The Solution Structure of the Complex Formed between α-Bungarotoxin and an 18-mer Cognate Peptide Derived from the α1 Subunit of the Nicotinic Acetylcholine Receptor from Torpedo californica*

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The region encompassing residues 181–98 on the α1 subunit of the muscle-type nicotinic acetylcholine receptor forms a major determinant for the binding of α-neurotoxins. We have prepared an 15N-enriched 18-mer amino acid sequence corresponding to the sequence in the subunit that has been implicated in binding. The root mean square deviation of the polypeptide backbone in the complex is 2.07 Å. This structure provides, to date, the highest resolution description of the contacts between a prototypic α-neurotoxin and its cognate recognition sequence.

The nicotinic acetylcholine receptor (nAChR)1 (1) has long been a prototype for ligand-gated ion channels. This receptor is involved in excitatory synaptic transmission at the neuromuscular junction and also plays an important role in the nervous system. The nAChRs are pentameric complexes composed of homologous subunits with subunits arranged around the central channel in a symmetrical manner. The muscle-type nAChR contains two α1 subunits and one each of the β1, γ(e), and δ subunits. The ligand binding sites are situated at the αγ(e) and αδ subunit interfaces. The muscle-type nAChR serves as an important model for the study of the structures and functions of related ligand-gated ion channels (for review, see Ref. 1).

The snake venom-derived α-neurotoxins fall into two categories, short and long neurotoxins, and act as high affinity competitive antagonists at the nAChR. Short neurotoxins (e.g. erabutoxin α) contain 60–62 amino acid residues and 4 conserved disulfide bridges. Long neurotoxins have 66–74 residues and 5 disulfide bonds, including four in a core region that are homologous in position to those found in the short neurotoxins. α-Bungarotoxin (Bgtx), obtained from the snake venom of Bungarus multicinctus, is a long α-neurotoxin that over the years has provided a powerful tool for the study of muscle-type nAChRs and which has come to be viewed as somewhat of a gold standard among the α-neurotoxins. A number of the α-neurotoxins have been heterologously expressed in recent years, allowing for investigations using site-directed mutagenesis (2–5).

From its x-ray structure, Bgtx is a relatively flat, slightly concave, disc-shaped protein with a characteristic three-finger folding motif consisting of three loops of structure (6). Previous NMR structural studies indicate that the solution structure of Bgtx, although generally consistent with the x-ray structure, does reveal some differences (7). Notably, the side chain of Trp28 in the two structures resides on opposite sides of the major plane of the molecule. In the solution structure, the Trp side chain is on the concave surface, as seen with most other α-neurotoxins containing this highly conserved residue. In contrast, the Trp side chain is located on the opposite face in the crystal structure (6). The structures of several other snake venom α-neurotoxins have been studied with NMR techniques (8–12), and all exhibit the characteristic three-finger structure.

Previous work indicates that the main determinants for Bgtx binding to the muscle-type nAChR lie between residues 173 and 204 of the α1 subunit (13), a region that coincides with one of three segments of the α subunit that have been implicated in correlation; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; r.m.s.d., root mean square deviation; NnmI, Naja mossambica mossambica I.
agonist binding (for review, see Ref. 1). Tyr<sup>190</sup>, along with Cys<sup>192</sup>, Cys<sup>193</sup>, and Tyr<sup>198</sup>, are selectively cross-linked with a variety of site-directed photoaffinity reagents (14). This region, termed segment C, contains a conserved pair of adjacent Cys residues, Cys<sup>192</sup>, Cys<sup>193</sup>, that form an unusual disulfide. Studies of synthetic peptides with sequences matching those in segment C have identified several peptides that bind Bgtx with affinities to the micromolar range (15, 16). A peptide fragment (α18-mer) with a sequence corresponding to amino acid residues 181–198 (α<sub>Y</sub>1818, RGKWKHWVVYTEPPT<sup>198</sup>) from the Torpedo californica nAChR binds Bgtx with an apparent KD of ~65 nM (17). Replacing the Tyr at position 190 with a Phe leads to a 60-fold decrease in Bgtx binding affinity for the altered peptide, suggesting an important role for this aromatic residue in complex formation (17). Mutations of Tyr<sup>190</sup>, when assessed in heterologous expression systems, also result in large decreases in α-neurotoxin binding (3, 18, 19). In addition, ligand gating is also dramatically affected by mutations in Tyr<sup>190</sup> (20, 21). Studies with recombinant receptor fragments corresponding to the α subunit from the mongoose nAChR, which is resistant to α-neurotoxins, suggest two subsites in the binding domain for Bgtx; one is a proline subsite consisting of Pro<sup>194</sup> and Pro<sup>197</sup>, and the other is an aromatic subsite involving positions 187 and 189 (15).

We previously described some of the structural features revealed by an NMR analysis of Bgtx complexed with a 12-amino acid peptide fragment (α12-mer) of the Torpedo nAChR α1 subunit (α<sub>K</sub>186, HWVVYYTCCPDPY<sup>196</sup>), which has an apparent KD of ~1.4 μM (17, 22). We now describe a more expansive NMR structural analysis of the higher affinity complex formed with the α18-mer. The structure of this complex may provide valuable information on the orientation of the contact residues in the native nAChR and may help in elucidating the essential interactions that direct the ability of the α-neurotoxins to recognize receptor sequences with remarkable affinity and specificity.

