α-Bungarotoxin Immobilized and Oriented on a Lipid Bilayer Vesicle Surface†

Bruce Babbitt and Leaf Huang*

Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37996-0840
Received May 16, 1984

ABSTRACT: We have developed a new method to assess the binding site on α-bungarotoxin (α-BGT) for the acetylcholine receptor. It involves the covalent attachment of a palmitic acid chain to the toxin molecule, generating monopalmitoyl-α-bungarotoxin (PBGT) which is then immobilized on the surface of a lipid vesicle by a process of spontaneous insertion via the acyl chain into preformed unilamellar vesicles (~800 Å in diameter). PBGT itself is able to bind specifically to Triton X-100 solubilized acetylcholine receptors with an association constant, K_A, of 5.56×10^6 M^{-1} which is approximately 20-fold lower in affinity than native α-BGT. Vesicle-associated PBGT binds to acetylcholine receptor enriched microsac membrane vesicles in aqueous buffer with a K_A for both lipid and protein of 4.26×10^7 M^{-1}. The putative site of acylation on the PBGT molecule is determined by extensive cleavage of a reduced and carboxymethylated PBGT with thermolysin. An acylated fragment is purified by hydrophobic column chromatography and identified by high-pressure liquid chromatography methods from the known primary sequence of the native toxin as a decapeptide including residues Thr_{47}–Glu_{56} [C. Y. Lee convention used; see Mebs, D., Narita, K., Iwanaga, S., Samejuma, Y., & Lee, C. Y. (1971) *Biochem. Biophys. Res. Commun. 44*, 711–716]. Sequential hydrolysis of the fragment from the carboxy terminus with carboxypeptidase Y indicates that Lys_{51} is the sole site of acylation. Immobilization of this chemically homogeneous acylated toxin derivative on the surface of a vesicle should therefore orient the toxin molecule in such a manner that amino acid residues proximal to and including Lys_{51} are unavailable for direct binding with the acetylcholine receptor. By comparison of our results to the three-dimensional structure of the toxin recently published by Agard and Stroud [Agard, D. A., & Stroud, R. M. (1982) *Acta Crystallogr., Sect. A A38*, 186–194], we are able to identify specific amino acid residues that do not significantly participate in the binding interaction with the receptor.

During the last 20 years, a considerable amount of work involving the effects of various chemical modifications of the potent postsynaptic neurotoxin α-bungarotoxin (α-BGT)[1] on its ability to bind to the acetylcholine receptor (AChR) at the neuromuscular junction producing a nondepolarizing postsynaptic block has been reported (Karlsson, 1979). When combined with other data such as those from chemical cross-linking studies (Karlin, 1980), the crystal structure of α-BGT determined by high-resolution X-ray crystallography (Agard & Stroud, 1982; Stroud, 1981), and a knowledge of the titratable amino acid side chains that are highly conserved evolutionarily between snake neurotoxins (Strydom, 1979), such structure–function relationship studies allow one to elucidate the amino acid side chains involved in the binding interaction of the toxin with the AChR (Kistler et al., 1982). An understanding of the binding site on the toxin for the receptor allows use of the toxin as a probe of the neurotransmitter–AChR interaction (agonist binding site) with the ultimate goal being the elucidation of the mechanism of AChR ion channel activity.

In our laboratory, we have previously reported the synthesis of a completely new type of chemically modified α-BGT achieved through the covalent addition of a single palmitic acid chain to the toxin molecule (Grant et al., 1982). This derivative, referred to as monopalmitoyl-α-bungarotoxin (PBGT), was shown to bind specifically to the AChR either when solubilized in Triton X-100 or when attached to the surface of a vesicle in aqueous buffer (Grant et al., 1982). Furthermore, we have recently reported that liposome-associated PBGT is physically homogeneous when studied by a variety of techniques including high-sensitivity differential scanning calorimetry, steady-state fluorescence spectroscopy, time-resolved phosphorescence and fluorescence spectroscopy, and fluorescence recovery after photobleaching (Babbitt et al., 1984).

In this paper, we report the determination of the precise location of the acyl chain in the PBGT molecule using conventional techniques of protein chemistry including protease digestion, thin-layer chromatography, and amino acid analysis by high-pressure liquid chromatography. This information is then used to determine the orientation of the PBGT molecule when immobilized on the vesicle surface, allowing us to predict the toxin binding site for the receptor.

Materials and Methods

Materials. α-BGT was purified from the lyophilized venom of *Bungarus multicinctus* (Miami Serpentarium, FL) as previously described (Lee et al., 1972) and radioiodinated with chloramine T (Hunter & Greenwood, 1962) to a final specific activity of 1.0×10^{13} cpm/mol. The purified toxin was found to contain a single band of 8000 daltons when electrophoresed on a 12% SDS–polyacrylamide gel and further shown to be pure by an analysis of the amino acid composition. Hexa-

† Supported by National Institutes of Health Grant GM 31724 and Research Career Development Award CA 00718 to L.H.

[1] Abbreviations: α-BGT, α-bungarotoxin; PBGT, palmitoyl-α-bungarotoxin; RM-PBGT, reduced and carboxymethylated PBGT; AChR, acetylcholine receptor; DOPC, dioleoylphosphatidylcholine; PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 1 mM Na_2HPO_4, pH 7.4); cpm, counts per minute; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PS, phenyl-Sepharose; SUV, small unilamellar vesicles; SDS, sodium dodecyl sulfate; NHSP, *N*-hydroxysuccinimide-activated ester of palmitic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

0006-2960/85/0424-0015$01.50/0

decyl[^{3}H]cholestanyl ether was synthesized and purified as described (Paltauf, 1968). The *N*-hydroxysuccinimide-activated ester of palmitic acid (NHSP) was synthesized according to Lapidot et al. (1967), recrystallized, and stored at room temperature in anhydrous dioxane. [^{3}H]NHSP was similarly prepared from [^{3}H]palmitic acid (New England Nuclear, Boston, MA) to a final specific activity of 6.2×10^5 cpm/μg. Dioleoylphosphatidylcholine (DOPC) was purchased from Avanti (Birmingham, AL). Carboxypeptidase Y was purchased from Worthington Diagnostic Systems Inc. and thermolysin type X from Sigma Chemical Co. Acetylcholine receptor and acetylcholine receptor enriched microsac membrane vesicles were isolated from the frozen electroplax tissue of *Torpedo californica* (Pacific Biomarine, Venice, CA) by a modification of the methods of Miller et al. (1978). All other chemicals were reagent grade.

