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published in
Journal of Biological Chemistry
2011

DOI (link to publisher)
10.1074/jbc.M110.188730

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)

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Download date: 10. May. 2022
Crystal Structures of a Cysteine-modified Mutant in Loop D of Acetylcholine-binding Protein

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Covalent modification of α7 W55C nicotinic acetylcholine receptors (nAChR) with the cysteine-modifying reagent [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET) produces receptors that are unresponsive to acetylcholine, whereas methyl methanethiosulfonate (MMTS) produces enhanced acetylcholine-gated currents. Here, we investigate structural changes that underlie the opposite effects of MTSET and MMTS using acetylcholine-binding protein (AChBP), a homolog of the extracellular domain of the nAChR. Crystal structures of Y53C AChBP show that MTSET-modification stabilizes loop C in an extended conformation that resembles the antagonist-bound state, which parallels our observation that MTSET-modification produces unresponsive W55C nAChRs. The MMTS-modified mutant in complex with acetylcholine is characterized by a contracted C-loop, similar to other agonist-bound complexes. Surprisingly, we find two acetylcholine molecules bound in the ligand-binding site, which might explain the potentiating effect of MMTS modification in W55C nAChRs. Unexpectedly, we observed in the MMTS-Y53C structure that ten phosphate ions arranged in two rings at adjacent sites are bound in the vestibule of AChBP. We mutated homologous residues in the vestibule of α1 GlyR and observed a reduction in the single channel conductance, suggesting a role of this site in ion permeation. Taken together, our results demonstrate that targeted modification of a conserved aromatic residue in loop D is sufficient for a conformational switch of AChBP and that a defined region in the vestibule of the extracellular domain contributes to ion conduction in an ion-selective Cys-loop receptors.

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2 The abbreviations used are: CLR, Cys-loop receptor; AChBP, acetylcholine-binding protein; nAChR, nicotinic acetylcholine receptor; MMTS, methyl methanethiosulfonate; MTSET, 2-(trimethylammonium)ethyl methanethiosulfonate; RMSD, root mean square deviation from ideal geometry.
peptides derived from apolipoprotein E non-competitively inhibited α7 receptors through hydrophobic interactions (9). In addition, when Trp-55 was mutated to alanine, the α7 W55A nAChR desensitized more slowly and recovered from desensitization more rapidly than wild-type receptor (10). Mutating Trp-55 to other aromatic residues (Phe or Tyr) had no significant effect on the kinetics of desensitization, whereas mutation to various hydrophobic residues (Ala, Cys, or Val) significantly decreased the rate of onset and increased the rate of recovery from desensitization (10). To gain insight into possible structural rearrangements during desensitization, we probed the accessibility of Trp-55 by mutating Trp-55 to cysteine (α7 W55C) and tested the ability of various sulfhydryl reagents to react with this cysteine (10). Modification with several positively charged sulfhydryl reagents, including [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET\(^{+}\)), produced α7 W55C nAChRs that became unresponsive to acetylcholine, whereas a neutral sulfhydryl reagent methyl methanethiosulfonate (MMTS) enhanced acetylcholine-activated currents by nearly 60% (10). These data suggested that Trp-55 plays an important role in both the onset and recovery from desensitization in the rat α7 nAChR, and suggested that Trp-55 may be a potential target for modulatory agents operating via hydrophobic interactions.

However, these data left unresolved how modification of loop D with MTSET\(^{+}\) or MMTS leads to two clearly distinct functional effects. MTSET\(^{+}\) modification renders α7 W55C receptors unresponsive to acetylcholine, whereas MMTS modification produces receptors with enhanced responses to acetylcholine. In this study, we used AChBP to determine x-ray crystal structures of the homologous Y53C mutant modified either with MTSET\(^{+}\) alone or MMTS in the presence of acetylcholine. We investigated whether structural changes of methanethiosulfonate (MTS)-modified AChBP correlate with the opposing functional effects of MTSET\(^{+}\) and MMTS observed on α7 W55C nAChRs.

