Crystal Structure of Thioflavin T Bound to the Peripheral Site of Torpedo californica Acetylcholinesterase Reveals How Thioflavin T Acts as a Sensitive Fluorescent Reporter of Ligand Binding to the Acylation Site

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Abstract: Acetylcholinesterase plays a key role in cholinergic synaptic transmission by hydrolyzing the neurotransmitter acetylcholine with one of the highest known catalytic rate constants. Hydrolysis occurs in a narrow and deep gorge that contains two sites of ligand binding: A peripheral site, or P-site, near the gorge entrance that contributes to catalytic efficiency both by transiently trapping substrate molecules as they enter the gorge and by allosterically accelerating the transfer of the substrate acyl group to a serine hydroxyl in an acylation site or A-site at the base of the gorge. Thioflavin T is a useful reporter of ligand interactions with the A-site. It binds specifically to the P-site with fluorescence that is enhanced ∼1000-fold over that of unbound thioflavin T, and the enhanced fluorescence is quenched 1.5- to 4-fold when another ligand binds to the A-site in a ternary complex. To clarify the structural basis of this advantageous signal change, we here report the X-ray structure of the complex of thioflavin T with Torpedo californica acetylcholinesterase. The two aromatic rings in thioflavin T are coplanar and are packed snugly parallel to the aromatic side chains of Trp279, Tyr334, and Phe330. Overlays of this structure with the crystal structures of Torpedo californica acetylcholinesterase complexes with either edrophonium or m-(N,N,N-trimethylammonio)-2,2,2-trifluoroacetophenone, two small aromatic ligands that bind specifically to the A-site, indicate that the phenyl side chain of Phe330 must rotate to sterically accommodate both thioflavin T and the A-site ligand in the ternary complex. This rotation may allow some relaxation of the strict coplanarity of the aromatic rings in the bound thioflavin T and result in partial quenching of its fluorescence.

Introduction

Acetylcholinesterase (AChE) terminates synaptic transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine with one of the highest known catalytic rate constants. Hydrolysis occurs in a narrow and deep gorge that contains two sites of ligand binding: A peripheral site, or P-site, near the gorge entrance that contributes to catalytic efficiency both by transiently trapping substrate molecules as they enter the gorge and by allosterically accelerating the transfer of the substrate acyl group to a serine hydroxyl in an acylation site or A-site at the base of the gorge. Thioflavin T is a useful reporter of ligand interactions with the A-site. It binds specifically to the P-site with fluorescence that is enhanced ∼1000-fold over that of unbound thioflavin T, and the enhanced fluorescence is quenched 1.5- to 4-fold when another ligand binds to the A-site in a ternary complex. To clarify the structural basis of this advantageous signal change, we here report the X-ray structure of the complex of thioflavin T with Torpedo californica acetylcholinesterase. The two aromatic rings in thioflavin T are coplanar and are packed snugly parallel to the aromatic side chains of Trp279, Tyr334, and Phe330. Overlays of this structure with the crystal structures of Torpedo californica acetylcholinesterase complexes with either edrophonium or m-(N,N,N-trimethylammonio)-2,2,2-trifluoroacetophenone, two small aromatic ligands that bind specifically to the A-site, indicate that the phenyl side chain of Phe330 must rotate to sterically accommodate both thioflavin T and the A-site ligand in the ternary complex. This rotation may allow some relaxation of the strict coplanarity of the aromatic rings in the bound thioflavin T and result in partial quenching of its fluorescence.

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Carbachol

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TMTFA

C₅H₃\textN\textC\textOH

Gallamine

Edrophonium

Figure 1. Chemical structures of AChE ligands discussed in the text.

The crystal structure of the complex of Torpedo californica AChE (TcAChE) with 4-oxo-N,N,N-trimethylpentanaminium (PDB code 2C5F) and with TMTFA (PDB code 1AMN) both indicate a tetrahedral adduct that nearly superimposes on a modeled structure of ACh in the A-site, and reveals that Trp84 indicates a tetrahedral adduct that nearly superimposes on a (PDB code 2C5F) and with TMTFA (PDB code 1AMN) both.

