How Do Short Neurotoxins Bind to a Muscular-type Nicotinic Acetylcholine Receptor?

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We investigated the interacting surface between a short curarimimetic toxin and a muscular-type nicotinic acetylcholine receptor, looking for the ability of various biotinylated Naja nigricollis α-neurotoxin analogues to bind simultaneously the receptor and streptavidin. All these derivatives, modified at positions 10 (loop I), 27, 30, 33, 35 (loop II), 46, and 47 (loop III) or the N-terminal (erabutoxin numbering), still shared high affinity for the receptor, and in the absence of receptor they all bound soluble streptavidin. However, the proportion of the toxin-receptor complex that bound to streptavidin-coated beads, varied both with the location of the modification and with the length of the linker between biotin and the toxin. In the receptor-toxin complex, the concave side of loops II and III was not accessible to streptavidin, unlike the N terminus of the toxin and, to a certain extent, loop I. On the convex face, loop III was the most accessible, whereas the tip of loop II, especially Arg-30, seemed to be closer to the receptor. The present data demonstrate that short toxins neither penetrate deeply into a crevice as proposed earlier nor lie parallel to the receptor extracellular wall. These data also suggest that they may not lie strictly perpendicular to the cylindrical wall of the receptor. These results fit nicely with three-dimensional models of interaction between long neurotoxins and their receptors and support the idea that short and long curarimimetic toxins share a similar overall topology of interaction when bound to nicotinic receptors.

The recent resolution of the structure of an acetylcholine binding protein (AChBP) by Brejc et al. (1) allowed positioning of α-bungarotoxin on AChBP (2, 3) and elaboration of an experimentally based three-dimensional model of α-cobratotoxin complexed to an αβ nicotinic acetylcholine receptor (nAChR) model (4). These studies positioned the long-chain curarimimetic toxins equatorially on the outside face of the cylinder formed by the five receptor subunits, almost perpendicularly to the cylinder wall, with loop III of the toxins looking “downward” to the membrane (in the case of nAChR). These long neurotoxins insert the tip of their second loop in the acetylcholine-binding site, explaining their antagonistic properties. In two models (2, 3), however, there were clashes between the AChBP and the toxin. In the other model (4), such inconveniences were avoided by moving slightly a binding loop of the receptor. It has been hypothesized that the AChBP might have been crystallized in a frozen “desensitized” state (5) and that the resting state could require a reorganization of the quaternary structure of the receptor. Because it has been suggested that curarimimetic toxins stabilize the resting state of the receptor (6–8), it may not be surprising that some structural adjustments are required for convenient docking experiments to be achieved.

Earlier data showed that long and short neurotoxins do not have identical binding determinants (9) and that they establish interactions with distinct amino acids of nAChR (10). Photoaffinity labeling experiments (11) have even suggested that the center of the binding site of a short neurotoxin may be proximal to the nicotinic channel axis, which would imply that the toxin enters into a deep crevice (12–14), in contrast to what is seen in three-dimensional models of complexes between long neurotoxins and their receptors (2–4). It has even been postulated that the passage entered by the short toxin is a narrow gorge (13). Such a crevice is not visible on the structure of the AChBP homooligomer but might exist in the resting state of the nAChR heterooligomer. To investigate whether short toxins bind differently from long toxins, we explored the accessibility of the two faces of a typical short neurotoxin free and complexed to the Torpedo acetylcholine receptor, drawing inspiration from the earlier work of Løbel et al. (15), who tested the ability of various biotinylcobratotoxin-nAChR complexes to bind avidin.

For this purpose, we synthesized various “cysteine analogues” of the toxin and coupled them to two different biotin derivatives, leading to a maximal distance of 11.5 and 16.5 Å, respectively, between the carbonyl function of biotin (coming from its carboxylic moiety) and the α-carbon of the modified cysteine. We checked that these derivatives bind independently to Torpedo nAChR and to streptavidin and looked for the ability of the binary biotinyltoxin-nAChR complex to be retained by streptavidin-coated beads. Our data demonstrate that a short-chain neurotoxin does not bind to Torpedo nAChR parallel to the receptor extracellular wall nor does it penetrate into a deep crevice. These data also suggest that they may not lie strictly perpendicular to the cylindrical wall of the receptor. All these features are globally comparable to those anticipated from three-dimensional models of interaction of long neurotoxins to their receptors.