Analytical Methods. Protein (α-BGT) was determined spectrophotometrically by using an absorption coefficient, $A^{1\%}_{280nm}$ of 12.0 (Chen et al., 1982). ^{125}I cpm were directly counted by using a Beckman Biogamma II counter. ^{3}H cpm were counted in a Beckman LS-230 liquid scintillation counter using a Triton X-100–toluene cocktail. All results were corrected for channel crossover if more than one isotope was employed.

Synthesis of Radiolabeled PBGT. [^{3}H]Palmitic acid at a specific activity of 4.0×10^3 cpm/nmol was covalently coupled to ^{125}I-α-BGT at a specific activity of 4.0×10^2 cpm/nmol by reaction of the iodinated toxin with [^{3}H]NHSP in deoxycholate as described previously (Grant et al., 1982). Mono-[^{3}H]palmitoyl-^{125}I-α-bungarotoxin was purified by hydrophobic column chromatography and stored at −20 °C in 50% ethanol. The purity of the PBGT preparation was checked by thin-layer chromatography on cellulose plates and found to contain a single spot of coincident ^{125}I cpm and ^{3}H cpm when a solvent system of 15:10:3:12 1-butanol:pyridine:glacial acetic acid:water was used.

Reduction and Carboxymethylation of α-BGT and PBGT. The procedure of Crestfield et al. (1963) was used with modification. To 1 mg of dry protein were added in succession 0.7 g of urea, 60 μL of disodium ethylenediaminetetraacetate (Na_2EDTA) solution (50 mg of Na_2EDTA/mL), 600 μL of 1.5 M Tris buffer, pH 8.6, and 20 μL of mercaptoethanol. The solution was made up to a final volume of 2.4 mL by addition of 820 μL of distilled water and finally 900 μL of a solution of 8 M urea in 0.2% EDTA. All reagents were bubbled with N_2 gas just prior to use so as to minimize the oxidation of (reduced) free sulfhydryls generated by this procedure. Following a 4-h incubation at room temperature, 200 μL of a freshly prepared solution of 0.268 g of iodoacetic acid in 1.0 mL of 1.0 N NaOH was added to the reaction mixture. The reaction was kept in the dark in order to prevent formation of iodine which may react with tyrosine, tryptophan, and histidine residues. Fifteen minutes after the addition of iodoacetate, the sample was applied to a 20-mL Bio-Gel P-4 column preequilibrated in 0.1 M NH_4HCO_3, pH 8.0. The reduced and S-carboxymethylated protein (hereafter referred to as RM-PBGT) eluted as a single peak in the void volume upon elution of the column with 3 bed volumes of 0.1 M NH_4HCO_3, pH 8.0. To assure full removal of reagents, especially EDTA, the protein was further dialyzed overnight at 4 °C in a Spectrapor-6 dialysis membrane (Spectrum Medical Industries, Los Angeles, CA) against 4000 volumes of 0.1 M NH_4NCO_3, pH 8, containing 0.02% sodium azide.

Enzymatic Hydrolysis and Isolation of an Acylated RM-PBGT Fragment. To 800 μg of protein in 0.1 M NH_4HCO_3, pH 8, was added 40 μL of a 0.1% solution of thermolysin type X stored at 0 °C in 0.1 M NH_4HCO_3, pH 8. The solution was incubated for 12 h at 37 °C and then dialyzed against 4000 volumes of PBS, pH 7.8, for 6 h. Following dialysis, the thermolysin-treated RM-PBGT was loaded onto a 5-mL phenyl-Sepharose column preequilibrated and washed with PBS. Acyl chain containing fragments were eluted with a step gradient of ethanol in distilled water in a range from 0 to 100% ethanol. One-milliliter fractions were collected; whole fractions were counted for ^{125}I cpm (protein), and 15-μL aliquots were counted for ^{3}H cpm (acyl chain) as previously described.

Characterization of Acylated RM-PBGT Fragments. Homogeneity of acylated fragments resulted from thermolysin hydrolysis of RM-PBGT was monitored by thin-layer chromatography using Eastman Kodak cellulose plates and a solvent system consisting of 15:10:3:12 1-butanol:pyridine-glacial acetic acid:water according to Dolly et al. (1981). The paper plates were thoroughly dried and then cut into 0.5-cm strips and counted for ^{3}H and ^{125}I cpm. Approximately 10 nmol of a purified peptide (eluting with 35% EtOH) fragment containing the fatty acid chain was lyophilized and then subjected to total acid hydrolysis for 15 h at 110 °C in 6 N HCl in a sealed, evacuated Pyrex tube. After hydrolysis, the sample was dried in vacuo and then stored at −70 °C until preparation for amino acid analysis (see below).

Hydroxylamine Treatment of Acylated RM-PBGT Fragment. Ten micrograms of the acylated fragment was incubated at room temperature overnight in 200 μL of 1 M NH_2OH·HCl (Aldrich), titrated to pH 6.6 with NaOH. Following the incubation, the sample was chromatographed over a 2-mL Bio-Gel P4 column preequilibrated and eluted with PBS. Fractions were counted for ^{3}H and ^{125}I cpm.

