LIGAND-INDUCED CONFORMATIONAL CHANGES IN THE ACETYLCHOLINE BINDING PROTEIN ANALYZED BY HYDROGEN-DEUTERIUM EXCHANGE MASS SPECTROMETRY

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Running Title: H/D Exchange Mass Spectrometry for AChBP

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Recent X-ray crystallographic studies of the acetylcholine binding protein (AChBP) suggest that loop C, found at the circumference of the pentameric molecule, shows distinctive conformational changes upon antagonist and agonist occupation. We have employed hydrogen-deuterium exchange mass spectrometry to examine the influence of bound ligands on solvent exposure of AChBP. Quantitative measurements of deuterium incorporation are possible for approximately 56% of the Lymnaea AChBP sequence, covering primarily the outer surface of AChBP. In the apo-protein, two regions flanking the ligand occupation site at the subunit interface, loop C (175-193) and loop F (164-171), show greater extents of solvent exchange than other regions of the protein including the N- and C-terminal regions. Occupation by nicotinic agonists, epibatidine and lobeline, and nicotinic antagonists, methyllycaconitine, α-bungarotoxin, and α-cobratoxin, all markedly restrict the exchange of loop C amide protons, influencing both rates and degrees of exchange. Solvent exposure of loop C and its protection by ligand suggest that in the apo-protein, loop C exhibits rapid fluctuations in an open conformation and bound agonist or antagonist restricts solvent exposure through loop closure with agonists and largely through occlusion of solvent with the larger antagonists. Loop F, found on the complementary subunit surface at the interface, also reveals ligand selective changes in amide proton exchange rates. Agonists do not affect solvent accessibility of loop F, whereas certain antagonists cause subtle accessibility changes. These results reveal dynamic states and fluctuating movements in the vicinity of the binding site for unligated AChBP that can be influenced selectively by ligands.

Receptors to neurotransmitters, hormones and various autacoids possess both recognition and signal transduction capabilities to confer signaling responses in the organism. Identifying the precise structural determinants governing selectivity and the conformational changes attendant to ligand binding in many cases has been hampered by limitations in structural characterization of integral membrane protein receptors at an atomic level of resolution. In the case of the pentameric ligand-gated ion channels, the discovery and crystallographic structural resolution of a soluble acetylcholine binding protein (AChBP) from snails that resembles the extracellular domain of the nicotinic acetylcholine receptor (nAChR) has provided a critical template for analyzing structure and conformation of the superfAMILY of pentameric ligand-gated ion channels (1,2).

AChBP shows sequence homology with the members of the Cys loop family of ligand-gated ion channels, and its three dimensional structure superimposes with the extracellular ligand binding region of the nicotinic receptor from Torpedo electroplaque (3,4). The pharmacologic selectivity for agonists and for alkaloid and peptide toxin antagonists of AChBP resembles that expected from studies with a large number of neuronal and muscle acetylcholine receptors (4-8).

The elucidation of structures of several complexes with alkaloid agonists and antagonists as well as peptidic toxins of differing size and structure (4,9,10), enabled one to compare conformations of the various complexes. Finally, the functionality of a chimera where a slightly modified AChBP was attached to the transmembrane spans of a related Cys loop receptor established that AChBP could serve as...
both a structural and functional surrogate of the ligand-gated ion channels (11).

Recently various physical methods, such as fluorescence spectroscopy (12,13) and computational (molecular dynamics) analysis (13,14), have been directed to examining the energetics of ligand association and the structural fluctuations that accompany ligand binding to the receptor or binding protein surrogate. Analysis of structure by mass spectrometry should prove to be an important addition to current structural analysis. Monitoring amide bond hydrogen exchange enables one to examine solvent exposure of the apo-protein and compare that with the many complexes that can form with the natural neurotransmitter acetylcholine, its congeners, the alkaloid agonists and antagonists and the several peptide toxins that associate with this protein. Mass spectrometry enables one to identify individual peptides in the subunit and therein ascertain by molecular mass changes in the extent of exchange occurring with a D₂O solvent over a particular time interval.

