**Variability among the Sites by Which Curaremimetic Toxins Bind to Torpedo Acetylcholine Receptor, as Revealed by Identification of the Functional Residues of α-Cobratoxin***

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α-Cobratoxin, a long chain curaremimetic toxin from *Naja kaouthia* venom, was produced recombinantly (rCbtx) from *Escherichia coli*. It was indistinguishable from the snake toxin. Mutations at 8 of the 29 explored toxin positions resulted in affinity decreases for Torpedo receptor with ΔΔG higher than 1.1 kcal/mol. These are R33E > K49E > D27R > K23E > F29A ≥ W25A > R36A ≥ F65A. These positions cover a homogeneous surface of approximately 880 Å² and mostly belong to the second toxin loop, except Lys-49 and Phe-65 which are, respectively, on the third loop and C-terminal tail. The mutations K23E and K49E, and perhaps R33E, induced discriminative interactions at the two toxin-binding sites. When compared with the short toxin erabutoxin a (Ea), a number of structurally equivalent residues are commonly implicated in binding to muscular-type nicotinic acetylcholine receptor. These are Lys-23/Lys-27, Arg-33/Arg-33, Lys-49/Lys-47, and to a lesser and variable extent Trp-25/Trp-29 and Phe-29/Phe-32. In addition, however, the short and long toxins display three major differences. First, Asp-38 is important in Ea in contrast to the homologous Glu-38 in α-Cbtx. Second, all of the first loop is insensitive to mutation in α-Cbtx, whereas its tip is functionally critical in Ea. Third, the C-terminal tail may be specifically critical in α-Cbtx. Therefore, the functional sites of long and short curaremimetic toxins are not identical, but they share common features and marked differences that might reflect an evolutionary pressure associated with a great diversity of prey receptors.

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In the course of the past 2 decades, a large number of curaremimetic toxins have been isolated from venoms of elapid and hydrophiid snakes (1). They are small proteins that all adopt a leaf-like shape, with three adjacent loops rich in β-pleated sheet which emerge from a small globular core where four disulfide bonds are invariably located (2–6). Despite this overall structural homogeneity, curaremimetic toxins can be divided into at least two major subfamilies on the basis of both their amino acid sequences (1) and functional properties (7). Thus, the long chain toxins have a longer C-terminal tail, an additional small loop cyclized by a fifth disulfide bond located at the tip of the central loop and a shorter first loop. One of these deviations, i.e. the fifth disulfide bond, has been associated with the unique ability of long chain toxins to bind to α7 receptors with high affinities (7). In addition, curaremimetic toxins isolated from the *Latíciauda colubrina* venom do not fall into either of these two major subfamilies. Such toxins have the size of long chain toxins (69 residues) but lack the fifth disulfide bond and bind with high affinity to muscular-type AChRs only (7). Therefore, in contrast to what has been believed during the past 2 decades, curaremimetic toxins do not form a homogeneous family of proteins. This conclusion raises, therefore, the possibility that short and long chain toxins may not achieve their comparable binding to muscular-type AChR by identical means. To approach this question, the sites where both types of toxins bind to the same type of AChR need to be elucidated.

Previously, mutational studies have been carried out with a view to understanding how short chain toxins recognize muscular-type AChRs. Initially, 10 residues were found to be important for erabutoxin a (Ea), a toxin from the sea snake *Latíciauda semifasciata*, to bind to AChR from Torpedo (8, 9). Among these, Lys-27, Trp-29, Asp-31, Arg-33, Glu-38, and Lys-47 are highly conserved in both short and long chain curaremimetic toxins, whereas Ser-8, Gln-7, Gln-10, and Ile-36 are more variable. In these studies, no attempt was made to identify the contribution of these functional residues to either one of the two toxin-binding sites. More recently, other studies also showed that the invariant residues Lys-27 and Arg-33, and to a lesser extent Lys-47, are important for a short chain toxin from *Naja mossambica mossambica* to bind to mouse muscle AChR and contribute differently to the two toxin-binding sites (10, 11). Therefore, the greater understanding of the functional sites of short chain toxins is emerging. In sharp contrast, and despite a number of preliminary studies based on numerous chemical modifications (12–17) and one genetic approach (18), the functional sites of long chain toxins remain unclear.

