Properties of Radiolabeled α-Bungarotoxin Derivatives and Their Interaction with Nicotinic Acetylcholine Receptors†

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ABSTRACT: Column-purified monoiodinated, diiodinated, and tritiated derivatives of α-bungarotoxin (α-Bgt) are distinguished on the basis of their ultraviolet absorption and circular dichroism (CD) spectra. The pattern of changes in CD spectra on incorporation of iodine into a single tyrosine residue of α-Bgt and the widespread wavelength distribution of these effects are interpreted as reflecting primary chemical modification of the tyrosine chromophore as well as vicinal and global secondary structural changes. Native and tritiated α-Bgt are shown to be more effective than iodinated α-Bgt derivatives in competing for specific toxin binding sites on putative nicotinic acetylcholine receptors (nAChR) derived from rat brain reflecting functional perturbation of the modified toxin. In contrast, both membrane-bound and solubilized nAChR from *Torpedo californica* electroplax display little or no specific binding preference for native toxin, nor are there significant differences in lethal potency of α-Bgt derivatives toward mice. These results suggest that peripheral and putative central nAChR may differ in their α-Bgt binding properties and suggest the usefulness of modified toxin in detecting those subtle differences.

Toward preparation of a suitable toxin label for the nicotinic acetylcholine receptor in mammalian brain, a systematic study of the properties of α-Bgt[1] and its radiolabeled derivatives was undertaken. Other investigators utilize iodinated α-Bgt for labeling putative central nervous system nicotinic acetylcholine receptor (Salvaterra et al., 1975; Salvaterra & Mahler, 1976; Lowy et al., 1976; Moore & Brady, 1976). However, an earlier report (Eterović & Bennett, 1974) suggested that iodinated α-Bgt might display anomalous affinity for binding sites in rat brain. A communication has appeared relating that iodination of a sea snake (*Hydrophiidae*) neurotoxin has the effect of abolishing lethal toxicity toward mice (Raymond & Tu, 1972). Modification of the single tyrosine residue of neurotoxin type I (short neurotoxin) from *Naja naja siamensis* (fraction 7C, Karlsson & Sundelin, 1976) or from *Naja haje* (Chicheportiche et al., 1972), or modification of both tyrosine residues of the type I neurotoxin from *Naja naja atra* (Chang et al., 1971) results in extensive loss of biological activity (see Tu, 1973). On the other hand, nitration of the single tyrosine residue of the long (type II) neurotoxin from *Naja naja* (Ohta & Hayashi, 1974) or that from *Naja naja siamensis* (fraction 3, Karlsson & Sundelin, 1976), or of the type I neurotoxin from *Naja nigricollis* (Karlsson & Sundelin, 1976) has little effect on toxic activity. As a long neurotoxin containing two tyrosine residues, α-Bgt differs from the toxins mentioned above. Toward a comprehensive understanding of the relationship between toxin structure and function, modification of the tyrosine residues on α-Bgt might provide information on their functional significance. Limited modification procedures might also be useful toward detection of subtle differences in the properties of putative nAChR from different nervous system loci, across species, or within a specific nAChR population possessing heterogeneous binding sites. The consequences of iodination of the tyrosine residues of α-Bgt on some of its physical chemical characteristics and on its interaction with membrane fractions derived from *Torpedo* electroplax and rat brain tissue are described.

Experimental Procedure

Crude venom from *Bungarus multicinctus* (Miami Serpentarium) was stored in lyophilized form at −20 °C, and α-Bgt was prepared according to Eterović et al. (1975b), with the following modifications. Purified α-Bgt was obtained by rechromatography of fraction 3 on a Sephadex G-50 column (7.5 × 24 cm), eluted with 0.02 M NH_4OAc, pH 7.0, at a flow rate of 12 mL/h, with subsequent rechromatography on Bio-Rex 70 (2.5 × 24 cm) eluted with a linear gradient of NaCl, 0 to 0.4 M, in 0.02 M NH_4OAc, pH 7.0 (1000 mL total volume). Concentrations of all α-Bgt derivatives were estimated by optical absorbance at 280 nm using an extinction coefficient, $\epsilon_{280}^{0.1\%}$ of 1.32 (Hanley et al., 1977). Using this technique, there is no systematic error introduced into monoiodo α-Bgt and diiodo α-Bgt concentration determinations, as indicated independently by nonspecific binding dilution experiments and by Lowry protein determinations, using BSA as a standard.

