Synthesis and Expression in *Escherichia coli* of a Gene for κ-Bungarotoxin†

James J. Fiordalisi,‡ Cynthia H. Fetter,‡ Alyssa TenHarmsel,‡,§ Regina Gigowski,‡ Vincent A. Chiappinelli,‖ and Gregory A. Grant*,‡,⊥

Department of Biochemistry and Molecular Biophysics and Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110, and Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, St. Louis, Missouri 63104

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ABSTRACT: A gene which codes for the 66-residue polypeptide of κ-bungarotoxin has been chemically synthesized by linking together 3 synthetic double-stranded oligonucleotides in a bacterial plasmid. The synthesis incorporated six unique silent restriction sites spaced throughout the gene for use in cassette mutagenesis. Direct expression of the κ-bungarotoxin polypeptide by itself in *Escherichia coli* failed to result in a stable product. The toxin polypeptide was stabilized and expressed in *E. coli* as part of a fusion protein with rat intestinal fatty acid binding protein under control of the nalidixic acid inducible recA promoter. Two fusion protein constructs were prepared that differed only in the cleavage site between the fatty acid binding protein and the toxin polypeptide. One contained a factor Xa cleavage site, and the other, since the toxin itself is devoid of methionine, contained a methionyl residue that served as a cyanogen bromide cleavage site. The fusion proteins were isolated by ion-exchange chromatography and reverse-phase HPLC. The construct containing the factor Xa cleavage site could not be cleaved under nondenaturing conditions. On the other hand, κ-bungarotoxin was efficiently cleaved from the methionyl fusion protein with CNBr. The toxin polypeptide was isolated by reverse-phase HPLC and ion-exchange chromatography and produced a complete and specific blockade of neuronal nicotinic acetylcholine receptors in chick ciliary ganglia which was indistinguishable from that produced by a comparable amount of venom-purified κ-bungarotoxin.

The venoms of proteoglyphous snakes, such as cobras, kraits, and sea snakes, contain polypeptide neurotoxins that are capable of selectively binding to nicotinic acetylcholine receptors and blocking nerve transmission (Endo & Tamiya, 1987). The α-neurotoxins, which comprise a large family (Endo & Tamiya, 1987; Mebs, 1985), have played a crucial role in characterizing the nicotinic acetylcholine receptors of vertebrate muscle and the muscle-derived electric tissue of electric fish (Changeux et al., 1984). More recently, the determination of the amino acid sequences of three distinct κ-bungarotoxins (Grant & Chiappinelli, 1985; Chiappinelli et al., 1990; Danse & Garnier, 1990) and κ-flavitoxin (Grant et al., 1988) and the obvious differences between them and the α-neurotoxins in both primary structure and function have established the existence of a new family of neurotoxins, referred to as the κ-neurotoxins.

The discovery of the κ-neurotoxins came from the observation that some, but not all, preparations of α-bungarotoxin blocked nicotinic transmission in the chick ciliary ganglion (Chiappinelli & Zigmond, 1978). It was eventually determined that this blocking activity was not due to α-bungarotoxin, which has no effect on transmission in chick ciliary ganglion, but to another agent (Chiappinelli et al., 1981). Subsequently, peptides which displayed this activity were purified from the venom of *Bungarus multicinctus* by several groups. These included Bgt 3.1 (Ravdin & Berg, 1979), κ-bungarotoxin (Chiappinelli, 1983), and toxin F (Loring et al., 1984). Although originally given different names by different groups, the available sequence data now indicate that they are probably all the same polypeptide (Grant & Chiappinelli, 1985; Loring et al., 1986) which is also referred to as neuronal bungarotoxin by some workers. Although κ-bungarotoxin is structurally related to α-bungarotoxin (Grant & Chiappinelli, 1985), another polypeptide neurotoxin found in the venom of *B. multicinctus*, its neurotoxic action is quite distinct (Chiappinelli, 1983; Chiappinelli & Dryer, 1984; Wolf et al., 1988). The α-neurotoxins, such as α-bungarotoxin, bind with high affinity and block the function of nicotinic acetylcholine receptors in vertebrate skeletal muscle and electric fish electric organ. The κ-neurotoxins, such as κ-bungarotoxin, are potent antagonists of a variety of neuronal nicotinic acetylcholine receptors that are unaffected by the α-neurotoxins.