**Experimental Procedures**

**Expression Construct**—We designed an oligonucleotide sequence to encode for residues 181–198 (YRGWKHWVVYYTCCPDPY) of the α1 subunit from the nAChR of T. californica. Three copies of this expression cassette were inserted downstream of a 125-amino acid ketosteroid isomerase gene and upstream of a His-tag sequence in plasmid pET-28.<sub>a</sub> (23). The final construct, ketosteroid isomerase-Met-(α18-mer)-His-tag, contained single Met residues separating the three cassettes from each other, from ketosteroid isomerase, and from the C-terminal His tag. The oligonucleotide sequence of this construct is available upon request. The plasmid, whose insert sequence was confirmed by DNA sequence analysis, was used to transform cells of the expression strain BL21 (DE3) (Novagen).

**Cell Growth**—Cell cultures were grown in standard M9 medium except that <sup>15</sup>NH<sub>4</sub>Cl was used as a replacement for normal NH<sub>4</sub>Cl. All cultures were supplemented with 100 μg/ml ampicillin (M9/Amp). A single colony from a fresh agar plate containing ampicillin was used to inoculate 100 ml M9/Amp medium and grown overnight at 37 °C. The overnight culture was added to 2 liters of M9/Amp medium in a VirTis benchtop fermentor. This culture was grown at 37 °C with stirring at 600 rpm until the A<sub>600</sub> was 0.7–0.8. Isopropyl-β-D-thiogalactoside was added to a concentration of 1 mM to initiate induction of the fusion protein. After 3 h, cells were harvested by low speed centrifugation.

**NMR Solution Structure of an α-Bungarotoxin-α18-mer Complex**

After resuspension in 40 ml of 20 mM Tris-HCl buffer (pH 7.9), cells were passed through a French pressure cell (SLM Instruments) at 15,000 p.s.i. Inclusion bodies were isolated by centrifugation, resuspended in “binding buffer” (6 M guanidine-HCl, 0.5 M imidazole, and 20 mM Tris-HCl (pH 7.9)), and applied to a column containing Ni<sup>2+</sup>-charged His-Link resin (Novagen) prepared according to the manufacturer’s specifications. After washing the resin with 10 column volumes of binding buffer and 5 column volumes of wash buffer (6 M guanidine-HCl, 40 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl pH 7.9), the ketosteroid isomerase fusion protein was eluted in 5 column volumes of elution buffer (6 M guanidine-HCl, 0.3 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9)). The C-terminal His tag was removed using a two-dimensional 15N heteronuclear single quantum correlation (1H-15N HSQC) (24–26) experiment. Amino acid spin systems were identified by two-dimensional total correlation spectroscopy (TOCSY) (24, 25, 27) and three-dimensional TOCSY-HSQC experiments (27–31) with a mixing time of 60 ms. The assignments of the NH and C<sup>a</sup>H protons and C<sup>a</sup>H protons of the amino acid spin systems of the peptide were further confirmed by a three-dimensional NHNA experiment provided the correlation between the <sup>15</sup>NH proton and the C<sup>a</sup>H proton of the same amino acid; these data help confirm the identification of the NH and C<sup>a</sup>H protons. Nuclear Overhauser effect (NOE) correlations (sequential, medium-range, and long range NOEs) were identified by two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) (24, 25) and three-dimensional NOESY-HSQC experiments (28–31) with a mixing time of 60 ms. Spectra were recorded at 25 °C to facilitate the assignment of resonances. All NMR spectra were recorded and analyzed with XwinNmr (Bruker), NMRPipe (34), and SPARKY (35).

In our comparing results with earlier, more preliminary assignments involving an unlabeled α18-mer peptide bound to Bgtx (36), we found that most Bgtx assignments are the same or similar (chemical shifts change less than 0.05 ppm) after calibration. However, the assignments of Val<sup>2</sup>, His<sup>4</sup>, Ser<sup>9</sup>, Ile<sup>11</sup>, Lys<sup>26</sup>, Cys<sup>29</sup>, Val<sup>40</sup>, Lys<sup>41</sup>, Lys<sup>42</sup>, Glu<sup>72</sup>, Arg<sup>72</sup>, and Gly<sup>73</sup> were significantly different; in most cases no comparable resonances were observed in our two-dimensional NOESY spectra. On the other hand, new resonance peaks appear elsewhere in the spectra. All these new resonances involving these residues were re-assigned based on sequential NOE connectivity. We think it most likely that the observed chemical shift differences between the two samples are caused by a difference in the ionic strength of the two samples even though both were prepared at pH 4.0 and spectra were acquired at 35 °C. Our present sample is dissolved in 50 mM potassium acetate, whereas the earlier sample was simply adjusted to pH 4.0 with the addition of HCl. A change in the ionic environment could have significant effects on eluting partial occupancy of charged residues, leading to changes in the chemical environment of a subset of spins. Similarly, it was necessary to re-assign most of the α18-mer peptide resonances. Of the eight previously assigned peptide residues, only Asp<sup>106</sup> and Thr<sup>126</sup> are unchanged between the two samples. However, the C<sup>α</sup>H proton of C<sup>α</sup>H proton of Thr<sup>126</sup> were erroneously assigned previously (36). The new swapped assignments incorpo...
rate the results from the three-dimensional HNHA experiment.

