We report a new, higher resolution NMR structure of α-bungarotoxin that defines the structure-determining disulfide core and β-sheet regions. We further report the NMR structure of the stoichiometric complex formed between α-bungarotoxin and a recombinantly expressed 19-mer peptide (128TPGKRTESFYECCKEPYDP166) derived from the α7 subunit of the chick neuronal nicotinic acetylcholine receptor. A comparison of these two structures reveals binding-induced stabilization of the flexible tip of finger II in α-bungarotoxin. The conformational rearrangements in the toxin create an extensive binding surface involving both sides of the α7 19-mer hairpin-like structure. At the contact zone, Ala7, Ser8, and Ile11 in finger I and Arg28, Lys38, Val39, and Val40 in finger II of α-bungarotoxin interface with Phe166, Tyr167, Glu168, and Tyr168 in the α7 19-mer underscoring the importance of receptor aromatic residues as critical neurotoxin-binding determinants. Superimposing the structure of the complex onto that of the acetylcholine-binding protein (19B), a soluble homologue of the extracellular domain of the α7 receptor, places α-bungarotoxin at the peripheral surface of the inter-subunit interface occluding the agonist-binding site. The disulfide-rich core of α-bungarotoxin is suggested to be tilted in the direction of the membrane surface with finger II extending into the proposed ligand-binding cavity.

The nicotinic acetylcholine receptor (nAChR) (1) is a ligand-gated ion channel that mediates excitatory transmission at the neuromuscular junction and at synapses in the central and peripheral nervous systems. It is the most intensely studied member of the ligand-gated ion channel superfamily and serves as a model for understanding the structure and function of related ion conducting channels including glycine, γ-aminobutyric acid type A, γ-aminobutyric acid type C, and type 3 serotonin receptors. nAChRs are pentameric complexes that assemble in the membrane with 5-fold symmetry. Each subunit contains an N-terminal extracellular domain about 200 amino acids long followed by four membrane-spanning segments (M1–M4) with an intracellular loop of variable length between M3 and M4. The second transmembrane region from each subunit contributes to the formation of the wall lining the channel pore.

In muscle and Torpedo electric organ, the subunit composition is (α1)2βδ and (α1)2βε in embryonic and adult tissue, respectively (for review, see Ref. 1). Neuronal nAChR subunits (α2-α10 and β2-β4) apparently can assemble in various combinations giving rise to multiple receptor subtypes. In heterologous expression systems, particular subunit combinations form functional hetero-pentamers (e.g. (α4)2β2) whereas the α7-α9 subunits form functional homo-pentamers (2, 3).

Snake venom-derived α-neurotoxins bind the muscle-type and, in some cases, homo-pentameric neuronal nAChRs with high affinity (4, 5) and are grouped into two families (4). Short chain neurotoxins have 60–62 amino acids. Long chain toxins have 66–74 amino acids and a fifth disulfide at the tip of the second loop. Solution NMR and x-ray crystallographic studies (e.g. Refs. 6–8) show that all α-neurotoxins share a tertiary structure known as the three-finger fold, a four-disulfide globular core from which emerge three loops or fingers and a C-terminal tail. An NMR dynamics study of a short α-neurotoxin reveals “disorder” at the tips of fingers I and II reflecting localized mobility on the picosecond to nanosecond time scales (9). The significance of this mobility is highlighted by evidence that mutation of residues at these locations in other related toxins produces large effects on binding (11).

α-Bungarotoxin (Bgtx), a long neurotoxin from the venom of Bungarus multicinctus, has been an important tool in many biochemical and functional studies of nAChRs including the homo-pentameric nAChRs. The three-dimensional structure of Bgtx, determined by x-ray crystallography (12), differed from solution and x-ray structures of related short and long toxins particularly in the length of the highly conserved central β-sheet in finger II (6–8). In contrast, an NMR-based investigation of the secondary structure in Bgtx indicated that the structure of Bgtx in solution most likely resembles that of other α-neurotoxins (13). Although this study paved the way to a
better understanding of the solution structure of Bgtx, an adequately high resolution structure of Bgtx has not been available until now. The high resolution three-dimensional structure of unbound Bgtx presented here greatly facilitates the investigation of conformational changes that Bgtx undergoes as it binds nACHR-derived and engineered sequences, of recent structural studies (14–20).

Photolabeling and mutagenesis studies indicate that the ligand-binding site on the nACHR is formed at subunit interfaces in the N-terminal extracellular region of the receptor (1). The major structural determinants of ligand binding are found on the α subunit with additional contributions made by residues of adjoining subunits. Strongly conserved residues from three discontinuous regions of the α subunit (designated loops A–C) contribute to the binding pocket. In the crystal structure of the acetylcholine-binding protein (AChBP), a snail homologue of the extracellular domain of a homo-pentameric nACHR, loops A–C contribute to a relatively hydrophobic cavity at the subunit interface (21).

Earlier studies (22) have shown that the major determinants of Bgtx binding lie between positions 173 and 204 on the α subunit, coincident with loop C. This region includes the highly conserved aromatic residues Tyr190 and Tyr198 and, in addition, Cys192 and Cys193 (in α numbering) which form an uncommon disulfide. Synthetic peptide binding studies have suggested that the Bgtx-binding site on the α7 subunit is in the homologous region (α7 178–196) (23). The importance of this region in toxin binding was further highlighted by the observation that six residues from this region of α7 could confer Bgtx sensitivity when placed into the corresponding positions of the Bgtx-insensitive α3 subunit (24). Additionally, mutation of loop C residues, Tyr187 and Tyr194 of α7, to Phe reduced Bgtx blockade of ACh-evoked currents in heterologously expressed receptors (25). This is consistent with a 1.5-fold reduction in Bgtx binding observed in synthetic peptide was lyophilized and stored at –20 °C.

Bathing Experiments—The Kd value for the α7 19-mer-Bgtx interaction was determined by measurement of inhibition of the initial rate of Bgtx binding to nACHR-enriched Torpedo membranes following preincubation of Bgtx with α7 19-mer (26). 125I-Labeled Bgtx (2.5 nM) was incubated over a wide range of concentrations of α7 19-mer in 0.2% bovine serum albumin, 30 mM sodium phosphate, pH 7.4, for 18 h at room temperature. 100 μl of the peptide toxin mixture was added to microfuge plate wells coated with 2 μg of Torpedo membranes that were pre-blocked with 200 μl of 2% bovine serum albumin, 30 mM sodium phosphate, pH 7.4. Torpedo membrane-bound 125I-Bgtx was measured in a γ counter. All measurements were done in triplicate. The effect of pH was also assessed by preparing samples in 30 mM sodium phosphate, pH 5.5, to replicate the buffer conditions of the NMR sample (see below).

NMR Sample Preparation—Hydrated Bgtx (Sigma) was used to re-suspend the α7 19-mer in order to facilitate formation of a 1:1 peptide–toxin complex. Uniformly 15N-labeled and uniformly 15N,13C-double-labeled α7 19-mer-Bgtx samples were prepared at a concentration of 1.5–2.0 mM. These samples contained 30 mM sodium phosphate and 50 μM sodium azide in 95% H2O, 5% D2O at pH 5.5. Sodium 3-trimethylsilylpropionate (Cambridge Isotope Laboratories) was added at 50 μM as an internal calibration standard. For deuterium exchange experiments, the complex was lyophilized and reconstituted in 99.9% D2O at pH 5.5 (isotope effect unaccounted). Preparation of the free Bgtx sample was described previously (13).

