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Brief Article

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Structures of Human Acetylcholinesterase in Complex with Pharmacologically Important Ligands

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Supporting Information

ABSTRACT: Human acetylcholinesterase (AChE) is a significant target for therapeutic drugs. Here we present high resolution crystal structures of human AChE, alone and in complexes with drug ligands; donepezil, an Alzheimer's disease drug, binds differently to human AChE than it does to *Torpedo* AChE. These crystals of human AChE provide a more accurate platform for further drug development than previously available.

INTRODUCTION

Acetylcholinesterase (AChE) degrades the neurotransmitter acetylcholine and is critically important for regulation of neurotransmission at synapses in all areas of the nervous system.[1] Defects in cholinergic neurotransmission are significant for the progression of Alzheimer's disease (AD), so cholinesterase inhibitors such as donepezil, galantamine, rivastigmine, and huperzine A have been extensively studied as symptomatic treatments for AD.[2,3] Large-scale inactivation of AChE has lethal consequences for any organism with a nervous system, so irreversible organophosphate inhibitors have been used as insecticides and as chemical warfare agents.[4]

Most crystallographic studies of AChE involve the mouse or the electric ray (*Torpedo californica*) homologues.[5] Of the over 100 AChE structures deposited in the Protein Data Bank, only four human AChE structures of the catalytic core have been solved to date, of which none are in complex with therapeutic drugs used for the treatment of disease. In addition, their resolutions have been limited to ∼3 Å. Crystallization of human AChE has generally required the presence of the snake venom toxin fasciculin-2 (Fas2),[5−7] an inhibitor which may impede the formation of drug complexes. Here we present a structure of recombinant human AChE (rhAChE), without Fas2, at a higher resolution of 2.15 Å in the unliganded state and in complex with donepezil and other drug molecules. We show that donepezil binds to rhAChE in a significantly different conformation than it does to *Torpedo californica* AChE (tAChE),[8] thus exemplifying how the human enzyme is more accurate for the study of drug binding. We also show that the crystals are amenable to the formation of drug complexes by cocrystallization or soaking and represent a general flexible platform for the rapid study of AChE inhibitors.

RESULTS AND DISCUSSION

The structures of rhAChE in various states are summarized in Table 1, and full diffraction data and refinement statistics are given in Supporting Information Table 1. Crystals of the apo (unliganded) state and of the (−)-huperzine A, (−)-galantamine, and donepezil (*R* enantiomer) complexes belong to space group $P3_121$ and are isomorphous to one another; each contains two molecules in the asymmetric unit (Figure 1). A dimer formed between helices $\alpha^3_{7,8}$ and α_{10} (secondary structure element naming as previously described[9]) of each molecule is similar to that seen in AChE of mouse,[10] *Drosophila*,[11] and *Torpedo*[12] and buries a total of 1932 Å^2 of solvent-accessible surface area. In addition, our rhAChE:Fas2 structure (Supporting Information Figure 1) is very similar to one previously reported,[7] but our higher resolution (2.6 Å versus 2.8 Å) improves the quality of the model.

Previous human AChE crystal structures show an active site that is partially blocked, either by binding of the black mamba toxin fasciculin-2[6,7] (Fas2), or by crystal contacts with another molecule of hAChE.[5] In contrast, our crystal lattice leaves the entire active site gorge open and accessible to small molecule substrates or inhibitors. We have determined the structure of rhAChE with three different inhibitors used to treat Alzheimer's disease[2,3] (Table 1, Supporting Information Table 1). The inhibitor complex structures are of similar resolution to the unliganded parent structure (Table 1) and show strong $F_o - F_c$ difference density, permitting unambiguous placement and refinement of the ligands, starting from a ligand-free model (Figure 2a−c). Y337, which has been described as a "swinging gate",[8] moves out of the active site gorge upon donepezil binding (Figure 2c).

There are no other significant changes in the active site in any of these structures (all-atom root-mean-square deviations less than 0.74 Å). The binding of (−)-huperzine A (Figure 2a) involves direct hydrogen bonds between O1 and N2 of the ligand with Y133 and Y337, respectively (See Supporting Information Figure 2 for ligand numbering). The interaction with Y337 (F330 in the tAChE:huperzine structure;[13] sequence alignment shown in Supporting Information Figure 3a) is critical for the inhibition of the human enzyme.[14] The binding of (−)-galantamine (Figure 2b) is similar to that observed in

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Table 1. Summary of Structures

	rhAChE (apo)	rhAChE:(−)-huperzineA	rhAChE:(−)-galantamine	rhAChE:donepezil
complex formation method	not applicable	soaking	cocrystallization	cocrystallization
d_{min} (Å)	2.15	2.30	2.40	2.35
space group	$P3_121$	$P3_121$	$P3_121$	$P3_121$
$R^a/R_{free}{}^b$ (%)	18.4/22.0	17.6/21.2	16.7/20.6	17.5/21.2
PDB code	4EY4	4EY5	4EY6	4EY7

$^aR = \sum||F_o| - |F_c||/\sum|F_o|$, where F_o and F_c denote observe and calculated structure factors, respectively. $^bR_{free}$ was calculated using 5% of data excluded from refinement.

