Reductive Methylation as a Tool for the Identification of the Amino Groups in α-Bungarotoxin Interacting with Nicotinic Acetylcholine Receptor†

Jose-Carlos Garcia-Borron,‡ Allan L. Bieber,§ and Marino Martinez-Carrion*,‡,∥

Department of Biochemistry, Virginia Commonwealth University, Richmond, Virginia 23298, and Department of Chemistry, Arizona State University, Tempe, Arizona 85287

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ABSTRACT: α-Bungarotoxin (αBgt) is a postsynaptic neurotoxin which blocks cholinergic transmission at the neuromuscular junction by binding tightly to the acetylcholine receptor (AcChR). The number of methylation sites in αBgt has been shown to decrease significantly upon binding of the toxin to the AcChR [Soler, G., Farach, M. C., Farach, H. A., Mattingly, J. R., & Martinez-Carrion, M. (1983) *Arch. Biochem. Biophys. 225*, 872–878]. We have compared the chemical reactivities of amino groups in free and AcChR-bound αBgt in an attempt to identify the regions in the αBgt molecule that become masked upon binding to the AcChR. Free αBgt and AcChR-bound αBgt were reductively methylated with formaldehyde and sodium cyanoborohydride, and the rate of modification of each one of the available amino groups was followed by cleaving the methylated toxin with V8 protease and resolving the resulting peptides by reversed-phase, high-performance liquid chromatography. Under conditions of limited reagent availability, five of seven amino groups in free αBgt reacted readily, whereas two other amino groups, probably those corresponding to Lys-51 and Lys-70, displayed lower reactivity. Upon binding to the AcChR, the rates of reductive methylation of residues Ile-1, Lys-26, and Lys-38 were considerably reduced (although to differing extents). The degree of protection was most pronounced for Lys-26. The rates of methylation of the amino groups in all other positions remained unchanged. These results allow further definition of the minimal binding surface of a representative neurotoxin.

The nicotinic acetylcholine receptor (AcChR)[1] is a complex membrane glycoprotein in the postsynaptic membrane of mammalian skeletal muscle which is also found in very high concentrations in the electric organ of certain fish (Conti-Tronconi & Raftery, 1982; Barrantes, 1983). The AcChR from *Torpedo californica* is a receptor protein of apparent molecular weight (M_r) 270000 (Martinez-Carrion et al., 1975) composed of four nonidentical but highly homologous subunits of M_r's 40000 (α), 50000 (β), 60000 (γ), and 65000 (δ) in a 2:1:1:1 stoichiometry (Reynolds & Karlin, 1978; Raftery et al., 1980). Binding of acetylcholine to a specific ligand site located on each α subunit triggers the opening of a short-lived cation channel. This response allows ion translocation through the membrane to occur, thus initiating postsynaptic membrane depolarization.

A number of snake venoms contain potent curaremimetic neurotoxins able to impair AcChR function by competitive inhibition of agonist binding (Chang & Lee, 1963; Weber & Changeux, 1974). Snake venom neurotoxins, because of their specific and tight binding, have been widely used to study AcChR structure and function. In fact, most of our knowledge of AcChR behavior has been derived from studies that used these exquisite probes. Thus, the characterization of the molecular basis for such a strong, quasi-irreversible interaction would contribute to a better understanding of the molecular structure of the AcChR.

It is widely agreed that the interaction of neurotoxins with AcChR involves large complementary surfaces, enabling the establishment of multipoint contacts. However, the information available on the nature and role of individual amino acid residues is still inconclusive. From sequence homology analyses, the highly conserved residues Lys-26, Trp-28, Arg-36, and Gly-37 (numeration based on the αBgt sequence) have been proposed to be essential for toxicity (Karlsson, 1979; Low, 1979). Unfortunately, chemical modification experiments designed to confirm and extend this hypothesis often lead to ambiguous conclusions. Due to the likely multipoint nature of neurotoxin binding, chemical modification of selected amino acids, or groups of identical side chains, usually leads to toxin derivatives that retain a considerable degree of biological activity. Consider, for instance, Lys-26 and Arg-36 which are thought to interact with the AcChR. Modification of Lys or Arg residues, even by techniques that abolish the original charge on the residue and/or introduce bulky substituents, results in toxin derivatives displaying considerable residual toxicity (Lobel et al., 1985; Martin et al., 1983). Also, acylation of αBgt Lys-51 decreases the apparent affinity of the toxin for the AcChR approximately 20-fold (Babbitt & Huang, 1985), whereas biotinylation of α-cobratoxin Lys-23 only reduces affinity by a factor of 10 (Lobel et al., 1985). However, Lys-51 has not been implicated in interactions with the AcChR, whereas cobratoxin Lys-23 (corresponding to αBgt Lys-26) has been suggested to contribute to the binding

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* Correspondence should be addressed to this author.

‡ Virginia Commonwealth University.

§ Arizona State University.

∥ Present address: School of Basic Life Sciences, University of Missouri—Kansas City, Kansas City, MO 64110–2499.

[1] Abbreviations: Bgt, bungarotoxin; Me-Bgt, reductively methylated bungarotoxin; M-Bgt, membrane-bound bungarotoxin; S-Bgt, free bungarotoxin; AcChR, acetylcholine receptor; CmC, (carboxymethyl)cysteine; DMK, dimethyllysine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; PITC, phenyl isothiocyanate; PTC, phenylthiocarbamyl; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DEAE, diethylaminoethyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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surface. On the other hand, binding of a neurotoxin to the AcChR surface is expected to lower the chemical reactivity of the amino acid side chains interacting with the AcChR, since the proximity of the receptor should make them less accessible to the reagents (Balasubramaniam et al., 1983). Therefore, a comparison of the chemical reactivities of selected side chains of toxin in free and bound form could be useful for the direct identification of the amino acids interacting with the AcChR.

α-Bungarotoxin (αBgt), a long-chain neurotoxin isolated from the venom of *Bungarus multicinctus*, has 74 residues in its sequence and contains 7 amino groups susceptible to reductive methylation. Previous work from this laboratory has shown that, unlike other chemical modifications, reductive methylation of αBgt only slightly reduces the binding ability of the toxin (Calvo-Fernandez & Martinez-Carrion, 1981). Moreover, when αBgt is bound to AcChR membranes, the number of methylation sites is reduced (Soler et al., 1983). Thus, methylation studies of soluble and receptor-bound αBgt seem well suited for the characterization of those structural regions of the toxin that are masked by binding to the AcChR. We have subjected αBgt, in its free and receptor-bound states, to reductive methylation. The methylated derivatives were then cleaved with V8 protease. The resulting peptides were isolated by reversed-phase HPLC (RP-HPLC), and the degree of methylation and rates of chemical modification were assessed and compared in an attempt to further characterize the structural domains and some of the specific residues involved in the binding to AcChR of a representative neurotoxin. The general methodology developed in this paper may be of interest for the study of the binding of other high-affinity polypeptide effectors to their corresponding membrane receptors.