We examine here the amide proton exchange behavior of AChBP and find that a peptide encompassing loop C shows rapid exchange in the apo form with 13 of 17 amides rapidly incorporating deuterium. Upon ligand binding, 6 out of 13 exchanging amide protons are occluded from the solvent. The extent of protection and exchange rates have been analyzed in terms of immobilization of loop C through capping of the small agonists and partial occlusion and/or immobilization of the loop by antagonists. Similarly, loop F found on the complementary subunit interface also reveals ligand selective changes in solvent exposure.

**MATERIALS AND METHODS**

*Materials* - (+)-Epibatidine, α-bungarotoxin (Bgtx), and α-cobratoxin (Cbtx) were purchased from Sigma. Methyllycaconitine citrate and α-lobeline were purchased from Tocris (Ellisville, MO). Deuterium oxide (D₂O; 99.9% deuterium) was purchased from Cambridge Isotope Laboratories (Cambridge, MA). Pepsin immobilized on 6% agarose was obtained from Pierce. α-Cyano-4-hydroxycinnamic acid (Aldrich) was recrystallized once from ethanol. All other materials were of the highest grade commercially available.

*Expression and purification of AChBP-AChBP from Lymnaea stagnalis* was expressed and purified as previously described (6). Briefly, N-terminal FLAG tagged AChBP was expressed in HEK 293 cells deficient in the glycosylation processing enzyme, N-acetylglucosylamine transporter I (GNT- cells) (15). Soluble AChBP secreted from media of the stable cell lines was purified by a FLAG antibody column followed by elution with 3XFLAG peptide in Tris-buffered saline (50 mM Tris HCl, 150 mM NaCl, pH 7.6). The eluate was concentrated to a stock solution containing ~260 μM AChBP in binding sites prior to deuterium exchange.

*Deuterium Exchange and Mass Spectrometry*- All exchange mixtures for AChBP contained 20-26 μM protein, 15 mM Tris HCl, 10 mM NaCl, with a final pH of 7.6 and 80% D₂O. Formation of the five AChBP complexes used a 1:1.2 fold molar excess of ligand and a five minute incubation. Deuterium exchange was initiated when the concentrated AChBP stock was diluted 10-fold into deuterated buffer. After 0, 0.5, 1, 2, 5, 10 minute intervals, the deuterium exchange reaction was quenched by a 10-fold dilution into a prechilled vial (0°C) containing a quench buffer composed of 2% TFA in H₂O, making the final solution approximately 0.04% TFA, pH 2.2. The quenched protein was then mixed with 50 μl of pepsin bead slurry (previously incubated on ice) with occasional mixing for 5 minutes, centrifuged for 15 seconds at 12,000 x g at 4°C, divided in aliquots, frozen in liquid N₂, and stored at -80°C until analyzed.

Samples were individually analyzed by MALDI-TOF mass spectrometry immediately after thawing to minimize off-exchange as described previously (16,17). Samples were mixed with cold matrix, spotted on a prechilled MALDI target plate, and dried under vacuum. The back exchange that occurred during the analysis was determined by carrying out control experiments where each of the protein samples was deuterated for 24 hours at 25°C.

To determine the number of deuterons incorporated in each peptide, mass spectra were quantified by subtracting the centroid of the undeuterated control from the centroid of the isotopic peak cluster for the undeuterated sample. All
values reported represent only the deuterons exchanged onto the backbone amide-hydrogen positions. The residual deuterium content that incorporated into rapidly exchanging side chain positions were subtracted as described previously (18). Finally, corrections were applied to account for the exchange condition of 80% D$_2$O, and back exchange of 21% (18). Kinetic plots of deuteration were typically fit to a double exponential model accounting for deuterons exchanging at a rapid rate (mainly solvent accessible amides) and those hindered through hydrogen bonding and solvent occlusion. The fit was implemented in Prism 4.0 (San Diego, CA).

Peptides produced by pepsin cleavage of AChBP were identified by a combination of sequence searching for accurate masses, and MS/MS sequencing by MALDI QqTOF mass spectrometer.