Iodinated α-Bgt was prepared using ICl in a simple nonenzymatic procedure. To a solution at a final concentration of 1.4 M HCl, containing 1 mol equiv of KIO_3, 2 mol equiv of $Na^{125}I$ (carrier free, New England Nuclear) and/or carrier KI are added, yielding iodine, which is rapidly converted, with yields in excess of 95%, to ICl. One volume of ICl mixture is then added to the appropriate quantity of α-Bgt in 5 volumes of 3.2 M NH_4Cl, pH 8.9, and incubated at 21 °C for 30 to 60 min. The molar ratio of α-Bgt to ^{125}ICl and the final pH of the iodination mixture may be varied to give enhanced yields of diiodo α-Bgt or monoiodo α-Bgt. Formation of diiodinated derivative is favored when α-Bgt is exposed to a greater than twofold molar excess of ^{125}ICl, and/or at pH lower than 8.0. Monoiodo α-Bgt is the primary product when there is an excess

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[1] Abbreviations used: α-Bgt, α-bungarotoxin; nAChR, nicotinic acetylcholine receptor(s); *Torpedo*, *Torpedo californica*; NH_4OAc, ammonium acetate; diiodo α-Bgt, diiodinated α-Bgt; monoiodo α-Bgt, monoiodinated α-Bgt; UV, ultraviolet; [^{125}I]-α-Bgt, ^{125}I-labeled monoiodinated α-Bgt; [$^{125}I_2$]-α-Bgt, ^{125}I-labeled diiodinated α-Bgt; [^3H]-α-Bgt, tritium-labeled α-Bgt; BSA, bovine serum albumin; CD, circular dichroism; CMF, crude mitochondrial fraction membranes; IC_{50}, concentration of competitor required to block 50% specific binding.

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of α-Bgt over ^{125}ICl, and at pH greater than 8.0. Typically, over 85% of available ^{125}I is incorporated into α-Bgt at stoichiometric molar ratios of ICl to α-Bgt. The reaction is terminated and the labeled α-Bgt is desalted by application of the mixture to a Sephadex G-10 column (1.5 × 35 cm) equilibrated in 5 mM Na_2HPO_4, pH 6.5. The first radioactive peak, corresponding to the labeled toxin, is pooled and applied to a Whatman CM-52 cation-exchange column (2 × 30 cm) equilibrated in 5 mM NaH_2PO_4, pH 6.5 (see, also, Bulger et al., 1977). Purified α-Bgt derivatives are eluted with a linear gradient, 0–80 mM NaCl, in 5 mM Na_2HPO_4, pH 6.5. Peaks are identified by UV absorption spectra (Cary 118 spectrophotometer) and by γ-ray counting of samples on a Nuclear-Chicago γ-well counter. [$^{125}I_1$]-α-Bgt typically has an initial specific activity of 100 dpm/fmol, which decays with the half-life of ^{125}I (60 days). Tritiated α-Bgt prepared from monoiodo α-Bgt essentially as described elsewhere (Eterović et al., 1975a), has a specific activity of ca. 25 dpm/fmol. Radiolabeled toxin fractions (1–25 μM) are stored at −20 °C in the presence of 1 mg/mL BSA (Sigma) to prevent adsorption to glass. Iodinated toxins retain ~80% of their binding activity through at least 2 half-lives, and preparations of tritiated toxin show greater than 93% activity for at least 1 year, as determined by quantities of specifically bound radioactivity when toxins are incubated with a large excess of *Torpedo* nAChR.

CD studies utilize the apparatus described by Sutherland et al. (1974). For far-ultraviolet measurements (190–250 nm), the slit width was 1.0 mm (giving a spectral bandwidth of 2.5 nm), the cell was 1.0 mm pathlength, and the samples were 0.01% protein in 5 mM Na_2HPO_4 buffer (pH 7.4). For near-ultraviolet measurements (250–350 nm), the slit width was 0.5 mm (giving a spectral bandwidth of 1.5 nm), the cell was 10 mm pathlength, and the samples were 0.1% protein in 10 mM NH_4OAc buffer (pH 7.4). For all calculations, α-Bgt was assumed to have a molecular weight of 8000 (Hanley et al., 1977).

Proteolytic digests of iodinated toxin derivatives were prepared by hydrolysis for 48 h at 21 °C with Viokase (Grand Island Biological Co.), 0.5 mg/mL of carrier BSA, 0.1 M Tris, 167 mM NaCl, adjusted to pH 8.0 with HCl. Hydrolysate product was separated from residual high molecular weight material on a UM-10 ultrafilter (Amicon). Paper chromatography was performed on 50 μL of the effluent in the presence of carrier monoiodotyrosine and/or diiodotyrosine (Sigma) using a solvent of 1-butanol:water:acetic acid (50:50:6). Radioactive spots were determined by autoradiography.