Materials and Methods

Excised electroplax tissue from *Torpedo californica* was purchased from Pacific Biomarine Supplies Co. (Venice, CA) and was stored at approximately −70 °C until needed. *Bungarus multicinctus* venom was obtained from Miami Serpentarium Laboratories (Salt Lake City, UT). ^{14}C-Labeled formaldehyde was from New England Nuclear. V8 protease was purchased from Miles Scientific (Naperville, IL). The lyophilized product was dissolved in water to give a protein concentration of 1 mg/mL and was stored frozen at −20 °C. Trifluoroacetic acid (TFA) and phenyl isothiocyanate (PITC), both Sequanal grade, were from Pierce. All other reagents were of the highest purity available from either Sigma, Aldrich, or Fisher, and unless otherwise indicated, they were used without any further purification.

Preparation of AcChR-Enriched Membranes. AcChR-enriched and alkaline-extracted membranes were prepared by a modification of published procedures (Neubig et al., 1979; Elliot et al., 1980; Lindstrom et al., 1980; Soler et al., 1984). Frozen electroplax tissue (150 g) was sliced into 1-cm cubes, placed in 300 mL of ice-cold homogenization buffer (10 mM Tris-HCl, 400 mM NaCl, 10 mM NaN_3, 5 mM EDTA, 5 mM iodoacetamide, and 5 mM PMSF, pH 7.6), and homogenized twice in a Brinkmann Polytron for 90 s at a power setting of 7. The homogenate was centrifuged for 10 min at 3500 rpm in a Sorval GS3 rotor. The pellet was homogenized again in 100 mL of buffer and centrifuged as before. The combined supernatants were filtered through four layers of cheesecloth and centrifuged for 30 min, at 30000 rpm, in a Beckman type 35 rotor. The supernatant solution was discarded and the pellet resuspended in 100 mL of 34% sucrose in 10 mM sodium phosphate, 0.2% NaN_3, 400 mM NaCl, and 1 mM EDTA, pH 7.4. From this suspension, 25-mL aliquots were layered onto 20 mL of 50% sucrose in the same buffer and centrifuged for 2 h at 30000 rpm in the Beckman 35 rotor. The membrane fragments collecting at the gradient interface were pooled, diluted 4-fold with 10 mM phosphate, 100 mM NaCl, and 0.02% NaN_3, pH 7.4, and centrifuged for 30 min at 30000 rpm in a type 35 Beckman rotor. The resulting pellet was resuspended in 10 mL of the latter buffer, diluted with 6 volumes of water, and adjusted to pH 11 by addition of 1 M NaOH while gently stirring. The alkaline suspension was continuously stirred for 1 h at 4 °C and then centrifuged in the Beckman 35 rotor for 30 min at 30000 rpm. The alkaline-extracted membrane fragments were resuspended in 100 mM Hepes buffer, pH 7.6, to a final protein concentration of 10 mg/mL. Protein concentrations were determined by the method of Lowry (Lowry et al., 1951), with bovine serum albumin as a standard. Specific αBgt binding activities were determined by the DEAE filter disk assay (Schmidt & Raftery, 1973) and typically ranged from 2 to 3 nmol of αBgt bound/mg of protein.

Purification of αBgt. αBgt was purified from *Bungarus multicinctus* venom according to a previously published procedure (Mebs et al., 1972). The procedure involves (i) chromatography on CM-Sephadex, (ii) desalting of the αBgt fractions on a Sephadex G-25 column, and (iii) rechromatography on a second CM-Sephadex column. Protein eluting from the second CM-Sephadex column as a single peak was lyophilized, redissolved in distilled water to a final concentration of 5 mg/mL, and stored frozen at −20 °C. The concentration of the toxin was determined spectrophotometrically by using an extinction coefficient at 279 nm of 10500 M^{-1} cm^{-1} and assuming a molecular weight of 8000. Typically, from 10 to 20 mg of toxin was obtained from 200 mg of venom. Toxin preparations obtained by this procedure were judged homogeneous by the following criteria: (i) amino acid analysis, (ii) appearance of a single band in 5–20% polyacrylamide gel electrophoresis, and (iii) appearance of a single peak on an analytical RP-HPLC column after reduction and carboxymethylation.

Reductive Methylation. Reductive methylation of αBgt was performed by previously published procedures (Calvo-Fernandez & Martinez-Carrion, 1981; Soler et al., 1983), with minor modifications. The commercial ^{14}C-labeled formaldehyde employed throughout this study had specific activities of 40–50 mCi/mmol and was freed of anionic impurities by chromatography on small (0.2–0.4 mL) columns of Dowex 1-X8 in the acetate form (Jentoft & Dearborn, 1979). Around 70% of the total radioactivity was recovered. Fresh solutions of unlabeled formaldehyde were prepared by hydrolysis of paraformaldehyde (Kodak). Sodium cyanoborohydride was recrystallized by a published procedure (Jentoft & Dearborn, 1979) and used immediately.

In a typical methylation experiment, the required amount of AcChR membranes (8–10 mg/mL protein in 100 mM Hepes, pH 7.5) was mixed with a 2-fold excess of αBgt. After 30-min incubation at room temperature, the reagents were added, and the membrane suspension was diluted with water to achieve the following final concentrations: 4–5 mg/mL protein, 60 mM $NaCNBH_3$, 35–40 mM CH_2O (specific activity 0.3–0.5 mCi/mmol), and 50 mM Hepes, pH 7.5. Care was taken to add $NaCNBH_3$ before formaldehyde. The reaction was allowed to proceed at room temperature, and then AcChR membranes were separated by centrifugation at 40000 rpm in a Beckman 50.2 Ti rotor. The supernatant solution, containing the unbound αBgt, was chromatographed in a Sephadex G-25 column equilibrated and eluted with 50 mM

ammonium acetate adjusted to pH 6.9 with acetic acid. The membrane pellet was washed by resuspension in 50 mM Hepes, pH 7.5, and centrifugation. The labeled, membrane-bound, toxin was released from AcChR by resuspension of the membrane pellet in 0.2 M glycine hydrochloride buffer, pH 2.3, incubation at room temperature for 60 min with occasional stirring, and centrifugation. The membrane pellet was discarded, and the supernatant containing the released toxin was chromatographed as described above.

When the kinetics of methylation were followed, aliquots were withdrawn from the reaction mixture at selected times, usually 5, 10, 15, 60, and 120 min, and the reaction was quenched by addition of 1.5 M Tris-HCl buffer, pH 7.9, to a final concentration of 0.6 M. Aliquots were kept at 4 °C until collection of samples was completed. Free αBgt and bound αBgt were obtained and treated as described above, except that excess reagent was removed by extensive dialysis against 50 mM ammonium acetate, pH 6.9, in SpectraPor 6 membranes, molecular weight cutoff 1000, rather than by gel filtration chromatography.