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The abbreviations used are: AChBP, acetylcholine-binding protein; nAChR, nicotinic acetylcholine receptor; HPLC, high performance liquid chromatography; Q10C-Biot11.5, analogue Q10C of N. nigricollis α-neurotoxin modified on Cys-10 with (biotinoyl)N-(iodoacetyl)ethylenediamine (the same convention has been used for the other cysteine analogues); Q10C-Biot16.5, analogue Q10C of N. nigricollis α-neurotoxin modified on Cys-10 with N-biotinyl-N′-6-maleimidohexanoyl)-hydrazide (the same convention has been used for the other cysteine analogues); [3H]Nter-Biot-K47C-NEM, K47C analogue of N. nigricollis α-neurotoxin modified on Cys-47 with N-[3H]ethylmaleimide and on the N terminus with biotinamidocaproate.
EXPERIMENTAL PROCEDURES

For the sake of clarity, we decided to use erabutoxin numbering for the amino acids of \textit{Naja nigrilollis} \(\alpha\)-neurotoxin as we did in a previous report (18).

Materials—\(N\)-(Biotinoyl)-\(N\)′-(iodoacetyl)ethylendiamine was from Molecular Probes. \(N\)′-Biotinoyl-\(N\)″-(6-maleimidohexanoyl)hydrazide, succinimidyl-6-biotinamidohexanate and soluble streptavidin were from Sigma Chemical Co. Streptavidin-coated beads (Dynabeads M280) were from Dynal. \(\alpha\)-\(\text{125I}\)Bungarotoxin (150–250 Ci/mmol) was from Amersham Biosciences. Live \textit{Torpedo marmorata} were from the Station Biologique d’Arcachon (France). Electrospray mass spectrometry was carried out using a Quattro II spectrometer from Micromass. Circular dichroism spectra were recorded on a Jobin Yvon CD6 discograph at 22 °C. Radioactivity was determined on a 1216-multigamma counter (Amersham Biosciences).

HPLC Conditions—Reverse-phase high performance liquid chromatography (HPLC) separations were performed using a Vydac C4 semi-preparative column (10 × 250 mm; flow rate, 4 ml/min; linear gradient, 5% to 18% B in 6 min, then 18% to 24% B in 19 min (A, H_2O-0.1% trifluoroacetic acid; B, CH2CN)). Gel filtration experiments were carried out with a Tosohaas TSK-Gel G2000 SWXL column (7.8 × 300 mm; flow rate, 1 ml/min; 50 mM sodium phosphate, pH 7.5).

Membrane Preparations—\(\alpha\)4nAChR-rich membranes from the electric tissue of \textit{T. marmorata} were prepared as described previously (16). The concentration of acetylcholine-binding sites was measured at equilibrium with \(\alpha\)-\(\text{125I}\)bungarotoxin (17).

Synthesis of Biotinyltoxin Derivatives—\textit{N}. nigrilollis \(\alpha\)-toxin analogues (Q10C, K27C, R30C, R33C, T35C, V46C, and K47C) were synthesized as described earlier (18). The structure of the refolded toxins was checked by electrospray mass spectrometry and circular dichroism. Each “cysteine analogue” (60 nmol in 50–300 μl of water) was then biotinylated on the single free cysteine with \(N\)-(biotinoyl)-\(N\)′-(6-maleimidohexanoyl)hydrazide (5 μl of a 20 mM solution in MeSO) buffered with 1 mM Tris/HCl at pH 8 (50 μl) or with \(N\)-(biotinoyl)-\(N\)″-(6-maleimidohexanoyl)hydrazide (5 μl of a 20 mM solution in MeSO) buffered with 0.5 mM Tris/HCl at pH 6.8 (50 μl), at room temperature for 1.5 h. For the \(N\)-terminal biotinyltoxin ([\(\text{3H}\)]Nter-Biot-K47C-NEM), after automated solid-phase peptide synthesis of K47C, the resin (190 mg) was taken up in 0.5 M methylpyrrolidone and reacted with succinimidyl-6-(biotinamido)hexanate (10 equivalents) in the presence of diisopropylthylamine (10 equivalents) for 24 h at room temperature. The resin was washed with N-methylpyrrolidone (3 × 10 ml), then dichloromethane (1 × 10 ml), and finally with methanol (1 × 10 ml) and dried, before classic deprotection and refolding of the peptide was carried out. Each biotinyltoxin derivative was purified by reverse-phase HPLC, as described above, and checked by electrospray mass spectrometry.

Binding Assays—Competition experiments were performed as described earlier (18), except for the receptor concentration, which was increased to 0.7 nM. \(\alpha\)-\(\text{125I}\)Bungarotoxin was used as the radioactive tracer, and equilibrium dissociation constants were determined according to Cheng and Prusoff (19).