**MATERIALS AND METHODS**

**AChBP-Y53C Expression, Purification, and MTSET\(^{+}\) Modification**—The mutant cDNA encoding Y53C AChBP from *Aplysia californica* was engineered using a QuickChange strategy (Stratagene) and verified by sequencing. Baculovirus was produced using the Bac-to-Bac expression system according to the manufacturer’s guidelines (Invitrogen). Untagged AChBP-Y53C was expressed in Sf9 insect cells and purified as previously described (11). In brief, medium from Sf9 cells was harvested 3–4 days after infection with P2 baculovirus and concentrated on Q Sepharose Fast Flow (GE Healthcare). Y53C AChBP was further purified by size exclusion chromatography on a Superdex 200 column and ion exchange on a monoQ column (GE Healthcare). C-terminally His-tagged Y53C AChBP was purified on a Ni-Sepharose column (GE Healthcare) and pentameric protein was isolated on a Superdex 200 gel filtration column. Protein was concentrated to 5–6 mg/ml on a Centriprep filter (Amicon) with a cut-off of 30 kDa.

[2-(trimethylammonium)ethyl] Methanethiosulfonate bromide (MTSET\(^{+}\)) and methyl methanethiosulfonate (MMTS) were purchased from Toronto Research Chemicals. MTSET\(^{+}\) was dissolved in DMSO at a concentration of 1 mM, and aliquots were stored at −20 °C. Dilutions of 100 mM MTSET\(^{+}\) were prepared fresh in double-distilled water and stored on ice immediately before starting covalent protein modification. Modification of Y53C AChBP with MTSET\(^{+}\) or MMTS was carried out on ice at a final concentration of 1 mM MTSET\(^{+}\) or MMTS during 1 h. To ensure maximal modification of AChBP-Y53C a second aliquot of MTS reagent was added and the reaction was extended for another hour. Covalent modification of Y53C AChBP with MTSET\(^{+}\) or MMTS was analyzed with mass spectrometry. Non-reacted MTS reagents or hydrolysis products were removed from the protein solution by dialysis prior to crystallization.

**Mass Spectrometry of MTS-modified AChBP-Y53C**—The AChBP protein sample (3 μg/μl) was mixed with 10 μl of dissolution buffer (0.5 mM TEAB; ABI) and 8 μl of trypsin solution (20 μg/ml) (sequencing grade from Promega). After incubation overnight at room temperature, the sample was loaded into a C18 ZipTip, and peptides were eluted with 2 μl of 80% acetonitrile/7.0 mM TFA. Of the eluant 0.5 μl was spotted on a MALDI metal plate, mixed with 1 μl of matrix (7 mg of α-cyano-4-hydroxycinnamic acid in 1 mL, 50% acetonitrile, 0.1% TFA, 10 mM ammonium phosphate), and analyzed on an ABI 4800 Proteomics Analyzer (Applied Biosystems). CID was performed at 2KV, the collision gas was air. MS/MS spectra were collected from 2500 laser shots. MALDI MS and MALDI/MS/MS analysis of the protein digests was used to identify and map the sites of modification. This mass spectrometry analysis showed that >75% of Cys-53 was modified by MTSET\(^{+}\), the remaining fraction is unmodified Cys-53 even in the presence of excess amounts of MTSET\(^{+}\). For MMTS-modified protein the analysis indicated that >90% of Cys-53 is covalently bound to MMTS.

**Crystallization and Structure Determination of MTSET\(^{+}\)-modified AChBP-Y53C**—Initial crystallization screens were carried out at 20 °C using nanoliter-dispensing robotics (TTP Labtech). Optimal crystals for AChBP Y53C-MTSET\(^{+}\) grew at 4 °C under the following chemical conditions: 150 mM KSCN, bistrispropane, pH 8.5, and 14% PEG3350. Cryoprotection was achieved by adding glycerol to the mother liquor at beamline ID14–1 of the European Synchrotron Radiation Facility (ESRF, Grenoble). The structure was solved by molecular replacement using MOLREP (12) and the unliganded *Aplysia* AChBP structure with PDB code 2UZ6. The model contains two pentamers in the asymmetric unit and was refined using PHENIX (13) with TLS and NCS restraints. Manual building was carried out using COOT (14). The MTSET\(^{+}\)-modified side chain at position 53 was built into the simple
Structure of MTS-modified AChBP

$F_o - F_c$ density map after refinement of a model in which the side chain at position 53 was omitted. Coordinates and topology file for MTSET$^+$ were obtained from the PRODRG server (15). The occupancy of MTSET$^+$ at each of the 10 binding sites was refined with PHENIX to an average value of 87%.