Just as α-bungarotoxin has played a major role in the isolation and study of neuromuscular nicotinic acetylcholine receptors, κ-bungarotoxin is an indispensable tool in the study of the neuronal nicotinic receptors and the neurophysiological processes in which they function. Although the difference in physiological activity of these two related polypeptides is distinct, the structural basis for the functional differences between the α- and κ-neurotoxins has not been determined. Elucidation of the structure–function relationships in these neurotoxins could lead to the development of new polypeptide probes capable of discerning new types or subclasses of receptors as well as previously unrecognized neurological mechanisms.

One of the major impediments to the progress of research with the κ-neurotoxins has been their availability. Not only is κ-bungarotoxin present in relatively low levels in venom (Loring et al., 1984) as compared to α-bungarotoxin but the

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* To whom correspondence should be addressed at the Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine.

‡ Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine.

§ Present address: P.O. Box 4912, Yale Station, Yale University, New Haven, CT 06520.

‖ Department of Pharmacological and Physiological Science, St. Louis University School of Medicine.

⊥ Department of Medicine, Washington University School of Medicine.

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venom is also increasingly more difficult to obtain. Moreover, snakes obtained from different geographical regions appear to produce κ-neurotoxins that display sequence heterogeneity (Chiappinelli et al., 1990). This paper describes the complete chemical synthesis of a gene which codes for κ-bungarotoxin and the successful expression and isolation of the protein product in *Escherichia coli*.

Materials and Methods

Restriction enzymes and DNA ligase were from Boehringer Mannheim, New England Biolabs, or Promega. Sequenase dideoxy sequencing kits were from U.S. Biochemical Corp., and nalidixic acid was from Sigma Chemical Co. Poly(vinylidene difluoride) (PVDF) membranes were either Immobilon-P from Millipore Corp. or Westran from Schleicher & Schuell, and nitrocellulose was from Schleicher & Schuell. Horseradish peroxidase coupled goat anti-rabbit IgG was from TAGO Immunologicals or Sigma Chemical Co. Sephacryl S-200 HR and Q-Sepharose were from Sigma. Native, venom-derived κ-bungarotoxin was purchased from Biotoxins Inc. Antibodies against κ-bungarotoxin were produced by Cocalico Inc. Factor Xa was kindly provided by Dr. John Matschiner of the University of Nebraska—Omaha. Factor Xa activity was monitored using Chromozym X from Boehringer Mannheim. pUC 18 and pKK233-2 were purchased from Pharmacia. pIFABP5840, native rat intestinal fatty acid binding protein, and rabbit antisera against fatty acid binding protein were kindly provided by Dr. J. I. Gordon and Dr. J. C. Sacchettini. Plasmids were grown in either *E. coli* strain JM101, MG1655, or GM2163. Strain GM2163 is a dam$^-$ host provided by New England Biolabs for the production of plasmids with non-methylated restriction sequences (i.e., *BclI*). Plasmid preparations for restriction digests and dideoxy sequencing were done according to the procedure of Kraft (Kraft et al., 1988). Restriction digests and ligations were done according to procedures described in Maniatis et al. (1982) and according to the suppliers recommendations.

Individual fragments of DNA were purified from agarose gels with Geneclean kits purchased from Bio 101 Inc. Overnight cultures of *E. coli* were started in 2 × TY broth plus 100 mg/mL ampicillin from single colonies. Large cultures in the same media were inoculated at 1% from overnight cultures. Protein expression from pKK233-2 was induced with IPTG (Maniatus et al., 1982). Protein expression from pIK and pMON was induced by the addition to the culture media of 10 mL/L of a 10 mg/mL solution of nalidixic acid in 0.1 N NaOH. Nalidixic acid was added when the 550-nm absorbance of the culture reached 0.6–1.0, and cells were harvested after 1–2 h of additional growth. Cells were lysed for 15 min with NaOH at a final concentration of 0.15 M and then neutralized.