**RESULTS**

Amide hydrogen/deuterium (H/D) exchange was designed to probe solvent accessibility of regions of AChBP and changes in solvent accessibility upon agonist and antagonist binding. To localize the observed changes, after incubation in buffer with or without ligand, AChBP was cleaved with pepsin under low pH quench conditions. Low pH substantially reduces amide exchange rates allowing one to measure prior exchange events at physiological pH. A total of 14 peptides from AChBP were analyzed from each MALDI mass spectrum (Fig. 1). The complete set of mapped (underlined) and unmapped regions is shown under the sequence of AChBP in Fig. 1. Incubation of AChBP with deuterium buffer led to an increase in mass for the envelopes associated with each identifiable peptide fragment (cf: Fig. 4A). Because deuterium labeling occurs when the protein is in its native state, the amount of deuteration reflects the solvent accessibility of a certain protein region. The rate and extent of the time-dependent incorporation of deuterium was quantified and plotted. Table I shows the analyzed data reported as the average number of deuterons incorporated after 10 minutes for three independent determinations.

**Amide Exchange Dynamics for Apo AChBP** - The 14 peptides analyzed, covering 56% of the total sequence, showed great differences in their exchange capacities over a ten minute interval. The crystal structure of AChBP shows a homopentameric structure with the five AChBP monomers surrounding a central channel. Apart from a N-terminal helix, beta structure forms much of channel lining and outer surface of the pentamer. In the apo-protein, two regions flanking the ligand binding site at the subunit interface (loop C, D175-E193; and loop F, Y164-F171) exchange rapidly with the solvent (Fig. 2, Table I). The loop C peptide is highly deuterated, with 13 out of 17 (~75%) possible amide protons deuterated, even after a short deuteration period of two minutes. Two peptides, covering the tip of loop F that is situated on the complementary subunit face of AChBP active site, were also highly deuterated. By contrast, the α1-β1 loop, and the N and C-terminal peptides have intermediate solvent accessibility; roughly 40-60% of amides were exchanged (Table I). It should be noted that the mapped C-terminal peptide included the C-terminal residues (206-210) for which electron density was not observed in the crystal structures. The most solvent excluded region of apo protein lies in the β-hairpin and β-strands of the extended β-sandwich. β2 (W53-T62) has two exchangeable amides out of nine, loop A (Y89-L102) region has two of 12 exchangeable protons. The more C-terminal portion of β5′(A103-L112) is virtually devoid of exchange after 10 minutes.

**Ligand Binding Markedly Reduces Amide Exchange in Loop C** - We examine here the amide proton exchange behavior of AChBP when associated with two nicotinic agonists, (+) epibatidine and lobeline, and three nicotinic antagonists, the alkaloid methyllycaconitine (MLA), and the peptidic toxins, Bgtx and Cbtx (Fig. 3). Occupation by epibatidine results in reduced exchange of six of the 13 rapidly exchanging amide backbone hydrogens in loop C (Fig. 4, Table I). The crystal structure of AChBP-epibatidine reveals close proximity (≤ 4 Å) for backbone residues of four loop C residues (Y183, C187, C188, Y192) with epibatidine. Therefore, solvent protection by epibatidine shows that loop C undergoes a loss of solvent accessibility and dynamics upon agonist binding. Similar results were obtained for the large agonist (lobeline) and alkaloid antagonist (MLA) bound AChBP (Table I, Fig. 5).
In the case of larger peptidic $\alpha$-neurotoxin antagonists, Bgtx and Cbtx, a greater number of amide hydrogens are protected. This is particularly manifest at short time periods (Fig. 6A). The higher affinity complex of AChBP with Bgtx approximates monophasic deuteration kinetics. By contrast, Cbtx produces a biphasic deuteration kinetic profile and a smaller degree of solvent protection after 10 minutes. This suggests that modes of interaction or the interfacial area between toxin-receptor complex differs slightly, even though both Bgtx and Cbtx are members of the long three-fingered $\alpha$-neurotoxins. Fig. 3B compares the amino acid residues on the toxin shown in the crystal structure (10) to be in contact with the AChBP subunit interface.

Interestingly, HEPES buffer, used in the initial crystallizations of AChBP (1) also results in solvent protection of loop C (Fig. 6B). Previous calorimetry studies show that the presence of HEPES lowers the apparent affinity of AChBP-nicotine interaction 6-fold, whereas another buffer, Tris-HCl at pH 7.0, had no effect (9). When we compare H/D exchange of AChBP in the presence of Tris and HEPES buffer at a comparable pH value (7.0), we find HEPES protects the loop C amide hydrogens suggesting HEPES occupation of the agonist-antagonist site in solution. This observation is consistent with the presence of HEPES in the binding site in the crystallized binding protein (1,2).

