Crystal Structure of *Lymnaea stagnalis* AChBP Complexed with the Potent nAChR Antagonist DHβE Suggests a Unique Mode of Antagonism

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**Abstract**

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels that belong to the Cys-loop receptor superfamily. These receptors are allosteric proteins that exist in different conformational states, including resting (closed), activated (open), and desensitized (closed) states. The acetylcholine binding protein (AChBP) is a structural homologue of the extracellular ligand-binding domain of nAChRs. In previous studies, the degree of the C-loop radial extension of AChBP has been assigned to different conformational states of nAChRs. It has been suggested that a closed C-loop is preferred for the active conformation of nAChRs in complex with agonists whereas an open C-loop reflects an antagonist-bound (closed) state. In this work, we have determined the crystal structure of AChBP from the water snail *Lymnaea stagnalis* (Ls) in complex with dihydro-β-erythroidine (DHβE), which is a potent competitive antagonist of nAChRs. The structure reveals that binding of DHβE to AChBP imposes closure of the C-loop as agonists, but also a shift perpendicular to previously observed C-loop movements. These observations suggest that DHβE may antagonize the receptor via a different mechanism compared to prototypical antagonists and toxins.

**Introduction**

Neuronal nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels present both in the central and peripheral nervous system. nAChRs belong to the Cys-loop superfamily and exist as homo or heteromeric receptors composed of either α subunits or β and α subunits in combination. The subunits are arranged symmetrically around a central ion channel pore. Each monomer possesses an N-terminal extracellular ligand-binding domain, a transmembrane region that forms the ion channel pore, and an extended intracellular loop [1–3]. These receptors are allosteric proteins that exist in a minimum of three different conformational states, termed the resting (closed), activated (open) and desensitized (closed) states. The balance between these states regulates the permeability of cations through the ion channel [4].

Insight into ligand binding and nAChR activation is rapidly emerging and structures of the acetylcholine binding protein (AChBP) have significantly aided this process. The mulloskan AChBP is a structural and functional homologue of the extracellular domain of nAChRs [5,6]. Previous studies have suggested that a closed C-loop is associated with agonist-bound structures of AChBP, and thus represents an active conformation of nAChRs, whereas an open conformation of the C-loop observed in antagonist-bound structures represents an inactive form of the receptor [7–10]. Likewise, a correlation between the degree of agonism and closure of the C-loop has been suggested [8,11]. In contrast to this, it was reported in a recent study that a series of agonists with 21–76% efficacy at α4β2 nAChRs displayed no variation in the degree of C-loop closure in *Lymnaea stagnalis* (Ls) AChBP [12].

The erythrina alkaloid dihydro-β-erythroidine (DHβE) (Fig. 1a) is a potent competitive antagonist at nAChRs that has been used extensively as a pharmacological tool compound to gain a better understanding of the involvement of these receptors in physiological processes. DHβE is a somewhat selective antagonist with preference for α4 containing receptors [13–16]. It inhibits α4β2 receptors with nanomolar affinity (Ki = 98 nM) [17] whereas affinities at α7 and α3β4 nAChRs lie in the micromolar range (Ki = 11 and 32 μM, respectively) [17,18].

To gain further insight into the inhibitory mechanism and binding mode of DHβE, we have determined the crystal structure of Ls-AChBP bound to DHβE. The structure reveals features that are unique to this antagonist.
Results and Discussion

DHβE binds at Ls-AChBP with an affinity comparable to that at α4β2 nAChRs

The binding affinity (K<sub>i</sub>) of DHβE at Ls-AChBP was determined to 52 ± 5 nM by replacement of [3H]-epibatidine binding using a recently reported assay where Ls-AChBP for reasons of compatibility with other medium throughput assays was fused to a 5-HT<sub>3</sub>A ion channel [12]. The affinity of DHβE at Ls-AChBP closely resembles that of α4β2 nAChRs, supporting previous observations that Ls-AChBP can be used as a structural surrogate for α4β2 receptors [12] to study how DHβE interacts with the receptor.

