Subunit Interface Selectivity of the α-Neurotoxins for the Nicotinic Acetylcholine Receptor*

(Received for publication, December 7, 1998, and in revised form, January 28, 1999)

Hitoshi Osaka‡, Siobhan Malany§, Joan R. Kanter, Steven M. Sine¶, and Palmer Taylor

From the Department of Pharmacology 0636, University of California, San Diego, La Jolla, California 92093, and the Receptor Biology Laboratory, Department of Physiology and Biophysics, Mayo Foundation, Rochester, Minnesota 55905

Peptide toxins selective for particular subunit interfaces of the nicotinic acetylcholine receptor have proven invaluable in assigning candidate residues located in the two binding sites and for determining probable orientations of the bound peptide. We report here on a short α-neurotoxin from Naja mossambica mossambica (NmmI) that, similar to other α-neurotoxins, binds with high affinity to αγ and αδ subunit interfaces (K_D ~ 100 pM) but binds with markedly reduced affinity to the αε interface (K_D ~ 100 nM). By constructing chimeras composed of portions of the γ and ε subunits and coexpressing them with wild type α, β, and δ subunits in HEK 293 cells, we identify a region of the subunit sequence responsible for the difference in affinity. Within this region, γ-Pro-175 and γ-Glu-176 confer high affinity, whereas Thr and Ala, found at homologous positions in ε, confer low affinity. To identify an interaction between γ-Glu-176 and residues in NmmI, we have examined cationic residues in the central loop of the toxin and measured binding of mutant toxin-receptor combinations. The data show strong pairwise interactions or coupling between γ-Glu-176 and Lys-27 of NmmI and progressively weaker interactions with Arg-33 and Arg-36 in loop II of this three-loop toxin. Thus, loop II of NmmI, and in particular the face of this loop closest to loop III, appears to come into close apposition with Glu-176 of the γ subunit surface of the binding site interface.

The nicotinic acetylcholine receptor (nAChR) is found in muscle is a pentamer composed of four homologous subunits present in the stoichiometry α2βγδ (fetal subtype) or α2βεδ (adult subtype). The subunits are arranged in a circular manner to surround a central channel in the order, αγδεβ or aεδβγ (1–3). The two binding sites for agonists, competitive antagonists, and the slowly dissociating α-neurotoxins are formed at interfaces between the αδ and αγ(ε) subunit pairs. The extracellular domain in each subunit is formed principally from the amino-terminal 210 amino acids, which is followed by four membrane-spanning domains. Residues within the amino-terminal 210 amino acids have been shown to be the major contributors to the ligand binding sites and for dictating the order of assembly of subunits.

Three segments of the α subunit, well separated along the linear sequence, harbor major determinants for ligand binding; these segments contain the key residues around Tyr-93, between Trp-149 and Asp-152, and spanning the region from Val-188 through Asp-200 (see Refs. 3 and 4 for reviews). Similarly, four discontinuous segments of the non-α subunits, appearing on the opposite face of the subunit, contain major determinants for ligand selectivity; in the γ subunit these segments contain the key residues Lys-34, between Trp-55 and Glu-56, between Ser-111 and Tyr-117, and between Phe-172 and Asp-174.

Since the early demonstration of irreversible neuromuscular blockade by the peptide from snake venom, α-bungarotoxin (5), and the use of labeled α-neurotoxins to identify the nAChR (6), these toxins have been the primary ligands employed for the identification and characterization of the muscle nAChR. Amino acid sequences are available for nearly 100 members of the α-neurotoxin family, which show a common basic structure consisting of three polypeptide loops emerging from a small globular core (7). α-Neurotoxins can be divided into the short (4 disulfide bonds and 60–62 residues) and long neurotoxins (5 disulfide bonds and 66–74 residues). Crystal and solution structure determinations reveal similar tertiary structures. Although these structurally well defined toxins are known to bind to the subunit interfaces (αε or αγε and αδ), typically with a K_D = 100 pM, little is known about their precise orientation with respect to the subunits that form the interfaces.

