Acrylodan-conjugated Cysteine Side Chains Reveal Conformational State and Ligand Site Locations of the Acetylcholine-binding Protein*

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We undertook cysteine substitution mutagenesis and fluorophore conjugation at selected residue positions to map sites of ligand binding and changes in solvent exposure of the acetylcholine-binding protein from Lymnaea stagnalis, a nicotinic receptor surrogate. Acrylodan fluorescence emission is highly sensitive to its local environment, and when bound to protein, exhibits changes in both intensity and emission wavelength that are reflected in the degree of solvent exclusion and the effective dielectric constant of the environment of the fluorophore. Hence, cysteine mutants were generated based on the acetylcholine-binding protein crystal structure and predicted ligand binding sites, and fluorescence parameters were assayed on the acrylodan-conjugated proteins. This approach allows one to analyze the environment around the conjugated fluorophore side chain and the changes induced by bound ligand. Introduction of an acrylodan-cysteine conjugate at position 178 yields a large blue shift with α-bungarotoxin association, whereas the agonists and alkaloid antagonists induce red shifts reflecting solvent exposure at this position. Such residue-selective changes in fluorescence parameters suggest that certain ligands can induce distinct conformational states of the binding protein, and that mutually exclusive binding results from disparate portals of entry to and orientations of the bound α-toxin and smaller acetylcholine congeners at the binding pocket. Labeling at other residue positions around the predicted binding pocket also reveals distinctive spectral changes for α-bungarotoxin, agonists, and alkaloid antagonists.

The nicotinic acetylcholine receptor (nAChR)1 is the prototypic member of the superfamily of pentameric ligand-gated ion channels (LGIC), that include γ-aminobutyric acid, glycine, and serotonin (5-HT3) receptors. These receptors are prevalent mediators of neurotransmitter signaling and targets of drug action. nAChR subtypes mediate fast neurotransmission both centrally as well as in the periphery by linkage to an intrinsic cation channel. In functional terms, AChBP shares virtually all of the ligand binding characteristics with the nicotinic receptor family, and reveals a structure largely consistent with the electron microscopy, image chemical modification, mutagenesis, and spectroscopic data. Based on the position of the associated HEPES buffer molecule (6), the ligand binding site at the subunit interface appeared to be formed from the side chain determinants of binding ascertained previously from mutagenesis and chemical modification (8–12). As a soluble entity of similar overall structure, AChBP provides new opportunities to investigate the structure and function of LGIC at the molecular level. Furthermore, should it be possible to couple AChBP to the receptor transmembrane-spanning region and achieve ligand gating of channel function, the binding protein would possess the conformational capabilities of the extracellular domain of receptors. Hence, conformational changes induced by ligand binding to AChBP may have global implications for ligand gating mechanisms for ion channels.

Another acetylcholine recognition protein with intrasubunit disulfide linkages, acetylcholinesterase (AChE), has multiple high resolution crystal structures of complexes for several ligands bound at its active center and peripheral site (13–15). Although these structures exhibit differences in conformation and side chain orientations, their overlay is likely to reveal only a small fraction of the conformational space of the AChE molecule in solution and the potential structural fluctuations and plasticity of the various ligand complexes (16, 17). Cysteine substitution mutagenesis is followed by acrylodan conjugation to the single introduced cysteine, which reveals changes in hydrophobicity and solvent exposure at various acrylodan positions on the AChE molecule associated with ligand binding.

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‡ The abbreviations used are: nAChR, nicotinic acetylcholine receptor; AChBP, acetylcholine-binding protein; LGIC, ligand-gated ion channel.
Conjugates of longer-lived fluorescein and anilinonaphthalene fluorophores with the introduced cysteines reveal distinct differences in segmental motion of certain structural domains of AChE upon ligand association (18). The results from these fluorescence-based studies in another system provide evidence for the value of the studies in solution described herein.

We undertook cysteine substitution mutagenesis and fluorophore conjugation at selected positions to describe the immediate environment surrounding the fluorophore, and examine regional flexibility of AChBP, as a nAChR surrogate. Ligand binding in the vicinity of the fluorophore may reveal changes in solvent exposure by ligand occlusion or through conformational changes induced by the ligand. Acrylodan fluorescence emission is highly sensitive to its local environment when bound to protein, and exhibits changes in both intensity and emission wavelength that reflect the effective dielectric constant of the environment around the fluorophore. Hence, cysteine mutants were generated based on the AChBP crystal structure and predicted ligand binding sites, and fluorescence parameters were assayed on the acrylodan-conjugated proteins. This approach allows one to analyze the environment around the fluorophore, conjugates of longer-lived fluorescein and anilinonaphthalene fluorophores with the introduced cysteines reveal distinct differences in segmental motion of certain structural domains of AChE upon ligand association (18). The results from these fluorescence-based studies in another system provide evidence for the value of the studies in solution described herein.

