Enhanced meta-analysis of acetylcholine binding protein structures reveals conformational signatures of agonism in nicotinic receptors

Spencer T. Stober and Cameron F. Abrams*

Department of Chemical and Biological Engineering, Drexel University, Philadelphia, Pennsylvania 19104

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Abstract: The soluble acetylcholine binding protein (AChBP) is the default structural proxy for pentameric ligand-gated ion channels (LGICs). Unfortunately, it is difficult to recognize conformational signatures of LGIC agonism and antagonism within the large set of AChBP crystal structures in both *apo* and ligand-bound states, primarily because AChBP conformations in this set are nearly superimposable (root mean square deviation < 1.5 Å). We have undertaken a systematic, alignment-free approach to elucidate conformational differences displayed by AChBP that cleanly differentiate *apo*/antagonist-bound from agonist-bound states. Our approach uses statistical inference based on both crystallographic states and conformations sampled during long molecular dynamics simulations to select important inter-C_α distances and map their collective values onto functional states. We observe that binding of (nAChR) agonists to AChBP elicits clockwise rotation of the inner β-sheet with respect to the outer β-sheet, causing tilting of the cys-loop away from the five-fold axis, in a manner quite similar to that speculated for α-subunits of the heteromeric nAChR structure (Unwin, J Mol Biol 2005;346:967), making this motion potentially important in transmission of the gating signal to the transmembrane domain of a LGIC. The method is also successful at discriminating partial from full agonists and supports the hypothesis that a particularly controversial ligand, lobeline, is in fact an LGIC antagonist.

Keywords: ligand-induced conformational change; molecular dynamics simulations; statistical inference; state determination

Introduction

The acetylcholine binding protein (AChBP) is a soluble protein released by glial cells that modulates neuronal transmission in cholinergic synapses of certain mollusks by binding acetylcholine (ACh) that has been excreted into the synaptic cleft. The sequence of the AChBP homopentamer is most ho-

mologous (23.9%) to the first 210 residues of the α subunit of the nicotinic acetylcholine receptor (nAChR) (Fig. 1) and is 15–18% homologous to the 5-hydroxytryptamine receptor 3A (5-HT_{3A}), GABA_A, GABA_C, and glycine receptors,^{3,4} indicating a common evolutionary ancestor for AChBP and ligand-gated ion channels (LGICs).⁵ High resolution *apo* and liganded crystal structures have been determined for AChBP from three species: *Bulinus truncatus*, *Lymnaea stagnalis*, and *Aplysia californica*. AChBP has been shown to bind ligands that are also known to bind nAChR and is often used as a surrogate for discovery of drugs active toward nicotinic receptors.^{6–11} Indeed, engineered chimeric LGICs in which AChBP replaces the wild-type extracellular domain (ECD) are functional (n.b. mutation of

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*Correspondence to: Cameron F. Abrams, Department of Chemical and Biological Engineering, Drexel University, Philadelphia, PA 19104. E-mail: cfabrams@drexel.edu

Table I. Scores For Crystal Structures Included in Calibration

Structure	Ligand	Classification	A	B	C	D	E	F	$\beta 1-\beta 2$	$\beta 8-\beta 9$	CYS	Mean
2BYQ	Epibatidine	Agonist	0.76	0.89	0.97	0.73	0.63	0.79	0.72	0.85	0.82	0.80
2WNL (1)	Anabaseine		0.65	0.69	0.68	0.76	0.66	0.68	0.66	0.77	0.70	0.69
3C79	Imidacloprid		0.67	0.81	0.93	0.89	0.77	0.87	0.76	0.78	0.83	0.81
3C84	Thiacloprid		0.70	0.85	0.93	0.91	0.81	0.82	0.70	0.78	0.76	0.81
2BR8	α -Ctx PnIA (A10L D14K)	Peptide antagonist	0.21	0.23	0.03	0.25	0.54	0.18	0.28	0.26	0.19	0.24
2UZ6 (1)	α -Ctx TxIA (A10L)		0.18	0.30	0.03	0.17	0.25	0.28	0.30	0.18	0.22	0.21
2BYP	α -Ctx ImI		0.17	0.10	0.01	0.30	0.22	0.28	0.29	0.30	0.39	0.23
2C9T (1)	α -Ctx ImI		0.19	0.32	0.04	0.30	0.14	0.35	0.28	0.51	0.27	0.27
2C9T (2)	α -Ctx ImI		0.20	0.28	0.04	0.27	0.26	0.30	0.26	0.15	0.23	0.22
2BYR (1)	Methyllycaconitine	Antagonist, $\alpha 7$ selective	0.34	0.40	0.15	0.42	0.16	0.35	0.24	0.51	0.48	0.34
2BYR (2)			0.30	0.31	0.06	0.26	0.18	0.52	0.33	0.56	0.20	0.30
2BYN	<i>Apo</i>		0.38	0.57	0.17	0.24	0.20	0.50	0.45	0.61	0.58	0.41
3GUA (1)			0.21	0.50	0.20	0.26	0.12	0.57	0.32	0.49	0.42	0.34
3GUA (2)			0.22	0.51	0.29	0.24	0.12	0.57	0.28	0.50	0.41	0.35

associated with agonist binding and their possible roles in the gating mechanism of the nAChR.