Acyl transfer to Ser200 is initiated. Crystal structures also in the A-site binds to the trimethylammonium group of ACh as modeled structure of ACh in the A-site, and reveals that Trp84 indicates a tetrahedral adduct that nearly superimposes on a (PDB code 2C5F) and with TMTFA (PDB code 1AMN) both.

AChE (AChE) with 4-oxo-N,N,N-trimethylpentanaminium. Acylation of the A-site is bound at the P-site. Strong evidence supporting this selectivity to the P-site of AChE, even though this site shows no indication of the \( \beta \)-structure typical of amyloid.

The fluorescence of ThT is increased ~1000-fold on binding to the P-site of human AChE; on further binding of an A-site ligand, such as EDR or TMTFA, to form a ternary complex, the fluorescence of the bound ThT is quenched 3–4-fold. These features make ThT a more sensitive and versatile fluorescent reporter of ligand interactions with AChE than propidium. The quenching of fluorescence in the ternary complex occurs even though there is no spectral overlap, nor any obvious steric overlap (based on an absence of thermodynamic interaction), between the bound ligands. This change in fluorescence is perhaps the most direct evidence for conformational interaction between the P- and A-sites that has been obtained. Certain substrates, including acetylthiocholine (ATCh), appear to take functional advantage of this conformational interaction. These substrates can bind to both the P- and the A-sites, as initially indicated by a competition between ATCh and fasciculin for binding to the P-site, and confirmed clearly in X-ray structures of several AChE complexes, including those with ACch, choline, ATCh, thiocholine and the nonhydrolysable ACh analogue, 4-oxo-N,N,N-trimethylpentanaminium. Acylation at the A-site is accelerated when a second substrate molecule is bound at the P-site. Strong evidence supporting this model of substrate activation was obtained from titrations with ThT. These titrations provided thermodynamic estimates of substrate affinities for the A- and P-sites that were in complete agreement with kinetic estimates of these affinities derived from the substrate activation model.

One unique advantage of fluorescent ligands that bind selectively to the P-site is their ability to report on molecular interactions occurring at the A-site. The fluorescence of propidium is enhanced nearly 10-fold when it binds to the P-site, and this increase is sufficient to conduct titrations that quantify propidium affinity for the P-site and detect the formation of ternary complexes with propidium bound at the P-site and selective ligands like edrophonium (EDR) (Figure 1) bound at the A-site. Thioflavin T (ThT) (Figure 1) is a fluorophore frequently used to detect amyloid structure in proteins, but ligand-binding data indicate that it also binds with high selectivity to the P-site of AChE, even though this site shows no indication of the \( \beta \)-structure typical of amyloid.

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In view of the sensitivity of ThT fluorescence to the conformation of residues that contact ThT, it is important to obtain three-dimensional structures of binary and ternary complexes of ThT with AChE. Such structures may provide insight into the molecular details of conformational interaction between the P- and A-sites.

**Materials and Methods**

Materials. Thioflavin T chloride (Sigma) was recrystallized as described.\(^{(22)}\) TcAChE was purified from the electric organ tissue of *T. californica*,\(^{(1,23)}\) and active site concentrations were determined by fluorogenic titration with N-methyl-(7-dimethylcarbamoxy)quinolinium iodide.\(^{(28)}\) Recombinant domain IV of mouse laminin β2, expressed in 293 cells,\(^{(29)}\) was a gift from Dr. Takako Sasaki (The Shriners Hospital for Children, Portland, OR).

