α-Bungarotoxin and the Competing Antibody WF6 Interact with Different Amino Acids within the Same Cholinergic Subsite†

Bianca M. Conti-Tronconi, Brenda M. Diethelm, Xiadong Wu, Fen Tang, and Tony Bertazzon
Department of Biochemistry, College of Biological Sciences, University of Minnesota, 1479 Gortner Avenue, St. Paul, Minnesota 55108

Bernd Schröder, Sigrid Reinhardt-Maelicke, and Alfred Maelicke*
Institut für Physiologische Chemie, Johannes Gutenberg-Universität, 6 Duesbergweg, D-6500 Mainz, West Germany
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ABSTRACT: In the nicotinic acetylcholine receptors (AChRs), the sequence segment surrounding two invariant vicinal cysteinyl residues at positions 192 and 193 of the α subunit contains important structural component(s) of the binding site for acetylcholine and high molecular weight cholinergic antagonists, like snake α-neurotoxins. At least a second sequence region contributes to the formation of the cholinergic site. Studying the binding of α-bungarotoxin and three different monoclonal antibodies, able to compete with α-neurotoxins and cholinergic ligands, to a panel of synthetic peptides as representative structural elements of the AChR from *Torpedo*, we recently identified the sequence segments α181–200 and α55–74 as contributing to form the cholinergic site (Conti-Tronconi et al., 1990). As a first attempt to elucidate the structural requirements for ligand binding to the subsite formed by the sequence α181–200, we have now studied the binding of α-bungarotoxin and of antibody WF6 to the synthetic peptide α181–200, and to a panel of peptide analogues differing from the parental sequence α181–200 by substitution of a single amino acid residue. CD spectral analysis of the synthetic peptide analogues indicated that they all have comparable structures in solution, and they can therefore be used to analyze the influence of single amino acid residues on ligand binding. Distinct clusters of amino acid residues, discontinuously positioned along the sequence 181–200, seem to serve as attachment points for the two ligands studied, and the residues necessary for binding of α-bungarotoxin are different from those crucial for binding of antibody WF6. In particular, residues at positions 188–190 (VYY) and 192–194 (CCP) were necessary for binding of α-bungarotoxin, while residues W_{187}, T_{191}, and Y_{198} and the three residues at positions 193–195 (CPD) were necessary for binding of WF6. Comparison of the CD spectra of the toxin/peptide complexes, and those obtained for the same peptides and α-bungarotoxin in solution, indicates that structural changes of the ligand(s) occur upon binding, with a net increase of the β-structure component. The cholinergic binding site is therefore a complex surface area, formed by discontinuous clusters of amino acid residues from different sequence regions. Such complex structural arrangement is similar to the "discontinuous epitopes" observed by X-ray diffraction studies of antibody/antigen complexes [reviewed in Davies et al. (1988)]. Within this relatively large structure, cholinergic ligands bind with multiple points of attachment, and ligand-specific patterns of the attachment points exist. This may be the molecular basis of the wide spectra of binding affinities, kinetic parameters, and pharmacologic properties observed for the different cholinergic ligands.

The nicotinic acetylcholine receptors (AChRs) are complex transmembrane proteins formed by homologous subunits [reviewed in McCarthy et al. (1986), Lindstrom et al. (1987), and Maelicke (1988)] which in peripheral tissues, such as fish electroplax and skeletal muscle, are assembled in a stoichiometry of $\alpha_2\beta\gamma\delta$ (Raftery et al., 1980; Conti-Tronconi et al., 1982a,b). The AChRs are among the best characterized ligand-gated ion channels. Binding to the AChR of acetylcholine or of other cholinergic agonists induces transient openings of the channel [reviewed in Sine and Taylor (1980), Conti-Tronconi and Raftery (1982), and Maelicke (1988)]. Antagonists compete with agonists for AChR binding, and block the agonist-induced activation of the channel.

The α subunits contain two cysteinyl (Cys) residues at positions 192 and 193 [reviewed in McCarthy et al. (1987) and Maelicke (1988)] which are labeled by the cholinergic affinity labels (Kao et al., 1984; Dennis et al., 1988). α-Neurotoxins from elapid snakes like α-bungarotoxin (αBTX) are high-affinity, slowly dissociating ligands of the AChR from peripheral tissues [reviewed in Klett et al. (1973), Blanchard et al. (1979), Sine and Taylor (1980), Wang and Schmidt (1980), Chang et al. (1984), and Maelicke (1988)]. Several studies of the binding of ^{125}I-αBTX and other similar neurotoxins to proteolytic, synthetic, or biosynthetic peptides suggested that a sequence segment of the AChR α subunit flanking and including Cys_{192} and Cys_{192} contains important constituent elements of the cholinergic site (Wilson et al., 1984; Oblas et al., 1986; Neumann et al., 1986a,b; Gershoni, 1987; Ralston et al., 1987; Gotti et al., 1988; Aronheim et al., 1988; Conti-Tronconi et al., 1988, 1990; Wilson & Lentz, 1988; McLane et al., 1990a,b; Guy et al., 1990; Ohana & Gershoni, 1990).

Anti-AChR monoclonal antibodies (mAbs) can be produced which bind with high affinity to the cholinergic binding site and compete with α-neurotoxins and small cholinergic ligands (Watters & Maelicke, 1983; Fels et al., 1986). These mAbs

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* Address correspondence to this author.

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$T\alpha 181\text{-}200_{unmod}$	YRGWKHWVYYTCCPDTPYLD
$T\alpha 181\text{-}200_{G181}$	GRGWKHWVYYTCCPDTPYLD
$T\alpha 181\text{-}200_{G182}$	YGGWKHWVYYTCCPDTPYLD
$T\alpha 181\text{-}200_{A183}$	YRAWKHWVYYTCCPDTPYLD
$T\alpha 181\text{-}200_{G184}$	YRGGKHWVYYTCCPDTPYLD
$T\alpha 181\text{-}200_{G185}$	YRGWGHWVYYTCCPDTPYLD
$T\alpha 181\text{-}200_{G186}$	YRGWKGWVYYTCCPDTPYLD
$T\alpha 181\text{-}200_{G187}$	YRGWKHGVYYTCCPDTPYLD
$T\alpha 181\text{-}200_{G188}$	YRGWKHWGYYTCCPDTPYLD
$T\alpha 181\text{-}200_{G189}$	YRGWKHWVGYTCCPDTPYLD
$T\alpha 181\text{-}200_{G190}$	YRGWKHWVYGTCCPDTPYLD
$T\alpha 181\text{-}200_{G191}$	YRGWKHWVYYGCCPDTPYLD
$T\alpha 181\text{-}200_{G192}$	YRGWKHWVYYTGCPDTPYLD
$T\alpha 181\text{-}200_{G193}$	YRGWKHWVYYTCGPDTPYLD
$T\alpha 181\text{-}200_{G194}$	YRGWKHWVYYTCCGDTPYLD
$T\alpha 181\text{-}200_{G195}$	YRGWKHWVYYTCCPGTPYLD
$T\alpha 181\text{-}200_{G196}$	YRGWKHWVYYTCCPDGPYLD
$T\alpha 181\text{-}200_{G197}$	YRGWKHWVYYTCCPDTGYLD
$T\alpha 181\text{-}200_{G198}$	YRGWKHWVYYTCCPDTPGLD
$T\alpha 181\text{-}200_{G199}$	YRGWKHWVYYTCCPDTPYGD
$T\alpha 181\text{-}200_{G200}$	YRGWKHWVYYTCCPDTPYLG

FIGURE 1: Codes and amino acid sequence of peptide α181–200 and the panel of homologous peptides used in this study. The amino acid residues are indicated with the single-letter notation.

and snake α-neurotoxins are high-affinity, nonirreversible proteinaccous ligands which offer a good alternative to irreversible affinity labels (Kao et al., 1984; Dennis et al., 1988) for mapping of cholinergic binding sites. An advantage of their use is that they may identify the amino acid residues on the AChR surface with which they interact, and answer the question as to whether the cholinergic binding site is formed by a very small area, i.e., by a single amino acid residue or a pair of residues, or whether it comprises a larger surface, formed by the interaction of clusters of several amino acid residues.