Diffraction data for Y53C-MMTS in complex with acetylcholine were collected to 2.7 Å at beamline ID23–2 (ESRF, Grenoble). The structure was solved by molecular replacement and refined using a strategy similar to the one described above. The model contains one pentamer in the asymmetric unit and was refined with BUSTER (16) using TLS and NCS restraints. Inspection of the simple $F_o - F_c$ density map revealed two noticeable blobs in each binding pocket. Acetylcholine molecules were manually placed into density with COOT using coordinates and restraints obtained from the PRODRG server. The MMTS-modified side chain was built into $F_o - F_c$ density map after refinement of the model in which the side chain at position 53 was omitted. Diffraction data were processed with MOSFLM for the Y53C-MTSET$^+$ data set and XDS for the Y53C-MMTS data set. Validation of the final model was carried out with MOLPROBITY (17, 18). All model figures were prepared with PYMOL (DeLano Scientific).

Competitive Binding Assays with AChBP-Y53C—Competition binding assays were performed in buffer (PBS, 20 mM Tris, 0.05% Tween, pH 7.4) in a final assay volume of 100 μl. [3H]epibatidine (GE Healthcare, specific activity 56 Ci/mmol) was used as the radioligand at 2.23 nM. Ligand was added to 10–50 ng His-tagged Y53C AChBP, mixed with 200 μg PVT Copper His-Tag SPA beads (GE Healthcare) and incubated for 1.5 h at room temperature under continuous shaking. The SPA beads were allowed to settle during 2–4 h and the radioactivity was measured in a Wallac 1450 MicroBeta liquid scintillation counter. All radioligand binding data were evaluated by a nonlinear, least-squares curve fitting procedure using Graphpad Prism (version 5, GraphPad Software, Inc., San Diego, CA). All data are represented as the mean ± S.E. from at least three independent experiments.

Single Channel Recordings from a1 GlyRs—Human embryonic kidney 293 (HEK) cells were cultured in DMEM medium supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin, in a humidified incubator at 37 °C and 5% CO2. HEK cells were co-transfected with GFP and wild-type or mutant a1 Glyr cDNA using a calcium phosphate transfection kit (Invitrogen) according to the manufacturer’s instructions. Cover slips containing transfected HEK cells were transferred to a chamber containing (in mM): 140 NaCl, 4 KCl, 2 CaCl2, 2 MgCl2, 10 glucose, and 10 HEPES; pH was adjusted to 7.4 with NaOH. Cells were visualized using a Nikon Eclipse TE300 microscope equipped for fluorescence detection. Borosilicate patch pipettes (3–6 MΩ) were filled with extracellular solution containing 100 μM glycine. Currents were recorded in the cell-attached configuration at −30, −60, and −90 mV. Experiments were performed at room temperature (22 °C) using an Axopatch-200A amplifier connected to a Digidata 1322A and pCLAMP software (version 10.1). Currents were filtered at 1 kHz and digitized at 10 kHz.

**TABLE 1**

<table>
<thead>
<tr>
<th>$K_i$ [nM]</th>
<th>$K_i$ [μM]</th>
<th>$K_i$ [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt Ac-AChBP</td>
<td>8.47 ± 1.93</td>
<td>0.77 ± 0.06</td>
</tr>
<tr>
<td>Y53C</td>
<td>26.76 ± 4.02$^a$</td>
<td>2.78 ± 0.47$^a$</td>
</tr>
<tr>
<td>Y53C-MMTS</td>
<td>19.11 ± 3.77$^b$</td>
<td>3.05 ± 0.28$^b$</td>
</tr>
<tr>
<td>Y53C-MTSET$^+$</td>
<td>NB</td>
<td>NB</td>
</tr>
</tbody>
</table>

$^a$ Different from wt p < 0.05.
$^b$ Different from wt p < 0.001.
NB, no binding.

RESULTS

X-ray Crystal Structures of MTS-modified Y53C AChBP—To investigate whether AChBP is a valid tool for structural studies of MTS-modified cysteine mutants, we investigated the pharmacological effects of MTSET$^+$ - and MMTS-modification on the homologous Y53C mutant in AChBP from Aplysia californica, Ac-AChBP (see Table 1). Mutation of Tyr-53 to cysteine leads to a reduction of [3H]epibatidine binding, whereas MMTS modification does not significantly alter binding. Nicotinic displacement is significantly lowered in both Y53C and its MMTS-modified protein. However, acetylcholine binding is selectively affected only in the MMTS-modified Y53C, suggesting that acetylcholine may address a different binding configuration. Upon modification with MTSET$^+$, no binding with [3H]epibatidine could be detected. This parallels the observation that α7 W55C nAChRs become unresponsive to acetylcholine after modification with MTSET$^+$ (10). Combining these data suggest that AChBP Y53C is sensitive to modification with MTS reagents and provides a model to understand the effects of MMTS and MTSET$^+$ modification at the structural level.

