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## Molecular docking study on the “back door” hypothesis for product clearance in acetylcholinesterase

Received: 31 July 2005 / Accepted: 23 September 2005 / Published online: 9 December 2005  
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**Abstract** Acetylcholinesterase (AChE) is one of the fastest enzymes known, even though the active site is buried inside the protein at the end of a 20-Å deep narrow gorge. Among the great variety of crystal structures of this enzyme, both in the absence and presence of various ligands and proteins, the structure of a complex of AChE with the pseudo-irreversible inhibitor Mf268 is of particular interest, as it assists in the proposal of a back door for product clearance from the active site. Binding of Mf268 to AChE results in the carbamylation of Ser200 and liberation of an eseroline-fragment as the leaving group. The crystal structure of the AChE-Mf268 complex, however, proves that eseroline has escaped from the enzyme, despite the fact that the Ser-bound inhibitor fragment blocks the gorge entrance. The existence of alternative routes other than through the gorge for product clearance has been postulated but is still controversially discussed in the literature, as an experimental proof for such a back door is still missing. We have used Monte Carlo-based molecular docking methods in order to examine possible alternative pathways that could allow eseroline to be released from the protein after being cleaved from the substrate by Ser200. Based on our results, a short channel at the bottom of the gorge seems to be the most probable back-door site, which begins at amino acid Trp84 and ends at the enzyme surface in a cavity close to amino acid Glu445.

**Keywords** Back door · AChE · Molecular docking · Mf268 · Eseroline

### Introduction

The regulation of signal transmission at cholinergic synapses relies on acetylcholine esterase (AChE, EC 3.1.1.7), which terminates the activity of the neurotransmitter acetylcholine (ACh). [1] This process is strongly associated with cholinergic deficiencies, characteristic for Alzheimer's disease (AD), a neurodegenerative disease of the central nervous system. [2] AD is currently treated by the inhibition of AChE in order to increase the reduced ACh-level in AD-patients, e.g. by galanthamine [3] (PDB code 1QTI [4] and 1DX6 [5]), E2020 [6] (PDB code 1EVE, [7] and rivastigmine [8] (PDB code 1GQR. [9] The study of the three-dimensional complex-structure of these inhibitors with *Torpedo californica* AChE, as well as of a great variety of other AChE-inhibitor complexes and of the uncomplexed AChE, provides us with a well-founded knowledge of the structural details of this enzyme. This is particularly true for the enzyme's active site and for the inhibition mechanism. (For a complete list of AChE-crystal structures see the Protein Data Bank. [10]) The rapid process of ACh-cleavage proceeds in a narrow, 20-Å deep pocket in the protein, the active-site gorge, in which four particular regions effect the ACh hydrolysis. These are the catalytic triad made up of amino acids Ser200, Glu327, and His440 at the bottom of the gorge, the anionic subsite at Trp84, which, for example, binds the choline during the cleavage of ACh, the acyl pocket at Phe288 and Phe290, and the oxyanion hole at Gly118, Gly119, and Ala201. In addition to these particular sites located inside the gorge, the peripheral anionic site at the entrance of the gorge (Trp279, Tyr70) is of special interest as it is believed to play a role in  $\beta$ -amyloid plaque formation as a key step in the development of AD. [11]

The AChE active site is located at the end of a 20-Å deep narrow gorge inside the protein. ACh is probably attracted to the active site by a steep electrostatic potential gradient along the gorge axis [12, 13] with the help of a breathing motion of the enzyme, which opens up the gorge wide enough for the ligand to pass through. [14] The products of ACh-hydrolysis, acetate and choline, may leave the active site through either the gorge-entrance, or through alter-