Digestion of the Acylated Fragment with Carboxypeptidase Y. A 14.1-μg sample (9.85 nmol) of acylated fragment was added to 200 μL of 10 mM sodium phosphate, pH 7 at 37 °C, and sonicated to yield a slightly turbid suspension. Thereafter, 5 μL (2.28 pmol) of a freshly prepared solution of carboxypeptidase Y in 10 mM sodium phosphate, pH 7, was added, and the suspension was mixed for 10 h at 37 °C. At the 10-hour time point, a 67-μL aliquot (3.3 nmol of total fragment and 0.75 pmol of enzyme) of the suspension was removed and diluted to 500 μL by the addition of 433 μL of phenyl-Sepharose gel preswollen in distilled water. The phenyl-Sepharose gel which is a hydrophobic derivative of Sepharose 4B was added in order to bind any acylated fragments and/or amino acids left behind after hydrolysis, allowing easy separation of hydrolyzed amino acids from the rest of the mixture. The suspension was mildly vortexed several times over a 30-min period and then spun at 3000*g* for 20 min to pellet all of the gel and associated materials. A control was performed in which acid-hydrolyzed fragment was added to the phenyl-Sepharose gel and was shown by high-pressure liquid chromatography methods (see below) not to bind to the gel under these same conditions (data not shown). The supernatant containing the hydrolyzed amino acids was removed, lyophilized, and stored at −70 °C until preparation for amino acid analysis (see below). After removal of the first 67-μL aliquot from the hydrolysis mixture after 10 h of incubation, the remaining suspension was resonicated, a second 5-μL aliquot of enzyme was added, and the mixture was incubated an additional 10 h. At the 20-h time point, a second 67-μL aliquot was removed and processed as before while a third 5-μL aliquot of enzyme was added. Finally, after 30 h of hydrolysis, the final 67-μL aliquot was removed and prepared for amino acid analysis as previously mentioned.

Identification of Amino Acid Residues Cleaved by Carboxypeptidase Y. Lyophilized supernatants of the fragment hydrolyzate were dissolved in 150 μL of 0.2 M sodium citrate buffer, pH 3.15, and 2–50-μL samples were injected onto a 4.6 mm i.d. × 25 cm Waters high-pressure liquid chromatography sulfonated styrene/divinylbenzene copolymer ion-exchange column for amino acid analysis. Samples were eluted at a flow rate of 0.45 mL/min via a concave stepwise ionic strength gradient (0.2–1 M sodium citrate and pH 3.15–7.4). Amino acid residues were identified by retention time and quantitated via postcolumn derivatization with *o*-phthaldehdye using Pierce amino acid standards "H" as external standards. The sensitivity of the system for quantitation of primary amines is 100 pmol (the presence of secondary amines such as Pro requires 500 pmol). To determine whether any acylated amino acid residue(s) or free palmitic acid had been produced by enzymatic cleavage of the fragment, a third 50-μL aliquot of the supernatant was assayed for ^3H cpm as previously described.

Identification of Uncleaved Amino Acids following Carboxypeptidase Y Treatment of Acylated Fragment. Amino acid residues still able to bind to the phenyl-Sepharose gel after the full 30-h treatment of the acylated fragment with carboxypeptidase Y were removed from the gel by incubation in 100% ethanol for 30 min followed by centrifugation of the suspension at 3000*g* for 20 min. The supernatant was removed and divided into three equal aliquots. One aliquot was dried under nitrogen, acid hydrolyzed as previously described, and analyzed for amino acid composition. Another aliquot was spotted onto a silica gel thin-layer chromatography plate and developed in a chloroform/methanol/water (65:35:4) solvent system. The plates were dried thoroughly and sprayed with ninhydrin and/or cut into 0.5-cm strips and counted for ^3H cpm to identify fragments/amino acid residues containing an acyl chain. The third aliquot was dried under nitrogen and counted for ^3H cpm as previously described.

Binding of α-BGT and PBGT to Detergent-Solubilized Acetylcholine Receptor. To measure binding affinities of the native toxin and its acylated derivative to the AChR, 13 μg (total protein) of acetylcholine receptor enriched microsac membrane isolated from *Torpedo californica* was dissolved in 50 μL of buffer containing 100 mM NaCl, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.2% Triton X-100, and 100 μg/mL bovine serum albumin and added to various concentrations of ^{125}I-α-BGT or ^{125}I-PBGT in a final volume of 150 μL. Samples were incubated at 24 °C for 3 h. The amount of radioactive toxin–receptor complex was assayed by the procedure of Damle & Karlin (1978) with *Whatman* DE-81 filters.

Vesicle Formation and Incorporation of PBGT into Lipid Vesicles. The detergent dialysis method of Enoch & Strittmatter (1979) was used with modification. Dioleoylphosphatidylcholine (DOPC) at 14 mg/mL with a trace amount of hexadecyl[^3H]cholestanyl ether (a nonexchangeable lipid marker) at a final specific activity of 1.25×10^4 cpm/nmol in PBS containing 0.02% sodium azide and 2.5 mM EGTA (PBS/N_3/EGTA), pH 8.0, was sonicated for 20 min at 4 °C in a bath sonicator (Laboratory Supplies Co., Hicksville, NY). A 4.9% solution of deoxycholate in PBS/N_3/EGTA was added to the SUV to yield a mixed micellar solution of 6 mg/mL in lipid and 0.82% in detergent. The resultant clear solution was then dialyzed at room temperature in a Spectrapor-2 dialysis membrane against 8000 volumes of PBS/N_3/EGTA, pH 8, for 48 h, followed by dialysis against 4000 volumes of PBS/N_3/EGTA at pH 7.4 for 24 h. All samples became turbid approximately 8–10 h after initiation of dialysis. Following the dialysis at pH 7.4, all samples were centrifuged at 3000*g* for 20 min in order to pellet residual soluble aggregated detergent. To remove any residual detergent from the vesicle suspension as well as SUV, the supernatant was fractionated through a Bio-Gel A50m column, and vesicles eluting in the void volume were pooled and stored under N_2 at 4 °C as our DOPC preformed vesicle stock. For incorporation of PBGT into the vesicles, 1-mL aliquots of DOPC vesicles adjusted to 6 mg/mL were mixed with 500-μL suspensions containing various amounts of PBGT in PBS/N_3/EGTA, pH 7.4, and incubated overnight at 37 °C to allow for equilibrium association of the acylated protein with the lipid bilayer (Babbitt et al., 1984). The PBGT vesicles were then rechromatographed over a Bio-Gel A50m column to remove unincorporated PBGT and small particles generated by the incorporation procedure. Void volume fractions were pooled and stored under N_2 at 4 °C until further use.