Crystallographic Analysis. Trigonal *P*\(_3\)121 TcAChE crystals, in which the active-site gorge is solvent-accessible, were grown by the batch-under-oil method,\(^{(30)}\) using a Douglas Instruments IMPAX 1–5 robot. In order to obtain the *P*\(_3\)121 TcAChE crystals, the protein solution used was a 1:4 mixture of *T. californica* CNS,\(^{(32)}\) and graphically fitted using COOT.\(^{(33)}\) Coordinates and structure factors were deposited in the PDB with entry code 2J3Q. X-ray data to 2.8 Å resolution were collected under cryogenic conditions, “in-house” at the Weizmann Institute of Science, on a Materials and Methods: X-ray crystallography using a Super Semi-Flex image plate area detector. Data collection and refinement statistics are shown in Table 1.

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*Number in parentheses is value for highest resolution shell 2.8–2.9 Å.*

**Results**

Fluorescence of ThT is Strongly Enhanced in Binary Complexes with TcAChE and Partially Quenched when an A-Site Ligand is Added to Form a Ternary Complex. We show in this report that a key residue involved in the binding of ThT to TcAChE is Phe330. Since the corresponding residue in human AChE (hAChE) is a tyrosine (Tyr337), it was important to confirm that TcAChE complexes involving TcAChE show fluorescence features similar to those involving hAChE. Titration of TcAChE with ThT (Figure 2A) gave the same 1200-fold increase in the relative fluorescence intensity of the bound ligand \((f_{\text{L}, \text{th}})/f_\text{L}\) previously observed with hAChE.\(^{(22)}\) However, at high concentrations of ThT, a decrease in fluorescence occurred that had not been seen with hAChE. We interpreted this decrease to reflect the low-affinity binding of a second molecule of ThT to the P-site of TcAChE (see Scheme 1). As noted in the Introduction, the binding of a ligand to the A-site in the ThT/hAChE complex would be expected to result in partial quenching of the fluorescence of thioflavin T bound at the P-site. The affinity of ThT for the A-site of TcAChE was at least an order of magnitude lower than that for the P-site, and incorporation spectrometer thermostatted at 25 °C. Samples were introduced into a Hi-Tech SFA 20 stopped-flow apparatus by mixing equal volumes (300 µl) of AChE and combined inhibitors, and the continuous fluorescence signal (F) was recorded for 3–4 min. Average F values were corrected for inner filter effects as described previously.\(^{(26)}\) Data were analyzed in accordance with Scheme 1, which assumes that AChE (E) can form the indicated binary and ternary complexes with ThT (L) and EDR (I). Ligand bound at the P-site in Scheme 1 is designated by the subscript P, while ligand bound at the A-site has no subscript. Rate equations corresponding to an equilibrium formulation of Scheme 1 were solved in the numerical integration program SCOP (Simulation Resources, Inc., Redlands, CA; version 3.5.2), and values of F were fitted in a two-step process.\(^{(22)}\) Initial fitting was conducted in the absence of EDR, with the known total concentrations \(E_{\text{tot}}\) and \(L_{\text{tot}}\) and the known fluorescence intensity coefficient for free ThT \((f_L)\) inserted as fixed input parameters, and \(K_{\text{LP}}\) and the intensity coefficient for thioflavin bound to the P-site \((f_{\text{PL}, \text{th}})/f_\text{PL}\) as the calculated output parameters. This analysis revealed small amounts of an apparent ternary complex involving ThT bound to both the P- and A-sites, so the fitting was extended to obtain \(K_{\text{PL}}\) and the intensity coefficient \(f_{\text{PL}, \text{th}}\) as additional output parameters. The fitting in principle also depended on \(K_0\) and the intensity coefficient \(f_{\text{EL}, \text{th}}\), but over a reasonable range of values \((K_0/K_{\text{PL}} > 0.25\) and \(f_{\text{EL}, \text{th}}/f_{\text{EL}} < 0.2\) the values of these parameters were unimportant (≤30% change in the output parameters). Greater uncertainty arose because \(K_{\text{PL}}\) and \(f_{\text{PL}, \text{th}}\) were highly correlated, preventing unique assignments. Consequently, in the second fitting step in the presence of EDR, a reasonable range of \(f_{\text{PL}, \text{th}}\) \((f_{\text{PL}, \text{th}}/f_\text{L} = 100–400)\) and the corresponding fitted value of \(K_{\text{PL}}\) \((34–5 \mu\text{M})\) were examined as fixed input parameters along with \(E_{\text{tot}}, L_{\text{tot}}, f_{\text{EL}, \text{th}}\), and \(f_{\text{PL}, \text{th}}\). Finally, \(K_{\text{LP}}, K_0, f_{\text{PL}, \text{th}}, f_{\text{EL}, \text{th}}\), and \(f_{\text{PL}, \text{th}}\) were obtained as fitted outputs.