Conformation Calculations—The cross-peak volumes in the two-dimensional NOESY spectra were integrated by the Gaussian fitting protocol using SPARKY. The cross-peaks were classified into three categories: strong, medium, and weak, with corresponding distance ranges of 1.8–3.0, 1.8–4.0, and 1.8–5.0 Å, respectively. The HN-H FCDIH coupling constants of the α18-mer peptide were obtained from the three-dimensional HNHA experiment (37). The FCDIH coupling constants were converted to dihedral angle restraints using previously described methods (38). For FCDIH < 6 Hz, the dihedral angle restraint was assigned to −60° ± 30°; for FCDIH > 8 Hz, the dihedral angle restraint was −120° ± 40°. The structures were calculated with distance geometry and simulated annealing protocols using the dg_sa.inp script of the NMR structure calculation program, CNSsolve (39). The following is the potential energy function used in these calculations: Ftotal = Fnoe + Fimp + Fvdw + Fnh where Fnoe relates to bond length, Fimp and Fvdw to bond angles, Fnoe relates to the van der Waals repulsion term, Fvdw relates to NOE distance constraints, and Fnh relates to dihedral angles. Pseudoatoms were used for protons that could not be stereospecifically assigned. The pseudoatom correction feature of CNSsolve was used to adjust the NOE distance constraint range automatically. In each batch of calculations, a different random seed number was used to initiate the calculation of a set of 50 structures. From the pool of calculated structures, only those structures lacking any NOE violation (<0.05 Å) were selected as “acceptable” for further analysis. As a result of the weighting of the Fnoe term in CNSsolve, none of the other energy terms were as critical as Fnoe in determining an acceptable structure. In total, 120 acceptable structures of the Bgtxα18-mer complex were obtained from 6 batches of independent calculations (i.e. 300 total structures), and 122 acceptable structures of free Bgtx were obtained from 8 batches of independent calculations (i.e. 400 total structures). The 20 lowest-energy structures out of the acceptable structures for free Bgtx and for the Bgtxα18-mer complex were selected to form an ensemble of representative final structures. The mean structure corresponding to each ensemble was calculated by a program written by Dr. Christian Rölz (40). The two mean structures (free Bgtx and Bgtxα18-mer complex) were further partially energy-minimized using DISCOVER (Molecular Simulations, Inc.) to create representative structures complete with side chains. All 20 structures depicted in Fig. 7 have been deposited into the Protein Data Bank, Research Collaboratory for Structural Bioinformatics. The four files corresponding to the mean and ensemble structures for the Bgtxα18-mer complex and for free Bgtx have been assigned the identifiers 1DGG, 1IDH, 1IDI, 1IDL.

We used RasMol (41), MOLMOL (42), and INSIGHT II (Molecular Simulations, Inc.) for the graphical analysis of the calculated structures. The surface charge potentials were calculated using MOLMOL. The contact surface areas of all the final individual Bgtxα18-mer complex structures were calculated by contacts of structural units (CSU) using CSU software (43). The energetically significant cation-π interaction analysis of the Bgtxα18-mer complex structures was performed using the CaPTURE program (44).

RESULTS

Preparation of 15N-peptide and Its Purification—To facilitate the assignment of the α18-mer peptide resonances while complexed with Bgtx, the peptide corresponding to Torpedo nAChR α1 subunit residues 181–198 was expressed heterologously in Escherichia coli as part of an insoluble fusion protein under conditions where [15N]Cl was used as sole source of nitrogen. After isolation of the fusion protein, CNBr cleavage at engineered Met sites was used to release the desired peptide, which contained the 18 residues of α1 subunit with an additional C-terminal residue derived from the engineered Met. As expected for CNBr cleavage of Met sites, the peptides isolated are a mix of the C-terminal homoserine form of the peptide and its corresponding dehydrated homoserine lactone form. HPLC analysis revealed three major peptide peaks which were further characterized (Fig. 1). Preparatory solid-phase binding studies indicated that all three peptide fractions bind Bgtx to an extent comparable with that obtained with a similar synthetic α18-mer peptide lacking the C-terminal homoserine (data not shown). All three peptide fractions were resistant to thiol alkylation with N-ethylmaleimide except after prior incubation of the peptide with dithiothreitol. These results suggest that the adjacent cysteines, Cys182 and Cys183, are in the disulfide state in the isolated peptides. Mass spectrometric analysis revealed that P2 corresponds to the C-terminal homoserine lactone form of the α18-mer, whereas P3 is the C-terminal homoserine form. The P2 peptide was chosen for the production of a Bgtxα18-mer complex.

The Formation of a Stoichiometric Bgtxα18-mer Complex—The pure α18-mer (P2) was resuspended in 50 mM perdeuterated potassium acetate buffer (pH 4.0) and analyzed with a two-dimensional 1H–15N HSQC experiment that is designed to acquire signal only from protons bound to 15N (Fig. 2A). An equimolar amount of Bgtx was then added, and the sample was again analyzed by HSQC (Fig. 2B). A comparison of these spectra (Fig. 2) clearly demonstrates the formation of a stoichiometric complex between the α18-mer and Bgtx. The free peptide (Fig. 2A) appears to be largely unstructured; all the NH resonances are poorly dispersed in chemical shift and vary in intensity. In contrast, after binding to Bgtx (Fig. 2B), nearly all the NH resonances undergo large chemical shift changes, and there is little evidence of any free peptide remaining based on the disappearance of resonances seen in the free peptide. These observations suggest that the α18-mer adopts a defined structure upon binding to Bgtx. Furthermore, in mole-ratio titration studies with less than stoichiometric concentrations of Bgtx, NH resonances corresponding to both the bound and the free peptide are present, and the chemical shift of the NH resonances for the bound peptide are fixed and do not vary with Bgtx concentrations (data not shown). These results indicate that the Bgtxα18-mer complex is in slow exchange.

