Relative Spatial Position of a Snake Neurotoxin and the Reduced Disulfide Bond \(\alpha(\text{Cys}^{192}-\text{Cys}^{193})\) at the \(\alpha\gamma\) Interface of the Nicotinic Acetylcholine Receptor

We determined the distances separating five functionally important residues (Gln\(^9\), Lys\(^{27}\), Trp\(^{29}\), Arg\(^{33}\), and Lys\(^{57}\)) of a three-fingered snake neurotoxin from the reduced disulfide bond \(\alpha(\text{Cys}^{192}-\text{Cys}^{193})\) located at the \(\alpha\gamma\) interface of the \textit{Torpedo} nicotinic acetylcholine receptor. Each toxin position was substituted individually for a cysteine, which was then linked to a maleimido moiety through three different spacers, varying in length from 10 to 22 Å. We estimated the coupling efficiency between the 15 toxin derivatives and the reduced cystine \(\alpha(192-193)\) by gel densitometry of Coomassie Blue-stained gels. A nearly quantitative coupling was observed between \(\alpha\text{Cys}^{192}\) and/or \(\alpha\text{Cys}^{193}\) and all probes introduced at the tip of the first (position 10) and second (position 33) loops of \textit{Naja nigricollis} \(\alpha\)-neurotoxin. These data sufficed to locate the reactive thiolate in a “croissant-shaped” volume comprised between the first two loops of the toxin. The volume was further restrained by taking into account the absence or partial coupling of the other derivatives. Altogether, the data suggest that \(\alpha\text{Cys}^{192}\) and/or \(\alpha\text{Cys}^{193}\), at the \(\alpha\gamma\) interface of a muscular-type acetylcholine receptor, is (are) located in a volume located between 11.5 and 15.5 Å from the \(\alpha\)-carbons at positions 10 and 33 of the toxin, under the tip of the toxin first loop and close to the second one.

The nicotinic acetylcholine receptor (nAChR\(^1\)) is a transmembrane protein, composed of five subunits \((\alpha_2\beta_2\gamma\delta)\) for the muscular type), which represents the prototype of the ligand-gated ion channels. It is quasi-reversibly blocked by snake curarimimetic toxins, a feature which helped in the isolation and purification of the receptor almost three decades ago. However, only recently has some light been shed on the molecular interaction between snake toxins and the nAChR. At least 10 residues of a sea snake toxin are involved in binding to the receptor from \textit{Torpedo marmorata}, i.e. Gln\(^9\), Ser\(^9\), Gln\(^{27}\), Lys\(^{27}\), Trp\(^{29}\), Asp\(^{33}\), Arg\(^{33}\), Ile\(^{36}\), Glu\(^{47}\), and Lys\(^{57}\) (1, 2). Receptor regions that interact with snake toxins have also been investigated in various studies. Some lines of evidence indicate that snake toxins can bind to two sites located at the interfaces of the \(\alpha\gamma\)- and \(\alpha\delta\)-subunits (3–5). Among the different receptor regions that may be implicated in toxin binding, the domain 180–200 of the \(\alpha\)-subunit is clearly an important determinant (for a review see Ref. 6). It is also involved in various other binding functions, because it is recognized by small organic agonists (7) and small antagonists (8–12). More recently, double mutant cycle analyses have revealed a number of contacts that may occur between nAChR and a cobra curarimimetic toxin (13–15). Thus, Arg\(^{33}\) of Nnml is coupled to the receptors \(\alpha\text{Val}^{118}\) and \(\gamma\text{Leu}^{119}\), whereas the toxin Lys\(^{27}\) interacts with \(\gamma\text{Glu}^{176}\), and to a lesser extent with \(\alpha\text{Tyr}^{192}\), \(\alpha\text{Pro}^{197}\), and Asp\(^{206}\). Although double mutant cycle analyses have evaluated the distances between various charged residues of this short-chain toxin and some residues of the receptor \(\gamma\)-subunit (15), the relative positioning of the toxin with respect to the receptor \(\alpha\)-subunit still remains unclear. To address this question, we have estimated the distances that separate several functionally important toxin residues from Cys\(^{192}\) and/or Cys\(^{193}\), two residues that belong to the critical binding region 180–200 of the two \(\alpha\)-subunits. More precisely, we used experimental conditions that allowed us to perform this study at a single binding site, namely, the site at the \(\alpha\gamma\) interface.