An analysis of inter- C_{α} distance changes with respect to a single crystal structure was previously used to analyze MD simulations of AChBP with and without a docked ACh ligand.¹⁷ The method we discuss here extends this analysis by (a) using multiple samples to calibrate state definitions and (b) demonstrating the ability to predict the states of a ligand-bound AChBP structures.

Results

We first present results pertaining to calibration of the method. We then present results from subjecting conformational states from outside the calibration test to scoring. Finally, we present results pertaining to predictions made by the method for structures with ligands of unknown or unclear function. A detailed presentation of the method itself appears in

the Materials and Methods section after some discussion of these results. Briefly, for each AChBP loop, the score associated with it appearing in Tables I–IV (Supporting Information Tables SI–SV) is an average and scaled distance between C_{α} s on that loop and C_{α} s elsewhere in the structure. These scores range from zero (indicating an nAChR-antagonist or *apo* like conformation) to one (indicating an nAChR-agonist like conformation). MD was conducted using three starting conditions: (1) the anabaseine-bound structure (2WNL),¹⁸ (2) the lobeline-bound structure (2BYS),¹⁰ and (3) the *apo* structure (2BYN).⁸ For (1) and (2), 70-ns trajectories were run, and we launched 55-ns branching simulations from each at 15-ns duration with deletion of the ligands. For (3), a 30-ns trajectory was run. The purpose of anabaseine-bound and *apo* trajectories was to provide calibration samples for the agonist-bound and antagonist/*apo* states, respectively. The anabaseine-

Table II. Scores for Crystal Structures Not Included in Calibration

Test Structures	Ligand	Classification	A	B	C	D	E	F	$\beta 1-\beta 2$	$\beta 8-\beta 9$	CYS	Mean
2WNL (2)	Anabaseine	Agonist	0.60	0.63	0.66	0.77	0.62	0.58	0.57	0.76	0.57	0.64
2WN9	4-OH DMXBA ^a	Partial agonist	0.55	0.62	0.50	0.25	0.31	0.64	0.54	0.60	0.64	0.52
2WNJ	DMXBA ^b		0.65	0.80	0.95	0.35	0.33	0.54	0.66	0.74	0.77	0.64
2W8E		<i>apo</i>	0.48	0.72	0.63	0.33	0.13	0.57	0.49	0.68	0.40	0.49
2BR7			0.49	0.68	0.66	0.57	0.51	0.41	0.32	0.57	0.34	0.51
2PGZ	Cocaine	Non-competitive antagonist	0.45	0.62	0.36	0.29	0.25	0.52	0.45	0.67	0.56	0.46
2W8G	<i>In silico</i> 35 ^c	Non-competitive and	0.42	0.54	0.14	0.54	0.29	0.33	0.38	0.53	0.25	0.38
2W8F (1)	<i>In silico</i> 31 ^d	competitive antagonist	0.39	0.67	0.40	0.39	0.21	0.60	0.50	0.62	0.37	0.46
2W8F (2)			0.39	0.66	0.20	0.37	0.20	0.58	0.40	0.57	0.31	0.41
2XYS	Strychnine	Competitive antagonist	0.40	0.60	0.54	0.19	0.20	0.59	0.46	0.19	0.29	0.38
2XYT (1)	d-Tubocurarine		0.33	0.56	0.47	0.21	0.20	0.55	0.57	0.44	0.29	0.40
2XYT (2)			0.42	0.67	0.31	0.56	0.42	0.59	0.53	0.27	0.23	0.45
2WZY (1)	13-Desmethyl spirolide C		0.40	0.40	0.16	0.60	0.69	0.45	0.35	0.54	0.47	0.45
2WZY (2)			0.37	0.34	0.20	0.59	0.81	0.56	0.36	0.57	0.41	0.47
2X00	Gymnodimine A		0.51	0.62	0.48	0.58	0.40	0.55	0.33	0.65	0.53	0.52
2UZ6 (2)	α -Ctx TxIA (A10L)	Peptide antagonist	0.18	0.30	0.03	0.17	0.25	0.28	0.30	0.18	0.22	0.21

^a The 4-hydroxy metabolite of DMXBA (4-OH 3-(2,4-dimethoxybenzylidene)-anabaseine).

^b 3-(2,4-dimethoxybenzylidene)-anabaseine (DMXBA).

^c (3-endo,8-anti)-8-benzyl-3-(10,11-dihydro-5h-dibenzo[a,d][7]annulen-5-yloxy)-8-azoniabicyclo[3.2.1]octane.

^d (3-exo)-3-(10,11-dihydro-5h-dibenzo[a,d][7]annulen-5-yloxy)-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane.

Table III. Comparison of the Response to Ligand Deletion for 2WNL and 2BYS MD Trajectories

Test Structures	Ligand	Classification	A	B	C	D	E	F	$\beta 1-\beta 2$	$\beta 8-\beta 9$	CYS	Mean
2WNC	tropisetron	$\alpha 7$ Partial agonist/non- $\alpha 7$ antagonist	0.63	0.64	0.92	0.37	0.16	0.84	0.60	0.69	0.71	0.62
2PH9	Galanthamine	Positive allosteric modulator/non-competitive antagonist	0.45	0.55	0.25	0.34	0.30	0.55	0.52	0.55	0.58	0.45
2BYS (1)	lobeline Y53C Mutant, MMTS with Ach Y53C Mutant, MTSET+		0.65	0.73	0.98	0.75	0.76	0.56	0.62	0.59	0.52	0.69
2BYS (2)			0.65	0.74	0.97	0.77	0.78	0.55	0.62	0.58	0.48	0.68
2XZ5			0.46	0.71	0.97	0.71	0.61	0.55	0.42	0.66	0.30	0.60
2XZ6 (1)			0.38	0.57	0.24	0.22	0.26	0.47	0.46	0.45	0.26	0.37
2XZ6 (2)			0.39	0.59	0.25	0.24	0.25	0.47	0.46	0.45	0.26	0.37

deleted and both lobeline-based trajectories provided conformational samples tested by the method.

