We delineated acetylcholine (ACh)-dependent conformational changes in a prototype of the nicotinic receptor ligand binding domain by molecular dynamics simulation and changes in intrinsic tryptophan (Trp) fluorescence. Prolonged molecular dynamics simulation of ACh-binding protein showed that binding of ACh establishes close register of Trps from adjacent subunits, Trp143 and Trp53, and draws the peripheral C-loop inward to occlude the entrance to the binding cavity. Close register of Trp143 and Trp52 was demonstrated by ACh-mediated quenching of intrinsic Trp fluorescence, elimination of quenching by mutation of one or both Trps to Phe, and decreased lifetime of Trp fluorescence by bound ACh. Occlusion of the binding cavity by the C-loop was demonstrated by restricted access of an extrinsic quencher of binding site Trp fluorescence by ACh. The collective findings showed that ACh initially establishes close register of conserved Trps from adjacent subunits and then draws the C-loop inward to occlude the entrance to the binding cavity.

Chemical signaling throughout the organism is initiated by molecular recognition of small biomolecules by receptor binding sites. Recognition is accompanied by conformational changes of the binding site, through induced fit, selection of pre-existing conformers, or a combination of the two, and these are amplified over distance and time to produce the biological response. In the case of rapidly gated synaptic receptors, the amplification propogates within the receptor macromolecule itself and occurs within as little as tens of microseconds (1). Structural counterparts of the initial recognition step likely evolved to provide this close spatial and temporal coupling. To understand the initial recognition step in rapidly gated synaptic receptors, we examined conformational changes due to agonist occupancy in AChBP, a prototype of the nicotinic receptor ligand binding domain (2).

The nicotinic receptor belongs to the Cys loop superfamily of rapidly gated synaptic receptors that transduce agonist binding into opening of an intrinsic ion pore. Although a complete atomic structure has not been determined for any member of the superfamily, atomic resolution data recently emerged for AChBP (3, 4), a close homolog of the ligand binding domain of the α7 neuronal nicotinic receptor. AChBP has proven to be a valuable model system for clarifying aspects of ligand recognition in nicotinic receptors, including structural modeling (5–7), dynamics of receptor ligand binding domains (8), computational docking of ligands (9, 10), and direct monitoring of ligand occupancy by changes in intrinsic Trp fluorescence (11, 12).

Here we delineated ACh-dependent conformational changes in AChBP by prolonged MD simulation and compared these with conformational changes detected by steady-state and time-resolved measurements of intrinsic Trp fluorescence. MD simulation revealed asymmetric relaxation of the AChBP homopentamer without ACh bound, but maintenance of the symmetrical structure with ACh bound. Differences between the ACh-free and ACh-bound structures revealed conformational changes linked to agonist occupancy. Conformational changes predicted by simulation were verified by measurements of intrinsic Trp fluorescence, showing that binding of ACh establishes close register of two Trp side chains from opposing subunits and draws the peripheral C-loop inward to occlude the entrance of the binding cavity.