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**EXPERIMENTAL PROCEDURES**

**Ligands and Labeling Reagents**—(+)-Epibatidine, gallamine, α-bungarotoxin, nicotine, and carbamylcholine were purchased from Sigma (Fig. 1). d-Tubocurarine chloride was purchased from ICN Pharmaceuticals, Inc. Methylycconitine citrate was purchased from Tocris (Ellicottville, MO). Metocurine iodide was a gift from the Eli Lilly Co. Acrylodan was obtained from Molecular Probes (Eugene, OR). 125I-α-Bungarotoxin (specific activity: 80 Ci/mmol) was a product of PerkinElmer Life Sciences, Inc. All other chemicals were of the highest grade commercially available.

**Expression, Mutagenesis, and Purification of AChBP**—Wild-type AChBP from *Lymnaea stagnalis* was expressed from a cDNA synthesized from oligonucleotides selected for mammalian codon usage, as previously described (19, 37). Briefly, the AChBP gene was inserted into a p3XFLAG-CMV-9 expression vector (Sigma) containing a preprotryptasin leader peptide followed by a NH2-terminal 3XFLAG epitope. A COOH-terminal His6 tag was attached for radioligand binding assays. Mutant AChBPs were generated by polymerase chain reaction-mediated standard mutagenesis procedures, and cassettes containing the mutation were subcloned into the wild-type vector and verified by double-stranded sequencing. Wild-type and mutant AChBP-transfected HEK-293 cells were selected with G418 to generate stably expressing cell lines. Dulbecco’s modified Eagle’s medium (MediaTech CellGro) containing 3% fetal bovine serum was collected at 3-day intervals from multiter flask for up to 4 weeks. Adsorption onto a FLAG antibody column followed by elution with the 3XFLAG peptide yielded purified protein in quantities between 0.5 and 2 mg/liter. Purity and assembly of subunits as a pentamer were assessed by SDS-PAGE and fast protein liquid chromatography.

**Radioligand Binding Assays**—A scintillation proximity assay (SPA, Amersham Biosciences) was adapted for use in a soluble radioligand binding assay. In 200-μl reaction vessels, AChBP (0.5 nM binding sites) was incubated with increasing concentrations of either 125I-labeled α-bungarotoxin or (+)-epibatidine in a solution of 0.1 mg/ml anti-His SPA beads. In competition assays, 125I-labeled α-bungarotoxin was held constant at 20 nM and epibatidine was added in variable concentrations. Radioactivity was measured on a Beckman LS 6500 liquid scintillation counter. All radioligand binding data are averages of at least three replicate experiments.

**Acrylodan Labeling**—The labeling reactions contained 20 μM AChBP (monomer concentration), 100 μM acrylodan, in 100 μl of 0.1 M NaPO4 buffer, pH 7.0. Labeling reactions ran for 90 min at room temperature, after which unreacted label was removed by buffer exchange (4 × 2-ml washes) in Centricron YM-30 spin columns (Amicon). The degree of specific labeling was assessed by comparison with labeling of wild-type AChBP where the four cysteines are disulfide linked as cystines, after normalization to protein concentration by the Bradford assay. Specific labeling for each mutant was as follows: W53C, 90%; L112C, 95%; M114C, 91%; K139C, 88%; E157C, 76%; Y164C, 78%; Q178C, 93%; S182C, 79%. Stoichiometry of labeling for each preparation was estimated from a comparison of acrylodan concentration (by absorption at 372 nm, extinction coefficient ~16,400 M–1 cm–1) and protein concentra-
tration (by absorption at 280 nm, extinction coefficient \( \epsilon_{280} \)) for each mutant was as follows: \( \epsilon_{280} = 55,79\% \); L112C, 59\%; M114C, 57\%; K139C, 55\%; E157C, 55\%; Y164C, 48\%; Q178C, 88\%; S182C, 49\%.

**Spectrofluorometric Assays**—Steady-state emission spectra were measured at room temperature using a Jobin Yvon/Spex FluoroMax II spectrofluorometer (Instrument S.A., Inc., Edison, NJ) with the excitation and emission band widths set at 5 nm. The excitation wavelength for acrylodan was set at 359 nm, and emission was monitored between 380 and 600 nm. Saturating ligand concentrations were set at \( \geq 10 \) fold over the \( K_d \) for that ligand, or \( \geq 5 \) fold over the concentration of binding sites, whichever was greater. Final ligand concentrations were 2.5 \( \mu \)M for epibatidine, gallamine, \( \alpha \)-bungarotoxin, \( \delta \)-tubocurarine, metcurine, nicotine, and methyllycaconitine, and 25 \( \mu \)M for carbamylcholine. The concentration of binding sites in the spectrofluorometric assays was 0.5 \( \mu \)M. Binding saturation was verified by observation that additional increments in ligand concentration did not result in a further chromic shift or change in quantum yield. For binding site titration experiments, the concentration of binding sites was held constant at 1 \( \mu \)M for epibatidine and \( \alpha \)-bungarotoxin titrations, and 3 \( \mu \)M for \( \delta \)-tubocurarine and gallamine. Ligand was added in incremental amounts to produce 0.1 \( \mu \)M (for epibatidine and \( \alpha \)-bungarotoxin) or 0.5 \( \mu \)M (for \( \delta \)-tubocurarine and gallamine) increases until saturation was achieved. Titrations data are based on duplicate experiments; all other fluorescence emission data are an average of at least three replicate experiments. Relative quantum yields were determined from the areas under the emission and excitation bands set at 5 nm. The excitation wavelength was measured by reacting the preformed complex with a large excess of wild-type, unlabeled AChBP to scavenge the dissociated ligand, and observing the time course of the change in fluorescence emission.