S-Carboxymethylation. Freeze-dried αBgt samples were dissolved in 3 mL of 8 M urea, 20 mM DTT, and 0.1 M Tris-HCl buffer, pH 8.0, to a final concentration of 0.05–0.1 mg/mL and incubated for 150 min, at room temperature, under an argon atmosphere. Solid iodoacetamide was added to a final concentration of 50 mM, and the samples were incubated under argon, in the dark, for 30 min. The reaction was then quenched by the addition of excess 2-mercaptoethanol. The carboxymethylated toxins were either chromatographed on Sephadex G-25 or dialyzed against 50 mM ammonium acetate buffer, adjusted to pH 6.9 with acetic acid, and lyophilized.

Protease Digestion of Methylated and S-Carboxymethylated αBgt. αBgt was dissolved in 0.05 M NaH_2PO_4, pH 7.8, at concentrations ranging from 0.2 to 1 mg/mL. The required volume of V8 protease (1 mg/mL in water) was added to give a final toxin to protease ratio of approximately 50:1. Digestion was allowed to proceed overnight at room temperature.

HPLC Separation of V8 Protease Digests. Resolution of V8 digestion peptides was achieved by reversed-phase HPLC, on an Altex gradient HPLC system, with a Bio-Rad HiPore 318 column (25 cm × 4.6 mm). The equilibration solvent, 0.1% trifluoroacetic acid (TFA), served as the first solvent for the gradient, and 0.1% TFA in acetonitrile was the second solvent. Gradient conditions are specified in the corresponding figure legends. Peptides were detected by their absorbance at 214 nm. The eluting peptides were collected manually, dried in a Speed Vac centrifuge, and stored at −20 °C until needed for amino acid analysis or for radioactivity measurements.

Amino Acid Analysis. Two procedures were employed. The first involved preparation of the phenylthiocarbamyl amino acids (PTC-amino acids) as described by Heinrikson and Meredith (1984). Derivatives were resolved at 45 °C on an Altex RP-PTH amino acid column (25 cm × 4.6 mm). Solvent system III of Heinrikson and Meredith was used. PTC derivatives were detected by the absorbance at 254 nm. The second procedure involved standard separation on ion-exchange columns and postcolumn derivatization with *o*-phthalaldehyde by a published procedure (Peterson et al., 1977). The analyses were performed on a Durrum MBF amino acid analyzer. In both cases, samples were hydrolyzed for 24 h at 105–110 °C in 6 N HCl containing 0.1% phenol.

Other Procedures. SDS–polyacrylamide gel electrophoresis was performed either on exponential gradient slab gels (5–20%) or in 12% gels, with a 3% stacking gel and a discontinuous buffer system (Laemmli, 1970). Absorption spectra were recorded by using a Cary 210 spectrophotometer. Radioactivity measurements were made in 5 mL of scintillation cocktail (3a70B, Research Products, Inc).

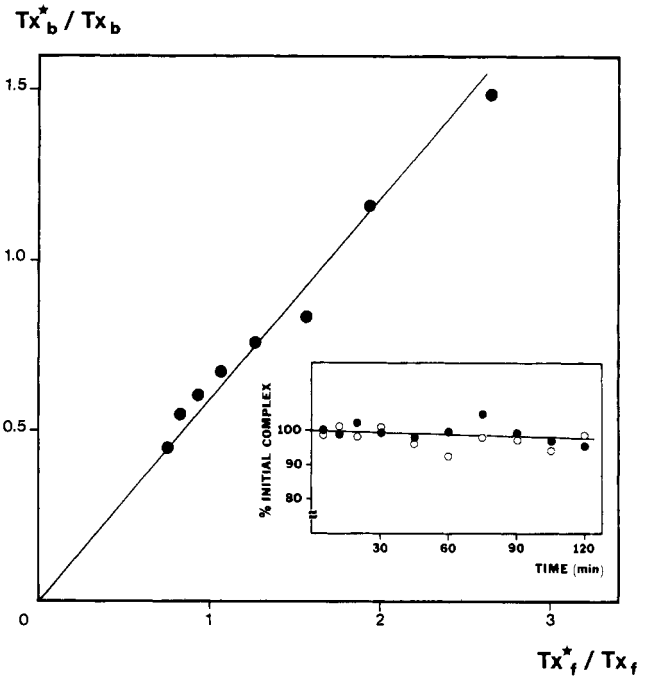

FIGURE 1: Competition of Me-Bgt and native toxin for binding to AcChR. Mixtures of 100 pmol of Me-Bgt and varying amounts of native toxin, ranging from 12 to 200 pmol, were incubated with AcChR membranes (35 pmol of toxin binding sites) for 2 h in a volume of 0.125 mL of 10 mM Hepes/100 mM $NaNO_3$, pH 7.5, containing 1.2% Triton X-100. The amount of Me-Bgt bound to the AcChR was determined by the DEAE filter disk assay. The amounts of free M-Bgt and free and bound native toxin were calculated from the known concentrations of total toxins, total receptor binding sites, and bound Me-Bgt. [Tx*] and [Tx] are the concentrations of methylated and native toxins, respectively. Subscripts b and f refer to receptor-bound and free toxins, respectively. Insert: Displacement of native and methylated receptor-bound Bgt by excess toxin. AcChR membranes were incubated with a 2-fold excess of either native or methylated toxin. Complexes were washed by centrifugation and resuspension in 10 mM Hepes buffer, pH 7.5, containing 100 mM $NaNO_3$. A 2-fold excess of native toxin was added to Me-Bgt–AcChR complexes, and similarly, native toxin–receptor complexes were treated with a 2-fold excess of Me-Bgt. At selected times, aliquots were pelleted in a Beckman Airfuge, for 10 min, at 30 psi. Me-Bgt displacements from AcChR complexes were determined by measuring the radioactiity released in the supernatant. Conversely, native toxin displacement by the Me-Bgt was followed by measuring the radioactivity associated with the membrane pellet. (Closed circles) Me-Bgt–AcChR complex.

RESULTS

Properties of Reductively Methylated αBgt. Treatment of αBgt free in solution (1–2 mg/mL) with 40 mM formaldehyde/60 mM sodium cyanoborohydride at pH 7.5 resulted in essentially quantitative modification of all amino groups available in the toxin. Accordingly, the amino acid composition of the modified toxin showed the disappearance of one Ile residue and six Lys residues (Table I). Monomethyllysine was not detected by any of the amino acid analysis methods employed. The methylation reaction was complete in less than 5 min.