**EXPERIMENTAL PROCEDURES**

*Molecular Docking and Dynamics Simulation—* We used the AMBER 7 program (13) to conduct molecular dynamics (MD) simulation of AChBP in the presence of explicit solvent molecules at room temperature, without ACh bound or with ACh bound to one, two, or all five binding sites. Atomic coordinates of AChBP (3) were downloaded from the Protein Data Bank (Protein Data Bank code 1I9B). To prepare the protein structure for docking and MD simulation, water, ions, and HEPES were first removed from the crystal structure. Partial atomic charges were then assigned to each atom of the protein using the restrained electrostatic potential charge model of the AMBER7 program. The crystal structure of ACh was used to prepare the ligand for docking. Partial atomic charges of ACh were obtained from electrostatic potential fitted charges from the HF/6–31+ quantam mechanics calculation. The AUTODOCK 3.0.3 program (14), which uses the Lamarckian genetic algorithm, was employed for docking simulation (grid sizes were 40 × 40 × 40, and grid spacing was 0.375 Å). The simulation yielded 10 docked conformations of ACh, of which the predominant conformation was chosen as the orientation in the binding site. AChBP without ACh or with one (at the interface of subunits c and d), two (at the interfaces of subunits c and d and subunits c and a), or five sites occupied was then solvated in a water box. The TIP3P model for water molecules was used, and the box was extended at least 10 Å in each direction from the solute. Na+ and Cl− counter ions were added to neutralize the system by employing the LEAP module of the AMBER.
7 program (parm 99 force field included). The system was energy minimized, followed by a 30-ps heating step to 298 K, and the Particle Mesh Ewald method was employed to calculate long-range electrostatic interactions. After the heating step, the system was maintained at 298 K, and MD simulation out to 45 ns was computed at 2.0-fs intervals, with frames collected every 1.0 ps. During the simulation, the SHAKE algorithm was turned on to constrain bonds involving hydrogen atoms, and the non-bonded interaction cutoff was set to 8.0 Å.

Plasmids and Mutagenesis—The previously described synthetic cDNA encoding AChBP (11) was subcloned into the cytomegalovirus-based vector pRBG4, and an MF2 epitope tag was constructed in-frame with the C-terminal coding sequence (9). Point mutations were constructed using the QuikChange kit from Stratagene. The final AChBP construct and all mutations were confirmed by automated dye terminator sequencing.

Expression and Purification of AChBP and Mutants—293 HEK cells were transfected with wild-type or mutant subunit cDNAs using calcium phosphate precipitation (15). Three days after transfection, wild-type or mutant AChBP secreted into the culture medium was collected with the addition of protease inhibitors (5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.002 mM aprotinin, and 0.01 mM pepstatin A), and the protein was purified by passing the medium through a mini-column with anti-MF2 coupled to an agarose gel (Sigma-Aldrich). The purified protein was obtained by elution with the FLAG peptide (100 μg/ml; Sigma-Aldrich) in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) and further purified by fast protein liquid chromatography using a Superdex 200 column (Amersham Biosciences) with Tris buffer (20 mM Tris, 150 mM NaCl, pH 7.4), and it was finally concentrated to the micromolar range using the Centricon YM-10 (Millipore).

Tryptophan Fluorescence Measurements—Protein samples in Tris buffered saline (150 mM NaCl, 5 mM Tris, pH 7.5) were excited at 294 nm and emission spectra were recorded from 315 to 430 nm (1 cm path length cuvette). Measurement were made at 298 K.

**FIG. 1.** Molecular dynamics simulation of AChBP without and with ACh bound to the five binding sites. A, absolute value of the distance change of each α carbon atom relative to the AChBP crystal structure (3) is indicated by color, from blue (no change) to red (large change), as a function of simulation time and residue number of each subunit (α–ε). B, superposition of ribbon structures of AChBP after 45 ns of MD simulation without ACh bound (brown) and with five ACh bound (blue) (subunits c and d are shown). The mobile C-loop is highlighted in red. C, close up view of superimposed C-loops from the 45-ns MD simulation of the ACh-free structure (brown), the 45-ns simulation with five ACh bound (blue), and the crystal structure with CCh bound (green). Side chains of Cys187, Cys188, and Tyr185 are shown together with ACh and Trp143 and Trp53 in orientations obtained by simulation with five ACh bound.
ACh-mediated Conformational Changes in AChBP