Binding of PBGT Vesicles to Microsac Membranes Enriched with AChR. To measure the binding affinity of vesicle-associated PBGT for AChR, PBGT vesicles at a protein to lipid molar ratio of 1.0×10^{-3} were prepared as previously described and added at various concentrations in 30 μL of PBS/N_3/EGTA, pH 7.4, to 4 μg (total protein) of AChR-enriched microsac membrane vesicles in 60 μL of PBS/EGTA/N_3, pH 7.4. Following incubation for 5 h at room temperature, 30-μL aliquots of the PBGT vesicle–microsac suspension were assayed for percent binding (cpm in pellet/cpm total) in triplicate by layering the sample over a 150-μL 5% sucrose solution in a 5 × 20 mm polyallomer tube and spinning at 165000*g* (generated at 30 psi air pressure) for 15 min in a 30° fixed-angle rotor empolying a Beckman Airfuge. Following centrifugation, the samples in the polyallomer tubes were frozen at −70 °C for at least 30 min and then sliced with a razor blade into top (120 μL, supernatant) and bottom (60 μL, pellet) portions and counted directly for ^{125}I cpm (protein). Thereafter, 380 and 440 μL of 10% SDS in distilled water were added to the supernatant and pellet fractions, respectively, and incubated overnight at 37 °C to dissolve the pellets. ^3H cpm (lipid) was then counted as previously described. All PBGT vesicle preparations were centrifuged alone under the aforementioned conditions to "preselect" for those vesicles that by themselves float on top of 5% sucrose, while microsacs were likewise preselected for their ability to pellet under the same centrifugation conditions.

RESULTS

Isolation of an Acylated Fragment of RM-PBGT. Two different acylated species were present following thermolysin treatment of RM-PBGT. One of the acylated products eluted from the phenyl-Sepharose column with 5% EtOH while the other more hydrophobic product eluted with 35% EtOH. The acylated species eluted with 5% EtOH is probably a hydrolytic precursor of the species eluted with 35% EtOH, or non-hydrolyzed RM-PBGT itself, since further treatment with thermolysin converted this peak into the 35% EtOH peak. In addition, the acylated species eluted with 5% EtOH migrated on thin-layer chromatography identically with RM-PBGT (see Table I), and RM-PBGT untreated with thermolysin eluted from the phenyl-Sepharose column with 5% EtOH (data not shown). The acylated fragment eluted with 35% EtOH could not be cleaved any further by thermolysin or by trypsin (presumably due to the presence of the acyl chain).

Finally, the acylated fragment eluted with 35% EtOH contained a 1:1 molar ratio of ^{125}I cpm (protein) to ^3H cpm (acyl chain), indicating that the fragment must include Tyr_{54}

Table I: Thin-Layer Chromatography of RM-PBGT Cleaved by Thermolysin

sample	spot no.	R_f	^3H cpm[a]	^{125}I cpm[b]	acyl:protein ratio
palmitic acid	1	0.93	32000		
RM-α-BGT	1	0.40		24000	
RM-PBGT	1	0.53	14720	1600	0.92
RM-PBGT	1	0.53	1864	141	1.32
thermolysin	2	0.80	7890	892	0.88
5% PS column	1	0.53	6750	837	0.81
35% PS column	1	0.80	8000	704	1.14

[a] Specific activity of 4.0×10^3 cpm/nmol. [b] Specific activity of 4.0×10^2 cpm/nmol.

Table II: Comparison of Amino Acid Composition of Acylated Fragment and Native α-BGT Thermolysin Fragment

amino acid residue	mol equiv theoretical[a]	mol equiv experimental
Ala	1	0.2
Thr	1	0.8
RM-Cys[b]	1	1.2
Pro	2	1.7
Ser	1	1.2
Lys	2	2.1
Tyr	1	0.9
Glu	2	2.2

[a] Native toxin thermolysin fragment containing Tyr_{54}: Ala_{46}-Thr_{47}-Cys_{48}-Pro_{49}-Ser_{50}-Lys_{51}-Lys_{52}-Pro_{53}-Tyr_{54}-Glu_{55}-Glu_{56} (Mebs et al., 1971). [b] Reduced and carboxymethylated cysteine.

which is well-known to be the preferentially iodinated residue in the toxin molecule (Vogel et al., 1972), and furthermore that the binding of the acyl chain to the protein is very stable under conditions of enzymatic cleavage, fragment purification and characterization, solubilization in detergents and 35% EtOH, and prolonged incubation at 37 °C at pH 8.

Characterization of the Acylated Fragments. (A) Thin-Layer Chromatography. In Table I are shown the results of an analysis of various RM-PBGT derivatives by thin-layer chromatography on cellulose plates. It is clear that the two fragments isolated after thermolysin treatment of RM-PBGT and binding to the phenyl-Sepharose gel are homogeneous and furthermore that the material eluted off of the phenyl-Sepharose gel with 5% EtOH is nondegraded RM-PBGT. Although the absolute amount of ^{125}I cpm under these conditions was quite low, it appeared that both peaks once again contained an approximate 1:1 molar ratio of protein fragment with iodinated Tyr_{54} to acyl chain.