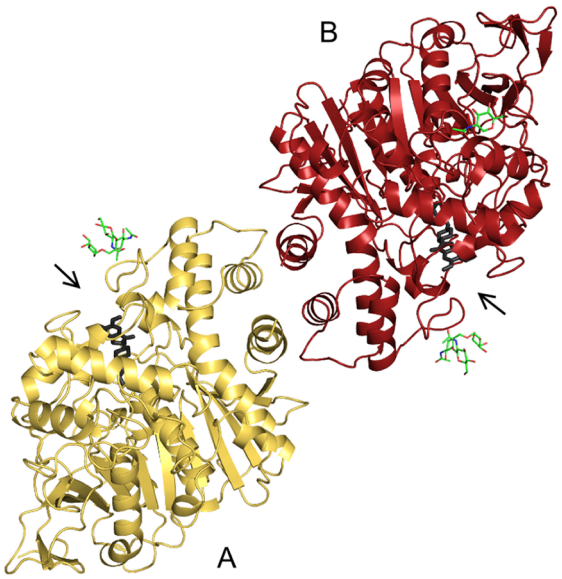

Figure 1. Structure of human acetylcholinesterase. The structure of rhAChE in complex with donepezil is depicted as a ribbon diagram. The two independent molecules A and B in the asymmetric unit of the crystal are colored yellow and red, respectively. Bound donepezil is shown in stick representation and colored black. Protein *N*-glycosylations are shown as sticks with carbon, oxygen, and nitrogen atoms colored green, red, and blue, respectively. Arrows depict the entrance of the active site gorge in the physiological dimer.

tAChE;[15,16] however, as with (−)-huperzine A, an additional hydrogen bond has formed between the tertiary amine (N10) of (−)-galantamine and Y337, which is in a different orientation than the corresponding F330 of tAChE.

The binding of donepezil (Figure 2c) is more complex and involves residues that span the length of the active site gorge. Donepezil is a racemic mixture of *R* and *S* enantiomers, both of which bind to human AChE with similar affinities.[17] Refinement of the *R* enantiomer produced a better match to our electron density than did the *S* enantiomer, so our model contains only the *R* form. Superposition of our rhAChE:donepezil complex with the tAChE:donepezil complex[8] reveals a broadly similar interaction but with one key difference (Figure 3a). The aromatic groups at the two ends of donepezil are in the same positions: the benzyl ring stacks against W86 (W84 in tAChE) in the active site, while the indanone ring stacks against W286 (W279 in tAChE) in the peripheral anionic site. However, the piperidine ring in the middle of the donepezil molecule is almost completely flipped over (142° rotation about the C10−C11 bond). If this ring is modeled and refined using the tAChE conformation, the fit to the electron density is inferior and some excessively close contacts with the protein are introduced. The piperidine ring flip permits a water-mediated hydrogen bond between N15 of donepezil and Y341 and Y377 of rhAChE. In the tAChE:donepezil complex, N15 makes water-mediated hydrogen bonds to Y121 and other residues on the opposite side of the active site gorge.[8] Inspection of electron density maps, calculated from structure factors deposited for the tAChE:donepezil complex, demonstrates