We have recently shown, studying the binding of αBTX and of three different monoclonal antibodies (mAbs) able to compete with αBTX and with other cholinergic ligands to a panel of overlapping synthetic peptides screening the complete sequence of *Torpedo* α subunit, that the latter model applies to the cholinergic binding site. Both αBTX and the mAbs bound to the same two discontinuous sequence regions of the AChR α subunit, and the relative contributions to ligand binding of each of these subsites varied in a ligand-specific fashion (Conti-Tronconi et al., 1990). Specifically, αBTX and mAb WF6 preferentially but not exclusively interacted with elements within the sequence region α181–200, while mAbs W2 and WF5 preferentially interacted with elements within the segment α55–74. Furthermore, we observed that binding of αBTX is facilitated by the existence of a disulfide bridge between cysteines α192 and 193, while WF6 binds with even higher affinity to the AChR (or to the synthetic peptide α181–200) when this disulfide bridge is reduced and the cysteines alkylated. These results, which are consistent with ligand-specific multipoint interactions between the AChR and cholinergic ligands, prompted us to further investigate the interaction of proteinic ligands with their subsites on the *Torpedo* AChR molecule.

As a first step into this direction, we have synthesized a set of peptides homologous to α181–200. Each of these peptides differs by just 1 amino acid exchange from the parent sequence, resulting in the set of 20 peptides shown in Figure 1. We have studied the binding of αBTX and mAb WF6 to these peptide analogues, using both solid-phase assays of the direct binding of αBTX to the peptides and competition assays of the ability of the peptides in solution to compete with native *Torpedo* AChR for binding to αBTX and WF6. The contribution to αBTX and WF6 binding of each individual amino acid residue within the sequence α181–200 was determined. We found striking differences in the patterns of amino acids which are crucial for interaction with αBTX and with WF6. Therefore, these ligands have distinct multipoint attachments even at the level of this subsite. This mechanism of binding may account for the species specificity and tissue specificity of the binding of αBTX and WF6.

Materials and Methods

Peptide Synthesis and Characterization. Peptides were synthesized according to Houghten (1985). Their purity was analyzed by high-pressure liquid chromatography (HPLC), amino acid analysis, and gas-phase sequencing as described before (Conti-Tronconi et al., 1990). The codes and the amino acid sequence of the peptides are reported in Figure 1.

Purification and Radiolabeling of α-Bungarotoxin (αBTX). αBTX was purified from *Bungarus multicinctus* venom (Biotoxins Inc., St. Clout, FL) according to Clark et al. (1972). The toxin was characterized and radiolabeled with ^{125}I as described before (Conti-Tronconi et al., 1990). It was separated from unreacted iodine and other low molecular weight contaminants by gel chromatography. The specific activity of ^{125}I-αBTX was determined according to Blanchard et al. (1979).

Purification of Monoclonal Antibody WF6. The production and properties of antibody WF6 have been described previously (Watters & Maelicke, 1983; Fels et al., 1986; Schröder et al., 1989). It was purified by chromatography on protein A–Sepharose 4B (Pharmacia) from supernatants of hybridoma cells raised in IgG-free medium. WF6 binds mutually exclusively with αBTX to membrane-bound or solubilized AChR from *Torpedo* electric tissue (Conti-Tronconi et al., 1990), but it blocks only one of the two acetylcholine binding sites per AChR monomer (Fels et al., 1986).

Preparation of AChR-Rich Torpedo Membrane Fragments. AChR-rich membrane fragments were prepared from *Torpedo californica* or from *Torpedo marmorata* electric tissue according to established procedures (Elliot et al., 1980; Neubig et al., 1979; Fels et al., 1986). The source of starting material and the level of enrichment did not affect the results reported here.

Reduction/Alkylation or Oxidation of the Cys_{192}–Cys_{193} Disulfide Bridge. Peptides (100–400 μM) in 10 mM sodium phosphate buffer, pH 8.0, were treated with dithiothreitol (final concentration 3.5 mM) for 1 h at room temperature under nitrogen. Iodoacetamide was added to a final concentration of 10 mM and incubated for a few minutes at room temperature. Finally, the mixture was dialyzed against 10 mM sodium phosphate buffer, pH 7.0, using dialysis tubing with a cutoff of 1000 Da (Spectraphor 1000).

For oxidation, the peptide solution (100–400 μM in 10 mM sodium phosphate buffer, pH 8.0) was made 1.75 mM in iodosobenzoic acid and was incubated for 1 h at room temperature under nitrogen, followed by dialysis as described above. The concentration of sulfhydryl groups in the native, reduced/alkylated, or oxidized peptide was determined by reaction with 5,5-dithiobis(2-nitrobenzoic acid) (Ellman et al., 1961) employing a molar extinction coefficient of the colored product of 13.600 at 420 nm.

Circular Dichroism. The ellipticity of the peptides as followed with a Jasco J 41 C spectropolarimeter (Jasco Inc., Easton, MD) interfaced by means of an Adalab A/D converter (Adalab-PC Interactive Microwave Inc., State College, PA) to an IBM-XT compatible personal computer. Water-jacketed, thermostated quartz cuvettes with path lengths of 0.5 cm, 1 mm, and 0.1 mm were obtained from Hellma (Forest

Hills, NY). Calibration of the instrument was done with (±)-10-camphorsulfonic acid. Peptides were solubilized at pH 11, and spectra of the random coil structure were taken. The pH was then lowered to 7.4 at which some peptides were partially insoluble. In these cases, the sample was centrifuged to eliminate undissolved material, and the supernatant was used for the recording of spectra. Protein concentration was assayed in the sample used to obtain the CD spectra. Absorption was recorded from 500 to 190 nm with a Perkin Elmer Lambda-5 spectrophotometer, with the following absorption coefficients used: 2.88 at 1 mM concentration for peptides containing two tryptophanyl and four tyrosyl residues, and 2.03 for peptides containing two tryptophanyl and three tyrosinyl residues.

The values of the CD spectra are expressed as the mean elipticity per residue, θ, which has the dimensions of degrees per square centimeter per decimole. The mean residue weight (MRW) used was 128.2 for α181–200, 126.1 for α181–200_{G188}, 122.9 for α181–200_{G189}, 126 for α181–200_{G191}, and 125.9 for α181–200_{G192} and α181–200_{G193}. The spectra of αBTX were taken in 5 mM Tris-HCl, pH 7.4. Solubilized toxin (2 mg/mL) was used to solubilize an equimolar amount of peptide, and the sample was then diluted to 1 mg/mL toxin concentration. The differential spectra of peptides in the bound form were obtained by subtracting the spectrum of the free toxin.

Digitized data were analyzed with a fitting program based on the reference spectra of Chang et al. (1978), and the approximate relative contents of types of secondary structure were normalized to 100.

Binding of αBTX to Synthetic Peptides. (*a*) *Dot Blot Assay.* The peptide was spotted onto nitrocellulose strips, and the assay was performed with four or five different concentrations of ^{125}I-αBTX, as indicated in the legends to Figure 4. All other conditions are as described in Conti-Tronconi et al. (1990). The dried strips were autoradiographed, and/or the dots were cut out and counted in a Beckman Gamma 5500 counter. Unspecific binding was determined in the presence of 80–100 μM unlabeled αBTX and was subtracted.

