

# Solution NMR of Acetylcholine Binding Protein Reveals Agonist-Mediated Conformational Change of the C-Loop

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## ABSTRACT

Previous X-ray crystallography, molecular dynamics simulation, fluorescence spectroscopy, and deuterium-hydrogen exchange of acetylcholine binding protein (AChBP) suggest that after binding of the agonist, the C-loop at the periphery of the binding site draws inward to cap the site and envelop the agonist. In this study, we use high-resolution solution NMR to monitor changes in the chemical environment of the C-loop without and with acetylcholine (ACh) bound. Substitution of [<sup>15</sup>N]cysteine for the native cysteines 123, 136, 187, and 188 provided intrinsic monitors of the chemical environments of the Cys- and C-loops, respectively. Two-dimensional transverse relaxation-optimized spectroscopy <sup>15</sup>N-<sup>1</sup>H HSQC spectroscopy of apo-AChBP revealed seven well resolved cross-peaks for the group of cysteines. The spectrum of AChBP with Ser

substituted for Cys 187 and 188 shows only two main cross-peaks, corresponding to Cys 123 and 136 from the Cys-loop, enabling resonance assignments. After binding of ACh, the five cross-peaks associated with cysteines from the C-loop condense into two predominant cross-peaks not observed in the spectrum from the apo protein, indicating a restricted range of conformations and change in chemical environment of the C-loop. The results show that isotopic cysteine can be incorporated into specified positions of AChBP expressed from a eukaryotic source, that the C-loop assumes multiple conformations without ACh, but that its conformation becomes restricted with ACh bound. The collective findings suggest a structural mechanism for agonist recognition in AChBP and related Cys-loop receptors.

Recognition of a neurotransmitter by its receptor is the first step in generating the postsynaptic response. Conformational changes associated with neurotransmitter recognition are expected in theory, but for rapidly activated synaptic receptors, they remain to be characterized experimentally. The crystal structure of AChBP (Brejc et al., 2001; Celie et al., 2004; Hansen et al., 2005) has emerged as a prototype for understanding structures of ligand binding domains from the Cys-loop superfamily of neurotransmitter receptors (Sine, 2002; Sixma and Smit, 2003). AChBP shows sequence homology to all members of the Cys-loop receptor superfamily, and its three-dimensional structure closely fits that of the extracellular ligand binding region of the acetylcholine receptor

from the *Torpedo marmorata* electroplaque (Unwin et al., 2002; Unwin, 2005). Furthermore, the pharmacology of AChBP is typical of a cholinergic receptor, binding small agonists and antagonists (Smit et al., 2001; Hansen et al., 2002),  $\alpha$ -conotoxins (Hansen et al., 2005; Celie et al., 2005), and  $\alpha$ -neurotoxins (Bourne et al., 2005). AChBP may therefore serve as a good model for investigating conformational changes associated with agonist recognition in Cys-loop receptors.

Detecting conformational changes presents special challenges for a protein the size of AChBP, which contains five identical 28-kDa subunits. Fluorescence spectroscopy provides high sensitivity and single-residue precision and has been used to monitor ligand-mediated changes in intrinsic Trp fluorescence of AChBP (Hansen et al., 2002; Gao et al., 2005) and in fluorophore-conjugated AChBP (Hibbs et al., 2004). However fluorescence studies require the fluorophore to be structurally compatible at the site of incorporation; for extrinsic fluorophores, the native structure is altered. Solu-

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**ABBREVIATIONS:** AChBP, acetylcholine binding protein; ACh, acetylcholine; TROSY, transverse relaxation-optimized spectroscopy; HSQC, heteronuclear single quantum correlation; HEK, human embryonic kidney; FPLC, fast-performance liquid chromatography.

tion NMR, on the other hand, maintains the native structure, and use of multiple dimensions allows resolution of resonances associated with any residue. Furthermore, the rapid signal decay inherent in large proteins can be overcome by the transverse relaxation-optimized spectroscopy (TROSY) technique, which selects the most slowly decaying resonances (Pervushin, 2000; Fiaux et al., 2002; Riek et al., 2002).