It is known that the disulfide bond \(\alpha(\text{Cys}^{192}-\text{Cys}^{193})\) can be selectively reduced without affecting antagonist binding (16) and hence specifically labeled with irreversible antagonists (8, 17, 18) or agonists (19). Treatment of the receptor with bromoacetylcholine or \([4-(N\text{-maleimido})\text{benzyl}]\text{trimethylammonium iodide}\) (MTBA) in the presence of a disulfide reducing agent leads to the covalent modification of one or both \(\alpha\)-subunits, depending on the concentration of affinity label (20–22). Even more interestingly perhaps, when the reducing agent is eliminated after the alkylation step, only one site is labeled by both probes, independent of their concentrations (22). This site has been demonstrated to correspond to the high affinity binding site of \(d\)-tubocurarine (23), which was later located at the \(\alpha\gamma\) interface of the receptor (11, 24). Using radioactive MTBA, it was further demonstrated that \(\alpha\text{Cys}^{192}\) and \(\alpha\text{Cys}^{193}\) are the exclusively labeled residues (8).

Our strategy was inspired from this site-directed alkylation procedure. First, we chemically engineered analogues of a cu-
rarriminetic snake toxin, by automated peptide synthesis. More precisely, we individually replaced each of the five functional residues Gln10, Lys27, Trp29, Arg33, and Lys47 by a cysteine residue. The reason for the choice of these residues was that they are both important binding contributors to toxin-receptor formation and are widely spread over the functional topography of the snake toxin (1, 2). Second, we linked each of them to three spacers of different lengths (10, 14, and 22 Å) that always ended in a maleimido group. Each, the 15 monomodified toxin derivatives was mixed with the receptor whose α(Cys182-Cys193) disulfide bond was selectively reduced and affinity-labeled under conditions where only the αγ interface was modified (23). Fourth, the coupling yield was estimated from gel electrophoresis experiments. Fifth, the data were exploited to build a model in which the toxin was positioned relative to cysteines 192 and/or 193 of the α-subunit at the αγ interface.

**EXPERIMENTAL PROCEDURES**

**Materials**—Peptides were synthesized with an Applied Biosystems 433 apparatus, using Fmoc-protected amino acids of the highest purity commercially available from NovaBiochem, N,N′-diphenylmaleimide and N,N′-bis[3-maleimidopropionyl]-2-hydroxy-1,3-propanediol from Sigma. [α125I]-Bungarotoxin (Bgtx) (200 Ci/mmol) was from Amersham Pharmacia Biotech. Live T. marmorata were from the Station Biologique d’Arcachon (France). Naja nigricollis horsera anti-venom serum was from the Pasteur Institute (France). Goat anti-rabbit (GAR-PO) and rabbit anti-horse (RAH-PO) IgG peroxidase conjugates were from Jackson Immunoresearch Laboratories (West Grove, PA). Electrospray mass spectrometry was carried out using a Quattro II spectrometer from Micromass. Circular dichroism spectra were recorded on a Jobiin Yvon CD6 dichrograph at 22 °C.

**HPLC Conditions**—Reverse-phase high performance liquid chromatography (HPLC) purifications were done with a Vydac C4 semipreparative column (10 × 250 mm; flow rate, 4 ml/min−1; linear gradient, 5% to 21% B in 6 min; then 21% to 27% B in 19 min (A, H2O − 0.1% trifluoroacetic acid; B, CH3CN − 0.06% trifluoroacetic acid)).

**Membrane Preparations**—nAChR-rich membranes from the electric tissue of T. marmorata were prepared, as described previously (25), in the presence of 20 mM N-ethylmaleimide to block all free cysteines. They were further purified by alkali treatment (26). The concentration of acetylcholine binding sites was measured at equilibrium with [3H]-acetylcholine using a Quattro II spectrometer from Micromass. Circular dichroism and its mass was determined by electrospray mass spectrometry. The mixture was left at room temperature for 30 min, except for the K27C derivatives, where the incubation time was prolonged to 8 h. Excess reactant was removed by exclusion chromatography (Biogel P2 300; Bio-Rad), with the toxin migrating in the void volume. Each maleimido-containing derivative was purified by HPLC as described above, and checked by electrospray mass spectrometry and circular dichroism.