NMR Assignments—Because only the peptide is 15N-enriched, 15N three-dimensional NMR experiments can be used to filter out Bgtx proton signals that are not correlated to 15N. Making use of this enrichment, three-dimensional TOCSY-HSQC, NOESY-HSQC, and HNHA experiments were obtained to make preliminary amino acid assignments of the α18-mer in its bound form. Fig. 3 illustrates a representative strip analysis used to identify the resonances of Lys185. Three-dimensional TOCSY-HSQC analysis is used to identify the resonances correlated by through-bound scalar connectivity to the 15N (i.e. C/H proton, C/H proton, etc.). Using these three-dimensional NMR experiments, we assigned the observable resonances for all of the amino acid residues in the α18-mer except for the
N-terminal Tyr\(^{181}\), which has an exchangeable NH, the C-terminal homoserine lactone, whose mobility may cause its signals to be too weak to be identified, and the two prolines, which lack amide protons (Fig. 2B). These assignments of the peptide resonances greatly facilitated the assignment of the Bgtx resonances in the homonuclear two-dimensional NMR data obtained with the complex.

Based on our three-dimensional NMR assignments, our two-dimensional NMR data (NOESY and TOCSY), and published Bgtx assignments (7, 36), we completed the assignment of the resonances obtained with the Bgtx\(^{\alpha 18}\)-mer complex. Fig. 4 summarizes the C\(^\alpha\)H proton to NH\(_{i-1}\) proton NOEs (sequential NOE), the C\(^\beta\)H proton to NH\(_{i+1}\) proton NOEs, and the NH proton to NH\(_{i+1}\) proton NOEs in the Bgtx\(^{\alpha 18}\)-mer complex used to complete the connectivity of the polypeptide backbone. In addition, 11 unambiguous intermolecular NOEs on the NH region of the peptide were assigned (Table I). Additional intermolecular NOEs in the C\(^\beta\)H proton and C\(^\gamma\)H proton regions were observed but have not as yet been unambiguously assigned. We performed a similar analysis on free Bgtx to generate the structure important for comparison of the free and bound forms of Bgtx.

Three-dimensional Structure Calculations and Comparison—The distance constraints resulting from the NOEs and the dihedral angle restraints obtained from the H\(^N\)-H\(^\alpha\) \(^J\) couplings (Table II) were then incorporated into CNS solve for
structure determination. These calculations utilized both distance geometry and simulated annealing protocols. Twenty structures of free Bgtx and of the Bgtx-18-mer complex with the lowest potential energy and no NOE violation larger than 0.5 Å are superimposed and shown as an ensemble in Fig. 5. In Fig. 6, the mean structure of each ensemble, partially energy-minimized, is shown to illustrate some of the key structural features. The overall backbone atomic root mean square deviation (r.m.s.d.) between the individual structures and the mean structure of free Bgtx is 2.03 Å, whereas that of the Bgtx-18-mer complex is 2.07 Å. The pairwise r.m.s.d. between the two mean structures is 6.05 Å.

We did not find any inter-residue NOE constraints involving the last two residues of Bgtx, Pro33 and Gly34. If these two residues are omitted from the r.m.s.d. calculation for Bgtx, the overall backbone r.m.s.d. is 1.98 Å for free Bgtx and 1.81 Å for bound Bgtx in the Bgtx-18-mer complex. A comparison of the r.m.s.d. determinations for various sequence segments within Bgtx indicates considerable regional variation in r.m.s.d. as summarized in Table II. The stem region of loop II is well defined (r.m.s.d. no greater than 0.90 Å in Bgtx-18-mer and 1.19 Å in free Bgtx) compared with loop II when considered in its entirety (r.m.s.d. of 1.56 Å in Bgtx-18-mer complex and 1.51 Å in free Bgtx). The tip of loop II can also be reasonably well superimposed (r.m.s.d. of 1.29 Å in Bgtx-18-mer complex or 1.14 Å in free Bgtx).

A comparison of free and bound Bgtx reveals that the proton resonances of a number of amino acids in Bgtx undergo large chemical shift changes upon peptide binding. These shift changes reflect significant alterations in the chemical environment of those protons. We find that Ala7 (C′H), Ser9 (C′H, C′H), Ile11 (NH, C′H, C′H, C′H), Trp28 (NH), Cys29 (NH, C′H), Asp30 (NH), Phe32 (NH), Cys33 (C′H), Arg36 (NH), Gly37 (NH, C′H), Lys38 (NH, C′H, C′H), Val39 (C′H), Val40 (NH, C′H), Lys52 (C′H), Val57 (C′H), Lys64 (C′H), Asn66 (C′H), Lys70 (NH, C′H), Gly71 (C′H), and Arg72 (C′H, C′H, C′H) all are characterized by chemical shift changes greater than 0.2 ppm upon binding. These amino acid residues are highlighted in red in the backbone structures shown in Fig. 5A. The changes in Ala7, Ser9, and Ile11 suggest that the outer tip of loop I participates in binding the α-Bgtx. This is substantiated by the intermolecular NOEs observed between the C′H and C′H protons of Pro10 and the NH protons of Asp195 and Thr196 in the peptide (Table I). Similarly, the chemical shift changes of Arg36-Val40 are correlated with intermolecular NOEs involving Lys38, Val39, and Val40 in Bgtx loop II. In the C-terminal tail region, the chemical shift changes in Lys70-Arg72 accompany intermolecular NOEs between the C′H protons of Pro69 and the NH of Cys192 in the peptide (Table I). In contrast to these examples of shift changes correlated with intermolecular contacts, several other shift changes in Bgtx residues appear to be due to conformational changes involving possible reorientations about the central triple-stranded β-sheet common to all α-neurotoxins. We believe that the chemical shift changes in the Trp28, Asp30, Phe32, Cys33 region and in Val57 are caused by general movement about the β-strand involving loop II and the proximal portion of loop III. Such secondary effects could also explain the chemical shift change of the side-chain C′H proton in Lys52 of loop III. Finally the chemical shift changes involving the side chains of Lys64 and Asn66 at the beginning of the C-terminal tail may reflect a change in chemical environment due to the apparent relocation of the distal C-terminal region to make a contact with the α-Bgtx peptide bridging the gap between Bgtx loop I and loop II (Fig. 5).