The structure of Ls-AChBP complexed with DHβE

The structure of Ls-AChBP was determined at 2.5 Å resolution (Table 1). The crystal belongs to space group P<sub>2</sub>₁<sub>2</sub>₁<sub>2</sub>₁ with a DHβE molecule bound at the interface of all ten monomers in the asymmetric unit of the crystal. The DHβE-bound structure reported here shows the same homopentameric assembly as previously determined AChBP structures (Fig. 1b) [7–12]. Each monomer consists of an N-terminal α-helix, two short α<sub>310</sub> helices and a 10-stranded β-sandwich core. The F-loop portion of the molecule (residues 154–160 in subunits B and E and 155–160 in subunits C, G, H, I, and J) is not completely modeled due to lack of clear electron density, signifying a greater flexibility of these parts of the protein.

The electron density map clearly demonstrate the existence of a single binding orientation for each of the ten DHβE molecules (Fig. 1c). DHβE binds underneath a closed C-loop at a position corresponding to that of nicotine in the Ls-AChBP crystal structure (PDB ID: 1uw6 [19]), Figs. 2a,b. The binding pocket is formed by the highly conserved aromatic residues Tyr89, Trp143, Tyr185, and Tyr192 from the principal side of the interface (Figs. 1c and 2a). This orientation is in agreement with a previous study where substitution of β2Trp82, α4Tyr126, α4Trp182, α4Tyr223, and α4Tyr230 in the α4β2 nAChR (corresponding to Trp53, Tyr89, Trp143, Tyr192, and Tyr195 in AChBP) for alanine were shown to decrease sensitivity to inhibition by DHβE [17].

Superposition of the nicotine-bound Ls-AChBP structure onto the DHβE-bound structure (C<sub>α</sub> atoms) gives a low RMSD value of 0.14 Å, indicating high structural similarity between the two structures. The main structural difference is the orientation of the C-loop (residues 185–192) (Fig. 2) capping the binding site. Also, the orientation of Met114 on the complementary side of the ligand-binding site differs between the two structures.

DHβE shows a similar hydrogen-bonding network to agonists

The protonated tertiary nitrogen of DHβE lies within hydrogen-bonding distance of the backbone carbonyl of Trp143 (2.7 Å, chain D) and is also in close contact with the hydroxyl group of Tyr89 from the B-loop (3.5 Å, chain D) (Fig. 2a). Hydrogen bonds to these two residues on the principal side of the subunit interface have been observed for other agonists bound to AChBP [12,19].

Figure 1. The structure of DHβE and Ls-AChBP complexed with DHβE. (a) Structure of DHβE. (b) Cartoon diagram showing homopentameric Ls-AChBP viewed along the five-fold symmetry axis. The five subunits are shown in different colors and DHβE in red spheres representation. (c) Ligand-binding pocket at the interface of two monomers formed by the highly conserved aromatic residues Tyr89, Trp143, Tyr185, and Tyr192 from the principal side of the interface (yellow) and Trp53 from the complementary side (limon). DHβE is shown in red and an omit 2Fo-Fc map is shown at 1σ. Hydrogen bonds between DHβE and its surroundings are shown as stippled lines. A blow-up of DHβE and the omit 2Fo-Fc map shown at 1σ is provided in Fig. S2.

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On the complementary side, DHβE interacts with the protein main chain via a water-mediated hydrogen bond (Fig. 2a). The oxygen of the methoxy group of DHβE interacts with a hydrogen bond from a water molecule, which is tightly coordinated to the backbone carbonyl oxygen of Leu102 and nitrogen of Met114. Similar water-mediated contacts have been previously reported in agonist-bound structures (Fig. 2b) [7–9,12,19,20]. In this way, the interface in a way comparable to that of agonists.

DHβE binds to Ls-AChBP under a shifted C-loop conformation

The conformation of the C-loop is very similar in all ten subunits (Fig. S1). In a previous study, the distance between the carbonyl oxygen atom of the conserved Trp residue in the A-loop (Trp143 in Ls-AChBP) and the γ-sulfur atom of the first Cys residue involved in disulfide bridge formation in the C-loop (Cys187 in Ls-AChBP) was used to quantify C-loop closure. This measurement was then correlated to the pharmacological profile of compounds co-crystallized with AChBP, suggesting a preference for antagonists to bind under open (extended) C-loops and agonists under closed (contracted) C-loops while partial agonists would bind under loops with intermediate closure [21]. Applying this metric to our DHβE-bound structure would classify the ligand as an agonist (Table 2), suggesting that it is insufficient to assess pharmacological fingerprints based on AChBP C-loop closure alone.