NMR Experiments—NMR experiments were carried out at 4 °H frequencies of 400 and 600 MHz on Bruker Avance spectrometers at Brown University and at 500 MHz on a GE spectrometer equipped either with a G console with a Nicolet computer or an Omega console with a Sun 3/160 computer at the University of California, San Francisco. For free Bgtx, the following spectra in H2O were acquired at 15, 25, and 35 °C, and pH 5.79: DQF-COSY (31), TOCSY (70 ms MLEV-17 spin-lock sequence) (32), and NOESY spectra (160-ms mixing time) (33) using the water suppression scheme described in Basus (34). No significant shifts in free Bgtx proton resonances were observed between samples at pH 5.5 and 5.79, suggesting that the chemical environment is comparable at both pH conditions. In addition, NOESY (160-ms mixing time) and E-COSY spectra (35) at 25 and 35 °C in D2O were acquired.

Distance restraints were calculated from experimental NOESY intensities using the program MARDIGRAS version 2.0 (36, 37) which uses the complete relaxation matrix to produce an upper and lower distance bound for each experimental intensity. To make use of this procedure that determines accurate distances from the integrated intensities of the NOESY cross-peaks, a value for the rotational correlation time, τr, must be defined. For free Bgtx the correlation time was determined by measurement of the 13C T1 and T2 relaxation times at natural abundance, using a sample dissolved in 99.96% D2O. These samples were carried out using a double-DEPT technique with proton detection for maximum sensitivity. For T1, we used the double-DEPT experiment with inversion recovery (38), and for T2 we used the double-DEPT sequence with a Carr-Purcell-Meiboom-Gill modification using a series of 180° pulses with a repetition rate of 1 ms to replace the single 180° refocusing pulse in the sequence of Nirmala and Wagner (39). The sum of the resonances at different portions of the spectra was used to determine the relaxation times. The data were analyzed by fitting to a single exponential decay function. Peak volumes from D2O...
and H₂O NOE spectra were obtained by fitting the peak or peaks to be integrated to a Gaussian line shape in Sparky. In the case of peaks with low signal to noise ratio, the points within a manually selected rectangular or elliptical area surrounding the cross-peak were summed using Sparky (40). In NOE spectra the JHN, coupling constants were determined by fitting anti-phase multiplets, followed by measurement of the separation between the simulated anti-phase multiple and the NOE peak. These values were used as minimum values, because the apparent coupling constant in NOE spectra will be smaller than the actual coupling constant. In DQF-COSY spectra, coupling constants were determined by line fitting the antiphase multiplets, followed by measurement of the separation between the simulated anti-phase multiple and the NOE peak. These values were used as minimum values, because the apparent coupling constant in DQF-COSY spectra is larger than the actual coupling constant due to the summation of anti-phase cross-peaks.

Exchange rates were measured at 25 °C for Bgtx lyophilized from H₂O and re-dissolved in D₂O. Immediately following this procedure, the sample was placed in the spectrometer, and one-dimensional and TOCSY spectra (40 ms mixing time) were alternately acquired. The first one-dimensional spectrum was acquired 14 min after dissolving the lyophilized powder in D₂O, and the first TOCSY spectrum was started 1 min later. Several spectra were obtained up to 36 h after starting the exchange, and further spectra were obtained and 2 weeks later with the sample maintained at 25 °C. The cross-peaks in the resulting spectra were integrated, and cross-peaks between α- and β-protons were integrated for use as intensity references to eliminate variations of the spectrometer conditions after the sample was removed and re-inserted in the magnet for the last time points.

For the α7 19-mer-Bgtx complex, H-13N three-dimensional NOEY-HSQC (120-ms mixing time) (41), H-15N three-dimensional TOCSY-HSQC (60-ms MLEV-17 spin-lock sequence) (41), and three-dimensional NHH (42) experiments were collected at 35 °C using a uniformly 15N-labeled α7 19-mer-Bgtx sample in order to assign resonances. To clarify ambiguities in the assignments, an HNCA experiment (43) was performed using uniformly 15N13C double-labeled α7 19-mer. H1 homonuclear NOEY (120-ms mixing time) (33) and TOCSY (60-ms MLEV-17 spin-lock sequence) (32) experiments were performed with 13N decoupling to assign bound Bgtx NOEY resonances. NOEY and TOCSY experiments were performed at 15, 25, and 35 °C and with different mixing times to resolve ambiguities and facilitate the assignment process. Water was suppressed using the WATERGATE method, incorporating a 3-9-19 refocusing pulse sequence with pulsed field gradients (44). Deuteration exchange was performed to identify slowly exchanging amide protons involved in secondary structure. Following a two-dimensional H-13N HSQC (45), five (sequential) homonuclear TOCSY experiments were collected over 16 mixing times for the α7 19-mer-Bgtx complex in D₂O. The stoichiometry of the α7 19-mer-Bgtx complex was determined in a mole ratio titration of the α7 19-mer using two-dimensional H-13N-HSQC experiments. NMR data were processed with XWIN-NMR (Bruker) or NMRPipe (46), and resonance assignments were made in Sparky (40). H1 chemical shifts were referenced to sodium 3-trimethylsilylpropionate (0.0 ppm). The H-13N, H-15N, and H-13C assignments will be deposited in the BioMagResBank chemical shift data base.

Structure Calculations—For free Bgtx, distance geometry calculations were performed on the Cray Y-MP at the San Diego Supercomputer Center using the distance geometry program VEMBED (47), a vectorized version of EMBED (48). Restrained molecular dynamics calculations were carried out with the four-dimensional modifications (49) to the GROMOS-87 programs (50) using a SparcIPX work station. The 37D4 force field was used, and all the calculations were performed in vacuo with all charged groups neutralized and with the mass of the hydrogens set to 12 atomic mass units. The distance restraint constant, Kdis, was set to 10,000 kJ mol⁻¹Å⁻², with an initial temperature of 800 K. Three ps of dynamics was run followed by 5 ps during which the three-dimensional projection force constant Kθ was increased from 0 to 5,000 kJ mol⁻¹Å⁻². At the same time, the temperature was slowly lowered in an annealing procedure over the next 11 ps with the final temperature set to 0.1 K. The time constant τθ for couplings to the thermal bath was set to 0.005 ps.