Antagonist Binding Alters Solvent Accessibility of Loop F - No significant differences in deuteration of loop F were observed when the agonists, epibatidine and lobeline, were bound (Fig. 7). By contrast, occupation of the antagonist MLA results in reduced exchange of loop F (Y164-F171) especially at the short deuteration periods up to 2 minutes (Fig. 7, Table 1). The crystal structure of the MLA-AChBP complex shows close proximity of MLA with Y164 of in loop F (4). Thus, MLA appears to lock the flexible loop F in place and reduce the dynamics of amide proton exchange.

In the presence of the large, three fingered peptide antagonists, Bgtx and Cbtx, in contrast to the other ligands, enhanced exchange of loop F was observed at short exchange times (Fig. 7). Accordingly, $\alpha$-neurotoxins enhance solvent accessibility to this region. Most of the increase in rate of deuteration for the neurotoxin complexes is lost with a shorter overlapping peptide fragment of the loop F, residues 165-171. Therefore, the increase in deuteration can be localized largely to the amide proton of F165, the most N-terminal amide in Y164-F171 peptide.

The crystal structure of Cbtx bound *Lymnaea* AChBP shows T155-Y164 segment of loop F more exposed toward solvent with r.m.s deviation of up to 3.5 Å for E163 (10). The enhanced exposure of the amide protons may reflect the C-terminal portion of this segment extending in a radial direction.

**Residues Y89-L102 Show Decreased Solvent Accessibility Upon Ligand Binding** - Because only 2 of 14 analyzed peptides show changes in exchange kinetics upon ligand binding during the 10 minute deuteration interval (Fig. 5), we extended the time-course of the deuteration exchange from 10 minutes to 25 hours. Even after 25 hours, the residues protected by epibatidine in loop C remain resistant to exchange appearing to be highly sheltered from solvent (data not shown). By contrast, differences in exchange with bound antagonists observed for loop F disappeared after extended exchange periods. Furthermore, residues 89-102 (loop A), a region highly protected from solvent in the apo form, showed decreased solvent accessibility upon epibatidine binding only at long deuteration periods extending to 25 hours (Fig. 8). The crystal structure shows the 89-102 loop to line the bottom of the binding site pocket and internal vestibule. The enhanced solvent protection indicates that enclosure of the loop C around the agonist in the binding site pocket has a slight influence on solvent exposure on the hydrogen-bonding network on the neighboring subunit (cf: Fig. 9).

**DISCUSSION**

**Solvent Exposure and Solution Conformation Assessed by H/D Exchange** - Amide H/D exchange with mass spectrometry, applied to full length AChBP in the apo, agonist, and antagonist bound states, provides a perspective on solution dynamics of AChBP and the conformational differences associated with ligand binding. The technique, employing differences in peptide mass with differential H$_2$O and D$_2$O exposure, allows one to analyze the native, unmodified structure in various aqueous buffers replicating physiologic
conditions. Studies in solution circumvent limitations that constrain the protein in the solid state or may be specifically induced by crystal packing or the position of the symmetry-related molecule in crystal structures.

H/D exchange coupled with Fourier transform infrared spectroscopy (FTIR) has been applied to the study of lipid vesicle reconstituted nicotinic receptors demonstrating that lipid composition affects exchange rates (19). Through the use of FTIR, overall exchange in α-helical and β-sheet regions can be distinguished, but the mass spectrometry approach (20) allows identification of exchange on particular peptides in the structure.

In this initial H/D exchange study, we were able to identify peptides that covered about 56% of the sequence. The structure of pentameric AChBP can be divided into an outer and inner shell and fortunately, following digestion, the outer shell to which agonist access yields about 75% of the peptides detectable in the mass spectrometry stream. Additional approaches modifying pepsin digestion conditions with low concentrations of denaturants or analyzing orthologous AChBP’s from Aplysia or Bulinus may provide means for enhancing coverage in this family of AChBP’s.