To conjugate the single free cysteine (Q10C, K27C, W29C, R33C, or K47C) of each toxin mutant with dimaleimide reagents, we first solubilized 0.7 mg (0.1 µmol) of toxin analogue in 200 µl of 250 mM sodium acetate buffer (pH 5.5/2.5 mM EDTA) and 100 µl of H2O. The solution was then poured on 20 equivalents of dimaleimide-containing reagent dissolved in either a mixture of dimethylformamide (40 µl/vacetone (160 µl) for the two shorter reagents (Mal10 and Mal14) or in 200 µl of sodium acetate buffer for the longest (Mal22) reagent, with rapid stirring. The mixture was left at room temperature for 30 min, except for the K27C derivatives, where the incubation time was prolonged to 8 h. Excess reactant was removed by exclusion chromatography, with the toxin migrating in the void volume. Each maleimido-toxin derivative was purified by HPLC as described above, and its mass determined by electrospray mass spectrometry.

**Binding Assays**—Binding competition experiments were performed at equilibrium, using [α125I]-Bgtx (200 Ci/mmol) as radioactive tracer. Varying amounts of toxins were incubated in Ringer’s buffer (5 mM sodium phosphate buffer, pH 7.5, 250 mM NaCl, 5 mM MgCl2, 4 mM CaCl2, 2 mM MgCl2, 0.02% NaN3, 0.01% Triton X-100), with 0.3 mM α-toxin binding sites and 9 nM [α125I]-Bgtx for 5 h at room temperature. The mixture was filtered through Millipore filters (HAWP), which had been soaked in the same buffer. The filters were rinsed twice with 5 ml of Ringer’s buffer and counted on a 1261-multigamma counter (Amer sham Pharmacia Biotech). Equilibrium dissociation constants were determined from IC50 values according to Cheng and Prusoff (31).
logues of \textit{N. nigricollis}.

![Image](image-url)

**Fig. 1:** \textit{N. nigricollis} \textit{a}-neurotoxin. The structure has been taken from Zinn-Justin et al. (51). The five residues, which were individually replaced by a cysteine, are shown in boldface font.

![Image](image-url)

**Fig. 2:** Circular dichroism spectra of the five “cysteine” analogues of \textit{N. nigricollis} \textit{a}-neurotoxin derivatives. The polypeptides were dissolved in water at 50 μM final concentration. The cell path length was 0.2 cm. (−−−) native toxin, (−−−−) Q10C, (−−−−−) K27C, (−−−−−−) W29C, (−−−−−−−) R33C, and (−−−−−−−−) K47C.

TABLE I

| Inhibition constants of \textit{N. nigricollis} derivatives | for \textit{T. marmorata} nAChR |
|------------------------------------------------------------|---------------------------------|
| \textit{K} \textsubscript{a} | \textit{K}/\textit{K} \textsubscript{wt} |
| Wild-type | 0.013 | 1 |
| Q10C | 0.056 ± 0.020 | 4.3 |
| Q10C-Mal10 | 0.13 ± 0.05 | 10 |
| Q10C-Mal14 | 0.07 ± 0.03 | 5.5 |
| Q10C-Mal22 | 0.08 ± 0.04 | 6 |
| K27C | 0.23 ± 0.08 | 17.5 |
| K27C-Mal10 | 0.38 ± 0.20 | 30 |
| K27C-Mal14 | 0.42 ± 0.20 | 33 |
| K27C-Mal22 | 0.07 ± 0.05 (high) | 5 |
| W29C | 1.9 ± 0.8 | 145 |
| W29C-Mal10 | >11 | >850 |
| W29C-Mal14 | >11 | >850 |
| W29C-Mal22 | >11 | >850 |
| R33C | 0.16 ± 0.07 | 12 |
| R33C-Mal10 | 0.07 ± 0.03 | 5.5 |
| R33C-Mal14 | 0.04 ± 0.02 | 3 |
| R33C-Mal22 | 0.02 ± 0.01 | 1.5 |
| K47C | 0.37 ± 0.13 | 28.5 |
| K47C-Mal10 | 0.20 ± 0.10 | 15.5 |
| K47C-Mal14 | 0.09 ± 0.04 | 7 |
| K47C-Mal22 | 0.11 ± 0.04 | 8.5 |

\* \textit{K}, inhibition constant for toxin derivative; \textit{K}/\textit{K} \textsubscript{wt}, inhibition constant for the wild-type toxin.

\* Due to a lack of points at high concentrations of ligand, the given \textit{K} value is only an estimate.

