

## COMMUNICATION

# A Neutral Molecule in a Cation-binding Site: Specific Binding of a PEG-SH to Acetylcholinesterase from *Torpedo californica*

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The crystal structure of acetylcholinesterase from *Torpedo californica* complexed with the uncharged inhibitor, PEG-SH-350 (containing mainly heptameric polyethylene glycol with a terminal thiol group) is determined at 2.3 Å resolution. This is an untypical acetylcholinesterase inhibitor, since it lacks the cationic moiety typical of the substrate (acetylcholine). In the crystal structure, the elongated ligand extends along the whole of the deep and narrow active-site gorge, with the terminal thiol group bound near the bottom, close to the catalytic site. Unexpectedly, the cation-binding site (formed by the faces of aromatic side-chains) is occupied by CH<sub>2</sub> groups of the inhibitor, which are engaged in C–H···π interactions that structurally mimic the cation–π interactions made by the choline moiety of acetylcholine. In addition, the PEG-SH molecule makes numerous other weak but specific interactions of the C–H···O and C–H···π types.

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**Keywords:** acetylcholinesterase; crystal structure; weak hydrogen bonding; C–H···π interaction; cation–π interaction

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The active site of acetylcholinesterase (AChE) is located at the bottom of a deep and narrow cleft called the active-site gorge.<sup>1</sup> Recognition of the quaternary ammonium ion of the substrate, acetylcholine (ACh), and possibly also substrate guidance down the gorge, is facilitated by cation–π interactions<sup>2</sup> with aromatic side-chains incorporated into the gorge wall. Crystal structures of the enzyme from *Torpedo californica* (TcAChE) have been published with numerous competitive inhibitors, invariably cations.<sup>3–6</sup> A common feature of

these structures is location of a cationic charge center in (or near) the quaternary ammonium-binding site associated with cation–π interactions. We have now prepared a complex with an uncharged inhibitor, polyethylene glycol (PEG) bearing a terminal thiol (SH) group (PEG-SH, dominant oligomeric fraction heptameric), and find positioning of CH<sub>2</sub> groups in the cation-binding site, forming C–H···π interactions with a geometry very similar to that of quaternary ammonium groups. In addition, there are numerous other weak but specific polar interactions with the gorge wall and with the active-site water molecules.

The present study was prompted by the observation that in some of our TcAChE crystal structures, in addition to the electron density corresponding to the charged inhibitors, elongated features of electron density are observed in the binding pocket. They resemble molecules of the PEG used in the crystallization protocol as a precipitant (PEG-200, i.e. with a dominance of tetramers), but could, at the given resolutions of 2–2.5 Å, be refined reasonably well as somewhat diffuse strings of water molecules. Hydrogen bond interactions of these “water molecules” are

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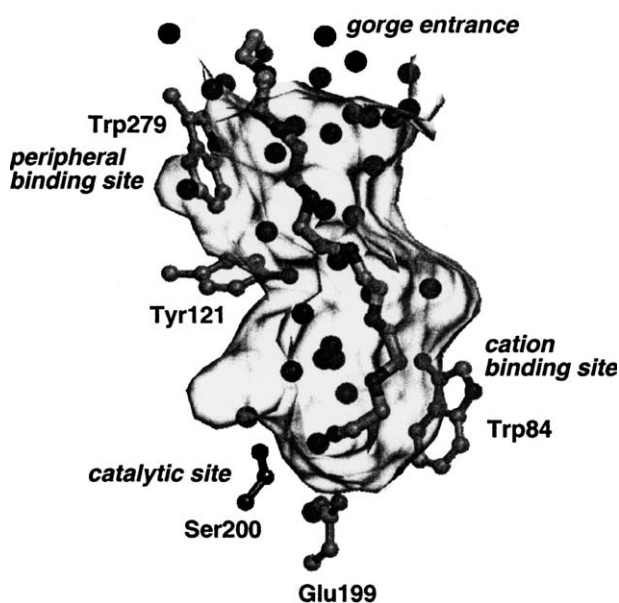
Abbreviations used: ACh, acetylcholine; AChE, acetylcholinesterase (EC 3.1.1.7); TcAChE, acetylcholinesterase from *Torpedo californica*; PEG, polyethylene glycol; PEG-200 etc., PEG with a mean molecular mass of 200 Da etc; PEG-SH, polyethylene glycol with a terminal SH-group; O<sub>w</sub>, water oxygen atom.