In the apo form, two segments (Y164-F-171, loop F; D175-E193, loop C) flanking the Lymnaea AChBP ligand binding site were highly deuterated even at the shortest exchange time, suggesting these two loop regions are well exposed to solvent and dynamic. In fact, these two loops appear to be at least as solvent accessible as the N- and C-terminal residues whose electron density are not visible in the crystal structure. The loop lining the channel opening, loop A (Y89-L102) showed a much slower exchange, reflecting a more ordered structure at channel lining. The slow exchange may also indicate that H2O molecules in the channel opening are more structured.

**Distinctive Changes in Loop C Behavior Associated with Agonist and Antagonists** - Recent crystallographic studies have revealed distinctive loop C conformations between the agonists and antagonists thus far studied (4,7,8,10). Agonists such as nicotine and carbamoylcholine (9), as well as the more selective agonists, epibatidine and lobeline (4), all reveal a closure or capping of the loop C around the ligand. Requirements for this capping might well arise from the small size of many agonists as well as their flexibility in terms of torsional movements of their respective ring systems. By contrast, complexes with the alkaloid antagonists, methyllycaconitine, and the peptidic α-conotoxins and α-cobratoxin reveal an extended loop C. In the case of methyllycaconitine, the distance between the loop C and core backbone is 7 Å, for α-cobratoxin 10 Å, and for α-conotoxin IM1 12 Å (4,10).

The distinction between an extended or closed loop C being associated with antagonist and closed loop C with agonist association was originally noted by Karlin (21) who found that bromoacetylcholine and m-maleimidobenzyl trimethylammonium (MBTA) when conjugated to the reduced vicinal disulfides, now known to reside on loop C, behaved as agonist and antagonist, respectively, on the intact receptor. The distance between the point of the sulphydryl-reactive electrophiles and the quaternary ammonium is 9 Å for bromoacetylcholine and 13 Å for MBTA.

In the case of agonist binding, the loop closure seen in the crystal structures likely restricts solvent access and exchange rates. Not only will surface occlusion by agonist itself exclude solvent interactions, but also amide bond associations between with interacting surfaces of the loop C and subunit core should diminish exchange. A comparison of the four agonist complexes available from crystal structure (4,9) suggests that their limited spatial dimensions and inherent torsional motion and flexibility should enable them to fit behind a completely closed loop C. Accordingly, both envelopment of the agonist and interactions between loop C and the backstop β structure behind the loop account for this lack of exchange of six additional hydrogens. We estimate four of loop C residues (Y185, C187, C188, Y192) are in van der Waals contact with the epibatidine agonist itself (4).

An equivalent reduction in exchangeable amide hydrogens for the MLA antagonist suggests that antagonists may also lock loop C in a more restricted position. Simple extension of loop C may serve to increase exposure of residues not in immediate proximity to the antagonist molecule, but this would be compensated for by occlusion of residue surfaces around the loop C by the larger antagonist. Thus, although epibatidine and methyllycaconitine show virtually identical protection of loop C amide hydrogens, the bulk of
the smaller epibatidine structure lies on the apical side of loop C, whereas the bulk of the MLA structure is on the membrane side (4). Also, epibatidine affects closure of loop C to envelop the ligand whereas MLA binds with the loop C in a more open conformation. Hence, restrictions in H/D exchange likely result from the associated ligand locking an apo structure with a flexible loop C into a more rigid conformation in the bound state.

A similar situation likely arises with the bound Cbtx and Bgtx where loop I and II of the toxin fingers cover portions of an extended loop C residues (S182-Y192). Although both toxins are members of the long α-neurotoxin family that contain an additional disulfide in the central loop (Loop II), sequence differences of certain residues (Fig. 3B) between the two toxins produce different rates and extents of solvent protection (Fig. 6). Although a Bgtx-AChBP crystal structure is not yet available for direct comparisons of the contacting residues, a difference in tertiary structure of the two toxins (Fig. 3A) suggest different interaction points for the flexible fingers on the α-toxin (22).

The capacity of HEPES buffer at 50 mM to limit amide hydrogen exchange in loop C is consistent with the crystal structure showing HEPES within the binding pocket is largely superimposed within the agonist occupied volume capped by loop C (1). Crystallization conditions using other buffer conditions and polyethylene glycol have shown a more open C-loop extending some 7 Å from the core structure [(4), Fig. 9]. Such a structure would allow for solvent exposure on the outer and inner surfaces of the loop C. Loop C in the absence of ligand may well exhibit rapid fluctuations in position as predicted from molecular dynamics (13,14). Hence, AChBP exchange dynamics are consistent with a more open solvent exposed loop C as seen in the absence of HEPES (4).