Inhibition constants were obtained by competition at equilibrium against \textit{α}-bungarotoxin (9 nM) on \textit{T. marmorata} nAChR (0.3 nM toxin binding sites). Values were estimated according to Cheng and Prusoff (31). Mean ± S.E. or standard errors for two or more measurements.

Consistent with its \textit{K} \textsubscript{a} value (36), and this value decreased 4-, 18-, 145-, 12-, and 29-fold upon use with mutations Q10C, K27C, W29C, R33C, and K47C, respectively. Despite the affinity decreases, the analogues still retained relatively high binding affinities.

**Introduction of Maleimide-containing Reagents into Cysteine-containing Toxins**—Three dimaleimides called Mal10, Mal14, and Mal22 (Fig. 3) were reacted with the additional cysteine introduced into each of the five toxin mutants. To avoid formation of toxin dimers and to favor formation of mono-

modified derivatives, we used a large excess of dimaleimides (20 eq). Excess reactant was subsequently eliminated by gel exclusion, and the 15 derivatives were purified by reverse-phase HPLC. All but the K47C-Mal10 derivative eluted as a single peak, which is strongly indicative of their homogeneity. The K47C-Mal10 derivative HPLC profile showed a slight but unexplained shoulder (not shown). All the derivatives displayed the expected mass (not shown) and were used as such for the labeling experiments.

All chemically modified derivatives but W29C displayed similar affinities for nAChR as compared with cysteine-containing derivatives (Table I). Due to a lack of material, we could not determine the IC\textsubscript{50} values for W29C maleimido analogues. Interestingly, the K27C-Mal22 derivative discriminated between the two toxin binding sites, one of them being recognized with almost the same affinity as for the wild-type toxin and the other with an affinity approximately two orders of magnitude lower.

**Affinity Labeling of the Reduced Receptor**—At first, all free cysteines of a membrane preparation of nAChR were blocked using 20 mM \textit{N}-ethylmaleimide. The nAChR possesses three types of disulfide bonds spread over the extracellular domains of the various subunits. These are Cys\textsuperscript{128}-Cys\textsuperscript{142} on all subunits, Cys\textsuperscript{128}-Cys\textsuperscript{183} on the α-subunits, and the Cys\textsuperscript{500} on the δ-subunits, linking two monomers of the receptor (37, 38). Under mild reducing conditions, only the α(Cys\textsuperscript{192}-Cys\textsuperscript{198}) disulfide bond is affinity-labeled (8), and if the reducing agent is eliminated before the alkylation step, only the αγ interface is labeled (23). We therefore carefully considered this procedure in a tentative attempt to generate selective labeling of the reduced α(Cys\textsuperscript{192}-Cys\textsuperscript{198}) bond at the αγ interface.

Incubation of the 15 maleimido-containing derivatives with the reduced nAChR was analyzed by SDS-PAGE and revealed by Coomassie Blue staining (Fig. 4A). The control lane with no derivative (lane 1) shows the four expected nAChR subunits.
ing reagents reduced receptor was treated with the well-known thiol blocking toxin molecule. No such clear-cut phenomenon was seen due to the maximal length between the cysteine moiety is left for reaction with the reduced receptor. The given distances correspond to the maximal length between the reactive carbon on the maleimide moiety.

Clear changes in intensity patterns were only seen for all derivatives modified at Q10C and R33C and for the derivative W29C-Mal22 (Fig. 4A; lanes 2, 3, 4, 10, 11, 12, and 13). In all these cases, the intensity of the α-subunit decreased. This change was concomitantly associated with an increased intensity in the β-subunit, whose apparent molecular mass was close to 46 kDa, which not only fits with the β-subunit but also with a complex consisting of an α-subunit molecule associated with one toxin molecule. No such clear-cut phenomenon was seen with the eight other derivatives. Neither was it seen in control experiments where the receptor was not reduced, nor when the reduced receptor was treated with the well-known thiol blocking reagents N-ethylmaleimide or 5,5′-dithiobis-(2-nitrobenzoic acid) (not shown). Moreover, when the reduced nAChR was pretreated with an excess of an analogous snake toxin, erabutoxin a, the effect vanished for all the derivatives, except for the Q10C analogues for which the protection from affinity labeling was around 80% (data not shown). These data indicate that the phenomenon (i) is associated with the presence of free cysteines 192 and 193, (ii) requires a maleimido-containing toxin derivative, and (iii) is specific to the introduced linkers located at positions 10, 29, and 33.