RESULTS

To delineate atomic-scale conformational changes associated with molecular recognition of ACh, we conducted MD simulations of AChBP in the presence of explicit water molecules and counter ions, either with or without ACh bound to the five binding sites. We generated the ACh-bound structure by computational docking of ACh to the AChBP crystal structure (3) and obtained a highly similar orientation of ACh compared with that of carbamylcholine (CCh) in complex with AChBP (4). Simulations out to 45 ns reveal clear ACh-dependent differences in the dynamics of local structural motifs at the ACh binding site, as well as differences distributed throughout the structure (Fig. 1A). With ACh bound, the hairpin joining β strands 9 and 10, known as the C-loop, occludes the entrance to the binding cavity, whereas without ACh, the C-loop moves away, opening up the cavity (Fig. 1B). The outward movement of the C-loop shows asymmetry across both space and time; it moves in two of the five subunits during the 45-ns simulation (subunits c and e), motion occurs in non-adjacent subunits, and the two C-loops move at different times. The analysis also reveals remarkably little motion of the global structure with ACh bound, as indicated by the preponderance of blue hues associated with little change from the initial 5-fold symmetrical structure (Fig. 1A). Thus, binding of ACh maintains an overall symmetrical conformation of AChBP, but without ACh, local regions, particularly the C-loop, relax asymmetrically.

Conformations of the C-loop with and without ACh are compared with the conformation in the crystal structure of AChBP with CCh bound (Fig. 1C). After 45 ns of simulation with ACh bound, the conformation of the C-loop is similar to that in the crystal structure of the CCh-bound complex. However, without ACh, the simulation reveals a large outward displacement of the C-loop compared with the ACh-bound structure; this displacement is much larger than that suggested by comparison of C-loop conformations in the HEPES- and CCh-bound crystal structures (4). The ACh-free structure obtained by simulation shows substantial disengagement of the disulfide-bonded cysteines, Cys187 and Cys188, and the conserved Tyr185 of the C-loop from the quaternary ammonium moiety of ACh (Fig. 1C).

We looked further for global structural changes by computing radius of gyration as a function of time for each subunit relative to the central vestibule (Figs. 2, A and B). The radius of gyration, calculated as displacement in the plane perpendicular to an axis passing through the central vestibule, measures the breathing motion of AChBP. With ACh bound to all five binding sites, the subunits show only small fluctuations in radius of gyration. However, without ACh bound, the subunits that showed motion of the C-loop increase radius of gyration, whereas the intervening subunit shows a decrease. By mapping distances between a carbon atoms and the central axis on the surface of the structure, changes in radius of gyration are seen to result from movement of local regions, rather than bulk movement of the subunit (Fig. 2C). These motions include outward displacement of the C-loop of non-adjacent subunits and inward displacement of the β8-β9 linker of the intervening subunit. The map further confirms that bound ACh restrains motion of the overall structure.

FIG. 2. Change in radius of gyration of each subunit of AChBP after MD simulation. A, radius of gyration (\(R_g = (\sum m_i r_i^2/\sum m_i)^{1/2}\), where \(m_i\) is mass of the atom, and \(r_i\) is the distance of the atom from the central axis) of each subunit from \(a\) to \(e\) is plotted against simulation time without ACh bound. \(B\), radius of gyration of each subunit is plotted against simulation time with five ACh bound. C, changes in displacement of each \(\alpha\) carbon atom from the central axis, relative to the crystal structure (3), are mapped on a surface representation of the opened up AChBP pentamer. Results from the last 5 ns of the 45-ns simulation are averaged and color-coded with blue for inward and red for outward displacement.

buffer (pH 7.4) were stored on ice and placed in a 3 x 3-mm cuvette for fluorescence measurements. All measurements were performed at 4 °C. Steady-state fluorescence was measured on a PTI fluorescence spectrometer (PTI, London, Ontario, Canada). Samples were excited at 298 ± 2 nm, and the emission spectrum was measured with 4-nm bandwidth resolution at wavelengths between 310 and 370 nm. The fluorescence intensity collected at 342 nm was used for analysis. For ligand titration experiments, the effects of dilution were corrected by dividing the steady-state fluorescence intensity decay were fitted by convoluting the observed lamp flash profile with an exponential fitting function of the form, \(F(t) = \sum A_i \exp(-t/\tau_i)\), where \(t\) is time, \(A_i\) is the decay amplitude, and \(\tau_i\) is the lifetime for the \(i^{th}\) component (16). Best fits were located using a nonlinear least squares protocol. We verified instrument calibration using \(N\)-acetyltryptophanamide, which has a 3-ns lifetime, in 0.1 M sodium phosphate at pH 7 and 20 °C (17). The observed \(N\)-acetyltryptophanamide lifetime was 2.95 ± 0.3 ns.