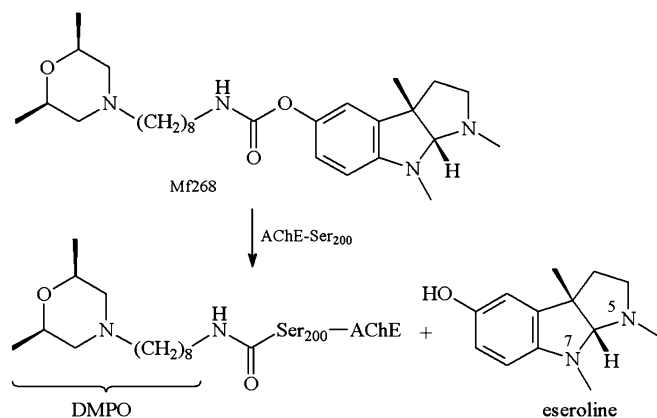
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native pathways that might open up due to conformational fluctuation of the protein. It might not always be possible for hydrolysis products to exit through the gorge entrance, as can be deduced from a crystallographic study of an AChE-complex with carbamate Mf268 (PDB code 1OCE [15]). This ligand (Fig. 1) is an analogue of physostigmine, with pseudo-irreversible inhibitory effect on AChE. [16] Interaction of Mf268 with AChE results in irreversible binding of the dimethylmorpholinooctyl (DMPO) fragment of the inhibitor to Ser200 and liberation of eseroline as the leaving group.

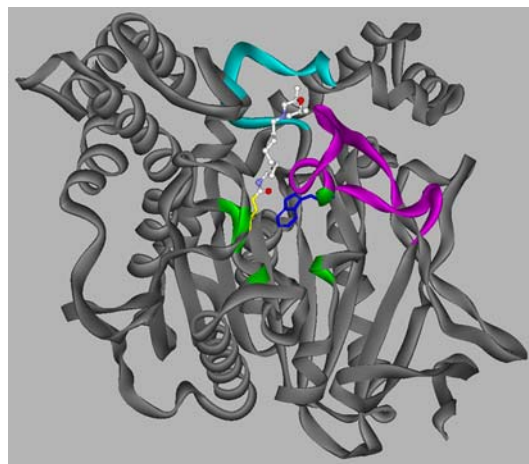
In the crystal structure of the AChE-Mf268 complex, [15] the leaving group eseroline is no longer present, and therefore must have left the complex during the handling involved in the process of enzyme inhibition and subsequent crystallography. Due to the bulkiness of the serine-bound DMPO-fragment, eseroline could not have escaped through the gorge opening, implying that alternative ways must exist for product clearance after substrate cleavage at the catalytic triad.

This experiment supports the theory of alternative passages other than through the gorge for the traffic of ligands to and from the AChE active site. [17–19] In this respect, three alternative routes have been discussed in the literature (Fig. 2). The first is a channel that begins at Trp84, accessible by a shutter-like in-plane motion of the Trp84 indole ring. This path leads to a cavity between Val129 and Tyr442, which finally emerges at a surface dimple near Glu445. [18, 20, 21] Trp84 also plays a role in the second alternative, the so-called  $\Omega$ -loop from Cys67 to Cys94, which perhaps could undergo a flap-like conformational transition so as to allow a leaving group to escape from the protein. [22, 23] Finally, a third alternative has been suggested in terms of a facial rearrangement of a loop between Trp279 and Ser291, which may produce a significant increase in the diameter of the gorge. [23]

Site-directed mutagenesis experiments, however, did not support the idea of alternative portals, as a participation of back-door traffic in catalytic activities of the enzyme was not detected either in human AChE [24] or in *Torpedo californica* AChE. [25] The only experimental evidence in favor of such an additional exit route, therefore, comes from



**Fig. 1** Pseudo-irreversible inhibition of AChE by carbamylation with Mf268



**Fig. 2** Proposed alternative exits from the active site of AChE: 1) back door (green) starting at Trp84 (blue) and stretching towards Glu445, 2)  $\Omega$ -loop stretching from Cys67 to Cys94 (purple), 3) gorge extension at Trp279-Ser291 (light blue). Ser200 (yellow) is carbamoylated with the DMPO-fragment of Mf268 (white)

the crystal structure (1OCE) of carbamoylated *Torpedo californica* AChE. [15] Additional proof is provided by the fact that an inhibitory monoclonal antibody of *Electrophorus* AChE is reported to bind to the region of the back door. [26] Furthermore, it was demonstrated in a recent crystallographic study that the loop between Trp279 and Ser 291 can be severely disrupted, producing a significant increase in diameter of the gorge. In this experiment an unusually bulky ligand could be placed into the AChE-binding site, suggesting facile rearrangement of this particular loop.