For the α7 19-mer-Bgtx complex, distance constraints were derived from a two-dimensional homonuclear NOEY experiment (120 ms mixing time; 35 °C). NOEs were manually selected as strong, medium, or weak according to intensity. The distance ranges corresponding to the categories are as follows: 1.8–3.0, 1.8–4.0, and 1.8–5.0 Å, respectively. When stereospecific assignment of methylene and methyl protons was not possible, a pseudoatom correction was employed. Slowly exchanging amide protons involved in a network of NOEs characteristic of β-sheets were identified as hydrogen bond donors and assigned the following constraints for residues (i,j): H=O, 1.6–2.5 Å and N=O, 2.5–3.3 Å. Dihedral angle constraints were based on SHINC, coupling constants calculated fromHNH data (42). For JHN, > 8 Hz, φ was restrained to −120 ° to 40°; for JHN, < 6 Hz, φ was restrained to −60 ° to 30°. The peptide bond between adjacent cysteines involved in a disulfide devi-}

RESULTS

 Determination of the Structure of Bgtx—Integrated volumes from cross-peaks in NOEY spectra of Bgtx were used in the program MARDIGRAS (36, 37) to calculate accurate distance constraints taking into account spin-diffusion effects. These calculations were carried out using a correlation time of 3.3 ns and were repeated at 3.7 and 2.9 ns to include the range of uncertainty in the correlation time determination. Based on the dipole-dipole relaxation mechanism due to the directly attached proton, the correlation time was determined from 13C T1 and 31P relaxation times. Indirect measurement of the 13C T1 relaxation time yielded an average value of 0.6 ± 0.06 s for the α-carbons; the 31P T1 relaxation time was 73 ± 7 ms. Extended starting structures were used in the distance calculations initially, and subsequent calculations began with structures obtained in the previous round of calculations. The largest distances thus calculated were used as the upper bound and the lowest as the lower bound for each distance restraint.

Initial structure calculations revealed a triple-stranded β-sheet whose hydrogen bonds were confirmed by H/D exchange experiments (Table I and see below). The nine most unambiguous hydrogen bonds were used as additional restraints in the final structure calculations, in addition to the 588 NOE restraints. Stereospecific assignments were obtained for 10 diastereotopic pairs of β-protons. From the HN-1H coupling constants, 43 β angle constraints were obtained, and from E-COSY spectra of α- and β-proton coupling constants were determined to yield constraints for 17 χ² angles. The relevant structural statistics are shown in Table II. From 20 distance geometry structures calculated, 13 were selected that had the correct global fold, as indicated by the lower energies and smaller number of distance violations. To overcome local barriers in the restrained molecular dynamics calculations, the four-dimensional version of GROMOS (49) was used. The final structures have a large r.m.s.d. (2.11 Å), when all backbone atoms are included, and several regions show large local r.m.s.d. If we exclude these regions, primarily the C-terminal segment and the end of finger II, the remaining region consisting of residues
Structures of α-Bungarotoxin and Its Complex with α7 19-mer

12409

Table I

| Donor   | τ_ex (days) | Error | Acceptor | Average energy (kcal/mol) | No. structures |
|---------|-------------|-------|----------|---------------------------|---------------|
| Val      | 0.11        | 0.02  |          | -2.8                      | 0             |
| Cys      | 1.15        | 0.09  |          | -3.0                      | 13            |
| Thr      | 0.22        | 0.03  | Ser      | -2.5                      | 13            |
| Thr      | 6.96        | 0.52  | Leu      | -2.5                      | 13            |
| Ser      | 10.7        | 1.7   | Thr      | -0.6                      | 10            |
| Val      | 1.34        | 0.04  | Cys      | -1.5                      | 13            |
| Cys      | 0.78        | 0.11  | Ile      | -2.8                      | 11            |
| Glu      | 0.34        | 0.01  | Pro      | -2.1                      | 7             |
| Leu      | 19.9        | 2.0   | Ala      | -3.0                      | 13            |
| Cys      | 20.3        | 1.7   | Cys      | -3.4                      | 13            |
| Tyr      | 28.3        | 4.8   | Gly      | -2.9                      | 13            |
| Arg      | 25.1        | 1.5   | Thr      | -2.0                      | 13            |
| Lys      | 43          | 13    | Val      | -2.4                      | 13            |
| Met      | 1.15        | 0.10  | Glu      | -2.9                      | 13            |
| Trp      | 0.28        | 0.01  | Glu      | -2.5                      | 13            |
| Asp      | <10 min b  |       | Val      | -2.4                      | 13            |
| Cys      | <10 min b  |       | Gly      | -2.1                      | 12            |
| Glu      | <10 min b  |       | Asp      | -0.6                      | 7             |
| Val      | 1.47 h      | 0.02  | Cys      | 1.0                       | 7             |
| Glu      | 5.46        | 0.59  | Lys      | -0.9                      | 9             |
| Leu      | 17.6        | 2.1   | Val      | -2.6                      | 13            |
| Gly      | 66          | 27    | Tyr      | -1.7                      | 13            |
| Cys      | 10–60 min   |      |          | 0                         |               |
| Ala      | 18.8        | 2.0   | Leu      | -2.0                      | 13            |
| Ala      | 11.4        | 1.1   | Gly      | -1.2                      | 10            |
| Thr      | 13.5        | 4.3   | Lys      | -2.9                      | 6             |
| Glu      | 1.26 h      |       | Met      | -3.1                      | 13            |
| Glu      | <10 min b  |       | Glu      | -0.9                      | 13            |
| Val      | <10 min b  |       | Arg      | -2.2                      | 13            |
| Thr      | 0.14        | 0.04  | Val      | -0.8                      | 11            |
| Cys      | <10 min b  |       | Cys      | -2.7                      | 13            |
| Cys      | 0.26        | 0.02  | Thr      | 0                        | 0             |
| Thr      | 2.4 h       | 0.6 h | Val      | 2.4                       | 13            |
| Lys      | 3.18        | 0.01 h| Thr      | 1.4                       | 13            |
| Cys      | 10–60 min   |      |          | 0                         |               |

H/D exchange time constant for free Bgtx at 25°C, pH 4.0

Table II

NOE restraints

| Structure | Free Bgtx | α7 19-mer - Bgtx |
|-----------|-----------|------------------|
| Total     | 588       | 671              |
| Intraresidue | 151       | 168              |
| Sequential (| j | j = 1) 193       | 160              |
| Medium range (| j | j = 4) 54       | 21               |
| Long range (| j | j ≥ 5) 190       | 94               |
| Intermolecular | NA a     | 28               |

Dihedral restraints

| Structure | Free Bgtx | α7 19-mer - Bgtx |
|-----------|-----------|------------------|
| Total     | 60        | 5                |
| φ         | 43         | 5                |
| N         | 17         | ND b             |
| Disulfide bonds | 5        | 6                |
| Hydrogen bonds | 9        | 10               |
| r.m.s.d. to average structure (Å) | | |

Backbone

| Structure | Free Bgtx | α7 19-mer - Bgtx |
|-----------|-----------|------------------|
| Well defined regions | 0.58 c | 1.04 c |
| Overall | 2.11       | 1.53             |

Heavy atoms

| Structure | Free Bgtx | α7 19-mer - Bgtx |
|-----------|-----------|------------------|
| Well defined regions | 0.96 c | 1.55 c |
| Overall | 2.58       | 2.22             |

Distance constraint violations >0.5 Å

| Structure | Free Bgtx | α7 19-mer - Bgtx |
|-----------|-----------|------------------|
|         | 0         | 0                |

NOTES

a Calculated from the final 13 Bgtx structures using DSSP (53).
b Not observed in the first TOCSY spectrum.

c Residues 1–16, 22–28, 39–48, 54–68 in Bgtx.
d Residues 1–16, 22–28, 39–48, 54–68 in Bgtx and 185–194 in the α7 19-mer.

1–16, 22–28, 39–48, and 54–68 can be matched together to yield, for the backbone atoms, an r.m.s.d. to the average of 0.58 Å (Fig. 1). Two regions with regular secondary structure are well defined, the β-sheet in finger I (residues 1–16) and the triple-stranded β-sheet formed by residues 22–28, 39–45, and 56–60. The poorly defined regions have few NOEs, and in particular, finger II has sequential NOEs that are smaller than expected, indicating the possibility of additional local motion in this region. Those regions poorly defined in free Bgtx are better defined in the α7 19-mer-Bgtx complex as indicated by the lower overall r.m.s.d. (Table II and see below).