The fusion proteins were purified by ammonium sulfate precipitation, gel filtration on Sephacryl S-200 HR, ion-exchange chromatography, and reverse-phase HPLC. The cell lysate protein precipitating between 0% and 70% ammonium sulfate saturation was recovered by centrifugation, dissolved in a minimal volume of 50 mM potassium phosphate, pH 7.0, and 150 mM NaCl, and loaded on a 100-cm column of Sephacryl S-200 HR and eluted with the same buffer. After ammonium sulfate precipitation of the appropriate pool, the sample is dissolved in a minimal volume of 50 mM ammonium bicarbonate, pH 8.0, dialyzed against that buffer, and loaded on a QAE-Sepharose column equilibrated in the same buffer. The fusion protein elutes in the breakthrough fraction while the bulk of the remaining protein is adsorbed to the resin. At this point, the fusion protein could either be purified further by reverse-phase HPLC or be submitted directly to cyanogen bromide digestion. The Sephacryl S-200 HR column could be eliminated if pure fusion protein was not a desired end product, since it is not necessary to obtain pure fusion protein as a prerequisite to obtaining pure κ-bungarotoxin.

For cyanogen bromide digests, the ammonium sulfate precipitate was dissolved directly in 70% formic acid, cyanogen bromide was added, and cleavage was allowed to proceed as described (Grant et al., 1980). The recombinant κ-bungarotoxin was purified from cyanogen bromide digests by reverse-phase HPLC. All reverse-phase procedures were performed with a Vydac C-18 column equilibrated in 0.1% trifluoroacetic acid and developed with a linear gradient of acetonitrile in 0.1% TFA. Cation-exchange HPLC was performed with a 200 × 4.6 mm Polyaspartic acid WCX column equilibrated in 25 mM sodium phosphate buffer, pH 6.0, with 10% (v/v) acetonitrile. The column was developed with a linear gradient using 500 mM sodium phosphate buffer, pH 6.0, with 10% (v/v) acetonitrile as the ending buffer.

Proteins were blotted onto PVDF or nitrocellulose with a Polyblot electrotransfer system purchased from American Bionetics according to the manufacturers procedure. Antibody screening of Western blots was performed according to established procedures (Cold Spring Harbor Laboratory, 1988), and sequencing blots were performed according to Matsudaira (1987).

Oligonucleotide synthesis, protein sequencing, and amino acid analysis were performed by the Washington University Protein Chemistry Laboratory on an Applied Biosystems 380B DNA synthesizer, an Applied Biosystems 470A or 477A protein sequencer, and a Beckman 6300 amino acid analyzer, respectively.

Bioassays for κ-bungarotoxin activity were performed by a standard assay using chick ciliary ganglia (Chiappinelli et al., 1990). Ciliary ganglia were dissected from newly hatched chicks and placed in an in vitro electrophysiological recording chamber. Suction electrodes were attached to the presynaptic oculomotor nerve and the postsynaptic ciliary and choroid nerves. Electrical stimulation of the oculomotor nerve results in a compound action potential recorded in the ciliary and choroid nerves which is blocked by κ-bungarotoxin. α-Bungarotoxin is ineffective in blocking the activity of this ganglionic nicotinic receptor (Chiappinelli, 1983).