On the other hand, the existence of transient openings between Trp84 and Glu445 (*Torpedo* numbering) has been demonstrated in several recent molecular dynamics simulations, suggesting the existence of a back door, particularly in this region. [20, 21, 27–30] Although this opening has been seen in only relatively few frames of extended MD simulations, it was detected independently of the species (*Torpedo* and mouse AChE), and has occurred in the presence and absence of an inhibitory ligand (huperzine A [29]), as well as in a fasciculin-AChE complex. [20, 28] In addition, a detailed study, which includes a number of unnatural ligands (cationic, neutral and anionic species) in a multiple-copy sampling MD-technique, implies that the protein matrix surrounding the gorge is quite porous to small ligands (like ammonium and methane), while larger ligands (like acetic acid or tetramethylammonium) can only leave through the gorge entrance, or are even trapped inside the gorge. This experiment contradicts the crystal-structure experiment with Mf268, in which the leaving group eseroline must have left the catalytic site after being cleaved from Mf268.

This discrepancy initiated our study, in which we have investigated the back-door hypothesis by molecular docking techniques, looking at possible locations of eseroline after its cleavage from Mf268 by AChE, and on its putative route to the enzyme surface through a pathway other than that of the gorge. Our results strongly support the back-door

**Table 1** Results of docking unprotonated (up), N<sub>7</sub>-protonated (N<sub>7</sub>-p) and N<sub>5</sub>-protonated (N<sub>5</sub>-p) eseroline using 1OCE. All energies are given in kJ mol<sup>-1</sup>. Underlined values are lowest of the respective column. For definition of column headings see [Materials and methods](#)

Entry	$E_{\text{ass}}$	LE	$E_{\text{nbd}}$	vdW	$E_{\text{est}}$	$E_{\text{cnt}}$	vdW+	Nhph	Nhb	$S_{\text{total}}$
Up	-28.0	0.3	-28.3	-8.7	-0.4	-19.1	1.1	7	0	3
N <sub>7</sub> -p	-26.2	0.1	-26.3	-8.0	-0.3	-18.1	1.0	7	0	2
N <sub>5</sub> -p	-26.1	3.7	-29.8	1.7	-12.3	-18.0	10.6	7	0	2

channel between Trp84 and Glu445, which was proposed more than 10 years ago, [17, 19] as the most probable alternative besides the gorge for product clearance in AChE.