To demonstrate that the toxin moiety was covalently associated with the new 46-kDa protein band, we blotted an SDS gel previously incubated with antitoxin antibodies present in a snake antivenom (Fig. 4B). Curiously, nonspecific detection was seen weakly at the level of the α-subunit and even more weakly around the β-subunit (Fig. 4B, lane 1). Nevertheless, strong differential labeling was readily observed around 46 kDa with W29C-Mal22 and all the derivatives modified at Q10C and R33C. For all these analogues, therefore, the toxin (molecular mass of 7 kDa) migrates with an apparent mass of approximately 46 kDa. No such detection was seen with other derivatives.

To establish whether the α-subunit is also present in the new 46-kDa band, we performed a similar blot experiment with an α-subunit-specific monoclonal antibody (33) (Fig. 4C). The control revealed an intense band migrating at the expected position of the free α-subunit (40 kDa). An additional band was seen around 46 kDa with the three derivatives modified at Q10C and R33C and with the longest derivative at W29C, confirming that, when these derivatives were present, the α-subunit migrated with a larger apparent molecular mass. In other words, these particular toxin derivatives comigrated with the α-subunit, a finding that strongly indicated their covalent association. We observed a similar though much weaker band with some other derivatives, in particular with K27C-Mal22, the two shorter W29C analogues, and the three K47C derivatives.

**Coupling Yields at the αγ Interface Binding Site**—To evaluate the coupling yield between toxin derivatives and the α-subunit at the αγ interface, we monitored the band intensities at both 39 and 46 kDa by densitometry of the Coomassie Blue-stained gel for experiments where the reduced receptor and the derivatives were incubated for 15 and 90 min. The densitometry data must obviously be considered with caution, as they only reflect rough estimates of coupling yields whose determination was further complicated by the overlap of the intensity of the β-subunit, which added to background errors. However, these coupling yields seem to be in good agreement with the sensitive α-subunit immunoblot detection (Fig. 4C). Though the novel band at 46 kDa was not detectable by immunoblot experiments for the two shorter derivatives at Lys27 (Fig. 4C, lanes 5 and 6), a small coupling yield (around 10%) was determined by gel densitometry (Fig. 5). We therefore considered this value as the threshold above which coupling was significant.

Irrespective of the position of the probe, no more than approximately half of the two α-subunits were labeled, showing that a single site was labeled under our experimental conditions, where the reducing agent was removed before reaction of the toxin (18, 21, 22, 39) (Fig. 5). Furthermore, it is known that low concentrations of bromoacetylcholine label the reduced receptor exclusively at the αγ interface (22, 23). In agreement with the view that the same site was probed by our derivatives, we found that preincubation with a low concentration (10 μM) of bromoacetylcholine fully inhibited labeling with R33C-Mal22 (Fig. 4D). Therefore, our data support the view that the observed coupling occurs selectively on the reduced disulfide at the αγ interface. The average 50% maximal coupling yield that was observed with respect to both α-subunits therefore corresponds to 100% labeling at the αγ interface (Fig. 5).

Fig. 5 shows that coupling yields obtained with Q10C-Mal22, W29C-Mal22, and the three derivatives of R33C were approximately 100% after both 15- and 90-min incubations. This suggests that the maximal coupling was reached within 15 min, in good agreement with an affinity-labeling process (39). Fig. 5 also shows that each modified toxin position behaved in a unique manner. At position 33, quantitative coupling was observed irrespective of the size of the spacer. At position 10, a 70–80% coupling yield was seen with the two shorter linkers and quantitative coupling with the Mal22 arm. At position 29, the coupling yield was 30% and 45% with the Mal10 and Mal14 spacers, respectively, and coupling was complete with the Mal22 spacer. Finally, the longest probe at position 27 and the three probes at position 47 led to at most 20–30% coupling, whereas the two shorter arms at position 27 led to virtually no coupling.
DISCUSSION

We prepared 15 derivatives of a snake toxin, each of them possessing a single maleimido moiety. We used these derivatives to localize the toxin spatially relative to cysteines 192 and/or 193 of the reduced α-subunit of the nAChR. Two lines of evidence indicated that only cysteines at the αγ interface of the nAChR were labeled. First, previous data showed that, under the same experimental conditions as those used in this study, only half of the toxin binding sites were labeled by a small toxin antagonist (8, 22, 40, 41). Accordingly, we observed a maximal coupling yield of the α-subunits of approximately 50%. Second, data from several laboratories (11, 22–24) demonstrated that the labeled site is at the αγ interface, this site being selectively discriminated by low concentrations of bromoacetylcholine (22, 41). In agreement with these findings, we observed that small amounts of the same reagent fully inhibit toxin labeling.