The aim of this paper was to identify residues that are important for α-cobratoxin (α-Cbtx), a long chain toxin from *Naja kaouthia*, in its binding to muscular-type AChR. To approach this question, we (i) constructed a cDNA encoding α-Cbtx; (ii) expressed it as a fusion protein in *Escherichia coli*;

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1 The abbreviations used are: AChR, nicotinic acetylcholine receptor; rα-Cbtx, recombinant α-cobratoxin; α-Cbtx, α-cobratoxin; Ea, erabutoxin a; PAG, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; α-Bgtx, α-bungarotoxin.

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(iii) showed that the unfused recombinant toxin (ra-Cbtx) is indistinguishable from the venom toxin; (iv) performed 35 mutations at 29 different positions covering the whole loop I of α-Cbtx, part of loops II and III, and its C-terminal tail. The data demonstrate that α-Cbtx and the short chain Ea share both similarities and marked differences in binding to the same muscular-type receptor.

EXPERIMENTAL PROCEDURES

Construction of the Synthetic Gene for α-Cobrotoxin—A synthetic gene for α-Cbtx was designed by back-translation of the primary amine acid sequence of native α-Cbtx and then optimizing the codon sequence for expression in E. coli. To obtain optimal expression of the recombinant α-Cbtx, we used a ZZ fusion protein strategy with the pCP vector, a pET3a derivative plasmid previously described (19). Expression of ZZ fusion proteins is inducible in the E. coli host strain BL21(DE3) which contains the structural gene for the T7 bacteriophage RNA polymerase under lac repressor control.

To create the desired synthetic gene, 10 oligomers ranging in length from 26 to 59 nucleotides were synthesized and purified by polyacrylamide/urea gel electrophoresis followed by elution in 0.5 M ammonium acetate. Equimolar mixtures of sense and complementary oligonucleotides were annealed at 95 °C for 5 min and cooled to room temperature overnight. The double-stranded oligonucleotides were then ligated by T4 DNA ligase (Appligene Oncor) in three steps. In a small quantity of the complete synthetic gene (213 base pairs) flanked by KpnI and BamHI restriction sites and containing the following sequence (sense strand) was obtained: 5′-ATCCGTTGCTTATTACCTCGTGATATACCTCCTCAAGGACTCTGATCTACACCAAGACTCTTGCGATGGTTCTGACTGTTGACATCCCTGTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTACACCAAGACTCTTGCTCAGTCTAGATATTACCTCCAAGGACGTCTGCTAC
venom toxins have identical amino acid compositions and N-terminal amino acid sequences. Third, the far UV CD spectra of the venom and recombinant toxins were virtually superimposable (Fig. 2). The two toxins have similar contents of β-sheet, the major structural component of α-Cbtx (5, 22). Fourth, electrospray mass analyses indicate the same mass value for both toxins: 7820.3, close to the theoretical value of 7821. Finally, the molar absorbance of the recombinant and folded toxin was 9670 m<sup>2</sup> cm<sup>-1</sup> M<sup>-1</sup>, a value identical to that determined for the venom toxin. The recombinant toxin therefore displays all the expected physicochemical characteristics of α-Cbtx. The final yield in toxin production was 1.2 mg/liter of culture.

Binding affinities of venom and α-Cbtx to Torpedo AChR were determined on the basis of competition experiments, using 3H-toxin α as a radioactive tracer. Typical competition binding curves obtained with the native and recombinant toxins are shown in Fig. 3. These data tally well with competition binding of the venom and recombinant toxins to the Torpedo receptor. Competition experiments with AChR from T. marmorata (4 nm) were performed at equilibrium, using α-H-toxin α (6.4 nm, 18 Ci/mol) as radioactive tracer as described under “Experimental Procedures.” Binding measurements were analyzed by the empirical Hill equation in order to define the Hill coefficient. A typical B<sub>max</sub> value was about 14,000 cpm, with a background always lower than 10% of the B<sub>max</sub> value. Each value indicated in the figure is the average of experiments done in triplicate. The functions describing competitive binding of α-Cbtx mutants to either a single site or two independent sites were fitted by nonlinear regression. Statistical improvement in the computer fit for a two-site model versus a one-site model was determined by the F test. The two-site model was accepted when the statistical comparison between the two models gave a value of p < 0.05.