\[\begin{align*}
&\text{Scheme 1} \\
&\quad EL \quad K_L \quad + \quad E \quad + \quad I \quad \overset{K_1}{\Rightarrow} \quad EI \\
&\quad L \quad L \quad L \quad \downarrow \quad \downarrow \quad K_{\text{LP}} \quad \downarrow \quad \downarrow \\
&\quad EL_pL \quad L \quad + \quad EL_p + \quad I \quad \overset{K_{\text{LP}}}{\Rightarrow} \quad EL_pI
\end{align*}\]
Figure 2. (A) Fluorescence titrations of Tc-AChE with ThT. Fluorescence values (F) were measured as outlined in the Methods at the indicated concentrations of ThT. The fluorescence intensity coefficient for the free ligand (fL) was obtained from the plot in the absence of AChE (Δ). The fluorescence F in the presence of a fixed concentration of Tc-AChE (170 nM) (C) was then analyzed as outlined in the Experimental Procedures. Values of Kp = 0.6 ± 0.3 µM and fL/fp = 1200 ± 300 were obtained. (B) EDR binding decreases the fluorescence of Tc-AChE-bound ThT. The fluorescence values (F) of mixtures of Tc-AChE (168 nM), ThT (C, 10 µM; ○, 3 µM; ◊, 1 µM), and the indicated concentration of EDR were determined. F values from all three data sets were fitted with the SCoP program simultaneously as outlined in the Experimental Procedures. The fitting gave Kp = 0.7 ± 0.2 µM for ThT; Kp = 190 ± 10 nM for EDR; Kp/Kp = 4 ± 1; and fL/fp = 3.4 ± 0.6. of this A-site binding did not obscure further thermodynamic analysis with the A-site ligand EDR, but did lower the precision with which some binding parameters could be determined (see Experimental Procedures).

We next examined the effect of increasing EDR concentrations on the fluorescence of bound ThT, and observed a decrease in fluorescence as EDR saturated the A-site (Figure 2B). Fitting of the data to Scheme 1 gave values of Kp for ThT and Kp for EDR, and indicated a slight decrease in the affinities of the ligands in the ternary complex relative to the binary complex (Kp/Kp = 4 ± 1). Almost no decrease in these relative affinities had been observed with Tc-AChE (Kp/Kp = 1.12 ± 0.02). However, the binding of EDR decreased the fluorescence of ThT in the ternary complex (fL/fp) by a factor of 3.4 ± 0.6, comparable to the decrease previously seen with Tc-AChE (2.76 ± 0.02). These data thus confirm that the fluorescence features of ThT complexes involving Tc-AChE are essentially the same as those involving hAChE.

X-Ray Structure of the ThT/TcAChE Complex. We earlier obtained structures of a number of complexes of Tc-AChE with A-site ligands,5,35 and with ligands spanning the A- and P-sites,6,36 by diffusing the ligands into the protein crystals in the P321 trigonal crystal form. In this crystal form, the P-site at the entrance to the active-site gorge is blocked by a segment of a symmetry-related Tc-AChE molecule. Hence, it was not possible to obtain structures of complexes with bulky P-site ligands using this approach with such crystals. A breakthrough was achieved when it was discovered that crystals of Tc-AChE could be grown in the presence of gallamine triethiodide, a P-site ligand with relatively low affinity, to yield a P321 trigonal crystal form in which the entrance to the active-site gorge is more open.37 Since then, several other crystallographic additives have been found to yield crystal forms of Tc-AChE in which the P-site is accessible to bulky ligands.38,39 In the present study, mouse recombinant domain IV of laminin β229 was used as a crystallization additive to obtain the P321 crystals. The rms deviation of the Cα chain between the P321 and the P321 crystal structures is only 0.3Å, indicating a virtually identical overall conformation of the two molecules. However, P-site ligands could be soaked into the P321 crystal form;37 indeed, a crystal structure at 2.8Å resolution was obtained upon soaking such Tc-AChE crystals with ThT (Figure 3).