(B) Amino Acid Composition Analysis. In Table II are shown the results of an analysis of the amino acid composition of the fast migrating acylated RM-PBGT thermolysin fragment that eluted from the phenyl-Sepharose column with 35% EtOH. In the footnote to the table is shown the linear sequence of a decapeptide containing Tyr_{54} known to be generated by thermolysin treatment of native toxin (Mebs et al., 1971). As can be seen in the table, the fit of the experimental data to the theoretical decapeptide composition is excellent. The fit was made even stronger since the decapeptide contained 100% of the ^{125}I cpm originally present in the intact acylated toxin; it is known that the toxin which contains only two tyrosine residues is selectively iodinated at Tyr_{54} (Vogel et al., 1972). Since the purified acylated peptide fragment consisted of several potential acylation sites including possible ester bonds at Thr_{47} and Ser_{50} and possible amide bonds at Lys_{51} and Lys_{52}, we decided to assay the fragment for the presence of an ester linkage by treatment with hydroxylamine. It has been shown that hydroxylamine is nucleophilic to certain carboxylic acyl derivs., in particular, esters, and upon reaction hydroxamic acids are formed (Yale, 1943). Following incubation with hydroxylamine, the fragment eluted intact with its acyl chain in the void volume of the Bio-Gel P4 column, maintaining an approximate 1:1 molar ratio of acyl chain to peptide fragment. In addition, the fragment rechromatographed over a phenyl-Sepharose column eluted intact, once again with 35% EtOH. Finally, thin-layer chromatography of the fragment eluted with 35% EtOH on silica gel showed no apparent change in the molecule, indicative that the acylated fragment was not cleaved by hydroxylamine and furthermore that the palmitic acid chain was probably not esterified to the decapeptide. (We also treated the fragment with 0.1 M KOH in 20% methanol with similar results, reinforcing the supposition that the acyl chain was not linked by an ester bond to the protein.) Therefore, it is most likely that the acyl chain is located on a lysine side chain.

Enzymatic Hydrolysis of the Acylated Fragment with Carboxypeptidase Y Hydrolyzed Amino Acid Residues. In order to identify which lysine residue(s) of the acylated fragment contained the palmitoyl chain, we decided to cleave the fragment sequentially from the carboxy terminus with carboxypeptidase Y since extensive treatment of the purified decapeptide with trypsin failed to generate any smaller peptides presumably due to steric hindrance by the acyl chain. In Table III are shown the results of extensive cleavage of the purified acylated decapeptide with carboxypeptidase Y. If the fragment is the decapeptide whose linear sequence is shown in Table II and if the hydrolysis by carboxypeptidase Y is quantitative, one would expect to see the appearance of Glu, Tyr, Pro, Lys, Ser, CM-Cys, and Thr (in that order) in the supernatant of the processed reaction mixture. Instead, it appears that the enzyme was able to cleave off amino acids from the carboxy terinus of the fragment toward the amino terminus only as far as Lys_{52} but evidently not past both Lys_{51} and Lys_{52} since no serine was cleaved. Probably the presence of the acyl chain and/or the aggregation of the fragment itself inhibited hydrolysis of the remainder of the amino acids on the amino-terminal side of Lys_{51}. Furthermore, a consideration

Table III: Amino Acid Composition of Hydrolyzed (Supernatant) and Nonhydrolyzed (Eluent from PS Gel) Residues of Carboxypeptidase Y Treated Acylated Fragment

	sample	mol equiv of Glu	Tyr	Pro	Lys	Ser	RM-Cys	Thr
theoretical		2	1	2	2	1	1	1
	supernatant							
	10 h	2[b]	0.94	0.68	0.56	0	0	0
experimental	20 h	2	0.97	0.72	0.70	0	0	0
	30 h	2	0.98	0.71	0.73	0	0	0
	eluent from PS gel							
	30 h	0[c]	0	0.96	1.42	1	0.78	1

[a] Reduced and carboxymethylated cysteine. [b] All values are normalized to Glu which was completely hydrolyzed by the enzyme. [c] All values are normalized to Thr and Ser.

of the appearance of the various residues in the supernatant as a function of time indicated that cleavage was occurring from the carboxy terminus (and reconfirms the identity of the acylated fragment itself). Most important to point out is the fact that during the 30-h incubation, although 681 pmol of lysine was cleaved off of 1867 pmol of total fragment (36% efficiency), a 50-μL aliquot of the same 30-h time point supernatant was found to contain essentially no acyl chain (^{3}H cpm), indicating that the lysine (and all other amino acids) cleaved off did not contain any palmitic acid. These results taken together indicate that the acyl chain is on Lys_{51}. Of course, unambiguous identification of Lys_{51} as the sole site of PBGT acylation necessitates the isolation of a palmitoyl adduct of Lys_{51} itself; we were unable to do this since complete cleavage of the decapeptide with proteases was apparently inhibited by the presence of the acyl chain. Furthermore, total acid hydrolysis of the decapeptide quantitatively removed the acyl chain from its amino acid attachment site. That Lys_{51} appears to be the preferred site for reaction of α-BGT with NHSP, possibly due to some type of hydrophobic interaction of the NHSP with toxin in detergent, aligning the reactive palmitoyl chain carbonyl carbon for reaction with the toxin Lys_{51} ε-amino group, is not unexpected since the use of a similar reagent, *N*-succinimidyl [2,3-^{3}H]propionate to label amino groups of α-BGT has previously been shown to yield mainly ε-propionyl-Lys_{51} (Dolly et al., 1981).