(*b*) *Competition Binding Assay.* ^{125}I-αBTX (2 pmol) was incubated with given concentrations of peptide in PBS (see Figure 5) in a final volume of 100 μL for at least 4 h at 4 °C. One picomole of membrane-bound AChR in 5 μL of PBS was added, and after incubation for 5 min at room temperature, the mixture was centrifuged at 13000 rpm in a Sorvall SH-MT rotor. The pellet was resuspended in PBS and washed twice by centrifugation as described above. The supernatant was carefully sucked off, and the amount of ^{125}I-αBTX bound to the membrane was determined by counting in a Beckman Gamma 5500 counter.

Binding of mAb WF6 to Synthetic Peptides. (*a*) *Dot Blot Assay.* The assay was performed as described before (Conti-Tronconi et al., 1990) using ^{125}I-labeled protein A [1.2 × 10^6 cpm/mL, labeled by the chloramine T method of Greenwood et al. (1963)] to monitor the concentration of bound WF6.

(*b*) *Solid-Phase Radioimmunoassay* (*SPRIA*). The assay protocol of Wilson and Lentz (1988) with the modifications of Conti-Tronconi et al. (1990) was employed.

(*c*) *Enzyme-Linked Immunosorbent Assay* (*ELISA*). Fifty microliters of peptide solution (0.05 mg/mL in PBS) was added to each well of flexible microtiter plates (Falcon) followed by 1 h of incubation at 37 °C. After the plates were washed and incubated with a solution of 3% bovine serum albumin (BSA) in PBS (150 mL/well, 1 h at 37 °C), 50 μL/well of WF6 in PBS was added, and the plates were incubated overnight at 4 °C. After three washes with PBS–Tween (PBS containing 0.05% Tween 20), the wells were treatd with 50 μL of a solution of peroxidase-linked rabbit anti-mouse IgG (Dakopatts), diluted 750-fold with 1% BSA–PBS. After two washes, 100 μL of substrate solution (0.15 M 1,2-phenylenediamine, 0.05% H_2O_2, and 0.1 M sodium citrate, pH 5.0) was added. The plates were incubated for constant periods of time, and the reaction was stopped by the addition of 25 μL of 4.5 M sulfonic acid. The absorption at 490 nm was read in a ImmunoReader NG-2000 (InterMed).

To quantify this assay, the peptide was applied at a concentration at which it is completely bound to the wells. The binding capacity of the wells was determined by titration, determining the amount of unbound peptide by ELISA after absorption to a second well. Complete immobilization of peptides up to 0.2 μg/well was observed.

(*d*) *Competition Binding Assay.* Fifty microliters of *Torpedo* membrane fragments (1:1000 in 0.1 M $NaHCO_3$, pH 9.0) was added to each well, and after incubation and several washes, the wells were further coated with BSA as described above. Aliquots of a solution of WF6 in PBS (2 nM) were incubated with various concentrations of synthetic peptides (0.16–5200 nM) for 2 h at 37 °C, after which time 50 μL of this mixture was added to the AChR-coated wells (triplicates). After 1 h of incubation at room temperature, the plates were washed as described above, followed by incubation with the peroxidase-linked second antibody. The enzyme reaction was performed at room temperature for 600 ± 5 s following the procedure described above.

To quantify this assay, the concentration of immobilized AChR was determined by the binding of ^{3}H-αBTX (specific radioactivity 4.48 TBq/mmol; Amersham). AChR-coated microtiter wells were incubated with various concentrations of ^{3}H-αBTX overnight at 37 °C, after which time the concentration of unbound αBTX was determined by counting in a scintillation counter the radioactivity of a 40-μL supernatant aliquot mixed with 5 mL of Quickzint 212 scintillation solution (Zinsser). After the plates were washed 3 times with PBS–Tween, the wells were cut off, and the concentration of bound ^{3}H-αBTX was determined as described above. Good agreement was observed between calculated and measured values for bound ^{3}H-αBTX.

Quantification of Antibody Binding Data. We assumed that the concentration of immobilized peptide is identical with that in the incubation mixture (see ELISA assay for details), while the concentration of purified antibody was determined by the UV absorption at 280 nm (1.4 OD_{280} = 1 mg/mL). The relative binding can be assayed by ELISAs applying identical conditions for each antibody concentration studied. This is not a true equilibrium binding assay, and thus the binding affinities determined in this way may be considerably lower than the real binding affinity.

The experimental conditions of the competition ELISA were too complicated to allow sophisticated data analysis. To obtain a rough estimate for the binding affinity of the mutated peptide analogues, we determined "apparent K_I" values, i.e., the concentrations of peptide at which the binding of WF6 to immobilized AChR was reduced by 50% under the experimental conditions used.

Results

Structural Properties of Synthetic Peptides. The amino acid sequences and the codes of the synthetic peptides used in this study are shown in Figure 1. The parent peptide Tα181–200_{unmod} corresponds to the sequence segment 181–200

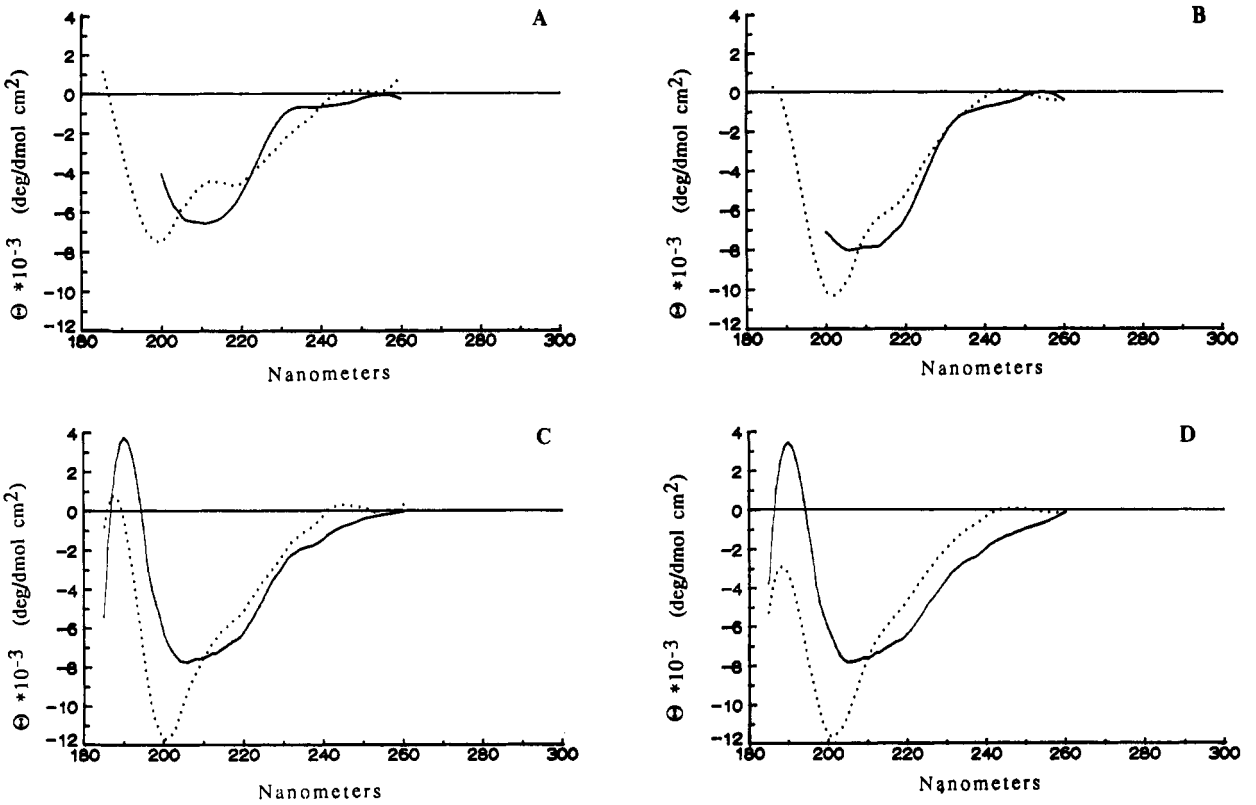

FIGURE 2: Far-UV CD spectra of $\alpha 181-200_{unmod}$ (panel A) and of three single-residue-substituted analogues from the panel illustrated in Figure 1 [$\alpha 181-200_{G191}$ (panel B), $\alpha 181-200_{G188}$ (panel C), and $\alpha 181-200_{G189}$ (panel D)]. Solid lines are spectra of the peptide in 5 mM Tris-HCl, pH 7.4; dotted lines are the spectra of the unfolded peptide in Tris-HCl, pH 11. Digitized data were analyzed by a fitting program based on the reference spectra of Chang et al. (1978). All four peptides had similar contents of β-pleated sheet structure (50–60%) and of random-coil structure (25–30%).

