Structural Determinants of α-Bungarotoxin Binding to the Sequence Segment 181–200 of the Muscle Nicotinic Acetylcholine Receptor α Subunit: Effects of Cysteine/Cystine Modification and Species-Specific Amino Acid Substitutions†

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ABSTRACT: The sequence segment 181–200 of the *Torpedo* nicotinic acetylcholine receptor (nAChR) α subunit forms a binding site for α-bungarotoxin (α-BTX) [e.g., see Conti-Tronconi, B. M., Tang, F., Diethelm, B. M., Spencer, S. R., Reinhardt-Maelicke, S., & Maelicke, A. (1990) *Biochemistry 29*, 6221–6230]. Synthetic peptides corresponding to the homologous sequences of human, calf, mouse, chicken, frog, and cobra muscle nAChR α1 subunits were tested for their ability to bind ^{125}I-α-BTX, and differences in α-BTX affinity were determined by using solution (IC_{50}s) and solid-phase (K_ds) assays. Panels of overlapping peptides corresponding to the complete α1 subunit of mouse and human were also tested for α-BTX binding, but other sequence segments forming the α-BTX site were not consistently detectable. The *Torpedo* α1(181–200) and the homologous frog and chicken peptides bound α-BTX with higher affinity (K_ds ~ 1–2 μM, IC_{50}s ~ 1–2 μM) than the human and calf peptides (K_ds ~ 3–5 μM, IC_{50}s ~ 15 μM). The mouse peptide bound α-BTX weakly when attached to a solid support (K_d ~ 8 μM) but was effective in competing for ^{125}I-α-BTX in solution (IC_{50} ~ 1 μM). The cobra nAChR α1-subunit peptide did not detectably bind α-BTX in either assay. Amino acid substitutions were correlated with α-BTX binding activity of peptides from different species. The role of a putative vicinal disulfide bound between Cys-192 and -193, relative to the *Torpedo* sequence, was determined by modifying the peptides with sulfhydryl reagents. Reduction and alkylation of the peptides decreased α-BTX binding, whereas oxidation of the peptides had little effect. Modifications of the cysteine/cystine residues of the cobra peptide failed to induce α-BTX binding activity. These results indicate that while the adjacent cysteines are likely to be involved in forming the toxin/α1-subunit interface a vicinal disulfide bound was not required for α-BTX binding.

α-Bungarotoxin (α-BTX),[1] from the venom of the Formosan banded krait, *Bungarus multicinctus*, has been exploited as a high-affinity ligand for the isolation of nicotinic acetylcholine receptors (nAChRs). The muscle-type nAChR, of which the *Torpedo* nAChR is the prototype, is composed of four homologous subunits (α1, β1, γ, δ) (Raftery et al., 1980; Conti-Tronconi et al., 1982). The N-terminal sequences of these subunits provided the basis for isolation of *Torpedo* nAChR subunit cDNAs (Noda et al., 1982, 1983a,b) and subsequently the cDNAs for muscle nAChR α1-subunits from various species, e.g., human (Noda et al., 1983c), calf (Noda et al., 1983c), mouse (Merlie et al., 1983; Boulter et al., 1985), chicken (Barnard et al., 1986; Nef et al., 1988), frog (Baldwin et al., 1988), and cobra (Neumann et al., 1989). The α1-subunit is implicated in forming ligand binding sites for agonists and antagonists, based on affinity labeling with acetylcholine analogues [e.g., see Karlin and Cowburn (1973), Moore and Raftery (1979), and Wolosin et al. (1980)] and the nicotinic antagonist *d*-tubocurare (Pederson et al., 1986; Pederson & Cohen, 1990). α-BTX competes for binding to the nAChR α1-subunit with other nicotinic ligands (Blanchard et al., 1979; Haggerty & Froehner, 1981; Gershoni et al., 1983), but studies using monoclonal antibodies that recognize different epitopes within the cholinergic binding site indicate that the sequence segments of the α1-subunit recognized by different ligands may only partially overlap (Watters & Maelicke, 1983; Mihovilovic & Richman, 1987; Chinchetru et al., 1989).

The sequence segments of the *Torpedo* nAChR α1-subunit that contribute to forming the cholinergic binding site have been mapped by (i) identification or sequencing of the peptides labeled with cholinergic affinity reagents in the intact nAChR (Kao et al., 1984; Kao & Karlin, 1986; Dennis et al., 1988; Moschovitz & Gershoni, 1988) and (ii) the binding of α-BTX to proteolytic fragments (Wilson et al., 1984, 1985; Pederson et al., 1986; Neumann et al., 1986a), synthetic peptides (Neumann et al., 1986b; Ralston et al., 1987; Wilson et al., 1988; Wilson & Lentz, 1988; Conti-Tronconi et al., 1988, 1989, 1990, 1991), and biosynthetic peptides (Barkas et al., 1987; Aronheim et al., 1988). Convergence of these findings indicates that a region between residues 173 and 204 of the *Torpedo* α-subunit forms a cholinergic binding site. Within this region, a pair of adjacent cysteine residues at positions 192 and 193, relative to the *Torpedo* α1 sequence, is common to all nAChR α-subunits and, on the basis of their labeling with cholinergic affinity ligands, is believed to lie in close proximity (~1 nm) to the acetylcholine binding site (Kao et

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[1] Abbreviations: α-BTX, α-bungarotoxin; nAChR, nicotinic acetylcholine receptor; IAA, iodoacetamide; DTT, dithiothreitol; IOBA, 2-iodosobenzoic acid; HPLC, high-pressure liquid chromatography; CM-Cys, *S*-(carboxymethyl)cysteine; KP, 10 mM potassium phosphate buffer, pH 7.4; PBS, phosphate-buffered saline.

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al., 1984). In the intact *Torpedo* nAChR or in peptide fragments generated with CNBr or proteolysis, alkylation of either of the cysteines requires their prior reduction, and on the basis of these results, the formation of a vicinal disulfide bond between Cys-192/193 was proposed (Kao & Karlin, 1986; Mosckovitz & Gershoni, 1988). To form a vicinal cystine, the peptide bond between adjacent cysteines must be nonplanar and in the cis conformation, and given the inherent degree of strain, it was thought to be an unlikely structure (Schultz & Schirmer, 1979; Thorton, 1981; Ovchinnikov et al., 1985, 1988; Al-Saleh et al., 1987). Recently, however, cystine formed by Cys-192 and -193 of the *Torpedo* nAChR α1-subunit has been directly observed (Kellaris et al., 1989). In addition to the region containing the adjacent Cys-192 and -193, other segments of the *Torpedo* nAChR α1-subunit, which may contribute to forming the cholinergic binding site, have been identified. These regions include sequences including Tyr-93 and Tyr-193, which were identified by affinity labeling studies (Dennis et al., 1988; Galzi et al., 1990), and the sequence segment *Torpedo* α1(55–74), identified by the binding of α-BTX and monoclonal antibodies that compete with nicotinic ligands (Conti-Tronconi et al., 1989, 1990). Thus, a model of the cholinergic binding site has emerged involving several peptide loops contained in the N-terminal portion of the α1-subunit. The isolated sequence segment containing residues α1(181–200), however, can bind α-BTX in the absence of the surrounding structural elements of the α-subunit (Neumann et al., 1986b; Ralston et al., 1987; Barkas et al., 1987; Wilson et al., 1988; Wilson & Lentz, 1988; Aronheim et al., 1988; Conti-Tronconi et al., 1989, 1990, 1991), forming what Wilson et al. (1988) have termed an α-BTX binding "prototope".