Although the tertiary structures of both short and long toxins nicely superimposed on their second and third loops, no structural fit was seen between their first loops, the tip of their loop II, and in their C-terminal tails (Fig. 4). To study the role of the tip of the first loop in α-Cbtx, we mutated all residues from Phe-4 to Asp-13 into Ala; only Lys-12 was changed into Glu. The role of the tip of the central loop was first explored by mutating all Asp-31, Arg-33, and Glu-38 which belong to the second loop of the toxin, and Lys-47 which is present in the third loop. Since long and short toxins exert comparable binding functions on muscular-type AChRs (1, 7), we first probed the role of the structurally homologous Lys-23, Trp-25, Asp-27, Arg-33, Asp-38, and Lys-49 in α-Cbtx. We performed mutations K23E, W25H, W25F, W25A, D27R, R33E, D38L, and K49E. We also mutated Tyr-21 because its structural role has been often proposed (1). The introduced mutations were Y21A and Y21F.

Although the tertiary structures of both short and long toxins nicely superimposed on their second and third loops, no structural fit was seen between their first loops, the tip of their loop II, and in their C-terminal tails (Fig. 4). To study the role of the first loop in α-Cbtx, we mutated all residues from Phe-4 to Asp-13 into Ala; only Lys-12 was changed into Glu. The role of the tip of the central loop was first explored by mutating all residues located within the disulfide bond Cys-26—Cys-30.
Plasticity of the Functional Site of Curaremimetic Toxins

The left part shows dissociation constants for wild-type and mutated α-Cbtx, as deduced from binding competition data. The right part shows dissociation constants for wild-type and mutated erabutoxin α (Ea), as previously reported (8, 9). Only mutations that caused affinity changes in Ea are shown. These mutation-sensitive positions are compared with those that occupy a structurally comparable position in α-Cbtx. Some mutations indicated the presence of two toxin-binding sites, whose $K_d$ values are indicated by $\Delta G$ values. $\Delta G$ values are the difference in free energy of binding between wild-type and mutant toxins. $\Delta G = \Delta G_{\text{mut}} - \Delta G_{\text{wt}} = RT \ln(K_d/K_t)$, with $R = 1.99$ cal/mol/K and $T = 293K$. The residues whose mutations caused a $\Delta G$ higher than 1.1 kcal/mol are in bold.

| Cbtx | $K_d$ ($\mu M$) | $K_d/K_t$ | $\Delta G$ (kcal/mol) | Ea ($K_d$) ($\mu M$) | $\Delta G$ (kcal/mol) |
|------|----------------|-----------|-----------------------|---------------------|-----------------------|
| α-Cbtx native | 39 ± 5 | 0.67 | −0.24 | 0 | 0 |
| α-Cbtx rec | 58 ± 7 | 1 | 0 | 70 | 0 |
| F4A | 63 ± 6 | 1.1 | 0.05 | 0 | 0 |
| I5A | 36 ± 9 | 0.62 | −0.28 | 0 | 0 |
| T6A | 51 ± 16 | 0.87 | −0.08 | 0 | 0 |
| F27A | 49 ± 6 | 0.85 | −0.09 | 0 | 0 |
| D5A | 50 ± 3 | 0.86 | −0.09 | 0 | 0 |
| D8R | 45 ± 12 | 0.77 | −0.15 | 0 | 0 |
| I9A | 40 ± 19 | 0.69 | −0.22 | 0 | 0 |
| T10A | 39 ± 12 | 0.68 | −0.22 | 0 | 0 |
| S11A | 56 ± 3 | 0.96 | −0.02 | 0 | 0 |
| K12E | 59 ± 1 | 1 | 0 | 0 | 0 |
| D13A | 67 ± 3 | 1.1 | 0.05 | 0 | 0 |
| Y21F | 69 ± 12 | 1.2 | 0.11 | 0 | 0 |
| K23E | 118 ± 31/1595 ± 374 | 2/27.5 | 0.40/1.94 | 2200/24,500 | 23/3.41 |
| W25F | 128 ± 40 | 2.2 | 0.46 | 4700 | 2.45 |
| W25H | 212 ± 43 | 3.6 | 0.75 | 600 | 1.25 |
| W25A | 641 ± 160 | 11 | 1.40 | 0 | 0 |
| D27N | 146 ± 12 | 2.5 | 0.53 | 0 | 0 |
| D27R | 1806 ± 62 | 31.1 | 2.00 | 100 | 0.23 |
| A28G | 47 ± 9 | 0.81 | −0.12 | 0 | 0 |
| A28R | 58 ± 21 | 1.0 | 1.0 | 0 | 0 |
| F29L | 720 ± 94 | 12.4 | 1.47 | 470 | 1.13 |
| F29W | 747 ± 2 | 1.29 | 1.15 | 0 | 0 |
| F29A | 175 ± 59 | 3 | 0.64 | 0 | 0 |
| C26S/C30S | 70 ± 6 | 1.2 | 0.11 | 0 | 0 |
| S31A | 65 ± 2 | 1.0 | 0.05 | 0 | 0 |
| I32A | 47 ± 3 | 0.81 | −0.12 | 0 | 0 |
| R33E | 44,500 ± 5200 | 767 | 3.87 | 830/48,300 | 1.44/3.81 |
| K35A | 64 ± 15 | 1.1 | 0.05 | 0 | 0 |
| R36A | 432 ± 13 | 7.4 | 1.17 | 0 | 0 |
| D38L | 149 ± 77 | 2.6 | 0.55 | 1730 | 1.87 |
| T47A | 65 ± 6 | 1.1 | 0.05 | 0 | 0 |
| K49E | 181 ± 43/3522 ± 654 | 3.1/32.6 | 0.66/2.31 | 1175/219,000 | 1.64/3.35 |
| D53K | 39 ± 6 | 0.67 | −0.20 | 0 | 0 |
| F65A | 394 ± 66 | 6.8 | 1.11 | 0 | 0 |
| F66A | 63 ± 9 | 1.1 | 0.05 | 0 | 0 |