Points of attachment of α-neurotoxin within the nAChR binding sites have been examined by cross-linking chemically modified (8, 9) or photoactivatable derivatives of α-neurotoxin (10–13) and by simple ultraviolet irradiation without chemical modification (14). These labeling studies have suggested contacts with both α and non-α subunits at the binding sites (see Refs. 2, 3, and 15 for reviews). Mutagenesis studies have also identified candidate residues in the principal loops of the α (16) and non-α subunits (17) that contribute to α-toxin binding. Although most α-toxins do not distinguish between the two sites on the receptor, an α-toxin from the venom of Naja mossambica mossambica (NmmI) distinguishes between the two sites of the Torpedo receptor (18). Thus NmmI emerges as a potentially valuable ligand for determining regions of close approach between α-toxins and the non-α subunits at the binding site. Previous work showed that the αε and αδ binding sites of the fetal mouse receptor exhibit similar affinities for NmmI (19). However, certain mutations in the NmmI toxin structure, and surprisingly also in the nAChR α subunit common to both sites, resulted in nonequivalent reductions in affinity at the αε and αδ binding sites (19). Here we examine binding of recombinant NmmI α-toxin to fetal and adult mouse AchRs and find that the affinity of NmmI for the αε interface is 3 orders of
magnitude lower than for the $\alpha\gamma$ and $\alpha\delta$ interfaces. Using subunit chimeras and site-directed mutations in $\gamma$ and $\epsilon$ subunits, we show that the enhanced affinity conferred by the $\gamma$ over the $\epsilon$ subunit arises from Pro-175 and Glu-176 in the $\gamma$ subunit. Mutant cycle analysis shows that Glu-176 interacts with cationic residues in loop II of the NmmI $\alpha$-toxin.

**EXPERIMENTAL PROCEDURES**

**Materials**—$\alpha$-Conotoxin MI was purchased from American Peptide Company. $^{125}$I-labeled $\alpha$-bungarotoxin ($\alpha$-BgTx) (specific activity $\approx 16\ \mu$Ci/ng) was a product of NEN Life Science Products.

**NmmI Expression and Purification**—A double-stranded synthetic NmmI cDNA containing the $\alpha$-erabutoxin signal sequence and strategically placed restriction sites was subcloned into pEZZ vector encoding two IgG binding proteins from Staphylococcal protein A. The Staphylococcal protein A-NmmI fusion protein was expressed using *Escherichia coli* HB 101, cleaved, and purified as described in Ackermann and Taylor (19).

**Construction of Mutant nAChR—cDNAs encoding mouse nAChR subunits were subcloned into a cytomegalovirus-based expression vector, pRBG4. All mutations were introduced using Quick Change Site-Directed Mutagenesis Kit (Stratagene) or by bridging two introduced or natural restriction sites with double-stranded oligonucleotides.**

**Cell Transfections—cDNAs encoding the wild type and mutant subunits were transfected into human embryonic kidney (HEK 293) cells using Ca.$\text{PO}_4$-phosphate. Cells were harvested in phosphate-buffered saline, pH 7.4, containing 5 mM EDTA, 2–3 days after transfection. They were briefly centrifuged, resuspended in potassium-Ringer’s buffer, and divided into aliquots for binding assays.**

**Ligand Binding Measurements—Cells were harvested in phosphate-buffered saline, pH 7.4, containing 5 mM EDTA, 2–3 days after transfection. They were briefly centrifuged, resuspended in potassium-Ringer’s buffer, and divided into aliquots for binding assays.**

**Rate Measurements—The association rate for $^{125}$I-labeled $\alpha$-bungarotoxin was measured using a $\alpha$-bungarotoxin concentration of 20 nM. At the specified time, cells were washed with 30 mM carbamylcholine in K$^+$-Ringers solution and then washed two times with K$^+$-Ringers solution alone and counted. For the measurement of dissociation rates, we equilibrated the surface receptor with 40 nM of $^{125}$I-labeled solution alone and counted.**