**Ligand Induced Changes in Loop F Exchange** - Residues in loop F are also proximal to the binding site, but arise from the complementary subunit interface. The loop F region shows substantial variability in sequence amongst the different subunits of the nAChR. This is also a region where sequence insertions are evident when subunits presenting a binding face are compared. For example, in the muscle nAChR subunits, γ, δ, ε show a sequence insertion in that region (3). Hence, the loop F region contains important residues that dictate selectivity for the binding site when this region of γ, δ, ε is paired with the principal face of α subunits (23). Thus, it is not surprising that this region exhibits considerable flexibility in solution. Since the surface for all agonist binding lies apical to loop C and hence distal to loop F, a lack of influence of agonist binding on exchange parameters of loop F might be anticipated.

Interestingly, the large α-neurotoxins, despite the bulk of their structure residing on the membrane side of the loop (10), increase exchange in this region. A suggestion of α-neurotoxins altering loop F position is evident in the recent crystal structure (10) where the 155-164 segment of the subunit becomes exposed. By contrast, the succinamidyl moiety of methyllycaconitine appears to cover and perhaps immobilize a portion of loop F region (Fig. 9). The binding surface of methyllycaconitine is unique among the alkaloid ligands studied to date in that much of its binding surface lies on the membrane side of loop C.

Outside of the differences in conformation of loops C and F, to date the H/D exchange data strongly suggest a minimum of conformational changes in other regions of the molecule, particularly those loops in the receptor that would be positioned near the membrane surface. In the crystal structures (4,8), only small differences in conformation are seen in the Cys loop and other regions that appear to interact with the M2-M3 loop region in the transmembrane span (11). This might arise from the sequence truncation in AChBP leaving its carboxyl terminal end limp as a flexible end. Indeed, a comparison of the carboxyl-terminal peptides 197-210 and 201-210 shows an equal degree of exchange suggesting that exchange is accentuated in the most carboxy terminal position of these peptides.

Our initial study of AChBP by H/D exchange shows that two loops (C and F) serve as flexible flaps controlling access of active site to ligands. H/D exchange with mass spectrometry reveals conformational states of AChBP not evident in a static crystal structure and thereby provides novel insight into the solution dynamics of AChBP.
REFERENCES

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The abbreviations used are: AChBP, acetylcholine binding protein; nAChR, nicotinic acetylcholine receptor; TFA, trifluoroacetic acid; Bgtx, \( \alpha \)-bungarotoxin; Cbtx, \( \alpha \)-cobratoxin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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FIGURE LEGENDS

FIG. 1:  (A) MALDI-TOF mass spectrum of AChBP after pepsin digestion. From this mass spectrum, a total of 14 peptides were identified via MS/MS sequencing. (B) Aligned sequences of AChBP from Lymnaea stagnalis showing all of the peptides generated by pepsin cleavage are underlined below. Fourteen pepsin digest peptides covering 56% of AChBP were identified and analyzed.

FIG. 2: Solvent exposure of apo AChBP assessed by H/D exchange. (A) All peptides analyzed in H/D exchange studies are mapped to the crystal structure of Lymnaea AChBP (PDB accession code 1UV6) and shown in yellow and orange. Key structural features and residues numbers are labeled. Those regions for which data could not be obtained are colored gray. (B) Deuteration trend of apo AChBP mapped onto the crystal structure of Lymnaea AChBP. The deuteration levels at 10 minutes are shown by different colors ranging from dark blue (˂5% deuteration) through red (>65% deuteration). Side view of the interface of two AChBP subunits and apical view of the pentameric complex are shown.

FIG. 3: (A) Structures of ligands used in the H/D exchange study. The crystal structures of \( \alpha \)-bungarotoxin and \( \alpha \)-cobratoxin are obtained from PDB accession code 2ABX and 2CTX, respectively. (B) Sequence alignment of \( \alpha \)-bungarotoxin and \( \alpha \)-cobratoxin. The asterisks denote Cbtx residues in van der Waals contact with AChBP(10).