The ability of methylated αBgt (Me-Bgt) to bind to AcChR was evaluated by competition with the native toxin and was only very slightly lower than that of native toxin after complete methylation (Figure 1). Moreover, native toxin was not able

Table I: Amino Acid Analysis of Reductively Methylated, Carboxymethylated Bungarotoxin[a]

amino acid	mol of amino acid/mol of methionine			
	actual	integer	known	difference
D	3.8	4	4	
E	5.4	5	5	
CmC	10.3	10	10	
S[b]	5.8	6	6	
G	3.8	4	4	
H	1.7	2	2	
T[b]	5.8	6	7	−1
A	5.1	5	5	
R	3.7	4	3	+1
DMK[c]	7.4	7	0	+7
P	9.2	9	8	−1
Y	1.9	2	2	
V[d]	5.3	5	5	
M	1.0	1	1	
I	1.1	1	2	−1
L	2.1	2	2	
F	1.0	1	1	
K	0.4	0	6	−6

[a] Determined by PITC precolumn derivatization. [b] Extrapolated to zero time from 24-, 48-, and 72-h hydrolyses. [c] Calculated on the basis of a proline standard. [d] Value for 40-h hydrolysis. Value at 24 h was 3.5, probably by resistance of the V–V bond at position 39–40 in the αBgt sequence.

to displace Me-Bgt from the receptor binding sites when incubated with a preformed AcChR–Me-Bgt complex over a 120-min period at room temperature (Figure 1, insert).

V8 Cleavage of Reductively Methylated αBgt. At pH 7.8, V8 protease is expected to cleave a polypeptide chain at the carboxylic side of both glutamic acid and aspartic acid residues (Drapeau, 1977). There are six possible cleavage sites along the αBgt molecule that should give rise to six different peptides, owing to the fact that two Glu residues are located side by side at positions 55 and 56.

Figure 2 shows the separation of the products obtained by V8 digestion of a fully methylated, reductively alkylated toxin obtained by RP-HPLC. Digestion was completed after overnight incubation at room temperature, as judged by the disappearance of the αBgt peak and by the stability of the peptide pattern to further additions of the protease. The peptide pattern was characterized by five major peaks, labeled I–V in the bottom trace of the chromatogram in Figure 2. Peptide fragments were identified by amino acid analyses of the manually collected peaks. The assignments were as follows: I, fragment 57–74; II, 31–41; III, 42–55; IV, 1–20; V, 21–30. Taking into account the appearance of five peaks, instead of six, and the amino acid composition of the resolved peptides, it is not likely that cleavage occurred to any significant extent at Asp-63. An alternative explanation, that peptides 57–63 and 63–74 are present but coelute, is unlikely since these two peptides differ markedly in hydrophobicity and a number of different gradients were used in unsuccessful attempts to isolate two fragments.

Reductive Methylation of αBgt in the Presence of AcChR Membranes. In order to study the possible protection of specific amino groups in αBgt when bound to AcChR, methylation was performed with mixtures containing a 2-fold excess of αBgt over AcChR total binding sites. After completion of the reaction, the labeled αBgt in solution (excess over AcChR binding sites, S-Bgt) was separated from Bgt–AcChR complexes by centrifugation. On the other hand, a number of treatments were tested to achieve the release of the membrane-bound, methylated toxin (M-Bgt). Thermal denaturation of the complexes at 64 °C resulted in low yields of released M-Bgt (Bieber et al., 1986). However, quantitative

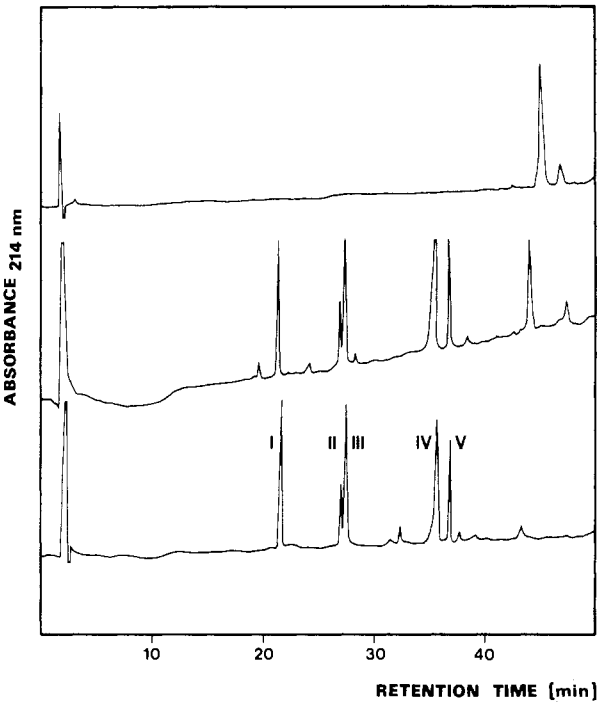

FIGURE 2: RP-HPLC separation of the products resulting from V8 digestion of Me-Bgt. Aliquots of undigested or V8-treated, reductively methylated, and S-carboxymethylated α-Bgt in 50 mM phosphate buffer, pH 7.8, were injected in a Bio-Rad HiPore RP 318 column (25 cm × 4.6 mm) operated at a flow rate of 1.5 mL/min. Gradient conditions: from solvent A (0.1% TFA) to 30% solvent B (0.1% TFA in acetonitrile) in 44 min; ramp to 100% B in 4 min, to 100% A in 4 min, and 8-min equilibration in 100% solvent A. Top chromatogram, undigested Bgt. Middle chromatogram, approximately 1 nmol of Me-αBgt digested with V8 protease (50:1 αBgt:protease ratio) for 3 h at room temperature. Bottom chromatogram, same after an overnight incubation.

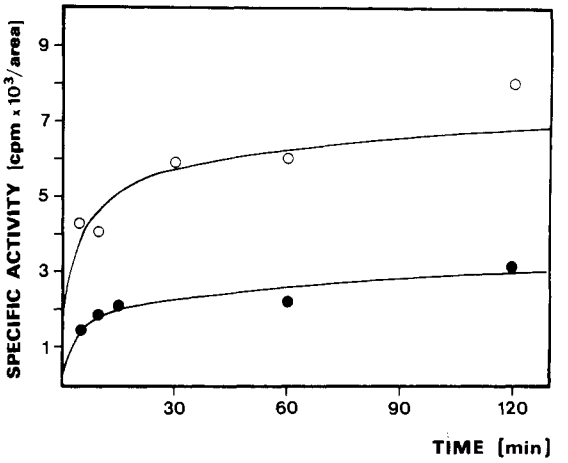

FIGURE 3: Kinetics and labeling of free (○) and AcChR-bound (●) αBgt. Aliquots of undigested M- and S-Bgt, containing approximately 1 nmol of toxin, were counted for radioactivity and submitted to RP-HPLC as shown in Figure 3. The specific activity of the toxin was calculated by taking the integrated area under the αBgt peak as an arbitrary measure of the concentration of toxin.

recovery of M-Bgt was approached by treatment of the complexes with 0.2 M glycine hydrochloride buffer, pH 2.3, for 60 min.