Comparison of DHβE-bound Ls-AChBP with previously determined structures of Ls-AChBP in complex with small molecule agonists shows a new conformational state of the C-loop, which is not reflected by the distance measurement discussed above. This conformational state has not been observed in previously reported AChBP structures, where a closed C-loop corresponds to an agonist-bound state and an open C-loop to an antagonist-bound state. The C-loop conformational change has been quantified by measuring the angle between the projection of a vector defined from the center of the C-loop to the Cα atom of Cys187 in the DHβE-bound structure, and the corresponding projection vector in the nicotine-bound structure, which has been used as reference (Table 2, Fig. 2d). For further explanation on projection vectors, see Materials and Methods. The angle in the DHβE-bound structure is 21.4°, while this number lies within the range of 5°–7.7° for all other Ls-AChBP structures complexed with different agonists. These measurements reveal that the C-loop undergoes a conformational movement, which is perpendicular to the previously observed C-loop movements in AChBP structures and thus could indicate that DHβE inhibits nAChRs by a unique mechanism.

To investigate if this C-loop movement could be due to crystal packing effects, we undertook a detailed analysis of the DHβE-bound structure. Only C-loops of five subunits (chains B and D of one pentamer and chains G, I, and J of the other pentamer) out of ten subunits of the Ls-AChBP structure in complex with DHβE are in contact (closer than 3.5 Å) with symmetry-related molecules. Furthermore, different loop regions (residues 23–28, 67–72 or 160–167) of the symmetry-related molecules are involved in those contacts. Therefore, it is unlikely that the difference in C-loop conformation of the DHβE-bound structure compared to other Ls-AChBP structures is determined by crystal packing forces. To investigate C-loop flexibility, we compared the average C-loop B-factor to the average B-factor of all protein atoms. The average B-factor of all ten C-loops in the DHβE-bound structure is 47 Å², compared to 37 Å² for all protein atoms. Thus, the average B-factor is slightly increased at the C-loop relative to the overall average B-factor. However, other Ls-AChBP structures in complex with agonists (PDB IDs: 1uw6, 3u8l, 2zju, 3u8k, 3u8m, 3u8n, and 2zvj) show the same trend as for the DHβE-bound structure, except for the complexes with nicotine (PDB ID: 1uw6 and NS3331 (PDB ID: 3u8j)). In these latter two structures, the C-loop has lower and equal values, respectively, compared to the overall average B-factor.

A similar hypothesis that DHβE inhibits nAChRs by a unique mechanism was previously raised by Bertrand et al. Based on electrophysiological data [22] it was shown that an L247T mutation in the α7 nAChR ion channel domain renders DHβE an agonist [22]. Since mutation of L247T also reduces desensitization, it was suggested that DHβE inhibits the activity of nAChRs by stabilizing the desensitized state rather than the non-activated state of the receptor. The unique conformation of the C-loop observed in the DHβE-bound structure of Ls-AChBP together with a hydrogen-bonding network similar to that seen for agonists supports a unique mode of antagonism for DHβE compared to prototypical antagonists and toxins.

### Table 1. Data collection and refinement statistics of the DHβE-bound Ls-AChBP structure.

| Space group | P2₁,2,2₁ |
|-------------|-----------|
| Unit cell:  |           |
| a, Å        | 119.25    |
| b, Å        | 121.31    |
| c, Å        | 152.07    |
| a = b = c, °| 90.00     |
| Resolution range, Å | 19.61-2.51 (2.64-2.51)² |
| Completeness, % | 98.5 (92.7) |
| Overall number of reflections | 304,953 |
| Number of unique reflections | 74,987 |
| Redundancy | 4.1 (4.0) |
| Rmerge, % | 7.4 (41.0) |
| l/σ | 15.9 (2.0) |
| Solvent, % | 43.7 |
| Number of atoms | 17,183 |
| Number of DHβE molecules | 10 |
| Number of DHβE atoms | 20 |
| Number of water molecules | 687 |
| Rwork, % | 20.0 |
| Rfree, % | 25.0 |
| Ramachandran plot, residues in most favored regions, % | 91.7 |
| Rmsd of bonds lengths, Å | 0.013 |
| Rmsd of bonds angles, ° | 1.4 |
| Average B-factor of protein main chains, Å² | 35 |
| Average B-factor of protein side chains, Å² | 39 |
| Average B-factor of water molecules, Å² | 36 |
| Average B-factor of DHβE molecules, Å² | 30 |
| Wilson B-factor, Å² | 45 |

*Numbers in parentheses represent the last resolution shell values.