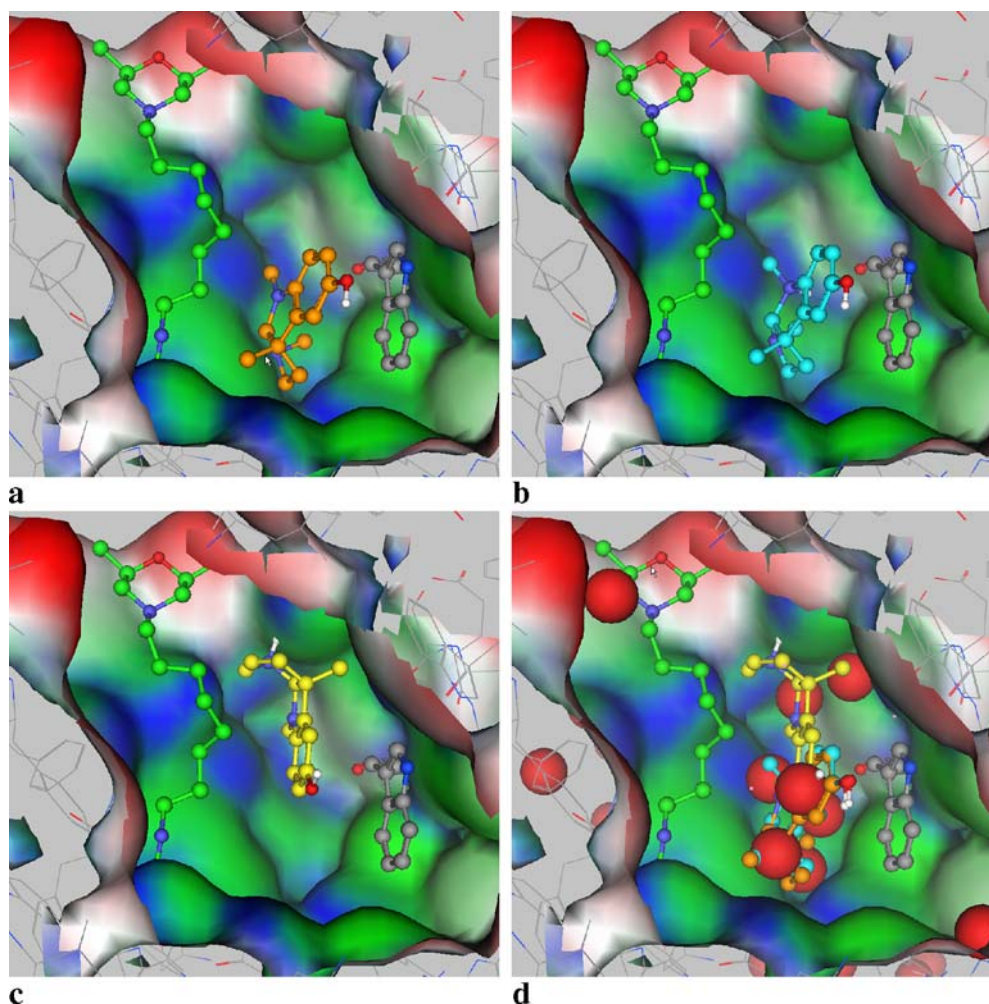
## Materials and methods

The three-dimensional structure of *Torpedo californica* acetylcholine esterase (*TcAChE*) complexed with Mf268 (PDB code 1OCE) was retrieved from the Protein Data Bank <http://www.pdb.org>. [10] Quick eXplore (QXP<sup>+</sup>), [31] which is based on the AMBER force field using full Monte Carlo (MC) as searching algorithm, was employed as our major docking tool. Furthermore, in order to limit the conformational search space, QXP<sup>+</sup> also provides a local Monte Carlo search (LMC) by restricting the implemented

rotational angle to a smaller interval, e.g. between 20–30°, rather than allowing a full 0–360° interval as in MC.

Based on this procedure, we have developed a “QXP-consensus scoring” method starting with a full MC run, and followed by a series of local Monte Carlo-docking runs (LMC-run). This multi-step docking procedure (MSD) will be described in detail elsewhere. [32] In short, the 25 results from an initial full MC-docking run were ranked according to all available calculated parameters ( $E_{\text{ass}}$  = total estimated binding energy; LE = conformation energy of the ligand;  $E_{\text{nbd}}$  = total energy of non-bonded interactions; vdW = van der Waals energy; vdW+ = positive van der Waals energy;  $E_{\text{est}}$  = electrostatic energy;  $E_{\text{cnt}}$  = contact energy of interactions; and  $N_{\text{hph}}$  = number of hydrophobic contacts). Ranking was achieved by assigning a score of 1 to all those

**Fig. 3** Docking result with neutral (a brown), N<sub>7</sub>-protonated (b light blue), and N<sub>5</sub>-protonated eseroline (c yellow). d displays an overlay of all three eseroline results with the crystallographic water molecules in the gorge of 1OCE as red balls. For comparison, Trp84 (grey) and the Ser200-bound DMPO-fragment (green) are also shown



data of a given result that lie within a range of  $\pm 2$  of the best value for the given parameter. This guarantees that all energetic parameters are taken into account. The single scores were then summed up to the total score ( $S_{\text{total}}$ ). For instance if  $E_{\text{nbd}}$  yields a lowest value of  $-35.6$  in a given set of the 25 docking answers, all those results from any of the other 24 docking answers that had  $E_{\text{nbd}}$  between  $-33.6$  and  $-35.6$  were also given a score of 1 for the  $E_{\text{nbd}}$  parameter. Positive numbers are treated likewise. If the docking result with the highest  $S_{\text{total}}$  is not the one with the lowest  $E_{\text{ass}}$ , the highest scored structure is taken as starting conformation for an LMC-run. This process is repeated until the hit with the lowest  $E_{\text{ass}}$  also shows the highest  $S_{\text{total}}$ .

Due to the limitation of the number of atoms to no more than 2,000 in QXP<sup>+</sup>, a spherical region of 17.0-Å radius (34.0-Å diameter) was cut around the hydroxyl oxygen of Tyr121, in order to make sure that the entire region of the AChE gorge is included in the selected sphere. Polar hydrogen atoms were then added, followed by optimization of their positions. This subset of the protein was used for docking experiments that typically involved 10,000 cycles per run.