To interpret our coupling data in terms of relative positions between the two receptor cysteines and the toxin, a number of assumptions had to be made. First, because the two shorter spacer lengths (Mal10 and Mal14) are highly rigid, the maleimides located at their tips were considered to describe a surface centered, respectively, on the α-carbon of the modified residue. Second, because we observed almost comparable full coupling with the two shorter arms, we assumed that at least one of the thiocarbonates of cysteines 192 and 193 is present in a volume comprised between two spheres with radii of approximately 11.5 and 15.5 Å (the distance between the reactive carbon and thiols is assumed to be approximately 1.5 Å). We called these volumes the “reactive volumes.” Third, bearing in mind the imprecision of the data shown in Fig. 5, we considered that the coupling yields with the two shorter arms at positions 10 correspond approximately to full coupling, like those at positions 33. Fourth, for partial but significant coupling, in the range of 30–50%, as observed with W29C derivatives, we considered that the reactive carbon was in proximity but not directly involved in the reactive volumes. Fifth, because the longer arm is highly flexible, we assumed it encompassed a large volume, which is difficult to predict, separated at most by 22 Å from the Ca of the considered residue.

In addition to these assumptions, a number of elements had to be considered. First, the coupling yield is influenced by the distance that separates the thiols and the maleimides and by the accessibility of the sulfhydryl which, for example, could be favorably (or unfavorably) orientated for the attack of the thiolate perpendicularly to the plane of the maleimide ring. Second, at the molecular level, only one of the two cysteines can effec-

FIG. 4. Visualization of the labeling of the α-subunit of T. marmorata nAChR. The receptor was reduced for 20 min with 1 mM DTT, at room temperature, under argon atmosphere, in phosphate buffer (100 mM, pH 7.6, 2.5 mM EDTA). After three steps of centrifugation (see “Experimental Procedures”), the reduced receptor (1 μM toxin binding sites) was incubated with the 15 toxin analogues (1 μM) for 15 min at room temperature, under argon atmosphere. A, Coomassie Blue staining of an SDS-PAGE gel of the reduced nAChR after coupling with the 15 toxin derivatives. 10A, 14A, and 22A represent the Mal10, Mal14, and Mal22 linkers, respectively, on each cysteine analogue. Reduced Torpedo membrane fragments (lane 1); same fragments after coupling with Q10C-Mal10 (lane 11); W29C-Mal14 (lane 14); R33C-Mal10 (lane 5); K27C-Mal14 (lane 16); R27C-Mal22 (lane 7); W29C-Mal10 (lane 8); W29C-Mal14 (lane 9); W29C-Mal22 (lane 10); R33C-Mal10 (lane 11); R33C-Mal14 (lane 12); R33C-Mal22 (lane 13); K47C-Mal10 (lane 14); K47C-Mal14 (lane 15); K47C-Mal22 (lane 16). Molecular weight markers (lane 17). B, immunoblot with a horse serum directed against N. nigricollis venom. The lanes correspond to the same derivatives as in A. C, immunoblot with monoclonal antibody 155, a monoclonal antibody directed against the nAChR α-subunit. The lanes correspond to the same derivatives as in A. D, inhibition of labeling by R33C-Mal22 with 10 μM bromoacetylcholine (BAC); reduced nAChR labeled with R33C-Mal22 (lane a), reduced nAChR labeled with 10 μM BAC (lane b), and reduced nAChR labeled with 10 μM BAC before reaction with R33C-Mal22 (lane c).
Location of $\alpha$(Cys$^{192}$-Cys$^{193}$) with Respect to an $\alpha$-Neurotoxin

Considering the rather large distance that separates the thiol(s) occupying the identified region of the reduced receptor and the closest toxin residues, Gln$^{45}$, Arg$^{33}$, and Ile$^{35}$, we suggest that the considered thiol(s) is(are) not fully hidden when the toxin is bound to its receptor. This situation could thus explain why the bound toxin does not fully protect labeling of the reduced receptor $\alpha$-subunit with bromoaceticholine (42) or N-ethylmaleimide. Probably, therefore, the reactive thiol(s) is (are) not in direct interaction with the toxin. Possibly also, the low coupling yields observed for the K27C and K47C derivatives (<30%) may be due to the flexibility and/or the accommodation of the molecules during the interaction between the receptor and toxin.