In Figure 2, we report whole-protein RMSD as a function of simulation time for the anabaseine-bound, anabaseine-deleted, lobeline-bound, and lobeline-deleted. After the RMSD has attained a steady state (about 20 ns, by inspection), it can be seen that the RMSD of the trajectories vary by ~ 0.5 Å, which is consistent with the RMSD differences between the various crystal structures (0.1–1.5 Å). In the case of lobeline-bound AChBP crystal structure, we see the Δ RMSD (RMSD with ligand bound vs. RMSD with ligand deleted) at less than 0.5 Å, whereas Δ RMSD for the anabaseine-bound structure is essentially zero. This indicates that the MD-equilibrated structures in the ligand-bound and *apo* forms are essentially superimposable, showing more similarity to one another than the RMSD over all AChBP crystal structures.

Evaluation of crystal structures included in calibration

Table I contains the scores for structures included in the calibration set. It has been shown that C-loop position varies from tightly closed for agonists, to an intermediate position for antagonists and *apo* structures, and propped open for the peptidic antagonists that are large enough to sterically hinder C-loop closure.^{10,18} Our analysis agrees with these findings: C-loop scores for agonists average 0.84, peptidic antagonists average 0.04, whereas those for the *apo* and

small antagonist average 0.21. In addition to the C-loop, the scores from the (+)-face and transmembrane domain (TMD) interface are also lower for the peptidic antagonists compared with *apo* and small antagonist structures. However, the structures in the (–)-face to score similarly among both antagonist types and the *apo* structure. This finding supports the view that small antagonists bind to residues within the binding pocket but evoke less change in conformation, compared with the *apo* state than do the larger peptidic antagonists.

Evaluation of crystal structures not included in calibration

Table II contains the scores for structures not included in the calibration set. The crystal structures 2PGZ,¹⁹ 2W8F,²⁰ and 2W8G²⁰ are bound to cocaine, (3-exo)-3-(10,11-dihydro-5h-dibenzo[a,d][7]annulen-5-yloxy)-8, 8-dimethyl-8-azoniabicyclo[3.2.1]octane (*in silico* 31), and (3-endo,8-anti)-8-benzyl-3-(10,11-dihydro-5h-dibenzo[a,d][7] annulen-5-yloxy)-8-azoniabicyclo[3.2.1]octane (*in silico* 35), respectively. These compounds are noncompetitive nAChR-antagonists (although some competitive activity has been reported for *in silico* 35).^{19,20} The method predicted scores of 0.38 – 0.46 for these three structures, which is consistent with an *apo* or small agonist structure.

2XYS,²¹ 2XYT,²¹ 2WZY,⁶ and 2X00,⁶ are bound to the competitive antagonists strychnine, *d*-tubocurarine, 13-desmethyl spirolide C, and gymnodimine A, respectively. The method predicted scores of 2XYS,

Table IV. Scores for Crystal Structures Containing Ligands of Unclear Function or Mutations

MD Simulations	Ligand	Classification	A	B	C	D	E	F	$\beta 1-\beta 2$	$\beta 8-\beta 9$	CYS	Mean
2WNL Crystal (Mean)	Anabaseine	Agonist	0.60	0.63	0.66	0.77	0.62	0.58	0.57	0.76	0.57	0.64
2WNL 5-15 ns	Ligand deleted at 15 ns		0.67	0.60	0.79	0.47	0.45	0.58	0.69	0.47	0.58	0.59
2WNL 60-70 ns			0.95	0.98	0.96	0.89	0.98	0.85	0.89	0.91	0.92	0.93
2WNL 60-70 ns			0.32	0.59	0.47	0.46	0.69	0.46	0.79	0.41	0.68	0.54
2BYS Crystal	Lobeline	Competitive antagonist	0.65	0.74	0.98	0.76	0.77	0.56	0.62	0.58	0.50	0.68
2BYS 5-15 ns	Ligand deleted at 15 ns		0.37	0.24	0.35	0.43	0.50	0.29	0.55	0.37	0.50	0.40
2BYS 60-70 ns			0.64	0.33	0.27	0.51	0.77	0.29	0.60	0.32	0.64	0.49
2BYS 60-70 ns			0.48	0.48	0.60	0.60	0.77	0.36	0.64	0.56	0.53	0.56

(1) and (2) for 2BYS and 2WNL indicate each of the crystals in the asymmetric unit cell.