**RESULTS**

**Insensitivity of the $\alpha\epsilon$ Site to NmmI**—Certain competitive antagonists distinguish between the two binding sites of the nAChR because of species or subtype differences in the non-$\alpha$ subunits that form the $\alpha\gamma$, $\alpha\delta$, and $\alpha\epsilon$ binding sites. We therefore compared binding of NmmI to form cell surface receptors with the composition $\alpha\beta\gamma\delta$ and $\alpha\beta\delta\epsilon$. $\alpha$-Neurotoxin (NmmI) binding was measured by competition with an initial rate of $\alpha$-bungarotoxin binding. $k_{ob}/k_{max}$ is the ratio of initial rates for $^{125}$I-labeled $\alpha$-bungarotoxin binding in the presence and absence of NmmI. Data are plotted according to the equation:

$$k_{ob}/k_{max} = \frac{k_{r_{bgd}}([\text{NmmI}][\text{NmmI}] + K_{bgd}) + 0.5 k_{r_{ae}}([\text{NmmI}] \langle K_{ae} \rangle)}{k_{r_{ae}} + k_{r_{bgd}}},$$

where $k_{r_{ae}}$ and $k_{r_{bgd}}$ are the $\alpha$-bungarotoxin association rates for the $\alpha\delta$ and $\alpha\epsilon$ sites, $K_{ae}$ and $K_{bgd}$ are the equilibrium dissociation constant for NmmI of the respective sites, and $k_{max} = 0.5 k_{r_{ae}} + 0.5 k_{r_{bgd}}$.

**Insensitivity of the $\alpha\gamma$ Site for NmmI**—Certain competitive antagonists distinguish between the two binding sites of the nAChR because of species or subtype differences in the non-$\alpha$ subunits that form the $\alpha\gamma$, $\alpha\delta$, and $\alpha\epsilon$ binding sites. We therefore compared binding of NmmI to form cell surface receptors with the composition $\alpha\beta\gamma\delta$ and $\alpha\beta\delta\epsilon$. $\alpha$-Neurotoxin (NmmI) binding was measured by competition with an initial rate of $\alpha$-bungarotoxin binding. $k_{ob}/k_{max}$ is the ratio of initial rates for $^{125}$I-labeled $\alpha$-bungarotoxin binding in the presence and absence of NmmI. Data are plotted according to the equation:

$$k_{ob}/k_{max} = \frac{k_{r_{bgd}}([\text{NmmI}][\text{NmmI}] + K_{bgd}) + 0.5 k_{r_{ae}}([\text{NmmI}] \langle K_{ae} \rangle)}{k_{r_{ae}} + k_{r_{bgd}}},$$

where $k_{r_{ae}}$ and $k_{r_{bgd}}$ are the $\alpha$-bungarotoxin association rates for the $\alpha\delta$ and $\alpha\epsilon$ sites, $K_{ae}$ and $K_{bgd}$ are the equilibrium dissociation constant for NmmI of the respective sites, and $k_{max} = 0.5 k_{r_{ae}} + 0.5 k_{r_{bgd}}$.

**Molecular Basis of Insensitivity of the $\alpha\epsilon$ Site for NmmI**—The $\gamma$ and $\epsilon$ subunits show high sequence identity in the extracellular domains (54% in mouse), and homologous residues should have virtually identical locations for their $\alpha$-carbon backbone positions. Yet, NmmI binds 1000-fold more tightly to the $\gamma$ than to the $\epsilon$ site. To determine the structural basis of NmmI selectivity, we constructed subunit chimeras containing portions of the $\gamma$ subunit substituted into the $\epsilon$ subunit. Each chimera was coexpressed with complementary $\alpha$, $\beta$, and $\delta$ subunits, followed by measurements of NmmI binding.
We first screened with chimeras containing γ sequence from the amino terminus to junctions ranging from positions 74 to 173 of the ε subunit. Each of these chimeras confers low affinity for NmmI, characteristic of the wild type αε site (Fig. 3), indicating that NmmI selectivity arises from residues carboxyl-terminal to position 173. By contrast, moving the chimera junction just four residues to position 177 increases NmmI affinity to that of the native αγ site (KD = 80 pM; Fig. 3). Thus residue differences between positions 174 and 177 confer NmmI selectivity for the γ over the αε site.