**RESULTS**

**Characterization of the Expressed Protein**—The expressed cysteine-substituted AChBPs were monitored to ascertain assembly as a pentamer of the appropriate molecular weight by elution volume on fast protein liquid size-exclusion chromatography and comparison with corresponding data on wild-type preparations (37). Mutants analyzed further had \( >90\% \) of the protein eluting as a pentamer rather than as a higher order oligomer or aggregate or as a monomer. Dissociation constants for each AChBP Cys-substituted mutant (see Table I) were determined for two standard ligands, \( \alpha \)-bungarotoxin and (+)-epibatidine, using the scintillation proximity assay (a version of a traditional radioligand binding assay). Dissociation constants (\( K_d \)) for the snake \( \alpha \)-toxin were determined by direct saturation binding with \( ^{125}\text{I}-\alpha \)-bungarotoxin, whereas \( K_d \) values for epibatidine were determined by competition with the radiolabeled \( \alpha \)-toxin. \( K_d \) values for all the cysteine substitution mutants were within a factor of 5 of wild-type AChBP for \( \alpha \)-bungarotoxin and epibatidine. An exception was the 15-fold change for M114C. Irrespective of these differences in \( K_d \), the ligands retained high affinity for the mutant proteins, indicating that cysteine substitution at the positions studied does not affect the overall fold of the subunits or their assembly.

To quantitate sites and dissociation constants after fluoro-

**Stopped-flow Kinetics**—Stopped-flow kinetic experiments were conducted using an Applied Photophysics SX.18MV (Leatherhead, UK) stopped-flow spectrophotometer. Acrylodan-conjugated AChBP mutants were excited at 372 nm, and a cut-off filter at 420 nm was used to collect the fluorescence signal. Rates of binding of \( \alpha \)-bungarotoxin were estimated from the slope of plots of the observed rate of fluorescence change versus ligand concentration. Rates of dissociation of \( \alpha \)-bungarotoxin were measured by reacting the preformed complex with a large excess of wild-type, unlabeled AChBP to scavenge the dissociated ligand, and observing the time course of the change in fluorescence emission.

**Acetylcholine-binding Protein Sites**

**TABLE I**

| AChBP mutant | \( K_d \) \( \alpha \)-bungarotoxin \( M \) | \( K_d \) epibatidine \( M \) |
|-------------|------------------|------------------|
| WT         | 1.8E-09          | 1.6E-10          |
| W53C       | 9.3E-09          | 1.8E-10          |
| L112C      | 4.1E-09          | 3.0E-10          |
| M114C      | 4.1E-09          | 2.4E-10          |
| K139C      | 15.5E-09         | 2.2E-10          |
| E157C      | 3.1E-09          | 2.1E-10          |
| Y164C      | 2.2E-09          | 3.6E-10          |
| Q178C      | 2.8E-09          | 2.3E-10          |
| S182C      | 3.3E-09          | 6.9E-10          |
| V183C      | 7.3E-09          | 1.7E-10          |

**FIG. 2. Emission spectra of Q178C-acrylodan in the unligated state and after saturation with epibatidine or \( \alpha \)-bungarotoxin.** With no ligand present (dashed line) the emission peak is at 485 nm. \( \alpha \)-Toxin association enhances quantum yield and shifts the emission maximum in the blue direction (hypsochromic shift), whereas epibatidine quenches the fluorescence and shifts the emission maximum in the red direction (bathochromic shift). Excitation wavelength \( = 359 \) nm.

with respect to the number of binding sites based on five sites per pentameric assembly (Table II). The approach to full ligand occupation at the 5 sites and no further fluorescence change, as shown in Fig. 3, was not always linear. This likely reflects the slightly different affinities of the acrylodan-labeled protein and the fractional labeling achieved for the particular acrylodan conjugate. The abrupt intersection of the extrapolated titration lines in Fig. 3B reflects the high affinity of the \( \alpha \)-toxin complex, because virtually all of the added \( \alpha \)-bungarotoxin binds until the five sites are fully occupied. In some cases, we have estimated the \( \alpha \)-bungarotoxin dissociation constant from its component rate constants, showing that it retains its high affinity for the acrylodan-conjugated AChBPs (Table II).