Figure 2. RMSD of four MD trajectories: light blue, 2BYS with ligands; dark blue, 2BYS trajectory after deletion of ligands at 15 ns; yellow, 2WNL with ligands; purple, 2WNL trajectory after deletion of ligands at 15 ns. Ligand deletion produces no distinguishable deviations in RMSD from the MD simulations where the ligand is not deleted. After the RMSD has attained a steady state (about 20 ns, by inspection), it can be seen that the RMSD of the trajectories vary by ~ 0.5 Å, which is consistent with the RMSD differences between the various crystal structures (0.1–1.5 Å). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

2XYT, and 2WZY are 0.38, 0.42, and 0.46, respectively (for 2XYT and 2WYZ this is the mean for both structures in the asymmetric cell). These scores also agree with their ligand classifications with respect to nAChR. 2X00 has a predicted score of 0.52, which is slightly higher than the other competitive antagonists but certainly not in the range of agonist crystal structures (0.69 – 0.81). This anomalous prediction could be the result of crystallization artifacts (as will be discussed below for lobeline), or a lack of method accuracy for this structure, as gymnodimine A has been shown to be a potent antagonist for a broad range of nAChR subtypes, including $\alpha 3\beta 2$, $\alpha 4\beta 2$, nAChR and chimeric $\alpha 7$ -5-HT₃ receptors.²²

2W8E²⁰ and 2BR7⁸ are *apo* crystal structures and the method predictions for these structures are 0.49 and 0.51, respectively, which agree with the *apo* crystal structures in the calibration set.

The 2WNJ and 2WN9 structures are bound to the partial agonists 3-(2,4-dimethoxybenzylidene)-anabaseine (DMXBA) and its 4-hydroxy metabolite (4-OH DMXBA), respectively.¹⁸ The predictions for these are 0.64 and 0.52, respectively. These scores are between the scores for the agonist-bound structures (mean = 0.75) and the *apo* structures (mean = 0.42), which is consistent with their classification as partial agonists.

Evaluation of crystal structures containing ligands of unclear function

This section contains analysis of structures containing ligands of unclear function (see Table IV, with

respect to what state they would be expected to elicit in nAChR, or structures that contain mutations. For example, ligands that have a dual or concentration dependent effect, such as 2PH9,¹⁹ fall into this category. The 2PH9 AChBP structure is bound to galanthamine (GAL), a concentration-dependent positive allosteric modulator or non-competitive antagonist for neuronal $\alpha 3\beta 4$ -, $\alpha 4\beta 2$ -, $\alpha 6\beta 4$ -, and $\alpha 7$ -nAChR.^{19,23} A positive allosteric modulator lowers the concentration of agonist required to activate a receptor. For instance, the EC₅₀ for ACh on the $\alpha 4\beta 2$ -nAChR is 20 ± 1.7 μ M, but in the presence of 0.5 μ M GAL is reduced to 10 ± 1.8 μ M. At concentrations of 0.1 – 1 μ M GAL acts as a positive allosteric modulator, but at concentrations >10 μ M it acts as a noncompetitive antagonist. The score for this structure is 0.45, which is slightly above the scores for the *apo* structures, but clearly not in the range of the agonists or partial agonists. This result supports the hypothesis that GAL may shift the equilibrium of the *apo* state toward a conformation that is more like an agonist-bound structure, which could conceivably lower the concentration of agonist required for activation, which is the hallmark of a positive allosteric modulator.

The 2WNC structure is bound to tropisetron, a partial agonist for $\alpha 7$ -nAChR and an antagonist for non- $\alpha 7$ -nAChR.¹⁸ The method prediction of 0.62 indicates that the state of AChBP is most similar to the partial agonists. This result indicates that AChBP may behave similarly to $\alpha 7$ -nAChR in its response to tropisetron. This result is not surprising as AChBP is 24% homologous with the ECD of the $\alpha 7$ receptor and 20 – 24% homologous to other nicotinic receptors,⁷ and is a homopentamer like the $\alpha 7$ -nAChR. This result supports the view that in addition to sharing binding characteristics with the $\alpha 7$ -nAChR, AChBP may exhibit conformational changes similar to those of the ECD of $\alpha 7$ -nAChR.

2XZ5²⁴ is an AChBP-Y53C mutant cocrystallized with ACh and methyl methanethiolsulfonate (MMTS), a cysteine-modifying reagent that potentiates ACh activation of a similarly mutated $\alpha 7$ -nAChR ($\alpha 7$ -nAChR-W55C). The method prediction of 0.6 for this structure is consistent with the expected agonist effect of methyl MMTS and ACh in combination with the Y53C mutation. However, the score for the C-loop of this structure is 0.97 (a result of the closed C-loop position elicited by MMTS), without this high score for C-loop, the average score is 0.55, well below the other agonists. Several unusual features in this crystal structure could explain these results: in each binding pocket, two ACh ligands are present, as this phenomenon has not been reported for other agonists, this may be a result of the mutation. Also, 10 phosphate ions form two rings centered on the five-fold axis of symmetry of the pentamer. These phosphates appear to interact with

residues Arg95 (in loop A), Glu47 and Asp49 (in the β 1- β 2-loop), and Lys40 (in the β 1 strand of the outer β sheet). The scores for loop A and the β 1- β 2-loop of 2XZ5 are 0.46 and 0.42, respectively, versus an average of 0.68 for both of these structures among the other agonists. It is conceivable that structural artifacts introduced from the presence of these phosphates (and additional ACh ligands) and their interactions attenuated the motions normally evoked by agonist binding.

2XZ6²⁴ is also an AChBP-Y53C mutant that is co-crystallized with (MTSET⁺), a cysteine-modifying reagent, that makes α 7-nAChR-W55C receptors unresponsive to ACh. The state prediction of 0.37 for both structures in the asymmetric unit cell is consistent with the antagonistic effect that this combination of mutation and ligand has on the α 7-nAChR.

