Functional Expression and Site-directed Mutagenesis of a Synthetic Gene for α-Bungarotoxin*

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In order to explore the structure-function relationships of the curaremimetic α-neurotoxins we have constructed and cloned a synthetic gene for Bungarus multicinctus α-bungarotoxin which is expressed in Escherichia coli. The recombinant α-bungarotoxin is expressed as a fusion protein with α-bungarotoxin linked to the COOH-terminal end of the T7 Gene 9-encoded coat protein. After treatment of the fusion protein with Factor Xa protease, a recombinant α-bungarotoxin is released that co-migrates with authentic α-bungarotoxin upon reverse-phase high performance liquid chromatography and ion-exchange chromatography. Final yields of active recombinant α-bungarotoxin were about 0.4 mg/liter of starting bacterial culture. The recombinant α-bungarotoxin contains 10 additional residues linked to the NH₂-terminal Ile of the α-bungarotoxin sequence due apparently to the inaccessibility of the engineered cleavage site to Factor Xa. Nevertheless, the recombinant α-bungarotoxin is capable of binding to the nicotinic acetylcholine receptor with an apparent affinity that is only decreased ~1.7-fold from that of authentenic α-bungarotoxin. Alanine substitution of a residue, Aspº, highly conserved among α-neurotoxins and previously suggested to play a key role in receptor recognition, resulted in a recombinant α-bungarotoxin whose receptor binding activity is indistinguishable from authentic α-bungarotoxin.

The snake venom-derived α-neurotoxins comprise a family of over 70 polypeptides of known primary amino acid sequence (Endo and Tamiya, 1987). The α-neurotoxins are traditionally classified as either "short," with 60-62 residues, or "long," with 70-74 residues, and produce their physiological effect, skeletal muscle paralysis, by binding to the postsynaptic nicotinic acetylcholine receptor (nAChR) thereby preventing the normal activation of this receptor by the neurotransmitter, acetylcholine. These curaremimetic α-neurotoxins, together with the κ-neurotoxins, which bind to a neuronal form of the nAChR (Chiappinelli, 1993; Oswald et al., 1991), and the more distantly related cardiotoxins constitute a large family of snake venom toxins with a conserved protein fold motif. These toxins share five invariant or conserved residues (Tyr-Phe⁴, Gly⁴³, Pro⁶⁵, Asp-Glu⁷⁵, and Asn⁷⁶) and four core disulfide bridges which together are presumed to be important in the formation of the characteristic tertiary structure consisting of three large main-chain loops (Endo and Tamiya, 1987; Ménez et al., 1992; Chiappinelli, 1993).

One of the most intensely studied of the long α-neurotoxins is α-bungarotoxin (Bgtx) which is obtained from the venom of the banded krait, Bungarus multicinctus. As a result of its extremely high affinity for the nAChR, Bgtx has provided a critically important biochemical tool for both the purification and the detailed functional and structural analysis of the nAChR (Stroud et al., 1990; Changeux et al., 1992; Chiappinelli, 1993). The x-ray crystal structure of Bgtx has been reported (Agard and Stroud, 1982; Love and Stroud, 1986) and refinements to the structure have been made based on two-dimensional NMR experiments (Basus et al., 1988; Kosen et al., 1988). Bgtx is 74 amino acids in length (molecular weight of about 8,000) and contains a fifth disulfide bond near the tip of loop 2. Bgtx, apart from the COOH-terminal tail, is a relatively flat molecule with all three main-chain loops roughly in one plane (e.g. see Fig. 6) covering an area of about 40 x 30 A with a depth of about 20 Å (Love and Stroud, 1986). The two strands of the middle polypeptide loop together with one strand from the third (COOH-terminal) loop form a triple-stranded, anti-parallel β sheet which is the major secondary structure found in the molecule and which is a common feature throughout this family of proteins.

There are 9 residues that are conserved in the short and long α-neurotoxins but not in the functionally unrelated cardiotoxins (Endo and Tamiya, 1987). These amino acids are believed to contribute either directly or indirectly to the high specificity and affinity of the neurotoxins for the nAChR. It has been proposed that two of these conserved residues, Asp³⁶ and Arg⁵⁶ in Bgtx, form an ion pair near the tip of loop 2 that mimics the structure of acetylcholine thus contributing to toxin specificity (Tseng et al., 1978). Sequence comparisons within this family of toxins and chemical modification studies have not, however, been able to determine conclusively the identity of all of the functionally relevant residues in the neurotoxins (Endo et al., 1987).
and Tamiya, 1987). More recently, several groups have taken advantage of heterologous expression systems in *Escherichia coli* to produce recombinant forms of neurotoxins and other ion channel toxins that would be amenable to site-directed mutagenesis studies of structure-function relationships (Howell and Blumenthal 1989; Ducancel et al., 1989; Boyot et al., 1990; Park et al., 1991; Fiordalisi et al., 1991, 1992; Gallagher and Blumenthal, 1992; Hervé et al., 1992; Pillet et al., 1993). In one such study of a number of site-directed mutations introduced into the short α-neurotoxin, erabutoxin α, it was shown that substitution of a His for the conserved Asp residue corresponding to Asp3' in Bgtx produces a 46-fold reduction in the apparent dissociation constant for the nACHr.