Thus, in addition to mutations made at positions 27, we introduced the mutations A28G, A28R and F29W, F29L, F29A. Then, the two half-cystines of the fifth disulfide bond were mutated into serine, and the vicinal Ser-31, Ile-32, Lys-35, and Arg-36 were mutated into alanine. The role of the C-terminal tail was probed by mutating the highly conserved Phe-65 and Pro-66 into alanine. Finally, we mutated Thr-47 and Asp-53 as a result of their spatial proximity to the invariant Lys-49.

Properties of the Mutants—35 mutants were prepared in satisfactory yields, not much different from that observed for the wild-type toxin. These mutants were purified by HPLC and displayed the expected mass, as determined by electrospray mass analysis. Only one difficulty was encountered with the mutant Y21A; it could be produced as an unfolded protein but failed to refold, suggesting a possible structural role for Tyr-21, as anticipated previously (1).

Competition binding experiments were performed with all mutants, and typical curves are shown in Fig. 3. Table I displays all the affinity constants and the corresponding difference in energy of binding ($\Delta G$) deduced from such curves.

As deduced from binding curves, the vast majority of the mutants was characterized by Hill coefficients of $1.1 \pm 0.2$, indicating similar affinities for the two AChR-binding sites. In contrast, the Hill coefficients calculated with the mutants K23E and K49E were equal to $0.6 \pm 0.1$, suggesting that these residues interact differentially with the two toxin-binding sites on Torpedo AChR. We then tentatively fitted these two binding curves using non-linear regression, assuming the presence of either a single site or two distinct sites. A comparison of these two models through statistical analysis indicated that the binding of the two mutants K23E and K49E fit better with the presence of two distinct classes of receptor sites ($p < 0.01$). These results agree with recent data reporting that mutations K27E and K47E in a short chain toxin ($N_{mm1}$) differentially influence the binding at the two receptor sites with, respectively, higher and lower affinities at the αδ and αγ sites (10, 11). Furthermore, the mutants K23E and K49E in α-Cbtx differ 14- and 17-fold, respectively, at the two sites, and these values are comparable to those found for the corresponding $N_{mm1}$ mutant. Differential binding may also occur with the α-Cbtx mutant R33E, as seen with the mutant R33E in $N_{mm1}$ (10), although the decrease in affinity was great, and material was insufficient to complete the binding curve.