To test the binding of α-Bgt derivatives to putative central nervous system acetylcholine receptors, crude mitochondrial fraction membranes derived from male Sprague-Dawley rat brains were prepared essentially as described previously (Eterović & Bennett, 1974) with an additional wash and resuspension in binding Ringers (115 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, 33 mM Tris, to pH 7.4 with HCl). These preparations contained approximately 40 fmol α-Bgt binding sites per mg of protein. In addition, membrane preparations from frozen *Torpedo* electroplax (Pacific Bio-Marine) were prepared from crude excitable vesicles (Hazelbauer & Changeux, 1974) by osmotic shock in 20 volumes of distilled water, followed by centrifugation at 17 000*g* for 10 min using a Sorvall SM-24 rotor in an RC-2B refrigerated (4 °C) centrifuge, with subsequent resuspension in buffer as indicated in the figure legends. Stock solutions of electroplax membranes contained ca. 1 μM α-Bgt binding sites or ~100 pmol per mg of protein.

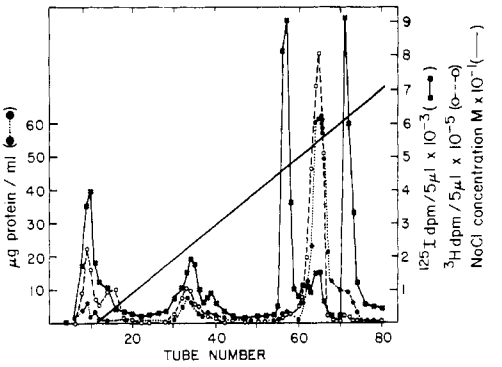

FIGURE 1: Characteristic chromatographic profile of radiolabeled α-Bgt derivatives. Mixture of [^{3}H]-α-Bgt, [^{125}I]-α-Bgt, and [$^{125}I_2$]-α-Bgt was applied to a CM-52 cation-exchange column equilibrated in 5 mM Na_2HPO_4, pH 6.5. After elution of one void volume, a linear gradient, 0–80 mM NaCl, was used to elute diiodinated, tritiated (or native), and then monoiodinated α-Bgt species. NaCl gradient (—), μg of protein/mL (●···●), ^{125}I dpm/5 μL × 10^{-3} (X—X), and ^{3}H dpm/5 μL × 10^{-5} (O- - - -O) are indicated. The first two radioactive peaks were discarded.

Purified *Torpedo* electroplax nAChR (1.9 nmol α-Bgt sites per mg of protein) was prepared according to Schmidt & Raftery (1972) and was a gift from Dr. David Devore. Samples of CMF (~100 pM) or electroplax membrane (~400 pM) are incubated in the presence of 0.6% BSA with appropriate concentration of radiolabeled α-Bgt for 60 min at 21 °C. For binding competition studies, samples containing a fixed concentration of radiolabeled toxin are coincubated with nonradiolabeled native or iodinated toxin at indicated concentrations, and nonspecific sites are chased with 10^{-6} M native toxin for 30 min. For binding saturation studies, specific binding represents the difference between test samples chased with 100-fold excess of nonradiolabeled toxin and blank samples that are pretreated for 30 min with a 100-fold excess of native toxin before incubation with radiolabeled α-Bgt, and subjected to the same chase procedure. All membranes are washed through four cycles of centrifugation at 17 800*g* and resuspension in binding Ringers over a period of 1.5–3 h. The final pellet is resuspended and transferred to Amersham/Searle γ-well counter tubes for counting ^{125}I on a Nuclear-Chicago counter, or to scintillation vials containing Aquasol-2 for ^{3}H liquid scintillation counting (Packard 3375). [^{125}I]-α-Bgt binding to purified electroplax nAChR was assayed according to Schmidt & Raftery (1972).

Membrane proteins are determined by the method of Lowry et al. (1951).

Results

Ion-Exchange Chromatography. Purified radiolabeled α-Bgt derivatives may be separated by ion-exchange column chromatography on Whatman CM-52 equilibrated at pH 6.5 in 5 mM Na_2HPO_4, by elution with a linear gradient, 0–80 mM NaCl (see also Bulger et al., 1977). A representative chromatogram (Figure 1) shows that [^{3}H]-α-Bgt is eluted subsequent to diiodinated, and prior to monoiodinated α-Bgt derivatives, at about 55 mM NaCl. The elution pattern of native α-Bgt is indistinguishable from that of tritiated toxin. The three classes of α-toxin exhibit unique ultraviolet absorption spectra (Figure 2). Native or [^{3}H]-α-Bgt has broad maximum absorbance at 272–278 nm and monoiodinated α-Bgt is characterized by a peak in absorbance at 280 nm, with shoulders at ~272 and ~288 nm.[2] The spectrum of diiodo

[2] The spectrum for monoiodinated α-Bgt was incorrectly attributed to diiodinated α-Bgt in a previous (Eterović et al., 1975a) publication.