As viewed in Fig. 6A, free Bgtx is oriented with loop I (blue) on the left, loop III on the right, and the tip of loop II (green) at the bottom. This view corresponds with the so-called “convex” or “concave” surface of the α-neurotoxins (6, 12). In the free form of Bgtx, the tip of loop II forms the lower rim of this concave surface, and the loop I and loop II of free Bgtx are well separated with no inter-loop NOE constraints. The distance between the N of Ser34 and the N of Pro10 in free Bgtx is ~31 Å. Upon peptide binding, both loop I and loop II interact with peptide residues (see below), as revealed by intermolecular NOEs (Table I). As a consequence of binding, loop I and II move closer to each other; the average distance between the N of Ser34 and the N of Pro10 narrows to ~24 Å. In addition, the tip of loop II switches to a convex conformation upon binding (Fig. 6 and Fig. 7, A-D). The C-terminal tail region of free Bgtx is relatively unconstrained in free Bgtx but participates in peptide interaction in the bound state (see below). The rest of the structure appears to change little upon complex formation (Fig. 6B). To provide a full comparison of binding-induced structural changes in Bgtx, we have superimposed the mean backbone structures (Fig. 7, B and D) and the full ensemble of structures (Fig. 7, A and C) as stereo images. The red traces correspond to the backbone of free Bgtx, whereas the blue traces refer to the backbone of bound Bgtx. The α-Bgtx backbone is colored green. In the front view (Fig. 7, A and B), loop I and loop II move toward each other and toward the peptide, whereas loop III is largely unaltered. In the left-profile view, the α-Bgtx has been removed for a better view of the changes in the Bgtx backbone (Fig. 7, C and D). The C-terminal tail is shifted to the right (toward the peptide) in the bound state. The tip of loop II moves left, highlighting the change in general orientation in this region from a concave to a convex surface.
To evaluate the significance of the observed change in the conformation of Bgtx upon binding, we compared the mean pairwise backbone r.m.s.d. within the ensemble of free structures with the mean pairwise backbone r.m.s.d. across ensembles (i.e., each bound structure to each free Bgtx structure). Such a comparison allows us to determine if the two data sets, the ensembles of free and bound structures, represent significantly different structures. The mean pairwise backbone r.m.s.d. among the 20 free Bgtx structures is 2.94 ± 0.38 Å. Similarly, among the 20 bound Bgtx structures, the pairwise r.m.s.d. is 2.82 ± 0.25 Å. In contrast, the mean pairwise backbone r.m.s.d. between the 20 bound Bgtx structures and the 20 free Bgtx structures is 6.05 ± 0.37 Å. Thus, the mean pairwise r.m.s.d. across the two ensembles differs by more than 6 S.D. from the mean pairwise r.m.s.d. for the ensemble of free (or bound) Bgtx structures. This analysis indicates that the ensemble of bound structures is indeed significantly different from the ensemble of free structures.

We observed five pairs of C-H-C-H long-range NOEs in the two-dimensional NMR study of the α18-mer complex. These included the following pairs of residues: Cys23-Cys44, Tyr24-Cys59, Arg25-Leu42, Lys26-Val57, and Met27-Val40. These NOEs were previously reported as evidence for an anti-parallel triple-stranded β-sheet in the solution structure of Bgtx (7), and similar β-sheet defining, inter-strand NOEs have been observed in the solution structures of other α-neurotoxins (12).

Binding Interactions—The structures indicate that Bgtx interacts with the α18-mer at three sites. These are the tip of loop I, the C-terminal tail region (intermolecular NOEs are obtained with Thr8, Pro10, and Pro69), and loop II residues facing loop I including Val39, Val40, and Lys38. Although Loop III of some α-neurotoxins (3) has been reported to be involved in binding to the nAChR, we did not observe any direct intermolecular NOEs between the α18-mer and loop III of Bgtx. The chemical shifts of most residues in loop III change little upon binding of the α18-mer. Only backbone traces are shown. The figures were prepared using the program MOLMOL (42).