A second emerging approach towards determining those toxin residues important for receptor recognition involves the direct structural elucidation of high affinity complexes formed between Bgtx and peptide fragments that correspond to the cognate sequence in the nACHr forming the major determinant of the toxin binding site (Wilson et al., 1992; Hervé et al., 1992). This study of a number of site-directed mutations introduced near the toxin residues important for receptor recognition involves the cognate sequence in the nACHr forming the major determinant (Basus et al., 1993). In this complex, 10 Bgtx residues, including Asp3', were shown on the complex that could be labeled metabolically with 15N in a simple heterologous expression system. Further NMR analysis and structural refinement of this complex as well as those formed with longer peptide fragments of the α-subunit would benefit from the availability of a recombinant Bgtx which could be labeled metabolically with 15N in a simple heterologous expression system. In this paper, we report the design and construction of a synthetic gene for Bgtx. We show that this synthetic gene can be expressed at high levels in *E. coli* as part of a fusion protein. Proteolytic cleavage of the isolated fusion protein results in a recombinant form of Bgtx that co-migrates with authentic Bgtx upon RP-HPLC and ion-exchange chromatography. We also utilize a "charged to alamine" scanning mutagenesis strategy (Gibbs and Zoller, 1991; Basus et al., 1991; Sells 1991) to explore the role of the charged side chain of Asp30 in directing receptor recognition.

**MATERIALS AND METHODS**

**Construction of the Synthetic Gene for a-Bungarotoxin—**A synthetic gene for Bgtx was designed by back- translating the primary amino acid sequence of native Bgtx and then optimizing the coding sequence for expression in *E. coli*. To obtain optimal expression of the Bgtx we used the Gene 9-fusion protein strategy first described by Howell and Blumenthal (1989). Plasmid pSB9 (Protein Polymer Technologies, Inc.), kindly provided by K. M. Blumenthal, is a derivative of pBR322 containing a copy of the structural gene for the Gene 9 coat protein of *T7* bacteriophage. Expression of Gene 9-fusion proteins are readily inducible in the *E. coli* host strain BL21(DE3) (Studier and Moffatt, 1986) which contains the structural gene for *T7* bacteriophage RNA polymerase integrated by λ into the host chromosome of BL21, and under lac repressor control through the lacUV5 promoter.

To create the desired synthetic gene a set of six overlapping oligodeoxynucleotides, A to F, ranging from 28 to 93 bases long were synthesized (Applied Biosystems 380A DNA synthesizer) and purified by polyacrylamide/urea gel electrophoresis followed by elution in 0.5 M ammonium acetate. Oligonucleotide A, a 28-mer (5'-CATCCGGTAACGCTGACAGCCGAGGGT), corresponds to the junction between the COOH-terminal polylinker of Gene 9 and the Factor Xa protease recognition site (5'AGC to GCC). Primer D30A1 (5'-GTGGTGC[w-2]-GACAAATGCAACCCGCACCCGAAAACGTCAGCCAGGTTAGM3') was designed to introduce a single base change (GAC to GCC). Primer D30A2 (5'-CCAGGGAGCTGACAGCCGAGGACCACAC-3') also encodes a silent mutation at the S34 codon (double underline region) to introduce a new diagnostic restriction site (PstI) that could be used for identification purposes. Each internal primer was added to the appropriate flanking primer to generate half the mutant by PCR. The two half-reactions were annealed, and the full product was amplified by PCR as with the construction of the Asp3'-Ala mutant was produced from pBGTXlM3 by recombinant PCR using two overlapping internal primers containing the desired mutation (GAC to GCC). Primer D90AI (5'-GTGGTGCGC[0(3)]CGCTTTC-TGC[AGCTCCCGTG-3']) and primer D90A2 (5'-CCACG GGGAGCTGACAGCCGAGGACCACAC-3') were also silent mutagenic oligonucleotides. The nucleotide sequence of the corrected construct, pBGTX1M3, was confirmed by double-strand DNA cycle sequencing. Isolated plasmid (Promega, MiniPrep kit) was used to transform the expression strain BL21(DE3) (Novagen) with pBGTX1M3. Expt for expression studies in BL21(DE3), the pBGTX class of plasmids were standardly maintained in the HB101 host strain for all genetic manipulations and for sequencing purposes.

**Construction of the Asp3'-Ala Mutant of a-Bungarotoxin—**The D30A mutant was produced from pBGTX1M3 by recombinant PCR using two overlapping internal primers containing the desired mutation. Single strand DNA cycle sequencing (Life Technologies Inc.) and confirmed by M13 single-strand DNA sequencing. The synthetic gene for Bgtx in pBGTX1M3 was found to contain a 10-base repeat located within the overlap region between oligonucleotides D and E. The altered sequence was corrected by recombinant PCR using two complementary oligonucleotides (25 and 24 bases long) containing the altered sequence. The nucleotide sequence of the corrected construct, pBGTX1M3, was confirmed by double-strand DNA cycle sequencing. Isolated plasmid (Promega, MiniPrep kit) was used to transform the expression strain BL21(DE3) (Novagen) with pBGTX1M3. Expt for expression studies in BL21(DE3), the pBGTX class of plasmids were standardly maintained in the HB101 host strain for all genetic manipulations and for sequencing purposes.