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**Table 1.** Data collection and model refinement statistics

<i>A. Data collection</i>	
Space group	Trigonal, $P3_121$
$a = b$ (Å)	111.2
$c$ (Å)	137.1
Radiation	Rotating anode Cu $K_{\alpha}$
Detector	300 mm MAR imaging plate
Temperature (K)	200
Resolution range (Å)	20–2.3
Completeness (%)	94.1
$R_{\text{symm}}$ (%)	4.1
<i>B. Model refinement</i>	
PDB entry	1JJB
$R_{\text{cryst}}$ (%)	17.7
$R_{\text{free}}$ (%)	22.5
rmsd from ideal bond lengths (Å)	0.006
rmsd from ideal bond angles (deg.)	1.3
Average $B$ -factor (Å <sup>2</sup> )	34.6
Estimated coordinate error from Luzzati (Å)	0.23
Estimated coordinate error from SIGMAA (Å)	0.26

*TcAChE* was crystallized as described<sup>1</sup> (36% PEG-200, 0.5 M Mes buffer, pH 5.8). Native crystals were soaked for five minutes in 40% PEG-SH 350† and 60% 0.1 M Mes buffer (pH 5.8), and subsequently cryocooled in liquid N<sub>2</sub>. Binding of PEG-200 to the *TcAChE* active site under crystallization conditions was corroborated by an increase in apparent  $K_m$  for the substrate acetylthiocholine from 0.080(±0.015) mM (no PEG) to 1.0(±0.3) mM (34% (w/v) PEG) at pH 5.8, 0.05 M Mes, at 25 °C. PEG decreased the apparent  $V_{\text{max}}$ . Plots of  $K_m/V_m$  were not linear across the entire range of inhibitor tested (0–2.5 M PEG-200), suggesting a non-competitive or complex mechanism of inhibition. The crystal structure was refined using the atoms of the protein molecule in the VX–*TcAChE* conjugate<sup>8</sup> as the starting model (one molecule per asymmetric unit, refinement with CNS,<sup>18</sup> model building with O<sup>19</sup>).



**Figure 1.** The active-site gorge of *TcAChE* filled with one heptamer PEG-SH molecule and 17 water molecules; some water molecules outside the gorge are also drawn‡.

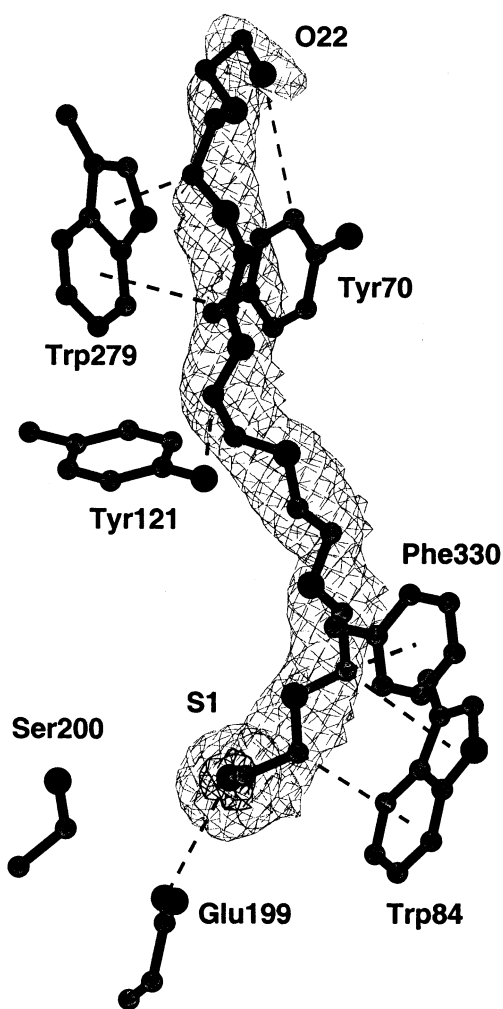
modest, but not unacceptable. Since the presence of PEG in a cation-binding pocket was not really expected, the string-of-water model was favoured. An explicit illustration has been published<sup>7</sup> for the VX–*TcAChE* conjugate.<sup>8</sup> In one case, however, a

structure model was assigned containing a PEG trimer positioned above the bound inhibitor within the active-site gorge.<sup>6</sup> The case for binding of PEG within the “active-site gorge” was strengthened by kinetic measurements showing inhibition of *TcAChE* by PEG at high concentrations (for details, see the legend to Table 1). To clarify the issue, we have, in the present study, prepared and crystallized the complex of *TcAChE* with PEG-SH-350, a primarily heptameric PEG species in which one terminal hydroxyl group has been replaced by a thiol group (for sample preparation, see the legend to Table 1). This thiol group can clearly be distinguished from a water molecule in an X-ray crystal structure, thus ruling out the string-of-water model when PEG is present.