Interaction of Biotinyltoxin Derivatives with Soluble Streptavidin—Each biotinyltoxin (5 nmol) was mixed with one equivalent of soluble streptavidin for 1.5 h at room temperature (50–100 μM final concentration). The mixture was then injected onto a Tosohaas TSK-G2000-SWXL gel-filtration column equilibrated with 100 mM sodium phosphate, pH 7.5, as described above. The concentrations of toxin and streptavidin were chosen to allow UV detection of the toxin on HPLC but also to match as best as possible the concentration of streptavidin used later when making the ternary complexes between the receptor, the biotinyltoxins, and the streptavidin-coated beads (16–22 μM).

Interaction of the Binary \(\alpha\)4nAChR-Biotinyltoxin Complexes with the Streptavidin-coated Beads—Torpedo membranes (980 pmol of toxin-binding sites) were incubated for 2 h, at room temperature, with an aliquot of \(\alpha\)-\(\text{125I}\)bungarotoxin (a concentration of labeled toxin was used to occupy 2.5% of the \(\alpha\)-bungarotoxin-binding sites) in 50 mM Tris/HCl, pH 7.5 (400-μl final volume), containing 3 mM EDTA and 1 mM EGTA (buffer A). After centrifugation (15 min, 4 °C, 21,913 × g), the supernatant was removed, and the pellet was washed three times with buffer A to eliminate radioactive impurities that did not bind to the receptor (commonly 30% of the radioactivity for purchased \(\alpha\)-\(\text{125I}\)bungarotoxin). The membranes were then solubilized in 1.2 ml of 10 mM Tris/HCl, pH 7.4, containing 10 mM NaCl, 1% Triton (buffer B), and centrifuged as described above. Aliquots of the supernatant (5 pmol of toxin-binding sites) were then incubated with each biotinyltoxin (20 pmol, 45-μl final volume) for 30 min. After use, magnetic streptavidin-coated beads (110 μl, 0.5–9 nmol/ml) were washed three times with 50 mM Tris/HCl, pH 7.6, containing 50 mM NaCl and 0.1% bovine serum albumin (200 μl) and twice with buffer B (200 μl). The solutions containing the \(\alpha\)4nAChR-biotinyltoxin complexes were mixed individually with the beads. After 30 min, the supernatants were removed and the beads were washed three times with buffer B. Finally, the amount of receptor bound to the beads was determined by γ-counting of the \(\alpha\)-\(\text{125I}\)bungarotoxin.

RESULTS

Preparation and Characterization of Toxin Derivatives—“Cysteine analogues” of \textit{N}. nigrilollis \(\alpha\)-neurotoxin were prepared as previously described (18). A cysteine residue was, respectively, placed on each toxic loop, on the concave side (where the critical binding residues are located), or on the convex side (opposite face) of the antagonist (Fig. 1). Thus, the previously described derivatives Q10C (loop I), K27C, R33C (loop II), and K47C (loop III) were selected to modify the concave side of the molecule. As for the other face of the toxin, we wanted to obtain an analogue with a cysteine on the convex side of loop I. Close to the tip of this loop, only two residues, Gln-6 and Pro-12, are located in such a position. Because Pro-12 might play a structural role in loop I, we discarded this residue and did not replace it with a cysteine. For Q6C, the synthesis of the linear peptide could be achieved, but it did not fold into a native-like structure. Hence, we were not able to obtain a cysteine analogue on the convex side of loop I. In contrast, we successfully obtained the R30C, T35C (loop II), and V46C (loop III) analogues (Fig. 1) and hence could test the proximity of the \(\alpha\)4nAChR to this side of the toxin, close to the tip of loop III and on both strands of the \(\beta\)-sheet near the tip of the second toxic loop. All our derivatives were purified by HPLC and checked by mass spectrometry and circular dichroism (data not shown). These cysteine analogues were further modified on their unique free sulfhydryl with two biotinylated derivatives, giving probes in which the \(\alpha\)-carbon of the cysteine was at 11.5 and 16.5 Å from the carbonyl moiety coming from the carboxylic group of biotin (Fig. 2). All derivatives were

FIG. 1. \textit{N}. nigrilollis \(\alpha\)-neurotoxin. All the amino acids shown in green were, respectively, replaced by a cysteine residue and further biotinylated, with the exception of the N-terminal leucine residue, which was coupled to biotin through an \(\varepsilon\)-amino-caproic linker during peptide synthesis. The structure has been taken from Zinn-Justin et al. (33). The arrow in the middle of the figure was drawn to define the height of the various residues, assuming that the tip of loop II is the bottom and the N terminus the top of the toxin. Note that the numbering of the residues corresponds to erabutoxin numbering.
tested for their ability to bind soluble streptavidin.