**Fusion Protein Expression—**BL21(DE3) cells were grown at 37°C to late log phase (A750 = 0.7) in Luria broth and induced with 0.5 mM isopropropyl-β-D-thiogalactoside (Life Technologies Inc., ultra-pure grade) for 3 h. Cells were centrifuged, washed in cold PBS (40 ml, all volumes based per liter of starting culture), and resuspended in 10 ml of 10 mM Tris, pH 8.0, containing 50 mM NaCl, 2 mM Na2EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 0.5 μM leupeptin, 0.2 μg/ml aprotinin, 1 mg/ml lysozyme, 2 mM DTT, and 50 mM MeSH. The suspension was passed through a French Pressure cell (SLM Instruments) at 20,000 psi. The cell debris was removed by centrifugation at 10,000 x g for 1 h at 4°C, and the supernatant was dialyzed overnight against 0.05 M Tris, pH 7.0, containing 0.6 M NaCl to remove nucleic acids. This step was used in place of streptomycin sulfate pre-
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cipitation (Howell and Blumenthal, 1989) which we observed to co-
precipitate the Bgtx-fusion protein. Saturated ammonium sulfate was
added at 4 °C to the nonadsorbed protein fraction to a final concentra-
tion of 50% and the precipitated protein was collected by centrifugation
at 8,000 × g for 10 min. Typically, we obtained about 150 mg of amm-
onium sulfate-precipitated protein/liter of starting culture. In this frac-
tion, 50% of the protein appeared to be the desired fusion protein as
indicated by SDS-polyacrylamide gel electrophoresis.

Protein Disulfide Refolding—In order to obtain refolded Bgtx by
simple dialysis, ammonium sulfate-precipitated fusion protein was re-
suspended in 50 mM Tris, pH 7, containing 5 mM MeSH and 2 mM
NaEDTA at a concentration of 1–4 mg/ml protein and dialyzed against
the same buffer at 4 °C. Following the third buffer change, the dialysis
was allowed to proceed overnight and no further MeSH was added. This
procedure led to functionally active fusion protein as measured by com-
petition binding assays but at about half the final yield achievable with
the procedure described below utilizing mixed-disulfide formation
(Seely and Young, 1991).

To facilitate a more efficient ref ormation of the correct disulfide pairs
via a mixed-disulfide intermediate, the ammonium sulfate-precipitated
fusion protein was resuspended in 50 mM Tris, pH 8.2, containing 100
mM NaCl and 5 mM DTT, to a final protein concentration of 1 mg/ml.
After 60 min at 4 °C, mixed disulfides were formed by adding solid
cysteine-HCl to 50 mM followed by the slow addition, with stirring, of
NaOH (1 M). After 10 min at pH 12, 50% of the further incubation for
10 min at 4 °C, the reaction mixture was diluted 6-fold into 50 mM Tris,
pH 8.2, 100 mM NaCl, 20 mM MeSH and the pH was adjusted to 8.2 with
HCl. The reaction mixture was purged with N2 and incubated at 4 °C
overnight.

Purification of Recombinant Bgtx—The refolded fusion protein could
be purified by chromatography on Pharmacia Q-Sepharose Fast-Flow
equilibrated in 50 mM Tris, pH 7.0, containing 50 mM NaCl. A linear
gradient of NaCl (50 mM to 0.6 M in 10 column volumes) was used to
elude the remainder of the adsorbed fusion protein. The major fraction
eluted from the column at a NaCl concentration of about 0.3 M is similar
to results reported by Howell and Blumenthal (1989). This fraction was
either dialyzed against 20 mM Tris, pH 8.0, containing 100 mM NaCl or
the buffer was changed using a Millipore Centriprep filtration unit.

Preliminary studies in which we monitored Factor Xa cleavage of fusion
protein, by the appearance of material co-migrating with Bgtx upon
reverse-phase HPLC, suggested an optimal enzymatic digestion time of
16 h. After digestion with Factor Xa protease (New England Biolabs; at 1%
enzyme/substrate at 23 °C for 16 h in the presence of 2 mM CaCl2), the
reaction mixture was applied to a Pharmacia Mono-Q column and the
material not retained by the column, containing the released recombi-
nant Bgtx which is a basic protein, was collected. This fraction was then
purified on reverse-phase HPLC using a C18 column.