Ligand-dependent conformational changes in AChBP have been inferred from crystal structures with bound agonists or peptide toxins. With either nicotine or carbamylcholine bound to AChBP, the C-loop at the periphery of the binding site is situated closer to the aromatic-rich center compared with the structure without agonist (Celie et al., 2004). However, the agonist-free structure contains HEPES in the binding site, which may serve as a ligand and promote a partial change in conformation, thus obscuring the true apo protein structure (Hansen et al., 2005). With either  $\alpha$ -conotoxins (Celie et al., 2005; Hansen et al., 2005) or  $\alpha$ -neurotoxin (Bourne et al., 2005) bound, the peripheral C-loop is displaced away from the binding site center, suggesting conformational mobility of the C-loop.

Starting with the HEPES-bound structure of AChBP, molecular dynamics simulation also reveals a large outward displacement of the C-loop, which is prevented when ACh is bound (Gao et al., 2005). This uncapped conformation of the C-loop is similar to that in the 4-Å resolution structure of the *T. marmorata* receptor (Unwin, 2005), to that from molecular dynamics simulation of a homology model of the homomeric  $\alpha 7$  receptor (Law et al., 2005), and to that in the crystal structure of the apo form of an AChBP ortholog from *Aplysia californica* (Hansen et al., 2005). Fluorescence of AChBP with acrylodan conjugated at Gln 178 at the base of the C-loop reveals a ligand-mediated change in environment from hydrophobic to hydrophilic, suggesting conformational change in this region (Hibbs et al., 2004). Finally, when ACh is bound to AChBP, a pair of essential Trp residues deep in the binding pocket shows reduced accessibility to an extrinsic quencher of Trp fluorescence (Gao et al., 2005), and five of the amide hydrogens in the C-loop show reduced exchange with solvent (Shi et al., 2006). Thus, the collective studies provide indirect evidence that the C-loop changes from uncapped to capped conformations upon binding the agonist. Herein, we used two-dimensional TROSY  $^{15}\text{N}$ - $^1\text{H}$  HSQC spectroscopy to directly monitor chemical environments of the C-loop with and without ACh bound.

## Materials and Methods

**Expression, Isotopic Labeling, and Purification of AChBP from HEK 293 Cells.** The previously described stable cell line expressing AChBP from *Lymnaea stagnalis* was used for large-scale protein expression (Hansen et al., 2002). It was generated from HEK 293 cells transfected with the expression vector CMV-9 (Sigma-Aldrich, St. Louis, MO) containing the AChBP coding sequence and an N-terminal 3 $\times$  FLAG tag sequence, followed by selection with G418 (Geneticin; Invitrogen, Carlsbad, CA). A cDNA encoding AChBP with the double mutation C187S and C188S was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), confirmed by dideoxy sequencing, and stably incorporated into HEK 293 cells in the same manner.

To incorporate  $^{15}\text{N}$ -labeled L-cysteine at selective sites in AChBP, cells from the stable cell line were maintained at 37°C in DMEM (Invitrogen) supplemented with fetal bovine serum [10% (v/v); Hy-

clone, Logan, UT] until 70% confluence was achieved. The medium was then replaced with Dulbecco's modified Eagle's medium lacking L-cysteine and L-methionine (Invitrogen) and supplemented with  $^{15}\text{N}$ -labeled L-cysteine (50 mg/l; Cambridge Isotopes, Andover, MA), unlabeled L-methionine (30 mg/l; Sigma-Aldrich), and dialyzed FBS [10% (v/v); Hyclone]. After incubation at 31°C for 6 days, the culture medium was collected, centrifuged, and passed through a column containing agarose-coupled anti-FLAG M2 antibody (Sigma-Aldrich). The protein was eluted in Tris-buffered saline (50 mM Tris HCl and 150 mM NaCl, pH 7.4) containing the 3 $\times$  FLAG peptide and further purified by FPLC size-exclusion chromatography (Superdex 200; Pharmacia, London, UK).