FIG. 4: Time course for incorporation of solvent deuterium into peptide fragment D175-E193 in the absence (A) and in the presence of the epibatidine (B). The time-dependence of exchange for this fragment is presented in panel C measured from the mass of the centroid. AChBP was deuterated in the presence of 15 mM Tris-HCl, 10 mM NaCl, with a final pH of 7.6 and 80% D₂O. The maximum number on the Y-axis denotes the total number of exchangeable amides in the peptide, and error bars represent the standard deviation of three independent determinations.

FIG. 5: Kinetics of deuterium incorporation into several peptide fragments in apo protein (black), the epibatidine (green), and MLA (orange) bound forms of AChBP. The total number of incorporated deuterons is plotted against time (minutes) for the following peptide fragments: D(-8)-L7 (N-terminal helix), W53-T62 (\( \beta \)2), Y89-L102 (loop A), A103-L112 (\( \beta \)5), Y164-F-171 (loop F), D175-E193 (loop C), and V197-L210 (C-terminus). Conditions are the same as in Fig. 4.

FIG. 6: (A) Kinetics of deuterium incorporation into the peptide fragment D175-E193 in apo (●), epibatidine (○), Bgtx (◇), and Cbtx (▽) bound forms of AChBP. The total number of incorporated deuterons is plotted against time. AChBP was deuterated in the presence of 15 mM Tris-HCl, 10 mM
NaCl, with a final pH of 7.6. (B) Kinetics of deuterium incorporation into the peptide fragment D175-E193 in the presence of buffer HEPES (open squares; 55 mM HEPES, pH 7.0) and Tris-HCl (closed circles; 15 mM Tris-HCl, 30 mM NaCl, pH 7.0).

FIG. 7: Kinetics of deuterium incorporation into the peptide fragment Y164-F171 (loop F) in apo (●), lobeline (○), MLA bound (△), and Cbtx bound (▽) complexes of AChBP. Incorporation of deuterons into the Bgtx-AChBP is not shown, but is virtually indistinguishable from the Cbtx complex. Conditions are the same as in Fig. 4.

FIG. 8: Kinetics of deuterium incorporation in the presence (○) and absence (●) of epibatidine over extended (25 hour) period for the peptide fragment Y89-L102 (loop A). Conditions are the same as in Fig. 4.

FIG. 9: Positions of bound ligand and their influence on AChBP exchange. The top panels show the crystal structures oriented to exhibit the AChBP subunit interface in (A) epibatidine with Aplysia AChBP [PDB ID: 2BYQ], (B) MLA with Aplysia AChBP [PDB ID: 2BYR], and (C) Cbtx with Lymnaea AChBP [PDB ID: 1YI5] complexes. The main chains from (+) and (-) faces of the subunit interface are in grey and gold respectively. Side chains in close proximity (within 4 Å) with ligand are labeled in black. Aplysia AChBP residues shown in Panels A and B correspond to their homologous residues in Lymnaea AChBP as follows: Y93:Y89 (loop A); S167: Y164 (loop F); Q186: V183; Y188: Y185; C190:C187; C191:C188; Y195:Y192. Loop C (D175-E193) is highlighted in blue, and loop F (Y164-F171) is highlighted in yellow, blue, and red depending on the complex. Note here loop C caps the ligand binding site in the presence of agonist, and is extended radially in the presence of antagonist. The bottom panels show changes in the H/D exchange probes for the same ligands bound to AChBP mapped onto an AChBP subunit interface. Sequence in dark blue (loop C) show greater protection in the presence of the respective ligand. Sequences in yellow are not protected by the ligands. Sequence in red (loop F) shows an increase exchange in the presence of the respective ligand. The crystal structure of lobeline with Aplysia AChBP [PDB ID: 2BYS] is shown in Supplemental Figure 1.
### TABLE I