Refolded fusion protein could also be purified in a batch procedure
using an anion-exchange resin (Macroprep Q, Bio-Rad). The eluted
fusion protein was concentrated by an Amicon pressure flow cell
by FPLC cation-exchange chromatography (S-Sepharose, Pharmacia
LKB Biotechnology Inc.). The recombinant toxin was eluted in 50 mM
NaOAc, pH 5.5, with a 5–15% gradient of 1 M NaCl. The protein fraction
eluting at about 132 mM NaCl was lyophilized and then desalted using a
PD-10 gel-filtration column (Pharmacia) pre-equilibrated with a 5-15%
gradient of 1 M NaCl. The final pure fractions were further analyzed by
desalting with a PD-10 gel-filtration column (Pharmacia) pre-equilibrated
with 0.1% trifluoroacetic acid and elution was performed using a
trifluoroacetic acid/acetonitrile/water gradient of 20–60% acetonitrile. The
final purified fractions were further analyzed for enzymatic activity by
mass spectrometry (Yale Cancer Center Mass Spectroscopy Facility). The
electrospray source (Analytica, Branford, CT) was used in conjunction with a
triple quadrupole instrument from

BG Biotech (Manchester, United Kingdom). The sample concentration
was 0.1 μg/ml in 50% methanol/water. NH-terminal sequencing
was performed using standard procedures by the W. M. Keck Foundation
Biotechnology Resource Laboratory at Yale University School of Medi-
cine.

The binding activity of recombinant toxin was determined in a com-
petition binding assay using 125I-labeled Bgtx and acetylcholine recep-
tor-enriched membrane preparations. The plasma membrane fraction
was obtained from frozen electric organs of T. californica (Pacific Bi-
omarine, Venice, CA) essentially as described by Czajkowski et al.
(1989). For use in binding assays, Torpedo membranes were diluted to
10 μg/ml in PBS. Flat-bottomed 96-well microtiter plates (Nunc, Immu-
nosorb) were pre-incubated with 0.1 ml of diluted membranes and centri-
fuged at 1,000 × g. The wells were washed three times with 100 μl of
PBS and quenched with 0.2 ml of 2% bovine serum albumin (Boehringer
Mannheim, Fraction V) in PBS for 1 h at room temperature. Wells were
washed twice with 200 μl of 0.2% bovine serum albumin in PBS and 0.1
ml of prepared samples or authentic Bgtx standards was added. Snake
venom-derived Bgtx was obtained from Miami Serpentarium (Miami,
FL). The serially diluted samples and standards were prepared in 0.2%
bovine serum albumin to a volume of 0.125 ml and added to 0.125 ml of
125I-labeled Bgtx (Amersham, 3000 Ci/mm) diluted to contain 100,000
cpm/well. The final concentration of 125I-labeled Bgtx in the assay is
about 3 nM. Following a 2-h incubation at room temperature, the wells
were washed 4 times with 0.2% bovine serum albumin in PBS. The
remaining 125I-labeled toxin bound to receptor was removed by the
addition of 100 μl of 2.5% SDS, 0.25 × NaOH, and washing the wells
twice with cotton swabs, and the bound radioactivity was determined in
a γ-counter. Nonspecific binding in the presence of a large excess of un-
labeled Bgtx was usually less than 10% of the total binding and the
binding data were not corrected for this low level of nonspecific binding.
The competition curves and resulting IC50 values to fit the data were
generated by nonlinear curve-fitting using the commercial program
ENZFITTER (Elsevier, The Netherlands) modified to analyze single-site
competition data.

RESULTS

Bacterial Expression Construct—We undertook to express
Bgtx in E. coli as a fusion protein appended to the COOH-
terminal portion of the T7 Gene 9 coat protein. This expression
system has the advantage that it avoids the formation of in-
soluble inclusion bodies commonly formed in many other bac-
terial overexpression systems. The Gene 9 protein is a highly
acidic protein which can be overexpressed to high levels in E.
coli and which can be readily isolated and purified by anion-
exchange chromatography (Howell and Blumenthal, 1989). The
Gene 9 protein offers the further advantage that it is free of
cysteine residues that could otherwise interfere with the refor-
mation of the five disulfides in Bgtx. Fig. 1 describes the major
features of the Gene 9-fusion protein expression system.
The specific design and construction of the synthetic gene for Bgtx
and its attachment to Gene 9 is detailed under "Materials and

FIG. 1. Construction of the Gene 9-α-bungarotoxin fusion in
plasmid pSR9. The DNA sequence for the synthetic gene encoding
Bgtx was derived from back-translation of the primary amino acid se-
cquence. Codons were optimized for use in E. coli BL21(DE3) cells. A
Factor Xa recognition site (IEGR) was engineered flanking the 5’-end
of the Bgtx sequence. The entire region was ligated into the pSR9 plasmid
after HindIII-SalI endonuclease digestion.
The final construct, pBGTX1M3, was designed to have the peptide sequence IEGR, placed immediately NH₂-terminal to the Bgtx sequence. The "restriction" protease, Factor Xa, has been shown in many other expression systems to cleave the peptide bond on the COOH-terminal side of the Arg residue within the sequence IEGR. Control experiments with authentic Bgtx indicated that Factor Xa did not produce any cleavage within the 74-amino acid sequence of Bgtx (data not shown).

Production of Recombinant Toxins—One of the major questions in the feasibility of the production of recombinant toxin was the efficiency and accuracy of the refolding of α-bungarotoxin's five disulfide bonds. To determine if refolding of Bgtx from the unfolded state was possible, authentic Bgtx was denatured in either urea or guanidine in the presence of 50 mM DTT and allowed to renature by dialysis against buffers containing either MeSH or DTT at 1–5 mM. In preliminary studies we determined that dialysis against MeSH or DTT led to a recovery of from 7 to 17% of the initial activity as measured by a binding displacement assay using Torpedo membranes enriched in nAChRs (data not shown). In contrast, recoveries following treatment with a redox mixture of glutathione or with air oxidation were ≤1–2%. Recoveries of activity following simple dialysis to remove denaturant in the absence of a reducing agent were typically less than 1%. The demonstration that reduced and denatured native Bgtx could be refolded with a yield of up to 17% made us more confident that a recombinantly expressed Bgtx could be successfully refolded.