H/D Exchange Rates for Free Bgtx—H/D exchange in a sample of lyophilized Bgtx dissolved in D2O at 25 °C and pH 4.0 was followed using TOCSY and one-dimensional spectra. A total of 30 amide protons were observed to be in slow exchange (Table I). The experimentally determined set of slow exchanging amide protons that denote potential hydrogen bond donors was compared with the set of predicted hydrogen bonds calculated by DSSP from the Bgtx coordinates (Table I). DSSP predicts hydrogen bonding based on the distance between hydrogen-bonding partners and their orientation. In general, there is good agreement between the hydrogen bonds detected in the structures and the amide protons slowly exchanging with deuterium in D2O. The only slowly exchanging amide proton with a long exchange time that did not form a hydrogen bond in the structures is Thr47-H28. Two hydrogen bonds detected in all structures with low hydrogen bond energy, which were not slowly exchanging, occur between Glu56 and Met27 and between Asp30 and Gly37. Mobility at the tip of finger II may explain this observation as both are found at the end of the triple-stranded β-sheet in finger II.

Expression and Purification of Metabolically Labeled α7 19-mer—Homonuclear NMR studies of Bgtx complexed with unlabeled synthetic peptides derived from the nAChR α1 subunit resulted in incomplete assignment of peptide proton reso-
to prepare NMR samples as homoserine and homoserine lactone are in pH-dependent equilibrium (56).

Affinity of the α7 19-mer-Bgtx Complex—The affinity of the α7 19-mer for Bgtx was determined in a solution-based assay that is rooted in the finding that synthetic peptides derived from the nAChR compete with intact Torpedo nAChR for binding Bgtx (24). Following equilibration of 15N-labeled Bgtx with varying concentrations of the α7 19-mer, the percentage of Bgtx that remained unbound was determined by the amount of binding to the nAChR under initial rate conditions. The concentration of α7 19-mer that diminished the initial rate of binding by 50% (K_D) was 30 μM (data not shown). The K_D value was identical at pH 7.4 and pH 5.5.

Stoichiometric Interaction between the α7 19-mer and Bgtx—CD studies of α7 19-mer alone in solution showed that the peptide structure is random coil (data not shown). Hence, we did not undertake NMR studies of free α7 19-mer and proceeded directly to determine its structure in complex with Bgtx. The stoichiometry of the α7 19-mer-Bgtx interaction was determined by titrating U-15N-labeled α7 19-mer with Bgtx and monitoring chemical shift changes in 1H,15N HSQC spectra. These NMR experiments track changes in the chemical environment of peptide amide protons as Bgtx is incrementally introduced. In the absence of Bgtx, the peptide amide proton resonances lie within a narrow chemical shift range, indicative of a lack of secondary or tertiary structure (Fig. 2A). Upon addition of Bgtx, virtually all the amide proton resonances undergo a change in chemical shift (Fig. 2, B and C). New peaks, corresponding to bound peptide, appear in previously unpopulated environments. The intensities of the “bound” peaks progressively increase as the added toxin approaches equimolar ratio leading to the conclusion that α7 19-mer and Bgtx form a 1:1 complex (Fig. 2, C and D).

Discrete sets of peaks corresponding to free and bound α7 19-mer indicate that the peptide-toxin complex is in slow exchange (57). This is further supported by the observation that the bound peak line widths and chemical shifts do not change upon incremental addition of Bgtx.

Assignment of α7 19-mer and Bgtx Resonances—We used U-15N-labeled α7 19-mer to distinguish unambiguously peptide resonances from those of Bgtx in the NMR spectrum fingerprint region. Three-dimensional TOCSY-HSQC and NOESY-HSQC spectra were collected to assign 15N-correlated resonances using the sequential resonance assignment strategy of Wüthrich (58). Additionally, correlation of α7 19-mer intraresidue amide and α-protons was confirmed in an HNHA experiment. Of the 20 amino acids of the peptide, only 16 were assignable by the 15N-edited experiments because the N-terminal NH is rapidly exchangeable and three residues are prolines with no amide proton. The sequential NOE assignments are summarized in Fig. 3.

Assignment of α7 19-mer resonances by heteronuclear NMR methods greatly simplified the assignment of Bgtx as several peptide resonances overlapped with those of Bgtx in homonuclear spectra. Bound Bgtx resonances were assigned by the sequential assignment strategy of Wüthrich (58) using two-dimensional homonuclear TOCSY and NOESY data (Fig. 3).

Structure Calculations—For the α7 19-mer-Bgtx complex, 671 NOE-derived distance constraints were used in distance geometry and simulated annealing calculations, including 548 intra-toxin, 95 intra-peptide, and 28 intermolecular constraints. Five HNHA-derived φ angle restraints for the α7 19-mer were introduced, as well as 20 hydrogen bond constraints within Bgtx based on assignment of hydrogen bond donors determined by deuteration exchange. The 10 lowest energy structures that fit the experimentally derived constraints

![Fig. 1. Stereo views of the well defined regions of free Bgtx.](image-url)
with no NOE violations exceeding a 0.5-Å cut-off are shown in Fig. 4. Relevant structural statistics are shown in Table II. The backbone r.m.s.d. value calculated between the 10 structures and the mean structure is 1.04 Å in the well defined regions of the complex as follows: Ile1-Cys18, Leu22-Trp28, Val102-Thr108, and Tyr24-His38 in Bgtx and Ser185–Tyr194 in the α7 19-mer. In Bgtx, these regions include slow exchanging amide protons and numerous long range NOEs; in the α7 19-mer, we find intermolecular and long range in-trapeptide NOEs (see below). The corresponding heavy atom r.m.s.d. is 1.55 Å. The backbone r.m.s.d. for α7 19-mer(Ser185–Tyr194) and the entire toxin is 1.38 Å, for α7 19-mer(Ser185–Tyr194) alone is 1.10 Å, and for the entire α7 19-mer and Bgtx is 1.53 Å.