The lobeline bound structure, 2BYS, is predicted to be in an agonist-bound state (score = 0.69 and 0.68 for each structure in the asymmetric unit cell) despite compelling evidence showing that lobeline is indeed an antagonist (α 7-nAChR IC₅₀ 8.5 μ M).^{25–27} It is surprising that the lobeline structure is predicted as clearly agonist-bound, and we therefore proceeded to investigate the 2BYS structure using MD simulation. As described in the Materials and Methods section, we launched an MD simulation of the 2BYS structure with lobeline bound. After 15 ns, one trajectory was continued uninterrupted and a second simulation was started from those coordinates but with the lobeline ligands deleted. Table III shows the results of predictions on this simulation compared with 2WNL (a known nAChR-agonist-bound structure). The scores for both 2WNL and 2BYS decrease from their crystal structure scores based on their time averaged coordinates from 5 to 15 ns, however the shift for 2BYS is much greater. After prolonged equilibration, time averaged coordinates at 60 – 70 ns for 2WNL clearly show that the structure is in an agonist-bound conformation (score = 0.93). However, if the ligand is deleted, the prediction average over 60 – 70 ns decreases by 0.39, as would be expected for removal of an nAChR-agonist. In 2BYS, in the 60 – 70 ns time period, the prediction of 0.49 is consistent with the other state-B structures and with the classification of lobeline as an antagonist in most of the literature. After deletion of the ligand, the 60 – 70 ns prediction is 0.56, which is only slightly higher than the scores seen for *apo* structures, but certainly not in the agonist range. This result also agrees with the most recent data indicating that lobeline is an antagonist. It is puzzling why the 2BYS crystal structure appears to be in an agonist-bound conformation (as predicted by our method). Perhaps artifacts of the crystallization process can account for this observation, in which case extended MD equilibration was able to relax the structure into a more contextual (i.e. dilute solution) conformation.

Structural insights on the nAChR gating mechanism

By selecting crystal structures or MD simulation frames that exhibit loop positions predicted by the algorithm to be associated with nAChR agonists or antagonists, we can gain insight into their possible positions in the activated or deactivated state of the nAChR by aligning these structures with respect to residues in the outer β sheet. From this analysis, we can see key ligand-induced motions in the loops A, B, and C that appear to contribute to the clockwise rotation of the inner β sheets and translation and twisting of the cys-loop that would allow outward tilting of the helix bundle.

It has been well documented that the C-loop closes on agonist binding, and we have also observed this in our analysis (Fig. 3). We have also observed (with respect to the nAChR-antagonist-bound or *apo* states) translation of the A-loop toward the center axis and similar, although smaller, translation of the B-loop in approximately the same direction with bound agonist. The cys-loop, when viewed from below (looking upward from the TMD) exhibits translation away from the central axis and anticlockwise twisting. These motions are reconciled in the following theory of ligand-induced conformational changes observed in AChBP, and how they may relate to gating motion in the ECD of LGICs. Motion from ligand-induced closure of the C-loop is transmitted along β 10, causing translation of the B-loop toward the pore through the coupling of β 10 to β 7 via the hydrogen bond network that forms the inner β sheet (specifically, β 10 is coupled to β 7 by the hydrogen bonds Val198-Phe144 and Leu200-Val142). This motion of B-loop could also be the result of interactions with the ligand. The hydrogen bonds between β 10 and β 7 act like a fulcrum which transmits this motion to the cys-loop causing its translation away from the pore. In addition, the translation of the A-loop toward the pore (which may also may be a result of ligand interactions) can transmit its motion to the cys-loop through β 6, which is also coupled to the A-loop via hydrogen bonds between Gln100 and Arg122. The sum of these changes in configuration can be conceptualized as a clockwise rotation of the inner β sheet with respect to the outer β sheet and outward translation and twisting of the cys-loop. This motion would not be easily identified via analysis of the crystal structures alone. The MD simulation of the 2WNL structure generated a stronger agonist-bound conformational signature than was seen in any of the crystals (Tables I and III). The attenuation of the agonist signature in the crystal structures may be an artifact of the crystallization process that relaxes on extended MD equilibration. Indeed, the generation of a more agonist like structure from MD simulation facilitated the identification of this mechanism.

This mechanism agrees with Unwin's analysis of structures of the nAChR that revealed a 15–16° anticlockwise rotation of the inner β sheets

Figure 3. (a) The position of the A-loop (green), B-loop (orange), C-loop (yellow), the cys-loop (black), the cys-loop disulfide bond (yellow CPK representation), the outer β sheets (tan), and the inner β sheets (light blue) are shown in the state-B conformation. The red translucent representation shows the position of these structures in state-A conformation revealing the clockwise rotation, with respect to the outer β sheet, and the tilting of the cys-loop in response. The motion of loops B and C, and to a lesser extent A, are also apparent. (b) Key hydrogen bonds have been rendered as purple springs connected to their corresponding residues that have been rendered in ball-and-stick representation. Structures of interest have also been labeled. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

compared with the outer β sheets in the α -subunits of nAChR when compared with non- α -subunits.²⁸ Comparison of Unwin's cryo-EM structure with those of agonist-bound AChBP has revealed that on agonist binding it is likely that nAChR α -subunits undergo a conformational rearrangement (clockwise rotation of the inner β sheets) so that they more closely resemble the non- α -subunits.²⁹ Ligand binding occurs at the interface between the (+)-face of an α -subunit with another subunit; therefore, it has been postulated that this relaxation of the distortion in the α -subunit is initiated by ligand binding and is a key component of the gating mechanism.²⁸ We propose here a mechanism in AChBP that couples ligand binding to clockwise rotation of the inner β sheets and subsequent translation and twisting of the cys-loop away from the pore axis, which may reflect changes that occur in the highly homologous ECD of nAChR during the gating reaction. The highly conserved cys-loop has been shown to be critical in the gating reaction of all cys-loop ligand gated ion channels.³⁰ In nAChR, translation of the cys-loop away from the pore axis could drive tilting of the helix bundle away from the pore axis, and this tilting has been postulated as a mechanism of channel opening.³¹