Over 6-fold affinity decreases occurred with the following mutations K23E, W25A, D27R, F29L, R33E, R36E, K49E, and F65A (Table I). To assess the structural consequences of the introduced mutations, we monitored the CD spectra of all the mutants. They were all similar, with three signals of comparable intensity found for each mutant, compared to α-Cbtx.
Plasticity of the Functional Site of Curaremimetic Toxins

The role of Lys-23 and Lys-49 in long chain toxins, as judged from previous chemical modifications, is somewhat controversial (12, 13, 17, 23). Our data show that mutations K23E and K49E differentially affected the binding of α-Cbtx at the two receptor sites, causing a weak effect at one site and 28- and 53-fold affinity decreases, respectively, for the other site (Table I). Therefore, positive charges at positions 23 and 49 are critical for the long chain toxin to bind at one of the two sites. It is known that α-conotoxins are appropriate ligands for identification of the subunit composition of the high and low affinity sites of muscle AChRs (10, 24–28). Nevertheless, the affinity ratio for the two sites is 46,000 and 19 for the muscle-derived cells and Torpedo AChRs, respectively (25). This low ratio observed with the Torpedo receptor did not allow us to identify which of the α9 or αδ interfaces was preferentially affected by mutations K23E and K49E.

Although highly conserved, the negative charge of Asp-27 is not in itself an important element for binding since the mutation D27N caused virtually no effect on toxin affinity. This is in agreement with the observation that mutation into alanine of the homologous Asp-30 in the long chain α-Bgtx had no effect on toxin affinity (18). However, we made a greater change by reversing its charge, so as to probe a possible role of Asp-27 in α-Cbtx. We found that mutation D27R caused a 31-fold affinity decrease (Fig. 3 and Table I). This strongly suggests that the residue is involved in the binding, although at the moment we are unable to explain how.

Three mutation-sensitive residues have an aromatic side chain (Table I; Fig. 3), and two of them, Trp-25 and Phe-29, are highly conserved among curaremimetic toxins. At present, some ambiguity exists in the literature concerning the role of Trp-25, its chemical modification being associated with controversial results (15, 29, 30). In the light of previous work (8, 9), we anticipated that mutations at Trp-25 would lower the affinity, and introduction in α-Cbtx of an alanine at position 25 did indeed reduce affinity 11-fold. However, neither mutation W25F nor W25H caused any detectable effect. The probable functional role of Trp-25 seems predominantly associated with its aromatic character. The role of an aromatic residue at this position in long chain toxins can be further illustrated with neuronal κ-bungarotoxin, a toxin that has a low affinity for muscular-type AChR. Substitution of its structurally homologous Glu-26 by a tryptophan caused a 5-fold affinity increase for the Torpedo receptor (31).

The role of Phe-29 in long chain toxins has not been investigated previously. Its aromatic character seems to be important since its substitution by leucine caused a 12-fold affinity decrease (Table I). In contrast, its mutation to the bulkier tryptophan (F29W) caused no effect on toxin binding activity. Surprisingly, the mutation F29A had no effect. We cannot explain this observation, although reducing the size of a functional residue may create a space that is compensated by vicinal side chains (32), and position 29 is sometimes naturally occupied by a threonine residue (1).

Mutation F65A caused a 7-fold affinity decrease, suggesting that the C-terminal tail of α-Cbtx may play some binding role, in agreement with previous data (33).

All other mutations in the second loop had little if any functional consequences. In particular, although Tyr-21, Ala-28, Cys-26—Cys-30, Ser-31, Lys-35, and Asp-38 are highly conserved among the sequences of long chain toxins, their mutation revealed that they contribute little if at all to the interaction with Torpedo AChR (Table I). None of the mutations introduced in the first loop at the 10 positions between Phe-4 and Asp-13 had any effect on the stability of the toxin-receptor complex. Clearly, this loop is not involved in the binding of α-Cbtx to Torpedo AChR. The long C-terminal tail is so specific to long chain toxins that it is not entirely involved in

![Backbone comparison of a long chain toxin (α-Cbtx in red) and a short chain toxin (Ea in green).](image-url)
the interaction with the muscular receptor type, since the mutation P66A caused no affinity decrease. Finally, although being located on loop III close to the critical Lys-49, Thr-47 and Asp-53 are unlikely to be functionally important since their respective mutations into alanine and lysine caused no significant affinity decreases (Table I).

**DISCUSSION**

We have identified residues of a long chain curaremimetic toxin whose mutations affect toxin binding to a muscular-type nicotinic acetylcholine receptor (AChR). The selected toxin was α-cobratoxin (α-Cbtx) from venom of the cobra *N. kaouthia*. It is a typical long chain curaremimetic toxin whose structural properties have been largely elucidated by x-ray diffraction (5, 22) and NMR spectroscopy (34) and whose functional properties have also been analyzed (35, 36).