over a 32-nm bandwidth centered at 335 nm. The fluorescence intensity
To examine how conformation of the C-loop depends on the extent of ACh occupancy, we carried out simulations with either one or two binding sites occupied by ACh (Fig. 3A). With one binding site occupied, the C-loops change conformation as if no ACh is bound; C-loops in two non-adjacent subunits again move outward at different times. Inward motion of the \( \beta8-\beta9 \) linker in subunit a is also seen, analogous to but somewhat greater than observed in subunit d in the ACh-free simulation (Fig. 1A). With two binding sites occupied, motion of the C-loop is highly restricted in all five subunits, similar to occupancy of all five binding sites. Thus, the restriction of C-loop motion by ACh shows allostery because occupancy of one binding site is not sufficient to restrict motion at that site, but rather occupancy of two physically separate binding sites is required. The structural changes accompanying this allosteric effect include outward motion of the C-loop (subunits c and e), inward motion of the \( \beta8-\beta9 \) linker (subunit d), and smaller motions distributed throughout the subunits (Figs. 1–3).

We sought to confirm the allosteric effect by comparing AChBP crystal structures (4) with one or two molecules of CCh bound (Protein Data Bank code 1UV6). In the AChBP pentamer with two molecules of CCh bound, all five C-loops are in the inward conformation shown in Fig. 1C. Although not mentioned by Celie et al. (4), in the complex with one CCh bound, one of the C-loops extends outward, away from the binding cleft, similar to our ACh-free structure after 45 ns of simulation (Fig. 1C), whereas the other C-loops are in the inward conformation of the HEPES- or CCh-bound structures. Thus, our MD simulations independently confirm the allostery suggested by crystal structures with one and two bound agonists. We then superimposed crystal structures of the subunit with the C-loop in the outward conformation and a subunit containing bound ACh-mediated Conformational Changes in AChBP

**Fig. 3. MD simulation of AChBP with one and two ACh bound.** A, absolute value of the distance change of each \( \alpha \) carbon atom relative to the AChBP crystal structure is indicated by color, from blue (no change) to red (large change), as a function of simulation time and residue number of each subunit (a–e). B, superposition of ribbon structures of subunit e from 45-ns simulation with one ACh bound (brown) and subunit c with two ACh bound (blue). C, superposition of \( \alpha \) carbons of the subunit with an "opened up" C-loop in the crystal structure with one CCh bound (brown) and a subunit from the crystal structure with two CCh bound (blue). Arrows indicate range of motion of the C-loop.
CCh (Fig. 3C) and compared that with superimposed ACh-free and ACh-bound structures obtained by simulation (Fig. 3B). The comparison shows a similar range of motion of the C-loop between the crystal structures and our simulated structures. Thus, simulation with one and two molecules of ACh bound reveals allosteric conformational changes of the C-loop that mirror those in the corresponding crystal structures.

Because little structural change is seen elsewhere in the subunit, the allosteric effect due to agonist occupancy appears to originate with conformational change of the C-loop. New contacts formed when the C-loop tilts inward include aromatic-quaternary and van der Waals contacts with the agonist, as well as hydrogen bonding between Ser186 of the C-loop and Glu163 of the β8-β9 linker of the adjacent subunit. More widespread conformational changes are expected to accompany the allosteric effects due to agonist occupancy, requiring even more prolonged MD simulation and analyses of the trajectory.