Discussion

The utility of AChBP crystal structures as model receptors for drug discovery relies on the ability to determine if a novel ligand will have the ability to elicit (or prevent) the conformational changes that

cause gating. It has been shown that traditional analysis of crystal structures is insufficient to elucidate the conformational changes unique to different classes of nAChR ligands. Our method uses α -carbon distance analysis, as described above, in conjunction with MD simulation to more accurately predict the effect of ligands on the conformation of AChBP, and may give insight to their action on the nAChR. It is apparent that this method could also be extended to be more broadly applicable to analysis of other protein/ligand systems.

This analysis has demonstrated that the ligand-induced conformation of AChBP is distinct for different classes of nAChR ligands. This supports the view that AChBP may undergo conformational changes similar to the ECD of nAChR on ligand binding. This method has also proven useful to study the effects of perturbations on MD simulations. We have shown that partial agonists evoke an intermediate conformational state between the *apo* and full agonist-bound states. AChBP has also been shown to share agonist-like conformational signatures (in addition to previously described bonding selectivity) with the $\alpha 7$ -nAChR ECD in its response to a highly $\alpha 7$ -nAChR specific agonist (tropisetron). We have examined lobeline through the use of our method, in conjunction with MD simulation, to confirm that although the crystal structure of AChBP bound to lobeline appears agonist-like, dynamics reveal that lobeline evokes an antagonist-like response in AChBP.

Finally, by examining how loop movements in AChBP contribute to predictions of agonist-like or

antagonist-like states, we have proposed a mechanism linking ligand binding to motion in the cys-loop, which may be similar to the gating reaction in the ECD of nAChR. Unwin postulated that the transition from closed to open configuration in the nAChR ECD comprises relaxation of the α -subunits into a configuration more like their non- α counterparts, by clockwise rotation of the inner β sheets. We postulate a similar stepwise mechanism in AChBP showing how the ligand binding event is transmitted through loops on the (+)-face, causing clockwise rotation of the inner β sheets. Furthermore, we showed how this rotation is coupled to motions in the cys-loop that, in a complete receptor, may transmit the ligand binding event to the TMD. Indeed, the ability to initiate rotation of the inner β sheets by interactions with the (+)-face loops may define the difference between nAChR agonists and antagonists.

Materials and Methods

The function of our method is to assign the state of any all-atom AChBP structure based on its location in a high dimensional space spanned by inter- α C distances, R_{ij} . Generally speaking, for a structure with N C_α s, there are $\frac{1}{2}(N)(N-1)$ unique R_{ij} 's, (only $3N-6$ of which must be specified to determine conformation). The major task in our method is identifying the best subset R_{ij}^* that reliably allows state assignment. The basic strategy involves calibration using various test inputs, which we now describe.

Paired C_α distance measurements and scaling

Distances R_{ij} between every nonredundant pair of C_α atoms were measured using MATLAB® (2010b, The MathWorks, Natick, MA). For each crystal structure of 210 residues and five subunits this amounts to 550,725 measurements. Statistical tests were used to determine the set of relevant measurements R_{ij}^* . In the crystal-based and hybrid calibration sets the two-sample Kolmogorov–Smirnov test³⁰ was used to determine which measurements exhibited a statistically significant difference between states A and B by setting a threshold of minimum statistical confidence that the mean of the state-A measurements $\bar{R}_{ijA}^*$ and state-B measurements $\bar{R}_{ijB}^*$ were different. This threshold can then be adjusted up or down to either increase or decrease the number of measurements deemed significant. Equation (1) defines $\bar{R}_{ijA}^*$, where k is a structure from the set of structures used to calibrate state-A and n_A is the total number of these structures.

$$\bar{R}_{ijA}^* = \frac{1}{n_A} \sum_{k \in A}^{n_A} (R_{ijA}^*)_k \quad (1)$$

For the MD trajectory-based calibration method, the data were filtered according to a more stringent

test as under independent MD simulation (2WNL and 2BYN) the structures and corresponding C_α measurements tend to diverge, leading to the detection of too many significant measurements by the Kolmogorov–Smirnov test. Here, measurements are discarded when the $\bar{R}_{ijA}^*$ and $\bar{R}_{ijB}^*$ measurements differ by less than a specified multiple of the combined standard deviations of these sets.