Nonhydrolyzed Amino Acid Residues of the Acylated Fragment. Also shown in Table III are the results of a high-pressure liquid chromatography analysis of the amino acid residues still able to bind (presumably mediated by the acyl chain) to the phenyl-Sepharose gel following extensive treatment of the fragment (30 h) with carboxypeptidase Y. The appearance of amino acids on the amino-terminal side of the fragment is quantitative and complementary to the results of the hydrolyzed residues. Although 73% of Lys_{52} had apparently been cleaved off, 100% of the ^{3}H cpm originally associated with the intact fragment remained in the nonhydrolyzed portion of the fragment bound to the phenyl-Sepharose gel. These data together with the results of the hydrolyzed amino acid residues shown in the previous sections support the designation of Lys_{51} as the site of acylation since the enzyme were able to cleave from the carboxy terminus up to and including an acylated Lys_{52} (see decapeptide linear sequence in Table II) the remainder of the fragment (Thr_{47}, Cys_{48}, Pro_{49}, Ser_{50}, and Lys_{51}) once separated from the acylated amino acid residue should have become available for cleavage. Furthermore, the remainder of the fragment should not have bound to the phenyl-Sepharose gel as "nonhydrolyzed amino acids". The possibility that the enzyme removed the acyl chain from the fragment, leaving free palmitic acid, or hydrolyzed an acylated lysine residue that remained bound to the phenyl-Sepharose gel can be discounted on the basis of our results obtained from thin-layer chromatography on silica gel of material eluted from the phenyl-Sepharose gel with 100% ethanol. The eluted material remained at or very near the origin (R_f = 0.04), while free palmitic acid moved with the solvent front (R_f = 0.96) and monoacylated lysine (prepared by reaction of free lysine with NHSP) moved a few centimeters away from the origin (R_f = 0.23). We conclude that the nonhydrolyzed material bound to the phenyl-Sepharose gel is an intact, partially hydrolyzed acylated fragment(s).

Binding Affinity of α-BGT and PBGT to Detergent-Solubilized AChR. In Figure 1 are shown the Scatchard analyses of the data for the binding of α-BGT and PBGT to detergent-solubilized AChR. The apparent association constant, K_A^{app}, is 5.56 × 10^6 M^{-1} for the PBGT–AChR interaction and 1.02 × 10^8 M^{-1} for the α-BGT-AChR interaction. Thus, the addition of one palmitoyl chain to α-BGT decreased the binding affinity of the molecule for the receptor by about 20-fold. It is also evident from Figure 1 that PBGT and α-BGT are bound to the same class of receptor, since both lines extrapolate to approximately the same point along the horizontal axis.

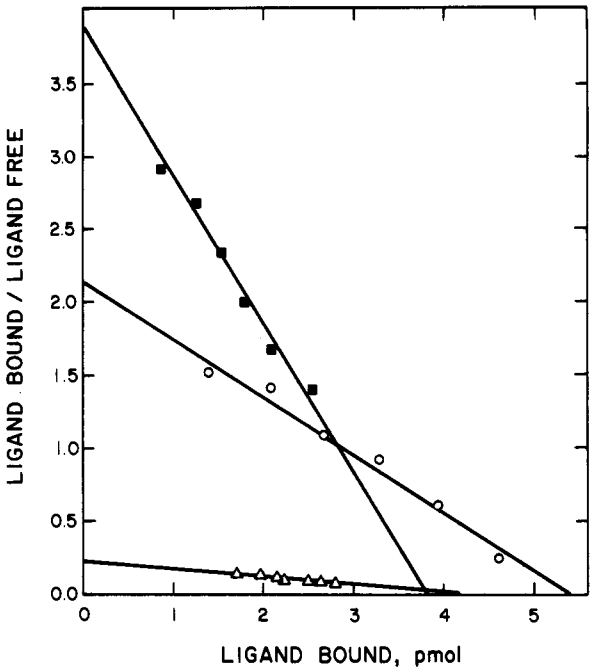

FIGURE 1: Scatchard analyses of the data for binding of α-BGT (■) and PBGT (△) to Triton X-100 solubilized AChR and of vesicle-associated PBGT (protein:lipid molar ratio = 1.0 × 10^{-4}) to AChR-enriched microsac membrane vesicles (○). Straight lines are least-squares fits of the data points with correlation coefficients greater than 0.97. Specific activity of the AChR-enriched membranes is 0.32 nmol of toxin binding sites/mg of protein.

Binding Affinity of Vesicle-Associated PBGT to Microsac Membranes Enriched with AChR. Also shown in Figure 1 is a Scatchard plot analysis of the data for the binding of PBGT vesicles at a molar ratio of protein to lipid of 1.0 × 10^{-3} to AChR-enriched microsacs. The apparent association constant for both lipid and protein is 4.26 × 10^7 M^{-1}. Therefore, PBGT is still capable of strong binding to microsacs when immobilized and oriented on a lipid bilayer surface. Furthermore, we have previously reported that vesicle-associated PBGT is able to inhibit agonist-dependent Na^+ influx of AChR-enriched microsacs, strongly indicating that the binding interaction is indeed mediated by specific interactions with AChRs (Grant et al., 1982).

Discussion

The results of this paper indicate that the acylation site on PBGT lies at residue Lys_{51} and consequently that PBGT is a chemically homogeneous hydrophobic derivative of α-BGT. Acylation of the toxin decreases its apparent affinity for detergent-solubilized AChR by approximately 20-fold, a result similar to that of Dolly et al. (1981), who measured a 5-fold decrease in the rate constant for toxin binding to AChR upon addition of a single propionyl group at Lys_{51}. Previously we have shown that the acylated toxin can be incorporated onto the surface of preformed phospholipid vesicles (liposomes) in such a way that they are interacting with the phospholipid bilayer principally via the fatty acid chain while the polypeptide region of the molecule extends outward from the bilayer

surface and does not interact strongly with the bilayer. Not surprisingly, therefore, we also found that the PBGT molecule exhibits a high degree of both rotational and lateral mobility on the surface of fluid bilayers and, more importantly, that the PBGT rotates and moves laterally as a single species, indicating a physically homogeneous interaction with the lipid bilayer. Furthermore, the fact that the stable association of PBGT with the bilayer surface [PBGT does not pull out of the vesicle upon interaction with the AChR (Grant et al., 1982)] does not prohibit specific binding (i.e., mediated by the PBGT–AChR interaction) of the PBGT vesicles to AChR-enriched microsacs strongly indicates that PBGT is associated with the vesicle in such a manner that the binding surface is exposed the remains available for binding to the AChR. It also appears that vesicle-associated PBGT is sufficiently flexible conformationally if indeed refolding of the binding surface is required upon interaction with the AChR as has been reported (Banks et al., 1974). Finally, PBGT maintains a conformation when self-aggregated in aqueous buffer, when solubilized in detergent, or when vesicle associated that is indistinguishable from that of the native toxin as seen by measurement of intrinsic tryptophan fluorescence spectra (data not shown).