## Results

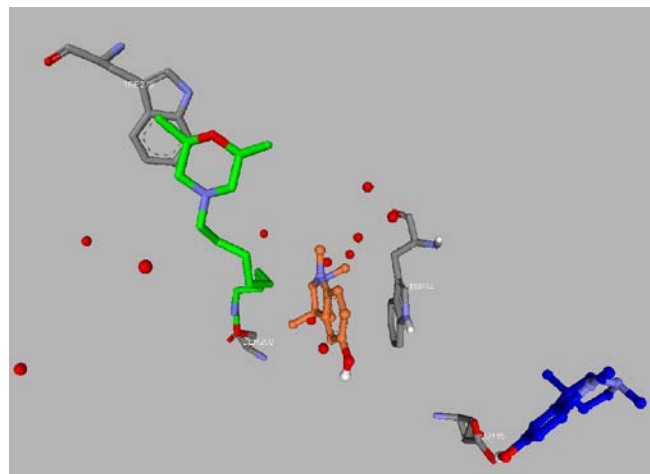
Interaction of Mf268 with AChE proceeds with carbamoylation of Ser200 and liberation of eseroline as the leaving group. In order to determine the fate of the leaving group after cleavage from the carbamate, we have investigated the location and orientation of eseroline under various conditions, using a QXP-based multi-step-docking procedure (MSD) and our newly developed QXP-consensus scoring method (for details see “Materials and methods”). This included docking experiments in the presence and absence of crystallographic water molecules as well as the various protonation states of eseroline. In addition, the reorientation of crystallographic water molecules in the presence of eseroline was examined in a simulated-annealing experiment.

### Docking of the DMPO-fragment

Initially starting from 1OCE, all water molecules and the DMPO-fragment including the linking NHCO-group were extracted from the crystal structure. Next, the unprotonated

**Table 2** Energies of interactions between unprotonated eseroline and surrounding amino acids from docking results with 1OCE in the absence of crystallographic water molecules

Amino acids	Interaction energy (kJ mol <sup>-1</sup> )
Ser122	-7.9
Gly118	-13.5
Gly117	-3.4
His440	-14.9
Glu199	-5.1
Trp84	-16.4



**Fig. 4** Docking solutions for unprotonated eseroline (blue) into 1OCE in the presence of movable water molecules (red balls) of the active site gorge. For comparison, the eseroline location from docking in the absence of water is also shown (brown). Four important amino acids (including Trp84) are shown in grey, while the covalently serine200-bound DMPO-fragment is green

and morpholino-*N*-protonated DMPO-fragment was individually docked into the ligand binding site of AChE. The resulting complex structures showed RMSD of 1.07 Å for the unprotonated fragment and a 180° flipped dimethyl-morpholino-ring in the case of a protonated structure with RMSD of 1.63 Å. This suggests that DMPO is not protonated in the bound state.

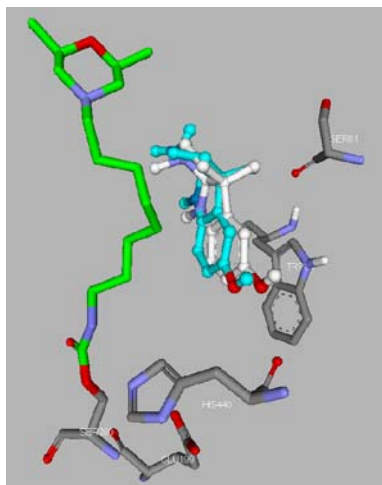
### Docking experiment with eseroline and 1OCE in the absence of crystallographic water molecules

The docking runs on the basis of 1OCE, i.e. in the presence of the DMPO-substituted Ser200, were carried out with three different protonation states of eseroline, i.e. with the neutral, N<sub>7</sub>-protonated and N<sub>5</sub>-protonated structures. The resulting QXP-data from MSD-runs are given in Table 1.