**Effect of \( \alpha \)-Bungarotoxin Binding on Acrylodan Fluorescence Emission**—Although the subunits in AChBP are identical, the subunit interface can best be related to the heteromeric receptor where the C-loop face, which contains the vicinal cysteines at 187 and 188, corresponds to the \( \alpha \)1 subunit in muscle and the complementary face corresponds to that of the muscle \( \gamma \), \( \delta \), or \( \epsilon \) subunit. These subunit positions are shown in gray (\( \alpha \)) and orange (\( \gamma \), \( \delta \), or \( \epsilon \)), respectively, in Fig. 4.

Changes in acrylodan fluorescence emission initiated by
binding of \( \alpha \)-bungarotoxin are listed in Table III. In these fluorescence studies, chromic shifts of 2 nm or more in emission maxima were considered significant. \( \alpha \)-Bungarotoxin binding produced a large blue shift (15 nm) in emission from the fluorophore side chain buried internally to loop C, W53C, a region of the putative binding pocket (6, 8). Interestingly, minimal (M114C) or small (L112C) shifts in the opposite direction, indicating greater solvent accessibility, are induced in acrylodan-conjugated residues located apical to the binding pocket at the subunit interface. These residues are found on the subunit face complementary to the C-loop. Data from residues on the membrane side of the pocket (E157C, Y164C, and Q178C) reveal emission shifts consistent with a significant decrease in solvent exposure for a residue located directly internal to the pocket (Y164C), as well as those around the exterior face of the subunits (E157C and Q178C). Little to no change in solvent exposure on the membrane side of the pocket on the C-loop face was observed (K139C; see Fig. 4 for structural orientation). Some-what surprisingly, the largest chromic shift among the labeled sites for any ligand was a 22-nm blue shift at Q178C, by \( \alpha \)-bungarotoxin. This indicates a potentially extensive interaction on the \( \alpha \)-toxin with an area on the C-loop side of the interface, but at some distance from the pocket and well removed from the predicted binding site region for small ligands (6, 21–25). All other ligands assayed induce a chromic shift to longer wavelength at the Q178C site. These data distinguish the binding site or conformation for the \( \alpha \)-bungarotoxin-bound complex from the conformations of the smaller agonists and competitive antagonists. A minimal change in solvent exposure around the E157C site of acrylodan conjugation, relative to other ligands, is consistent with the greater solvent occlusion by \( \alpha \)-bungarotoxin on the C-loop positioned subunit face than the complementary face.

**Effect of Agonist Binding on Acrylodan Fluorescence Emission**—Epibatidine, nicotine, and carbamylcholine (Table IV) all induced blue shifts in acrylodan emission wavelength at the W53C and Y164C sites. Carbamylcholine, having more torsional flexibility in its bonds and occupying a smaller molar volume than the other two agonists, generally produced smaller shifts in emission wavelength. Epibatidine and nicotine decreased solvent exposure in the apical region of the pocket at the L112C position, whereas carbamylcholine did not influence this region. This class of ligands also exerted marked effects on solvent exposure at two positions of fluorophore conjugation presumed to be outside of the pocket; all three agonists significantly decreased polarity of the acrylodan environment at the E157C position, and increased polarity at the Q178C position. These changes in emission wavelength for residues distant from the pocket were also seen for the antagonists, with the emission shift of \( \alpha \)-toxin on the Q178C position being the notable exception. Little change in emission wavelength is evident for acrylodan conjugated at S182C and V183C. These side chains reside in a hydrophobic environment and presumably stay fixed in their locations after ligand binding.

**Effect of Alkaloid Antagonist Binding on Acrylodan Fluorescence Emission**—Gallamine, d-tubocurarine, metocurine, 

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**Table II**

| Mutant | Ligand       | Chronic shift | [Ligand]/[sites] | \( k_{on} \) | \( k_{off} \) | \( K_D \) |
|--------|--------------|---------------|-----------------|-------------|-------------|---------|
| W53C   | \( \alpha \)-Bungarotoxin | -15          |                | 1.095       | 2.0E + 05   | 8.0E - 03 | 40.0    |
| W53C   | Gallamine    | -11          |                | 1.133       | 1.098       | 8.0E - 03 |         |
| L112C  | Epibatidine  | -16          |                | 1.089       | 1.090       | 8.0E - 03 |         |
| E157C  | Epibatidine  | -10          |                | 1.095       | 6.0E + 05   | 8.0E - 03 | 13.0    |
| Y164C  | Epibatidine  | -9           |                | 1.095       | 6.0E + 05   | 8.0E - 03 |         |
| Y114C  | d-Tubocurarine | -22         |                | 1.095       | 6.0E + 05   | 8.0E - 03 |         |
| Q178C  | \( \alpha \)-Bungarotoxin | -22         |                | 1.095       | 6.0E + 05   | 8.0E - 03 |         |