In the crystal structure (2.3 Å resolution, crystallographic details are given in Table 1), there is, in fact, a continuum of electron density along the gorge that can be modelled readily as a PEG-SH heptamer (Figures 1 and 2). The molecule extends throughout the the gorge, even protruding slightly from the entrance. In addition, there are at least 17 water molecules present in the gorge, located similarly to the water molecules identified in various other crystal structures.<sup>7</sup> In particular, there is a continuous chain of hydrogen bonded water molecules reaching from the oxyanion hole out to the exterior water. The thiol terminus of PEG-SH can be identified clearly, with an electron density maximum more than twofold higher than that observed for the O atoms of water or of ethylene glycol (Figure 2). The other terminus does not exhibit higher electron density than typical O atoms, showing that the PEG-SH displays a unique

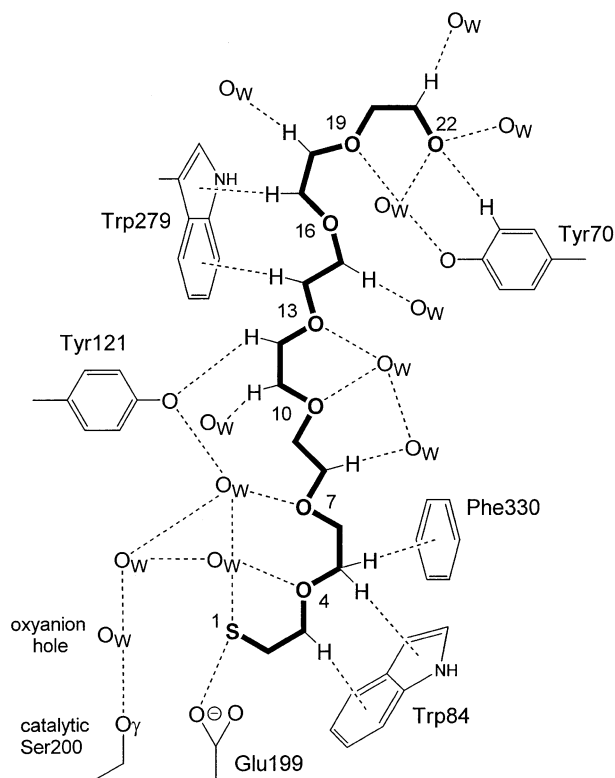
† Obtained from [www.pegshop.co.kr](http://www.pegshop.co.kr)

‡ Picture prepared with DINO: A. Philippsen (1999) <http://www.bioz.unibas.ch/~xray/dino>



**Figure 2.** Difference electron density map after exclusion of the inhibitor and subsequent refinement. Light grid:  $3\sigma$  level, enclosing the whole PEG-SH molecule. Dark grid:  $10\sigma$  level, showing a single peak representing the sulfur atom. Weak polar interactions with the wall of the active-site pocket are shown as broken lines. For clarity, water molecules are not shown.

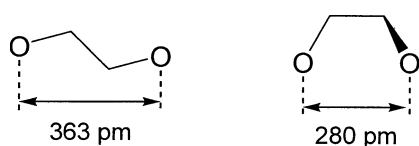
orientation with its thiol terminus being bound at the bottom of the gorge. The displacement parameters increase slightly but systematically by about 20% from the bottom to the top end of the chain. Somewhat surprisingly, the bound PEG-SH can be well refined as a pure heptamer, despite the heterogeneity of the preparation. This could be due to preferential binding of the heptamer, or simply mean that partial occupancy by other oligomers cannot be distinguished at the given resolution. Comparison of Figure 2 with the diffuse water chains displayed for the structure of VX-TcAChE<sup>5</sup> reveals that the latter occupy the same space as the upper (opposite Trp279) and lower (opposite Trp84) segments of PEG-SH. This suggests that in VX-TcAChE, and probably also in several of the other AChE structures obtained from crystals grown in PEG-200,<sup>3-6</sup> short