The results of all three experiments showed a positioning of eseroline at the lower end of the gorge, between the Ser200-bound DMPO-fragment and Trp84 in a cavity, which in the crystal structure is occupied by water molecules Wat811, Wat812, Wat814, Wat819, Wat839, Wat840, Wat842, and Wat903. However, while the neutral and the N<sub>7</sub>-protonated eseroline have almost the same orientation, the N<sub>5</sub>-protonated eseroline is to be found in a position that

**Table 3** Energies of interactions between unprotonated eseroline and surrounding amino acids from docking results with 1OCE in the presence of crystallographic waters molecules of the gorge

Amino acids	Interaction energy (kJ mol <sup>-1</sup> )
Glu445	-25.6
Tyr458	-2.5
Asn429	-7.5
Val431	-4.6
Leu430	-6.5



**Fig. 5** Position of eseroline in the simulated annealing experiment (*white*). In addition, the eseroline location from docking the  $N_5$ -protonated eseroline is also shown (*light blue*) together with the serine200-bound DMPO-fragment (*green*), and some amino acids of the active site, including Trp84

is rotated in the molecule plane and flipped over in comparison to the other two structures. This results in an edge-on interaction of the aromatic ring of  $N_5$ -protonated eseroline with the five-membered ring of the Trp-indole, which are about 3.6 Å apart. The other two eseroline structures are perfectly stacked over the center of the complete indole system in a distance of about 3.5 Å. (Fig. 3).

As can be seen from Table 1, the neutral eseroline shows the lowest total estimated binding energy. A detailed investigation of the interaction of this structure in association with its surrounding amino acids verifies the importance of Trp84 for eseroline binding, as it shows the highest interaction energy with  $-16.4 \text{ kJ mol}^{-1}$  (Table 2). Eseroline, in this position, is perfectly set up for departure from the active site through the back-door channel, which could be gated by a shutter-like motion of Trp84.

#### Docking experiment with eseroline and 1OCE in the presence of crystallographic water molecules

To consider the effect of water molecules on the location of the leaving group, eseroline was docked into the completely

**Table 4** Energies of interactions between unprotonated eseroline and surrounding amino acids, or water molecules from simulated annealing experiment with 1OCE

Components of the binding site interacting with eseroline	Interaction energy ( $\text{kJ mol}^{-1}$ )
Ser81	-9.4
Trp84	-13.2
Wat 840	-9.5
Wat 842	-7.5
Wat 903	-11.2

water-filled binding site of 1OCE, i.e. in the presence of crystallographic waters Wat801, Wat805, Wat811, Wat812, Wat819, Wat831, Wat839, Wat840, Wat842, Wat884, Wat892, and Wat903. Multi-step docking was then performed with neutral eseroline and movable water molecules, in order to allow for readjustment of water molecules in the presence of the leaving group. In contrast to the docking experiment without water, eseroline is not placed within the active site in this experiment, but is located behind Trp84 with respect to the cleavage site at Ser200 (Fig. 4).

Eseroline in this position chiefly interacts with Glu445, Tyr458, Asn429, Val431, and Leu430, of which Glu445 has the most prominent interaction energy (Table 3). This is due to the close distance of the carboxyl group of Glu445 and the eseroline oxygen.

#### Docking experiment under simulated annealing conditions

The influence of the flexibility of both protein and water on the favorable location of eseroline was investigated by a simulated-annealing experiment using the gorge structure of 1OCE, including all crystallographic water molecules. The highest and the lowest temperatures of the system were 600 and 30 K, respectively.

As the result of the simulated annealing conditions, eseroline is located in an almost identical position as the  $N_5$ -protonated structure in the previous experiment. Eseroline is stacked with its aromatic ring in an edge-on fashion onto the five-membered ring of Trp 84 in a distance of 3.3 Å. The OH-group shows a distance of about 8 Å to the carbamate group from which it had been cleaved off (Fig. 5). In addition, the experiment also identifies water molecules Wat811, Wat839, Wat840, Wat842, and Wat903 as those that hold eseroline in place, of which Wat840, Wat842, and Wat903 show the most prominent interactions. Trp84 and Ser81 are the amino acids that contribute measurable interaction energies to the stabilization of eseroline in this location (Table 4).