**Summary of Hydrogen/Deuterium Exchange Data for AChBP**

<table>
<thead>
<tr>
<th>Region of AChBP</th>
<th>Number of Amides Deuterated after 10 Minutes</th>
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<tr>
<td>Loop F</td>
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<tr>
<td>F165-F171</td>
<td>MH+ 934.44 6 3.66 ± 0.06 3.63 ± 0.13 3.60 ± 0.10 3.82 ± 0.03</td>
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<tr>
<td>Y164-F171</td>
<td>1097.50 7 4.81 ± 0.01 4.84 ± 0.09 4.46 ± 0.09 5.00 ± 0.06</td>
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<tr>
<td>β5</td>
<td>A103-L112 1044.57 9 0.20 ± 0.02 0.17 ± 0.01 0.25 ± 0.05 0.15 ± 0.04</td>
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<td>β2</td>
<td>W53-T62 1308.60 9 2.44 ± 0.08 2.54 ± 0.01 2.63 ± 0.07 2.50 ± 0.01</td>
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<tr>
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<tr>
<td>Y89-L102</td>
<td>1572.85 11 2.62 ± 0.08 2.53 ± 0.06 2.54 ± 0.05 2.53 ± 0.06</td>
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<tr>
<td>C-term Tail</td>
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<tr>
<td>V197-L210</td>
<td>1646.97 12 4.51 ± 0.24 4.38 ± 0.06 4.62 ± 0.22 4.46 ± 0.08</td>
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<td>N-term Helix</td>
<td></td>
</tr>
<tr>
<td>D(-8) - L7</td>
<td>1809.85 14 8.43 ± 0.28 8.59 ± 0.25 8.79 ± 0.27 8.58 ± 0.13</td>
</tr>
<tr>
<td>D(-8) - Y8</td>
<td>1972.91 15 9.55 ± 0.03 9.43 ± 0.10 9.70 ± 0.10 9.55 ± 0.24</td>
</tr>
<tr>
<td>Loop C</td>
<td></td>
</tr>
<tr>
<td>D175-E193</td>
<td>2162.88 17 12.5 ± 0.29 7.54 ± 0.12 7.64 ± 0.14 4.53 ± 0.05</td>
</tr>
<tr>
<td>α1- β1 loop</td>
<td></td>
</tr>
<tr>
<td>Y8-A28</td>
<td>2482.31 17 9.62 ± 0.17 9.53 ± 0.01 9.93 ± 0.20 9.59 ± 0.09</td>
</tr>
<tr>
<td>Y8-S30</td>
<td>2668.43 19 11.18 ± 0.23 11.22 ± 0.15 11.01 ± 0.23 11.06 ± 0.19</td>
</tr>
</tbody>
</table>

Data are reported as means ± standard deviations of deuterons incorporated after ten minutes for three independent determinations. Peptides are grouped on the basis of their location in the crystal structure.
Figure 1

A

Counts

1000 1200 1400 1600 1800 2000 2200 2400 2600
Mass (m/z)

11000 10000 9000 8000 7000 6000 5000 4000 3000 2000 1000

B

α1

DYKDDKLDRADILYN

β1

IRQTSRPDVITQRDRPVAVSVLKFINILEVNEITNEVD

β2

VVFQWTNSDRTLAWNSSHSPDVSPVSVPISLWVPDLAAY

β3

β4

loop A β5 β5' β6 β6' Cys loop

β7

β8

loop F

β9

loop C

β10

TESGATCRIKGSWHHSREISVDPTTDDESEYFSQYS

RFEILDVTQKKNSVTSCPEAYEDEVEVLNFRKKQREIL
Figure 2

Deuteration Level

- >65%
- >35%
- <30%
- <5%
Figure 3

A

Methyllycaconitine

Lobeline

HEPES

α-Bungarotoxin

α-Cobratoxin

Epibatidine

B

```
Bgtx  IYCVTBATFD EAVTCPIPEDNL EYFVRACDACRSSRGRV WELGCATCPSKGKPYEEVT CSTEKRKR---- 74
Cbtx  IMGP1PLDAP---- KDPMFHVYATRACDACSSRGRMLGCAATCPVTGVDI DCCSTENDNPFFTKR---- 71
```

* * *
Figure 4

A. Apo

B. Epibatidine

C. Residues 175-193
Figure 6

A  Residues 175-193

B  Residue 175-193

Deuterons Incorporated

Time (min)
Figure 7

Residues 164-171

Deuterons Incorporated

Time (min)
Figure 8

Residues 89-102

Deuterons Incorporated

Time (hr)
Figure 9
Ligand-induced conformational changes in the acetylcholine binding protein analyzed by hydrogen-deuterium exchange mass spectrometry

Jianxin Shi, Julia R. Koepppe, Elizabeth A. Komives and Palmer Taylor

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