To assess whether biotinylated toxins bind similarly to streptavidin-coated beads and to soluble streptavidin, a radioactive biotinyltoxin derivative ([3H]Nter-Biot-K47C-NEM) was synthesized. The solid phase-bound, K47C-protected peptide received a biotin moiety on its N-terminal end through an ε-aminocaproic linker. Then the deprotected N-terminal-biotinylated peptide was refolded, and its single free sulfhydryl (Cys-47) was coupled to N-[3H]ethylmaleimide. The distance between the N-terminal atom of N. nigricollis α-neurotoxin and the carbonyl group of biotin (stemming from its carboxylic moiety) was 10 Å, which is the shortest length of all the linkers used in this study.

All probes were purified by reverse-phase HPLC and checked by mass spectrometry and circular dichroism (data not shown).

Interaction of Biotinyltoxins with nAChR—Competition experiments with α-[125I]-bungarotoxin on Torpedo nAChR showed that all chemically engineered toxin analogues have high affinity for the receptor (Table I). Although the concentration of receptor used in this study was slightly higher than in a previous analysis (18), the affinities of Q10C, K27C, R33C, and K47C remained on the same order of magnitude. On the convex side, the affinity of R30C was the most affected, with an almost 7-fold increase in the $K_i$ value compared with the wild-type toxin. This is consistent with previous data obtained with the homologous Lys-23 of the long α-cobratoxin (15). The affinity of R33C and K47C derivatives decreased by less than one order of magnitude, whereas the biotinylated Q10C analogues and, as expected, all the convex biotinylated toxins retained approximately a wild-type affinity. The affinity of the [3H]Nter-Biot-K47C-NEM was similar to that obtained for all the K47C derivatives (this study and Ref. 18), showing that modification of the N terminus had no influence on the toxin affinity for the nAChR. Clearly, our compounds still retained high affinity for the acetylcholine-binding sites.

Interaction of Biotinyltoxins with Soluble Streptavidin—The absence of radioactivity in our toxin derivatives did not allow us to determine directly their affinity for streptavidin. Therefore, we checked their ability to bind soluble streptavidin by gel filtration experiments on HPLC. By injecting first our analogues one by one, we observed that, despite their common overall structure (as checked by circular dichroism), they were not retained identically on the TSK column (Table II), indicating that, in our conditions, interactions between the biotinyltoxins and the column phase depend on the position and nature of the modified residue and do not only depend on the stokes radii of the various derivatives. All retention times varied between 14 and 20 min and were much longer than the streptavidin retention time (about 7 min) (Fig. 3).

In the presence of one equivalent of streptavidin binding sites, the peak characterizing each biotinyltoxin derivative was expected to be shifted and in fact to join the one resulting from the tetramer of streptavidin. Due to the low capacity of resolution, above 70 kDa, of the type of column used in this study, streptavidin and toxin-bound streptavidin eluted at the same retention time. Therefore, binding of toxin to streptavidin could only be followed by the partial or full disappearance of the toxin.

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### Table I

| Derivative                   | $K_i$ ($\text{pM}$) | $K_i/K_{\text{mut}}$ |
|-----------------------------|---------------------|-----------------------|
| Wild type                   | 13 ± 2              | 1                     |
| Q10C                        | 21 ± 3              | 1.6                   |
| Q10C-Biot11.5               | 16 ± 6              | 1.2                   |
| Q10C-Biot16.5               | 22 ± 11             | 1.7                   |
| K27C                        | 125 ± 5             | 9.6                   |
| K27C-Biot11.5               | 120 ± 40            | 9.2                   |
| K27C-Biot16.5               | 65 ± 9              | 5                     |
| R30C                        | 88 ± 9              | 6.8                   |
| R30C-Biot11.5               | 26 ± 8              | 2                     |
| R30C-Biot16.5               | 28 ± 7              | 2.2                   |
| R33C                        | 50 ± 13             | 3.8                   |
| R33C-Biot11.5               | 59 ± 14             | 4.5                   |
| R33C-Biot16.5               | 11 ± 3              | 0.8                   |
| T35C                        | 25 ± 2              | 1.9                   |
| T35C-Biot11.5               | 17 ± 5              | 1.3                   |
| T35C-Biot16.5               | 14 ± 6              | 1.1                   |
| V46C                        | 15 ± 3              | 1.2                   |
| V46C-Biot11.5               | 26 ± 8              | 2                     |
| V46C-Biot16.5               | 25 ± 5              | 1.9                   |
| K47C                        | 170 ± 6             | 13.1                  |
| K47C-Biot11.5               | 53 ± 1              | 4.1                   |
| K47C-Biot16.5               | 97 ± 10             | 7.5                   |
| Nter-Biot-K47C-NEM          | 111 ± 53            | 8.5                   |