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Conclusions

In this study, we have determined the crystal structure of Ls-AChBP in complex with DHβE, which is a potent competitive antagonist of nAChRs. The structure reveals three main features that are unique to this antagonist: (i) DHβE introduces a C-loop closure compared to that of agonists, (ii) the C-loop undergoes a conformational shift perpendicular to the previously observed C-loop movements, and (iii) the hydrogen-bonding network of DHβE is similar to that of agonists. Thus, DHβE seems to prevent receptor activation via a mechanism different from that of prototypical antagonists and toxins.

Materials and Methods

Protein purification and crystallization

Recombinant Ls-AChBP was expressed using the Bac-to-Bac baculovirus expression system in Sf9 insect cells and purified as
Crystal Structure of Ls-AChBP Complexed with DHβE

Described previously [12,19]. The protein solution was incubated with 50 mM DHβE prior to crystallization. DHβE-bound crystals were obtained using the hanging drop vapor diffusion method at 20°C. Crystallization drops were made by mixing 1 μl of a 4.9 mg/ml protein:DHβE solution in 20 mM Tris Base (pH 8.0) and 20 mM NaCl with 1 μl of crystallization solution containing 0.1 M HEPES (pH 7.5), 25% v/v polyethylene glycol (PEG) 400, and 0.2 M MgCl₂. Crystals grew within 3 weeks to a final length of 0.2 mm.

Crystallographic data collection, refinement, and model building

The crystal was mounted in a cryo-loop and flash-cooled in liquid nitrogen after brief immersion in a cryo-protectant composed of mother liquor supplemented with 25% (v/v) glycerol. X-ray data were collected at 100 K on beamline I911-3 at the MAX-lab synchrotron, Lund, Sweden, using a marmosaic 225 detector at a wavelength of 0.997 Å. Data were processed and scaled using XDS [23] and Scala [24], respectively. Five percent of the data were set aside during the scaling process as test set for calculation of Rmerge.

The structure was solved by the molecular replacement method using the program Phaser [25]. A pentamer of Ls-AChBP (in-house structure; to be published) was used as the search model. The refinements were performed with Phenix [26] using non-crystallographic symmetry (NCS) and rebuilt interactively using Coot [27]. Residues in the F-loop (154–164) were excluded from NCS restraints. The input structure of DHβE was generated using Maestro [28] and MacroModel [29]. Low energy conformations of DHβE with a protonated tertiary nitrogen were generated using the Monte Carlo molecular mechanics method with an energy cutoff set to 13 kJ/mol and used to generate geometry restraints after selection of the low energy conformer with the best visual fit to the electron density map. Water molecules were added during the refinement using Phenix. The starting Rwork and Rfree of the structure were 39.1% and 40.8%, respectively, which were improved to the final Rwork and Rfree of 20.1% and 24.2%, respectively. Data collection and refinement statistics are summarized in Table 1.

The quality of the final model was assessed by examination of the detailed stereochemistry using Procheck [30] and Molprobity [31]. The Ramachandran plot of the structure shows that 91.7% of the residues are in the most favored regions by the Procheck criteria, 8.1% in additionally allowed regions and 0.2% in the generously allowed regions.

Structure analysis

The structure and ligand analyses were performed using Coot [27] and PyMOL [32]. The figures were generated using PyMOL.

The superposition of the DHβE-bound structure onto the nicotine-bound Ls-AChBP structure was performed on Cα atoms employing the “super” command in PyMOL. The projection vector (shown in green, Fig. 2d) belonging to the nicotine-bound structure was defined as follows: (i) First, a reference plane was defined at the C-loop position (residues 185–192) in the nicotine-bound structure was performed on Cα atoms. The Ramachandran plot of the structure shows that 91.7% of the residues are in the most favored regions by the Procheck criteria, 8.1% in additionally allowed regions and 0.2% in the generously allowed regions.