Next, we determined crystal structures of Ac-AChBP in which the homologous aromatic residue Tyr-53 was mutated to cysteine and subsequently modified with either MTSET$^+$ or MMTS. The crystal structure of Ac-AChBP Y53C with MTSET$^+$ was solved from diffraction data to a resolution of 3 Å (Table 2). The asymmetric unit contains two pentamers, which are in an upside-down orientation relative to each other and interact through an interface that involves two neighboring C-loops (Fig. 1A). As a result of this crystal contact, one of these C-loops is more contracted compared with the conformation of all other C-loops in this pentamer (see supplemental Fig. S1). Mass spectrometry of MTSET$^+$-modified Y53C and inspection of difference electron density maps indicated the attachment of an MTSET$^+$ side chain to Y53C (Fig. 1A). Contact analysis of surrounding residues in the ligand-binding pocket shows that the MTSET$^+$ side chain interacts with the side chains of Gln-36 and Ser-165 in loop F and Tyr-91 in loop A (Fig. 1A). Superposition of each of the 5 monomers shows a variable conformation of the MTSET$^+$ side chains (see supplemental Fig. S1). Together with partial covalent modification, flexibility of the MTSET$^+$ side chain might account for the weak electron density observed at some positions in the crystal structure. High flexibility of the MTSET$^+$ side chain and partial density was also observed in
the crystal structure of MTSET\textsuperscript{−}-modified Sortase B from *Staphylococcus aureus* (PDB code 1QWZ), despite the higher resolution (1.7 Å) of the diffraction data (19).

The conformational state of loop C and its relative contraction was quantified by measuring the distance between the carbonyl oxygen atom of the conserved Trp-145 and the γ-sulfur atom of Cys-188 in all subunits (magenta, Fig. 2). In comparison, this distance is 7.9 Å, compared with 12.45 Å for loop C in an extended conformation of the published nicotine-bound structure of AChBP (green, Fig. 2 and Ref. 20) and 14.38 ± 0.13 Å for loop C in an extended conformation of the α-conotoxin-bound structure (red, Fig. 2 and Ref. 11). The published crystal structure for apo AChBP revealed a disordered loop C (yellow, Fig. 2 and Ref. 21), which is most likely due to an intrinsic mobility of this loop in the unliganded state (22). These data indicate that MTSET\textsuperscript{−}-modification of Cys-53 stabilizes Ac-AChBP in a conformational state that is similar to the antagonist-bound complexes with α-conotoxins (Fig. 2). This observation parallels the effect of MTSET\textsuperscript{−}-modification on α\textsubscript{7} W55C nAChRs, which produces receptors unresponsive to saturating concentrations of acetylcholine.

To gain more insight into the potentiating effect of MMTS-modification of α\textsubscript{7} W55C nAChRs upon application of acetylcholine, we also solved a crystal structure of MMTS-modified Y53C-AChBP in complex with acetylcholine. Mass spectrometry analysis of MMTS-modified Y53C AChBP indicated that >90% of the protein was covalently modified in the presence of excess amounts of MMTS. The crystal structure for MMTS-Y53C in complex with acetylcholine was solved from diffraction data to a resolution of 2.8 Å (Fig. 1B and Table 2). Difference electron density maps indicate that the cysteine side chain at position 53 was modified and that acetylcholine molecules occupy each of the five binding pockets (ACh1, Fig. 1B) at a position that is almost identical to the published carbamylcholine-bound structure of Ls-AChBP (Fig. 1D, CCh and Ref. 20). The quaternary amine group of ACh1 forms a cation-π interaction with the conserved aromatic residues of the principal face (shown in yellow in Fig. 1B). Unexpectedly, electron density near the MMTS-modified side chain indicates the occupancy of a second acetylcholine molecule in the same binding pocket (ACh2, Fig. 1B). Contact analysis shows that the MMTS side chain closely interacts with this second acetylcholine molecule, together with residues of the complementary face, namely Thr-34, Gln-36, and Ser-165 (Fig. 1B). The conformation of loop C in this crystal structure was compared with the MTSET\textsuperscript{−}-modified Y53C structure by measuring the distance between carbonyl oxygen atom of Trp-145 and the γ-sulfur atom of Cys-188, which amounts to 7.18 ± 0.02 Å (blue, Fig. 2). Together, these data show that the conformational state of MMTS-Y53C in complex with acetylcholine is distinct from MTSET\textsuperscript{−}-Y53C and offer a possible explanation for the potentiating effect of MMTS on acetylcholine-evoked currents in α\textsubscript{7} W55C nAChRs.