*The numbering of the modified residues corresponds to erabutoxin numbering.*
peak. In all cases but one, only one major peak at about 7 min, corresponding to the streptavidin retention time, could be detected (one example is shown in Fig. 3A). No free toxin could be observed, indicating that each biotinyltoxin was fully complexed to streptavidin and that this binding was stable in our conditions. The only exception was the K27C-Biot11.5 derivative, where only a decrease in the toxin peak absorbance could be observed (Fig. 3B). However, when adding a second equivalent of streptavidin binding sites, the K27C-Biot11.5 peak disappeared (Fig. 3B). These data confirm that all our derivatives except one (K27C-Biot11.5) bind efficiently to streptavidin. K27C-Biot11.5 also binds to streptavidin but more weakly. The same phenomenon was observed earlier (15) for the Lys-23-biotinylated position on α-cobratoxin, which had a K<sub>app</sub> of only 10 μM for avidin as compared with the 10<sup>−10</sup> M value observed with free biotin. The lysine residue (27 on the short toxin or 23 on the long toxin) is central on the concave face of the toxin, and even a linker 11.5 Å long is probably not sufficient for easy binding to streptavidin, whereas lengthening this arm by 5 Å suffices to allow an efficient binding to toxin derivative (K27C-Biot16.5).

To assess further the validity of our binding experiments, the radioactive [3H]Ner-Biot-K47C-NEM toxin analogue (10-Å linker) was directly tested on the streptavidin-coated beads. We observed that all radioactivity was quantitatively retained on the beads (data not shown). Therefore, we had in hand the appropriate tools to investigate whether biotinyltoxin-receptor complexes would bind to streptavidin-coated beads and, hence, to probe the accessibility of the receptor-complexed, toxin-bound biotin moiety to streptavidin.

**nAChR-Biotinyltoxin-Streptavidin Ternary Complexes**—To quantify the receptor in solution or bound to the beads, we labeled *Torpedo* membranes with an aliquot of α<sup>-125I</sup>-bungarotoxin (equivalent to 2.5% of the total α-bungarotoxin-binding sites). Furthermore, we checked that neither α<sup>-125I</sup>-bungarotoxin nor the Triton-solubilized receptor and nAChR/non-biotinylated toxin complex were retained by the beads (data not shown).

When comparing the percentage of ternary complex formation (Fig. 4), we found clear differences depending on the face of the toxin, which was modified or the length of the linker between the toxin and biotin. Thus, although the linker was the shortest (10 Å) of all tested analogues, the N-terminal biotinyltoxin complexed to the nAChR bound streptavidin efficiently (about 72% of the nAChR amount), confirming previous results indicating that this position was solvent-accessible (22). For the analogues with the 11.5-Å linker, only one receptor-toxin complex was able to bind to streptavidin. This was V46C-Biot11.5 (convex face of loop III), which shows 26% binding to the nAChR, showing that residue 46 is the second most accessible position of all the tested derivatives. All toxins harboring a 16.5-Å linker on the convex face could bind both the receptor and streptavidin, weakly for R30C-Biot16.5 (27%) and highly for T35C-Biot16.5 (62%) and V46C-Biot16.5 (79%). In contrast, when the 16.5-Å linker was located on the concave side, K27C-Biot16.5, R33C-Biot16.5, and K47C-Biot16.5 complexed to nAChR did not bind streptavidin. Interestingly, however, the receptor-Q10C-Biot16.5 complex bound efficiently to the streptavidin-coated beads (71%), whereas the complex made with the shorter Q10C-Biot11.5 derivative was not able to bind streptavidin. Hence, the results obtained for the Q10C derivatives differ from the findings noted with Lys-27 and Lys-47, which are also concave-side residues and which are located at the same “height” of the toxin (Fig. 1).