FIG. 3. *Titration of binding sites and kinetics of association for \( \alpha \)-bungarotoxin at Q178C-acrylodan.* Equilibrium emission spectra are presented in A. The ratio of species in B was determined from the following equation: \( [I_{acq} - I_{acq}] - [I_{acq} - I_{acq}] = [I_{acq} - I_{acq}] / [I_{acq} - I_{acq}] \), where \( I \) is intensity at the specified wavelength and the subscripts \( 0, \infty \) denote the absence of ligand and saturating ligand conditions. In C the rate of association as measured by stopped-flow kinetics of \( \alpha \)-bungarotoxin association with Q178C-acrylodan was calculated from the slope plotted as the observed rate of fluorescence change versus concentration of \( \alpha \)-bungarotoxin.
methyllycaconitine, and metocurine (Table V) induced a blue shift in acrylodan emission wavelength from the W53C position, consistent with the bound ligand displacing solvent from this location. Metocurine induced a larger blue shift at this location, consistent with the bound ligand displacing solvent from the W53C position. This ligand decreases solvent accessibility to areas in the pocket (W53C and Y164C), as well as immediately apical to the pocket likely because of its large size (L112C and M114C), but appears to open up the regions examined between the pocket and the membrane (Q178C and K139C) at both subunit interfaces.

**Table III**

Fluorescence emission parameters of acrylodan-labeled AChBP mutants in the presence of α-bungarotoxin

| AChBP mutant | Acrylodan emission maxima (nm) | Chromic shift | Relative quantum yield |
|--------------|--------------------------------|---------------|-----------------------|
|              | (−) -Ligand α-Bungarotoxin |
| W53C         | 489 447                     | −15           | 1.46                  |
| L112C        | 499 503                     | 4             | 1.30                  |
| M114C        | 486 487                     | 1             | 1.04                  |
| K139C        | 491 492                     | 1             | 1.13                  |
| E157C        | 508 503                     | −5            | 1.36                  |
| Y164C        | 508 503                     | −5            | 1.43                  |
| Q178C        | 485 463                     | −22           | 1.36                  |
| S182C        | 482 484                     | 2             | 0.99                  |
| V183C        | 476 477                     | 1             | 0.98                  |

DISCUSSION

**Characteristics of Fluorescence Emission from Acrylodan-conjugated Cysteine Residues**—Employing cysteine substitution mutagenesis and selective fluorophore conjugation at the introduced cysteine in crystallographically defined protein templates expands structural analyses to solution-based considerations. When the endogenous cysteines in a protein are disulfide linked as cystines, the relatively selective tethering of acrylodan through a Michael addition to an introduced single cysteine nucleophile provides a site-directed and regionally localized spectroscopic handle. Upon excitation of acrylodan to form an excited-state dipole, its fluorescence emission is sensitive to reorientation of local neighboring or solvent dipoles. These polarity-sensitive fluorescence characteristics give rise to distinctive Stokes shifts for acrylodan conjugated to the protein, revealed in the difference between excitation and emission wavelengths (27). Whereas the number of cysteine substitutions on AChBP is still limited to regions around the ligand binding site ascertained from the crystal structure and a variety of mutagenesis and chemical modification studies, several interesting observations emerge that are predictive of the binding determinants of the site and potential conformational changes accompanying ligand binding.

**Fluorescence Characterization of Residues in the Unliganded Protein**—In the absence of ligand, emission maxima from the nine described acrylodan-labeled mutants reveal the Y164C and E157C positions to be in the most hydrophilic environment, with peak emission wavelengths at 508 nm. The crystal structure shows both of these native side chains to be on the surface of the protein largely exposed to the solvent. The fluorescent moiety of acrylodan is tethered through an extended thioether linkage and can explore a wider range of positions than the natural side chain. Presumably, extensive interaction with solvent molecules or proximal polar residues on the protein lowers the excited state energy of the fluorophore to yield longer wavelength emission.

In contrast to these relatively hydrophilic positions, acrylodan...
molecules tethered at M114C, Q178C, S182C, and V183C have emission maxima at the blue end of the spectrum, indicative of a hydrophobic environment and far lower solvent exposure. Referring to the crystal structure, the Q178C position is buried between two β-sheets and points somewhat inward to the protein core, and M114C in the apical entry area of the binding pocket is wedged into the subunit interface. S182C is located on the outside of the C-loop where it could fold over the pocket, and V183C is adjacent to S182C but points into the pocket. The crystal structure reveals a high degree of solvent accessibility at the S182C site, however, the fluorescence emission data for acrylodan conjugated at S182C suggest otherwise. In this case, it seems likely that the fluorophore is oriented toward the core of the protein, packing into a more hydrophobic area. This position is consistent with a minimal change in emission wavelength or quantum yield at this site upon ligand binding.