The time course of methylation of M- and S-Bgt was followed by quenching the reaction at selected times by addition of excess Tris-HCl buffer. After carboxymethylation, the specific activity of the toxin samples was determined by counting radioactivity and taking the area under the Bgt peak in an HPLC chromatogram as an arbitrary measure of the

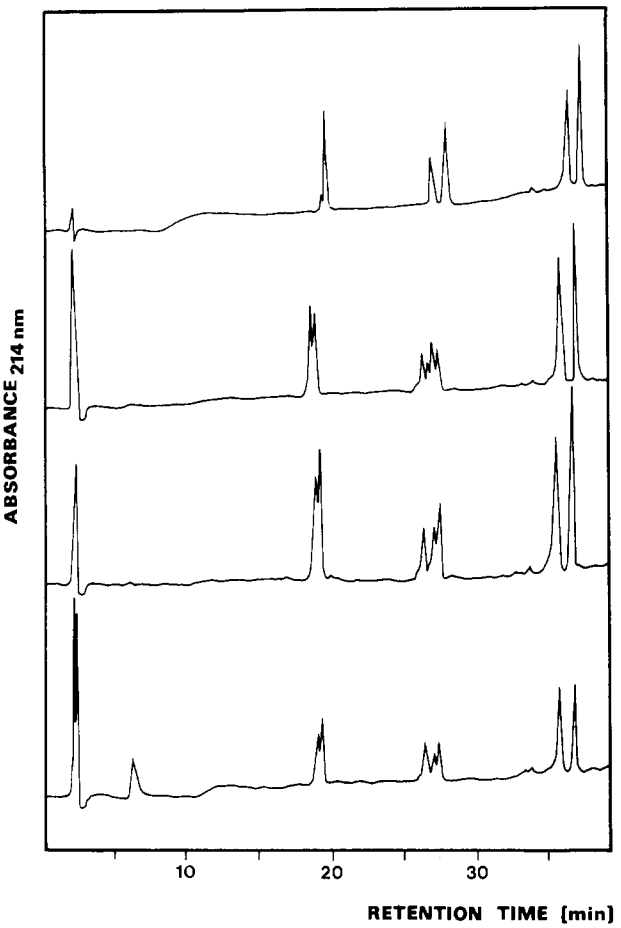

FIGURE 4: RP-HPLC separation of the products resulting from V8 digestion of M- and S-Bgt. A slightly different gradient than the one described in Figure 2 was used to achieve a better separation of peaks II and III: from 0% to 5% solvent B in 2 min; ramp to 15% B in 15 min and to 30% B in 15 min; ramp to 100% B in 4 min and then back to 100% A in 4 min followed by 8-min equilibration. The remaining experimental conditions are identical with those in Figure 3, except that V8 digestion was allowed to proceed overnight for all samples. From top to bottom, control αBgt labeled in solution, excess Bgt labeled for 10 min in the presence of AcChR membranes, same after 120 min of reductive methylation, and membrane-bound αBgt labeled for 120 min.

toxin concentration. As shown in Figure 3, the specific activity of the soluble toxin was higher than that of the receptor-bound Bgt, by a factor that varied roughly from 2 to 3, depending on the reaction time.

V8 Cleavage of M- and S-Bgt. The peptide patterns obtained by V8 cleavage of methylated and reductively alkylated M and S toxins were more complex than those obtained for the toxin modified in the absence of membranes. Moreover, these patterns were dependent on both the reaction time and the concentrations of formaldehyde and cyanoborohydride employed in the methylation reaction. Peptides I, II, and III appeared as doublets. Amino acid analyses of the individual peaks showed that the increased complexity of the peptide pattern was not due to the release of proteins from AcChR membranes nor to an altered protease cleavage. Instead, the doublets corresponded to different degrees of methylation of the same peptide. For each pair, the peak eluting at the longer time corresponded to a more extensively methylated species. Accordingly, for each one of the doublets, the relative proportions of the two components varied with the reaction time, the contribution of the second peak increasing as the reaction proceeded (Figure 4). Moreover, when the concentrations of formaldehyde and cyanoborohydride were increased, the amounts of the more extensively methylated species were higher at a given reaction time. The lower contribution to the overall hydrophobicity of the peptide made by the introduction of two methyl groups can account for the lack of resolution of two or more components for peaks IV and V, since the original hydrophobicity of these peptides is considerably higher than that of peptides I–III.

By contrast, in the absence of AcChR membranes, Bgt was fully methylated under similar reaction conditions. Hence, the appearance of partially methylated peptides certainly reflects the presence of AcChR membrane proteins and amino lipids that readily react with formaldehyde, thereby limiting the availability of substrate for labeling amino groups in the Bgt molecule.

Cleavage at Asp-63 was not found in any of the samples analyzed. Furthermore, cleavage did not occur at this position in S-carboxymethylated Bgt that had not been reductively methylated, so the presence of a dimethyllysine residue at position 64 cannot be the primary cause of the protection of this potential cleavage site. Failure of V8 to cleave other proteins at predicted positions has been previously noticed by others (Drapeau, 1977).

Kinetics of Methylation of Individual αBgt Amino Groups. The kinetics of methylation of amino groups in αBgt, whether free in solution or AcChR bound, were followed over a period of 2 h by either of two strategies, depending on the residue studied. αBgt samples methylated for different times were S-carboxymethylated and cleaved with V8 protease, and the resulting peptides were resolved by RP-HPLC. For those peptides that could be resolved according to their degrees of methylation, the progress of the methylation reaction was assessed by following the increase in the relative amount of the second peak of the doublet which corresponds to the fully methylated peptide. On the other hand, peptides eluting as a single peak were collected, dried, and resuspended in 50 mM phosphate buffer, pH 7.8. An aliquot was then rechromatographed to determine both the recovery and purity of the peptide, and a second aliquot was counted for radioactivity. Reaction progress was assessed by the increase in specific activity of the peptide as a function of time, calculated by dividing the total radioactivity of the sample by the area under the corresponding chromatographic peak.

Peptides II (Lys-38), IV (Ile-1 amino terminus), and V (Lys-26) reacted less rapidly in the receptor-bound toxin than in free αBgt (Figure 5). However, the degree of this protection on binding to AcChR membranes was different in each case. All three residues reacted rapidly in the soluble toxin. The protection of residues Ile-1 and Lys-38 was evident at short reaction times, but the specific activity of the membrane-bound residues approached that of the soluble ones as the reaction proceeded, so that after 30 min of reaction, the extent of methylation was very similar for both free and membrane-bound samples. The reactivity of Lys-26 was affected more dramatically upon binding to AcChR, since the specific activity of the membrane-bound residue remained considerably lower than that of unbound toxin even after 120 min of reaction.