Structure of α7 19-mer-bound Bgtx—The overall α-neurotoxin three-finger fold is preserved in α7 19-mer-bound Bgtx, as shown in Fig. 4. This view of the complex presents the concave surface of Bgtx with finger I on the left and finger III on the right. The central three-stranded β-sheet that is conserved among α-neurotoxins is evidenced by a network of long range Hα-Hα and Hβ-HN NOEs, as well as 10 slowly exchanging amide protons. Similarly, a two-stranded antiparallel β-sheet in finger I is observed in many calculated structures. Notably, α7 19-mer binding induces chemical shift perturbations greater than 0.2 ppm in Bgtx resonances for at least 15 residues consistent with their involvement in intermolecular contacts or conformational rearrangements (Table III). Fig. 5A illustrates the conformational changes that occur in Bgtx upon α7 19-mer binding. In comparison to unbound Bgtx, the tip of finger I in bound Bgtx is extended and parallel to the central β-sheet, owing to intermolecular contacts in this region (Table IV). A hydrogen bond between Ser29-O and Ile11-HN, absent in the uncomplexed form, stabilizes the tip of finger I in 8 of the 10 ensemble structures. The flexible tip of finger II undergoes two significant and stabilizing conformational changes upon binding. The fifth disulfide loop (Cys29–Cys33) adopts a convex conformation, whereas Gly34–Lys38 move closer to the α7 19-mer, placing Arg36 and Lys38 in position to make intermolecular contacts. These qualitative observations are quantitated in a comparison of the calculated per residue r.m.s.d. for free and α7 19-mer-bound Bgtx using the respective average structures (Fig. 5B).
mer exhibits no regular secondary structure upon binding Bgtx according to the NMR data as no long range intrapeptide backbone NOE networks nor slowly exchanging peptide amide protons characteristic of α-helical or β-sheet structure were observed. Rather, the α7 19-mer is primarily constrained by contacts with Bgtx over a 9-residue stretch from Phe186 to Tyr194 (see below) and long range intrapeptide NOEs involving backbone proton interactions between Ser185 and Tyr194 and between Phe186 and Tyr194. We also observe long range intrapeptide ring proton NOEs between Tyr187 and Tyr194 and between Phe186 and Tyr194. We also observe strong backbone NOE networks involving Tyr187/Cys189 and Gly187/Cys189 constraint the α7 19-mer describing a hairpin-like turn about the vicinal disulfide. Only sequential NOEs are observed for residues flanking the Ser185-Tyr194 region, leaving the two ends of the α7 19-mer unconstrained.

We find that the sequential NOEs connecting Cys189 and Cys190 are characteristic of a trans peptide bond conformation. We observe strong δi,δi-1 and δi,δi+1 NOEs typical of a trans conformation, and there is no evidence for δi,δi+2 and δi,δi+3 NOEs that would be expected for a cis conformation (58). The α7 19-mer-Bgtx Interface—An extensive network of intermolecular NOEs ranging from Phe186 to Tyr194 on the α7 19-mer positions the α7 19-mer between fingers I and II of Bgtx on the concave face of the toxin and forms a contact area of ~650 Å² (Fig. 4 and Table IV). The aromatic ring of Phe186 alone is involved in contacts with Ala7, Ser3, and Ile11 of the first finger in Bgtx (Table IV). Interestingly, we observe a 0.6 ppm upfield shift of the Ile11 δ methyl protons (Table III). Our structures indicate that these protons are close to the δ ring protons of the Phe186 aromatic ring, suggestive of a ring current-induced shift. A similar shift in the Ile11 Hα/Hδi signal involving the corresponding residue Tyr194 in a1 was observed in the α12-mer-Bgtx structure (14) and is thought to suggest a hydrophobic contact. More expansive contacts are observed between the α7 19-mer and the side of finger II proximal to finger I involving Phe186–Glu188 of the α7 19-mer and Lys38–Val50 in Bgtx. Phe186 and Tyr187 make hydrophobic contacts with Val39 and Val40. This hydrophobic patch is continuous with additional intratoxin hydrophobic contacts between Val39 and Trp58 and between Val40 and His68. Glu188 makes contacts with Lys38, possibly forming an electrostatic pair and, in half of the ensemble structures, donates its amide proton in a backbone hydrogen bond with Arg36. Furthermore, we observe NOEs involving the γ protons of Arg36 at the tip of finger II in Bgtx and the γ protons of both Phe186 and Tyr194 in α7 19-mer, suggestive of additional hydrophobic contacts. Cation–π interactions have been suggested to stabilize ligand interactions with the nAChR (59). NOEs that are characteristic of cation–π interactions (60) are not observed between Bgtx and the α7 19-mer. The positive charge of Arg36 points away from the α7 19-mer, suggesting that it may make contact with residues elsewhere in the intact α7 receptor (e.g., Trp149). No intermolecular NOEs are observed involving Cys38 and Cys190 of the α7 19-mer, consistent with biochemical evidence that Bgtx binding does not depend on these residues (61). Finally, the N terminus of Bgtx is distant from the intermolecular contact zone, consistent with the observation of identical apparent affinities of N-terminal polyhistidine-tagged recombinant Bgtx and venom-derived Bgtx for both Torpedo and mouse muscle nAChRs (62).

**DISCUSSION**

**Structure of Free Bgtx**—The three-dimensional structure of Bgtx presented here builds on an earlier NMR structural study aimed primarily at determining elements of secondary struc-

### Table III

**Chemical shift perturbations in Bgtx induced by α7 19-mer binding**

| Residue | Proton | Free δ | Bound δ |
|---------|--------|--------|--------|
| Ser9    | HN     | 8.27   | 7.93   |
| Ser9    | H      | 4.59   | 4.86   |
| Ile33   | Hδi    | 4.13   | 3.92   |
| Ile34   | Hδ    | 1.69   | 1.41   |
| Ile34   | Hδi   | 0.91   | 0.34   |
| Trp28   | HN     | 7.54   | 7.96   |
| Cys29   | H      | 5.14   | 5.37   |
| Asp30   | Hδi    | 8.51   | 9.41   |
| Asp30   | Hδ    | 3.26   | 3.53   |
| Phe32   | HN     | 8.31   | 8.80   |
| Cys33   | Hδi   | 4.05   | 4.28   |
| Cys33   | Hδ    | 3.60   | 3.80   |
| Ser34   | Hδi   | 4.03   | 3.68   |
| Ser34   | Hδ    | 3.96   | 3.65   |
| Gly37   | HN     | 7.25   | 7.69   |
| Lys38   | HN     | 8.16   | 9.35   |
| Val39   | H      | 3.58   | 3.37   |
| Val39   | HN     | 7.59   | 8.08   |
| Val39   | H      | 4.68   | 4.35   |
| Val39   | Hδi   | 1.73   | 1.48   |
| Glu41   | HN     | 9.44   | 9.14   |
| His48   | Hδi   | 6.76   | 6.46   |
| Gln71   | Hδi   | 8.09   | 8.35   |
The present structure provides a high resolution view of the disulfide core and \(\beta\)-sheet regions that are the structure-defining features of three-finger toxins (6–8). This structure, with a backbone r.m.s.d. from the mean of 0.58 \(\AA\) in the structured regions (residues 1–16, 22–28, 39–48, 54–68), improves on a recent NMR solution structure of Bgtx that has a backbone r.m.s.d. of 1.76 \(\AA\) for the corresponding regions (16; Research Collaboratory for Structural Bioinformatics Protein Data Base code 1IDL). Additionally, the new structure now makes apparent the structural disorder at the tip of finger II that is thought to be important in recognition of the nAChR, as illustrated in the \(\alpha7\) 19-mer-Bgtx complex (see below).