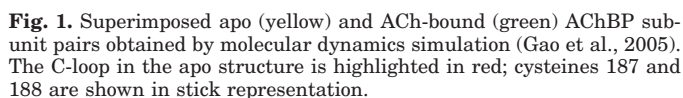
**NMR Spectroscopy of AChBP.** AChBP was concentrated to 10 mg/ml in a volume of approximately 300  $\mu\text{l}$  containing 50 mM Tris HCl, 150 mM NaCl, and 5% (v/v)  $\text{D}_2\text{O}$ . All NMR experiments were run at the National Magnetic Resonance Facility at Madison (NMR-FAM; Madison, WI), on a Varian Inova spectrometer operating at 800 MHz and equipped with a cryogenic probe. The sample was placed in a Shigemi sample tube, and two-dimensional spectra were recorded at 31°C using a TROSY- $^{15}\text{N}$ - $^1\text{H}$  heteronuclear single quantum correlation (HSQC) pulse sequence (Kay et al., 1992; Weigelt, 1998) from the Varian BioPack library of sequences and optimized at NMR-FAM for use with the cryogenic probe. By and large, 256 to 512 scans were averaged for each of the 72 complex points collected in the indirect  $^{15}\text{N}$  dimension, resulting in a total acquisition time of 12 to 24 h. The proton carrier frequency was set at the water resonance, whereas the nitrogen carrier was set at 127 ppm, and the  $^{15}\text{N}$  spectral window narrowed to 22 ppm to optimize the resolution with the least number of points. The spectra were processed and analyzed using nmrPipe/nmrDraw software (Delaglio et al., 1995).

**Tryptophan Fluorescence Measurements.** Wild-type or mutant AChBP in 50 mM Tris HCl and 150 mM NaCl, pH 7.4, was stored on ice and placed in a 3  $\times$  3 mm cuvette in a fluorescence spectrometer (Photon Technology International, Birmingham, NJ) and maintained at 4°C as described previously (Gao et al., 2005). Steady-state fluorescence was measured using an excitation wavelength of 298  $\pm$  2 nm and an emission wavelength of 342 nm. The normalized fluorescence intensity change ( $F$ ) was plotted as a function of ACh concentration.

## Results

Molecular dynamics simulation of AChBP revealed a progressive opening of the binding pocket in which the C-loop that caps the pocket in the HEPES-, nicotine-, and carbamylcholine-bound structures (Celie et al., 2004) moves outward to uncapped the pocket (Gao et al., 2005). The tip of the C-loop harbors a pair of disulfide-linked cysteines, 187 and 188, that are expected to encounter substantially different environments between uncapped and capped conformations (Fig. 1). Only two other cysteines are present in each subunit, at positions 123 and 136, but these localize to the Cys-loop remote from the binding pocket. We therefore reasoned that selective labeling of AChBP with [ $^{15}\text{N}$ ]L-cysteine should produce NMR spectra with a small number of cross-peaks and that a subset of these should change chemical shift after binding of the agonist.

A two-dimensional  $^{15}\text{N}$ - $^1\text{H}$  TROSY HSQC spectrum from apo AChBP labeled with [ $^{15}\text{N}$ ]L-cysteine was recorded for 12 h at 31°C using an 800-MHz spectrometer (Fig. 2). The protein was stable under the temperature and buffer conditions employed, as revealed by FPLC size-exclusion chromatography, which showed a single mono-disperse peak that was superimposable before and after recording multiple spectra (Fig. 3). The apo form of AChBP exhibited seven strong



Calculated  $^{15}\text{N}$  chemical shifts for cysteines in AChBP

The program PROSHIFT (Meiler, 2003) was used to compute  $^{15}\text{N}$  chemical shifts based on the AChBP crystal structure (PDB code 1I9B).

