitting of Eq. (1) to the experimental data in Figure 3

| | W.t. | W121A | W359L |
|---|---|---|---|
| $k_i[M^{-1}s^{-1}]$ | $1.21 \times 10^8 \pm 0.35 \times 10^8$ | $7.42 \times 10^7 \pm 0.91 \times 10^7$ | $4.88 \times 10^7 \pm 0.35 \times 10^7$ |
| $k_3[s^{-1}]$ | $2004 \pm 41$ | $952 \pm 35$ | $2192 \pm 115$ |
| $K_3$[μM] | $6.43 \pm 1.99$ | $19.8 \pm 6.0$ | $46.4 \pm 5.4$ |
| $K_4$[mM] | $31.1 \pm 2.6$ | $19.9 \pm 7.5$ | $29.5 \pm 3.5$ |
| $a$ | 0 | 0 | 0 |
| $b$ | $0.1755 \pm 0.0443$ | $0.0105 \pm 0.0035$ | $0.0453 \pm 0.0089$ |

portions were measured. The kinetic model, as described previously was used[5] (see Scheme 1), and Eq. (1) was employed in the initial rate analysis.

$$v_0 = \frac{E_0 k_3 (S)[1 + a(S)/K_4]}{S[1 + (S)/K_4] + k_3[1 + a(S)/K_4][1 + (S)/K_3]/[k_i(1 + b(S)/K_3)]}. \quad (1)$$

The values of the relevant kinetic constants were obtained by the fitting of this equation to the initial rates obtained from the curves for the time course of product formation using the non-linear regression program developed by R.G. Duggleby.[10] The results are shown in Table I.

## Materials

All experiments were done at 25°C in 10 mM phosphate buffer, pH 7.0, with a total ionic strength of 0.2 M obtained by addition of NaCl. The wild type enzyme and the mutants were produced in baculovirus infected cells,[11] purified by affinity chromatography and titrated with MEPQ synthesized according to Levy and Ashan.[12] Acetylthiocholine iodide and 5,5′-dithio-bisnitrobenzoic acid (Ellman's reagent) were obtained from BDH Biochemicals. All substances were reagent grade. The measurements were carried out on a stopped-flow apparatus PQ-SF 53 (Hi-Tech Ltd., Salisbury, UK).

## RESULTS AND DISCUSSION

Several alignments of the residues from DM-AChE with those from T-AChE are found in the literature.[13] In the original one, reported by Hull and Spierer,[6] residues 4-534 of T-AChE are aligned with residues 41-611 of DM-AChE. Among seven insertions and deletions, a 33 amino acid peptide after residue 142 missing in the Torpedo enzyme was shown to be unimportant for the enzyme's catalytic activity.[11] Consequently, a G-G-G tripeptide was used instead, to keep the distance of the two $\beta$-structures while performing the modeling. During the modeling it was seen that the other six differences were also not essential for the general course of the polypeptide backbone, since they are situated mainly at the surface of the molecule. However, it also appeared that the original alignment[6] between C330 and P372 (C254 and P292) was incorrect. The course of the backbone and the residues in this region were in a very unusual position as suggested by the CHECK module in WHATIF. Manual correction (Figure 1) reveals an additional deletion of two residues in DM-AChE just after W359, the residue known to be a part of the peripheral anionic site in acetylcholinesterases but lacking in butyrylcholinesterases.[14] Therefore, the architecture in this region could account for the differences in kinetics of DM-AChE compared to the *Torpedo* enzyme.

### Modeling of DM-AChE

The first step was to change all residues in the T-AChE refined 2.5 Å X-ray structure (Brookhaven access code 2ACE)[15] differing from those in DM-AChE. This was done by using the BLDPIR command in WHATIF followed by the steepest descent (s.d.) refinement with CHARMM. The constraints for the position of the catalytic triad residues (S276, E405, H518) and the three disulfides (104-131, 330-345, 480-598) were applied in the refinement. After checking the obtained structure with the FSTCHK option in the CHECK module, bad backbone portions were substituted with the original parts from the T-AChE X-ray structure, mutated with the MUTATE command in WHATIF, and refined again with CHARMM (without constraints). This step was repeated several times until the check score approached the score of the homology built human AChE model (Brookhaven access code 1CLJ) by Sussman *et al.*[15] Additionally, molecular dynamic calculations were performed twice during this procedure as follows: a coat of approximately 800 water molecules was added to the model