Sequence comparison of the γ and ε subunits reveals only two mismatched residues between positions 174 and 177 (Fig. 4A). We therefore constructed point mutations at these two positions of the γ subunit and measured NmmI binding to the resulting mutant receptors. The point mutations γP175T and γE176A reduce affinity to values intermediate to those of the wild type αγ and αε sites (P175T, KD,wt/KD,mt = 36; E176A, KD,wt/KD,mt = 16; Fig. 4, Table I). Combining the two mutations into a single γ subunit reduces affinity to approach that of the wild type αε site (Fig. 4, Table I). The converse double mutation in the ε subunit, εT176P and εA177E, increases NmmI affinity to equal that of the wild type αγ site (Fig. 4, Table I). Thus, the residue pairs at homologous positions γPro-175/εThr-176 and γGlu-176/εAla-177 fully account for the 1000-fold selectivity of NmmI for the αγ over the αε site.

Sequence alignment shows that the δ subunit contains the same residues in this region as the γ subunit, consistent with high affinity of the αδ site. We therefore attempted to produce a low affinity αδ site by introducing the residue determinants in ε that reduce γ affinity into equivalent positions of the δ subunit. The mutations δP181T and δE182A, singly or combined, do not affect appreciably NmmI affinity (Table I), indicating that substitutions of other residues unique to the ε subunit into the δ subunit are required to decrease affinity of NmmI for the αδ site.

*Residues in NmmI That Interact with Selectivity Determinants in the γ and ε Subunits—* Because Pro-175 of the γ subunit likely orient Glu-176 to come into close apposition with a cationic residue on the NmmI toxin, we asked whether an anionic residue at the homologous position to 176 in the ε subunit stabilizes a cationic residue in the central loop of NmmI. Because Coulombic interactions can be effective over relatively long distances, and both attractive (opposite charges) and repulsive (like charges) forces can be generated, we measured binding of the mutant toxins, R27E, R33E, and R36E to receptors containing the mutation γE176K. Each pair of receptor-toxin mutations is equivalent to a charge reversal between receptor and toxin, and should the distance relationships be appropriate, charge reversal could preserve a stabilizing interaction, if Coulombic forces prevail.

Among the cationic residues in NmmI, Lys-27 showed the strongest interaction with γGlu-176. The receptor mutation

---

**Fig. 2.** Kinetics of 125I-labeled α-bungarotoxin association with the nAChR expressed as αβγδ and αβδε in HEK cells. Top panel, association of 5 nM 125I-labeled α-bungarotoxin with 200 pM wild type αβγδ (●) and αβδε(CO) receptors. The data for αβγδ are fit by a single exponential approach to equilibrium with a k on of 3.6 ± 0.7 × 10^6 M⁻¹ min⁻¹, whereas for αβδε a two-exponential fit of equal amplitudes is used k on of 3.9 ± 0.5 × 10^6 M⁻¹ min⁻¹ and 1.0 ± 0.2 × 10^6 M⁻¹ min⁻¹. Bottom panel, dissociation of 125I-labeled α-bungarotoxin after equilibration of 40 nM toxin with 20 pM receptor, washing twice with K⁺-Ringers solution, and resuspending with dilution in K⁺-Ringers solution. Averaging three such dissociation experiments yields k off = 2.5 ± 0.5 × 10⁻⁶ min⁻¹ for αβγδ and 2.6 ± 0.6 × 10⁻⁶ min⁻¹ for αβδε.