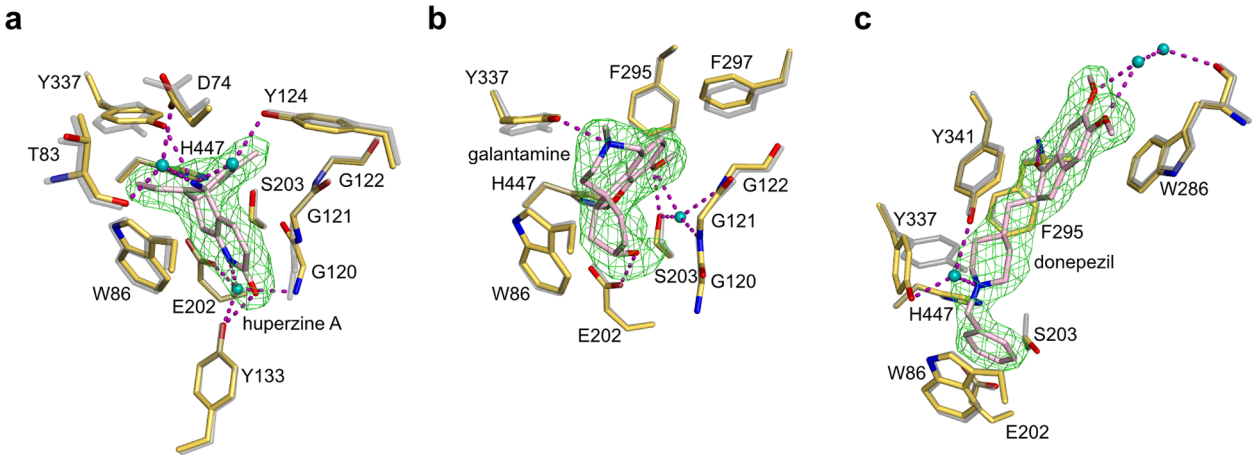

Figure 2. Active site bound ligands. (a−c) Close up views of the rhAChE active site with bound (−)-huperzine A, (−)-galantamine, and (R) donepezil, respectively. Carbons are colored pink in the ligands and light yellow in residues of the complex. Residues of the apo state are shown superimposed and colored semitransparent gray. All oxygen and nitrogen atoms are colored red and blue, respectively. Waters are shown as turquoise spheres, and hydrogen bonds are drawn as purple dashes. An $F_o - F_c$ simulated-annealing omit map, contoured at 4σ and colored green, is shown around the ligands.

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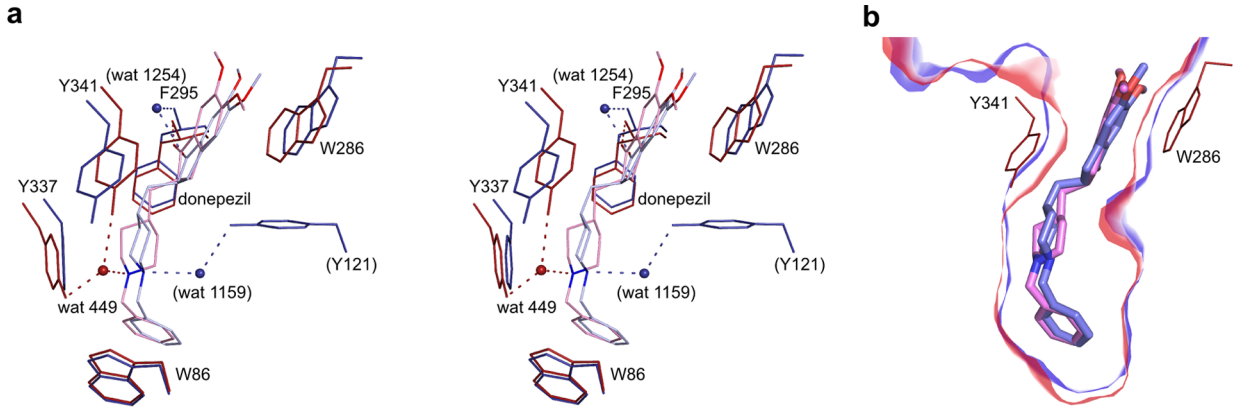

Figure 3. Donepezil binding site differences. (a) A region near the active site of the human actylcholinesterase:donepezil complex is shown in stereo view, with residues from *Torpedo* acetylcholinesterase (solved at 2.5 Å[8]) superimposed. Ligand and protein atoms are shown as sticks, with waters shown as spheres. Protein residues, waters, and hydrogen bond dash representations are colored red in the human structure and blue in the *Torpedo* structure. Donepezil nitrogen and oxygen atoms are colored blue and red, respectively, while carbon atoms are colored pink in the human-bound conformer and light blue in the *Torpedo*-bound conformer. Residue numbering refers to human acetylcholinesterase, with equivalent numbers for tAChE in parentheses. (b) The active site gorge of the superimposed human and *Torpedo* AChE:donepezil complexes is shown; the red and blue "ribbons" represent a 0.5 Å wide slice of the solvent-accessible surface of the human and *Torpedo* structures, respectively. The bound donepezil molecules for both structures are depicted as in (a). Residues W286 and Y341 of the human structure are shown in stick form; the equivalent tAChE residues are omitted for clarity.

that the orientation of the piperidine ring in the tAChE:donepezil complex is a superior fit for that structure than our ring orientation would be. In addition, the atomic *B*-factors of the piperidine ring in both the human and *Torpedo* AChE structures are comparable with that of atoms in the rest of ligand and surrounding protein side chains, indicating that the entire ligand is well ordered in each structure.