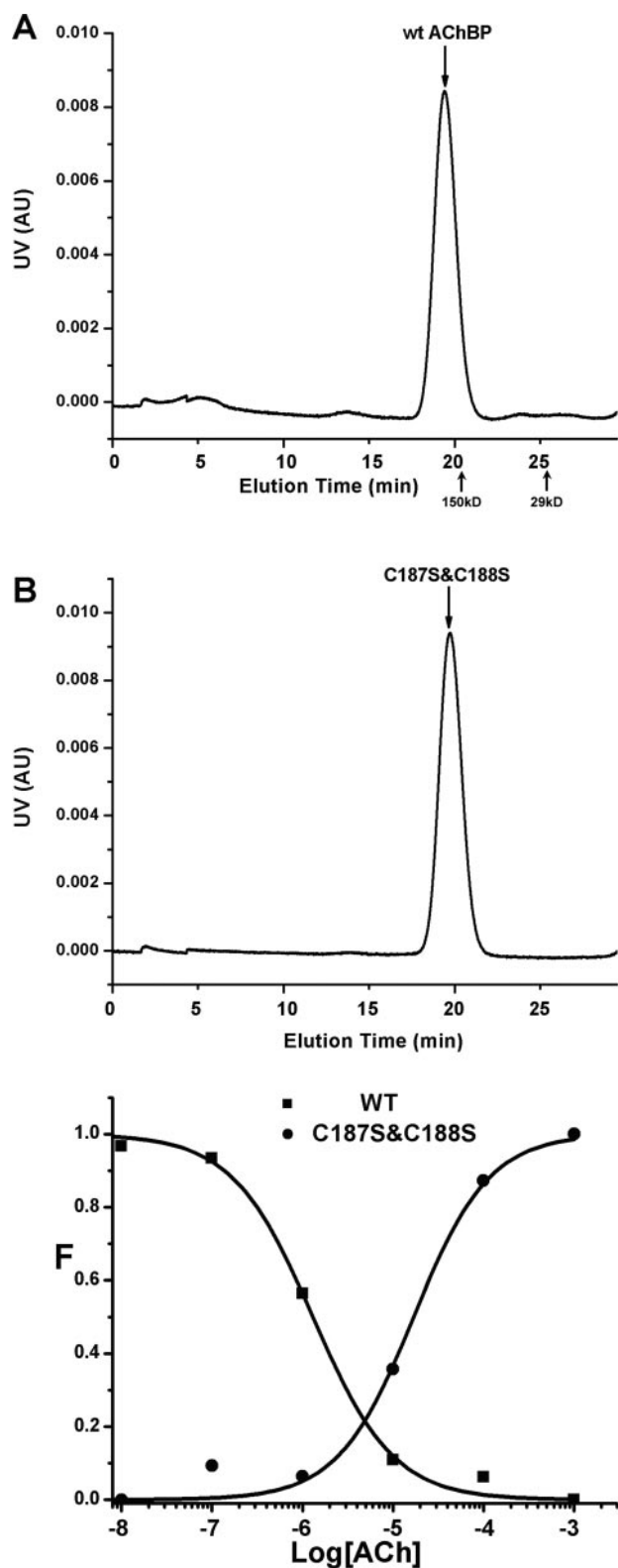
Residue Number	Subunit A	Subunit B	Subunit C	Subunit D	Subunit E	Average
				<i>ppm</i>		
Cys-123	121.46	120.76	120.06	120.38	120.07	120.55
Cys-136	123.72	124.61	123.85	124.26	124.66	124.22
Cys-187	116.43	115.92	116.61	115.90	116.52	116.28
Cys-188	115.84	115.73	115.82	115.63	115.47	115.70

To distinguish the cross-peaks corresponding to cysteines in the C- and Cys-loops, we generated isotopically labeled AChBP in which Ser was substituted for cysteines 187 and 188 in the C-loop. FPLC size exclusion chromatography revealed identical elution profiles for the mutant and wild-type proteins (Fig. 3), and the elution profile of the mutant was unchanged after acquisition of the spectra, as observed for wild-type AChBP. Moreover, measurements of intrinsic Trp fluorescence showed that the mutant protein retained the ability to bind ACh, although with lower affinity. Fluorescence of the mutant protein unexpectedly increased upon binding ACh, whereas fluorescence of the wild type protein decreased (Fig. 3).