Our laboratory has studied the structural requirements for α-BTX binding to the sequence segment *Torpedo* α1(181–200) using synthetic peptides corresponding to single amino acid substitution analogues (Conti-Tronconi et al., 1991). In the present study, we take advantage of naturally occurring amino acid substitutions, and compare the ability of the homologous sequence segments from the human, frog, calf, chick, cobra, and mouse muscle nAChR α1-subunits to bind α-BTX using two types of assays: (i) a solid-phase toxin blot assay, where peptides are blotted onto nitrocellulose and the relative ability of ^{125}I-α-BTX to bind to peptides is compared by varying the concentration of toxin (Conti-Tronconi et al., 1989, 1990, 1991); and (ii) a competition assay, where peptides at different concentrations are tested for their ability to sequester ^{125}I-α-BTX in solution, reducing the amount of α-BTX available for binding to native *Torpedo* nAChR (McLane et al., 1990; Conti-Tronconi et al., 1991). We demonstrate that the sequence segment 181–200 of muscle nAChR α1-subunits of different species forms a universal α-BTX binding site. Our results complement those of Ohana and Gershoni (1990), who have used fusion proteins containing the sequence segment 183–204 of different muscle nAChR α1-subunits. In addition, we are able to determine and chemically alter the disposition of the adjacent Cys residues at positions 192/193 of our synthetic peptides and, thus, assess the effects of cystine/cysteine modification on α-BTX binding. By scanning the entire nAChR α1-subunit sequences of mouse and human using overlapping synthetic peptides, we were also able to determine if additional sequence segments contribute to forming the α-BTX binding site.

Materials and Methods

Peptide Synthesis and Characterization. Peptides were synthesized by manual parallel synthesis (Houghten, 1985).

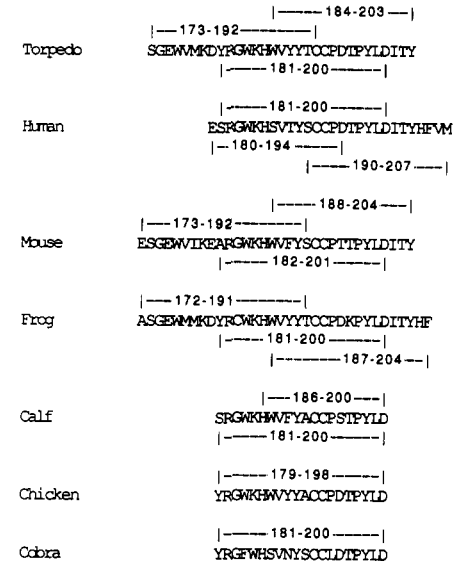

FIGURE 1: Sequences of overlapping synthetic peptides corresponding to an α-BTX binding domain between positions 172 and 207 on α1-subunits. Overlapping peptides corresponding to the *Torpedo* nAChR α1-subunit sequence between residues 173 and 204, and homologous sequences for the muscle nAChR α1-subunit of different species, were synthesized and characterized as described under Materials and Methods. The corresponding sequence segments for the *Torpedo* electric organ nAChR α1-subunit (Noda et al., 1982) and the muscle nAChR α1-subunits from calf and human (Noda et al., 1983c), mouse (Boulter et al., 1985), chicken (Nef et al., 1988), frog (Baldwin et al., 1988), and cobra (Neumann et al., 1989) are given. The sequences of the peptides are indicated, and the numbers correspond to the positions of the amino acid residues relative to each α1-subunit sequence. In the text, the peptides are referred to by species, subunit, and amino acid residues, for example, "*Torpedo* α1(181–200)".

The purity was assessed by reverse-phase HPLC (high-pressure liquid chromatography) using a C18 column (Ultrasphere ODS) and an acetonitrile/water gradient (5–70%) containing 0.1% trifluoroacetic acid, and the major peak consistently accounted for 65–85% of the total absorbance at 214 nm. The amino acid composition of all peptides was determined by derivatization of amino acid residues released by acid hydrolysis with phenyl isothiocyanate, followed by separation on a reverse-phase HPLC column (PICO.TAG) as described by Heinrickson and Meredith (1984). These results gave satisfactory correspondence between experimental and theoretical values. The sequence and purity of all peptides corresponding to the homologous sequences α1(181–200) of muscle nAChRs of different species, and randomly selected peptides corresponding to other sequences, were verified by gas-phase sequencing (Applied Biosystems), indicating that contamination by truncated peptides was less than 5–15%. The sequence and codes of some of the peptides are reported in Figure 1. Additional synthetic peptides used to screen the complete sequences of the human and mouse α1-subunits corresponded to the following sequence segments. For the human α1-subunit, these peptides corresponded to residues 1–14, 7–22, 19–34, 32–51, 48–67, 63–80, 76–93, 98–105, 101–120, 118–137, 135–154, 151–168, 166–185, 203–218, 214–234, 230–249, 246–264, 261–280, 280–297, 293–308, 304–322, 320–337, 339–347, 343–356, 352–368, 364–380, 376–393, 387–406, 403–421, and 419–437. For the mouse α1-subunit, overlapping peptides corresponded to residues 1–14, 10–27, 23–41, 32–51, 48–67, 51–70, 63–80, 76–93, 82–97, 98–105, 103–122, 118–137, 135–154, 142–162, 154–172, 168–187, 191–209, 203–218, 216–235, 230–249, 246–264, 261–280, 277–296, 293–308, 304–323, 322–341, 331–350, 343–362,

352–368, 364–380, 373–392, 389–408, 403–421, 414–433, and 423–437.

Modification of Cysteine/Cystine Residues. Synthetic peptides (0.5 mg/mL) in 100 mM potassium phosphate buffer, pH 8.5, were treated with either 2-iodosobenzoic acid (IOBA) (0.1 mM) or dithiothreitol (DTT) (1.5 mM) for 5 h at room temperature. Samples of untreated, oxidized, and reduced peptides were alkylated with iodoacetamide (IAA) (6 mM) overnight at 4 °C. The reactants and peptides were separated on a Sephadex G10 column (0.9 × 4 cm) as reagents for binding assays. Samples were chromatographed by using a Bio-Rad P6 column (1.8 × 47 cm) for determination of the ratio of monomer to dimer. Both columns were equilibrated and eluted with 10 mM potassium phosphate buffer, pH 7.4. The P6 column was calibrated with bradykinin (1060 daltons), porcine renin substrate tetradecapeptide (1759 daltons), human β-endorphin (3465 daltons), human growth hormone releasing factor (5041 daltons), and blue dextran 2000. Carboxymethylation of free sulfhydryl groups was assessed by amino acid composition analysis, as described above. The (carboxymethyl)cysteine (CM-Cys) derivative standard elutes after the Asp and Glu derivatives, and prior to the Ser derivative, using 140 mM sodium acetate buffer (pH 6.4) containing 0.05% triethylamine and 6% acetonitrile.

Preparation and Calibration of Radiolabeled α-BTX. α-BTX was isolated from *Bungarus multicinctus* venom (Biotoxins Inc.) by the method of Clark et al. (1972). For some experiments, commercial preparations of α-BTX (Biotoxins Inc., lots ABT 88A and 88B) were used. The purity of α-BTX, as assessed by gas-phase sequencing, indicated that contaminating sequences, if present, were below the level of detectability (<3–5%). α-BTX was radiolebeled with carrier-free ^{125}I (Lindstrom et al., 1981) and calibrated as described by Blanchard et al. (1979) using membrane-bound nAChR prepared from *Torpedo californica* electric organ. The specific activity of the ^{125}I-α-BTX used in experiments was 4–120 Ci/mmol, and low specific activity preparations were isotopic dilutions.