Table 2. Quantification of the C-loop conformational change.

| Ls-AChBP complexed with | Trp143(O) - Cys187(S) distance (Å)a | Angle between projection vectors (°)b |
|-------------------------|-------------------------------------|--------------------------------------|
| Agonist: nicotinec      | 7.3                                 | -                                    |
| Agonist: carbamylcholinef| 7.3                                 | 5.0                                  |
| Agonist: 1-(5-phenylpyridin-3-yl)-1,4-diazepanef| 7.5| -4.2                                      |
| Agonist: imidaclopiridf| 10.8                                | 0.1                                  |
| Agonist: 1-(5-ethoxy pyridin-3-yl)-1,4-diazepanef| 7.8| 0.1                                      |
| Agonist: 1-(6-bromopyridin-3-yl)-1,4-diazepanef| 7.3| 0.2                                      |
| Agonist: 1-(6-bromo-5-ethoxy pyridin-3-yl)-1,4-diazepanef| 7.5| 2.2                                      |
| Agonist: 1-(5-phenylpyridin-3-yl)-1,4-diazepanef| 7.5| 4.7                                      |
| Agonist: clothianidinẽ| 7.3                                 | 7.7                                  |
| Antagonist: DHβE        | 7.5                                 | 21.4                                 |

aQuantification of C-loop closure by the method of Brams et al. [21].
bFor explanation on projection vectors, see Materials and Methods.

cPDB ID (chain A):
d1uv6;
e3u8l (chain B);
f2zju;
g3u8k;
h3u8m;
i3u8n;
j3u8j;
k2zjv;
l3uw6 (chain B);
m2wl6;

For explanation on projection vectors, see Materials and Methods.

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| Agonist: 1-(5-phenylpyridin-3-yl)-1,4-diazepanef| 7.5| -4.2                                      |
| Agonist: imidaclopiridf| 10.8                                | 0.1                                  |
| Agonist: 1-(5-ethoxy pyridin-3-yl)-1,4-diazepanef| 7.8| 0.1                                      |
| Agonist: 1-(6-bromopyridin-3-yl)-1,4-diazepanef| 7.3| 0.2                                      |
| Agonist: 1-(6-bromo-5-ethoxy pyridin-3-yl)-1,4-diazepanef| 7.5| 2.2                                      |
| Agonist: 1-(5-phenylpyridin-3-yl)-1,4-diazepanef| 7.5| 4.7                                      |
| Agonist: clothianidinẽ| 7.3                                 | 7.7                                  |
| Antagonist: DHβE        | 7.5                                 | 21.4                                 |
Acceison Numbers

Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 4alx.

Supporting Information

Figure S1  The conformation of the C-loop is very similar in all ten subunits. The subunits of the DH\(\beta\)E-bound structure have been superimposed (shown in different colors). (TIF)

Figure S2  An omit 2Fo-Fc map for DH\(\beta\)E shown at 1\(\sigma\) with DH\(\beta\)E modeled in. Three different views of the electron density are shown. (TIF)

Figure S3  Vector representation showing the conformational change of the C-loop due to DH\(\beta\)E binding to Ls-AChBP. The DH\(\beta\)E-bound structure (red) has been superimposed onto the nicotine-bound Ls-AChBP structure (green). \(\text{Ob}\) is the distance between the center of the C-loop and C\text{\textsuperscript{2}} atom of Cys187 in DH\(\beta\)E-bound Ls-AChBP structure, and \(\text{Oa}\) is the corresponding projection vector. \(\text{Oc}\) is the distance between the center of the C-loop and C\text{\textsuperscript{2}} atom of Cys187 in nicotine-bound Ls-AChBP structure, and \(\text{Od}\) is the corresponding projection vector. (TIF)

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Author Contributions

Conceived and designed the experiments: AS JSK JLK MG TB. Performed the experiments: AS EON. Analyzed the data: AS JSK EON MG TB. Contributed reagents/materials/analysis tools: JLK. Wrote the paper: AS JSK TB.

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