**Fig. 3.** NmmI α-toxin association with the nAChR formed by transfection of cDNAs encoding chimeras of the γ and ε subunits, along with α, β, and δ subunits. Left, schematic representation of chimeric cDNAs formed between the γ and ε subunits. (γ74ε) denotes that the amino-terminal 74 amino acids are constructed from γ subunit (shaded), and residues carboxyl-terminal to this position come from ε subunits (nonshaded). M1-4 denotes the putative transmembrane regions. Right: The dashed vertical line represents the K D for NmmI binding to the wild type αγ interface. The dashed bars show the actual K D for each chimera (top ruler) and the log [KD,mt/KD,wt] (bottom ruler). ΔΔG values can be obtained from multiplying by 2.3 RT.
Dissociation constants were calculated from competition with the initial rate of the $^{125}$I-labeled $\alpha$-bungarotoxin binding. Receptor was expressed as $\alpha$, $\beta$, or $\delta$ by transfection of cDNAs encoding four respective sets of subunits. $K_D$ is dissociation constant for $\alpha$, $\beta$, or $\delta$ sites by fitting a two-site analysis. The ratios of dissociation constants of mutant (mt) to wild type (wt) were calculated using an average or mean value of at least two measurements involving separate transfections. $\Delta G$ is free energy of binding calculated from Equation 1 in the text. $\Delta G_{INT}$ was calculated using Equation 2 in the text. Values less than unity were inverted and indicated with a minus sign.

### Table I

| Receptor | Toxin | $K_D$ | $K_D$ | $\Delta G$ | $\Delta G_{INT}$ |
|----------|-------|-------|-------|-----------|-----------------|
|          |       | $\alpha \beta \delta$ |       |           |                 |
| WT       | WT    | 0.14  | 0.14  | 1.0       | 0.0             |
| $\gamma$P175T | WT | 0.14  | 5.1   | 0.49      | 0.0             |
| $\gamma$E176A | WT | 0.068 | 2.2   | 1.6       | -0.42           |
| $\gamma$E176K | WT | 0.22  | 93    | 0.36      | 0.27            |
| $\delta$P173E176A | WT | 0.05  | 41    | 0.93      | -0.60           |
| $\delta$E182A | WT | 0.13  | 0.13  | 0.93      | -0.04           |
| $\delta$P181T/E182A | WT | 0.13  | 0.13  | 0.93      | -0.04           |
| $\delta$P181T/E182/S34K | WT | 0.11  | 0.11  | 0.79      | -0.14           |
| WT       | R27E  | 13.8  | 54    | 13        | 1.5             |
| WT       | R33E  | 95    | 2,200 | 680       | 3.9             |
| WT       | R36E  | 30    | 3500  | 210       | 3.1             |
| $\gamma$E176K | WT | 1.3   | 1.3   | 1.3       | -5.9            |
| $\gamma$E176K | R27E | 13    | 1.3   | 1.3       | -2.7            |
| $\epsilon$P173E176A | R27E | 13    | 1.3   | 1.3       | -2.2            |
| $\alpha\beta\delta$ | R27E | 0.14  | 0.11  | 1.6       | 0.0084          |

### Fig. 4

Assignment of the residues contributing to $\epsilon$ subunit insensitivity to NmmI binding. A, top, the junctions of two chimeras, $\gamma$173 and $\epsilon$177. The shaded bar represents $\gamma$ subunit sequence fol- lowed by the nonshaded $\epsilon$ subunit. Junctional amino acids are shown by the bar. Amino acids from $\epsilon$ are designated in italics. Numbering is for $\gamma$ subunit. Only two differences at positions 175 and 176 ($\epsilon$176/177) exist in this region. A, bottom, mutations (*) were introduced at $\gamma$ or $\epsilon$ subunits to examine the contributions at these two positions. $K_D$ and the $K_D$/mt are shown in a logarithmic scale as described in Fig. 2 B, sequence alignment around 175 and 176 in the $\gamma$ subunit. The super- scripts in italics show reported determinants for binding of various li- gands. Superscripts are: waglerin, W (30); $\alpha$-conotoxin MI, CM (29); anionic residues for agonists, A (25, 26), and acetylcholine, ACh (38). $\gamma$E176K decreases affinity of the $\alpha\gamma$ site for NmmI by nearly 3 orders of magnitude. Similarly, the NmmI mutation K27E decreases affinity of NmmI for the $\alpha\gamma$ site by approximately 2.5 orders of magnitude. However, combining both $\gamma$E176K and K27E results in a complex that is more stable by 4.5 orders of magnitude. The loss of energy, $\Delta G$, arising from substitution from wild type into mutant is calculated from the dissociation constant ($K_D$) as follows.

$$\Delta G = RT \ln \frac{K_D^m}{K_D^{wt}}$$

(Eq. 1)