We next focused on the aromatic-rich ACh binding cavity and examined dynamics of two conserved Trp residues, Trp143 and Trp53. These Trps are located deep in the binding cavity and project toward each other from adjacent subunits (Fig. 4A). Analogous Trps are found at binding sites of every subtype and species of nicotinic receptor, indicating they are essential for binding ACh. The distance between closest carbon atoms on the indole rings of Trp143 and Trp53 remains around 3.3 Å throughout the simulations of both ACh-free and ACh-bound structures (data not shown). However, the angle defined by the planes of the two indole rings fluctuates considerably without ACh and becomes fixed at around 90° with ACh bound (Fig. 4, B and C). This edge-to-face stacking is observed for many Trp-Trp pairs in structures deposited in the Protein Data Bank (17). Thus, binding of ACh maintains close register of these two principal components of the binding site, but without ACh, conformational mobility of the indole side chains increases.

To look for experimental counterparts of the conformational changes observed by MD simulation, we measured intrinsic Trp fluorescence of AChBP in solution, with and without ACh. The initial studies asked whether tryptophans Trp143 and Trp53, from opposing subunits, are responsible for ACh-dependent quenching of intrinsic Trp fluorescence (11). Thus, we generated pure AChBP, the mutants W53F and W143F, and the corresponding double mutant and measured steady-state fluorescence as a function of ACh concentration. ACh quenches about 35% of the total Trp fluorescence of wild-type AChBP, with the concentration dependence of quenching well described by occupancy of a single binding site (Fig. 5). However, ACh fails to quench intrinsic Trp fluorescence when Phe is substituted for either Trp53 or Trp143 but rather enhances fluorescence about 10–20% in a concentration-dependent manner (Fig. 5). When Phe is substituted for both Trp53 and Trp143, ACh neither quenches nor enhances fluorescence. Thus, ACh-mediated quenching of Trp fluorescence originates from interaction between Trp143 and Trp53.

**FIG. 5. Steady-state fluorescence change after addition of ACh to wild-type AChBP and the mutants W53F and W143F.** All fluorescence intensities are corrected for dilution due to addition of ACh. Intensity change is normalized according to the following formula: $F = (F_{\text{ACh}} - F_0)(F_{\text{max}} - F_0)$, in which $F_{\text{ACh}}$ is the observed fluorescence intensity for a given concentration of ACh, $F_0$ is fluorescence before addition of ACh, and $F_{\text{max}}$ is the asymptotic fluorescence intensity when the concentration of ACh approaches infinity.
Time-resolved fluorescence is a highly sensitive indicator of protein conformational change involving a fluorophore and quencher, such as the pair of intrinsic binding site Trps in AChBP. Quenching results from changes in electronic configurations of ground or excited states of the interacting Trps, and these can be distinguished with changes in fluorescence amplitude or lifetime. Perturbation of the ground state alters the amplitude of fluorescence relaxation, whereas perturbation of the excited state alters the decay rate (18). Measurements of time-resolved fluorescence reveal that binding of ACh to wild-type AChBP shortens Trp lifetime, but lifetime is unaltered when Phe is substituted for either Trp53 or Trp143 (Fig. 6, Table I). In all cases, a single relaxation is observed, even though three additional Trps remote from the binding site are present; fluorescence decay of the non-binding site Trps cannot be distinguished as distinct components and likely falls into the 2–3-ns range observed for the overall decay. Thus, in wild-type AChBP, ACh promotes interaction between Trp53 and Trp143 that quenches fluorescence through an excited state mechanism.

Among possible excited state quenching mechanisms, proton transfer is least likely because it requires precise alignment and van der Waals contact of the amide groups of the two Trps. Electron transfer, on the other hand, is the most likely mechanism because it occurs through space and is sensitive to separation distance and orientation of the interacting fluorophores (19, 20). The basic conditions for observing electron transfer, as well as for a change in the rate of transfer on binding ACh, are accommodated by our MD simulation of AChBP; the two indole rings are highly mobile in the unbound state, but they maintain close register with ACh bound (Fig. 4).