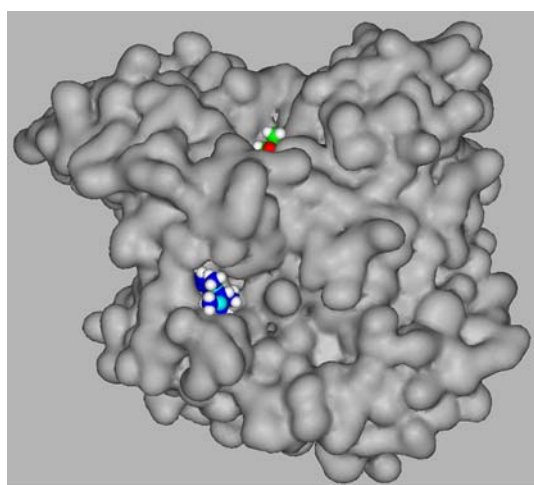
## Discussion

In our molecular-docking study, we have investigated the possible fate of the leaving group eseroline, liberated during the AChE-cleavage process as the result of inhibiting AChE with the pseudo-irreversible ligand inhibitor Mf268. Our investigation is based on the crystal structure of the carbamoylated AChE by Bartolucci et al. (1OCE), which resulted from covalent binding of the inhibitor Mf268 to AChE. As the leaving group of this process, the alcohol eseroline, was not detected in the crystal structure of the complex, eseroline must have left the enzyme during the processes of enzyme inhibition and subsequent handling of the complex. Furthermore, as the presumed entrance route for the inhibitor, the AChE gorge, is blocked by the serine-bound DMPO-fragment of the inhibitor, eseroline must have left the gorge through an alternative route rather than through

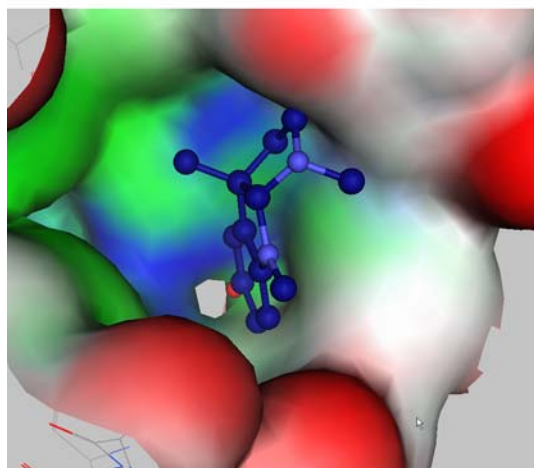
the gorge opening. Of the three alternative portals that have been proposed for product clearance from the active site, [15, 20, 22, 23] our results favor the back-door theory, in which a channel is proposed that begins with Trp84 as the entrance gate, and ends with Glu445 in a small cavity at the enzyme surface.

The docking experiments with eseroline and the carbamoylated AChE protein 1OCE disclosed two possible locations for the leaving group. When crystallographic water molecules are omitted from the AChE structure, there is enough room between the carbamate cleavage site at Ser200 and the putative channel entrance at Trp84 to accommodate eseroline in an energetically favored position parallel to the Trp-indole ring. The leaving group in this case is surrounded by amino acids Trp84, Gly117, Gly118, Ser122, Glu199, and His 440. In the crystal structure 1OCE this space is occupied by eight water molecules.

In contrast, in the presence of the twelve water molecules present in the AChE gorge in 1OCE, there is no room for



**a**



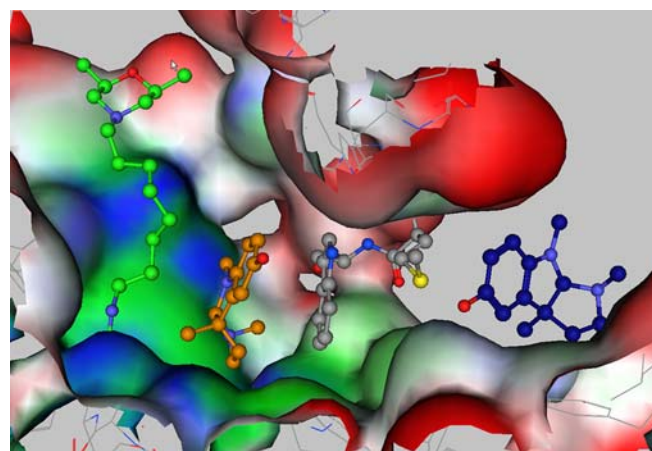
**b**

**Fig. 6** **a** Surface view of AChE showing the position of eseroline (blue with light blue N-atoms and white H-atoms) when crystallographic water molecules of the gorge are included in the docking experiment. At the entrance of the gorge, a part of the DMPO-fragment is also seen (green with red oxygen and white H-atoms). **b** Cavity near Glu445 which is the putative exit site of eseroline