The structure of α-BGT determined by X-ray crystallography at a resolution of 3.5 Å (Agard & Stroud, 1982; Stroud, 1981) reveals that the toxin is a flat "open hand" shaped molecule consisting of three disulfide cross-linked loops of polypeptide chain. Previous studies comparing sequence homologies (Strydom, 1979), three-dimensional X-ray crystal structures (Stroud, 1981; Tsernoglou et al., 1978; Walkinshaw et al., 1980), and extensive chemical modification literatures (Karlsson, 1979) of the various "long" and "short" neurotoxins, especially α-BGT, erabutoxin b, and α-cobratoxin, when combined with the results of immunological (Klymkowsky & Stroud, 1979) and binding studies have revealed that the toxin contains an extended 20 × 30 Å concave binding surface for acetylcholine receptor (as opposed to a simple one- or two-point interaction) involving both hydrophobic and charged contact points including all of the highly conserved "functional" amino acid residues. In particular, Tyr_{24}, Lys_{26}, Glu_{42}, and Lys_{52} are all highly conserved residues whose side chains lie proximal to each other along the binding surface (Kistler et al., 1982; Strydom, 1979). In addition, the side chains of highly conserved Asp_{31}, Arg_{37}, and Gly_{38} which are believed to mimic acetylcholine (Karlsson, 1979; Low, 1979; Tsernoglou et al., 1978) are oriented toward each other along the concave binding surface and are thought to direct/orient the toxin to the acetylcholine binding site on the receptor. Met_{28} (Dionne et al., 1978) and Tyr_{55} (Tsernoglou & Petsko, 1976) are the only other residues found protruding into the binding surface (Stroud, 1981) and perhaps represent hydrophobic contact points on the interaction surface with AChR (Kistler et al., 1982). Finally, although the side chain of invariant Trp_{29} is rotated completely away from the extended binding surface in α-BGT (Kistler et al., 1982), it has been suggested that it moves into the binding site upon refolding of the toxin molecule [which contains very little conventional intramolecular secondary structure (Kistler et al., 1982), allowing a dynamic and flexible binding surface in solution (Lauterwein et al., 1978)] during interaction with AChR (Banks et al., 1974).

Our results indicate that upon association of PBGT with the lipid vesicle surface we are able to immobilize and orient the toxin molecule in a homogeneous and predictable fashion. Since our results indicate that the acyl chain is located through an amide linkage at Lys_{51}, we can conclude that the area of the toxin molecule in close proximity to Lys_{51} and therefore in close association with the vesicle surface cannot be an integral part of the binding site for AChR. This conclusion is consistent with the model of the X-ray crystal structure (3.5-Å resolution) of the toxin recently published by Stroud (1981) in which Lys_{51} lies at the periphery of the extended concave binding site so that a single-point attachment and anchorage of the toxin to the vesicle surface by insertion of the acyl chain into the outer phospholipid monolayer would not be expected to inhibit the ability of a PBGT molecule that is completely free to rotate and move laterally to present its binding site to the AChR. In addition, covalent modification of Lys_{51} by other amino-specific reagents has resulted in a reduction but rarely an abolition of the toxin binding activity (Dolly et al., 1981; Sugiyama & Yamashita, 1981) as opposed to modification, for example, dansylation, of Lys_{26} which is located in the center of the concave binding site and results in complete loss of reactivity without any apparent change in toxin conformation (Karlsson, 1979). In particular, although the acylated toxin molecule should exhibit a high degree of rotational/motional freedom along the extended side chain of Lys_{51}, it appears that amino acid residues Ala_{45}, Ala_{46}, Thr_{47}, Cys_{48}, Pro_{49}, Ser_{50}, Lys_{51}, Lys_{52}, and Pro_{53} which all lie in approximately the same plane at the very edge of the molecule (Stroud, 1981; R. Love, personal communication) should not be available for direct interaction with the AChR when PBGT is associated with the vesicle. Therefore, the only amino acid residue believed to contribute directly to the binding with receptor that is unable to present itself to the receptor in vesicle-associated PBGT is Lys_{52}, and it has been shown that specific monoacetylation of Lys_{52} in α-cobratoxin increased the lethal dose in mice approximately 1.5-fold (Karlsson & Eaker, 1972), indicating that it is not an integral part of the binding site. Furthermore, the removal of the positive charge from the highly conserved Lys_{52} in long toxins had a large effect on lethality only in combination with several other changes in the molecule, and neutralization of only a single lysine charge had the same effect irrespective of the position of the residue in the toxin structure (Karlsson, 1979). It is interesting to note that a portion of this same stretch of amino acids, specifically Ala_{45}–Ser_{50}, is very highly conserved in all neurotoxins and is believed to interact with AChR through hydrophobic and hydrogen-bonding interactions. Loss of these contacts together with the lack of access of Lys_{52} to the AChR in Triton X-100 detergent may explain the approximate 20-fold decrease in K_A occurring upon addition of the acyl chain to the toxin molecule. In addition, the hydrophobicity of this stretch of amino acids may also explain the unique labeling of Lys_{51} when α-BGT is reacted with NHSP in detergent. All of the other amino acid residues mentioned above as being part of the toxin binding surface especially those of Asp_{31}, Arg_{37}, and Gly_{38}, which together mimic acetylcholine, should be fully exposed to AChR in the vesicle-associated PBGT since they are sufficiently far away from the vesicle surface itself. All that is required of the acylated toxin molecule is that it be able to flip-flop along the unexposed Ala_{45}–Pro_{53} "hinge" region in order to expose all of the necessary amino acid side chains of the extended binding surface; there should be no other constraints since the toxin molecule exhibits a high degree of conformational flexibility (with little intramolecular secondary structure as mentioned previously) which should allow a close apposition of toxin and receptor complementary binding surfaces.