It was previously suggested that the positively charged quaternary ammonium ion of the antagonist MBTA is located at about 10 Å from the $\alpha$(Cys$^{192}$-Cys$^{193}$) disulfide bond (8). Based on circumstantial evidence, it was also proposed that ammonium cations of small organic ligands may be mimicked by the positive charge of Arg$^{33}$ of curarimimetic toxins (43, 44). Considering the present data, Arg$^{33}$ is indeed the only positively charged toxin residue that is consistent with the proposed mimicry, the positive charges of the two other candidates (Lys$^{27}$ and Lys$^{47}$) being too remote from the disulfide bond.

How does our conclusion fit with previous experiments (13, 15, 45–47) designed to position a short or a long-chain toxin with respect to nAChR? An NMR study of a complex between the long-chain $\alpha$-Bgtx and a dodecapeptide of the Torpedo receptor (a185–196) indicated that the region a186–190 is located between the first and the second loops of the toxin (46). Another NMR study of a complex between $\alpha$-bungarotoxin and a library-derived peptide sharing some similarity with the receptor peptide $\alpha$187–199 showed that this 13-mer peptide also

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2 P. Kessler, personal observation.
binds roughly in between the first and second toxin loops. Both studies clearly agree with our findings that the receptor region comprising a(Cys192-Cys193) is located in between the first and second loops of a snake toxin. In one study, the peptide residues that are homologous to cysteines 192 and 193 were too mobile to be located (46). In the other study (47), these residues do not fit in the reactive region, but are located much more to the back of the plane defined by the large b-sheet of a-bungarotoxin. More precisely, the distance that separates the region colored violet (Fig. 6) from those peptide residues is approximately 15 Å. This situation might result from differential binding between short (this study) and long toxins (46, 47). Two lines of evidence support this view. First, a derivative of a long-chain toxin modified on the residue homologous to Lys27 with a mercurial compound similar in length to our Mal14 derivative, slightly coupled to the reduced cysteines of the a-subunits (45), in contrast to our own findings. Second, recent data have indicated that the binding topographies of short- and long-chain neurotoxins for Torpedo AChR are not strictly identical (48). It could also be argued that the small peptides used in NMR studies might behave differently when free or integrated in the whole receptor. More work is needed to clarify this question.

Recently, double mutant cycle analyses have shown that when another short toxin, NmmI, binds at the a/g interface of the non-reduced mouse muscular receptor, Arg63 of the toxin is in proximity to aVal188 and aTyr190 (13). This result fully agrees with our proposed location of the disulfide bond a(Cys192-Cys193) at the same interface (Fig. 6). In addition, it was observed that Arg63 is also in close proximity to Trp55 and Leu119 of the g-subunit of the muscular receptor (15). Assuming that the Torpedo and mouse receptors are comparable, our data combined with those of Osaka provide a molecular basis for the positioning of a short-chain toxin relative to residues of the a- and g-subunit at the a/g interface.

It is currently assumed that the sequential homology of gAsp174 and δAsp180 is associated with an identical position of the two residues in their respective sites (49). Thus, a site-directed labeling experiment has shown that δAsp180 is located 1 nm from the reduced disulfide bond a(Cys192-Cys193) (38, 50). Furthermore, γAsp174 was recently located near Lys47 and Lys199 on the third loop of NmmI toxin (15). Interestingly, our model (Fig. 6) shows that the homologous Lys47 on N. nigricolis toxin a, and therefore the interacting γAsp174 (15), are over 10 Å from the disulfide bond at the aγ interface. This evidence thus suggests that the binding sites at the aγ and aδ interfaces are structurally distinct.
In conclusion, the data reported in this paper describe the respective spatial positioning of an element of the nAChR and a snake toxin, a potent receptor antagonist. Together with recent double mutant cycle analyses (15), it offers a suitable molecular basis for building up the remaining receptor elements that interact with the toxin.

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Location of α(Cys192-Cys193) with Respect to an α-Neurotoxin

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