Toxin Blots and Scatchard Analysis. Nitrocellulose strips were spotted with 1 μL of each peptide solution [0.25 mg/mL in 10 mM potassium phosphate buffer, pH 7.4 (KP buffer)] and dried at room temperature. The strips were blocked with 10 mg/mL cytochrome *c* in KP buffer for 2 h at room temperature. Cytochrome *c* (5 mg/mL), which has charge properties similar to α-BTX, was added to solutions of ^{125}I-α-BTX to reduce the nonspecific binding. The blocked nitrocellulose strips were incubated with 0.1–10 μM ^{125}I-α-BTX in PBS (10 mM sodium phosphate/100 mM NaCl, pH 7.2) for 1–4 h at room temperature, washed 8 times with 3 mL of PBS containing 0.1% Tween-20 (2.5 min/wash), mounted for autoradiography using Kodak X-Omat film, and exposed at −70 °C. The concentration dependence of ^{125}I-α-BTX binding was determined by counting triplicate samples (5-mm squares) in a γ counter. At each concentration of ^{125}I-α-BTX, nonspecific binding was determined by preincubation of replicate strips with 100 μM unlabeled α-BTX for 2 h at room temperature prior to addition of ^{125}I-α-BTX, and was found to vary approximately linearly with the concentration of ^{125}I-α-BTX (5–25% of the total ^{125}I-α-BTX bound). The total amount of ^{125}I-α-BTX bound to peptides did not exceed 3% of the radioactivity added. The relative affinities of peptides bound to nitrocellulose were compared by calculating an apparent K_d using the programs EBDA and LIGAND (Munson & Rodbard, 1980; McPherson, 1983).

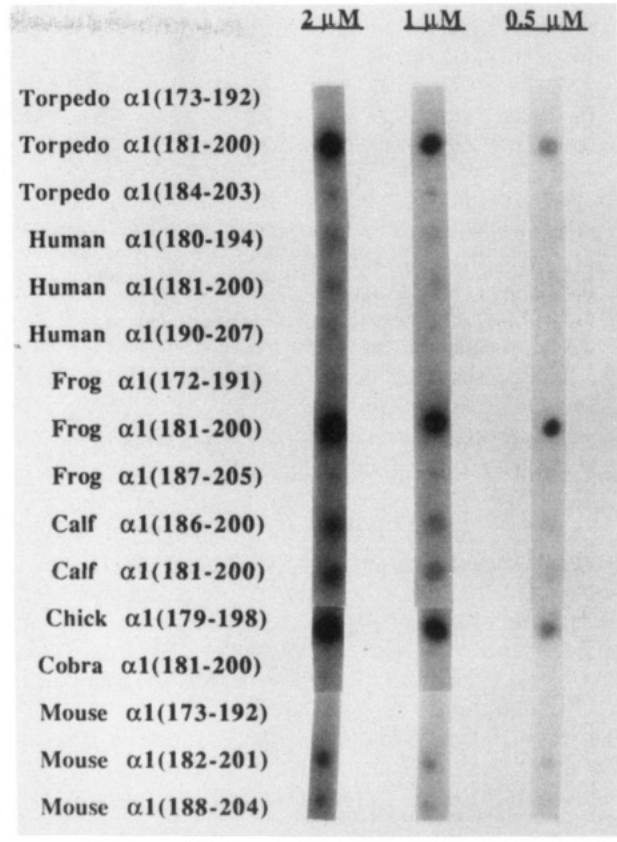

FIGURE 2: Toxin blot assay of overlapping synthetic peptides between amino acid residues 172 and 207 of *Torpedo* electric organ and vertebrate muscle nAChR α1-subunits. Peptides solutions (250 ng) were spotted onto nitrocellulose strips and allowed to dry at room temperature. The strips were blocked by incubation with 10 mg/mL cytochrome *c* for 2 h, followed by incubation with ^{125}I-α-BTX (0.5, 1, or 2 μM; 4.4 Ci/mmol) in the presence of 5 mg/mL cytochrome *c* for 4 h at room temperature with agitation. The strips were washed as described under Materials and Methods. Autoradiographs were exposed for 16 h at −70 °C.

Competitive Inhibition of ^{125}I-α-BTX Binding by Peptides. Peptides (5–500 μg/mL) were preincubated with ^{125}I-α-BTX (0.5–2 pmol) in KP buffer containing 10 mg/mL cytochrome *c* overnight at 4 °C. To 100 μL of the peptide/toxin solution was added 0.2–1 pmol of membrane-bound *Torpedo* nAChR (Neubig et al., 1979; Elliot et al., 1980). After incubation for 3 min at room temperature, the assay tubes were centrifuged at 14000*g* for 45 min, washed with PBS, and recentrifuged. The pellet was counted in a γ counter. Nonspecific binding (<5%) was determined by preincubation of *Torpedo* nAChR with 20 nM unlabeled α-BTX for 10 min prior to addition to the radiolabeled toxin solutions. Binding of ^{125}I-α-BTX in the presence of peptides was compared with values obtained with toxin preincubated with only buffer. The IC_{50} values were determined by logit–log analysis using the program EBDA (McPherson, 1983; Rodbard & Frazier, 1975) and graphically represented by a Hofstee plot, where the negative slope is equal to the IC_{50} (Molinoff et al., 1981).

RESULTS

Comparison of α-BTX Binding Sequences of Different Species. (A) Toxin Blot Assays. A panel of peptides, 15–21 amino acids in length, corresponding to overlapping sequence segments homologous to the *Torpedo* nAChR α-BTX binding site, were compared for their ability to directly bind ^{125}I-α-BTX. In Figure 2, the results of a typical toxin blot assay (n = 6) are shown for three different concentrations of ^{125}I-α-BTX (2, 1, and 0.5 μM). The strongest signals were obtained

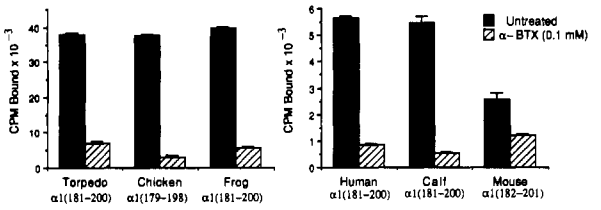

FIGURE 3: Binding of α-BTX to peptides: specificity of toxin blot assay. Toxin blot assays were performed essentially as in Figure 2, except nitrocellulose strips were incubated with or without unlabeled α-BTX (100 μM) during the blocking period, and incubations with ^{125}I-α-BTX (4 μM, 4.5 Ci/mmol) were performed for 1 h. Strips were washed as described under Materials and Methods and counted in a γ counter. The results are presented as the mean of triplicate determinations, and the error bars are standard deviations.

for peptides *Torpedo* α1(181–200), frog α1(181–200), and chick α1(179–198). Moderate to weak signals were obtained for peptides *Torpedo* α1(184–208), calf α1(181–200), calf α1(186–200), mouse α1(182–201), mouse α1(188–204), frog α1(182–205), and the human nAChR peptides α1(180–194) and α1(181–200). For the different species, the peptides that were found to bind ^{125}I-α-BTX most efficiently were those homologous to the sequence segment 181–200 of the *Torpedo* nAChR α1-subunit, which the exception of the cobra peptide α1(181–200), which did not bind ^{125}I-α-BTX detectably. For this reason, further studies were primarily focused on peptides *Torpedo* α1(181–200), chick α1(179–198), frog α1(181–200), human α1(181–200), calf α1(181–200), and mouse α1(182–201).

The specificity of ^{125}I-α-BTX binding to peptides was determined by preincubating toxin blots in the presence or absence of unlabeled α-BTX (100 μM). As shown in Figure 3, unlabeled α-BTX substantially reduced the binding of ^{125}I-α-BTX (0.6 μM) to peptides. The level of nonspecific binding for the homologous peptides *Torpedo* α1(181–200), chick α1(179–198), frog α1(181–200), human α1(181–200), calf α1(181–200), and mouse α1(182–201) was ~10–25%. Preincubation with other nicotinic ligands at concentrations up to 10 mM (e.g., *d*-tubocurare, carbamylcholine, and nicotine) did not significantly reduce ^{125}I-α-BTX binding (data not shown). Other investigators using peptides of similar size have reported difficulties in demonstrating competitive inhibition of α-BTX binding by cholinergic ligands, and IC_{50} values, when attainable, have been in the range of 10–100 mM [e.g., see Neumann et al. (1986b) and Griesmann et al. (1990)]. Using a 32-mer corresponding to *Torpedo* nAChR α1(173–204), Wilson and Lentz (1988) have, however, convincingly demonstrated inhibition of α-BTX binding by *d*-tubocurare (IC_{50} ~ 100 μM), and it is possible that our peptides are of insufficient length to include the residues necessary for the binding site of other ligands in addition to α-BTX.