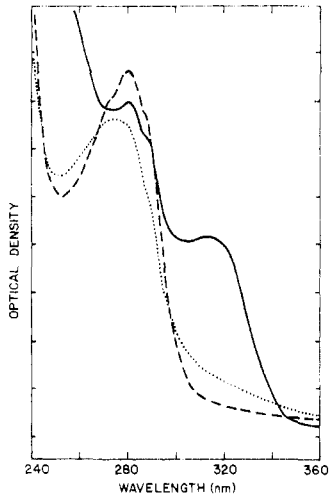

FIGURE 2: UV absorption spectra of cold native α-Bgt (25 μM, ····), monoiodinated (65 μM, - - - -), and diiodinated (123 μM, —) α-Bgt derivatives. Note that optical density is plotted on an arbitrary scale, and the spectra are not normalized. Spectra run on samples in 5 mM Na_2HPO_4 (pH 7.4) in 1-cm cuvettes, at 0.5 nm/s scan speed, using the auto slit mode of a Cary 118 spectrophotometer.

α-Bgt is markedly altered, with enhanced absorption at wavelengths less than 270 nm, obliterating the characteristic trough in absorption seen for native and monoiodinated toxins. The spectrum for diiodo α-Bgt also displays the characteristic peak and shoulder profile of monoiodo α-Bgt at 270–290 nm, as well as a broad local maximum centered at 320 nm.

Circular Dichroism Characteristics. These large differences in UV spectra and the unexpected order of elution of toxin species on ion-exchange chromatography suggested that iodination of α-Bgt might induce changes in the toxin structure. To explore this possibility, CD spectra were examined. In the near-UV (Figure 3b) diiodo α-Bgt has a positive CD band at 315 nm, while neither monoiodo nor native α-Bgt exhibit a signal. In the mid-UV (Figure 3a) both native and monoiodo α-Bgt exhibit a band at ~228 nm that is absent in the diiodinated derivative. The negatively dichroic band at ~280 nm is increased in intensity with monoiodination, and increased further with incorporation of the second iodine into α-Bgt. The CD spectra for the α-Bgt derivatives differ over the range from 190 to 220 nm (Figure 4). Native, monoiodo and diiodo α-Bgt display, in common, a negative band at ~210 nm, which is about 50% stronger for native toxin than for iodinated species. The spectra for diiodo α-Bgt turn slightly positive at ~198 nm, and are negatively dichroic at lower wavelengths, while the positively dichroic bands for monoiodo and native α-Bgt occur at 195 and 193 nm, respectively.

Identification of Radiolabeled Residues. Monoiodo and diiodo α-Bgt were subjected to extensive proteolytic hydrolysis to identify the amino acid group(s) labeled with ^{125}I. Hydrolysis products were separated by paper chromatography; radioactive spots comigrated with monoiodotyrosine for [^{125}I]-α-Bgt, and diiodotyrosine for [$^{125}I_2$]-α-Bgt. Thus, a single tyrosine residue on α-Bgt is labeled in both monoiodo and diiodo α-Bgt. There are trace quantities of monoiodotyrosine in [$^{125}I_2$]-α-Bgt.

Binding Properties. Iodination of α-Bgt is found to perturb the affinity of α-toxin for binding sites in crude mitochondrial fractions derived from rat brain. Native α-Bgt, when coincubated at concentrations from 10^{-10} to 10^{-6} M with 10^{-8} M [^{125}I]-α-Bgt or [$^{125}I_2$]-α-Bgt, competes for radiolabeled iodinated toxin binding sites more effectively than monoiodo and diiodo toxin, as determined by IC_{50} values on log–probit

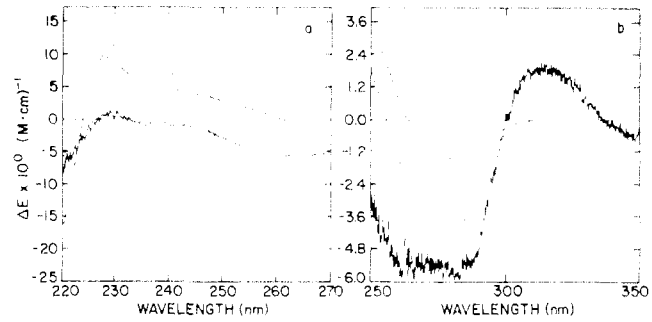

FIGURE 3: CD spectra of native (- - - -), monoiodinated (·····), and diiodinated (——) α-Bgt in the near- and mid-ultraviolet range. Note the differences in ordinate scale. Samples were used no longer than 2 months after purification, having been stored at −20 °C in the interim. No alterations in spectra were observed over at least 6 months for samples treated in the same manner.