The 2D TROSY  $^{15}\text{N}$ - $^1\text{H}$  HSQC spectrum of the mutant AChBP showed two main cross-peaks. One of these fell within the spectral region displayed in Fig. 4 (left), whereas the other was off scale and corresponded to the solitary peak centered at 135.3 ppm ( $^{15}\text{N}$ ) and 7.7 ppm ( $^1\text{H}$ ) of the wild-type AChBP spectrum in Fig. 2. Computation of  $^{15}\text{N}$  chemical shifts based on the AChBP crystal structure predicts an 8- to 9-ppm chemical shift of the  $^{15}\text{N}$  resonance of Cys-136 from the Cys-loop relative to resonances of cysteines in the C-loop (Table 1), in qualitative agreement with the spectrum in Fig. 2. Thus, the two cross-peaks in the mutant spectrum correspond to cysteines 123 and 136 of the Cys-loop. Moreover, these two cross-peaks coincide with two cross-peaks observed in the wild-type spectrum (Fig. 4), enabling assignment of resonances corresponding to cysteines in the C-loop of wild-type AChBP. The presence of only two cross-peaks in the spectrum from the mutant AChBP shows that the five cross-peaks assigned to the C-loop in wild-type AChBP did not result from metabolic scrambling of the cysteine.

After addition of ACh to wild-type AChBP, cross-peaks in the 2D TROSY  $^{15}\text{N}$ - $^1\text{H}$  HSQC spectrum changed markedly (Fig. 4). The five cross-peaks assigned to the C-loop in the apo-AChBP spectrum condensed into two sharp cross-peaks with strong intensity and a third cross-peak with weak intensity; chemical shifts of these resonances were distinct from any observed in the apo-AChBP spectrum (Fig. 4, compare two upper panels). Two additional cross-peaks were observed, but these coincided precisely with cross-peaks observed in the mutant spectrum after addition of ACh (Fig. 4,





**Fig. 3.** FPLC of wild-type and mutant AChBP and fluorescence quenching or enhancement by ACh. A and B show size-exclusion chromatography (0.7 ml/min) of wild-type and mutant AChBP after acquisition of NMR spectra. Bottom, change in steady-state fluorescence intensity of wild-type and mutant AChBP after titration of ACh, normalized according to  $F = (f_{\text{ACh}} - f_0)/(f_{\text{max}} - f_0)$ , where  $f_{\text{ACh}}$  is the intensity at a given concentration of ACh,  $f_0$  is the intensity before addition of ACh, and  $f_{\text{max}}$  is the asymptotic intensity as the concentration of ACh approaches a maximum. For wild-type AChBP,  $f_{\text{max}}/f_0$  was 0.65 (Gao et al., 2005) and that for the mutant was 1.3.

right, compare upper and lower panels), and thus correspond to cysteines in the Cys-loop.

The spectral changes with ACh bound provide direct evidence that the apex of the C-loop of AChBP changed chemical environment in response to binding the agonist. In the apo-AChBP spectrum, the multiple cross-peaks show that the C-loop experiences a variety of distinct environments, perhaps as a result of several interchangeable conformations. However, in the ACh-bound spectrum, the reduced number of cross-peaks indicates that the apex of the C-loop experienced more limited conformational mobility.

## Discussion

We used solution NMR to assess changes in chemical environment of the C-loop at the periphery of the ligand binding pocket of AChBP in response to binding ACh. The experiments were made possible by large-scale expression of AChBP from a stable eukaryotic cell line, selective incorporation of  $^{15}\text{N}$  cysteine into the C- and Cys-loops, and high-resolution 2D heteronuclear TROSY spectroscopy. The results complement those from X-ray crystallography of liganded AChBP (Celie et al., 2004, 2005; Bourne et al., 2005; Hansen et al., 2005), molecular dynamics simulation of AChBP (Gao et al., 2005), fluorescence of acrylodan-conjugated AChBP (Hibbs et al., 2004), ACh-mediated changes in accessibility of binding site Trps to an extrinsic fluorescence quencher (Gao et al., 2005), and changes in deuterium-hydrogen exchange in response to ligand binding (Shi et al., 2006). The collective findings clarify how the C-loop of AChBP contributes to agonist recognition and suggest how this may translate into a picture of agonist recognition in Cys-loop receptors.