TABLE II
Structural statistics for free Bgtx and for the Bgtx α18-mer complex

|                        | Bgtx · α18-mer complex | Free Bgtx |
|------------------------|------------------------|-----------|
| Total number of distance constraints | 530                    | 457       |
| Intraresidue (|i − j| = 1) | 303                    | 289       |
| Sequential (|i − j| = 1) | 150                    | 116       |
| Medium range (|i − j| ≤ 4) | 11                     | 12        |
| Long range (|i − j| > 4) | 55                     | 40        |
| Backbone heavy atoms (Å) to mean backbone structure | 2.07                  | 3.29      |
| Backbone heavy atoms (Å) (residues 1–72) | 1.81                  | 1.98      |
| Backbone heavy atoms (Å) (residues 1–74) | 1.95                  | 2.03      |
| All heavy atoms (Å) (residues 1–72) | 2.98                  | 3.21      |
| All heavy atoms (Å) (residues 1–74) | 3.03                  | 3.23      |
| Backbone r.m.s.d. for different regions |                        |           |
| Residues 1–16 (Å) (loop I) | 1.44                  | 1.71      |
| Residues 17–21 (Å) | 0.99                  | 0.73      |
| Residues 22–28 (Å) (stem I of loop II) | 0.81                  | 0.75      |
| Residues 29–36 (Å) (tip of loop II) | 1.29                  | 1.14      |
| Residues 37–44 (Å) (stem II of loop II) | 0.90                  | 1.19      |
| Residues 45–60 (Å) (loop III) | 1.44                  | 1.53      |
| Residues 61–72 (Å) (C-terminal tail) | 1.32                  | 1.49      |
| Residues 181–198 (Å) (α18-mer) | 2.19                  |           |

FIG. 5. Structural comparison between free-Bgtx and the Bgtx α18-mer complex. A, twenty superimposed structures calculated based on distance constraints in free Bgtx. The red lines mark those Bgtx regions that undergo large chemical shifts upon α18-mer binding. B, twenty superimposed structures calculated based on distance constraints for the Bgtxα18-mer complex. The blue lines correspond to Bgtx, and the green lines signify the α18-mer. The black lines and the labels N and C mark the N and C termini of Bgtx. The labels N' and C' mark the N and C termini of the α18-mer. Only backbone traces are shown. The figures were prepared using the program MOLMOL (42).
The α18-mer, consistent with a lack of involvement of Bgtx loop III in α18-mer binding.

The α18-mer residues responsible for the intermolecular NOEs with the Bgtx sites are Tyr189, Tyr190, Thr191, Cys192, Asp195, and Thr196. The contact zone is ~18 Å in length (from N of Tyr189 to N of Thr196), and the total van der Waals contact surface areas range from 500 to 690 Å², with a mean of 580 Å². It is noteworthy that there is a cluster of four positive charged Bgtx residues (Arg36, Lys38, Lys70, Arg72) near the contact zone. In close apposition in the complex are found peptide residues Trp187, Tyr189, Tyr190 (Fig. 8). This arrangement suggests the possibility of cation-π interactions (44) contributing energetically to the formation of the Bgtx-α18-mer complex. A cation-π interaction analysis carried out on the 120 Bgtx-α18-mer-calculated structures revealed that a total of 24 individual structures, including two from the 20 best structures, have energetically significant cation-π interactions as determined by CaPTURE (44). In this analysis, cation-π interactions are selected as energetically significant if the electrostatic energy is <−2.0 kcal/mol or, alternatively, if the electrostatic energy is <−1.0 kcal/mol and the van der Waals interaction for the pair is <−1.0 kcal/mol (44). As shown in Table III, the following candidate cation-π pairings were observed: Lys38/Tyr190, Lys38/Tyr189, Lys70/Trp187, Arg36/Tyr189, Arg36/Trp187, and Lys70/Tyr190. The Lys38/Tyr190 pairing was observed in 12 of these 24 structures, and one of these structures is shown in Fig. 9.

DISCUSSION

NMR studies of complexes formed between peptides and peptide binding domains have been very instrumental in many other systems in elucidating mechanisms of molecular recognition (45). In seeking to understand the structural basis for the
FIG. 7. Stereo views comparing the polypeptide backbone traces of bound and free Bgtx. A and C, a view of the ensemble of structures for free Bgtx and for the Bgtx-α18-mer complex. B and D, a view of the two mean structures calculated from the ensembles. In A and B, the views correspond to the concave surface of Bgtx. In C and D, Bgtx is rotated -90° to the right as compared with the view in A and B (the α18-mer is not shown). The red traces correspond to free Bgtx, the blue traces correspond to Bgtx in the Bgtx-α18-mer complex, and the green traces correspond to the α18-mer. The figures were prepared using the program MOLMOL (42).
relatively high affinity ($K_D \approx 65$ nM) binding observed between Bgtx and the $\alpha_{18}$-mer, we identified a number of NOE distance constraints that reveal important information about the interaction between the $\alpha_{18}$-mer and Bgtx and that also define conformational changes in Bgtx upon complex formation. These constraints have been used to generate the structural features presented in this study. The disc-like shape of Bgtx and of the complex limits the total number of long-distance NOEs available (NOEs between residues distant in primary sequence), and this in turn limits the final resolution of the structures obtained (Table II). This limitation is applicable to all members of the $\alpha$-neurotoxin family (11, 12). Finally, we compare the structural features of the binding between Bgtx and the cognate $\alpha_{18}$-mer to the biochemical- and mutagenesis-based observations made with the intact nAChR.

One of our most striking observations is the binding-associated re-orientation in several segments of Bgtx that normally are not in contact with one another. Loop I and the C-terminal tail segment alter their configuration with respect to the main body of the protein, and the tip of loop II undergoes a change in its relative curvature and shape (Fig. 7). Together, these changes suggest a considerable coordinated reconfiguration of Bgtx upon interacting with receptor sequences. Such a reconfiguration is consistent with the extensive flexibility that has been noted in these and other protein toxins. It has been suggested that such flexibility may have evolved to serve important functional purposes (e.g., Ref. 46).