of the α subunit of the AChR from *Torpedo californica* and *Torpedo marmorata*. The other peptides are analogues which differ from the parent peptide by single amino acid substitutions. The individual residues of the sequence Tα181–200 were changed to a glycine residue. The residue Gly_{183} or the parent sequence was changed to alanine. The purity of the peptides, assessed as described before (Conti-Tronconi et al., 1990), was 85% or higher.

In order to be suitable structural representations of the main cholinergic subsite of the AChR, the structure in solution of the parent peptide should not be random, and the peptide structure should not change substantially as a consequence of these single-residue substitutions. These requirements were tested by CD spectral analysis. Figure 2 shows the far-UV CD spectra of equimolar amounts of $\alpha 181-200$ and of three peptides containing single-residue substitutions ($\alpha 181-200_{G188}$, $\alpha 181-200_{G189}$, and $\alpha 181-200_{G191}$). Analysis according to Chang et al. (1978) yielded for $\alpha 181-200_{unmod}$ approximately 50% of β-pleated sheet structure and only 30% of random-coil structure. The three modified peptides, which were selected because of their different ability to bind αBTX (see below), had similar structural contents as the parent peptide. These findings suggest that any differences found in the binding properties of the substituted peptides, as compared to peptide $T\alpha 181-200_{unmod}$, are not due to gross changes in the peptide structure but rather reflect the specific contribution to ligand binding of the substituted residue.

The above conclusion does not exclude the possibility of conformational adjustments upon ligand binding. Indeed, as shown in Figure 3, the CD spectra of a mixture of peptide $\alpha 181-200_{unmod}$ and of αBTX is not simply the sum of the two independent spectra but rather show an increase of 20–25% in β structure at the expense of random-coil structure. Similar effects were observed for peptides $\alpha 181-200_{G186}$ (Figure 3C), $\alpha 181-200_{G191}$ (not shown), and $\alpha 181-200_{G199}$ (not shown), and were absent in mixtures of αBTX with peptides which do not mimic cholinergic subsites (Figure 3D). Thus, the observed increase in β-pleated sheet structure was the result of peptide/αBTX interactions, and it may have been caused either by limited conformational adjustments of the peptide (or αBTX, or both) in the course of complex formation or by the very nature of the interaction between two polypeptides of mainly β structure (see Discussion). From these results, irrespective of their alternative interpretations, we conclude that peptide $\alpha 181-200_{unmod}$ and the set of single-residue-substituted analogue peptides used here are suitable models for the main cholinergic subsite and its single-residue mutations.

Binding of αBTX to Synthetic Peptides. To study the binding of αBTX to the panel of homologous synthetic peptides, we employed the same two assays previously developed for mapping of the sequence regions of *Torpedo* AChR α subunit which form the cholinergic binding site (Conti-Tronconi et al., 1990). In the "direct binding" assay, the peptides were immobilized on nitrocellulose strips, and after the strips were incubated with ^{125}I-αBTX and washed, they were autoradiographed and/or cut and counted in a γ counter. Figure 4 shows the results of a representative experiment using four different concentrations of ^{125}I-αBTX. Consistently, the apparent K_D values for αBTX binding to the peptides either remained unchanged (i.e., approximately 1 μM) or went up by a factor of 4–6, or there was no measurable binding under the experimental conditions used. From the B_{max} values (not shown), it appeared that at least peptide $\alpha 181-200_{G196}$ did not bind to nitrocellulose as well as the other peptides, and this may cause an overestimate by this assay of the influence of residue T_{196} on αBTX binding. The same may apply to some

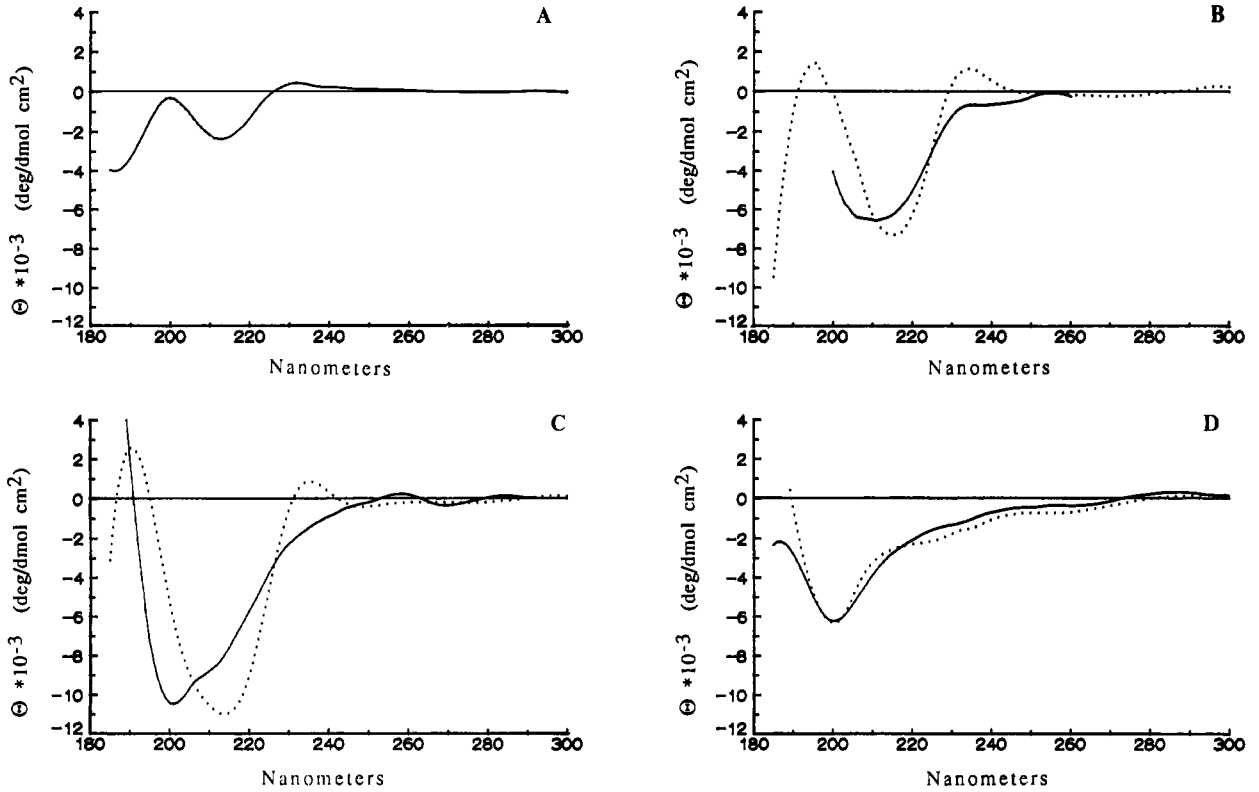

FIGURE 3: Effect of binding to αBTX on the far-UV CD spectra of $\alpha 181-200_{unmod}$ and $\alpha 181-200_{G186}$. αBTX was dissolved at a concentration of 1 mg/mL in 5 mM Tris-HCl, pH 7.4, and the CD spectrum was obtained (panel A). To obtain the CD spectrum of $\alpha 181-200_{unmod}$ bound to αBTX, the toxin was dissolved at a concentration of 2 mg/mL, an equimolar amount of the peptide was added, and the sample was diluted to 1 mg/mL toxin concentration. The differential spectrum of bound $\alpha 181-200_{unmod}$ (dotted line in panel B) was obtained by subtracting the spectrum of free αBTX from that of the reaction mixture. For comparison, the spectrum of the peptide in the absence of αBTX is also shown (solid line in panel B). Panel C shows the CD spectra of free and bound $\alpha 181-200_{G186}$ obtained as described above. The CD spectra of a control peptide (α91–110) in the absence and presence of αBTX are shown in panel D. The subtracted spectra for $\alpha 181-200_{unmod}$ and $\alpha 181-200_{G186}$ show red shifts of their negative maxima which calculate to an increase of 20–30% in β- structure at the expense of random-coil and β-turn structure.