A different situation was found for peptides I (Lys-64,70) and III (Lys-51,52). The specific activity of the components in each doublet remained constant throughout the time period studied, but the relative amounts of the second peptide in each doublet increased with time. The kinetics of methylation of the membrane bound and free species were identical, within experimental error. Amino acid analyses of the individual peptides in each of the doublets were consistent with the second

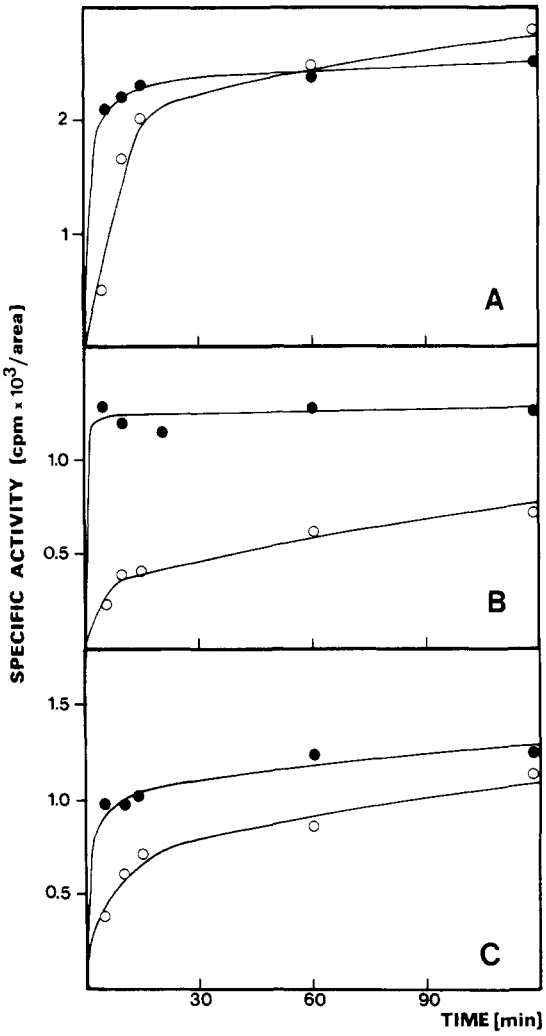

FIGURE 5: Kinetics of labeling of Lys-38 (A), Lys-26 (B), and Ile-1 (C) in M- and S-Bgt. The specific activities of peptides II, V, and IV were determined as described in Figure 3. Open symbols refer to AcChR-bound Bgt and closed symbols to the unbound excess Bgt in solution.

peak in doublet I being a fully methylated species, containing two dimethyllysine derivatives, whereas the second peak in doublet III seemed to contain a dimethyllysine and a monomethylated derivative. However, an accurate quantitation was difficult due to the overlap of the peaks in each doublet, and the limited resolution of Lys from the methyl derivatives in the amino acid analyses. In any case, both the peptide pattern and the amino acid analyses data obtained for peptides I and III are consistent with the presence in each peptide of one Lys residue of lower reactivity than the one observed for the Lys residues in peptides II and V and for the amino-terminal group of αBgt in peptide IV.

Discussion

The curaremimetic neurotoxins have been classified according to length into two groups: the short toxins are 60–62 residues long whereas the long toxins consist of 71–74 amino acids. Toxins belonging to both groups show considerable sequence homology [reviewed in Dufton and Hider (1983)]. They are disk-shaped molecules whose backbone chain is arranged in three loops that extend roughly parallel from a cross-linked core consisting of four highly conserved disulfide bridges. The long toxins have a C-terminal extension of 9–14 residues that contains an additional disulfide linkage, forming a pentapeptide loop. This disulfide bridge can be selectively reduced, at least in α-cobratoxin (Martin et al., 1983). However, the overall conformation of long and short neurotoxins, as well as the microenvironment of most of the residues thought to be important for binding to the AcChR, seem to be very similar.

Many attempts have been made to characterize the binding domains of snake neurotoxins by chemical modification techniques [reviewed in Karlsson (1979) and Low (1979)]. Two general approaches have been employed in these studies. On one hand, purified neurotoxins have been subjected to selective chemical modification of certain amino acid residues, and the toxicity and/or affinity for AcChR of the derivatives have been analyzed. Alternatively, different types of spectroscopic probes have been introduced in known regions of the toxin molecules, and the behavior of the free and AcChR-complexed toxins has been compared. However, these approaches present several inherent problems:

(i) In most cases, an adequate characterization of the conformational effects of chemical modification on the toxin structure has not been attempted. Even if neurotoxins are generally thought to be rigid molecules owing to their highly cross-linked core, many residues, especially those located on the surface and thus able to interact with AcChR, should retain considerable conformational freedom.

(ii) Binding of neurotoxins to AcChR is thought to involve multiple contacts and, possibly, rather large regions in the toxin and receptor molecular surfaces. Thus, toxin derivatives usually retain various degrees of biological activity even after the modification of "essential" amino acids.

(iii) A quantitative assessment of the effects of chemical modification of the activity of neurotoxins is by itself a complex problem. Many chemically modified toxins retain affinities for the AcChR characterized by dissociation constants in the subnanomolar to submicromolar range. Measuring the affinity of such molecules by competition binding experiments does not reflect an equilibrium situation but rather differences in the kinetics of binding. Several factors such as structural rigidity (Endo et al., 1981) and overall electrical charge of the toxin (Endo et al., 1986) have been invoked as factors that influence the rate of toxin association with AcChR. These factors could be altered by chemical modification, especially if the positive charges of Lys residues are neutralized or converted to negative charge by derivatization. Thus, an interpretation of competition binding experiments following chemical modification in terms of equilibrium constants can be misleading.

(iv) The use of spectroscopic techniques to study the behavior of AcChR-complexed toxins after labeling at specific positions with spectroscopic probes is complicated, in many cases, by the lack of appropriate theoretical models to rationalize the experimental data obtained for such a complex system. These problems have been recently emphasized in a study of solute accessibility to fluorescein isothiocyanate labeled cobratoxin bound to AcChR (Johnson & Yguerabide, 1985).