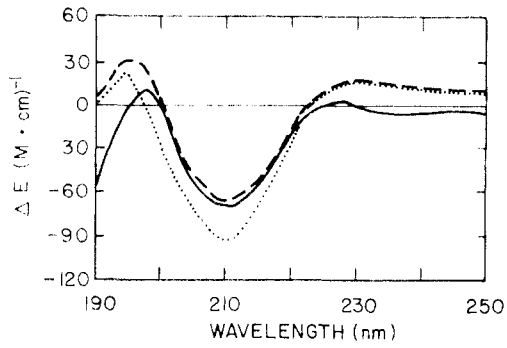

FIGURE 4: Far-UV CD spectra of native (···), monoiodinated (- - -), and diiodinated (——) α-Bgt derivatives.

analysis (Figure 5a). In the representative experiment illustrated, IC_{50} is 4.5 nM for native toxin, 28 nM for monoiodo α-Bgt, and 65 nM for α-Bgt. The slope of the log–probit plot for native α-Bgt is somewhat steeper than that for monoiodo and diiodo α-Bgt, and very nearly approximates that expected for mass action binding to a homogeneous population of sites, i.e., 90% bound at 0.1 × IC_{50} and 10% at 10 × IC_{50}. In contrast, the slopes of the log–probit plot for monoiodo and diiodo α-Bgt are more shallow, diverging from the data for native α-Bgt at higher concentrations. When rat brain CMF [^3H]-α-Bgt binding sites are challenged with native, monoiodo, and diiodo α-Bgt (Figure 5b), differences in competition effectiveness are not manifest and there is no obvious difference in slope of the log–probit plot for the different α-Bgt derivatives. Data from several experiments as exemplified in Figures 5a and 5b are summarized in Table I. Relative ratios of apparent IC_{50} values for competition against [^{125}I]-α-Bgt and [$^{125}I_2$]-α-Bgt binding sites in rat brain CMF show that native α-Bgt is three to five times more effective than iodinated α-Bgt. However, when [^3H]-α-Bgt is used to label sites, there are no significant differences evident in binding to rat brain CMF in the presence of native, monoiodo, or diiodo α-Bgt. An additional feature of the data (Table I) is that native toxin is about two times more effective in competing for [^{125}I]-α-Bgt sites than for [^3H]-α-Bgt sites, and about five times more effective in competition for [$^{125}I_2$]-α-Bgt specific binding.

When toxin binding to membrane fractions from *Torpedo* electroplax tissue is examined, no gross differences in the effectiveness of native, monoiodo, and diiodo α-Bgt toward [^{125}I]-α-Bgt binding are detected (Figure 6). As seen in Table I, this result is characteristic of competition studies using electroplax membranes, independent of the form of the radiolabeled α-Bgt derivative used for binding. The results of a