of the modified peptides that do not show any measurable binding of αBTX. The opposite (i.e., somewhat larger B_{max} values for the modified peptides) was observed for peptides in which any of the residues Y_{181}, G_{183}, W_{184}, H_{186}, and W_{187} had been substituted.

In the "competition binding" assay, binding of ^{125}I-αBTX to the peptides occurred in solution, and the concentration of unbound ^{125}I-BTX was determined by binding to an excess of *Torpedo* AChR (see Materials and Methods). Although it is less sensitive than the direct binding assay described above, the competition assay avoided ambiguities resulting from variations in the peptides attachment to solid supports. Figure 5 depicts the results of one such experiment, at three different concentrations of inhibiting synthetic peptides. Because of the very large differences in the binding affinity of αBTX to native AChR, as compared to its binding affinity to the peptides, rather large concentrations of peptides must be used to observe measurable effects. A clear pattern of more or less essential residues for αBTX binding emerged from these experiments, which was largely consistent with the results of the dot blot assays (Figure 4). Peptide $T\alpha 181-200_{unmod}$, as well as the analogues with single-residue substitutions at positions 181–187, 191, and 195–200, at concentrations as low as 0.1 mg/mL, efficiently inhibited ^{125}I-αBTX binding to native AChR. On the other hand, substitution of any of residues V_{188}, Y_{189}, Y_{190}, C_{192}, C_{193}, or P_{194} drastically affected the ability of the peptide to compete with AChR for αBTX binding. The latter peptides did not inhibit ^{125}I-αBTX binding to native AChR, or did so to a much lesser extent than peptide $T\alpha 181-200_{unmod}$. Therefore the interaction of αBTX with the main cholinergic subsite formed by the sequence segment α181–200 appears to involve at least six amino acid residues (V_{188}, Y_{189}, Y_{190}, C_{192}, C_{193}, and P_{194}. A few other residues seem to have an accessory role, because the effects of their substitution, although significant, were of lesser importance.

Table I: Structural Contents of Synthetic Peptides Obtained by CD Spectral Analysis[a]

amino acid exchange	α helix	β sheet	β turn	random coil
$(V = G)_{188}$	0.09	0.67	0.00	0.24
$(Y = G)_{189}$	0.06	0.74	0.00	0.20
$(C = G)_{192}$	0.05	0.35	0.26	0.34
$(C = G)_{193}$	0.03	0.64	0.07	0.27

[a] CD spectra were taken as described under Materials and Methods and in the legend of Figure 2. They were analyzed according to Chang et al. (1978).

Binding of Antibody WF6 to Synthetic Peptides. Four assays were developed (see Materials and Methods) and were performed independently in both laboratories. Figure 6 depicts the results of a dot blot experiment using four different dilutions of antibody. Again, as shown in Figure 4 for αBTX binding, substitution of some amino acids profoundly affected the affinity of the peptides for the antibody, while other substitutions did not significantly affect WF6 binding. Simular results were obtained with the ELISA and SPRIA assays described under Materials and Methods (Figure 9). To quantify the variations in binding affinity for the antibody induced by single amino acid substitutions within the sequence α181–200, the ELISA assay was performed under conditions

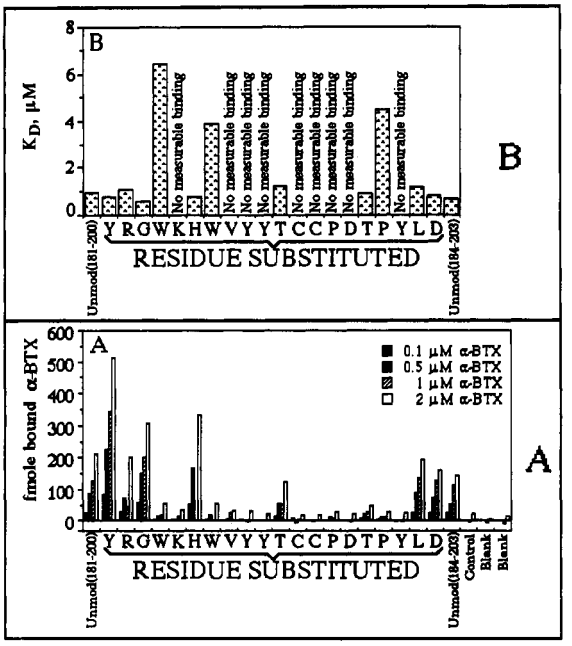

FIGURE 4: Dose dependency of the binding of ^{125}I-αBTX to the panel of synthetic peptides, as investigated by dot blot assay. (A) Four concentrations of ^{125}I-αBTX (0.1, 0.5, 1.0, and 2 μM) were used. Unspecific binding was determined for each point in the presence of an excess of unlabeled αBTX and was subtracted. Control refers to ^{125}I-αBTX binding to a nonrelated peptide; blank refers to ^{125}I-αBTX binding to nitrocellulose without any peptide attached. We also determined ^{125}I-αBTX binding to the overlapping peptide α184–203$_{unmod}$. Consistent with the finding that substitution of one each of the first three amino acids of α181–200$_{unmod}$ does not significantly alter αBTX binding, α184–200$_{unmod}$ binds the toxin to a similar extent and with similar affinity (see panel B) as α181–200$_{unmod}$. (B) K_D values obtained from Scatchard analysis of the dot blot assays depicted in (A).

Table II: Apparent Equilibrium Dissociation Constants of the Binding of Antibody WF6 to the Panel of Synthetic Peptides

	direct ELISA		competition ELISA	
peptide	K_D (μM)	rel affinity	K_I (μM)	rel affinity
unmod	0.17	1.0	0.35	1.0
Y181	0.13	1.3	0.44	0.8
R182	0.20	0.9	0.42	0.8
G183	0.06	2.8	0.36	1.0
W184	0.11	1.5	0.6	0.7
K185	0.10	1.7	0.39	1.0
H186	0.18	0.9	0.76	0.5
W187	0.38	0.4	>100	<0.01
V188	0.17	1.0	0.63	0.6
Y189	0.10	1.7	0.70	0.5
Y190	0.12	1.4	2.1	0.2
T191	>1.5	<0.1	>100	<0.01
C192	0.04	4.2	0.89	0.4
C193	0.6	0.3	27	0.01
P194	>1.5	<0.1	38	0.01
D195	>1.5	<0.1	>100	<0.01
T196	0.48	0.4	0.95	0.4
P197	0.42	0.4	1.5	0.2
Y198	0.90	0.2	>100	<0.01
L199	0.50	0.3	0.49	0.7
D200	0.22	0.8	0.37	1.0

of known concentrations of immobilized peptide and antibody (see Materials and Methods). Because of the solid-phase nature of this assay, the "K_D" values thus obtained (Table II) should be considered upper limits of the true equilibrium dissociation constants of the antibody/peptide interaction. Figure 7 summarizes one set of such data in terms of relative affinities of binding. Three ranges of affinities were apparent, as follows. Changes of less than a factor of 2 were considered