Results and Discussion

Design and Construction of the Gene for κ-Bungarotoxin. The amino acid sequence of κ-bungarotoxin was used as the basis for the gene sequence (Grant & Chiappinelli, 1985). The gene was synthesized in 3 pieces employing 6 synthetic oligonucleotides ranging in length from 65 to 85 bases and constructed in pUC 18 by successive splicing between the *Bam*HI and *Hin*dIII sites of the polylinker as depicted in Figure 1. The *Spe*I site depicted in segment II does not correctly code for the κ-bungarotoxin sequence. It was used specifically for ease of assembly of the construct and was subsequently replaced with the correct coding sequence by cassette mutagenesis of pIII. After each splicing event, the new construct was transformed into *E. coli*, and ampicillin-resistant colonies were selected. The plasmids obtained were screened by testing for the presence of the unique restriction endonuclease site, shown in boxes in Figure 1, and the entire sequence of the insert was verified by dideoxy sequencing. The final construct which consisted of the complete coding sequence of κ-bungarotoxin, minus the *Spe*I site, was named pFFG. Other than the noncoding *Bam*HI and *Hin*dIII sites at the ends of the synthetic gene, six silent restriction sites, which are

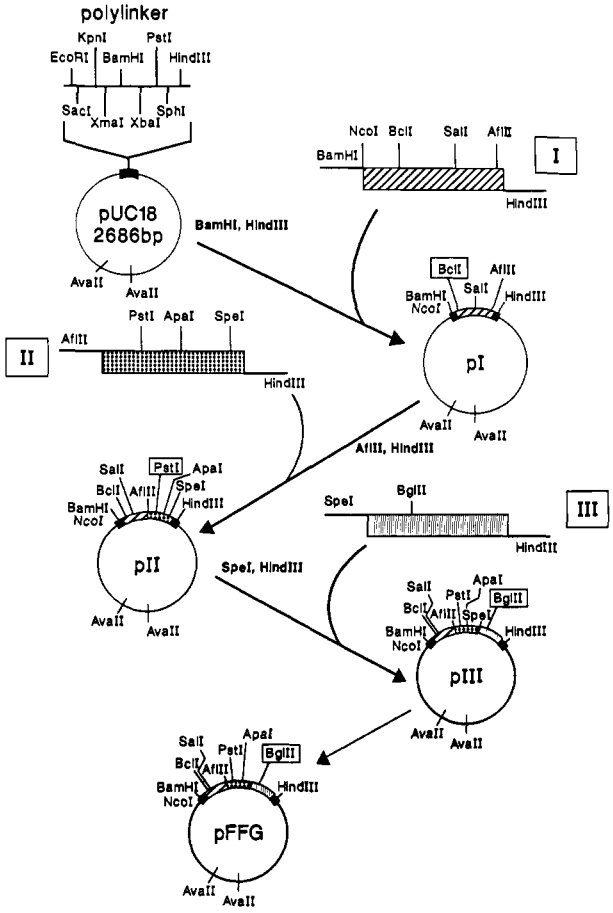

FIGURE 1: Construction of the κ-bungarotoxin gene. Details are presented in the text. Unique restriction sites used for screening of each plasmid are boxed.