The Bgtx-containing fusion protein was purified further by anion-exchange chromatography on a Mono-Q column. Although the run-through fraction had detectable nAChR binding activity and appeared to contain intact fusion protein as assessed by SDS-gel electrophoresis, there was no increase in specific binding activity after Factor Xa digestion of this material. No further characterization of this material was attempted. From a 1-liter culture grown in a Fernbach shaker flask, we typically recovered approximately 35 mg of highly enriched fusion protein following salt elution from the anion-exchange column. The Bgtx-containing fusion protein represents the majority of the total protein in this fraction as judged by Coomassie staining of SDS-polyacrylamide gels (data not shown). The Bgtx-fusion protein at this stage of purification has demonstrable nAChR binding activity as determined by competition of 125I-Bgtx binding. In these assays, the Bgtx-fusion protein exhibited an IC₅₀ of 2.1 × 10⁻⁸ M in comparison to authentic Bgtx with an average IC₅₀ of about 1.5 × 10⁻⁷ M under the assay conditions used. The slope of the binding displacement curves suggested that the active fusion protein was apparently homogenous in its binding activity (data not shown). The 1,000-fold reduction in binding affinity of the recombinant Bgtx-containing fusion protein is greater than the 70–100-fold reduction in the affinity reported for a recombinant erabutoxin-fusion protein (Ducancel et al., 1989). The greater reduction in activity seen with the Bgtx-Gene 9 fusion protein may be due to electrostatic interactions between the numerous basic residues in Bgtx and the highly negatively charged Gene 9 protein.

The Bgtx-fusion protein was treated with Factor Xa to release the Bgtx. Initially, we further purified the Bgtx, which is a basic protein, by applying the Factor Xa reaction mixture to a Mono-Q column and collecting the material that did not absorb to the column. The Bgtx from the "run-through" fraction was then purified further by RP-HPLC with authentic Bgtx as a standard. The results shown in Fig. 2 demonstrate that the Bgtx eluted at a position in the acetonitrile gradient nearly identical to that of authentic Bgtx. Upon RP-HPLC, the authentic Bgtx, whose mass and purity have been verified by...
mass spectroscopic analysis, elutes as two major peaks of protein. The two forms isolated by HPLC may represent two conformers of Bgtx that are resolvable by this chromatographic method or the material eluting later in the gradient may represent partially unfolded Bgtx (Corran, 1989). In any case, the rBgtx elutes similarly upon RP-HPLC. To compensate for possible variations in sample injections, authentic and rBgtx were mixed in a 1:1 ratio and the mixture analyzed by RP-HPLC (Fig. 2). The similarity of the eluted protein peak profiles for the mixture as compared to both authentic and rBgtx suggests that the rBgtx is very similar to authentic Bgtx in its retention and elution characteristics upon RP-HPLC. In a further comparison of the relative ratio of the material in the two eluted peaks, a larger proportion of the rBgtx was found in the second peak eluting at the slightly greater acetonitrile concentration. Recovery of the eluted samples followed by reconstitution into aqueous buffer allowed us to determine that both the early and late-eluting peaks of protein from both authentic and rBgtx were able to bind to the nAChR with comparable high affinity as determined by a competition binding assay using Torpedo electric organ-derived membranes (data not shown).

A definitive comparison of specific binding activities of the recombinant toxins isolated after RP-HPLC purification was not possible as additional SDS-polyacrylamide gel electrophoresis analysis revealed the presence of a significant level of low molecular weight protein contaminants. We therefore decided to use cation-exchange (S-Sepharose) chromatography as a final purification step. The recombinant Bgtx eluted at the same position as authentic Bgtx (132 mM NaCl) on S-Sepharose, whereas the D30A mutant eluted at a slightly higher salt concentration (143 mM NaCl), consistent with the removal of a negative charge. SDS-polyacrylamide gel electrophoresis of the recombinant proteins purified by S-Sepharose chromatography indicated that the final fractions were highly purified and essentially devoid of contaminating proteins as shown in Fig. 3.

Disulfide Refolding and Final Yields—A significant amount of rBgtx refolding, as evidenced by final recovery of binding activity, could be attained simply by dialyzing the fusion protein against MeSH either before or after the anion-exchange chromatography step. When the FPLC-Mono Q purified fusion protein was simply dialedyzed against 10 mM Tris, pH 7, 100 mM NaCl, and 1 mM MeSH, the final yields of the rBgtx following Factor Xa cleavage and HPLC isolation were 83 µg/liter of bacterial culture. Refolding of the fusion protein before FPLC under similar conditions increased the final yield slightly to 93 µg/liter of culture. The use of L-cystine to form mixed disulfides as the initial step of refolding has been reported as an important determinant of final yield (Kohno et al., 1990). The cystine-based refolding scheme that we finally settled on is based on a protocol developed for the refolding of recombinant secretory leukocyte protease inhibitor, which contains 8 disulfide bonds (Seely and Young, 1991). Using this method and RP-HPLC for final purification, final yields were increased 3-fold to about 300 µg/liter of bacterial culture. With S-Sepharose chromatography replacing the RP-HPLC and with a batch anion-exchange column in place of FPLC, we were able to obtain 452 µg of toxin/liter of starting culture.