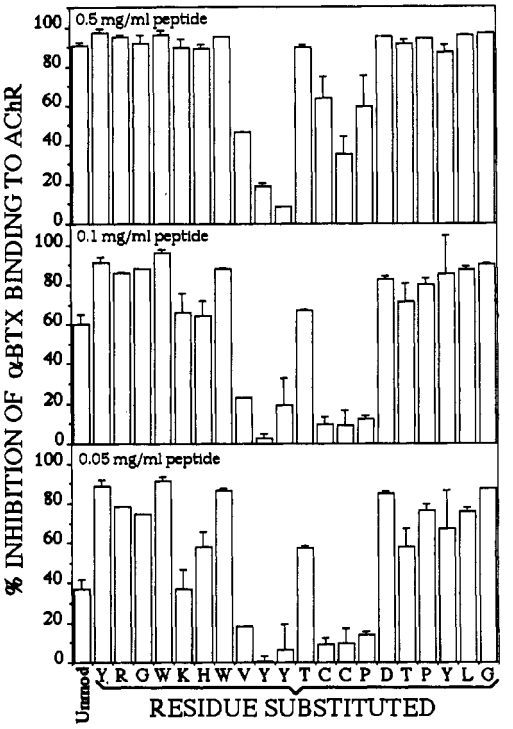

FIGURE 5: Inhibition by synthetic peptides of the specific binding ^{125}I-αBTX to native *Torpedo* AChR. Three concentrations of added peptides were employed (0.5, 0.1, and 0.5 mg/mL, as indicated). The peptides used are identified along the abscissa, by indicating the residue substituted in the peptide. "Unmod" refers to the peptide corresponding to the unmodified sequence segment α181–200. It clearly appears that the peptide Tα181–200$_{unmod}$, as well as the analogues with single-residue substitutions at positions 181–187, 191, and 195–200, at concentrations as low as 0.1 mg/mL, efficiently inhibited ^{125}I-αBTX binding to native AChR. On the other hand, substitution of any of residues V_{188}, Y_{189}, Y_{190}, C_{192}, C_{193}, or P_{194} drastically affected the ability of the peptide to compete with AChR for αBTX binding. The latter peptides did not inhibit ^{125}I-αBTX binding to native AChR, or did so to a much lesser extent than peptide Tα181–200$_{unmod}$. All the experimental conditions are described in detail under Materials and Methods. Tha data are the average of triplicate samples. The standard deviations are indicated. The unspecific binding ^{125}I-αBTX binding to native *Torpedo* AChR, determined in the presence of an excess of unlabeled αBTX, was consistently <3% of the total binding and was subtracted. In this particular experiment, the total amount of ^{125}I-αBTX bound by the sample, in the absence of any peptide, was 2.25 pmol; the unspecific binding was 0.067 pmol.

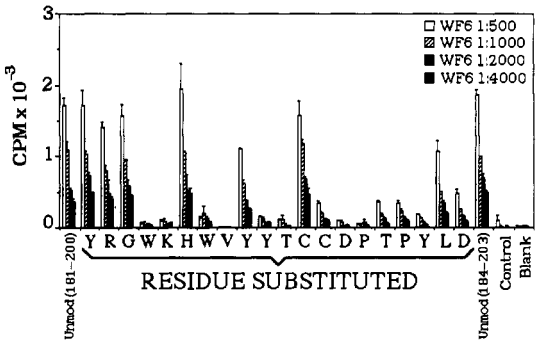

FIGURE 6: Dose dependency of the binding of antibody WF6 to the panel of synthetic peptides, as determined by dot blot assay. Four different dilutions of the WF6 stock solution (1 mg/mL) were employed (1:500, 1:1000, 1:200, and 1:4000). Unspecific binding of ^{125}I-labeled protein A was determined for each point in the absence of the mAb and was subtracted. "Blank" refers to protein A binding (to WF6) in the absence of immobilized peptide; "control" refers to protein A binding (to WF6) in the presence of an unrelated peptide.

insignificant, i.e., within the experimental range of error. Amino acid substitutions which caused affinity changes of up to a factor of 5 were considered "influential", those causing

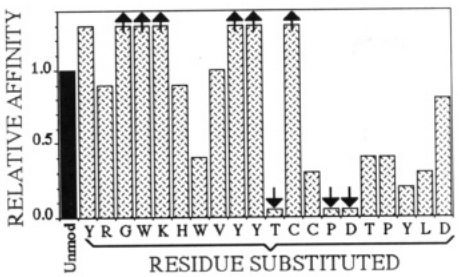

FIGURE 7: Semiquantitative ELISA of the binding of antibody WF6 to the panel of synthetic peptides. ELISAs (triplicates) were performed for several different concentrations of antibody, and titration curves and Scatchard plots were constructed. The corresponding "K_D" values are listed in Table II. The figure depicts "relative affinities", i.e., reciprocal K_D values divided by that for the parent peptide Tα181–200$_{unmod}$. Arrows denote off-range values.

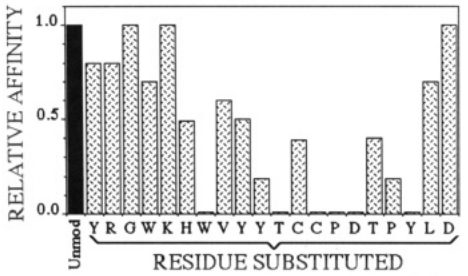

FIGURE 8: Inhibition by synthetic peptides of the binding of WF6 to immobilized AChR, as determined by competition ELISA. Antibody WF6, preincubated in the absence and presence of various concentrations of synthetic peptides, was applied onto microtiter wells coated with a fixed concentration of *Torpedo* AChR. The relative concentration of AChR-bound antibody was assayed by ELISA as described under Materials and Methods. Titration curves were constructed, and the concentration of the respective peptide inhibiting 50% of the binding of WF6 to AChR was deduced ("K_I" value). The corresponding data are listed in Table II. The figure depicts "relative affinities" of WF6 binding.

even larger changes in affinity (including "no measurable binding") were considered "essential". Following these criteria, we identified residues T_{191}, P_{194}, D_{195}, and Y_{198} as essential, and W_{187}, C_{193}, T_{196}, P_{197}, and L_{199} as influential for the interaction with antibody WF6. However, as discussed before for αBTX binding, the peptides vary considerably in their ability to adhere to the plastic surface (and possibly also in their conformation and acessibility when attached) and may compromise the conclusions drawn from direct binding studies.

To avoid such ambiguity, we investigated the ability of the peptides to bind mAb WF6 by the competition binding assay. After the test peptide and antibody WF6 were incubated in solution, the mixture was distributed into the wells of a microtiter plate which had been coated with a fixed concentration of *Torpedo* AChR. The relative concentration of WF6 available for AChR binding was then assayed by ELISA (for details, see Materials and Methods). From the resulting plots, the concentration of peptide which reduced by 50% the binding of WF6 to immobilized AChR was deduced. This apparent "K_i" value is related to the binding affinity of peptide and antibody in solution (Table II).