The relative affinity of the homologous peptides was determined by varying the concentration of ^{125}I-α-BTX used in the toxin blot assays from 0.1 to 10 μM. The peptide blots were counted in triplicate at each concentration and corrected for nonspecific binding, and Scatchard analysis was performed by using the programs EBDA and LIGAND (Munson & Rodbard, 1980; McPherson et al., 1983). The results of three experiments are summarized in Table I. The apparent K_ds for α-BTX binding to peptides homologous to *Torpedo* α1(181–200) ranged between 0.8 and 8 μM and indicated a rank order of affinity for α-BTX (from highest to lowest) of frog ~ chick ~ *Torpedo* ≫ calf ~ human ~ mouse.

(B) Competition Assays. Peptides corresponding to the homologous sequence segments 181–200 of *Torpedo* α1-subunit were also tested for the ability to bind α-BTX using a competition assay, as described under Materials and Methods. The results of these experiments (n = 6) confirmed those obtained with the toxin blot assay. The peptide sequences corresponding to amino acid residues 181–200 of *Torpedo*, human, calf, and frog α1-subunits, and the homologous sequences mouse α1(182–201) and chick α1(179–198), were found to inhibit the binding of ^{125}I-α-BTX to native *Torpedo* nAChR by ~80–95%, compared to binding in the absence of peptide. The cobra α1(181–200) peptide, however, inhibited <10% of the binding of ^{125}I-α-BTX to native *Torpedo* nAChR. Other overlapping sequences between residues 173 and 204 were less effective in inhibiting the binding of ^{125}I-α-BTX to native *Torpedo* nAChR than the sequences homologous to *Torpedo* α1(181–200).

The relative affinities for ^{125}I-α-BTX of peptides in solution were determined by varying the concentration of peptide used in the competition assay from 5 to 250 μg/mL, and the results of these experiments (n = 3) are summarized in Table II. A typical experiment is represented in Figure 4 as a Hofstee plot, where the IC_{50} values for the peptides can be directly compared as the negative slopes of the linear regression lines. The IC_{50} values ranged from 0.7 to 20 μM, and defined two groups of peptides—those with high affinity for α-BTX (IC_{50}s 1–2 μM), such as frog α1(181–200), *Torpedo* α1(181–200), chick α1(179–198), and mouse α1(182–201), and peptides with low affinity for α-BTX (IC_{50} 10–20 μM), represented by human α1(181–200) and calf α1(181–200). The IC_{50}s indicate the same relative affinities as found for peptides in the solid-phase assays, with the notable exception of the peptide mouse α1(182–201), which binds α-BTX with low affinity when bound to nitrocellulose, but with high affinity in solution. The results indicate that solid-phase assays may constrain a peptide to a limited set of conformations, which is not representative of its conformational repertoire in solution. For this reason, we

Table I: Scatchard Analysis of Peptides[a]

peptide	apparent K_d (μM)
Torpedo α1(181–200)	2 (0.7)
human α1(181–200)	5 (2.5)
calf α1(181–200)	3 (1.7)
mouse α1(182–201)	8 (1.4)
chicken α1(179–198)	0.9 (0.7)
frog α1(181–200)	0.9 (0.4)

[a] Peptides (250 ng) were applied to nitrocellulose strips. After being blocked with 10 mg/mL cytochrome *c*, the strips were incubated with ^{125}I-α-BTX (0.1–10 μM). Scatchard analysis was performed by using the analysis programs EBDA and LIGAND (Munson & Rodbard, 1980; McPherson, 1983). The values for the K_ds, the apparent dissociation constants, are the means of three independent experiments, and the standard deviations are in parentheses.

Table II: IC_{50} Analysis: Competition of Peptides for ^{125}I-α-BTX Binding with Native *Torpedo* nAChR[a]

peptide	IC_{50} (μM)
Torpedo α1(181–200)	1.9 (0.2)
Human α1(181–200)	15 (2.3)
calf α1(181–200)	16 (1.2)
mouse α1(182–201)	1.4 (0.4)
chick α1(179–198)	2.1 (0.7)
frog α1(181–200)	1.0 (0.2)

[a] Peptides (5–250 μg/mL) were incubated with ^{125}I-α-BTX (0.5–2 pmol) overnight at 4 °C prior to addition of native membrane-bound *Torpedo* nAChR (0.2–1 pmol). The membrane pellets were centrifuged and washed, as described under Materials and Methods, and counted in a γ counter. The IC_{50} values are the means obtained from three independent experiments and were determined by using the computer program EBDA (McPherson, 1983), and the standard deviations are in parentheses.

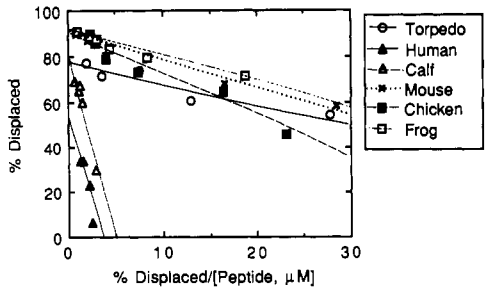

FIGURE 4: Competition of peptides for ^{125}I-α-BTX with native membrane-bound *Torpedo* nAChR: concentration dependence. Peptides (5–500 μg/mL) were incubated overnight at 4 °C with 1.2 pmol of ^{125}I-α-BTX (100–150 cpm/fmol) in a final volume of 100 μL of 10 mM potassium phosphate buffer, pH 7.4, containing 10 mg/mL cytochrome *c*. *Torpedo* membrane-bound nAChR was added, and after being mixed, the solution was centrifuged at 14000*g* for 45 min at 10 °C. The pellet was washed with PBS and counted in a γ counter. The assays contained 2 pmol of ^{125}I-α-BTX and 0.2–0.3 pmol of membrane-bound *Torpedo* nAChR. The results are expressed as the mean of triplicate determinations at each concentration of peptide. The graphical representation is a Hofstee plot (Molinoff et al., 1981), in which the IC_{50} values can be directly compared as the negative slopes of the linear regression lines. The peptides and IC_{50} values are as follows: *Torpedo* α1(181–200), 0.83; human α1(181–200), 17; calf α1(181–200), 16; mouse α1(182–201), 1.3; chicken α1(179–198), 1.7; frog α1(181–200), 1.3.

believe that the *Torpedo* nAChR competition assay is a more reliable assay than solid-phase assays.

Effects of Modification of Cysteine/Cystine Residues on α-BTX Binding. Common to all nAChR α-subunits is pair of cystine residues at positions 192/193 relative to the *Torpedo* nAChR α-subunit sequence. In order to assess the importance of the redox state of these cysteine residues for α-BTX binding, we treated peptides homologous to *Torpedo* α1(181–200) with sulfhydryl reagents. The peptides were treated in the following ways: (1) oxidation with 2-iodosobenzoic acid (IOBA) and alkylation of any remaining sulfhydryl groups with iodoacetamide; (2) reduction with dithiothreitol, followed by alkylation of free sulfhydryls with iodoacetamide (DTT/IAA); and (3) alkylation of the free sulfhydryl available in untreated peptide (IAA). The latter treatment was used to determine the ratio of reduced to oxidized forms present in solutions of untreated peptide. The products of the reactions were assessed by amino acid composition analysis, following acid hydrolysis and derivatization with phenyl isothiocyanate, as described under Materials and Methods. *S*-(Carboxamidomethyl)cysteine, the product of IAA treatment, is converted to *S*-(carboxymethyl)cysteine (CM-Cys) by acid hydrolysis, and its phenyl isothiocyanate derivative elutes on reverse-phase HPLC between the Asp (D) derivative peak and the peaks for Ser (S) and Gly (G) derivatives. The HPLC elution profiles for peptides which have been alkylated (IAA) or reduced and alkylated (DTT/IAA) are shown in Figure 5 for only peptides human α1(181–200) and frog α1(181–200), and the results for all peptides are summarized in Table III. The expected position of the CM-Cys peak, determined with a standard, is indicated with an arrow.