We previously reported the solution structure of the complex formed between Bgtx and a dodecapeptide corresponding to amino acid residues 185–196 from the $\alpha$ subunit of the Torpedo nAChR (22). The apparent affinity of Bgtx for the $\alpha_{12}$-mer is about 15–20-fold lower than for the $\alpha_{18}$-mer. In both structures, Tyr189 and Tyr190 lie in a similar position, close to Val39 and Val40 of Bgtx. In addition, in both structures the peptides are relatively elongated. There are significant differences, however, in the orientation of the two peptides relative to Bgtx. In the Bgtx-$\alpha_{12}$-mer complex, the polypeptide backbone of the five identified amino acid residues is between loop I and loop II, in a position roughly parallel to loop II. In the Bgtx-$\alpha_{18}$-mer complex, the peptide is in a more perpendicular orientation with respect to loop II and the tip of loop I (Fig. 6).

The Bgtx-$\alpha_{12}$-mer complex may represent an intermediate

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**FIG. 8.** Stereo view of the surface charge profile of the Bgtx-$\alpha_{18}$-mer complex. Surface charge potentials were calculated as described under “Experimental Procedures.” Blue regions show positive charge, and red regions show negative charge. See Fig. 5B for orientation. The figure was prepared using the program MOLMOL (42).

**FIG. 9.** Orientation of a suggested Tyr$^{190}$-Lys$^{38}$ cation-$\pi$ interaction. The two side chains are taken from one of the 20 ensemble Bgtx-$\alpha_{18}$-mer structures depicted in Fig. 5. a, the distance between the NZ of Lys$^{38}$ and the CE2 of Tyr$^{190}$ is 5.49 Å; b, the distance between the NZ of Lys$^{38}$ and the CE1 of Tyr$^{190}$ is 5.85 Å; c, the distance between the CG of Tyr$^{190}$ and the NZ of Lys$^{38}$ is 5.53 Å.

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**Table III**

| Cation-$\pi$ interaction pair | Number of structures observed |
|--------------------------------|------------------------------|
| Lys$^{38}$/Tyr$^{190}$        | 12                           |
| Lys$^{38}$/Trp$^{197}$        | 8                            |
| Lys$^{38}$/Tyr$^{189}$        | 3                            |
| Arg$^{36}$/Tyr$^{190}$        | 1                            |
| Arg$^{36}$/Trp$^{197}$        | 1                            |
| Lys$^{70}$/Tyr$^{189}$        | 1                            |

CaPTURE (44) was used to analyze all 120 acceptable structures of the Bgtx-$\alpha_{18}$-mer complex for cation-$\pi$ interactions. Two of the 24 structures identified contained two cation-$\pi$ interaction pairs. In both cases, the two pairs were Lys$^{38}$/Tyr$^{190}$ and Lys$^{70}$/Trp$^{197}$. The Bgtx-$\alpha_{18}$-mer complex may represent an intermediate...
stage in binding with the aromatics of the peptide, Tyr^{189} and Tyr^{190}, forming a nucleation site for further interactions. Although the Bgtx-\alpha18-mer complex is more energetically favored, the total contact surface areas are very similar, ~600 Å², for the two complexes. A library-derived peptide selected for its ability to bind Bgtx with high affinity also contains two adjacent Tyr residues, and these residues contribute the largest contact area in the complex (47). Although this 13-residue library-derived peptide adopts a more globular or sphere-like conformation, it is also found localized to the cleft formed between loop I and loop II with close apposition of the C-terminal region (47).

In the Bgtx-\alpha18-mer complex, the intermolecular NOEs between Pro^{10} and both Asp^{195} and Thr^{196} and between Thr^{8} and Cys^{192} (Table I) demonstrate the involvement of Bgtx loop I in the formation of the peptide complex. This finding is consistent with mutagenesis studies of erabutoxin a, a short \( \alpha \)-neurotoxin. Loop I mutations, S8T and Q10A, in erabutoxin a result in a very large reduction in binding affinity for the Torpedo nAChR (2). Recently, a double-mutant cycle analysis involving the related short \( \alpha \)-neurotoxin, \textit{Naja mossambica mossambica} I (NmmI), and the mouse nAChR has revealed an interaction between Ser^{6} in NmmI and Tyr^{196} on the \( \alpha \) subunit at the \( \alpha \gamma \) site (3). In contrast, no evidence of an interaction was observed between Ser^{6} and Val^{198} in the NmmI study. In addition, Val^{198} can be energetically coupled to Arg^{33} and Arg^{36} in NmmI (19). Based on sequence alignment, the corresponding Bgtx residues would be Arg^{36} and Lys^{38} respectively. These observations are entirely consistent with the structure of the Bgtx-\alpha18-mer complex; the tip of loop I is in closer proximity to the peptide residues C-terminal to Thr^{196} (Tyr^{196} is located at the extreme left in Fig. 6B), whereas Val^{198} is removed from loop I and in close proximity to the loop II residue, Val^{199} (Table I).

Mutational analysis of loop I residues in the long \( \alpha \)-neurotoxin, \( \alpha \)-cobrotoxin from \textit{Naja kaouthia} venom, failed to detect a significant role for this region in binding to the Torpedo nAChR (2). Because the sequence of the loop I region differs greatly between Bgtx and this \( \alpha \)-cobrotoxin and because loop I is two residues longer in Bgtx (12 versus 10 between the corresponding Cyx residues delimiting loop I), it is possible that these two toxins differ in this region in their mode of interaction with the nAChR. In addition, recent comparisons of short and long \( \alpha \)-neurotoxins suggest significant differences between these two families of toxins in their detailed mode of interaction with the nAChR (2, 18).