For both Phe mutants, ACh enhances fluorescence in a concentration-dependent manner but causes negligible change in fluorescence lifetime. These observations suggest that when ACh binds to each mutant AChBP, a conformational change occurs that affects ground-state electronic configurations of Trp143 and Trp53, enhancing fluorescence. This conformational change is likely analogous to that which produces fluorescence quenching when both Trp143 and Trp53 are present. However, in the Phe mutants, electron transfer quenching of Trp fluorescence is not observed because of disparate energies of Phe and Trp excited states.

MD simulation reveals that (a) ACh maintains close register of the Trp pair deep in the binding cleft and (b) ACh draws the C-loop inward to occlude the entrance to the binding cavity. To obtain experimental evidence for conformational change of the C-loop, we used the pair of binding site Trps as reporters of binding cavity occlusion, and we measured Trp fluorescence in the presence of the Stern-Volmer collisional quencher acrylamide (21). Without ACh, acrylamide readily quenches Trp fluorescence of AChBP, as expected from accessibility of Trp143 and Trp53 when the C-loop is in its outward conformation (Fig. 7A). However, when ACh is bound, quenching is strongly attenuated.

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**TABLE I**

|                  | Lifetime* | Amplitude | χ² | Steady-state intensity |
|------------------|-----------|-----------|----|------------------------|
| WT apo           | 3.1       | 1         | 2.0| 1                      |
| WT + ACh         | 2.3       | 0.97      | 1.2| 0.72                   |
| W53F apo         | 3.2       | 1         | 1.4| 1                      |
| W53F + ACh       | 3.3       | 1.16      | 1.4| 1.2                    |
| W143F apo        | 2.8       | 1         | 1.7| 1                      |
| W143F + ACh      | 2.6       | 1.22      | 1.9| 1.09                   |

* After removal of a small scattering component present in all the curves, tryptophan fluorescence decay was fitted with a single exponential function giving the favorable reduced χ² indicated. Lifetime error is ±0.1 ns. Apo isoform decay amplitudes and steady-state intensities (amplitude × lifetime) are normalized to unity. Amplitude error is ±0.05. Steady-state intensity changes upon ACh binding compare favorably with those observed directly. WT, wild-type; apo, unbound.
ated, as expected from restricted access due to inward movement of the C-loop.

Our interpretation that the C-loop occludes the binding cavity when ACh is bound is clear-cut if quenching of binding site Trps is partial. In that case, fluorescence of the binding site Trps contributes to the overall fluorescence signal but does not change across acrylamide concentrations, whereas the fluorescence of the three non-binding site Trps is quenched by acrylamide. The net result is a smaller slope of the Stern-Volmer plot in the presence of ACh compared with its absence.

To eliminate uncertainty in the extent to which ACh quenches fluorescence of binding site Trps, we examined collisional quenching of the W53F and W143F mutants, in which ACh enhances rather than quenches fluorescence. Both mutants show similar ACh affinities to wild-type AChBP (Fig. 5), suggesting similar conformational changes upon agonist binding. For W53F without ACh, acrylamide readily quenches Trp fluorescence, whereas with ACh, quenching is attenuated (Fig. 7B); the corresponding quenching constants are very close to those observed for wild-type AChBP. Thus, for the W53F mutant in the presence of ACh, the clear explanation for attenuated collisional quenching is restricted access of the remaining binding site Trp$^{143}$.

Analogously, ACh attenuates collisional quenching in the W143F mutant (Fig. 7C), although the attenuation is not as large as that observed for wild-type or W53F. Differences in fluorescence intensities of the individual Trps may be responsible for quantitative differences in collisional quenching constants. However, in the presence of ACh, the steeper slope in the Stern-Volmer plot may result from incomplete closure of the C-loop or the more peripheral location of the remaining Trp$^{53}$. Reduced closure of the C-loop is likely because substitution of Phe at Trp$^{143}$ should impair coordination of ACh, which is necessary to attract the electron-rich aromatic cloud of Tyr$^{185}$ located on the C-loop (4).