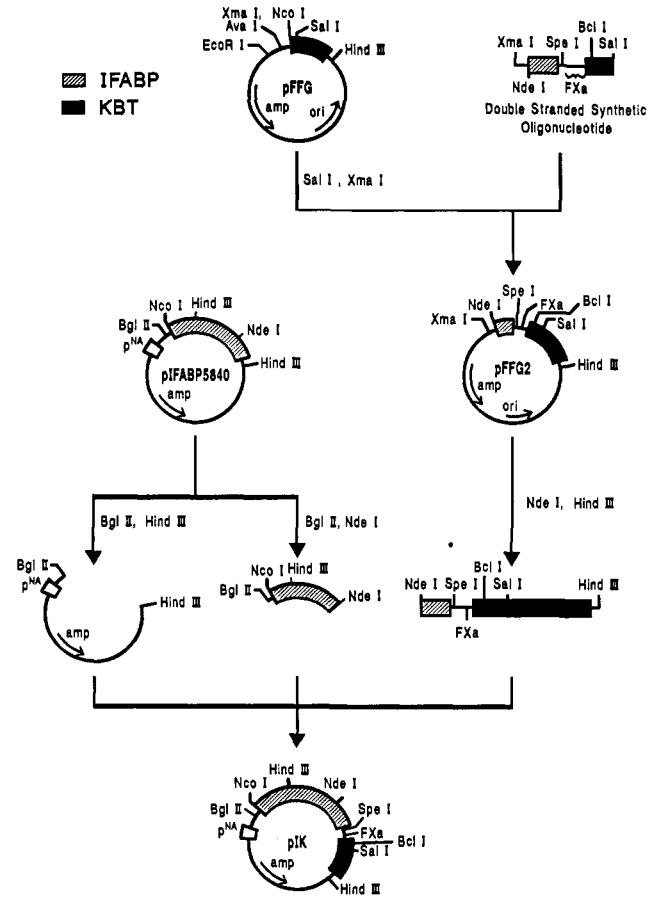

FIGURE 2: Construction of the fatty acid binding protein–κ-bungarotoxin gene. Details are presented in the text.

unique to pFFG, were incorporated. The *Nco*I and *Hin*dIII sites were used for transfer to the expression vectors. In order to incorporate the *Nco*I site, the first residue of κ-bungarotoxin was changed to a valine. It is unlikely that this would affect activity since this residue occurs at this position in many active neurotoxins. Nontheless, this residue was later changed back to the original arginine in the fusion protein construct (see below) when the *Nco*I site was no longer needed.

Initial attempts at expression of the synthetic κ-bungarotoxin gene were made using *E. coli* and the plasmid expression vectors pKK233-2 (Amann & Brosius, 1985) and pMON 5840 (Olins & Rangwala, 1990). Expression of protein was followed by SDS gel electrophoresis of bacterial lysates, before and after induction, by inspection for the appearance of a protein product corresponding to κ-bungarotoxin. Native, venom-purified, or a chemically synthesized κ-bungarotoxin polypeptide was used as a standard for judging migration position on the gel. Under the conditions used for electrophoresis, no native bacterial protein comigrated to this area, and therefore any significant production of κ-bungarotoxin polypeptide should be clearly visible. Unfortunately, neither expression vector was successful in producing an intact κ-bungarotoxin polypeptide at a level that could be detected with this technique. In both cases, the insoluble debris from the bacterial lysate was also devoid of detectable κ-bungarotoxin. The most likely explanation for these results is that the κ-bungarotoxin polypeptide was being rapidly degraded after synthesis. The available literature indicates that most small eukaryotic proteins are successfully expressed in a procaryotic host only as part of a larger fusion protein (Marston, 1986) and attempts by others at expression of another unrelated neurotoxin also showed similar results (Howell & Blumenthal, 1989).

Construction of a Fusion Protein Gene Containing κ-Bungarotoxin. In order to stabilize the κ-bungarotoxin polypeptide in the bacterial host, a fusion protein gene was prepared. The construct for the fusion protein gene consists of the gene for rat intestinal fatty acid binding protein (IFABP) followed by the synthetic κ-bungarotoxin gene in register with the strong nalidixic acid inducible promoter of the plasmid vector pMON. This particular construct was chosen because (a) the IFABP gene in pMON was readily available (pIFABP5840), (b) this construct efficiently produces larger amounts (30 mg/L) of stable IFABP in *E. coli*, (c) high-titer antisera against IFABP were also available for detection of the fusion protein, (d) the three-dimensional structure of IFABP was known to high resolution (Sacchettini et al., 1989) and indicated that the C-terminal tail of IFABP, which extends into solution from the orthogonal β-sheet structure of the protein, provided an accessible attachment point for the neurotoxin, and (e) the size of the fusion product (22 000 kDa) would be quite managable.