The relative yields of CM-Cys, determined by the ratio of the peak heights for peptides alkylated with or without prior reduction (IAA versus DTT/IAA), were used to determine the proportion of peptide existing in solution in the reduced state. The results of this analysis are summarized in Table III. The peptides *Torpedo* α1(181–200, calf α1(181–200), chick α1(179–198), cobra α1(181–200), and frog α1(181–200) were found to exist predominantly (~70–80%) in reduced form, whereas the peptides mouse α1(182–201) and human α1(181–200) were found to be ~65% and ~95% oxidized,

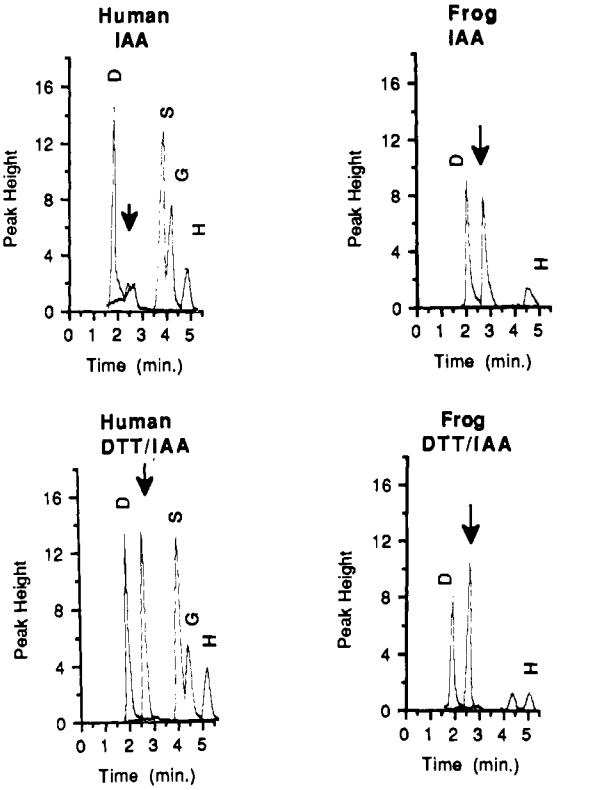

FIGURE 5: Carboxymethylation analysis of peptides: alkylation with IAA with and without prior reduction with DTT. The reduction and/or alkylation of peptides, and the preparation of peptides for amino acid analysis, are described under Materials and Methods. The phenyl isothiocyanate derivatives of the amino acids released by acid hydrolysis were analyzed on a reverse-phase HPLC column (PICO.TAG). The results represent the partial elution profile in 140 mM sodium acetate buffer (pH 6.4) containing 0.05% triethylamine and 6% acetonitrile. The arrow marks the expected elution time of the phenyl isothiocyanate derivative of carboxymethyl-Cys (CM-Cys). The other markers are the derivatives of other amino acids: D = Asp; D,N = Asp/Asn; G = Gly; E = Glu; S = Ser; H = His. An explanation of abbreviations, IAA and DTT/IAA, is found in the text. The degree of reduction of the untreated peptide was determined by comparing the peak heights for CM-Cys before and after reduction with DTT. These results are summarized in Table III.

Table III: Carboxymethylation Analysis of Peptides[a]

peptide	molar ratio of CM-Cys IAA	molar ratio of CM-Cys DTT/IAA	% reduced
Torpedo α1(181–200)	1.90	2.60	73.1
human α1(181–200)	0.12	2.26	5.3
calf α1(181–200)	1.94	2.35	82.6
mouse α1(182–201)	0.58	1.67	34.7
chicken α1(179–198)	1.91	2.52	75.8
frog α1(181–200)	2.15	2.93	73.4

[a] Peptides (0.5 mg/mL) were alkylated with IAA (1.5 mM) with (DTT/IAA) or without (IAA) prior reduction with DTT (6 mM), and amino acids released by acid hydrolysis were derivatized with phenyl isothiocyanate. Samples were analyzed for CM-Cys [*S*-(carboxymethyl)cysteine] as described under Materials and Methods. The molar ratio of CM-Cys was calculated as the total picomoles indicated by the CM-Cys peak height on the HPLC profile (Figure 5) divided by the total yield of amino acids obtained from amino composition analysis. The relative molar ratios for amino acid composition analysis of IAA-versus DTT/IAA-treated peptides were used to calculate the percent of the untreated peptide available in reduced form under assay conditions.

respectively. The CM-Cys peaks were absent in the elution profiles for peptides treated with iodosobenzoic acid prior to alkylation (IOBA), indicating that oxidation of the free sulfhydryl groups was essentially complete under the reaction conditions (data not shown). Other oxidation products, e.g.,

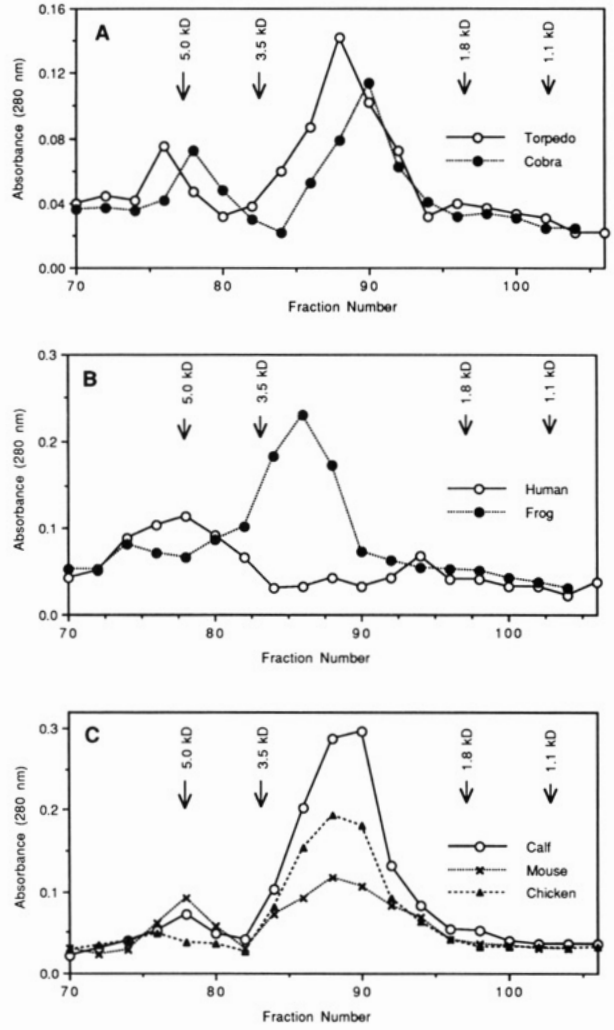

FIGURE 6: P6 chromatography of peptides for determination of the monomer:dimer ratio found for untreated peptides in solution. The Bio-Rad P6 (1.8 × 47 cm) column was calibrated with bradykinin (1060 daltons), porcine renin substrate tetradecapeptide (1759 daltons), human β-endorphin (3465 daltons), human growth hormone releasing factor (5041 daltons), and blue dextran 2000. The following peptides (50–150 μg) were applied and eluted in 10 mM potassium phosphate buffer (pH 7.4), 5–7 mL/h: *Torpedo* α1(181–200), cobra α1(181–200), human α1(181–200), frog α1(181–200), calf α1(181–200), mouse α1(182–201), and chicken α1(179–198).