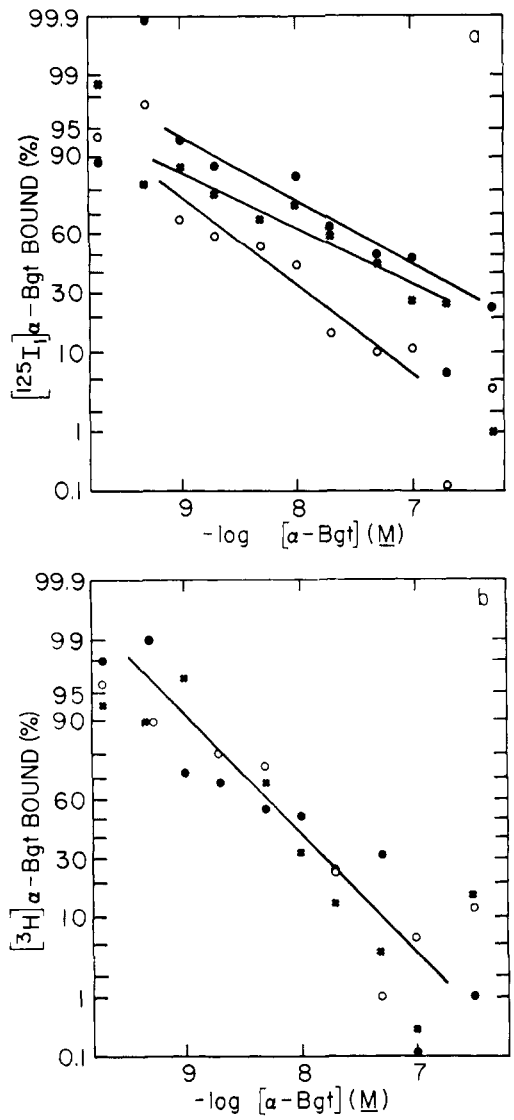

FIGURE 5: (a) [$^{125}I_1$]-α-Bgt binding competition for rat brain CMF sites. Percent specifically bound [^{125}I]-α-Bgt is plotted on a probability scale against the concentration of competing α-Bgt on a logarithmic scale. Assays done in 0.6 mg/mL BSA, 1.06 mg of CMF, 33 mM NaCl, 3 mM $NaHPO_4$, pH 7.4, 10^{-8} M [^{125}I]-α-Bgt, with coincubation in the presence of native (○), monoiodinated (X), or diiodinated (●) α-Bgt derivatives for 60 min at 21 °C followed by 30 min chase in 10^{-6} M native α-Bgt. Solid lines represent least-squares linear regression analysis of data for each toxin derivative. See Table I for average estimate of error in IC_{50} value. (b) [^{3}H]-α-Bgt binding competition for rat brain CMF sites. Percent specifically bound [^{3}H]-α-Bgt is plotted on a probability scale against concentration of competing α-Bgt on a logarithmic scale. Assays in 30 mM NaCl, 2 mM KCl, 0.6 mM $CaCl_2$, 0.4 mM $MgSO_4$, 11 mM Tris, pH 7.4, 0.6 mg/mL BSA, 0.85 mg of CMF, 10^{-8} M [^{3}H]-α-Bgt, coincubated for 60 min with native (○), monoiodinated (X), or diiodinated (●) derivatives, followed by a 30-min chase with native α-Bgt at 10^{-6} M, at 21 °C. Solid line represents least-squares linear regression of pooled data. See Table I for average estimate of error in IC_{50} value for each derivative.

single experiment examining competition effectiveness of α-Bgt derivatives toward [^{125}I]-α-Bgt (6.2 nM) binding to solubilized nAChR from *Torpedo* electroplax membranes indicate that native α-Bgt is only marginally more effective than either of the iodinated derivatives. IC_{50} values are 5.0 nM for native toxin, 6.0 nM for monoiodo α-Bgt, and 7.5 nM for diiodo α-Bgt.

Data were also acquired on the affinity of radiolabeled α-Bgt derivatives for specific binding sites in brain CMF. Least-squares linear regression analysis of double-reciprocal plots for binding saturation data pooled from two experiments yield

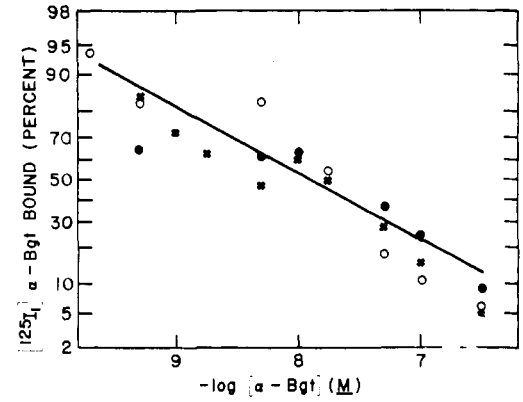

FIGURE 6: [^{125}I]-α-Bgt binding competition for *Torpedo* electroplax membranes. Percent specifically bound [^{125}I]-α-Bgt is plotted on a probability scale against concentration of competing α-Bgt on a logarithmic scale. Assays in 33 mM NaCl, 3 mM Na_2HPO_4, pH 7.4, 2 μg of membrane protein, 0.6 mg/mL BSA, 1.7×10^{-8} M [^{125}I]-α-Bgt, coincubated with native (○), monoiodinated (X), or diiodinated (●) α-Bgt derivatives for 60 min at 21 °C followed by 30 min chase with native α-Bgt. Membranes washed in presence of 50 mg of inert membranes from rat spleen, liver, and blood. Solid line represents least-squares linear regression of pooled data. See Table I for average estimate of error in IC_{50} for each derivative.

values of apparent K_D (nM ± standard error) of 0.9 (±0.2) for [^{3}H]-α-Bgt, 1.1 (±0.1) for [^{125}I]-α-Bgt, and 7.7 (±1.4) for [$^{125}I_2$]-α-Bgt. Corresponding K_D^{app} values for toxin binding to electroplax membranes are 7.9 (±1.3), 9.0 (±2.2), and 7.9 (±0.9) nM for [^{3}H]-α-Bgt, [^{125}I]-α-Bgt, and [$^{125}I_2$]-α-Bgt, respectively.

Lethality. Another control for the appropriateness of radiolabeled α-Bgt as a specific tag for putative nAChR sites is to test biological activity by animal lethality. The results of these studies (Table II) show no significant difference in toxicity of [^{3}H]-α-Bgt, monoiodo α-Bgt, or diiodo α-Bgt relative to native toxin, and certainly not the three- to fourfold difference as determined by binding studies to brain CMF.

Discussion

Column-purified native, monoiodinated, diiodinated, and tritiated α-Bgt derivatives may be distinguished on the basis of their UV and CD spectra, and in their apparent affinity for rat brain α-Bgt binding sites. In contrast, the α-Bgt derivatives show no significant differences in animal lethality studies or in their affinity for nAChR sites in *Torpedo* electroplax. The effects of modification by iodination of exposed tyrosine residues on α-Bgt are apparently reversible, since tritiated α-Bgt prepared from monoiodo α-Bgt possesses spectroscopic and functional properties indistinguishable from those of native toxin.