Figure 8 reports the related data in terms of relative affinities of binding. Consistent with the direct binding data, T_{191}, P_{194}, D_{195}, and Y_{198} were identified as "essential" amino acids. In addition, substitution of residues W_{187} and C_{193} strongly affected the binding affinity for WF6, while substitution of residues Y_{190}, T_{196}, and P_{197} had much smaller effects. The differences between the results of the dot blot assay and the competition ELISA assay may be due both to the higher sensitivity of the former assay and to its potential pitfalls, as

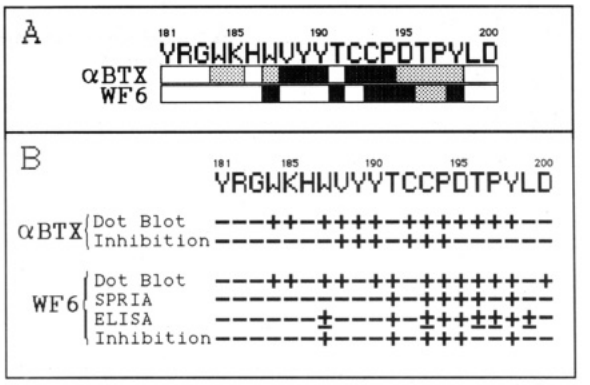

FIGURE 9: Schematic representation of the amino acid residues within peptide Tα181–200$_{unmod}$ which were found to be "essential" or "influential" for the binding of αBTX and WF6. (Panel A) Cumulative summary of the effect of single-residue substitutions on αBTX and WF6 binding. Black squares refer to "essential residues" and dotted squares refer to "indfuential" residues. (Panel B) Summary of the results obtained with the different experimental approaches used in this study to assess the effect of single-residue substitutions on αBTX and WF6 binding. Minuses indicate that substitution of that residue did not affect the ability of the peptide to bind the ligand; pluses indicate a profound effect; ± indicates a lesser but significant effect.

discussed above. Figure 9 depicts schematic representation of the effect of single-residue substitution on WF6 binding, measured with the different techniques reported.

In summary, binding of the antibody WF6 to the main cholinergic subsite appears to involve at least six amino acid residues discontinuously distributed within the sequence α181–200. These residues only partially overlap those crucial for αBTX binding.

Discussion

We previously showed that the cholinergic binding site on the α subunit of *Torpedo* AChR is not a single narrow sequence region but rather it comprises a rather large area formed by at least two sequence segments folded together in the native structure of the AChR (Conti-Tronconi et al., 1990). As a conclusion of that study, we suggested that the interaction between AChR and αBTX is not mediated by a single amino acid residue or pair of them, as may occur for the active site of enzymes, but that it involves instead a complex interaction of several residues on both the AChR and the αBTX molecules, thus explaining the very high affinity with which this interaction occurs [K_D of the order of 10^{-12} M; reviewed in Lee (1979)] . In the present paper, we demonstrated that this is indeed the case, because, even within the "subsite" formed by the sequence segment α181–200, the αBTX/AChR interaction is mediated by at least six amino acid residues discontinuously distributed along the sequence region which forms this subsite. These results are in excellent agreement with earlier suggestions, based on the structure of α-neurotoxins (Walkinshaw et al., 1980; Kistler & Stroud, 1982; Basus et al., 1988) and the properties of their binding to AChR (Martin et al., 1983), that these ligands recognize a large area on the extracellular AChR surface. Furthermore, the residues essential for ligand binding are different for the two proteinic ligands investigated (Figure 9). Different spectra of ligand-specific attachment points may therefore provide the molecular basis for the well-established specificity in ligand recognition and primary response of nicotinic receptors [reviewed in Maelicke (1984)].

The experimental approach used for this study, which utilizes synthetic peptides as structural models for selected

regions of receptors (Conti-Tronconi et al., 1989), deserves some comments. Its successful application requires a sufficiently high affinity of the model peptides for the ligands. Albeit several orders of magnitude lower than the affinity of native AChR for αBTX [reviewed in Lee (1979)] and WF6 (Fels et al., 1986), peptide α181–200 still binds these ligands with affinities comparable to ACh binding to native AChR (Conti-Tronconi, 1990; Figure 4). This is probably due to the relatively high content of secondary structure of α181–200 in solution (Figure 2). The binding affinity of peptide Tα181–200 for αBTX is within the range found by other laboratories for αBTX binding to denatured α subunit or proteolytic/synthetic peptides from the α subunit (Haggerty & Frohener, 1981; Wilson et al., 1984; Neumann et al., 1986a,b; Gershoni, 1987). CD spectral analysis indicates that the content of secondary structure is not severely compromised by the single-residue substitutions (Figure 2), so that our studies may indeed identify the individual contributions to ligand binding of each amino acid within the sequence α181–200. This may be an unusually fortunate situation, and it may not apply to other sequence regions of the AChR or other receptors.

The sequence α181–200 may form a part of the AChR molecule endowed with unusually high structural stability and resistance to denaturation also in the native form of the AChR. This is suggested by (i) the ability of α-neurotoxins and WF6 to bind to the denatured α subunit (Haggerty & Frohener, 1981; Fels et al., 1986; Conti-Tronconi et al., 1990; Neumann et al., 1986a,b); (ii) the finding that newly synthesized α subunit can bind αBTX at a much earlier stage of structural maturation than small cholinergic ligands (Merlie, 1984; Carlin et al., 1986), and (iii) partially digested AChR preparations retain the ability to bind cholinergic ligands (Lindstrom et al., 1980; Fels et al., 1982; Conti-Tronconi et al., 1982b) while the ion channel contained within the AChR molecule not always can be activated under these conditions (Boheim et al., 1981). The structural stability of the sequence region α181–200 may be responsible for the residual binding activity of denatured or partially digested AChR preparations, while channel activation requires concerted binding to all subsites of the cholinergic binding region, including less stable ones, and is therefore lost upon denaturation/digestion of the AChR molecule.

The observed interaction of antibody WF6 with several residues even within this particular cholinergic subsite is in excellent agreement with the described interaction between antibodies and their protein antigen, as deduced by X-ray diffraction studies of antigen/antibody complexes. Antibodies bind to large areas (690–750 Å), and 14–16 residues on the protein antigen are directly involved in antibody binding [reviewed in Davies et al. (1988)]. Although in all cases studied so far antibody epitopes are formed by juxtaposition of several discrete segments of the antigen sequence, brought in contact as a result of the teriary folding of the protein, long segments may occur, as in the case of a lysozyme epitope which is formed by 16 residues arranged in 2 9-residue loops. A similar situation seems to occur for the AChR area to which αBTX and antibody WF6 bind, where several residues are in contact with WF6 or αBTX, six of which (Val_{188}, Tyr_{189}, Tyr_{190}, Cys_{192}, Cys_{193}, and Pro_{194} for αBTX, Trp_{187}, Thr_{191}, Cys_{193}, Pro_{194}, Asp_{195}, and Tyr_{198} for WF6) are within the segment α181–200 and have been identified here. Other not yet identified residues should reside within the segment α55–74, identified by our previous study (Conti-Tronconi et al., 1990) as contributing to the binding site of αBTX and WF6.

Table III: Alignment of Sequence Segments Homologous to Tα181–200 from Different AChRs, and Ability of the Corresponding AChRs To Bind αBTX

species and tissue	sequence[a,b]	αBTX binding
Torpedo electroplax	Y R G W K H W V Y Y T C C P D T P Y L D	+
Xenopus laevis muscle	Y R C W K H W V Y Y T C C P D K P Y L D	+
Chicken muscle	Y R G W K H W V Y Y A C C P D T P Y L D	+
Bovine muscle	S R G W K H W V F Y A C C P S T P Y L D	+
Human muscle	S R G W K H S V T Y S C C P D T P Y L D	+
Mouse muscle	A R G W K H W V F Y S C C P T T P Y L D	•
Cobra muscle	Y R G F W H S V N Y S C C L D T P Y L D	-
Rat neurons: α2	A T G T Y N S K K Y D C C A E - I Y P D	-
Rat neurons: α3	A P G Y K H E I K Y N C C E E - I Y Q D	-
Rat neurons: α4	A V G T Y N T R K Y E C C A E - I Y P D	-

[a] The sequences are from Noda et al. (1982, 1983), Boulter et al. (1985, 1986), Isenberg et al. (1986), Goldman et al. (1987), Wada et al. (1988), and Neumann et al. (1989). [b] Conserved and conservatively substituted residues (as compared to the *Torpedo* sequence) are shown in boxes.