These data taken together with previously reported data referenced in this report should allow a more rigorous determination of the binding site on the toxin for the receptor. This

approach, which to our knowledge is the first of its kind involving the immobilization and orientation of a chemically homogeneous acylated protein onto some surface (in this case onto the surface of a lipid vesicle) in a physically homogeneous manner, may be useful (and complementary to other more conventional methods) for the determination of the binding interfaces of other biologically significant protein–protein (ligand–receptor, enzyme–substrate, etc.) interactions. It holds a distinct advantage over the traditional immobilization (cross-linking) approaches in which detailed analysis of the cross-linking site(s) on the protein is not possible with the consequence that the orientation of the immobilized protein is unknown.

The present study is also important from the standpoint that it relates to a model system we have developed to study the basic physicochemical parameters governing membrane interactions mediated by specific membrane-associated receptors and ligands (Grant et al., 1982; Babbitt & Huang, submitted for publication). We chose to study the binding of microsac membrane vesicles highly enriched with nicotinic acetylcholine receptors to vesicles with incorporated PBGT. The advantage of employing this receptor–ligand (AChR–α-BGT) pair is its high specific binding affinity and well-characterized interaction from both a biochemical and a biophysical standpoint. We have found that vesicle-associated PBGT mediates the association of lipid vesicles with microsac membrane vesicles by specific interaction with the AChR. It is possible to study the thermodynamics of this membrane–membrane interaction as a function of the multivalency of the vesicle-associated PBGT, and the results will be published elsewhere (Babbitt & Huang, submitted for publication).

ACKNOWLEDGMENTS

We thank Dr. Jorge E. Churchich for his helpful suggestions concerning the use of carboxypeptidase Y, Dr. Robert Love for many helpful discussions concerning the three-dimensional structure of α-BGT, and Phil Sieg and Dr. Kuang-Tzu Davis Lin for determination of amino acid compositions.

Registry No. α-BGT, 11032-79-4; acetylcholine, 51-84-3.

REFERENCES

Agard, D. A., & Stroud, R. M. (1982) *Acta Crystallogr., Sect. A A38*, 186–194.

Babbitt, B. P., & Huang, L. (1985) *Biochemistry* (in press).

Babbitt, B. P., Huang, L., & Freire, E. (1984) *Biochemistry 23*, 3920–3926.

Banks, B. E. C., Miledi, R., & Shepoline, R. A. (1974) *Eur. J. Biochem. 45*, 456–468.

Chen, Y. H., Tai, J. C., Huang, W. J., Lai, M. Z., Hung, M. C., Lai, M. D., & Yand, J. T. (1982) *Biochemistry 21*, 2592–2600.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.

Damle, V. M., & Karlin, A. (1978) *Biochemistry 17*, 2039–2045.

Dionne, V. E., Steinbach, J. H., & Stevens, C. F. (1978) *J. Physiol. (London) 281*, 421–444.

Dolly, J. O., Nockles, E. A. V., Lo, M. M. S., & Barnard, E. A. (1981) *Biochem. J. 193*, 919–923.

Enoch, H. G., & Strittmatter, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 145–149.

Grant, S. W., Babbitt, B. P., West, L. K., & Huang, L. (1982) *Biochemistry 21*, 1274–1279.

Hunter, W. M., & Grenwood, F. C. (1962) *Nature (London) 194*, 495–496.

Karlin, A. (1980) in *The Cell Surface and Neuronal Function* (Cotman, C. W., Poste, G., & Nicolson, G. L., Eds.) pp 191–260, Elsevier/North-Holland Biomedical Press, New York.

Karlsson, E. (1979) *Handb. Exp. Pharmacol. 52*, 158–212.

Karlsson, E., & Eaker, D. (1972) *Tai-wan I Hsueh Hui Tsa Chih, 71*, 358–371.

Kistler, J., Stroud, R. M., Klymkowsky, M. W., LaLancelle, R. A., & Fairclough, R. H. (1982) *Biophys. J. 37*, 371–383.

Klymkowsky, M. W., & Stroud, R. M. (1979) *J. Mol. Biol. 128*, 319–334.

Lapidot, Y., Rappoport, S., & Wolman, Y. (1967) *J. Lipid Res. 8*, 142–145.

Lauterwein, J., Lazdunski, M., & Wuthrich, H. (1978) *Eur. J. Biochem. 92*, 361–371.

Lee, C. Y., Chang, S. L., Kau, S. T., & Luh, S. H. (1972) *J. Chromatogr. 72*, 71–82.

Low, B. W. (1979) *Handb. Exp. Pharmacol. 52*, 213–257.

Mebs, D., Narita, K., Iwanaga, S., Samejuma, Y., & Lee, C. Y. (1971) *Biochem. Biophys. Res. Commun. 44*, 711–716.

Miller, D. L., Moore, H.-P. H., Hartig, P. R., & Raftery, M. A. (1978) *Biochem. Biophys. Res. Commun. 84*, 632–640.

Paltauf, F. (1968) *Monatsh. Chem. 99*, 1277–1280.

Stroud, R. M. (1981) in *Biomolecular Stereodynamics* (Sarma, R. H., Ed.) Vol. II, pp 55–73, Adenine Press, New York.

Strydom, D. J. (1979) *Handb. Exp. Pharmacol. 52*, 258–275.

Sugiyama, H., & Yamashita, Y. (1981) *J. Biochem. (Tokyo) 90*, 729–735.

Tsernoglou, D., & Petsko, G. A. (1976) *FEBS Lett. 68*, 1–4.

Tsernoglou, D., Petsko, G. A., & Hudson, R. A. (1978) *Mol. Pharmacol. 14*, 710–716.

Vogel, Z., Sytkowski, A. J., & Nirenberg, M. W. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3180–3184.

Walkinshaw, M. D., Saenger, W., & Maelicke, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2400–2404.

Yale, H. L. (1943) *Chem. Rev. 33*, 209–256.