**Figure 3.** A representation of the specific intermolecular interactions formed by the PEG-SH molecule. The glycol units O7–O10 and O13–O16 are in *trans* conformation, and the others are in  $\pm$  *gauche*. Of the 17 water molecules in the active-site gorge, only those that are in direct contact with PEG-SH, and those that form a link to the catalytic Ser200 are drawn. Some relevant intermolecular distances: S··Glu199O<sup>e1</sup> = 3.67 Å; S··W1380 = 3.52 Å; all water–O distances < 3.2 Å; C3·· $\pi$ (6-ring, midp.)Trp84 = 3.86 Å; C5·· $\pi$ (5-ring, midp.)Trp84 = 3.75 Å; C5·· $\pi$ (midp.) Phe330 = 3.97 Å; C14·· $\pi$ (6-ring, midp.) Trp279 = 3.96 Å; C17·· $\pi$ (5-ring, midp.) Trp279 = 3.92 Å; C12··O<sup>n</sup>Tyr121 = 3.50 Å; O22··C<sup>e1</sup>Tyr70 = 3.80 Å; all C··O<sub>w</sub> distances < 3.8 Å.

PEG fragments are present in at least partial occupancy.

Apart from these overall features, interesting information is obtained by close inspection of the binding geometries, as illustrated in Figure 3 (numerical data are given in the Figure legend). The SH group is in hydrogen-bond distance of the carboxylate group of Glu199 adjacent to the catalytic Ser200 (3.67 Å, a typical value for S–H··O<sup>–</sup> bonds).<sup>9</sup> This is the only conventional hydrogen bond contact between PEG-SH and the enzyme, but there are, in addition, a large number of weak specific interactions. In particular, two CH<sub>2</sub> groups of PEG-SH, linked by an ether O atom, are located in the quaternary ammonium-binding site, and form C–H·· $\pi$  interactions with the faces of Trp84 and Phe330 (C·· $\pi$ (midp.) distances around 3.9 Å). In substrate recognition, these two side-chains participate in cation– $\pi$  interactions (N<sup>+</sup>–CH<sub>3</sub>·· $\pi$ ) of similar configuration and geometry.<sup>3</sup> Also, at



**Scheme 1.** The two low-energy conformations of an ethylene glycol unit. The  $\pm$ gauche conformers (right) ideally have an O...O separation of 2.80 Å, and can easily be mistaken for a pair of water molecules.

the peripheral cation-binding site (adjacent to Trp279 near the gorge entrance), PEG-SH forms C-H... $\pi$  contacts analogous to the cation- $\pi$  interactions observed, e.g. in the binding of the bis-quarternary ligand, decamethonium.<sup>3</sup> This means that the weak but specific C-H... $\pi$  interactions, which have recently attracted attention in protein structures,<sup>10–12</sup> here play a structural role analogous to that of the much stronger cation- $\pi$  interactions. Furthermore, there are protein-ligand C-H...O contacts displaying the typical geometry of weak hydrogen bonds,<sup>9,13–17</sup> which involve the side-chains of Tyr70 and Tyr121.

The O atoms of PEG-SH all face away from the gorge wall, and all but one are in good hydrogen bond distance of water molecules present in the active-site gorge. In addition, there are five C...O<sub>w</sub> (O<sub>w</sub> = water oxygen atom) contacts of PEG-SH that are suggestive of weak C-H...O hydrogen bonds. Of the seven glycol units, two are in a *trans* conformation, characterized by an O...O separation of around 3.6 Å, and the other five are in  $\pm$ gauche conformations with O...O around 2.8 Å (Scheme 1). The latter distance is the same as that between between hydrogen-bonded water molecules, which explains why PEG seen at moderate resolution can so easily be mistaken for a water chain. If only the O atoms are included in a structure model and are refined as water molecules, a fairly reasonable hydrogen bond coordination will be obtained.

In conclusion, the PEG-SH-TcAChE structure demonstrates that a neutral molecule can be bound to an aromatic cation recognition site by C-H... $\pi$  interactions that structurally mimic the cation- $\pi$  interactions formed in substrate binding. This is an issue that is of general importance, reaching far beyond its immediate relevance for AChE structures.

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