Characterization of Recombinant Toxins—As indicated in Fig. 3, the relative migration of authentic and recombinant toxins upon SDS-polyacrylamide gel electrophoresis suggests that the recombinant toxins appear to have a greater molecular weight than authentic Bgtx. When analyzed by electrospray mass spectrometry, authentic Bgtx has an apparent mass of 7,974.5 daltons. The rBgtx contained no material with the mass of authentic Bgtx and instead contained a major species with an apparent mass of 9,032.9 daltons (data not shown). NH₂-terminal sequencing of the purified rBgtx revealed the presence of 10 additional amino acids at the NH₂ terminus of the Bgtx sequence. These results suggest that the engineered IEGR protease recognition site may not be accessible to enzymatic cleavage. Fig. 4 illustrates the expected arginine cleavage site, following the recognition sequence IEGR, as well as the experimentally observed major cleavage site, 10 amino acids upstream within the Gene 9 polylinker coding region. We suspect that the NH₂-terminal Ile of Bgtx is not sufficiently exposed to allow access by Factor Xa protease. This is consistent with the observation that the purified rBgtx can be extensively retracted with Factor Xa with no further change in apparent molecular weight. We did not attempt to use purified trypsin to cleave at the IEGR site of the fusion protein as authentic Bgtx appears to be sensitive to trypsin digestion as seen by SDS-polyacrylamide gel electrophoresis (data not shown). Tryptic release of the COOH-terminal tetrapeptide from Bgtx has previously been shown to reduce toxicity about 13-fold and further digestion caused peptide cleavage after Arg⁶ (Wu et al., 1983).

The binding activities of the recombinant toxins were assayed by a competition binding assay using Torpedo nAChR-enriched membranes and 125I-labeled native toxin. Unlabeled authentic Bgtx was used as a standard and all binding determinations were performed at least in triplicate. Fig. 5 summarizes a direct comparison of the competition data obtained for authentic Bgtx, rBgtx, and the D30A mutant. From a comparison of the mean values for the IC₅₀ values, it appears that the rBgtx is slightly less active than authentic Bgtx but the difference in IC₅₀ values was clearly less than 2-fold. From four trials, the IC₅₀ of authentic Bgtx was 15.1 ± 4.4 nM (±S.E.) while the IC₅₀ of the purified rBgtx was 25.2 ± 6.5 nM suggesting a statistically significant difference in the binding activities of these two preparations at a confidence level of 95% (Student’s t test). In contrast, the IC₅₀ of the D30A mutant was 10.5 ± 2.8
remimetic α-neurotoxin to be prepared as a recombinant protein strategy than the one described here. Erabutoxin fusion to protein A. The final yield of HPLC-purified, active rBgtx, contains five disulfides, has been produced in receptor-enriched membranes, we chose to use the observed IC₅₀ values if the dissociation constant for 125I-labeled Bgtx but they did appear to influence somewhat the equilibrium between the two Bgtx conformers separable by HPLC. Furthermore, the NH₂-terminal addition of the 10 amino acids had a minimal but nevertheless detectable effect on receptor binding, increasing the measured IC₅₀ by about 1.7-fold. This finding is consistent with chemical modification studies showing that trinitrophenylation of the amino terminus of the related α-neurotoxin, cobrotoxin, had little effect on biological toxic activity (Chang et al., 1971). It also is consistent with the work of Ménez and co-workers (Ménez et al., 1984) who have suggested that the NH₂-terminal part of the short α-neurotoxin, erabutoxin, is not directly involved in the "neurotoxic" site. In addition, the inability of Factor Xa to cleave the IEGR sequence, even in the purified Bgtx, suggests that protease access may be hindered by surrounding residues and thus the NH₂ terminus is unlikely to participate directly in receptor recognition. The present study of rBgtx argues that not only is the free α-amino group not important for receptor binding but also that additional NH₂-terminal residues do not produce a major steric interference to receptor binding. Our findings suggest that the additional NH₂-terminal residues in the Bgtx may provide a useful site for the preparation of fluorescent or other chemical conjugates of rBgtx with minimal effects on receptor binding activity.

Asp⁵⁰ is a highly conserved residue among both short and long-type α-neurotoxins (Endo and Tamiya, 1987). Based on the x-ray crystal structure of a sea snake α-neurotoxin related to Bgtx, it has been suggested that Asp⁵⁰ may interact with Arg⁶⁰ to form a hydrogen-bonded ion pair that structurally mimics the α-subunit of the nAChR.