The recombinant toxin (r-α-Cbtx) was chemically, functionally and structurally indistinguishable from the venom toxin and was produced with a yield of approximately 1.2 mg per liter of culture. Other long chain toxins have been previously produced recombinantly. A few years ago, α-bungarotoxin (α-Bgtx) from venom of the krait *Bungarus multicinctus* (37) was produced in *E. coli*, using another expression system (18). However, the recombinant toxin possessed 10 extra residues in its N-terminal sequence, and the yield was substantially lower. More comparable to our results were the data on the production of the recombinant neuronal α-bungarotoxin (α-Bgtx) (31). By using a similar approach based on the production of a fusion protein, an active α-Bgtx was produced in *E. coli*. We suggest that the recombinant system described in the present paper may be of general applicability for small proteins rich in disulfide bonds.

**On the Residues by Which α-Cbtx Binds to Torpedo AChR**—If we consider as functionally important the residues for which at least one mutation decreased by more than 1.1 kcal/mol the affinity toward at least one of the two toxin-binding sites, the binding surfaces of α-Cbtx to Torpedo receptor then include Arg-33 > Lys-49 > Asp-27 > Lys-23 > Phe-29 > Trp-25 > Arg-36 ≈ Phe-65. These residues cover a surface of approximately 880 Å², with their side chains located on the concave face and pointing in a similar direction (Fig. 4). Thus, if one looks at the toxin structure with the concave side facing the viewer and the three loops hanging down (see Fig. 5), they form a stretched surface that essentially crosses the second and third loops, with a large contribution of positively charged and aromatic residues.

Examination of residues that are insensitive to mutations reinforced this delineation. Thus, Fig. 5 shows that the left upper region of the molecule is uncrritical for binding, since none of the residues of the first loop were sensitive to mutation. The lack of functionality of this loop was unexpected for three reasons. First, various residues, such as Phe/Tyr-4, Thr-6, Pro-7, and Ser-11, are found in more than 80% of the long chain toxins, and such a conservation is usually considered to reflect some functional and/or structural involvement, although this is clearly not the case here. Second, an NMR study of the complex between α-Bgtx and a receptor fragment (Ta185–196) indicated an interacting role of the toxin residues Thr-5, Thr-6, Ala-7, and Ile-11 (2). However, α-Bgtx is an atypical toxin, especially regarding the constitution of its first loop. Furthermore, the toxin might establish differential interactions with a receptor fragment or the whole receptor. Third, as will discussed below, the first loop of erabutoxin α, another curaremimetic toxin, is critically involved in binding to the same receptor (9).

Other regions of the toxins were also excluded from the receptor binding region. Thus, the base and part of the tip of the central loop seem to be excluded as judged from the unimportance of Tyr-21, Asp-38, Ala-28, Ser-31, and Ile-32. That the convex face of the toxin is excluded is suggested by the observation that Lys-35, whose side chain points toward this face, is insensitive to mutation. Although the role of the C-terminal tail is not yet completely understood, part of it is not implicated as shown by the insensitivity of the mutation P66A. Finally, the region on the right of the molecule is also poorly functional since of the three residues probed in the third loop, only Lys-49 is sensitive to mutation (Fig. 5B).

Therefore, the data showing both the positions that are sensitive to mutations and those that are insensitive delineate the
site by which α-Ctx recognizes the Torpedo AChR. This site includes the residues shown in Fig. 5B, some of them being involved in one or both toxin-binding sites.

Comparison of Functional Sites of Long and Short Chain Toxins—It is well documented and rather puzzling that the family of snake curaremimetic toxins include long and short members to achieve the same type of action, high affinity blockade of AChR (1). The present data obtained with the long α-Ctx and those previously reported for the short Ea (8, 9) allow us to examine how the two types of toxins exert this common function. Such a comparison is all the more appropriate as follows: (i) the studies of α-Ctx and Ea were based on similar competition binding assays, using similar preparations of AChR from T. marmorata, and (ii) the equilibrium dissociation constants are quite similar for the two toxins, 70 and 60 pM for Ea and α-Ctx, respectively.