Differences in the shape of the binding site between rhAChE and tAChE are evident from analysis of solvent-accessible surface boundaries (Figure 3b). The channel volume (calculated by the 3V program[18]) in rhAChE is 683 Å^3, while that of tAChE is 727 Å^3. The entrance of the active site gorge in rhAChE is markedly narrower, as Y341 and W286 lie ∼1.5 Å closer to each other than do the corresponding residues in tAChE. The overall conformations of rhAChE and tAChE are very similar (RMSD = 0.93 Å over 522 C^{α} positions), and the residues lining the active site gorge are highly conserved. However, the second shell of residues, which pack against the active site gorge residues, are not so well conserved (e.g., P446 in rhAChE corresponds to I439 in tAChE), and the different packing environments produce the different gorge shape.

The narrower gorge in rhAChE, created by different positions of Y337 and Y341 (Y334 and F330 in tAChE), results in a conformation where C15 and C16 of the piperidine ring (Supporting Information Figure 2) pack against the hydrophobic portions of the side chains of Y337, F338, and H447. Packing in this region is quite tight, and only the smallest substituents (e.g., H → F) might be accommodated. In contrast, the other side of the piperidine ring (atoms C12 and C13) face a negatively charged and hydrophilic environment, including the hydroxyl group of Y124 and the side chain of D74, as well as several bound water molecules. This region is much more open; a substituent on C13 could extend nearly 10 Å in the general direction of A127. Although it was discovered early in the development of donepezil that derivatives possessing the piperidine ring showed greatly lowered IC_{50} values,[17] further SAR studies concentrated on the ends of the molecule, where the indanone and benzyl rings are in donepezil, and modification of the piperidine ring was not tested.[19] The effects of substitutions as described above remain to be explored.

Differences in the binding of flexible ligands that span the length of the active site gorge in acetylcholinesterases of related species (Supporting Information Figure 3b) have been documented before,[20] and the cause of such differences is likely attributed to the types of subtle changes in gorge shape that we observe in our analysis. It would be expected that the differences in gorge shape may generally affect the binding of long inhibitors capable of conformational variability, such as dual AChE inhibitors, which have moieties separated by a tether that simultaneously bind to both the peripheral and catalytic site.[21,22]

CONCLUSIONS

In summary, our structures reveal that the binding site of human AChE is significantly different than that of *Torpedo* AChE and leads to the binding of donepezil in different conformations that may be of consequence toward further design of next generation derivatives. It follows that the human enzyme is a better starting model than the *Torpedo* enzyme for structure-based drug design, modeling, docking, and molecular dynamics computations. As a platform for further experimental studies, we show that this crystal form permits soaking and cocrystallization of hAChE with a wide variety of ligands, yielding the high-resolution structures necessary for such efforts.

EXPERIMENTAL SECTION

General Methods. Using standard cloning protocols, a cleavable octahistidine tag was engineered into a construct based on a previously reported soluble hAChE construct containing a C-terminal truncation.[23] Recombinant protein was expressed from a stable HEK 293-H (Invitrogen) cell line, purified by nickel-affinity and gel filtration chromatography on an AKTAxpress system (GE Healthcare) and subjected to tag cleavage and removal before crystallization experiments. Crystals of ligand-free rhAChE and the rhAChE:Fas2 complex were grown from conditions containing polyethylene glycol (PEG) 3350 and potassium nitrate, or ammonium sulfate buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), respectively,

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via the sitting drop or hanging drop vapor diffusion method. The drugs (−)-huperzine A and (−)-galantamine were obtained from Sigma-Aldrich, and donepezil was obtained from Santa Cruz Biotechnology. Crystals of ligand-bound rhAChE were obtained by cocrystallization or soaking, and following data collection, structures were solved by molecular replacement. For detailed methods, please refer to the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for cloning, protein expression, purification, crystallization, ligand soaking, data collection, and structure determination. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The atomic coordinates and structure factors for the structures of ligand-free rhAChE, and rhAChE in complex with huperzine A, galantamine, donepezil, and Fas2 have been deposited in the Protein Data Bank with codes 4EY4, 4EY5, 4EY6, 4EY7, and 4EY8, respectively.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AChE, acetylcholinesterase; AD, Alzheimer's disease; Fas2, fasciculin-2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, polyethylene glycol; rhAChE, recombinant human acetylcholinesterase; RMSD, root-mean-square deviation; tAChE, *Torpedo californica* acetylcholinesterase

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