αBTX binds to all known AChRs from vertebrate muscle but not to cobra muscle AChR, nor to several rat neuronal AChRs, whose constituent α subunits have been sequenced (rat α_2, α_3, and α_4 subunits). WF6 cross-reacts with the AChRs of the two peripheral tissues tested [*Electrophorus electroplax* and rat muscle, see Watters and Maelicke (1983)]. Comparison of the sequence segments α181–200 from the different AChRs, in light of the results reported here, should explain the spectrum of binding of αBTX and WF6 at the level of the single amino acid residues. Table III shows an alignment of this sequence segment from *Torpedo* AChR, from different vertebrate muscle AChRs, including cobra, and from the three rat neuronal α subunits known not to bind αBTX. Conserved residues and conservative substitutions are shown in boxes. It clearly appears that the six residues crucial for αBTX binding (indicated in Table III with an x) are all conserved or conservatively substituted in all vertebrate muscle AChRs, with the only exception being cobra muscle AChR, where both Tyr_{189} and Pro_{194} are nonconservatively changed (Tyr → Asn, Pro → Leu). In the neuronal subunits which do not bind αBTX, three of these crucial residues (Val_{188}, Tyr_{189}, and Pro_{194}) are consistently substituted nonconservatively. In the human muscle α subunit, Tyr_{189} is changed to threonine. This change, although conservative, may explain the described lesser ability to bind αBTX of synthetic and biosynthetic peptides corresponding to the human sequence (Lentz et al., 1987; Wilson & Lentz, 1988; Ohana & Gershoni, 1990; Griesmann et al., 1990). The good affinity of αBTX for native human muscle AChR is well explained by the multipoint attachment reported here, so that substitution of one or a few of the many residues involved in αBTX may be functionally silent. Of the six residues crucial for WF6 binding (indicated in Table III with an downward arrow), four are conserved in all muscle AChRs but not in cobra AChR where both Trp_{187} and Pro_{194} are nonconservatively changed. The other two residues (Thr_{191} and Asp_{195}) are also nonconservatively changed in some muscle AChR, and one may predict that WF6 may not be a "universal" probe for all peripheral nicotinic receptors.

The observed multipoint attachment of antibody WF6 to the main cholinergic subsite explains well why anti-AChR antibodies are rather species specific and subunit specific, in spite of the extensive sequence homology existing among AChRs from different species and the subunits of individual AChRs [reviewed in McCarthy et al. (1986), Lindstrom et

al. (1987), and Maelicke (1988)]. As shown here, the exchange of a single residue in the epitope sequence may already reduce the binding affinity by a factor of 20–100 (Table II, Figures 7 and 8). Similarly, variations in distance between essential residues may strongly influence antibody binding. Thus, antibodies can discriminate between homologous sequences much more efficiently than may be expected on the basis of linear sequence differences. A similar explanation may apply to the specificity of different α-neurotoxins for different AChR subtypes (Loring et al., 1984; Grant & Chiapinelli, 1985; McLane et al., 1990).

The observed multipoint attachment of αBTX to the main cholinergic subsite explains well the inability of previous studies to identify within the α-neurotoxin molecule single amino acid residues [or pairs of them as essential for neurotoxicity (reviewed in Low (1980)]. Studies on the binding properties of chemically modified α-cobratoxin have shown that none of its positively charged residues, nor tyrosine or tryptophan, nor the integrity of its disulfide bridges is essential for binding to the AChR (Martin et al., 1983). Because the chemical modification of single amino acid residues or groups of residues induced only graded changes in binding affinity, it was concluded that several structural elements of the toxin must be involved in binding. Those studies, and the detailed structural information obtained from X-ray and NMR studies (Walkinshaw et al., 1980a,b; Kistler & Stroud, 1982; Basus et al., 1988), suggested that a large area of the α-neurotoxin molecules formed by three sequence segments, i.e., both sides of the central loop and the lower tip of loop III, is involved in AChR binding. A corresponding large area on the AChR surface, formed by several residues within the sequence segments α181–200 and α55–74 (Conti-Tronconi et al., 1990), should make contact with the α-neurotoxins. The high affinity [$K_D = 10^{-12}$–10^{-10} M; reviewed in Lee (1979)] and very slow reversibility [$K_{off} \sim 2 \times 10^{-4}$ to 5×10^{-5} and 4×10^{-3} to 10^{-4} min^{-1} for αBTX and αCTX, respectively; e.g., see Blanchard et al. (1979) and Kang and Maelicke (1980)] of the binding of these toxins are consistent with interaction with the AChR via large surfaces, with formation of a large number of attachment points. In view of the results presented here, a testable model of the interaction between the sequence regions (and their essential residues) of neurotoxins and AChR within their complexes may soon be feasible.

A result of the present study which deserve further comment is the increase in β-pleated sheet structure determined for complexes of αBTX with the main cholinergic subsite. As shown by X-ray crystallographic analysis and NMR studies (Walkinshaw et al., 1980a,b; Kistler & Stroud, 1982; Basus et al., 1988), the "long" α-neurotoxins have a similar structure, consisting of a globular head, the folding of which is determined by four disulfide bridges, and three loops hanging down from the head arranged side-by-side in a β-sheet configuration. These loops are rather flexible, and their regular β configuration can be disrupted as in crystals of αBTX, where the regular β-sheet configuration of the region proposed to interact with the AChR is disrupted by an irregular orientation of residue Lys_{27} (Love & Stroud, 1986). These results suggested that α-neurotoxins may change their conformation upon binding to the AChR (Kistler & Stroud, 1987). Indeed spectral changes of fluorescent residues upon binding to AChR strongly support the possibility of conformational adjustments of the α-neurotoxins induced by the binding (Kang & Maelicke, 1980; Johnson et al., 1984; Cheung et al., 1984). The results reported here further support the formation of extended β structures between the sequence loops forming the binding area on the α-neurotoxin and the sequence regions of the AChR forming the cholinergic site, involving some of the amino acids identified here as attachment points between those molecules. Structural rearrangement of protein ligands upon binding has been described for the interaction between a monoclonal antibody and lysozyme (Amit et al., 1986).

Registry No. $T\alpha181\text{–}200_{unmod}$, 117861-08-2; $T\alpha181\text{–}200_{G181}$, 130905-08-7; $T\alpha181\text{–}200_{G182}$, 130905-09-8; $T\alpha181\text{–}200_{A183}$, 130905-10-1; $T\alpha181\text{–}200_{G184}$, 130905-11-2; $T\alpha181\text{–}200_{G185}$, 130905-12-3; $T\alpha181\text{–}200_{G186}$, 130905-13-4; $T\alpha181\text{–}200_{G187}$, 130905-14-5; $T\alpha181\text{–}200_{G188}$, 130932-90-0; $T\alpha181\text{–}200_{G189}$, 130905-15-6; $T\alpha181\text{–}200_{G190}$, 130905-16-7; $T\alpha181\text{–}200_{G191}$, 130905-17-8; $T\alpha181\text{–}200_{G192}$, 130905-18-9; $T\alpha181\text{–}200_{G193}$, 130905-19-0; $T\alpha181\text{–}200_{G194}$, 130905-20-3; $T\alpha181\text{–}200_{G195}$, 130905-21-4; $T\alpha181\text{–}200_{G196}$, 130905-22-5; $T\alpha181\text{–}200_{G197}$, 130905-23-6; $T\alpha181\text{–}200_{G198}$, 130905-24-7; $T\alpha181\text{–}200_{G199}$, 130905-25-8; $T\alpha181\text{–}200_{G200}$, 130905-26-9; α-bungarotoxin, 11032-79-4.

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