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SELLVNTKSGKVMGTRVPVLSSHISAFLGIPFAEPPVGNMRFRRPEPKKPWSGVWN
DRLVVQTSSGPVRGRSVTVQGREVHVYTGIPYAKPPVEDLRFRKPVPAEPWHGVLD
  * * * ** * *  * *         *** * ***   *** * *  ** **
4
41
ASTYPNNCQQYVDEQFPGFSGSEMWNPNREMSEDCLYLNIWVPS------------
ATGLSATCVQERYEYFPGFSGEEIWNPNTNVSEDCLYINVWAPAKARLRHGRGANG
*      * *   * ****** * ****   ****** * * *
       67       76       84        94    100
       104      113      121       131   137
--------------------PRPKSTTVMVWIYGGGFYSGSSTLDVYNGKYLAYT
GEHPNGKQADTDHLIHNGNPQNTTNGLPILIWIYGGGFMTGSATLDIYNADIMAAV
                                  *******  ** *** **    *
                                  114    121
                                  184    191      200
EEVVLVSLSYRVGAFGFLALHG-------SQEAPGNVGLLDQRMALQWVHDNIQFF
GNVIVASFQYRVGAFGFLHLAPEMPSEFAE-EAPGNVGLWDQALAIRWLKDNAHAF
  *   *  ********* *           ******** **  *  *  **   *
                     160                       179
                     230                       255
GGDPKTVTIFGESAGGASVGMHILSPGSRDLFRRAILQSGSPNCPWASVSVAEGRR
GGNPEWMTLFGESAGSSSVNAQLMSPVTRGLVKRGMMQSGTMNAPWSHMTSEKAVE
** *   * ******  **     **  * *  *   ***  * **
           200                       226    233
           276                       302    309
RAVELGRNLNCN----LNSDEELIHCLREKKPQELIDVEWNVLPFDSIFRFSFVPE
IGKALINDCNCNASMLKTNPAHVMSCMRSVDAKTISVQQWNS--YSGILSFPSAPD
    •    ***             * *           **      *  *   *
         254             265           279        290
         330             345           359        368
DFVIDGEFFPTSLESMLNSGNFKKTQILLGVNKDEGSFFLLYGAPG-FSKDSESKI
KYTIDGAFLPADPMTLMKTADLKDYDILMGNVRDEGTYFLLYDFIDYFDKDDATAL
   *** * *            *   ** *   ***  ****     * **
                         319     327    334   339
                         397     405    412   418
SRMSGVKLSVPHANDLGLDAVTLQYTDWMDDNNGIKNRDGLDDIVGDHNVICPLMH
PRLEIMNNIFGKATQAEREAIIFQYTSWEG-NPGYQNQQQIGRAVGDHFFTCPTNE
 •          *      *   *** •   • *  *       ****   **
                       378            390         402
                       457            468         480
FVNKYTKFGNGTYLYFFNHRASNLVWPEWMGVIHGYEIEFVFGLPLVKELNYTAEE
YAQALAERGASVHYYYFTHRTSTSLWGEWMGVLHGDEIEYFFGQPLNNSLQYRPVE
         •     * * ** *   * ***** ** ***  ** **   * *   *
                  425       435  440
                  503            518
EALSRRIMHYWATFAKTGNPNEPHSQESKWPLFTTKEQ--KFIDLNTEPM-KVHQR
RELGKRMLSAVIEFAKTGNPA--QDGEE-WPNFS-KEDPVYYIFSTDDKIEKLARG
  *  *       *******      *  ** *  **     *        *
           476             492      501       511
           554             569      578       588
LRVQMCVFWNQFLPKLLNAT
PLAARCSFWNDYLPKVRSWA
     * ***  ***
     521          535
     598          612
```

FIGURE 1 Comparison of amino acid sequence of T-AChE (first line) and DM-AChE (second line). Residues 4-534 seen in the X-ray structure of T-AChE are compared. Stars denote identical residues.

FIGURE 2 Stereo view of the superimposed backbone tracings of residues 4-534 of T-AChE X-ray structure (red line) and of DM-AChE homology built model (blue line). Ω loop containing W121 is in yellow. Side chains of S276, W359, W121 (S200, W279, W84, *Torpedo* numbering) and D413 are shown as ball and stick models. W359 and D413 are at the rim while S276 and W121 are at the bottom of the gorge. See Color Plate III.

structure of DM-AChE (from 2.5 to 5 Å); water molecules were relaxed by s.d. refinement with constrained protein, followed by 10 ps dynamics at 300 K; heating by 30 K in the intervals of 2 ps was performed during 20 ps dynamics from 0 to 300 K without constraints; equilibration and dynamics for 20 ps each, resulting in a structure which was once again checked for bad backbone portions, corrected as described above, and refined.