With the above consideration in mind, comparison of the chemical reactivities of selected side chains of neurotoxins in their free and receptor-bound states seems to be a reasonable alternative approach for studies of the intermolecular interactions involved in binding to the AcChR. This type of study should allow a direct identification of the side chains masked upon binding to the AcChR. Reductive methylation of amino groups with formaldehyde and sodium cyanoborohydride (Jentoft & Dearborn, 1979) is a suitable chemical modification technique for attempting such a comparison. It can be per-

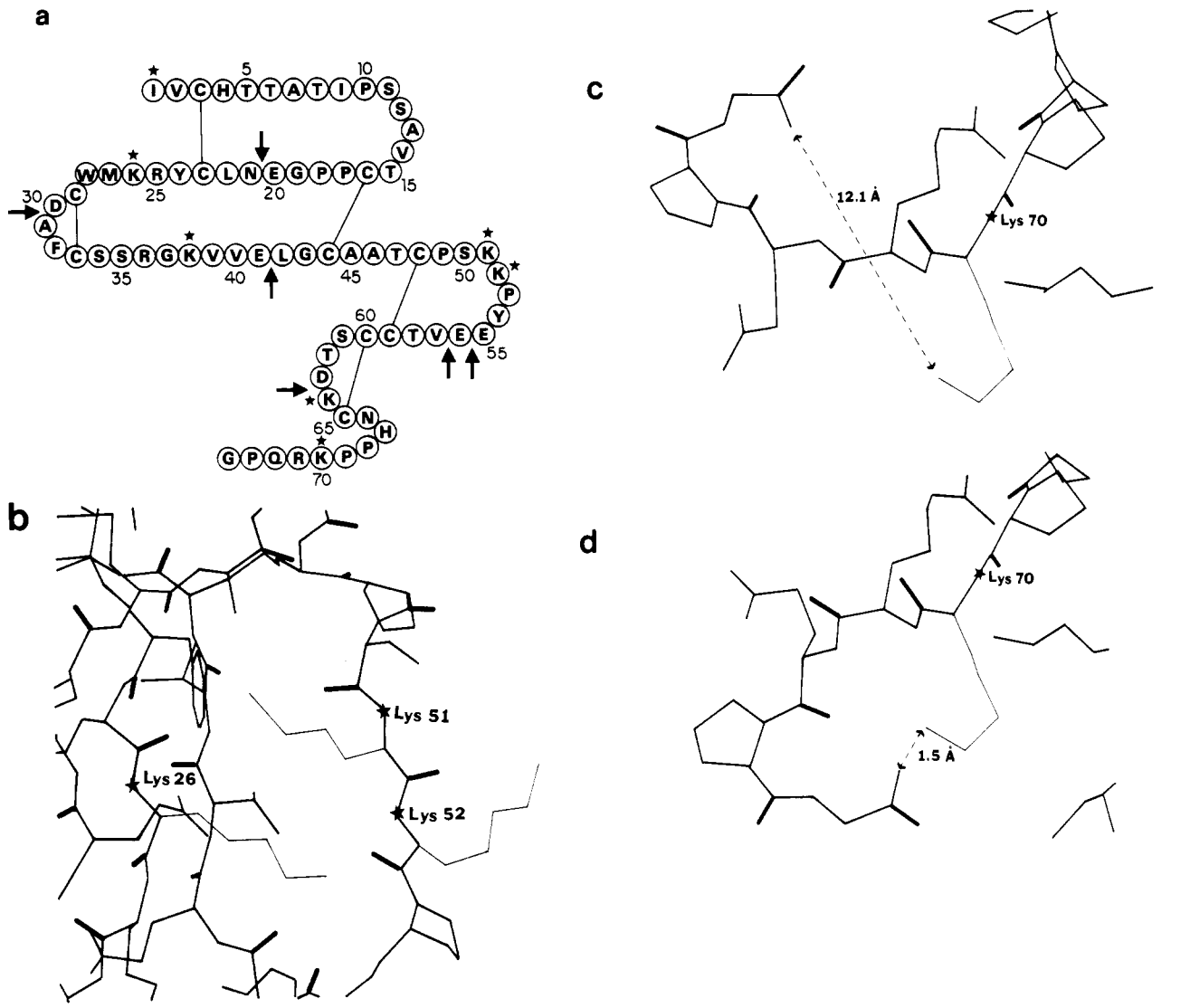

FIGURE 6: (a) Sequence of αBgt (Mebs et al., 1972). Arrows indicate the possible V8 cleavage points at pH 7.8. The reductively methylatable residues are marked by a star. (b) Detail of the crystal structure of Bgt, as deduced from the crystallographic data of Agar and Stroud (1982), showing the pocket containing Lys-26 and -51. Lysine side chains are drawn with a thinner trace, and the thicker trace corresponds to peptidic carbonyls. The position of the amino acidic nitrogen atom of the lysine residues is indicated by a star. (c) Crystal conformation of the C-terminal region of the toxin. (d) Possible conformation of the C-terminal tail of the toxin in solution. The broken line indicates the distance between the ϵ-amino group of lysine-70 and the C-terminus. The figures were generated in an Evans & Sutherland Co. PS330 picture system.

formed under mild conditions; it is highly specific and causes only minor changes in the pK_a values of modified amino groups (Jentoft & Dearborn, 1983). Moreover, all α-type neurotoxins are rich in Lys residues, so that it is possible to simultaneously probe several regions encompassing the total length of the toxin molecule. Finally, binding of αBgt to the AcChR has been shown to result in a reduction of the potential methylation sites in the toxin (Soler et al., 1983). Ideally, the methylation experiments should be performed in a mixture containing AcChR–toxin complexes and excess of unbound toxin in solution, in order to have reaction conditions for soluble and bound neurotoxin that are nearly identical. Such an approach is actually possible, since the binding of αBgt, either native or methylated, is tight enough to prevent any significant exchange between free and bound toxins during the time course of the experiment.

Figure 6a depicts the amino acid sequence of αBgt, and selected regions of the three-dimensional structure of αBgt deduced from the X-ray analysis of Agar and Stroud (1982) are shown in Figure 6b–d. Bgt is a flat molecule with overall dimensions of 40 Å × 30 Å × 20 Å. The dimensions of the three loops characteristic of neurotoxins are 20, 25, and 10 Å with the two longer loops held together by β-sheet hydrogen bonds (Fairclough et al., 1983). Residues with free amino groups are located on the first loop (Ile-1), on both stretches of the second loop (Lys-26 and Lys-38) along the third loop (Lys-51,52 and -64), and, finally, in the C-terminal tail (Lys-70). The free amino groups of Ile-1 and Lys-38, -52, -64, and -70 are on the surface of the molecule, clearly exposed to the solvent. On the other hand, Lys-26 lies in a cavity, where its accessibility to bulky molecules might be restricted. A similar situation is found for the homologous Lys-23 residues of α-cobratoxin. Steric hindrance might account for the low affinity of the biotinylated α-cobratoxin Lys-23 for avidin (Lobel et al., 1985). The uniqueness of the environment of this residue is also reflected by its hyperreactivity toward biotin (Lobel et al., 1985), acetic anhydride (Balasubramaniam et al., 1983), and fluorescein isothiocyanate (Johnson & Taylor, 1982) and the abnormally low pK_a of 7.9 that has been assigned to the homologous Lys-27 in *Naja naja siamensis* neurotoxin III (Tsetlin et al., 1982). Similarly, residue 51 is pointing toward the core of the molecule, where it could be held in position by hydrogen bonds established between one or more of three peptidic carbonyl groups pointing toward the

amino group and located at distances from 2.5 to 3.5 Å (Figure 6b). This residue can be selectively acylated with *N*-hydroxysuccinimide-activated palmitic acid (Babbitt & Huang, 1985).