The Bgtx NMR structure is most similar to the X-ray structure of the long chain toxin, \(\alpha\)-cobratoxin (Cbtx) (8), with an average backbone atom r.m.s.d. between Cbtx and the 13 Bgtx structures of 1.33 \(\AA\), for the segment of the well defined regions of Bgtx that are best matched to the corresponding regions in Cbtx (residues 1–6, 11–16, 22–28, 39–48, and 54–68 in Bgtx; residues 1–6, 9–14, 19–25, 36–45, and 51–65 in Cbtx). A comparison of the sequences reveals that finger I in Bgtx is two residues longer than in Cbtx. These additional residues appear as an extra bulge in finger I without changing the overall conformation of the finger. The sequences of Bgtx and Cbtx at the tip of finger II (residues 29–38 in Bgtx; 26–35 in Cbtx) are nearly identical with only two residues different out of ten. Although the overall structure of Bgtx suggests this region is disordered (Fig. 5A), a match of Bgtx residues 29–38 indicates that it has elements of autonomous structure. Furthermore, the local structure between residues 29 and 38 agrees well with the X-ray structure of Cbtx (Fig. 6) with an average backbone r.m.s.d. of 1.74 \(\AA\). The local structure of the corresponding loop of the long neurotoxin LSIII from \(Laticauda\) \(semifasciata\) (9) also shows a similar structure, with an average backbone r.m.s.d. of 1.6 \(\AA\) between Cbtx and LSIII for residues 26–35 in both toxins. Like the Bgtx and LSIII solution structures, the NMR solution structure of Cbtx (7) also exhibits a large r.m.s.d. for finger II with respect to the overall structure, with a local structure for residues 26–35 similar to its crystal structure. This suggests that the tip of finger II may move as a rigid body relative to the triple stranded \(\beta\)-sheet to which it is connected.

The \(\alpha7\) 19-mer-Bgtx Interaction—The structure of the \(\alpha7\) 19-mer-Bgtx complex reveals several important intermolecular contacts that form the structural basis for the high affinity interaction between nAChRs and \(\alpha\)-neurotoxins. Previously, indirect structural information as determined in mutagenesis studies has helped identify \(\alpha\)-neurotoxin binding determinants on the neuronal nAChR (24, 63). The results presented here provide the first direct structural evidence for the contact zone involving an \(\alpha\)-neurotoxin and a neuronal nAChR sequence. Additionally, the structure obtained may represent an energetically favorable conformation for a region of the nAChR important in agonist and antagonist binding and provide an understanding of differences between \(\alpha\)-neurotoxin recognition of muscle-type and neuronal nAChRs.

The \(\alpha7\) 19-mer on its own lacks stable secondary structure as demonstrated by its CD profile (data not shown) and narrow dispersion of its HN resonances (Fig. 2A). Bgtx binding induces a transition whereby the \(\alpha7\) 19-mer takes on a hairpin-like structure (Fig. 4) that is temporally stabilized, meaning that on the NMR time scale, the \(\alpha7\) 19-mer does not rapidly exchange between the free and bound states (Fig. 2B). Such slow exchange kinetics, although often associated with complexes of higher affinity than the 30 \(\mu\)M \(K_D\) of the \(\alpha7\) 19-mer (57), has also been observed in the interaction between cyclin-dependent kinase 2 and a fragment of its inhibitor p21\(^{\text{War14Clp15Sad1}}\) (Fig. 5A), which is characterized by an affinity in the 10–3 \(\mu\)M range (64). The p21 fragment undergoes a binding-induced transition from its native unfolded state to a folded bound state. Disorder-to-order folding transitions have been observed recently in other proteins that are involved in protein-protein or protein-nucleic acid interactions such as fibronectin-binding protein (65) and the transcriptional activation domain of the herpesvirus protein VP16 (66). The binding domains of these proteins are unfolded in the native state and form \(\alpha\)-helical or \(\beta\)-hairpin structures upon interacting with their respective target mole-

**Table IV**

| Observed Intermolecular NOEs |
|----------------------------|
| Proton designations follow IUPAC-recommended nomenclature. |

| \(\alpha7\) 19-mer | Bgtx |
|-----------------|-------|
| Ph168 | H\(^{16}/H^{13}\) \(a\) | Ala\(^7\) | H\(^a\) |
| Ph168 | H\(^{16}/H^{13}\) \(a\) | Ala\(^7\) | H\(^a\) |
| Ph166 | H\(^{16}\) | Ala\(^7\) | H\(^a\) |
| Ph166 | H\(^{16}\) | Ala\(^7\) | H\(^a\) |
| Ph166 | H\(^{16}/H^{13}\) \(a\) | Ser\(^9\) | H\(^b\) |
| Ph166 | H\(^{16}/H^{13}\) \(a\) | Ser\(^9\) | H\(^b\) |
| Ph166 | H\(^{16}/H^{13}\) \(a\) | H\(^a\) |
| Ph165 | H\(^{16}/H^{13}\) \(a\) | Ile\(^{11}\) | H\(^c\) |
| Ph165 | H\(^{16}/H^{13}\) \(a\) | Ile\(^{11}\) | H\(^c\) |
| Ph166 | H\(^{16}/H^{13}\) \(a\) | Ile\(^{11}\) | H\(^c\) |
| Ph166 | H\(^{16}/H^{13}\) \(a\) | Ile\(^{11}\) | H\(^c\) |
| Ph166 | H\(^{16}/H^{13}\) \(a\) | Ile\(^{11}\) | H\(^c\) |
| Ph166 | H\(^{16}/H^{13}\) \(a\) | Arg\(^{26}\) | H\(^{17}/H^{15}\) \(a\) |
| Ph166 | H\(^{16}\) | Val\(^{39}\) | H\(^d\) |
| Try\(^{17}\) | H\(^{17}/H^{15}\) \(a\) | Val\(^{39}\) | H\(^d\) |
| Try\(^{17}\) | H\(^{17}/H^{15}\) \(a\) | Val\(^{39}\) | H\(^d\) |
| Try\(^{17}\) | H\(^{17}/H^{15}\) \(a\) | Val\(^{39}\) | H\(^d\) |
| Try\(^{17}\) | H\(^{17}/H^{15}\) \(a\) | Val\(^{39}\) | H\(^d\) |
| Glu\(^{38}\) | H\(^e\) | Lys\(^{38}\) | H\(^f\) |
| Glu\(^{38}\) | H\(^e\) | Lys\(^{38}\) | H\(^f\) |
| Glu\(^{38}\) | H\(^e\) | Lys\(^{38}\) | H\(^f\) |
| Glu\(^{38}\) | H\(^e\) | Lys\(^{38}\) | H\(^f\) |
| Glu\(^{38}\) | H\(^e\) | Lys\(^{38}\) | H\(^f\) |
| Glu\(^{38}\) | H\(^e\) | Lys\(^{38}\) | H\(^f\) |
| Tyr\(^{19}\) | H\(^e\) | Arg\(^{26}\) | H\(^{17}/H^{15}\) \(a\) |
| Tyr\(^{19}\) | H\(^e\) | Arg\(^{26}\) | H\(^{17}/H^{15}\) \(a\) |
| Tyr\(^{19}\) | H\(^e\) | Val\(^{39}\) | H\(^d\) |
| Tyr\(^{19}\) | H\(^e\) | Val\(^{39}\) | H\(^d\) |

\(^a\) Resonances that have not been assigned individually. Distance constraints for such NOEs were determined using a pseudoatom approach described under “Experimental Procedures.”

![Fig. 6. Structure of finger II. Stereo view showing residues 29–38 corresponding to finger II for 10 of the 13 structures of Bgtx (gray) matched to the corresponding region comprising residues 26–35 of the X-ray structure of Cbtx (black). The labels indicate the location of the \(\alpha\)-carbons of some of the residues in this loop for the Bgtx structures. With the exception of the cysteines of Bgtx, only the backbone atoms are shown.](image-url)
from long toxins by the increased length of their first finger. Mutations at the tip of the first finger of the short toxins erabutoxin a and Naja mossambica mossambica I (Nm1) cause a decrease in affinity for the Torpedo nAChR (11, 69).