To facilitate data analysis, a linear transformation is applied to each relevant bond length of a structure under investigation R_{ij}^* such that $\bar{R}_{ijA}^*$ maps to a constant κ_A and $\bar{R}_{ijB}^*$ maps to κ_B . This relevant, scaled measurement is Ξ_{ij} . The linear transform is shown in Eq. (2)

$$\Xi_{ij} = \left(R_{ij}^* - \bar{R}_{ijA}^* \right) \frac{\kappa_B - \kappa_A}{\bar{R}_{ijB}^* - \bar{R}_{ijA}^*} + \kappa_A \quad (2)$$

The set of relevant, scaled C_α measurements Ξ_{ij} were then analyzed to determine if they represent a loop in state-A or state-B. To do this a nonparametric continuous probability distribution function (CDF) is constructed describing the set Ξ_{ij} from a particular structural area (e.g., loop A). Kernel smoothing was used to construct the CDF in MATLAB so no assumption was made regarding the distribution of the set Ξ_{ij} . Taking $[\kappa_A, \kappa_B]$ symmetric about zero,

$$\Psi = \Pr(\bar{\Xi}_{ij} > 0) = 1 - \text{cdf}(\Xi_{ij}) \quad (3)$$

$\Pr(\cdot)$ is the probability and $\text{cdf}(\cdot)$ is the value of the CDF when evaluated at the argument. This probability Ψ is then the score for the particular structural area under analysis. Ψ approaches 1 for 100% probability of state A and 0 for 100% probability of state-B.

Three types of calibration were evaluated to determine which most effectively predicted the correct state of a test set of crystal structures and the states visited during an MD-trajectory. These methods are:

1. AChBP crystal structures in agonist-bound state comprise the state-A calibration set, whereas antagonist-bound and *apo* structures comprise the state-B calibration set.
2. Frames from an equilibrated MD simulation of AChBP bound to an agonist and an *apo* simulation are used to calibrate state-A and state-B, respectively.
3. A hybrid of these two methods in which the calibration set in 1 is augmented by an equal number of time averaged frames from the MD simulations in 2.

Crystal structures

Crystal structures of *Aplysia* AChBP were used to form a calibration set based on their being bound to a nAChR-agonist, nAChR-antagonist, or *apo*. If a

ligand would be expected to activate a nAChR (e.g., an agonist) we assign it to the so-called state-A set, otherwise it is classified as state-B. PDB codes 2BYQ,¹⁰ 2WNL,¹⁸ 3C79, and 3C84³³ are nAChR-agonist-bound and are used to calibrate state-A. PDB codes 2BR8,⁸ 2BYR, 2BYP,¹⁰ 2C9T,¹¹ and 2UZ6³⁴ are antagonist-bound and PDB codes 2BYN,¹⁰ and 3GUA³⁵ are *apo*; these structures were used to calibrate state-B.

The test set of crystal structures are also from *Aplysia* AChBP. These structures are not included in the calibration of the method and are used to evaluate the ability of the method to correctly predict ligand classification (e.g. agonists should be predicted as state-A). PDB codes 2PGZ,¹⁹ 2W8F, and 2W8G²⁰ have been crystallized with noncompetitive nAChR-antagonists bound. 2XYS, 2XYT,²¹ 2WZY, and 2X00⁶ contain competitive nAChR-antagonists. Therefore, we expect a successful method to classify these structures as state-B. PDB code 2W8E²⁰ and 2BR7⁸ are *apo* and we therefore expect the prediction of state-B for this structure as well. PDB codes 2WN9 and 2WNJ¹⁸ are crystallized with partial agonists and we expect these to be classified as mixtures of state-A and B by the method since in nAChR they often activate the receptor, but to a lesser extent than a full agonist. The 2WNL and 2UZ6 structures each contained two AChBP pentamers in the asymmetric unit cell and we were therefore able to include these structures in both the training and validation sets. In this case, chains designated A to E were assigned to the training set and chains F to J were included in the test set.

In addition to these structures, there are additional crystal structures containing ligands that cannot be classified because the response these ligands elicit in the nAChR remains equivocal. These structures are investigated once the optimal calibration method has been identified. PDB code 2PH9¹⁹ contains the ligand GAL, a positive allosteric modulator at low concentration (0.11 μ M) and a noncompetitive antagonist at higher concentration (>10 μ M).²³ 2WNC is bound to tropisetron, a partial agonist for $\alpha 7$ -nAChR and antagonist for non- $\alpha 7$ -nAChR.¹⁸ 2BYS is crystallized with lobeline, mentioned above.

2XZ6 and 2XZ5 are mutants of *Aplysia* AChBP where Tyr53 (in the D-loop) has been mutated to cysteine (AChBP-Y53C).²¹ 2XZ6 has been cocrystallized with MTSET. 2XZ5 has been cocrystallized with ACh and MMTS, another. Although we expect the method to predict state-B for 2XZ6 and A for 2XZ5, as the receptors have been mutated we cannot be certain if they will share the same conformational ensembles as wild-type AChBP.

MD trajectories

The 2WNL crystal structure with agonist, anabaseine,¹⁸ was equilibrated under NVT conditions for 70

ns and the *apo* crystal structure, 2BYN,¹⁰ was equilibrated for 30 ns. The coordinates used in the MD calibration method and to augment the crystal structures in the hybrid calibration method (mentioned above) were frames randomly chosen from the last 5 ns of the agonist-bound and *apo* simulations that were then time averaged over a 100 ps window to reduce thermal noise.

From the coordinates of the 2WNL simulation at 15 ns, another identical MD simulation was launched with the nAChR agonist, anabaseine, deleted from the structure. This simulation was continued to 70 ns under the same conditions as the first 2WNL simulation. The second simulation was used to test the ability of the method to detect the transition from an agonist-bound (state-A) to an *apo* (state-B) state as deletion of the agonist would be expected to elicit this transition.