High-resolution NMR provides several advantages over X-ray crystallography and fluorescence spectroscopy for detecting protein conformational changes. The protein is maintained in soluble form where it can freely explore conformational space. In contrast, the conformation in a crystal may differ from those predominant in solution, and the different conformational states may be difficult to arrest in crystal form. Fluorescence relies on either intrinsic Trp residues, incorporation of unnatural fluorescent amino acids, or covalent conjugation of thiol- or amino-reactive fluorophores. NMR, on the other hand, allows selection of the amino acid for isotopic labeling, and maintains the native structure. In AChBP studied here, cysteine is present at only four positions per homomeric subunit of the native structure, and two positions localize to a region thought to undergo a substantial change in conformation.

The spectrum from apo AChBP shows five distinct cross-peaks associated with cysteines 187 and 188 of the C-loop, whereas only two cross-peaks are expected. The presence of multiple cross-peaks indicates a range of distinct conformations, presumably similar in nature to the uncapped conformation observed by X-ray crystallography and molecular dynamics simulation (Fig. 1). *Cis-trans* isomerization about the disulfide bond (Otting et al., 1993) or different conformations of the equivalent subunits could also give rise to multiple cross-peaks in apo AChBP. Prolonged molecular dynamics simulations show that bound ACh reduces asymmetry and restricts mobility of the main chain of AChBP (Gao et al., 2005) and the  $\alpha 7$  ligand binding domain (Henchman et al.,

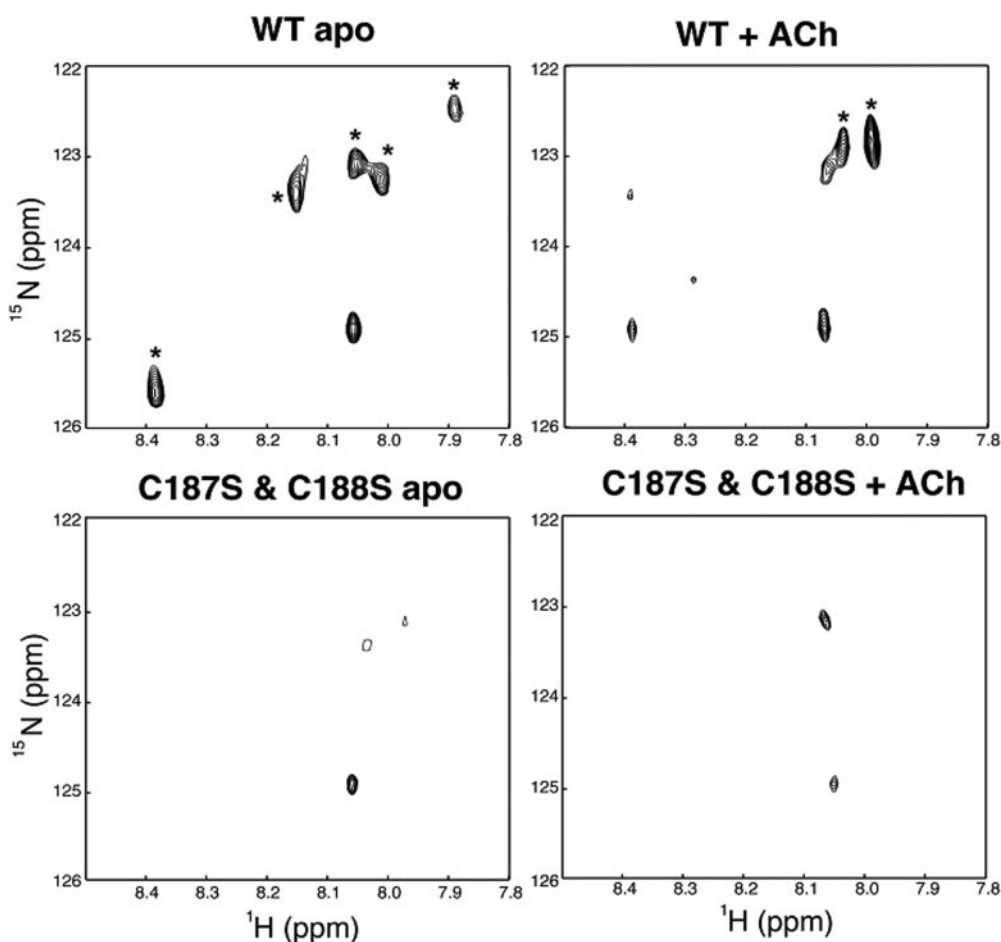
2003), which may explain the restricted range of conformations indicated by the presence of only two main cross-peaks in the ACh-bound spectrum.