The expression vector containing the fusion protein gene, pIK, was constructed from pIFABP5840 and pFFG as outlined in Figure 2. In order to place the gene for κ-bungarotoxin in the proper reading frame at the 3′ end of the IFABP gene, the vector pFFG was first altered to incorporate several features. The first of these is the addition of a short segment of the 3′ end of the IFABP gene starting at a unique restriction site (*Nde*I) found 36 bases upstream from its stop codon. Second, the stop codon for the IFABP gene was eliminated. Finally, a segment coding for a specific protein cleavage point was added for the purpose of cleaving the neurotoxin poly-

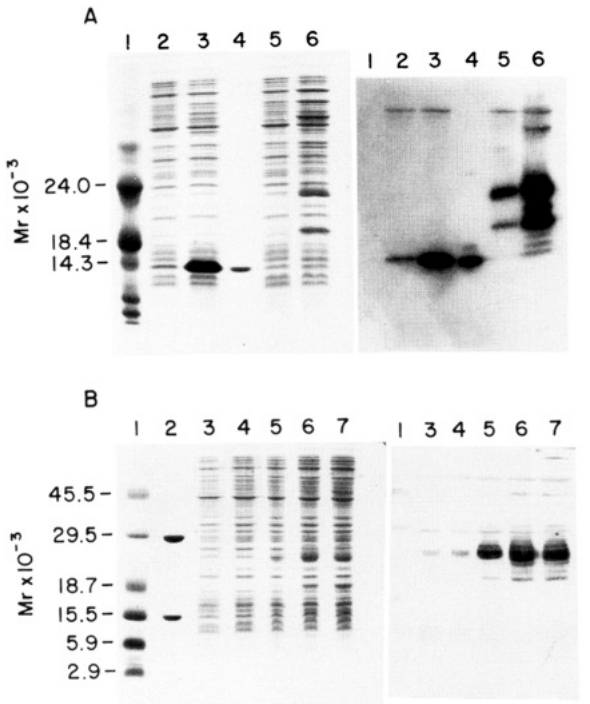

FIGURE 3: SDS gel electrophoresis (left) and Western blot (right) of the pIK fusion protein. (Panel A) Anti-IFABP antisera: lane 1, molecular weight standards; lane 2, uninduced extract of *E. coli* containing pIFABP5840; lane 3, induced extract of *E. coli* containing pIFABP5840; lane 4, purified rat intestinal fatty acid binding protein; lane 5, uninduced extract of *E. coli* containing pIK; lane 6, induced extract of *E. coli* containing pIK. (Panel B) Anti-κ-bungarotoxin antisera: lane 1, molecular weight standards; lane 2, IFABP and carbonic anhydrase standards; lanes 3 and 4, uninduced extract of *E. coli* containing pIK; lanes 5–7, induced extract of *E. coli* containing pIK at 30 min, 1 h, and 2 h after induction, respectively.

peptide from the fusion protein. Initially, the factor Xa cleavage sequence was chosen for this purpose (Nagai & Thogerson, 1987) but was later changed to methionine for cyanogen bromide cleavage. In addition to the above-mentioned features, an additional unique restriction site (*Spe*I) was included after the IFABP sequence and before the cleavage site sequence. This served the dual purpose of providing a unique site that was used for restriction endonuclease screening during construction of the plasmid and as a convenient site for cassette mutagenesis if the nature of the protein cleavage sequence needed to be changed later on. As mentioned previously, the amino-terminal arginine residue was also restored because the *Nco*I site was no longer needed. The intermediate plasmid, pFFG2, was constructed by placing a synthetic double-stranded oligonucleotide, containing all of the features mentioned above, between the unique *Xma*I and *Sal*I sites of pFFG as outlined in Figure 2.

The final construct, pIK, was produced by ligating the three segments obtained from pFFG2 and pIFABP5840 as shown in Figure 2. This three-piece ligation was necessary because the IFABP gene contains an internal *Hin*dIII site. The result was a complete fatty acid binding protein–κ-bungarotoxin fusion gene under control of the recA promoter.

Expression of the κ-Bungarotoxin Fusion Protein. pIK was transformed into *E. coli* MG1655, and synthesis of the fusion protein under control of the recA promoter was induced with nalidixic acid. Induction time courses were monitored by Coomassie blue staining and Western blotting of SDS gels of total bacterial protein using anti-IFABP and anti κ-bungarotoxin antisera. A control induction of the IFABP gene alone (pIFABP5840) with this expression system was run in parallel.