The coupling energy, $\Delta G_{INT}$, is defined in terms of the respective dissociation constants ($K_D$) of the complexes,

$$\Delta G_{INT} = RT \ln \frac{K_D^{mt} \times K_D^{wt}}{K_D^{wt} \times K_D^{mt}} = \frac{K_D^{wt} \times K_D^{mt}}{K_D^{mt} \times K_D^{wt}} - \ln \frac{K_D^{wt} \times K_D^{mt}}{K_D^{mt} \times K_D^{wt}}$$

(Eq. 2)

where the $\Delta G^*$ values are the standard free energies for formation of the toxin/receptor complex. If the mutations do not interact, the two differences in standard free energies should be equal because the effect of mutating the receptor should be independent of whether or not toxin is mutated (Equation 3). Similarly, mutating the toxin should be independent of the receptor mutations (Equation 4). On the other hand, if the two
mutations interact, the bracketed differences should not be equal. When applied to the γE176K/K27E pair, mutant cycle analysis reveals a substantial free energy of interaction of −5.9 kcal/mol (Table I). Similar analysis of the NmmI mutations, R33E and R36E, reveal modest interaction-free energies of −2.7 and −2.2 kcal/mol, respectively (Table I). The overall results indicate close approach of cationic residues in the central loop of the NmmI toxin and γGlu-176 of the binding site, with the most proximal charged residue being Lys-27 of the toxin.

The α-neurotoxins are a family of three-fingered peptide toxins found in venom of elapid snakes (7). They have proven to be invaluable tools for the isolation and study of the nAChR because of their high affinities and slow rates of dissociation from the receptor (5, 6). Although the isolated α-subunit of the receptor retains the capacity to bind α-BgTx, whereas isolated β, γ, or δ subunits do not, the α-toxins bind with far lower affinity to the α subunit than to the intact receptor. Moreover, small agonists and antagonists do not compete with α-toxin binding to the isolated α subunit at expected concentrations (23). These observations point to a predominant, but not sole, contribution to the α-neurotoxin binding coming from the α subunit. Our previous work showed that Val-188, Tyr-190, Pro-197, and Asp-200 of a subunit contribute to NmmI binding (19). Also glycosylation at positions 189 and 187, yielding oligosaccharides uniquely found in cobra and mongoose nAChR, reduced α-BgTx binding substantially (16).

Although the α subunit appears to be the predominant site of α-toxin binding, chemical cross-linking and mutagenesis studies show that non-α subunits are close to the site of α-neurotoxin binding (Refs. 8–14, 16, 17, and see Refs. 3 and 15 for reviews). The results described here further illustrate the role of neighboring non-α subunits in contributing to high affinity α-toxin binding, as NmmI binds to αε interfaces of the adult type of nAChR (αβδε) with 3 orders of magnitude lower affinity than to the αγ and αδ interfaces of the fetal receptor (αβγδ). Binding studies, initially using chimeras and subsequently point mutants, show that εThr-176/eAla-177 (Pro/Glu in γδ) contribute entirely to insensitivity of the αε interface to NmmI. The observation that α-bungarotoxin association is only slightly affected by the γ and ε sequence differences suggests that this region of the γ, ε, and δ subunits is not used equivalently for stabilization of the entire family of bound α-neurotoxins. At the present time, it is unclear whether stabilization from this region is unique to some of the short α-neurotoxins, or the long α-neurotoxins, such as α-bungarotoxin, acquire the bulk of their stabilization energy from other portions of the structure. Distinct differences in specificity between the short and long neurotoxins have been noted for the α7 subtype of nAChR (24).

Residues at the γ175/176 positions were previously unrecognized as determinants of ligand binding. However, they are immediately adjacent to γAsp-174, which was shown by cross-linking to be ~9 Å away from Cys-192/193 in the α subunit (25,

![FIG. 5](image1.png) **Structure of an α-neurotoxin from Naja mossambica mossambica (NmmI) and mutations studied.** An energy minimization model of NmmI described in (19), is shown with the mutated side chains of loop II. The concave face of the toxin is facing the viewer.