**Shifts in Emission Maxima Induced by Ligand Binding**—A comparison of the shifts induced by the three agonists (Table IV) and the four synthetic and natural alkaloid antagonists (Table V) does not reveal a pattern distinctive for agonists versus antagonists. Perhaps this is not unexpected, as the binding affinities of the various ligands and the structure of AChBP might best reflect that of a desensitized state of the receptor (5, 19). Nevertheless, several distinctive characteristics of ligand-induced changes in the emission spectra are evident. First, blue shifts are evident for residues positioned on the surface complementary to the C-loop containing subunit, Trp53 and Leu112 (Fig. 4, in orange). These positions of fluorophore conjugation are also found apical to the C-loop. Amino acid residues in the muscle γ- and δ-subunits conserved as γTrp55 and γLeu119, and δTrp57 and δLeu122, that are homologous to Trp53 and Leu112 in AChBP, appear as important determinants of agonist and antagonist binding (23). Hence, these residues are likely to be occluded from solvent when ligands bind, either by the ligand itself or through displacement by the side chain position of acrylodan. The spectroscopic changes observed are consistent with the Trp53 and Leu112 surface being within the proximal ligand binding region as seen for the HEPES site in the crystal structure (6).

A consistent change for the binding of all of these ligands is the 5–9-nm red (bathochromic) shift for acrylodan at the Q178C position. This shift is indicative of increased exposure to

### Table IV

| AChBP mutant | Acrylodan emission maxima (nm) | Chromic shift | Relative quantum yield |
|--------------|--------------------------------|---------------|------------------------|
| W53C         | 489                            | 484           | −5                     | 1.09                   |
| L112C        | 499                            | 483           | −16                    | 1.92                   |
| M114C        | 486                            | 487           | 1                      | 1.08                   |
| K139C        | 491                            | 489           | −2                     | 1.08                   |
| E157C        | 508                            | 498           | −10                    | 1.72                   |
| Y164C        | 508                            | 499           | −9                     | 1.70                   |
| Q178C        | 485                            | 493           | 8                      | 0.90                   |
| S182C        | 482                            | 484           | 2                      | 0.97                   |
| V183C        | 476                            | 478           | 2                      | 0.95                   |

### Table V

| AChBP mutant | Acrylodan emission maxima (nm) | Chromic shift | Relative quantum yield |
|--------------|--------------------------------|---------------|------------------------|
| W53C         | 489                            | 478           | −11                    | 1.20                   |
| L112C        | 499                            | 496           | −3                     | 1.02                   |
| M114C        | 486                            | 485           | −1                     | 1.05                   |
| K139C        | 491                            | 493           | 2                      | 0.96                   |
| E157C        | 508                            | 505           | −4                     | 1.01                   |
| Y164C        | 508                            | 505           | −3                     | 1.01                   |
| Q178C        | 485                            | 492           | 7                      | 1.00                   |
| S182C        | 482                            | 484           | 2                      | 0.93                   |
| V183C        | 476                            | 477           | 1                      | 0.94                   |

| AChBP mutant | Acrylodan emission maxima (nm) | Chromic shift | Relative quantum yield |
|--------------|--------------------------------|---------------|------------------------|
| W53C         | 489                            | 483           | −6                     | 1.09                   |
| L112C        | 499                            | 496           | −3                     | 1.02                   |
| M114C        | 486                            | 485           | −1                     | 0.97                   |
| K139C        | 491                            | 490           | −1                     | 1.08                   |
| E157C        | 508                            | 487           | −21                    | 1.55                   |
| Y164C        | 508                            | 499           | −9                     | 1.35                   |
| Q178C        | 485                            | 490           | 5                      | 1.03                   |
| S182C        | 482                            | 483           | 1                      | 0.97                   |
| V183C        | 476                            | 476           | 0                      | 0.97                   |

| AChBP mutant | Acrylodan emission maxima (nm) | Chromic shift | Relative quantum yield |
|--------------|--------------------------------|---------------|------------------------|
| W53C         | 489                            | 482           | −4                     | 0.94                   |
| L112C        | 499                            | 495           | −4                     | 1.15                   |
| M114C        | 486                            | 485           | −1                     | 1.06                   |
| K139C        | 491                            | 492           | 1                      | 1.05                   |
| E157C        | 508                            | 495           | −13                    | 1.43                   |
| Y164C        | 508                            | 501           | −7                     | 1.30                   |
| Q178C        | 485                            | 491           | 6                      | 1.04                   |
| S182C        | 482                            | 483           | 1                      | 0.95                   |
| V183C        | 476                            | 476           | 0                      | 0.94                   |

| AChBP mutant | Acrylodan emission maxima (nm) | Chromic shift | Relative quantum yield |
|--------------|--------------------------------|---------------|------------------------|
| W53C         | 489                            | 482           | −7                     | 1.17                   |
| L112C        | 499                            | 490           | −9                     | 1.53                   |
| M114C        | 486                            | 482           | −4                     | 1.20                   |
| K139C        | 491                            | 497           | 6                      | 1.05                   |
| E157C        | 508                            | 500           | −8                     | 1.54                   |
| Y164C        | 508                            | 503           | −5                     | 1.38                   |
| Q178C        | 485                            | 491           | 6                      | 1.01                   |
| S182C        | 482                            | 484           | 2                      | 0.98                   |
| V183C        | 476                            | 477           | 1                      | 0.95                   |
Disulfide linkages for model (23).