The X-ray structure of the ThT/Tc-AChE complex refined to an R factor of 19.6% and an R-free of 25.1%. Residues 4–536 of Tc-AChE were traced in the electron density map, as well as one molecule of ThT, 121 water molecules, and 3 N-acetylgalcosamine moieties attached to Asn residues 59, 416 and 457. Two bound Mg2+ ions, derived from the mother liquor, are seen in the electron density map. One of these Mg2+ ions is in contact with Glu268Oε1 and Oε2 and with His264Nε2 (2.22, 3.34, and 2.66 Å, respectively); the second with Asp392Oε2, Asp326Oε1 and a water molecule (2.35, 2.43, and 2.36 Å, respectively). The positions of the two Mg2+ ions superimpose, respectively, on those of a Mg2+ ion and a Zn2+ ion in a structure of Tc-AChE crystallized in the presence of MgCl2 (Anne Nicolas, unpublished results).

Figure 3 shows that the planar ThT molecule is lodged in the upper part of the active-site gorge, within the P-site, making nonbonding interactions with four of the aromatic side-chains that line the gorge surface, viz. Tyr70, Tyr121, Trp279 and Phe330. Superposition of the ThT/Tc-AChE structure on that of native Tc-AChE (PDB code 1EA5) yields rms = 0.33 Å (for 532 Cα atoms), and shows that all the gorge side-chains maintain their native conformation in the complex and that the ThT molecule fills the space within the gorge previously occupied by four water molecules.

More detailed inspection reveals that the benzothiazole ring of ThT is stacked against Trp279, and that its dimethylami-
nophenyl moiety is nearly coplanar with the phenyl group of Phe330 at a distance of 3.5 Å (Figure 3). Other interactions between ThT and the protein include a 3.2 Å contact from Tyr121OH to the dimethylaminophenyl moiety and 3.4 and 3.7 Å contacts from Tyr70 and Tyr334 to the benzothiazole ring, respectively.

Overlay of the ThT/TcAChE Structure with the Crystal Structures of A-Site Complexes. Since, as mentioned in the Introduction, the A-site ligands, EDR and TMTFA, quench the fluorescence of the ThT/TcAChE complex, we overlaid both EDR and TMTFA from the EDR/TcAChE complex (PDB code 2ACK), with a model of ThT taken from the ThT/TcAChE crystal structure. From these overlays it is apparent that the ThT/TcAChE structure can sterically accommodate both EDR and TMTFA at the A-site, the aromatic rings of both EDR and TMTFA, in their respective complexes, are nearly coplanar with Trp279/ThT/Phe330 in the ThT/TcAChE complex. However, if one does the reverse, and overlays ThT from the ThT/TcAChE structure on the crystal structures of either EDR/TcAChE (Figure 4B) or TMTFA/TcAChE (not shown), it can be seen that there is a clash of the dimethylaminophenyl moiety of the ThT molecule with the phenyl side-chain of Phe330. The reason for this clash is that in both the EDR/TcAChE and TMTFA/TcAChE structures the phenyl ring is rotated ~115° relative to its orientation in both the native TcAChE and ThT/TcAChE structures.