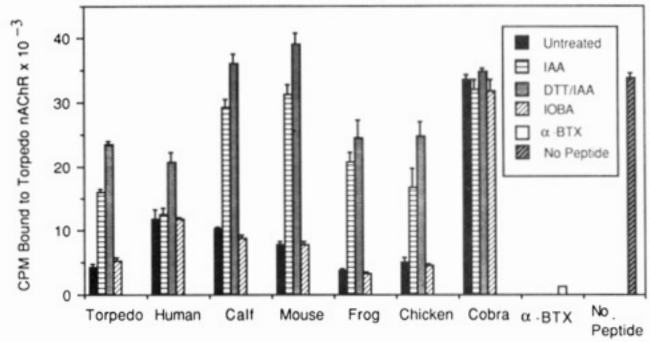

FIGURE 7: Modification of cysteine/cystine residues of peptides: effect on competition with native *Torpedo* nAChR for ^{125}I-α-BTX binding. Untreated peptides and peptides oxidized, reduced, and/or alkylated were separated from reactants on a Sephadex G10 (0.9 × 4 cm) column. *Torpedo* competition assays were performed as described under Materials and Methods and in Figure 4. Peptides (~100 μg/mL) were incubated with 0.5 pmol of ^{125}I-α-BTX (150 cpm/fmol) for 4 h at room temperature, prior to addition of 0.2 pmol of *Torpedo* nAChR. "α-BTX" refers to the binding of ^{125}I-α-BTX to *Torpedo* nAChR that was preincubated with 20 nM unlabeled α-BTX for 10 min prior to its addition to ^{125}I-α-BTX, and the positive control (100% binding) is the ^{125}I-α-BTX binding to *Torpedo* nAChR in the absence of peptide ("No Peptide"). The peptides correspond to *Torpedo* α1(181–200), cobra α1(181–200), human α1(181–200), frog α1(181–200), calf α1(181–200), mouse α1(182–201), and chicken α1(179–198).

cysteic acid (Fontana et al., 1981) were not detected.

At least two different disulfide bonds could account for the oxidized forms of the peptides, that is, monomers containing a vicinal disulfide bond or dimers cross-linked across the pair of adjacent cysteine residues. In order to determine whether the oxidized forms of the peptides represented monomers or dimers, samples of treated and untreated peptides were chromatographed on a P6 (Bio-Rad) column that had been calibrated with standards as described under Materials and Methods, and the results for untreated peptides are shown in Figure 6. Untreated peptides *Torpedo* α1(181–200) and cobra α1(181–200) (Figure 6A) had similar chromatograms, eluting predominantly as monomers (MW 2.7K and 3.0K, respectively), and a dimer form (~5K) accounted for <30% of each peptide. These results, in addition to those from carboxymethylation analysis, indicated that the inability of peptide cobra α1(181–200) to bind α-BTX was not due to differences in the redox state of the Cys residues. The elution profiles for untreated peptides of the muscle nAChR α1-subunit from the other species are shown in Figure 6A,B. Peptides frog α1(181–200), calf α1(181–200), and chicken α1(179–198) eluted predominantly as monomers (MW 2.5–3.3K), with a small dimer peak (~5K) accounting for ~10–20% of the peptide. The elution profile of the untreated mouse α1(182–201) peptide indicated a monomer:dimer ratio of 2:1 (Figure 6C), and peptide human α1(181–200) (Figure 6B) was found to exist predominantly (~80%) in the dimer form (MW ~4.5K). Following reduction and alkylation, only monomer peaks were observed for each peptide, demonstrating that the peaks at ~5 kD_a were disulfide-linked dimers and not merely aggregates (data not shown). Following oxidation with iodosobenzoic acid, the distribution of monomer/dimer remained the same for each peptide, which indicated that chemically induced oxidation produced primarily monomers.

In order to determine the effects of cysteine/cystine modification on the α-BTX binding activity, untreated peptides and peptides that had been modified by oxidation, reduction, and/or alkylation were tested by using the competition binding assay. The results of a typical experiment ($n = 3$) are shown in Figure 7. The ability of the peptide cobra α1(181–200) to bind α-BTX was unaffected by sulfhydryl modifications, i.e., under all conditions, the peptide did not bind α-BTX. The α-BTX binding activity of the peptides corresponding to the *Torpedo* α1(181–200) sequence and the homologous muscle α1-subunits of other species were unaffected by oxidation with iodosobenzoic acid, but alkylation of the free sulfhydryl groups in untreated or reduced peptides drastically altered the ability of the peptides to bind α-BTX. The ability of the human peptide to bind α-BTX was not altered by alkylation with IAA without prior reduction, consistent with the results of carboxymethylation analysis, which indicated that the untreated peptide was ~95% oxidized.

Scanning of Complete α1-Subunits for α-BTX Binding Sequences. In order to determine if other sequence segments of the vertebrate nAChR α1-subunits are able to form prototopes for α-BTX, we selected the human and mouse nAChR α1-subunits as exemplary sequences. Overlapping peptides corresponding to the complete human and mouse nAChR α1-subunits were synthesized and tested for inhibition of ^{125}I-α-BTX binding to native *Torpedo* nAChR in competition assays (the corresponding amino acid residues are listed under

Materials and Methods). The peptides tested included the human and mouse sequences α1(48–67) and α1(51–70), which overlap α1(55–74), a sequence segment that is homologous to a peptide of the *Torpedo* α1-subunit that we have previously shown to compete for α-BTX (Conti-Tronconi et al., 1989, 1990). At a concentration of 100 μg/mL, four peptides consistently competed for binding of ^{125}I-α-BTX to native *Torpedo* nAChR, i.e., the human peptides α1(181–195), α1-(181–200), and α1(190–207) and the peptide corresponding to mouse α1(182–201) (data not shown). Other peptides were found to inhibit ^{125}I-α-BTX binding to *Torpedo* nAChR, if the peptide concentration was increased to 200 μg/mL (~80 μM), but the inhibition observed was small (<30%) and inconsistent between experiments. These results indicate either (i) that our panel of peptides did not sufficiently represent all potential α-BTX binding prototopes or (ii) that additional prototopes are not present in the human and mouse α1 sequences.

Discussion

Our laboratory has used synthetic peptides to determine the structural requirements for α-BTX binding to nAChR α-subunits (Conti-Tronconi et al., 1989, 1990, 1991; McLane et al., 1990). We have previously defined a sequence segment of the *Torpedo* nAChR α1-subunit at positions 181–200 that is able to bind α-BTX (Conti-Tronconi et al., 1989, 1990), and have determined the effects of single amino acid substitutions of Gly for each residue of this sequence (Conti-Tronconi et al., 1991). In order to study the effects of all possible amino acid substitutions on α-BTX binding, a panel of 400 different synthetic peptides would be required. The naturally occurring substitutions provide a point of departure from which to examine the effects of different substitutions on the affinity of α-BTX binding, and at the same time maintain a structure that is relevant to a functional acetylcholine binding site. In the present study, synthetic peptides corresponding to the homologous sequence segments of muscle nAChR α1-subunits from different species were compared for their ability of to bind α-BTX. Our experiments were designed to answer three questions: (i) Do peptides corresponding to the α1-subunits of various species differ in their affinities for α-BTX, and, if so, can these differences be correlated with amino acid substitutions? (ii) Are the adjacent cysteine residues, which are conserved in all nAChR α1-subunits at approximately positions 192 and 193, important for α-BTX binding, and, if so, do α-BTX binding peptides contain a vicinal disulfide bond? (iii) Do other sequence segments of vertebrate muscle nAChR α1-subunits, in addition to residues 181–200, contribute to forming the cholinergic binding site?