DISCUSSION

The results presented indicate that we have developed a system for producing relatively high yields of a Bgtx which has a binding affinity comparable to authentic, snake venom-derived Bgtx. The final yields of active rBgtx (450 μg/liter of culture) are comparable to those obtained for recombinant charybdotoxin, a 37-residue scorpion venom polypeptide with three disulfides (Park et al., 1991), and to those reported for anthopleurin B, a 49-residue sea anemone venom polypeptide also with three disulfides (Gallagher and Blumenthal, 1992). In contrast, recombinant α-bungarotoxin, which is related in sequence to the long-type α-neurotoxin family and which, like Bgtx, contains five disulfides, has been produced in E. coli at a final yield of about 4 μg of active toxin/liter based on 50-liter fermentations (Fiordalisi et al., 1991, 1992). Recombinant α-bungarotoxin was prepared, however, using a different fusion protein strategy than the one described here. Erabutoxin a, a 62-residue polypeptide with four disulfides and the first curaremimetic α-neurotoxin to be prepared as a recombinant protein (Ducancel et al., 1989), was produced as a CNBr-cleavable fusion to protein A. The final yield of HPLC-purified, active recombinant erabutoxin a was about 33 μg/liter of shaker culture (Ducancel et al., 1989; Boyot et al., 1990).

If we normalize the yield of active rBgtx (452 μg) to the amount of rBgtx-fusion protein at the ammonium sulfate precipitation step where we have about 75 mg of protein (per liter of culture), then our final yield is about 3% of that expected. This value represents only a minimal estimate of the overall refolding efficiency, however, as other factors such as fusion protein aggregation, possible endogenous protease contamination, and inappropriate cleavages by the added Factor Xa protease may contribute to the overall losses. Nevertheless, the particular refolding strategy utilized remains an important consideration as overall yields of active rBgtx varied by about 5-fold depending on the refolding procedure. To test whether yields could be improved if another restriction protease were used in place of Factor Xa, we constructed a Bgtx-fusion protein in which the substrate site (DDDDK) for enterokinase-III (Biozyme) replaced the apparently Factor Xa-inaccessible IEGR sequence. Analysis of the enterokinase-III-cleaved rBgtx-fusion protein by mass spectroscopy revealed a minor peak with the appropriate molecular weight expected for authentic Bgtx (data not shown). The presence, however, of large quantities of other protein fragments in the digest made further purification from this mixture impractical. We are currently investigating other nonenzymatic strategies for site-specific peptide bond cleavage in Gene 9-fusion protein constructs. Chemical cleavage could offer a significant cost advantage for the production of the relatively large amounts of rBgtx, metabolically labeled with 13C, that would be required for heteronuclear NMR investigations of toxin structure and dynamics.

Although we did not intentionally set out to create a Bgtx with 10 additional amino acids at the NH₂ terminus, the properties of the BGTX1M3 construct, which does indeed contain the sequence GSRRVDEIEGR appended to the NH₂ terminus of the Bgtx sequence, provide valuable new information on Bgtx structure and function. The 84-residue rBgtx showed chromatographic behavior, on reverse-phase HPLC, similar to that of authentic Bgtx (Fig. 2). The additional residues therefore did not affect the HPLC "footprint" of Bgtx but they did appear to influence somewhat the equilibrium between the two Bgtx conformers separable by HPLC. Furthermore, the NH₂-terminal addition of the 10 amino acids had a minimal but nevertheless detectable effect on receptor binding, increasing the measured IC₅₀ by about 1.7-fold. This finding is consistent with chemical modification studies showing that trinitrophenylation of the amino terminus of the related α-neurotoxin, cobrotoxin, had little effect on biological toxic activity (Chang et al., 1971). It also is consistent with the work of Ménez and co-workers (Ménez et al., 1984) who have suggested that the NH₂-terminal part of the short α-neurotoxin, erabutoxin, is not directly involved in the "neurotoxic" site. In addition, the inability of Factor Xa to cleave the IEGR sequence, even in the purified Bgtx, suggests that protease access may be hindered by surrounding residues and thus the NH₂ terminus is unlikely to participate directly in receptor recognition. The present study of rBgtx argues that not only is the free α-amino group not important for receptor binding but also that additional NH₂-terminal residues do not produce a major steric interference to receptor binding. Our findings suggest that the additional NH₂-terminal residues in the Bgtx may provide a useful site for the preparation of fluorescent or other chemical conjugates of rBgtx with minimal effects on receptor binding activity.