**Phosphates Ions Are Bound in the Vestibule of AChBP**—Surprisingly, we also found peaks in the difference map that are
localized in the vestibule of AChBP (Fig. 3, A and B). These peaks were interpreted as phosphate ions, which were present in the crystallization solution at a concentration of 200 mM. In total, there are ten phosphate ions bound in the vestibule, which are arranged in two pentagonal layers. The upper layer contains five phosphate ions at a distance of 9 Å apart (Fig. 3D, ring 1) that occupy the same positions as sulfate ions observed in a previous study (23). These sulfates are stabilized through interaction with Arg-95, which is in an equivalent position to a conserved positively charged residue in anion-selective Cys-loop receptors and a conserved negatively charged residue in cation selective receptors (for example, Asp-97 in the muscle α1 nAChR subunit). This residue, which lies near the transmembrane interface, was therefore suggested to stabilize cations before entry into the channel pore (23). However, in the MMTS-Y53C crystal structure, we find a second layer of phosphate ions in a pentagonal arrangement at a closer distance of 7 Å apart (Fig. 3D, ring 2). Phosphates in this lower ring interact directly with Lys-40 or through water molecules involved in hydrogen bonds with Glu-47 and...
Asp-49 in AChBP. A detailed interaction of residues and surrounding water molecules involved in hydrogen bonding of phosphate ions is shown in Fig. 3C.

The observation that sulfates as well as phosphates bind in a defined location of the vestibule of AChBP suggests that this region functions as a general anion-binding site. Therefore, we questioned whether chloride anions may interact in a similar manner in the vestibule of anion-selective Cys-loop receptors, such as GABA$_{\alpha}$-R and GlyR. Sequence alignment with other Cys-loop receptors indicates that a residue of ring 1 (Arg-95 in Ac-AChBP) matches a conserved negatively charged residue, for example Asp-97 in $\alpha$1 nAChR (Fig. 4A).

In contrast, anion-selective Cys-loop receptors contain 1 or 2 adjacent positively charged residues at this position, for example Lys-105/Lys-106 in the $\alpha$1 GABA$_{\alpha}$-R and Lys-104/Gly-105 in the $\alpha$1 GlyR. Additionally, alignment of residues Glu-47 and Asp-49 in ring 2 of AChBP indicates the conservation of a negative charge in anion-selective Cys-loop receptors, for example Glu-59 in the $\alpha$1 GABA$_{\alpha}$-R and Asp-57 in the $\alpha$1 GlyR (Fig. 4A).

We investigated whether residues of ring 1 and ring 2 have a conserved role as anion-binding sites in the anion-selective $\alpha$1 GlyR. Therefore, we mutated positions K104A and G105D (ring 1) and D57I and R59T (ring 2) to the corresponding residues in the $\alpha$1 nAChR. Expression into Xenopus oocytes showed that these mutants were functional and had a reversal potential that was identical to wild-type $\alpha$1 GlyR (data not shown), indicating that ion selectivity is not affected. Next, we investigated whether charge alterations affect the conductance of Cl$^-$ ions and recorded single channel activity evoked by 100 $\mu$M glycine in the cell-attached configuration on HEK293 cells expressing $\alpha$1 GlyR mutants (Fig. 4, B–E).

Compared with wild-type GlyR, the mutants show a decreased current amplitude at all potentials tested (Fig. 4, B–E). A current amplitude versus potential plot is linear (data not shown), allowing us to calculate the unitary conductance (slope of the line). The single channel conductance was decreased by 40% for mutant ring 1, by 60% for mutant ring 2, and by 70% for the quadruple mutant ring 1 + 2 (Fig. 4F). The effects of these mutations in ring 1 and ring 2 on single channel conductance were not additive since the quadruple mutation shows conductance levels similar to mutant ring 2. Furthermore, the ring 2 mutations result in the loss of activity at $\pm 30$ mV suggesting that these residues are not only critical for channel conductance, but may also be involved in gating. These results demonstrate that conserved charges at two adjacent sites in the extracellular domain of the $\alpha$1 GlyR contribute to ion selection and permeation. Coordinates and structure factors for Y53C-MTSET$^+$ and Y53C-MMTS with acetylcholine have been deposited in the Protein Data Bank with accession codes 2XZ6 and 2XZ5, respectively.
DISCUSSION