N. mossambica (energy minimized sensitive to the structures of the bound ligand (Tables III shift, its peak emission wavelength appears to be the most in all cases acrylodan at position 157 shows a hypsochromic shift. However, the region of E157C (155–160) is unresolved in the crystal structure suggesting a lack of structural landmarks and binding determinants on the two molecules. In α-bungarotoxin, disulfides link residues 3 with 23, 29 with 33, 16 with 44, 48 with 59, and 60 with 65. In the N. mossambica mossambica α-toxin, disulfides link residues 3 with 24, 17 with 41, and 43 with 54. α-Bungarotoxin is a 74-amino acid peptide, whereas N. mossambica mossambica has 62 amino acids.

### Table VI

**Sequence alignments of interacting residues from AChBP and nAChR subtypes**

| Segment A     | Segment B     | Segment (loop) C |
|---------------|---------------|-----------------|
| AChBP α7 (chick) | 89YNNAISKEPV97 | 14WHHSHREIS151  |
| AChBP α1 (mouse) | 92YNNASDER196 | 14WTYGG152      |
| N. mossambica mossambica α-toxin | 93YNNADGD199 | 14WTYDGHSV156    |
| N. mossambica mossambica β-toxin | 89YNNAISKEPV97 | 176VTQK8NSVTSYCCPE-AYED194 |
| N. mossambica mossambica γ-toxin | 92YNNASDER196 | 178PGKRTESFYECCKEPYPP196 |
| N. mossambica mossambica δ-toxin | 93YNNADGD199 | 180EARSGWKHWVFGCCPTPILYDIT200 |
| N. mossambica mossambica ε-toxin | 89YNNAISKEPV97 | 188, 190, 193 in the C-loop-containing subunit and 53, 112, and 198, and with residues 55, 119, and 176 on the complementary face, are in close proximity, it is

The bathochromic shift in emission at the Q178C position might also be compared with the hypsochromic shift at E157C. The same clockwise subunit rotation alluded to above could cause the E157C side chain to become buried or less exposed to solvent. In general, whereas the Q178C-acrylodan conjugate shifts in a red direction, acrylodan at E157C shows a blue, hypsochromic shift. However, the region of E157C (155–160) is unresolved in the crystal structure suggesting a lack of structural rigidity and thermal stability (6). Moreover, lysine substitution mutagenesis also reveals that this region lacks a defined β-sheet structure (29). Interestingly, whereas in all cases acrylodan at position 157 shows a hypsochromic shift, its peak emission wavelength appears to be the most sensitive to the structures of the bound ligand (Tables III–V).

**α-Bungarotoxin Induced Shifts in Acrylodan Emission**

α-Bungarotoxin induced shifts in acrylodan emission suggest a kinetic limitation in formation of a distinct conformational state. The slower rates, reported long ago for the muscle type receptor (30, 31), are also observed with AChBP (19). No evidence for wavelength shifts in the direction of solvent exclusion upon α-bungarotoxin binding is found for residues in the region apical to the C-loop (L112C and M114C). If anything, these two residues apical to the binding pocket show an increase in solvent exposure. In contrast, unusually large hypsochromic shifts are observed for acrylodan at W53C and Q178C. Smaller shifts are seen for the E157C and Y164C side chains. Taken together, these findings reveal that α-bungarotoxin binds from the membrane side of the C-loop in contrast to the small ligands that enter from the apical side.

Our findings on the distinctive changes in acrylodan emission induced by α-bungarotoxin at multiple locations around its binding site might be compared with studies on residue proximity to the α-toxin binding site on the muscle receptor and the neuronal α7 receptor. Thermodynamic mutant cycle analysis has been used to analyze pairwise interactions between α-toxin (Naja mossambica mossambica) residues and receptor subunit determinants that influence binding affinity (23, 32). These studies, which analyze the interaction or linkage free energy between paired residues, reveal that the tip of loop II of the N. mossambica mossambica α-toxin, with its extended Arg23 side chain (see Fig. 5), interacts with α-subunit residues 188, 190, and 198, and with residues 55, 119, and 176 on the γ-subunit. Because the homologous residues in AChBP (Table VI), 183, 188, and 193 in the C-loop-containing subunit and 53, 112, and 171 on the complementary face, are in close proximity, it is

![Acetylcholine-binding Protein Sites](image-url)

Fig. 5. Structures of α-bungarotoxin (NMR, Protein Data Bank code 1ID) and N. mossambica mossambica α-toxin from N. mossambica mossambica (energy minimized model (23)). Disulfide linkages for α-bungarotoxin and for N. mossambica mossambica α-toxin are shown along with residues that are structural landmarks and binding determinants on the two molecules. In α-bungarotoxin, disulfide link residues 3 with 23, 29 with 33, 16 with 44, 48 with 59, and 60 with 65. In the N. mossambica mossambica α-toxin, disulfide link residues 3 with 24, 17 with 41, and 43 with 54. α-Bungarotoxin is a 74-amino acid peptide, whereas N. mossambica mossambica has 62 amino acids.