![FIG. 6](image2.png) **Linkage of the free energy of binding between charge modifications on the ligand and receptor.** A and B, inhibition of the initial rate of 125I-labeled α-bungarotoxin binding to cell surface NαChR expressed as αβγδ by wild type NmmI (A) and K27E mutant NmmI (B). C and D, inhibition of the initial rate of 125I-labeled α-bungarotoxin binding to cell surface NαChR expressed as αβγE176Kδ by wild type (C) and K27E mutant (D) NmmI α-toxin. The dashed line in D is the predicted curve when the coupling energy (ΔΔG_Npc) between γE176K and K27E is 0. The deviation of observed affinity (D) from that predicted by no linkage between the residues at the αγ site (dashed line) produces a large coupling energy of −5.9 kcal/mol.
26) and was shown to influence the affinity of quaternary agonists and antagonists (27, 28). Moreover, the adjacent residues of γPhe-172 (δAsp-178, εLe-173) are known to confer site-selectivity to the smaller competitive peptide inhibitors such as α-conotoxin MI (29) and waglerin (30). The equivalent region of the α7 subunit (ASP-163, Ile-164, and Ser-165), which presumably forms a homomeric pentamer of subunits, constitutes part of a putative Ca$^{2+}$ binding region that faces the ligand binding site (31). At the α subunit interface of the binding site, both aromatic (Tyr-190, Tyr-198) and anionic (Asp-200) residues were mapped to the α-toxin binding surface (19). Here, we identify another anionic residue in the γ subunit, Glu-176, perhaps restricted in its position by a neighboring secondary amino acid Pro-175, on the γ subunit, as a crucial residue for binding.

The significant linkage between Glu-176 and cationic residues in loop II of the toxin suggests that an electrostatic interaction contributes to the tight binding of the Nmml/NaChR complex. Because the linkage obtained from charge reversal is greater for Lys-27 than for Arg-33 and Arg-36, one would predict that the portion of loop II proximal to loop III of the toxin, is closest to the γ subunit (cf. Fig. 5). In this analysis, the loss of free energy ($\Delta G$) associated with a single charge mutation results from all interactions between the charged residue and its multiple neighboring residues. The pairwise interactions ($\Delta G_{\text{INT}}$) resulting in charge reversal of specified residues in the interacting molecules should then highlight the strength of interaction coming from the paired charged residues. In the absence of significant changes in conformation or strength of interaction coming from the paired charged residues, the pairwise interactions from Equation 2 should largely reflect the Coulombic interaction between the respective paired residues (32).

Extensive studies on a related short neurotoxin, erabutoxin a, involving mutations at 36 toxin positions clearly revealed the importance of the tips of loops situated on the concave face of the toxin (33, 34). These investigations showed that the K27E mutation of erabutoxin a decreases its affinity more than 100-fold for Torpedo nAChRs. Photo-activatable p-azidobenzoyl and p-azidosalicyl groups attached to Lys-26 (analogous position at Lys-27 of Nmml) of neurotoxin II labeled γ and δ subunits of the receptor upon photolysis (11, 12). Three different photocativatable groups attached to the equivalent residue Lys-23 of a long neurotoxin, toxin 3, also labeled predominantly the γ and δ subunits in preference to the α subunit (13). Thus mutagenesis and chemical labeling studies showed a crucial role of lysine at position 27 and its proximity to γ and δ subunits. Here, our mutant cycle studies delineate the interaction between Glu-176 of the γ subunit and Lys-27 of Nmml toxin. The largest linkage in loop II between K27E and γE176K ($\Delta G = -5.9\, \text{kcal/mol}$) and smaller linkages ($\Delta G_{\text{INT}} = -1.6 \text{ to } -3.0 \, \text{kcal/mol}$) found previously between K27E and α subunit residues of Val-188, Tyr-190, Pro-197, and Asp-200 (35) correlate well with the labeling studies. A more complete elucidation of α-toxin-receptor interactions should enable us to orient a docked α-toxin with respect to the subunit interfaces, as well as refine existing models of the structure of the extracellular domain of the receptor (4).