Substituted cysteine accessibility scanning (SCAM) is a widely used method to study the relationship between structure and function of ion channels (24). With the availability of an increasing number of x-ray crystal structures for ion channels, SCAM is now frequently employed to test the validity of three-dimensional models in the context of channel dynamics and their lipid membrane environment (25–27). SCAM has powerful advantages in that the method allows real-time monitoring of the reaction speed between the MTS reagent and the targeted cysteine residue for the closed, open, or desensitized state of the channel. However, this reaction is typically followed indirectly by measuring changes in the functional properties of ion channels such as ion conduction, voltage dependence of activation or ligand activation. Because of the indirect nature of this method, it is not always possible to establish a clear relationship between the observed functional effects of MTS modification and the underlying conformational changes of the channel.

In this study, we report the first x-ray crystal structures of an MTS-modified cysteine mutant of the nAChR homolog AChBP. We used the homologous mutant of $\alpha_7$ W55C nAChRs, which is oppositely regulated by two different MTS reagents, namely MTSET and MMTS (10), to investigate the correlation between structure and function. We previously demonstrated that modification of $\alpha_7$ W55C nAChRs with MTSET produces receptors that become unresponsive to saturating concentrations of acetylcholine (10). The crystal structure of MTSET-modified Y53C AChBP shows that loop C is stabilized in a conformational state that resembles the antagonist-bound state. This result suggests that the unresponsive behavior of MTSET-modified $\alpha_7$ W55C nAChRs may arise from a similar stabilization of the receptor in an antagonist-bound state. This also suggests that the conformational change of loop C is correlated with the activational state of the receptor. In contrast to MTSET-modified $\alpha_7$ W55C nAChRs, we previously showed that modification of $\alpha_7$ W55C nAChRs with MMTS enhances acetylcholine-evoked...
currents by nearly 60% (10). The crystal structure of MMTS-modified Y535C AChBP shows that loop C is strongly contracted, similar to other agonist-bound structures. We also observed that two acetylcholine molecules occupy the binding pocket, which possibly explains the potentiating effect of acetylcholine on MMTS-modified α7 W55C nAChRs. One of these acetylcholine molecules faces the principal binding site and occupies a position that overlaps with the binding mode observed in the related carbamylcholine-bound structure of AChBP. The second acetylcholine molecule interacts with residues of the complementary face, including the MMTS-modified side chain of Cys-53. Therefore, we propose that the potentiating effect of MMTS on α7 W55C nAChRs arises from a favorable interaction with the MMTS-modified side chain that stabilizes two acetylcholine molecules in the ligand-binding site. Together, both AChBP crystal structures parallel our observations from functional studies on α7 W55C nAChRs, and offer possible explanations for the opposing effects of MTSET⁺ and MMTS. Our results suggest that targeted modification of a single residue in loop D is sufficient to trigger conformational changes of AChBP.

The functional importance of residues in loop D has been demonstrated by mutagenesis studies in different CLR.s. Mutation of the homologous W55 residue in the GABA₅-R γ2-subunit, F77C, abolished binding by [³H]Ro15–1788 and the benzodiazepine [³H]flunitrazepam. Mutation of neighboring residues in loop D, A79C and T81C, caused a 10-fold reduction in the affinity of the tranquilizers eszopiclone and zolpidem (28). In the GABA₅-R α1-subunit, it was shown that for the homologous Trp-55 subtle changes were caused by unnatural amino acid mutations (29). In the muscle nAChR it was shown that mutation W57F in the δ-subunit and W55F in the ε-subunit have only minor effects on acetylcholine-sensitivity but mainly affect channel gating by reducing the channel opening rate (30). In insect nAChRs, it was shown that the basic Arg and Lys residues at positions +2 and +4 of the homologous W55 residue are responsible for the high affinity of the insecticide imidacloprid and related neonicotinoids (31). Mutation of loop D residues N55S and V56I in the human nAChR δ-subunit, which are at positions −2 and −1 of the homologous Trp-55 residue, abolishes sensitivity to TMAQ, a novel agonist for β4-containing nAChRs (32). In a related study it was shown that mutations N55S, V56I, T59K, and E63T in the human nAChR β2-subunit reduce the affinity of acetylcholine and nicotine, while having little to no effect on the affinity of epibatidine and dimethylphenylpiperazinium (DMPP) (33).