On finger II of Bgtx, at Val39 and Val40, we find that multiple contacts are made with Phe186 and Tyr187 in the α7 19-mer. Our structural data confirm the importance of similar hydrophobic interactions observed between aromatic residues at α1 homologous receptor positions 189 and 190 and Val39 and Val40 of Bgtx in complexes with the α12-mer, α18-mer, LLPep, and HApep (see Refs. 14 and 16–18; Fig. 8). Taken together, these results indicate that Val39 and Val40 in Bgtx are important for binding multiple nAChR subtypes through interactions with these adjacent aromatic residues, highly conserved among Bgtx-sensitive α-subunits. As hydrophobic and, in particular, aromatic residues are directly involved in many protein-protein interactions (70), these residues may provide binding stability and specificity and may serve as a nucleating site for subsequent nAChR subtype-specific interactions.

The hairpin-like backbone trace of the structured region of the α7 19-mer (Ser185–Tyr194) resembles the β9–β10 hairpin in the homologous region of the AchBP (21). Whereas Bgtx-bound HApep, HAP, and op25mer, a peptide including α1 182–202 (Fig. 8) (19), form a β-hairpin, we failed to observe any slowly exchanging amide protons or extensive long range intrapeptide NOE networks that would indicate a β-sheet component in the structure of Bgtx-bound α7 19-mer. In contrast with the above-mentioned Bgtx-bound peptides, the turn that is observed in the corresponding region of the α18-mer (16) in its complex with Bgtx is more extended (Fig. 7). The α18-mer ends before Asp200 (α1 numbering), which is a residue that participates in formation of a β-sheet in the op25-mer, apparently stabilizing the β-hairpin turn. The absence of Asp200 in the α18-mer may help explain the lack of any NMR evidence to date for a β-sheet in the α18-mer. Interestingly, an unnaturally introduced constraint that generates a hairpin turn in the α18-mer results in no violation of the NMR-derived constraints for the α18-mer-Bgtx complex.

Differential Affinity of Short and Long Chain Neurotoxins for the α7 nAChR—Short neurotoxins bind the α7 nAChR with lower affinity than long neurotoxins, whereas both bind the muscle-type nAChR with equally high affinity (5). A key structural difference between the long and short toxins is a fifth disulfide located at the tip of finger II in long toxins. The necessity of a fifth disulfide for high affinity interaction with α7 was clearly demonstrated by disruption of this disulfide bridge in the long toxin Cbtx. Although reduced Cbtx bound the Torpedo nAChR with subnanomolar affinity, comparable with unmodified Cbtx, its affinity for the α7 nAChR was decreased 104-fold (5). The importance of the fifth disulfide was later confirmed in mutational studies (63). Interestingly, NMR studies have shown that the end of finger II in α-neurotoxins is a dynamic entity (9, 10). Our structural analysis of the α7 19-mer-Bgtx complex reveals that the fifth disulfide loop (Cys29–Cys53) moves as an independent unit upon binding to re-orient onto the convex face of Bgtx (Figs. 4 and 5A). The chemical shift perturbations from Cys29 to Cys53 and the appearance of at least four new long range intratoxin NOEs involving these residues support this conformational rearrangement. The conformational rearrangement induced by the α7 19-mer may therefore help explain the importance of the fifth disulfide in long toxin-α7 interactions. Accompanying the movement of the fifth disulfide loop, there also is a change in curvature of the neighboring segment at the tip of finger II (Fig. 5A). Arg27 is

2 Q. Shi and E. Hawrot, manuscript in preparation.

3 H. Zeng and E. Hawrot, unpublished observations.
repositioned enabling it to make intermolecular contacts with Tyr^{187} and Tyr^{194} of the a7 19-mer. This is consistent with a double mutant cycle analysis of Cbtx and the a7 nAChR that found Arg^{36} (homologous to Arg^{36} in Bgtx) proximal to Tyr^{187} and Pro^{195} (63).

A variety of mutations in Cbtx have been examined for effects on binding muscle-type and a7 nAChRs (63, 71). It was concluded that Cbtx uses a common set of residues to recognize both receptor subtypes as well as non-overlapping sets for binding one or the other subtype. It was argued that Arg^{36} in Cbtx (homologous to Lys^{38} in Bgtx) is a a7-specific binding residue. This was somewhat surprising as Cbtx-Arg^{36} lies on the convex face of the toxin, whereas toxin-nAChR interactions until then were thought to involve only residues on the concave toxin face. The a7 19-mer-Bgtx structure confirms the importance of the position occupied by Arg^{36} in Cbtx and Lys^{38} in Bgtx. Our structural data suggest a possible electrostatic interaction between Lys^{38} on the convex face of Bgtx and Glu^{188} of the a7 19-mer. Supporting the importance of this receptorsubtype interaction, a negatively charged residue at position 188 is conserved in a7 subunits across species but not in the muscle-type a1 subunit (Fig. 8).

Conformation of the Vicinal Disulfide Peptide Bond—A disulfide bridge between two adjacent cysteine residues in nAChR a subunits is an unusual structural feature found in only a small number of other proteins, e.g. methanol dehydrogenase (72) and atracotoxin (51). The significance of this disulfide is underscored by its conservation among all nAChR sequences (27). Those residues shaded in dark gray are conserved across species and between a1 and a7 nAChR subunits. They also have been localized to the binding site by chemical modification or photoaffinity labeling (1). A, alignment of nAChR and AChBP sequences. Glu^{188} (dark gray shading), a residue that interacts with Lys^{38} on the convex side of Bgtx, is conserved among a7 subunits of various species. B, alignment of Bgtx-binding peptides studied by NMR and x-ray crystallography. a12-mer (14), a18-mer (15, 16), and op25 (19) sequences are derived from the Torpedo a1 subunit. LLPep is a phage display library-derived peptide isolated for its ability to bind Bgtx (17), and HAPep and HAP are high affinity engineered peptides based on the library lead (18, 20).

Reorientation of Bgtx Bound to the nAChR—The recent crystal structure determination of AChBP coupled with the structure of the a7 19-mer-Bgtx complex presented here permits an initial juxtaposition of Bgtx onto this structural model for the extracellular domain of the nAChR (21). As the AChBP is homologous to members of the nAChR family and binds nAChR agonists and antagonists, its structure serves as a model for the nAChR fold. Although an x-ray structure of a mimotope peptide-Bgtx complex was recently superimposed onto the AChBP structure (20), we predict the orientation of receptor-bound Bgtx based on an authentic receptor sequence.

The highly constrained region of the Bgtx-bound a7 19-mer (Ser^{185}–Tyr^{194}) superimposes onto the corresponding region of the AChBP with a backbone r.m.s.d. of 1.93 Å (Fig. 9A). As a first approximation of the orientation of Bgtx about the nAChR extracellular domain, we anticipated and observed side chain clashes between the Bgtx and AChBP structures, but notably, the two backbone traces do not intersect (Fig. 9B). As the AChBP crystal structure is hypothesized to represent a "desensitized" state that is more compact than the resting state that Bgtx stabilizes (3), Bgtx would have even easier access to its binding site.