Finally, results from the double mutant W53F/W143F further support our interpretation that the C-loop occludes the binding cavity when ACh is bound. No difference is observed in the ability of acrylamide to quench fluorescence in the presence or absence of ACh (Fig. 7D), as expected because both binding site Trps are replaced by Phe. The corresponding slope of the Stern-Volmer plot is 1.7, which is greater than the slope of 1.2 observed for wild-type AChBP in the presence of ACh; the slope for wild-type AChBP is lower because the binding site Trps are not fully quenched and are less accessible to the collisional quencher than the Trps outside the binding site. The same reasoning applies for the W53F mutant in the presence of ACh, which shows a slope of 1.3, indicating occlusion of the binding cavity by the C-loop. Finally, the greater slope of 1.9 for the W143F mutant in the presence of ACh indicates that the remaining Trp$^{53}$ in the binding site is only partially sequestered from solvent and contributes to collisional quenching by acrylamide. Thus, our results from MD simulation and fluorescence quenching by acrylamide demonstrate an ACh-mediated con-
formational change of the C-loop that occludes the entrance of the binding cavity.

**DISCUSSION**

We delineated ACh-mediated conformational changes in a prototype of the nicotinic receptor ligand binding domain by correlating MD simulation of AChBP with measurements of intrinsic tryptophan fluorescence. The findings extend the insights from crystal structures by examining conformational dynamics of AChBP in solution. The collective results provide a picture of the initial interaction of ACh with the binding site to form a stable complex. Initially, ACh entered the site and brought into close register conserved Trp residues from adjacent subunits. Upon stabilization of the Trp pair by ACh, the peripheral C-loop tilted inward to occlude the entrance to the binding cavity, enclosing the agonist. Residue side chains in the C-loop provided additional stabilization of ACh and included Cys137, Cys188, Tyr185, and Tyr192. This motion of the C-loop was revealed in reverse chronology by MD simulation of AChBP without ACh bound and is allosteric because motion was restricted by ACh occupancy of not one but two physically separate binding sites. Thus, the initial binding of ACh led to synchronous conformational changes between subunits as well as within a single subunit.

With the C-loop in the open conformation, ACh diffused with little hindrance into the aromatic-rich binding cleft. Once ACh was present, Trp143 and Trp52 from opposing subunits were brought into close register. This close register was revealed by shortening of Trp lifetime by ACh and could correspond to a shortening of the inter-Trp distance, a change in relative orientations of the indole side chains, or both. The shortening of Trp lifetime does not distinguish which of these takes place, only that a change in conformation of Trp143 and Trp52 has occurred. However, MD simulation showed increased mobility of the indole side chains in the absence of ACh and restricted mobility when ACh was bound, suggesting the shorter lifetime corresponds to a change from a mobile to a fixed, perpendicular orientation of the two Trps. The orientation of the two Trps in the ACh-bound simulation was virtually identical to those in the CCh- and nicotine-bound crystal structures of AChBP (4), suggesting a change in orientation is the root of the Trp lifetime change when ACh is bound.

Nevertheless, the possibility remains that a change in inter-Trp distance caused the decrease in Trp fluorescence lifetime. In such a scenario, the increased mobility of indole side chains detected by MD simulation may be the precursor to separation of the Trp pair as the structure relaxes. Longer simulation times are needed to look for a possible increase in inter-Trp distance that could explain changes in Trp fluorescence lifetime.