The result of this modeling procedure is shown in Figure 2 in the form of a DM-AChE model backbone superimposed on the X-ray backbone of T-AChE. The most striking difference in the two chains is seen in the loop building the acylation part of the active site (opposite to the Ω loop). Together with the helix which starts with E405 from the catalytic triad, it makes the rim of the gorge oval compared to T-AChE. Additionally, Asp72, a part of the peripheral site in the *Torpedo* enzyme [cf. Reference 4], is substituted by Tyr in DM-AChE, and another Asp (D413) approximately at the same height but away from the center of the gorge entrance may take over its role. In this case we can speculate that the additional hollow at the rim of the gorge could accept small charged ligands more firmly and differently oriented than the corresponding surface in the Torpedo enzyme.

It should also be mentioned that in the loop starting with W359 there is a Leu at position 366. It corresponds to F288 in the Torpedo enzyme which is believed to be a residue co-responsible for the very poor turnover of butyrylcholine.[14] Since the F288L-F290V double mutant of T-AChE is still relatively less effective (40% of the activity towards acetylcholine[14]) than the wild type of *Drosophila* enzyme (65%), there should be another mechanism which contributes to the substrate specificity of cholinesterases.

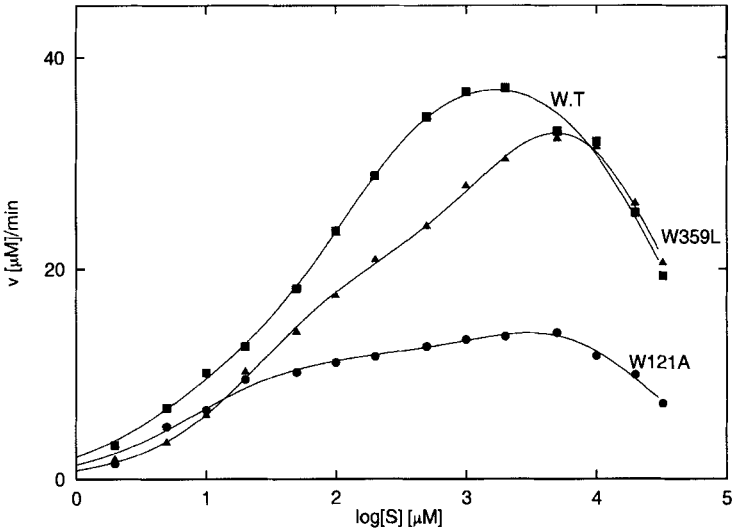


FIGURE 3 Direct plots of initial rates vs. logarithm of substrate concentration for the hydrolysis of acetylthiocholine catalyzed by wild type and two mutant *Drosophila* acetylcholinesterases.

An indication can perhaps be given by the kinetics of the wild type enzyme in comparison with W121A and W359L mutant enzymes. Figure 3 shows direct plots of initial rates vs. logarithm of substrate concentration for the three enzymes and the kinetic constants are shown in Table I. Although the two mutated tryptophans are approximately 15 Å apart, the lowering of the rate optimum with pronounced apparent activation at intermediate substrate concentrations is the common change in the curve patterns with both mutants. This suggests the coordinate action of hydrophobic residues in the active site gorge during the catalytic process. It seems that the coordination is disrupted by a lack of either of them. If this is so, the position of important hydrophobic residues forming the active site gorge during the catalytic process could also be connected with a large substrate affinity change at the peripheral site upon substrate–enzyme complex formation and/or acylation of active serine (S276): while the first substrate molecule is being processed, a coordinate flip-flop of active site gorge residues make the enzyme resistant to another substrate molecule by changing the affinity at the peripheral site. Similar kinetics was shown for W86A mutant mouse AChE[16] (corresponding to W121A of DM) but wild type vertebrate enzymes display no apparent activation at intermediate

substrate concentrations,[17] suggesting relative rigidity of the gorge. Moreover, Scheme 1, verified under a number of different experimental conditions,[5] contains all the facets in this hypothesis and by setting $K_3 = K_4$ and $b = 1$ it also explains kinetics of vertebrate wild type enzymes.

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## APPENDIX

A coordinate file of the homology built model of acetylcholinesterase from *Drosophila melanogaster* can be downloaded from the following WEB site: http://www2.mf.uni-lj.si/~stojan/stojan.html.