**DISCUSSION**

Experimental data showed that short and long neurotoxins do not have identical binding determinants (9) but do establish interactions with distinct amino acids on the nAChR (10). Photolabeling experiments suggested that short neurotoxins penetrate into a deep crevice of the nAChR (11–14), whereas recent three-dimensional models of long α-neurotoxins complexed to their receptors showed that only the tip of the central loop of the toxins penetrates into the receptor (2–4). To determine the binding orientation of short neurotoxins and their penetration into the nAChR, we studied the ability of various biotinyltoxin derivatives to make a ternary complex with nAChR and streptavidin. We probed various positions (i) on the concave side of a typical short toxin and known to be involved in the nAChR-binding site (10, 27, 33, and 47), (ii) on the convex side (30, 35, and 46), and (iii) on the “top” of the molecule (N-ter) (Fig. 1). Therefore, we prepared 15 biotinyltoxin derivatives from chemically engineered cysteine analogues of a short neurotoxin, introducing a single biotin moiety at the mentioned positions and using three linkers of 10, 11.5, and 16.5 Å in length. Because all our derivatives could bind separately to streptavidin and nAChR, the lack of formation of a ternary complex between streptavidin and the toxin-nAChR complex was likely to reflect the inaccessibility of biotin to the tetramer of streptavidin, attributable to steric hindrance due to the presence of the nAChR.

**Like Long Neurotoxins, Short Neurotoxins Do Not Penetrate into a Deep Crevice**—We showed that the convex side of loop III of receptor-bound toxin remained quite accessible to the bulky tetramer of streptavidin (67 kDa), because the biotinyltoxin with the 11.5-Å-long linker on position 46 could make the ternary complex, although less efficiently than with the longest linker (Fig. 4). Interestingly, with the latter, we obtained opposite results for positions 46 and 47, the first facing the convex side (efficient binding) and the other facing the concave side (no binding) of the toxin. These data could be compared with previous results on Lys-49-modified α-cobratoxin (equivalent to Lys-47 in our short toxin), which was able to make the ternary complex but with a diminished ability (15). Lobel et al. (15) concluded that the ternary complex was strained. The slight discrepancy obtained between these studies, for equivalent positions, might be due to the difference in length of the linkers separating biotin from the toxin or to slight differences in the binding orientation of the toxins. However, all data demonstrate that the convex face of loop III is much more accessible to the bulky tetrameric streptavidin than its concave side and that, as for long neurotoxins, loop III does not penetrate at all into the receptor. The T35C-Biot16.5 derivative was one of the most efficient probes in our ternary complex formation exper-

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**TABLE II**
Retention times (t<sub>r</sub>) of *N*. nigricollis biotinyltoxin derivatives on a TSK-G2000-SWXL gel-filtration column

| Derivative          | t<sub>r</sub> (min) |
|---------------------|---------------------|
| Q10C-Biot11.5<sup>a</sup> | 16.5                |
| Q10C-Biot16.5       | 16.7                |
| K27C-Biot11.5       | 14.3                |
| K27C-Biot16.5       | 14.9                |
| R30C-Biot11.5       | 16.0                |
| R30C-Biot16.5       | 15.3                |
| R33C-Biot11.5       | 18.4                |
| R33C-Biot16.5       | 14.6                |
| T35C-Biot11.5       | 17.6                |
| T35C-Biot16.5       | 15.1                |
| V46C-Biot11.5       | 20.0                |
| V46C-Biot16.5       | 18.6                |
| K47C-Biot11.5       | 18.0                |
| K47C-Biot16.5       | 15.6                |

<sup>a</sup>The numbering of the modified residues corresponds to erabutoxin numbering.
Fig. 3. Binding of biotinyltoxins to soluble streptavidin determined by gel filtration. 5 nmol of biotinyltoxins, streptavidin binding sites or a 1:1 mixture of both (50–100 μM final concentration; 90-min incubation at room temperature) were, respectively, injected into a TosohHaas TSK-G2000-SWXL gel-filtration column equilibrated with 100 mM sodium phosphate, pH 7.5 (flow rate, 1 ml/min). Note that the numbering of the modified residues corresponds to erabutoxin numbering. A, biotinyltoxin modified on position 33 with the 11.5-Å-long linker (continuous line) and the same molecule plus one equivalent streptavidin binding sites (dotted line), the same molecule plus one equivalent streptavidin binding sites (discontinuous line), the same molecule plus two equivalents streptavidin binding sites (discontinuous line).