The intermolecular NOE between Pro^{69} and the backbone NH of Cys^{192} together with the chemical shift changes observed within the C-terminal tail region upon formation of the Bgtx-\alpha18-mer complex clearly indicate that the C-terminal tail region of Bgtx also plays a role in peptide binding. This observation is consistent with biochemical and mutagenesis studies examining the role of the C-terminal tail region in binding to native nAChRs where binding affinity was decreased by 7–15-fold when C-terminal residues were removed (4, 48).

The proximity of the positively charged Bgtx residues (Lys^{38} and Arg^{36}) with the aromatics of the peptide (Tyr^{189} and Tyr^{190}) in the Bgtx-\alpha18-mer complex suggests an important functional contact. Mutagenesis studies in Bgtx and related toxins also point to important roles for Arg^{36} and Lys^{38}. Ala-substitution of Arg^{36} in Bgtx leads to a 90-fold decrease in Bgtx binding affinity as measured with heteroexpressed mouse nAChR (4), whereas charge reversal studies in \( \alpha \)-cobrotoxin demonstrate that R33E (position corresponds to Arg^{36} in Bgtx) causes a 767-fold decrease in binding affinity for Torpedo nAChR (2). Ala-substitution at Arg^{36}, the position corresponding to Lys^{38} in Bgtx, reduces binding affinity by 7.4-fold (2).

An important contact role for Tyr^{189} is consistent with chimeric analysis and toxin footprinting studies of nAChR \( \alpha \) subunits. The \( \alpha 3 \) subunit, which shows no sensitivity to Bgtx block, acquires a significant sub-micromolar affinity for Bgtx with a single point mutation, K189Y, involving the introduction of an aromatic residue at position 189 (49). In a footprinting protection study using Cys-substituted mutations in the heterologously expressed mouse \( \alpha 1 \) subunit, the introduced thiol of \( \alpha 1 F189 C \) is protected by Bgtx from reaction with a hydrophilic biotinylmaleimide (18). Similarly, the functional importance of Tyr^{190} in \( \alpha \)-neurotoxin binding is supported by mutagenesis studies where large decreases in Bgtx and NmmI toxin binding affinity are observed (3, 18, 19). A double-mutant cycle analysis with NmmI toxin revealed pairwise contacts between Tyr^{190} and R33E and R36E, with a greater coupling energy to R36E (3, 19). In the present study, we document an intermolecular NOE constraint between Lys^{38} (position corresponds to Arg^{36} in NmmI toxin) and Thr^{191}, one residue removed from Tyr^{190} in the Bgtx-\alpha18-mer complex.

Cation-π interactions are interactions between a cationic group, such as the side chain of Arg and Lys, with the electronegativity of an aromatic π cloud (44). Cation-π interaction pairs within the Bgtx-\alpha18-mer complex were identified using CaPTURE (44). Of the 120 acceptable structures, 24 showed evidence of cation-π interactions including two of the 20 best structures (Table III). One of these latter two structures was chosen to illustrate one such candidate cation-π interaction (Fig. 9). In this example, the NZ nitrogen of Bgtx Lys^{38} is oriented within 6 Å of the aromatic ring of Tyr^{190}. We speculate that cation-π interactions may be involved in Bgtx binding to the nAChR just as cation-π interactions involving cTrp^{149} may be important in the binding of acetylcholine to the nAChR (50). Additional high resolution structures with better resolution of the side-chain positions would be needed to test this proposal further.

In the Bgtx-\alpha18-mer complex, Cys^{192} and Cys^{193} of the peptide are located in between loop I and loop II and adjacent to the C-terminal tail of Bgtx. This position correlates well with recent biochemical cross-linking data concerning the spatial orientation of the reduced disulfide bond between Cys^{192} and Cys^{193} at the \( \alpha \gamma \) interface of the Torpedo nAChR (51). Several Cys-substituted mutants of \textit{Naja nigricollis} \( \alpha \)-neurotoxin, a short-chain neurotoxin, were cross-linked to the Torpedo \( \alpha \) subunit with various efficiencies depending on the spacer length of the dimaleimide derivative used and the site of toxin mutagenesis. It was concluded that Cys^{192} and Cys^{193} were located under the tip of the first loop, ~11.5 Å from the \( \alpha \)-carbon at toxin position 10, and close to the second loop, ~15.5 Å from the \( \alpha \)-carbon at toxin position 33 (51). This orientation is entirely consistent with the structure shown in Fig. 6B.

In summarizing the contact information, we find that Bgtx interacts with the \( \alpha 18 \)-mer primarily through three contact regions. Arg^{36}-Val^{198} in loop II appears to be a core binding site that is common to both the Bgtx-\alpha18-mer complex and the earlier Bgtx-\alpha12-mer complex. We also find that the tip of loop I is involved in peptide binding. Lys^{38} and Arg^{72} of the C-terminal tail provide the third binding region that serves to function as a physical continuum between the two other binding sites. As described above, there is a remarkable correlation between the conclusions drawn from various mutagenesis and biochemical cross-linking studies and the structures presented here for the Bgtx-\alpha18-mer complex. Although it is clear that additional receptor contacts are required to achieve the Bgtx binding affinity observed with the native receptor, the evidence to date nevertheless suggests that the \( \alpha 18 \)-mer complex with Bgtx may serve as a useful and accessible model for studying
of the structural and energetic basis of the high affinity toxin-receptor interaction.

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The Solution Structure of the Complex Formed between α-Bungarotoxin and an 18-mer Cognate Peptide Derived from the α1 Subunit of the Nicotinic Acetylcholine Receptor from Torpedo californica

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