The results suggest that progressive iodination of an available tyrosine on α-Bgt may cause an alteration in the conformation of the molecule. The primary evidence for this conclusion comes from examination of the CD spectra. At wavelengths from 250 to 350 nm, the CD spectral properties of proteins are dominated by nonpeptide side-chain chromophores, namely, tyrosine, tryptophan, and disulfide residues. From 250 to 290 nm, the CD spectra for α-Bgt undergo a progressive shift to a more negative $\Delta\epsilon$ on addition of one, and then a second iodine to the native molecule. This result suggests that a near-UV circular dichroism is altered progressively as iodine atoms are incorporated into the tyrosine ring structure, and the CD changes would appear to be attributable to primary effects of iodination on tyrosine. However, from 290 to 350 nm, the distinctive CD spectra for diiodo α-Bgt emerge from that

TABLE I: Binding Competition Parameters.

Membrane fraction	Radiolabeled derivative	Nonradiolabeled competitor[a] IC_{50} Monoiodinated	Nonradiolabeled competitor[a] IC_{50} Diiodinated	Native toxin[b] $(IC_{50})^{-1}$
Rat brain CMF	[^{3}H]-α-Bgt	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1
	[^{125}I]-α-Bgt	2.9 ± 1.1	7.4 ± 3.5	1.6 ± 0.4
	[$^{125}I_2$]-α-Bgt	4.0 ± 1.5	5.2 ± 2.4	4.5 ± 2.2
Torpedo electroplax	[^{3}H]-α-Bgt	1.1 ± 0.3	1.5 ± 0.8	1.0 ± 0.2
	[^{125}I]-α-Bgt	0.8 ± 0.2	0.9 ± 0.3	1.0 ± 0.5
	[$^{125}I_2$]-α-Bgt	0.8 ± 0.5	1.5 ± 0.5	1.5 ± 0.4

[a] IC_{50} values are normalized to the apparent IC_{50} value of 1.0 for native α-Bgt competition toward radiolabeled toxin. An apparent IC_{50} value of 4.0 indicates that competitor is four times less effective in preventing radiolabeled toxin binding than native toxin. [b] $(IC_{50})^{-1}$ values are normalized to the apparent $(IC_{50})^{-1}$ value of 1.0 for native α-Bgt competition toward [^{3}H]-α-Bgt. An apparent $(IC_{50})^{-1}$ value of 4.0 indicates that native toxin is four times more effective toward preventing radiolabeled iodinated toxin binding than it is toward preventing [^{3}H]-α-Bgt binding. Hence reciprocal IC_{50} is recorded to facilitate comparison with data in columns 3 and 4 since it conserves competition potency relationships. Relative IC_{50} values are determined from least-squares linear regression analysis of log-probit plots, for binding competition effectiveness of nonradiolabeled α-Bgt derivatives toward radiolabeled α-Bgt derivative specific binding. All normalized IC_{50} values are mean ± standard error of the mean for no fewer than three replicate experiments. Solvent conditions varied between the extremes as given in Figures 5 and 6, but were not found to perturb apparent IC_{50} values significantly.

TABLE II: Lethality.[a]

	Native	Mono-I	Di-I	[^{3}H]
Time to death (min)	39.4 + 9	45.8 ± 9.7	36.8 ± 7.8	38.3 ± 5.1
Apparent dose (μg/g)	0.54 ± 0.17	0.41 ± 0.13	0.57 ± 0.14	0.53 ± 0.09

[a] Summary of toxicity studies using labeled α-Bgt derivatives. Thirty- to forty-gram mice (n = 8) were injected subcutaneously with α-toxin at 0.5 μg/g of mouse and time to death was recorded. From a standard curve for log time to death vs. dose of native α-Bgt generated from data taken for 50 mice, which is linear over the range from 0.1 to 2.0 μg/g, apparent dose (μg/g) is determined.

for monoiodo and native toxin abruptly. Thus, the observed changes in near-UV dichroic properties could be due to superposition of several effects, including local disruption of the tryptophan chromophore, and long-range structural perturbations as well as modification of the tyrosine chromophore.

The origin of the unique CD band seen at 230 nm for α-type neurotoxins has been attributed to non-peptide chromophore and/or β-turns in α-toxin peptides (Chen et al., 1976). The positively dichroic signal seen for α-Bgt in this region is obliterated on iodination to diiodo α-Bgt, in a manner which might reflect partial, localized denaturation of α-Bgt, as is seen upon selective reduction of the disulfide group responsible for maintaining the tight extra loop in the long neurotoxins (Hanley, unpublished results). Presently, we cannot distinguish this possibility from those in which the dichroism effects at 230 nm are fully attributable to a primary effect of iodination on tyrosine itself, or where there are shifts in counteracting chromophore contributions that produce the observed iodinated toxin spectra.