Discussion

Crystal structures of TcAChE complexes with A-site ligands, with the P-site ligands fasciculin and propidium, show remarkably few differences in conformation of the entire active-site gorge relative to native TcAChE. These observations indicate that the binding of these ligands does not induce large (and potentially slow) conformational changes in the active site, as might be expected for an enzyme like AChE that is built for unusually high catalytic speed. The structure of the ThT/TcAChE complex also shows few changes from that of native TcAChE, and the basis of the enhanced ThT fluorescence in this complex and its partial quenching in ternary complexes are of interest. The fluorescence of ThT has been shown to depend on solvent viscosity, with a relationship previously described for a class of fluorescent dyes called molecular rotors. These dyes show increased fluorescence when introduced into high-viscosity media due to a decreased torsional relaxation. The X-ray structure of the ThT/TcAChE complex presented here shows the ThT rings system in a coplanar position packing snugly parallel to Trp279, Tyr334 and Phe330. This packing causes a decrease in torsional relaxation and, hence, the increase in fluorescence.

The structures of complexes of TcAChE with bis-quaternary and other gorge-spanning ligands confirm that they bridge the A- and P-sites and traverse the region occupied by the bound ThT. In some of these structures the bifunctional ligands display contacts with Trp279 and Phe330 of TcAChE that bear some similarity to those made by ThT. Thus, in the BW284C51/TcAChE complex (PDB code 1E3Q), one quaternary phenylammonium group of the symmetric bifunctional ligand interacts with Trp279, with the two aromatic rings being coplanar, and an allyl group at its other extremity makes a strong hydrophobic interaction with Phe330. The Alzheimer disease drug donepezil (E2020 or Aricept) also spans the A- and P-sites. Its indane ring stacks against the indole ring of Trp279, and the charged nitrogen of a piperidine ring, separated by one methylene group from the indane ring,
makes a cation-π interaction with the phenyl ring of Phe330.\textsuperscript{16} In the crystal structure of the complex of \textit{Ta}AChE with the anticancer prodrug CPT-11 (PDB code 1U65), another ligand that spans the A- and P-sites,\textsuperscript{37} the planar camptothecin moiety superimposes on the planar benzothiazole ring of ThT and stacks against Trp279. The terminal piperidine moiety of CPT-11 occupies the same space as the quaternary ammonium moiety of EDR at the A-site, and the penultimate piperidino ring stacks against the phenyl ring of Phe330. However, in all three of these complexes this phenyl ring rotates away from its position in the ThT/\textit{Ta}AChE complex to allow the ligand to span the A- and P-sites.

The packing of ThT with its rings snugly parallel to Trp279, Tyr334, and Phe330 reveals a precise ligand location that has not been previously observed in X-ray structures of AChEs complexed with other ligands specific for the P-site. In the complex of mouse AChE (mAChE) with the P-site ligand gallamine (Figure 1) (PDB code 1N5M), the aromatic moiety of the ligand, which is the only part of the gallamine molecule seen in the structure, is positioned similarly to ThT, making stacking contacts with both Trp286 and Tyr341 but not Tyr337 (Trp 279, Tyr 334, and Phe 330, respectively, in \textit{Ta}AChE).\textsuperscript{10} In structures of mAChE complexes with two other ligands that bind selectively to the P-site, propidium and decidium, the aromatic rings or that it imposes a smaller rotation of the phenyl ring of Phe330 from coplanarity with the dimethylaminophenyl moiety of ThT in the ternary complex.\textsuperscript{26} while the A-site ligand carbachol quenches ThT fluorescence by only 35%. (T.L. Rosenberry, unpublished data). Carbachol (Figure 1), an ACh analog, probably produces a change in conformation of Phe330 similar to that seen in the crystal structures of the complexes with ATCh and thiocholine,\textsuperscript{24} which in turn, is similar to those produced by EDR and TMTFA. The smaller quenching produced by carbachol, compared to the much larger quenching produced by EDR and TMTFA, may be due to the fact that it does not participate in a comparable stacking array involving stacking of the ThT with the \textit{Ta}AChE aromatic rings or that it imposes a smaller rotation of the phenyl ring of Phe330 from coplanarity with the dimethylaminophenyl moiety of ThT in the ternary complex.

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