* Peptides (residues 178–196) studied as described in Ref. 22.

* Peptides from Torpedo californica (residues 181–198 and 182–202) studied as in Refs. 21 and 33.

* Segment F*, whereas not recognized in all models, is in spatial proximity to segment F and these two regions have a synergistic influence on α-conotoxin association (20, 36).
likely that this long loop intersects or bisects at the subunit interface. Similar coupling was found for the arginine at the tip of loop II in α-cobratoxin in its interaction with C-loop residues in the α7 nicotinic receptor (25).

A similar conclusion was reached through crystallographic and NMR studies (21, 22, 24, 33), which examined the structure of α-subunit peptides of 13 and 21 amino acid residues from the C-loop when bound to the α-toxin. Although the limited segment of peptide studied enabled a direct examination of only a small fraction of the potential interacting surface of the receptor subunits with α-toxin, the position of the C-loop with respect to the surrounding subunit interface allowed constraints to be placed on the positions of neighboring residues not on the C-loop itself. Hence, it was proposed that Arg26 at the loop tip in α-cobratoxin and the choline moiety in acetylcholine are sandwiched between the aromatic groups of the C-loop at α Tyr176 and α Tyr190 and the complementary face at γ Trp57 and γ Leu139 (33). Other NMR studies with α-bungarotoxin and a C-loop peptide from the α7 subunit of 19 residues show similar interactions with the C-loop extending from the loop tip at Arg26 to residue 40 (22). These studies with the synthetic peptide also identify a potential contact zone for loop I in the α-toxin.

Mutant cycle analysis with N. mossambica mossambica α-toxin allows for an analysis of interacting residue pairs in additional loops or regions of the α-toxin structure and the receptor subunit interface. Strong interactions were found between Lys47 and Arg33 on loop II of N. mossambica mossambica α-toxin with γ Glu176, γ Leu119, and γ Trp57 of the muscle receptor (Table VI). Arg26 in N. mossambica mossambica α-toxin does not partner with γ-subunits, but with the α-subunit face (23, 32). Hence, with N. mossambica mossambica α-toxin, residues N-terminal to Arg26 and proximal to loop III (Fig. 5) have a primary interaction with the γ-subunit, whereas residues C-terminal to Arg26 and proximal to loop I interact with the α-subunit. Finally, interaction energies are found between loop I (Glu176 and Glu150) and residues α Val119, α Tyr190, and α Tyr176, as well as between loop III (Lys176 and Lys150) and γ Glu176, γ Asp184, and γ Trp55. Thus, the disc-shaped α-toxin likely lies with an orientation close to parallel with the membrane rather than perpendicular to it. This toxin orientation is also consistent with the structure-activity studies of Menez and colleagues (34) with the short α-neurotoxins. In fact, the proposed residue placement of Samson et al. (35) places loop I and the portion of loop II facing loop I in the vicinity of Gln178, consistent with N. mossambica mossambica residue pairs.

Our findings suggest that acetylradon extending from the Q178C may be occluded from solvent by loop I interactions. The α-carbon positions of Gln178 and Trp53 are ~26 Å apart as measured through space, revealing that α-toxin binding covers a substantial area of the receptor subunit interface. α-Bungarotoxin acquires much of its interaction energy through van der Waals contact with the subunit face bearing the C-loop rather than the complementary γ, δ surface. The data presented here support recent NMR-based models of α-bungarotoxin binding (22, 33), where the primary interaction surface with α-toxin arises from the subunit face bearing the C-loop and involves loop I and the position of loop II that faces loop I.

An alternative explanation of our data that should be considered relates to the α-bungarotoxin actually perturbing the C-loop position so that it protrudes radially. In this case, the side chain on residue 178 is forced in the direction of the protein core with the partial opening of the C-loop cover. A potential hinge region in the vicinity of residue 178 would be influenced by the large α-bungarotoxin molecule dislodging and opening the C-loop or flap, whereas the flap may close down upon the smaller ligands when bound, exposing the side chain in the 178 hinge region. Such an explanation would be consistent with the opposing directions of the emission wavelength shift of acrylodan at Q178C elicited by small ligands and α-toxin (Fig. 2).

Whereas several docking models of the α-toxin-receptor interaction have been proposed (21–23, 25, 33, 35), experimental data positioning the α-toxin or other antagonists and agonists are only beginning to emerge. The fluorescence approach, while lacking atomic level resolution, provides spectroscopic parameters useful for further monitoring the interaction and local conformational changes induced around the inserted fluorophore of the complex in solution. Such solution-based studies should also allow for subsequent analyses of torsional and segmental motion in the respective localized regions of the interacting molecule (18). Finally, through a comparison of the spectral perturbations achieved by various classes of alkaloid and peptide ligands, the positions of the bound ligand and differences in AChBP conformation associated with ligand binding can be deduced.

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