While there is more uncertainty as to whether the CD differences evident in the near- and mid-UV are attributable to shifts in nonpeptide chromophores, or to disruptive effects on local polypeptide structure, the changes in spectra in the far-UV are largely dominated by secondary structure contributions and thus are more appropriate to monitor conformational changes. The 50% reduction in signal strength at 210 nm on iodination of α-Bgt would be consistent with diminution in secondary structure. The most striking effect is seen for diiodo α-Bgt, which exhibits markedly negative dichroism at 190–198 nm, consistent with a significant addition of random coil to the observed spectrum (Greenfield & Fasman, 1969). While the possibility remains that there may be chromophoric group contributions to the observed CD differences, given the magnitude and wavelength distribution of these effects, we favor the interpretation that iodination of α-Bgt introduces both local structural changes in the vicinity of nonpeptide chromophores, and perturbations in secondary structure.

That the iodination-induced structural changes in α-Bgt, suggested by the spectroscopic analysis, produce alterations in the functional characteristics of the toxin is demonstrated by the results of α-Bgt binding experiments. Iodinated toxin is less effective than native α-Bgt toward competition for [^{125}I]-α-Bgt and [$^{125}I_2$]-α-Bgt binding sites in rat brain crude mitochondrial fraction membranes, suggesting either that iodinated α-Bgt is occupied at nonspecific sites, thus reducing the free iodotoxin concentration, or that it has diminished affinity for specific sites in brain. Although there may be complicating effects due to the higher nonspecific adsorptivity of iodinated toxins to membrane preparations derived from rat brain, the binding saturation experiments described above demonstrate the *specific* nature of the alteration in binding characteristics of α-Bgt induced by iodination, particularly for incorporation of two iodine atoms. One of several simple mechanisms that might account for these observations and serve as a tentative model for a central nervous system nAChR assumes that there are two toxin binding sites in close proximity per functional receptor unit (see, also, Maelicke et al., 1977). While ternary iodinated toxin–receptor–iodinated toxin complexes are not strictly forbidden—specific levels of [^{125}I]-α-Bgt and [$^{125}I_2$]-α-Bgt binding at equilibrium are virtually identical with levels of [^{3}H]-α-Bgt binding to rat brain CMF fractions—they are unstable relative to ternary complexes including one or no iodinated toxin molecules. Thus, at a given concentration of radiolabeled iodinated α-toxin, native toxin would appear to be more potent than nonradiolabeled iodinated α-Bgt toward competition for specific binding sites. Since [^{3}H]-α-Bgt closely resembles native toxin, ternary [^{3}H]-α-Bgt–receptor–[^{3}H]-α-Bgt complexes will also be stable, and no *detectable* differences in competition potencies of native and nonradiolabeled iodinated toxin will be manifest. One feature of the mechanism is that ternary complex formation itself might be responsible for the formation of long-lived, "irreversible" toxin–receptor complexes; while toxin sites are homogeneous on free receptor molecules, apparent positive cooperativity of α-Bgt binding or apparent evidence for

preexisting nonequivalent sites may not actually reflect functional properties of the receptor molecule per se, but reflect instead the relative stability of toxin–toxin interaction-mediated ternary complexes over binary toxin–receptor complexes. The implication is that modification of α-Bgt at sites removed from the receptor binding active site might cause loss of the "irreversible" nature of toxin binding by hindering the stabilizing toxin–toxin interaction. Experiments that may distinguish this possibility from one where the modifiable tyrosine residue is at or near the receptor binding active site, such as identification of the precise location of that residue in the primary sequence of α-Bgt and characterization of the kinetics of α-Bgt derivative binding to peripheral and central nAChR, are in order.

While iodinated toxin is less effective than native α-Bgt in binding to putative CNS nAChR in both competition and binding saturation experiments, native and monoiodo α-toxin are only marginally, but not significantly, more effective than diiodo α-Bgt toward binding *Torpedo* nAChR. Taken together, these results suggest that the two receptor types might differ in their toxin binding properties.

In summary, modification by iodination of tyrosine on α-Bgt may cause structural alterations in the toxin, affecting its biological activity. Chemical derivatives of α-toxin may be used as subtle probes for characterization of nicotinic acetylcholine receptor site microheterogeneity and differences between central and peripheral nAChR.

Acknowledgments

The authors would like to express appreciation to Ms. Marie Hebert for technical assistance in the lethality studies and in preparation of α-Bgt, to Mr. Ray Aune of the Health Chemistry Division of the Lawrence Berkeley Laboratory for assistance in tritiation of α-Bgt, to Dr. K. Sauer for a critical reading of the manuscript, to Mr. Hiromi Morimoto for useful discussion, and to Ms. Beth Klingel and Lois Soulé for typing the manuscript.

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