Fig. 4. Binding of the nAChR-biotinyltoxin complex to streptavidin-coated beads. Torpedo nAChR (5 pmol), solubilized in buffer B (Tris/HCl (10 mM), pH 7.4, 10 mM NaCl, 1% Triton) and for which 2.5% of the acetylcholine-binding sites were occupied by α-125I-bungarotoxin as a tracer, were incubated with various biotinyltoxins (20 pmol, 45-μl final volume) for 30 min at room temperature. The nAChR-biotinyltoxin complexes were mixed afterward individually with magnetic streptavidin-coated beads (110 μl, 6.5–9 nmol/ml), washed three times with 50 mM Tris/HCl, pH 7.6, 50 mM NaCl, 0.1% bovine serum albumin (200 μl) and washed twice with buffer B (200 μl). After 30 min, the supernatant was removed, the beads were washed three times with buffer B, and the amount of receptor bound to the beads was determined by γ-counting of the α-125I-bungarotoxin. The error bars correspond to the S.E. for three different experiments on the same batch of α-125I-bungarotoxin-traced receptor. For some complexes that do not bind to the beads, the error was too small to be visualized on the histogram. Note that the numbering of the modified residues corresponds to erabutoxin numbering.

imments. Considering the position of Thr-35 on the toxin, this result demonstrates that, if the toxin penetrates into the receptor, only the tip of loop II is able to do it. This conclusion is depicted in Fig. 5, in which the biotinyl carboxylate was positioned at 16.5 Å from the α-carbon of Cys-35, in the direction of the “top” of the toxin (in the opposite direction from the putative receptor-penetrating area of the toxin). The less cumbersome region of streptavidin was oriented toward the putative receptor-penetrating area of the toxin (“bottom” of the molecule), and streptavidin was positioned to avoid most clashes with the toxin. Although this figure is a simple illustration, it explains why only the tip of the second loop of the short toxin may penetrate into the receptor (to give an idea of the receptor surface, the backbone of AChBP was drawn in colored ribbons beside the toxin/streptavidin pair). Therefore, this result does not support the earlier proposition, based on photoaffinity labeling, that short toxins penetrate into a deep crevice of the receptor (11–14).

Like Long Neurotoxins, Short Neurotoxins Do Not Bind Parallel to the Receptor Surface—Previous site-directed mutagenesis of erabutoxin-a (20, 23) demonstrated that only concave-side residues of short neurotoxins are critical for binding to nAChR. Such a result could be interpreted as a demonstration that their convex face is fully accessible to the solvent and that the bound toxins lie more or less parallel to the receptor surface, orienting their concave face toward the nAChR. If this is the case, all the convex-modified biotinyltoxins would be expected to bind streptavidin and nAChR simultaneously independent of the length of the linker between the toxin and biotin. Clearly this is not the case, because the derivatives with the 11.5-Å-long linker on the convex side cannot make the ternary complex with streptavidin and nAChR. Moreover, the N-terminal-modified toxin is one of the most efficient probes, although it harbors the shortest linker. A similar result was obtained with the long α-cobratoxin modified on Lys-12 (15). Our result is also in agreement with the observation that a monoclonal antibody (Mo1) can interact with an epitope located around the toxin N terminus (24) even when the toxin is bound to the receptor (22, 25). Therefore, although the convex face of the toxin is not fully accessible to streptavidin in the biotinyltoxin-receptor complex, the N-terminal area is instead accessible to macromolecules such as streptavidin or antibodies. These observations agree with the models depicting the three-dimensional structure of long neurotoxins bound almost perpendicularly to the receptor surface (2–4).