These unique microenvironments of individual lysines resulted in differences in the kinetics of methylation. When methylation was performed in the presence of AcChR membranes, two types of amino groups could be distinguished by their kinetic behavior. Residues Ile-1, Lys-26, and Lys-38 reacted rapidly and almost quantitatively. This is consistent with Ile-1 and Lys-38 being exposed to the solvent, and with Lys-26 being a reactive residue with an unusually low pK_a. However, peptide III, containing Lys-51 and -52, and peptide I, containing Lys-64 and -70, showed kinetic behavior reflecting rapid methylation of one of the two Lys residues in each peptide, and a much slower methylation of the remaining ϵ-amino group. Moreover, the extent of methylation of the slow reacting Lys residue was different in each case. Amino acid analysis data are consistent with one of the residues in peptide III reacting with only one formaldehyde molecule to give a monomethyllysine derivative, whereas the methylation of the slow reacting residue in peptide I proceeded to yield the dimethyllysine derivative.

These observations can be rationalized in relation to the crystal structure of αBgt. Figure 6b shows the region comprising residues Lys-51 and -52. It seems likely that Lys-52 is the residue that reacts more rapidly since this side chain seems completely exposed to the solvent, with no restrictions to its accessibility. Conversely, the location of Lys-51 in the interior of a cavity could account not only for a lower methylation rate but also for the appearance of a stable monomethylated species. Due to the presence of neighboring side chains, the formation of a second Schiff's base, after the initial addition of a formaldehyde molecule, could easily be prevented by the considerable degree of steric hindrance of the hydrogen-bonded ϵ-amino group. On the other hand, the situation found for peptide I, containing Lys-64 and -70, is less clear, since both residues appear from the crystal structure of the toxin to be exposed to the solvent and protrude from the surface of the molecule. However, Lys-64 is located near the end of the third loop, in a region of the molecule unlikely to display significant conformational freedom, whereas Lys-70 is located in the C-terminal tail, at a distance approximately 12 Å from the carboxyl terminus (Figure 6c). The C-terminal tail of some neurotoxins has been proposed to be a mobile region (Dufton & Hider, 1983), and in the case of αBgt, it is disposed in such a way that rotation around the C_α–C bond corresponding to Arg-71 could bring the carboxyl group of the terminal Gly-74 to within about 2 Å of the ϵ-amino group of Lys-70. This would allow formation of a salt bridge that could further stabilize the solution conformation of the toxin (Figure 6d). Assuming the conformation shown in Figure 6d to be the preferred conformation of the C-terminal region in solution, the increased pK_a of Lys-70 resulting from ionic interaction with the C-terminal carboxylate could easily account for the decreased rate of methylation. In any event, even after the establishment of a salt bridge, the degree of steric hindrance of Lys-70 should be much lower than that found for Lys-51, so that its methylation could proceed to the formation of the dimethyl derivative.

Comparison of the specific activities and rates of methylation of the free amino groups in M- and S-Bgt clearly shows that Lys residues 51, 52, 64, and 70 react to a similar extent and with similar kinetics in both free and AcChR-bound toxin. Therefore, neither the exterior stretch of loop III nor the C-terminal tail of the molecule seems to be implicated in binding to the AcChR. Similar conclusions have been reported by others (Tsetlin et al., 1982; Babbit & Huang, 1985). Moreover, this observation is consistent with the general idea of similar topology in binding to the AcChR of both long and short toxins since the latter do not have the C-terminal extension present in αBgt.

On the other hand, residues Lys-26 and Lys-38 are both protected when the toxin is bound to the AcChR, although the extent of this protection is different, being much more pronounced for Lys-26. Therefore, these residues, located on both stretches of loop II, along with other highly conserved residues thought to be important for binding, seem to be included in the binding surface of the toxin.

The binding surface of neurotoxins is thought to be primarily formed by the concavity formed by loops II and III (Low, 1979). Our results support this view since this region of the toxin includes Lys-27 and -38. However, we have obtained evidence that implicates the amino-terminal Ile-1 in binding to the AcChR. Interestingly, Ile-1 maps near the hinge region which connects loops II and III, and in the crystal structure, the free amino group points in the same direction as the side chain of Lys-38.

Taken together, the results presented allow identification of a minimal binding surface of αBgt as a triangle defined by residues Ile-1, Lys-26, and Lys-38, that encompasses most of the central loop of the toxin. On the other hand, neither the C-terminal extension nor the external stretch of loop III seems to contribute significantly to αBgt binding to AcChR.

The binding of snake toxins to AcChR is an excellent example of a high-affinity interaction between two heterologous proteins. Binding of antibodies to peptidic antigens and peptide hormone recognition by their specific receptors are other examples of such specific, strong interactions. Each of these biological events is thought to involve multiple contacts between the complex interacting molecules. Therefore, we believe that the techniques described in this paper will provide a useful strategy for the study at the molecular level of other heterologous polypeptide interactions.

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Calcium-Promoted DNA Cleavage by Eukaryotic Topoisomerase II: Trapping the Covalent Enzyme–DNA Complex in an Active Form†

Neil Osheroff* and E. Lynn Zechiedrich
Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232
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ABSTRACT: The effects of calcium ions on interactions between *Drosophila melanogaster* topoisomerase II and DNA were assessed. Although the divalent cation could not support DNA strand passage, it was able to promote high levels of enzyme-mediated DNA cleavage. Moreover, sites of cleavage on plasmid pBR322 generated in calcium-promoted reactions were similar to those obtained in the presence of magnesium. When calcium-containing enzyme–DNA mixtures were treated with ethylenediaminetetraacetic acid, cleaved nucleic acids could be generated in the absence of sodium dodecyl sulfate (SDS) or other denaturing detergents. The product of this SDS-independent calcium-promoted reaction was a covalent topoisomerase II–DNA complex. Enzyme molecules trapped in such complexes were found to be kinetically competent. Therefore, calcium should be a valuable tool for studying the enzymology of topoisomerase II mediated DNA cleavage.

Eukaryotic type II topoisomerases alter the topology of DNA by passing an intact helix of DNA through a transient, enzyme-bound, double-stranded break made in a second helix (Wang, 1982, 1985; Vosberg, 1985). The mechanics of the double-stranded DNA passage reaction necessitate that the enzyme be able to cleave and rejoin the nucleic acid backbone in a concerted fashion.

Since the cleavage/religation cycle is central to the function of topoisomerase II, an assay has been developed that allows the DNA cleavage reaction to be studied in vitro (Sander & Hsieh, 1983; Liu et al., 1983). In this assay, enzyme-mediated DNA cleavage is induced by the rapid addition of a denaturing detergent, such as sodium dodecyl sulfate (SDS),[1] to mixtures

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