The peptide *Torpedo* α1(181–200) and the homologous peptides for the muscle nAChR α1-subunits of human, calf, mouse, chicken, and frog were found to bind ^{125}I-α-BTX, whereas the peptide cobra α1(181–200) did not detectably bind α-BTX. This result is physiologically significant, as the unique insensitivity of snakes and advanced lizards to α-neurotoxins has been correlated with the inability of ^{125}I-α-BTX to bind their native muscle nAChRs (Burden et al., 1975). We compared the relative affinities of the α-BTX binding peptides from other vertebrate species using both solid- and solution-phase assays. The apparent K_ds from solid-phase assays were 1–2 μM for peptides corresponding to *Torpedo* α1(181–200), chick α1(179–198), and frog α1(181–200) and 3–8 μM for the peptides human α1(181–200), calf α1(181–200), and mouse α1(182–201). The solution-phase competition assay clearly distinguished peptides of different affinities for α-BTX; i.e., the *Torpedo*, chick, frog, and mouse peptides competed

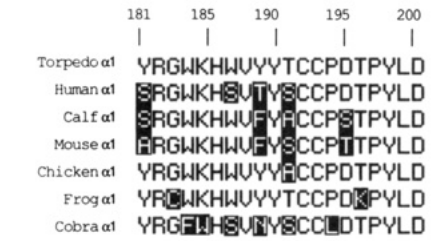

FIGURE 8: Comparison of aligned sequence segments. The peptides *Torpedo* α1(181–200), human α1(181–200), calf α1(181–200), mouse α1(182–201), chicken α1(179–198), frog α1(181–200), and cobra α1(181–200) are aligned and numbered according to the *Torpedo* nAChR α1-subunit sequence. The highlighted amino acids represent nonconserved residues.

with IC_{50}s ~ 1–2 μM for ^{125}I-α-BTX binding to native *Torpedo* nAChR, whereas IC_{50}s ~ 15 μM were obtained for the human and calf peptides. Our results confirm and extend those obtained by Ohana and Gershoni (1990), who expressed the sequence segment 183–204 from the nAChR α1-subunit of different species as trpE fusion proteins. Their Scatchard analyses of bacterial extracts indicated the following rank order of affinities (K_ds ~ 0.1–6 μM) for α-BTX: *Torpedo* > chick > frog > mouse > calf > human. We find, however, that the peptide frog α1(181–200) binds ^{125}I-α-BTX with affinity comparable to the *Torpedo* α1(181–200) peptide. The discrepancies with our results may reflect differences between the sequences used and/or the contribution of the trpE protein to determining the conformation of the peptide segments. Our results also confirm those previously obtained by Wilson and Lentz (1988), who reported that peptides corresponding to the sequence segments 173–204 of calf and human nAChR α1-subunits bind ^{125}I-α-BTX with low affinity (K_ds ~10–100-fold higher than the homologous *Torpedo* peptide, respectively).

The sequences for peptides corresponding to the muscle nAChR α1-subunits of different species are numbered and aligned with respect to the *Torpedo* α1-subunit in Figure 8, and the amino acid residues that differ from the *Torpedo* sequence are highlighted. Inspection of these sequences reveals that six amino acids of the cobra sequence differ from the other α1-subunits and these may represent important amino acids for α-BTX binding. Notable nonconservative substitutions in the cobra α1(181–200) sequence segment include the replacements of Lys-185, Trp-187, Tyr-189, and Pro-194 by Trp, Ser, Asn, and Leu, respectively. The minimal α-BTX binding sequence defined for the *Torpedo* α1-subunit has been reported to be contained in residues α1(186–196) (Wilson et al., 1988), which suggests that the cobra α1 sequence substitutions for Trp-187, Tyr-189, and Pro-194 are most likely to be critical. In our previous studies, we have demonstrated, using single substitution analogues of the *Torpedo* α1(181–200) peptide, that two clusters of amino acids within this minimal sequence segment are important for α-BTX binding, i.e., the amino acids at positions 188–190 (VYY) and those at positions 192–194 (CCP) (Conti-Tronconi et al., 1991). Tyr-190 and Cys-192/193 are conserved in the cobra α1-subunit and neuronal α-subunits (α2, α3, α4) that do not bind α-BTX (Deneris et al., 1987; Wada et al., 1988; Nef et al., 1988; McLane et al., 1990), and this indicates that the presence of these residues is not sufficient for α-BTX binding. Furthermore, Val-188 is conserved in the cobra α1 sequence, and both Pro-194 and Val-188 are substituted nonconservatively in the α-subunit sequence of the neuronal α-BTX binding proteins (Schoepfer et al., 1990; Couturier et al., 1990; McLane et al., unpublished results). The convergence of these results implicates only Tyr-189 as a critical residue in the interaction of α subunits from different nAChRs with α-BTX.

Comparison of the vertebrate muscle and *Torpedo* sequences in Figure 8 indicates that the sequence VYY at positions 188–190 is common to the peptides that bind α-BTX with high affinity (the *Torpedo*, frog, and chick peptides) and that amino acid substitutions occur at position 189 for peptides found to bind α-BTX with lower affinity. In the calf and mouse α1-subunit sequences, Tyr-189 is substituted by Phe, a conservative substitution. Additional amino acid residues at positions 181, 191, and 195 differ for the calf and mouse peptides, but how they confer differences in the affinity for α-BTX is difficult to surmise. In the human and cobra α1 sequences, Tyr-189 is nonconservatively replaced by Thr and Asn, and Trp-187 is substituted by Ser, which may be related to the low α-BTX binding activity of these peptides. The finding that aromatic residues are important for α-BTX binding is congruent with models for α-BTX binding, which propose that the contacts between α-BTX and the nAChR involve primarily hydrophobic and hydrogen bonding, with only a few electrostatic interactions (Love & Stroud, 1986). The ability of the human α1 peptide to bind α-BTX, despite the nonconservative substitutions of Tyr-189 and Trp-187, may result from the ability of secondary nonconservative substitutions to compensate for each other, as has been documented for other proteins studied by site-directed mutagenesis (Blacklow & Knowles, 1990), whereas the additional substitutions in the cobra sequence may prevent such a compensating effect. Our results provide the basis for testing this hypothesis using systematic amino acid substitutions of these residues. In conclusion, despite the high degree of conservation of the sequence segment 181–200 of the nAChR α1-subunits of different species, a few amino acid substitutions may account for the apparent differences in affinity of peptides for α-BTX. How these differences in α-BTX binding correlate with phylogenetic sensitivity remains to be determined.

The pair of cysteine residues at positions 192 and 193 is conserved in all muscle nAChR α-subunits and is believed to form a vicinal disulfide in the intact *Torpedo* nAChR (Kao et al., 1984; Kao & Karlin, 1986; Mosckovitz & Gershoni, 1988; Kellaris et al., 1989). Given the unusual structure that a cystine would confer to this sequence segment, we were interested in determining the disposition of the adjacent cysteines of the peptides used in the present study. Carboxymethylation analysis was performed to determine the availability of free sulfhydryl groups for alkylation with IAA in peptides before and after reduction with DTT. The yield of *S*-(carboxymethyl)cysteine on amino composition analysis indicated that untreated peptides corresponding to *Torpedo* α1(181–200) and the homologous sequences of frog, chick, calf, and cobra were ~70–80% reduced, whereas the mouse and human peptides showed a tendency to dimerize and were ~60% and ~90% oxidized, respectively. To further assess the role of the vicinal disulfide bond in α-BTX binding, peptides were treated with IOBA, converting them to >90% oxidized monomers. Oxidation of the peptides, however, did not affect their α-BTX binding activity, as assessed by using the competition assay. In contrast, the ability of peptides to compete for ^{125}I-α-BTX with native *Torpedo* nAChR was greatly diminished by alkylation of free sulfhydryl groups with IAA. These results agree with previous reports of the effects of alkylation on α-BTX binding to peptides corresponding to the *Torpedo* α1-subunit (Ralston et al., 1987; Neumann et al., 1986b; Conti-Tronconi et al., 1990). In conclusion, our present studies indicate that both reduced and oxidized forms of the peptides are able to bind α-BTX and that a vicinal disulfide bond is not a critical structural element of the α-BTX binding site. Despite the apparent indifference of α-BTX to the redox state of Cys-192 and -193, these residues do appear to be involved in forming the peptide/toxin interface, as suggested by the marked reduction of α-BTX binding upon their alkylation.