From a global perspective, our model shows Bgtx localized to the interface of two receptor subunits (Fig. 9C) with the concave surface facing the "plus side" of the subunit interface (21), similar to the mimotope/Bgtx superposition (20). The disulfidel–rich globular core of the toxin is proximal to the membrane with fingers I–III extended toward the AChBP-binding cavity.
Fig. 9. Model of Bgtx bound to the nAChR. The structured region of the α7 19-mer (Ser185–Tyr194) was superimposed onto the corresponding region of the AChBP (Val185–Tyr192) to model the orientation of Bgtx relative to the nAChR. The most representative structure from the α7 19-mer-Bgtx ensemble, as calculated by NMRCLUST (82), was chosen for superposition. A, superposition of backbone atoms of α7 19-mer (Ser185–Tyr194) in blue and AChBP (Val185–Tyr192) in red. The first of the adjacent cysteines is green in both segments. B, ribbon diagram of Bgtx at the subunit interface. Two adjacent subunits of the AChBP pentamer are shown with the β-sheet in blue on the plus side and yellow on the minus side. Bgtx, between the subunits, is in magenta. C, global view of the predicted Bgtx-nAChR interaction. A surface model of the AChBP is shown with each subunit colored differently. This view of the AChBP is perpendicular to the 5-fold axis with the synaptic side on top and membrane side below. Bgtx is shown as a red stick model with the unstructured C-terminal tail removed. The α7 19-mer is not shown for clarity. The χ1 angles of Gln567 and Tyr986 of the AChBP subunit forming the complementary Bgtx-binding site (in white) were rotated to prevent clashes with Bgtx. Neither residue participates in secondary structure elements.

at about a 45° angle to the vertical axis of the receptor. Fingers I and II are near binding loops on the AChBP that are believed to form the principal ligand-binding site on the plus side of the subunit interface. This region includes binding loop C that overlaps with the continuous segment (α1 173–204) that composes the major muscle receptor determinant for binding α-neurotoxins (22). Finger II, in particular, extends deep into the subunit interface approaching binding loops A and B. Fingers II and III are in position to interact with the complementary ligand-binding site on the adjoining subunit. For example, residues homologous to Trp55, Leu119, and Glu176 of the nAChR γ subunit are in position to interact with finger II, consistent with mutational studies that demonstrated the significance of these nAChR residues in α-neurotoxin recognition. Double cycle mutant analysis of the short α-toxin NmmI and the nAChR γ subunit identified the interacting pairs γTrp55/NmmI-Arg33, γTrp55/NmmI-Lys27, γLeu119/NmmI-Arg33 and γGlu176/NmmI-Lys27 (80). Additionally, Bgtx binding to the muscle-type nAChR was blocked by chemical modification of the γLeu119 → Cys mutant and the homologous residues in the δ and ε subunits with a methanethiosulfonate compound bearing a quaternary ammonium group (81).

This view of receptor-bound Bgtx suggests that α-neurotoxins approach their binding site from the receptor periphery, rather than from the vestibule. A similar superposition of α18-mer residues forming the α18-mer-Bgtx contact zone (Tyr189–Thr196) (16) also places Bgtx on the AChBP periphery, suggesting that the path Bgtx travels to bind the nAChR is similar for multiple receptor subtypes. Furthermore, these models provide a new perspective on the α-neurotoxin-nAChR interaction. Based on a number of previous studies, the prevalent model placed Bgtx at the crest of the synaptic side of the receptor with finger II extending into the vestibule (14). A strikingly different picture of the α-neurotoxin-nAChR interaction emerges from our model of Bgtx proximal to the membrane with its loops extended toward the binding cavity at the subunit interface. Based on structural data and consistent with recent biochemical findings, this predictive model forms a solid basis for future studies.

Acknowledgments—We thank Dr. Dale Mierke and Dr. Dusan Uhrin for valuable discussions, Dr. Qing-Luo Shi for assistance in CD studies, and Quochao Pham for assistance in recombinant expression. We gratefully acknowledge Nathan Walsh for excellent assistance in data processing and structure calculations. We thank M. Day for the NMR processing program for free Bgtx studies and Dr. R. C. van Schaik and Dr. W. F. van Gunsteren for the four-dimensional molecular dynamics program and GROMOS and also for assistance in the use of their software. We acknowledge the San Diego Supercomputer Center for the use of the Cray-YMP. The stereo pair images in Fig. 1 were generated with the MidasPlus program from the Computer Graphics Laboratory, University of California, San Francisco (supported by National Institutes of Health Grant RR-01081). NMR instrumentation was funded by Grant RR08240 from the National Institutes of Health and Grants DBI-9723282 (to E. H.) and DMB-9104794 (to V. J. B.) from the National Science Foundation.

REFERENCES
1. Karlin, A., and Akabas, M. H. (1995) Neuron 15, 1331–1344
2. Sargent, P. (1993) Ann. Rev. Neurosci. 16, 403–443
3. Grutter, T., and Changeux, J.-P. (2001) Trends Biochem. Sci. 26, 459–462
4. Chiapinelli V. A. (1999) in Natural and Synthetic Neurotoxins (Harvey, A., ed) pp. 65–128, Academic Press, New York
5. Servent, D., Winckler, D. V., Hu, H. Y., Kessler, P., Drevet, P., Bertrand, D., and Ménez, A. (1997) J. Biol. Chem. 272, 24279–24286
6. Zinn-Justin, S., Roumestand, C., Gilquin, B., Bontems, F., Ménez, A., and Toma, F. (1999) Biochemistry 38, 11335–11347
7. Leroy, E., Miko, A., Yang, Y., and Guettet, E. (1994) J. Biomol. Struct. Dyn. 12, 1–17
8. Betzel, C., Lange, G., Pal, G. P., Wilson, K. S., Maelicke, A., and Saenger, W. (1991) J. Biol. Chem. 266, 21530–21536
9. Connolly, M. J., Stern, A. S., and Hoch, J. C. (1996) Biochemistry 35, 418–426
10. Guenneugues, M., Drevet, P., Pinkasfeld, S., Gilquin, B., Ménez, A., and Zinn-Justin, S. (1997) Biochemistry 36, 16097–16108
11. Trénaré, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Ducancel, F., Boulain, J.-C., and Ménez, A. (1995) J. Biol. Chem. 270, 9362–9369
12. Love, R. A., and Stroud, R. M. (1986) Protein Eng. 1, 37–46
13. Basus, V. J., Biliter, M., Love, R. A., Stroud, R. M., and Kuntz, D. L. (1988) Biochemistry 27, 2763–2771
14. Basus, V. J., Song, G., and Hawrot, E. (1993) Biochemistry 32, 12290–12298
15. Gentile, L. N., Basus, V. J., Shi, Q.-L., and Hawrot, E. (1995) Ann. N. Y. Acad. Sci. 737, 222–237
16. Zeng, H., Mous, L., Grant, M. A., and Hawrot, E. (2001) J. Biol. Chem. 276, 22830–22840
17. Schefer, T., Balass, M., Fuchs, S., Katschke-Katzer, E., and Anglister, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6059–6064
18. Schefer, T., Kasher, R., Balass, M., Fridkin, M., Fuchs, S., and Katschke-Katzer, E. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6629–6634
19. Samson, A. O., Chill, J. H., Rodriguez, E., Schefer, T., and Anglister, J. (2001) Biochemistry 40, 23381–23389