The residues by which Ea and α-Ctx interact with Torpedo receptor are shown in Fig. 5. A number of these functionally important residues are identical in nature and located at homologous positions in the two toxins (Fig. 5, Table I). These are, in Ea and α-Ctx, respectively, Lys-27/Lys-23, Thr-29/Thr-25, Asp-31/Asp-27, Phe-32/Phe-29, Arg-33/Arg-33, and Lys-47/Lys-49. These residues are also conserved in most other short and long chain toxins and so may constitute a common functional core for most curaremimetic toxins. Since Arg-33, Lys-27, and Lys-47 of the short toxin Nmn1 bind close to the α-subunit region 188–200 of the muscle receptor (11, 38), we suggest that the functionally homologous residues of the long chain toxin recognize a similar region of the receptor.

Beside their remarkable functional similarities, the two toxins display a number of differences that can be appreciated at different levels. Thus, mutations at homologous residues of the functional core may not be followed by fully identical functional consequences. This is probably the case of the positively charged residues. Recent analysis performed with recombinant mouse muscle AChR subunits expressed in HEK cells indicated that the two sites can be differentiated by some toxin mutants (27). Although discrimination of the two binding sites is less straightforward with the Torpedo receptor than with mammalian muscle-type AChRs (25), some site selectivity could be identified from binding competition curves for the mutants Lys-23E and K49E of α-Ctx (Fig. 3 and Table I) and perhaps with its R33E mutant. Our previous competition data obtained with Ea mutants were initially treated by assuming that the two binding sites of the long toxin (Fig. 3 and Table I), mutation at Ser-8 caused large affinity decreases in Ea, especially for one of the two binding sites (Table I), mutation at Thr-6 had no effect on the affinity of α-Ctx. Ser-8 and Thr-6 are therefore not only functionally distinct but more generally a threonine and a serine cannot always be considered theoretically to play comparable roles, even when they occupy a comparable location in two structurally and functionally similar proteins.

With hindsight, that the tip of the first loop does not play equivalent roles in short and long toxins is unsurprising. The first loop is substantially longer in the short toxin, precluding a superimposition with that of the long toxin (Fig. 4). Thus, considering the common and highly functional residue Arg-33 as a common binding reference for the two toxins, the closest residues at the tip of loop I in Ea and α-Ctx are located at quite different distances. Thus, CorS33 is at 12.4 Å from CaS8 in Ea and at 17.8 Å from CaT6 in α-Ctx. We suggest that the shortness of the first loop in α-Ctx makes its residues somewhat inaccessible to the interactive receptor surface. In contrast, the mutation-sensitive Phe-65 in α-Ctx is not only closer to Arg-33 (CoxR33-CoxF65 = 13.5 Å) but is located at a distance that is comparable to that between Arg-33 in Ea and the tip of the first loop (see above). In other words, the C-terminal tail in α-Ctx might be functionally equivalent to the critical residues located on the first loop of Ea.

At first sight, the short toxin seems to have more functional residues than the long toxin (Fig. 5 and Table I), but 74% of the positions have been probed in Ea (40) and only 41% in α-Ctx. Possibly, therefore, a number of functional residues in α-Ctx remain to be identified, in particular within the C-terminal tail and the third loop, which have not been fully explored. It is also possible that the backbone contributes differentially in the two toxins and of course the mutational approach is uninformative about this. Further studies are needed to clarify these points. It is not inconceivable that the two toxins use a different number of residues to interact with the same receptor, even with a nearly identical high affinity. This would only imply that the individual binding contributions of the residues in the two toxins are different. We are unable to assess this point in the present analysis, where the introduced mutations did not necessarily reflect the genuine binding contribution of the original side chain, as evidenced by the differential effects of the introduction of different residues at the same position. Some mutations like those in which the original charged was reversed might have considerably amplified the effective binding contribution.

Concluding Remarks—In conclusion, the present study shows that long and short curaremimetic toxins exploit a common core composed of Lys-27/23, Trp-29/25, Asp-31/27, Phe-32/29, Arg-33/33, and Lys-47/Lys-49, for binding to one or both
toxin recognition sites of Torpedo AChR. However, these toxins also differ functionally in two ways. First, the binding energy contribution of some residues of the common core may be different in the two toxin families. Second, additional residues may be functionally important in one toxin only. Therefore, two curaremimetic toxins recognize the same receptor with comparably high affinities, through substantial variations around a common binding core. We are now investigating whether the same strategy occurs when the same toxin binds with high affinities to two different receptors, as observed with long toxins that bind to both muscular-type and α7 neuronal receptors (7).

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