Orientation of Short Neurotoxins with Respect to the Receptor Surface—The three-dimensional structure of the long chain α-bungarotoxin is particularly flexible in its central loop, as observed in complexes with nicotinic receptor peptides or motopes (2, 3, 26, 27). Thus, the flexibility of the tip of the second loop of α-bungarotoxin was demonstrated by NMR spectroscopy and is mostly related to the fifth-disulfide loop of the toxin, which was shown to move as an independent unit upon binding to a receptor peptide (3). However, short curarrimimetic toxins do not possess this extra disulfide loop. Even if no
structure of a complex with a short toxin has ever been published, it has been shown that fasciculin, another short three-finger toxin, interacts with its receptor, acetylcholinesterase, almost as a key in a lock (28–30). Interestingly, the structure of this complex (28, 29) together with the recent three-dimensional models between neurotoxins and their receptors (2–4) show that similar domains of the toxins (between loop I and loop II) interact with their respective receptors. Therefore, it may not be unreasonable to assume that short-chain curarimimetic toxins interact with muscular-type nicotinic receptors in a “lock-and-key” manner and do not change their overall structure upon binding. The shape of AChBP (1) allows us to assume that the extracellular domain of the nAChR resembles a cylinder. Therefore, binding of our short toxin through the tip of its second loop, strictly perpendicular to the receptor surface, would result in equivalent steric hindrance on both the concave and convex faces. As a consequence, the ability to make ternary complexes with the receptor and streptavidin should be comparable for residues located at equivalent distance or height (as defined in Fig. 1) from the cylinder wall. Obviously, this is not the case. Although residues 27, 46, and 47 occupy roughly such a position on the toxin, analogues modified at the concave face at positions 27 and 47 could not bind streptavidin and the receptor simultaneously, irrespective of the length of the linker, whereas the V46C-Biot16.5 and even the V46C-Biot11.5, on the convex side, were able to do it (Fig. 4). Furthermore, ternary complex formation was possible with the longest linker, when modification occurred at the comparable positions 30 and 35, which are also on the convex face but much closer to the tip of loop II. Clearly, the convex face is much more accessible, at least in loops II and III, than the concave face. This conclusion agrees with previous mutagenesis studies on another short neurotoxin (20, 23), which showed that convex-side residues of erabutoxin-a were not involved in binding to the receptor. A reasonable explanation of the predominant access of the bulky streptavidin (67 kDa), on the convex face of the toxin bound to the receptor, is that the angle between the receptor surface and the concave face of the short toxin is smaller than the angle made with the convex face. Such a conclusion fits well with three-dimensional models of interaction between long toxins and their receptors (2, 4), which are fairly similar in their toxin orientation and positioning. Fig. 6, derived from the report of Fruchart-Gaillard et al. (4), illustrates this view. We simply overlaid N. nigricollis α-neurotoxin on cobratoxin complexed to the α7-receptor and afterward deleted the long neurotoxin (only two subunits of the α7-receptor model were shown). Clearly, the angle between the surface of the receptor and the concave face of the toxin is acute, whereas it is obtuse with the convex face.

Orientation of the Loops of Short Neurotoxins with Respect to the Membrane—Previous affinity labeling data showed that αCys-192 and/or αCys-193 of the reduced Torpedo receptor are located at a distance comprised between 11.5 and 15.5 Å from the α-carbons of Gln-10 and Arg-33 of N. nigricollis α-neurotoxin (18). This observation is consistent with the recent three-dimensional models of interaction between long toxins and their receptors (2–4), orienting loop I toward the apical surface of the receptor, orienting loop III toward the membrane, and introducing the tip of loop II into the acetylcholine binding site. This particular orientation of the toxin may account for the differential behavior of analogues modified at positions 10 and 47 with the longest linker, at the same height, on the concave face and involved in the binding site. The probe at position 10 on loop I is particularly efficient for the ternary complex formation, unlike position 47 (loop III), which was unable to bind both proteins. If one were to replace the long toxins with our short toxin, in the different three-dimensional models of interaction with nicotinic receptors, then in one model (3) position 10 would be accessible unlike position 47. In the two other models (2, 4), the 16.5-Å linker at position 10 could orient streptavidin above the apical face of the receptor. Such a way out is impossible for position 10 with the short linker and for position 47 regardless of the linker we used.

In conclusion, the present data demonstrate that binding location and orientation of a short neurotoxin on a muscular-type receptor can be anticipated from the recent three-dimen-
sional models of long α-neurotoxins bound to their receptors (2–4). Like long neurotoxins, short curarimimetic snake toxins neither penetrate deeply into a crevice as stated earlier (11–14) nor lie parallel to the receptor surface. If short toxins conserve their overall structure upon binding, then the angle between the concave face of the toxin and the receptor surface should be smaller than the same angle with respect to the convex face. Such an orientation of short neurotoxins fits also to previous affinity labeling experiments (18) and agrees with most of the results obtained by mutant cycle analysis (31, 32), with some exceptions, in particular concerning some non-α residues that were shown to be close to lysines on loops II and III on the concave face of another short neurotoxin (31). However, the insertion of additional residues in the γ- and δ-subunits (before loop F) may have a structural impact on the acetylcholine binding site, and the resting state of the muscular receptor may own structural differences with respect to AChBP, precluding any conclusion about these results. Like long neurotoxins, short toxins would then compete with acetylcholine by introducing the positive charge of Arg-33 in the aromatic cage and be forced to stay in that position, as a result of the whole set of interactions between the toxin and the receptor, these ligands therefore become quasi-irreversible antagonists. In the last months, an increasing number of reports concerning toxin-receptor interactions were published. Yet, docking of a short α-neurotoxin on a muscular-type receptor still awaits a model for fruitful discussions.

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