Asp⁵⁰ is a highly conserved residue among both short and long-type α-neurotoxins (Endo and Tamiya, 1987). Based on the x-ray crystal structure of a sea snake α-neurotoxin related to Bgtx, it has been suggested that Asp⁵⁰ may interact with Arg⁶⁰ to form a hydrogen-bonded ion pair that structurally mimics the α-subunit of the nAChR.
the stereocchemical features of the agonist, acetylcholine, and which may thus be important for increased binding and specificity (Tsernoglou and Petako, 1976; Tsernoglou et al., 1978; Low, 1979). In an attempt to determine the relative importance of the negative charge at position 30, we chose to mutate this residue to an alanine, adopting the charged to alanine scanning mutagenesis strategy utilized so effectively by Wells, Zoller, and co-workers (Bass et al., 1991; Gibbs and Zoller, 1991; Wells, 1991) to dissect other ligand-receptor and protein-protein interactions. Alanine, besides being found in all types of secondary structure and in both buried and exposed regions of proteins, has the further advantage that it does not introduce new hydrogen bonding possibilities nor is it sterically bulky, especially in comparison to aspartate (Wells, 1991). Using alanine to substitute for Asp30 provides important information on the specific role of the carboxyl group beyond the β-carbon common to both Ala and Asp. As clearly demonstrated in Fig. 5, the D30A mutant showed no significant change in binding affinity for the nAChR in comparison to the native toxin. This finding strongly suggests the carboxyl group of Asp30 in native Bgtx does not play a major functional role as a mimic of the ester and carboxyl oxygens of acetylcholine as had previously been proposed (Tsernoglou et al., 1978). In addition, as the final yield of the D30A mutant was comparable to that of the parental rBgtx (data not shown), the D30A substitution does not appear to affect adversely expression levels, protein folding, or disulfide formation.

Our results with the D30A mutant are in general agreement with experiments performed with a 13-mer peptide fragment of loop 2 from Bgtx (Met27-Val36) in which the residue corresponding to Asp30 was mutated to Ala with no change in the peptide’s binding affinity (Lentz, 1991). Similarly, when six carboxyl groups in cobrotoxin were simultaneously modified by the addition of a glycine methyl ester to the carboxyl groups only a 25% decrease in lethality was seen (Chang et al., 1971). These results, as well as our studies of the D30A mutant, appear somewhat contrary to a report that a Asp30-His mutation in recombinant erabutoxin a, leads to a 46-fold decrease in binding affinity (Pillet et al., 1993). To explain these findings, we propose that the chemical and steric properties of the functional group beyond the β-carbon at residue 30 in Bgtx does indeed influence binding, although a negative charge per se does not appear to be required for high affinity binding activity. The NMR solution structure of the stoichiometric complex formed between Bgtx and a dodecapeptide corresponding to nAChR α-subunit residues 185-196 (α185-196) has led to the identification of a number of Bgtx residues that are within ~4 Å of receptor peptide residues as evidenced by observable intermolecular NOEs (Casus et al., 1993). A NOE cross-peak is observed between the amide NH of Asp30 and the CbHs of the peptide residue corresponding to the conserved Tyr160 found in all functional α-subunits. Furthermore, the amide NH, the CaH, and one of the CbHs of Asp30 show a large chemical shift (≥0.12 ppm) in resonance positions upon receptor-peptide binding indicating a large change in chemical environment (Casus et al., 1993). Together, these findings suggest that the side chain of Asp30 is indeed in close proximity to the bound receptor peptide, at least part of which appears to lie in the cleft formed between the NH2-terminal loop and the long middle loop of Bgtx (Fig. 6). The larger size of the His side chain in the Asp→His mutant, possibly together with the hydrogen bonding characteristics of the imidazole ring, may contribute to the considerable effect of this particular substitution on receptor recognition in contrast to the D30A mutant results reported here. Simple removal of the carboxyl group from position 30 in Bgtx (Fig. 6), leaving the “truncated” β-carbon found in both Asp and Ala, does not inhibit receptor binding, whereas the possible protrusion of the large and planar His side chain into the toxin’s “binding cleft” may explain the 46-fold reduction in binding affinity seen with the recombinant erabutoxin a (Pillet et al., 1993). Our findings with the D30A mutant clearly indicate that the negative charge at this position is not critical for binding.

The D30A mutant has a slightly but significantly higher apparent affinity (2.4-fold) for the nAChR than the parental rBgtx, both of which contain the additional 10 amino acids at the NH2 terminus. As the parental rBgtx exhibits a 1.7-fold lower affinity in comparison to authentic Bgtx, the D30A mutation may alleviate some steric hindrance introduced by the NH1-terminal addition. It is possible that residues in the NH2-terminal addition may interact electrostatically with Asp30 in the parental rBgtx leading to a decrease in binding affinity. Removal of the negative charge at position 30 may therefore prevent this mode of interference. Alternatively, the D30A mutation may itself lead to an increase in binding affinity by removing a charged group that normally interferes somewhat with binding. In fact, several alanine substitution mutations have been observed in other systems to produce 2-4-fold increases in apparent binding affinities (Wells, 1991).

The availability of an active rBgtx addresses to some extent concerns about the future availability of authentic snake venom-derived Bgtx due to the endangered status of Bungarus multicinctus. The final yields of active rBgtx that we have obtained compare favorably with other expressed neurotoxins (Howell and Blumenthal, 1989; Boyot et al., 1990; Park et al., 1991). Furthermore, the construct reported here should provide an important biochemical tool to probe the structure-function relationship of Bgtx residues through site-directed mutagenesis. A recombinant form of Bgtx also opens up the opportunity to label the protein metabolically with 15N and 13C. Heteronuclear NMR studies of toxin structure and dynamics should help to reveal more molecular details of the protein-protein recognition events underlying α-neurotoxin recognition and
specificity towards the nAChR. Finally, the recombinant neurotoxins may provide an important molecular scaffold (protein-folding motif) from which we can probe and engineer new receptor recognition sites.

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REFERENCES