Recent affinity labeling studies of the intact *Torpedo* nAChR have indicated that the cholinergic binding sites on the α-subunits are formed by several peptide loops, i.e., sequence segments containing Tyr-93, Tyr-149, and Cys-192/193 (Dennis et al., 1988; Galzi et al., 1990). Furthermore, we have demonstrated that in addition to the peptide *Torpedo* α1(181–200), the sequence segment *Torpedo* α1(55–74) binds α-BTX poorly in toxin blot assays but competes for α-BTX with native *Torpedo* nAChR and is recognized by a monoclonal antibody that blocks α-BTX binding (Fels et al., 1986; Conti-Tronconi et al., 1989, 1990). The corresponding sequence segment of the vertebrate muscle nAChR subunit, however, is relatively divergent when compared to the sequence α1(181–200). In order to determine if other sequence segments of the vertebrate muscle nAChR α1-subunit are able to form independent prototopes that bind α-BTX, we tested panels of overlapping peptides corresponding to the complete human and mouse α1-subunits, but were unable to consistently detect α-BTX binding to peptides other than the sequence between positions 180 and 207. Therefore, if other sequence segments contribute to the formation of a high-affinity α-BTX binding site on the human or mouse α1-subunits, they either are incapable of forming a prototope or have not been properly represented by our panel of synthetic peptides.

The use of synthetic peptides to study ligand binding has certain advantages over bacterially expressed fusion proteins, which contain other unrelated protein sequences that may alter the conformation of the sequence segment of interest. These include the ease of selective modification of amino acid residues and secondary structural analysis. Both approaches to the mapping of ligand binding segments also share several inherent limitations. First of all, the ligand binding sites must reside on continuous sequence segments that are able to attain an active conformation in the absence of other neighboring peptide loops found in the native protein, i.e., a prototope. Second, the isolated peptide sequence must be able to bind the ligand with sufficiently high affinity to be detectable by using available assay systems. And third, given that a high-affinity prototope exists on a protein subunit, a panel of synthetic or biosynthetic peptides designed to map this site may not contain the prototope due to insufficient length of the peptides or insufficient overlap. This latter limitation may be represented in this study by our inability to demonstrate competitive binding of α-BTX with other cholinergic ligands, which may have only partially overlapping binding sites (Watters & Maelicke, 1983; Mihovilovic & Richman, 1987; Chinchetru et al., 1989). On the other hand, we and other investigators have been fortunate in our ability to define α-BTX binding segments on the α-subunits of the *Torpedo* and vertebrate muscle nAChRs using small peptides.

In conclusion, we demonstrate that a sequence segment corresponding to *Torpedo* α1(181–200) and the homologous sequence segments from the muscle nAChR of vertebrate species contain the amino acid residues required for a universal α-BTX binding site. By comparison of the relative of affinities for α-BTX of the synthetic peptides, a model of the amino acid residues required for high-affinity binding can be formulated. In addition, we have been able to demonstrate that a vicinal disulfide bond between the highly conserved cysteine residues at positions 192 and 193, relative to the *Torpedo* nAChR, is

not a requirement for α-BTX binding. Although the sequences of the cholinergic binding site have been largely conserved between the α1-subunits of different species, some nonconservative amino acid substitutions must be tolerated to allow agonist binding. The snake muscle nAChR α1-subunit has undoubtedly reached a level of divergence that optimizes this degree of flexibility, yet induces a conformation of its cholinergic site, which is incompatible with α-BTX binding.

Registry No. Cys, 52-90-4; SGEWVMKDYRGWKHWVYYYTC, 133295-47-3; WVYYTCCPDTPYLDITY, 133295-48-4; YRGWKHWVYYTCCPDTPYLD, 117861-08-2; SRGWKHSVTYSCCPDTPYLD, 133295-49-5; ESRGWKHSVTYSCCPD, 133295-50-8; SCCPDTPYLDITYHFVM, 133295-51-9; WVFYSCCPTTPYLDITY, 133295-52-0; ESGEWVIKEARGWKHWVFYS, 133295-53-1; ARGWKHWVFYSCCPTTPYLD, 133322-53-9; ASGEWMMKDYRCWKHWVYYT, 133322-54-0; YRCWKHWVYYTCCPDKPYLD, 133322-55-1; WVYYTCCPDKPYLDITYHF, 133322-56-2; HWVFYACCPSTPYLD, 133322-57-3; SRGWKHWVFYACCPSTPYLD, 133295-54-2; YRGWKHWVYYACCPDTPYLD, 133295-55-3; YRGFWHSVNYSCCLDTPYLD, 133295-56-4; α-bungarotoxin, 11032-79-4.

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Essentiality of the Molecular Weight 15 000 Protein (Subunit IV) in the Cytochrome b–c_1 Complex of *Rhodobacter sphaeroides*†

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ABSTRACT: The cytochrome b–c_1 complex from *Rhodobacter sphaeroides* was resolved into four protein subunits by a phenyl-Sepharose CL-4B column eluted with different detergents. Individual subunits were purified to homogeneity. Antibodies against subunit IV (M_r = 15 000) were raised and purified. These antibodies had a high titer with isolated subunit IV and with the b–c_1 complex from *R. sphaeroides*. They inhibited 95% of the ubiquinol–cytochrome c reductase activity of the cytochrome b–c_1 complex, indicating that subunit IV is essential for the catalytic function of this complex. When detergent-solubilized chromatopores were passed through an anti-subunit IV coupled Affi-Gel 10 column, no ubiquinol–cytochrome c reductase activity was detected in the effluent, and four proteins, corresponding to the four subunits in the isolated complex, were adsorbed to the column. This indicated that subunit IV in an integral part of the cytochrome b–c_1 complex. No change in the apparent K_ms for Q_2H_2 and for cytochrome c was observed with anti-subunit IV treated complex. Antibodies against subunit IV had little effect on the stability of the ubisemiquinone radical in this complex, suggesting that they do not bind to the subunit near its ubiquinone-binding site.

The cytochrome b–c_1 complex of the photosynthetic bacterium *Rhodobacter sphaeroides*, which catalyzes electron transfer from ubiquinol to cytochrome c_2 has been purified and characterized in several laboratories (Gabellini et al., 1982; Takamiya et al., 1982; Yu et al., 1984; Ljungdahl et al., 1986; Andrews et al., 1990; McCurley et al., 1990; Purvis et al., 1990). In spite of the fact that different bacterial strains and different isolation procedures were used, all the cytochrome b–c_1 complex preparations from *R. sphaeroides* contain four protein subunits with apparent molecular weights of 43 000, 30 000, 21 000, and 15 000. The three larger molecular weight proteins are identified as cytochromes b and c_1 and iron–sulfur protein, respectively. The M_r = 15K subunit has recently been identified as one of the ubiquinone (Q)[1]-binding sites in the

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[1] Abbreviations: DTE, dithioerythritol; PBS, phosphate-buffered saline; Q, ubiquinone; Q_2H_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol; SDS, sodium dodecyl sulfate; TMD, 50 mM Tris-HCl, pH 8.0, containing 1 mM $MgSO_4$ and 0.01% dodecyl maltoside.

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