A recent study examined deuterium-hydrogen exchange of AChBP, without and with bound agonists, followed by enzymatic digestion and mass spectrometric identification of a fragment containing the C-loop (Shi et al., 2006). In agreement with the present results, the C-loop of apo AChBP exhibited faster and more extensive incorporation of deuterium compared with agonist-bound AChBP. The present study complements the findings from deuterium-hydrogen exchange by providing single-residue precision and localizing a region of conformational mobility to the vicinal cysteines at the apex of the C-loop.

The present results directly demonstrate a change in chemical environment of the C-loop after binding of ACh in solution, but they do not indicate the direction or magnitude of the change. However, our results combine with results from X-ray crystallography, molecular dynamics simulation, and fluorescence spectroscopy to clarify our picture of how the C-loop contributes to molecular recognition of the agonist. In the agonist-free conformation, the C-loop assumes a range of interconvertible uncapped conformations, allowing access of small agonists, antagonists, and peptide toxins. When the ligand is a peptide such as  $\alpha$ -conotoxin or  $\alpha$ -neurotoxin, the C-loop remains uncapped and the toxins fill the binding pocket, preventing access of small ligands. When the ligand is a small agonist, the C-loop moves into a capped position, locking the agonist in place. This capping mecha-

nism is reminiscent of the clamshell mechanism for agonist binding to ionotropic glutamate receptors (Gouaux 2003).

In Cys-loop receptors, the capping motion of the C-loop not only aids in binding the agonist, but it may also initiate downstream conformational changes that trigger gating of the channel (Sine and Engel, 2006). The ability of an AChBP/5-HT<sub>3</sub> chimera to open the channel in response to agonist shows that AChBP has the capacity for conformational change that opens the channel (Bouzat et al., 2004). The uncapped conformation of AChBP obtained by MD simulation shows a salt bridge between Asp 194 at the base of the C-loop and Lys 139 just after the Cys-loop (F. Gao and S. M. Sine, unpublished observations), which is also present in the crystal structure of AChBP with bound HEPES (Brejc et al., 2001; Celie et al., 2004). In the agonist-bound crystal structures, a new salt bridge forms between Tyr 185, located near the apex of the C-loop, and Lys 139, and the distance between Asp 194 and Lys 139 increases (Celie et al., 2004). In the muscle nicotinic receptor, mutations of Asp 200, Lys 145, and Tyr 190 (equivalent to Asp 194, Lys 139, and Tyr 188, respectively, in AChBP) all profoundly impair gating of the channel, suggesting that the capping motion of the C-loop relays the initial conformational change from the ACh binding site toward the channel gating machinery (Mukhtasimova et al., 2005). Thus the conformational changes of the C-loop detected here not only contribute to molecular recognition of the agonist but also might initiate the cascade of structural rearrangements that opens the channel.



**Fig. 4.** Two-dimensional TROSY HSQC- $^{15}\text{N}$ - $^1\text{H}$  spectra of [ $^{15}\text{N}$ ]cysteine-labeled wild-type and mutant AChBP in the apo and ACh-bound (20 mM acetylcholine chloride) forms recorded at 800 MHz at 31°C. In all four spectra, the solitary peak at 135.3 ppm ( $^{15}\text{N}$ ) and 7.7 ppm ( $^1\text{H}$ ), visible in Fig. 2, was off scale. \*, major cross-peaks associated with cysteines 187 and 188 of the C-loop.

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