eseroline, even though the water molecules were allowed to move during the docking process. Instead, eseroline, under these conditions, is found in a cavity at the protein surface close to Glu445, which may be the site of the putative exit of the back door channel (Fig. 6). The exit door is part of a cavity at the enzyme surface that is composed by amino acids Asn429, Leu430, Val431, Glu445, and Tyr485. In the crystal structure 1OCE, the water molecules Wat807, Wat823, Wat830, Wat869, Wat897 are located in this cavity.

Under simulated-annealing conditions, however, when eseroline is immersed into the twelve gorge water molecules, the system can adjust to accommodate eseroline in front of Trp84 parallel to the Trp-indole ring. The eseroline position is again very similar to that resulting from the docking experiment in the absence of water. This confirms the argument that eseroline moves towards Trp84, the putative gate to the back door channel, after being liberated from Mf268. The distance from this point to the enzyme surface it is only about 8 Å. The channel behind Trp84 consists of a narrow pass, which is only blocked by Met83 and a few water molecules, as depicted in the crystal structure of 1OCE (Fig. 7). It is, however, wide enough to allow eseroline to pass through, presumably assisted by conformational fluctuations of the protein.

Our docking studies do not account for any positioning of eseroline in the vicinity of the two other proposed alternative portals, i.e. in the area of the Cys67 to Cys94 loop, and the Trp279-Ser291 loop, respectively. Our results, therefore, suggest the alternative channel from Trp84 to the vicinity of Glu445 at the surface as the most probable path for product clearance in AChE from the lower end of the gorge. Trp84, in this mechanism, serves as the gate that could be opened by a shutter-like movement of the Trp-indole ring, in order to allow a leaving group to escape. Much like the swinging gate Phe330 that controls the entrance of the natural substrate Ach, or an inhibitor to the gorge, the Trp-shutter only has to function every 0.1 ms or



**Fig. 7** Cross-section view of the gorge and the alternative channel with (from left to right): DMPO-fragment (green) in the gorge, eseroline (brown) in front of the back door channel after it has been cleaved off from Mf268, Trp84 (grey) as the putative gate to the channel, Met83 (grey) as the only amino acid between the back door and the exit of the channel, eseroline (blue) in the cavity that represents the exit site of the channel

less. [33] It is, therefore, well in accord with an almost diffusion-controlled reaction time for the cleavage mechanism of AChE.

Our findings contradict results obtained from a multiple-copy sampling MD study in which it was predicted that only very small species like ammonium ions and methane would be allowed to leave the protein through the back door, as only limited movement of the Trp84 side-chain could be observed. [21] On the other hand, our data is in accordance with recent MD-simulation studies from the McCammon-group, [20, 34, 35] which demonstrated that the back door is the most probable site of product clearance from the gorge in comparison to the “side door” in the  $\Omega$ -loop from Cys67 to Cys94. The side-chain of Trp84 has been shown to open the gate to the back door by sweeping through an angle of almost 90° to a position where it interacts with Tyr442. In addition, docking experiments with choline, the leaving group from the initial step of an ACh-cleavage by AChE, showed this fragment to be right in place for leaving through the back door towards the enzyme surface. [35] Eseroline in our docking study is even closer to the back door entrance, which further supports a possible clearance through this alternative exit.

One should bear in mind, however, that our molecular docking experiments only show static pictures of this fascinatingly quick biological process and, therefore, can only suggest mechanistic details of the product clearance through an AChE back door. Similarly, MD experiments have also to be interpreted with caution, because even a simulation of 10 ns covers only a time-fraction of the catalytic cycle of AChE. Therefore, the observation of open back-door conformations in MD simulations is also not a guarantee for the involvement of the back door in AChE catalytic function. Unequivocal proof of such a procedure could only be obtained through experimental data, and perhaps through time-resolved crystallographic studies.

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