To investigate the effect of lobeline on the conformation of AChBP, a pair of simulations on 2BYS was also performed under conditions identical to those described for 2WNL (one simulation with ligand for 70 ns and one simulation branching from the first where the ligand is deleted at 15 ns). MD simulations were performed using NAMD 2.7b2³⁶ on TeraGrid (and in-house) resources using the CHARMM 22 force field with CMAP corrections.^{37,38} Force fields for ligands were created by combining relevant structural elements from the force fields above. Simulations were performed using a Langevin thermostat at 310 K in an NVT ensemble simulation (although an NPT ensemble is used for initial equilibration of cell size) with a timestep of 1 fs. An 8.5 Å switching distance and 9.0 Å cutoff distance has been used for nonbonded interactions. The particle-mesh Ewald grid spacing was 1.0 Å. Each system was solvated with TIP3P water using the VMD³⁹ SOLVATE plugin and ionized to a neutral charge at physiological ionic concentration (150 mmol/L NaCl). The final system volume was ~ 750 nm³ and contained $\sim 70,000$ atoms. Each system was minimized for 500 steps with fixed C $_{\alpha}$ atoms, then for 500 steps with no fixed atoms. The system was then equilibrated in an NPT ensemble at 310 K for 1 ns before NVT production simulations.

Calibration method evaluation

The set of relevant, scaled C $_{\alpha}$ measurements Ξ_{ij} were divided into groups according to key structures for AChBP:

1. Structures containing residues from the (+)-face involved in ligand binding⁷: loops A, B, and C.
2. Structures containing residues from the (−)-face involved in ligand binding⁷: loops D, E, and F.
3. Loops that would form the interface between the ligand-binding domain and TMD in an LGIC that have been shown to be responsible for transmission

of gating motion and possibly ligand affinity¹¹. Loops $\beta 1$ – $\beta 2$, $\beta 6$ – $\beta 7$ (the cys-loop), and $\beta 8$ – $\beta 9$.

For each of the key structures above, Ξ_{ij} was filtered so that each Ξ_{ij} contained one C_α from a key structure and one C_α from elsewhere in the protein (but not any of the other key structures).

The three training options were evaluated according to the extent to which they enable the method to correctly classify the conformation of AChBP as belonging to state-A with ligands presumed to activate nAChR, or to state-B otherwise. Also, the method's ability to identify the conformational changes induced by deletion of the agonist during the MD simulation of 2WNL was examined. These cases were subdivided into three performance criteria:

1. Test crystal classification: the ability of the method to correctly classify crystal structures from the test set (not included in calibration of the models), mentioned above.
2. MD frame classification: the ability of the method to correctly classify frames from the 2BYN (state-B) and 2WNL (state-A) simulations, which were not included in calibration set.
3. Dynamic MD trajectory classification: the ability of the method to discern the conformational change resulting from the deletion of the nAChR-agonist in the 2WNL MD simulation.

Supporting Information Tables SI and SII show the scores for the test crystals evaluated by using each of the three calibration methods (performance criterion 1, above). Mean scores for nAChR agonists or partial agonists were 0.60, 0.50, 0.55 for the hybrid, MD, and crystal based calibration methods, respectively. Mean scores for nAChR antagonists or *apo* structures were 0.43, 0.46, 0.39 for the hybrid, MD, and crystal based calibration methods, respectively. The MD calibration method shows little difference in the score for state-A and state-B structures in this evaluation.

Supporting Information Tables SIII–SV show the scores for frames chosen from 2BYN (state-B) and 2WNL (state-A) simulations (performance criterion 2, above). Mean scores for the 2WNL trajectory frames were 0.90, 1.00, 0.52 for the hybrid, MD, and crystal based calibration methods, respectively. Mean scores for 2BYN trajectory frames were 0.10, 0.00, 0.43 for the hybrid, MD, and crystal based calibration methods, respectively. Both the MD and hybrid calibration methods are able to correctly classify frames from both simulations. The crystal calibration method shows only a slight difference between the agonist and *apo* trajectories.

Supporting Information Table SVI shows the scores for performance criterion three. Here, the inter- C_α distance measurements were averaged over

the last 60–70 ns of the 2WNL MD trajectories where in one case the agonist had been deleted at 15 ns, and in the other the ligand was included for the entire simulation. As the agonist is presumed to elicit state-A, if the ligand is deleted a successful model should predict a decrease in the score for the trajectory where the ligand has been deleted. The scores decrease on deletion of the ligand from 0.93 to 0.54 for the hybrid calibration method, from 0.99 to 0.69 for the MD calibration method, and 0.52 – 0.51 for the crystal-based calibration method. The crystal based calibration method is unable to resolve the change elicited by deletion of the agonist, however both the MD and hybrid calibration methods clearly have the ability to detect ligand deletion.

The hybrid calibration method was successful in each of the above tests (and is therefore used as the calibration method for the analysis), while the other calibration methods had poor performance in at least one of the tests. One possible explanation for the success of the hybrid calibration is that although the composition of the calibration set determines the transform that maps the open and closed states, it also effects what C_α measurements meet the confidence threshold for inclusion in the analysis. This dual effect means that by including MD generated and crystal structure derived coordinates, the C_α distances deemed significant by statistical analysis are more likely to contain measurements that are significant under both crystallographic and dynamic conditions.

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