In addition to loop D, accumulating evidence implicates loop C as a structural component that is key to both ligand binding and subsequent conformational changes underlying CLR activation, inhibition, and desensitization (34). The important role of loop C derives from a large body of work using x-ray crystallography (11, 35–37), molecular dynamics simulation (38–40), site-directed mutagenesis (41–44), and electrophysiology (45, 46). The data from these studies demonstrates that loop C is flexible in the non-liganded form (22, 37) and adopts distinct conformations upon agonist or antagonist binding. Loop C assumes a contracted configuration with agonists bound (corresponding to either the open or desensitized state of the receptor) and takes on an extended configuration with antagonists bound (corresponding to the closed state of the channel (11, 35, 36). More recent work suggests that the degree of loop C movement may correspond to agonist efficacy (37). Movement in the ligand binding domain is thought to propagate from the extracellular domain to the pore region to allow activation or inhibition of ion flux. This transduction of signal can be understood both as a sequence of chemical events (47) or as coordinated movements or rotations of the whole extracellular domain (40, 48, 49).

Unexpectedly, we observed electron density that could be interpreted as phosphate ions in the vestibule of AChBP at a location that is very near to the interface with the transmembrane domain in integral Cys-loop receptors. The contribution of rings of charged amino acids to channel conductance has previously been demonstrated in the Torpedo nAChR (50), but detailed insight into the mechanism of ion conductance was lacking. Structural insight into selection of ions in the extracellular domain of Cys-loop receptors was obtained from a crystal structure containing 5 sulfate ions near residue Arg-95 in the vestibule of AChBP (23). Hansen et al. demonstrated that this residue, which corresponds to a highly conserved Asp in cation-selective Cys-loop receptors, affects single channel conductance upon charge reversal mutations in the muscle nAChR. In our structure, we observe 10 phosphate ions bound to a cluster of charged residues that involve Lys-40, Glu-47, Asp-49, and Arg-95. The phosphate ions are arranged in two pentagonal layers separated by a distance of less than 4 Å. The 5 phosphate ions in the upper layer (ring 1) occupy the same positions as the sulfate ions in the study from Hansen et al. and are arranged at a distance of 9 Å apart. Phosphate ions in the lower layer (ring 2) are spaced at a distance 7 Å apart and interact with Lys-40, Glu-47, and Asp-49. The observation that sulfates as well phosphates bind in a defined location of the vestibule of AChBP suggests that this region functions as a general anion-binding site. One of the binding site residues, Glu-47, is strictly conserved as a negatively charged amino acid (Asp or Glu) in anion-selective Cys-loop receptors, and hydrophobic (M, I, or V) in cation-selective Cys-loop receptors. Therefore, we hypothesized that chloride anions may interact in a similar manner with conserved negatively charged residues in anion-selective Cys-loop receptors, such as GABA₅-R and GlyR. We demonstrated that mutation of homologous residues in ring 1 and ring 2 of the α1 GlyR causes a pronounced reduction in the single channel conductance. This result suggests a functional role of the extracellular domain of α1 GlyR in selection and permeation of anions. Our observation fits with molecular dynamics simulations of the nAChR (51) and the bacterial homolog GLIC (52), which demonstrated the existence of one or more cation reservoirs in the extracellular domain of these cation-selective Cys-loop receptors.

In conclusion, our study shows a good correlation between structure and function, and offers possible explanations for the opposite effects of MTSET⁺ and MMTS on α7 W55C nAChRs. We show that targeted modification of loop D plays a key role in defining the conformational state of AChBP.
Structure of MTS-modified AChBP

This hints at a contribution of a conserved aromatic residue in loop D that goes beyond its established role of shaping the ligand-binding site. This residue may contribute to a structural switch that discriminates between the activated and non-activated state of Cys-loop receptors. In addition, the unexpected observation of phosphate anions bound in the vestibule of AChBP parallels the functional role of two rings of charged residues in the extracellular domain of the α1 GlyR that contribute to ion selection and permeation.

Acknowledgments—We thank the ESRF and local contacts for assistance during data collection. We thank Titia Sixma for access to crystallization robotics during initial stages of the study. We thank Pattie Lamb for technical assistance.

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