Once ACh established close register of the inter-subunit Trp pair, Trp143 and Trp52, the C-loop tilted inward, occluding the entrance to the binding cavity. Alignment of the Trp pair appeared to precede inward tilt of the C-loop because the Trps were already fully aligned in a subunit in which the C-loop was in the open conformation in the crystal structure with one CCh bound (Fig. 3C). Using fluorescence of the Trp pair as a reporter of binding site accessibility, we demonstrated inward motion of the C-loop by the impaired ability of acrylamide to quench fluorescence when ACh was bound. Occlusion of the binding cavity by the C-loop was further revealed by the simulated conformation of AChBP with ACh bound (Figs. 1–3) and confirmed by the conformation of the C-loop in the crystal structure with CCh bound (4).

Our simulation revealed a much larger conformational change of the C-loop than suggested by the difference in conformation between the HEPES- and CCh-bound structures of AChBP (Fig. 1C). In the simulation without ACh bound, the outward relaxation of the C-loop reached an apparent steady state; this conformation may represent a stable intermediate because it is similar to the open conformation of the C-loop in one subunit in the crystal structure of AChBP with one bound CCh (Fig. 3, B and C). Similarly, in the simulation with ACh bound, the inward contraction of the C-loop was nearly complete because it approached the conformation found in the crystal structure of AChBP with CCh bound (Fig. 1C). The greater conformational range of the C-loop detected here explains not only how agonists and antagonists can penetrate into the binding pocket, but also how they are stabilized in the bound complex.

The simulations also revealed the remarkable ability of bound ACh to maintain the 5-fold symmetrical structure of AChBP throughout a prolonged MD simulation. On the other hand, without ACh bound, AChBP relaxed asymmetrically, showing conformational change of two of the five C-loops. The asymmetric relaxation of AChBP is analogous to the asymmetric relaxation observed from MD simulation of a structural model of the α7 receptor ligand binding domain (8). One difference, however, was absence of an outward tilt of the C-loop over the 10-ns simulation of the α7 binding domain. α7 is 24% homologous to AChBP and likely changes conformation with a different time course; 10 ns may not be long enough to capture the expected relaxation of the C-loop in α7. Similarly, in AChBP, longer simulations are required to observe relaxation of all five C-loops, which is expected because the stoichiometry of ACh binding is five per pentameric unit (11).

Both our MD simulations and Trp fluorescence measurements provided strong evidence that the conformations of the C-loops differ between unliganded and fully liganded forms of AChBP; two or more C-loops were in the open conformation in the unliganded state, whereas most (if not all) were in the closed conformation in the fully liganded state. Furthermore, we observed allosteric by MD simulation: with one ACh bound, full outward relaxation of the C-loop was observed; but with two ACh bound, outward motion of the C-loop was prevented. Comparison of simulations with one versus two ACh bound indicated allosteric because the second ACh enabled the first to prevent motion of the C-loop. However, unlike the difference in C-loop conformation between unliganded and fully liganded forms, the allosterity we detected on the nanosecond time scale does not have an experimental counterpart. Potentially, the allosterity we detected on the nanosecond time scale would not be present at microsecond or millisecond times closer to physiologic time scales; two bound ACh might merely slow the rate at which the C-loops relax compared with the rate with one ACh bound. We therefore inspected crystal structures of AChBP with either one or two molecules of CCh bound and found a similar conformational range of the C-loop between the two structures (Figs. 3C). Because the crystal structures likely represent stable conformations, the similarity between simulation and crystal structure conformations of the C-loop suggests the allosterity we observed is a true phenomenon.

Conformational change of the C-loop likely initiates allosteric communication of agonist occupancy between the binding sites. Communication between the binding sites, by necessity, traverses the subunit interface, and this is likely mediated by new contacts that bridge the interface. These include hydrogen bonds between side chains in the C-loop and the opposing β8-β9 linker, as well as contacts among the agonist, the C-loop, and the Trp pair from adjacent subunits.

Although AChBP does not contain an ion channel, the conformational changes described here are likely relevant to conformational changes mediated by agonist in the receptor-coupled channel. When AChBP is linked to an ion channel and
engineered for compatibility at the junction between binding and channel domains, ACh triggers opening and closing of the channel (22).

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