Table I: Amino Acid Analysis of Recombinant κ-Bungarotoxin Proteins

	r-IFABP–toxin fusion protein		r-κ-bungarotoxin	
residue	calculated	expected	calculated	expected
Asp	24.0	24	6.9	7
Thr	15.0	17	5.3	6
Ser	8.2	11	3.8	6
Glu	24.8	23	5.9	6
Pro	4.6	5	5.2	5
Gly	16.9	16	4.1	3
Ala	9.8	8	2.9	2
Cys	nd[a]	10	nd	10
Val	12.5	13	2.2	2
Met	nd	4	nd	0
Ile	12.0	12	3.2	4
Leu	14.1	13	3.6	4
Tyr	4.9	5	0.8	1
Phe	10.4	11	2.6	3
His	1.7	2	0.9	1
Lys	18.0	17	2.6	2
Arg	12.5	11	3.7	4
Trp	nd	2	nd	0

[a] nd, not determined.

The Coomassie-stained gels show that upon induction of pIK, a protein of approximately 23 kDa appears which is unique to this vector (Figure 3A, lane 6; Figure 3B, lanes 5–7) and which reacts positively to both anti-IFABP and anti-κ-bungarotoxin antisera. This molecular weight is in good agreement with the expected molecular weight for the fusion protein of approximately 22 000. This band is not present in the induction of pIFABP (Figure 3A, lane 3). The appearance of an additional band at approximately 19 kDa is also seen. This band reacts strongly with anti-IFABP antisera but only very weakly with anti-κ-bungarotoxin antisera. This is consistent with a fusion protein species which is truncated at the C-terminus and contains only a portion of the toxin polypeptide.

The identity of the 23- and 19-kDa proteins was confirmed by electroblotting a similar SDS gel onto a PVDF membrane and analyzing the stained proteins by automated microsequencing. The sequence determined for both the 23- and 19-kDa proteins was AFDGTWKV, which is identical with the amino-terminal sequence of rat IFABP (r-IFABP) produced in *E. coli* (see Table II).

Analysis of SDS gels of soluble cell extract compared to insoluble cellular components, after lysis with a French press, demonstrated that a significant portion of the fusion protein remains soluble. While positively reacting material of the correct molecular weight is found associated with the sedimented cellular components, perhaps in the form of inclusion bodies, the majority of the total cellular fusion protein, based on band intensity, is found in the soluble extract.

Purification of the IFABP–κ-Bungarotoxin (pIK) Fusion Protein. The 23-kDa IFABP–κ-bungarotoxin fusion protein could be purified from the soluble cellular lysate by gel filtration chromatography on Sephacryl S-200 HR followed by QAE-Sepharose and reverse-phase HPLC. SDS–polyacrylamide gel electrophoresis of this product showed a single major band that migrated with the correct molecular weight. The identity of the purified fusion protein was verified by amino acid compositional analysis (Table I) and amino-terminal sequence analysis (Table II). That the isolated fusion protein was complete and intact is evident from inspection of the values for proline and histidine in Table I. All five proline residues and one of the histidine residues are from κ-bungarotoxin. Moreover, the single histidine residue in κ-bungarotoxin is its C-terminal residue.

Table II: Automated Sequence Analysis of Recombinant Proteins

	r-IFABP–κ-bungarotoxin fusion protein[a]		r-κ-bungarotoxin	
cycle	expected	found (pmol)	expected	found (pmol)
1	Ala	Ala (22.4)	Arg	Arg (24)
2	Phe	Phe (15.5)	Thr	Thr (60)
3	Asp	Asp (10.9)	Cys	nd[b]
4	Gly	Gly (13.3)	Leu	Leu (160)
5	Thr	Thr (4.8)	Ile	Ile (140)
6	Trp	nd[c]	Ser	Ser (54)
7	Lys	Lys (4.0)	Pro	Pro (148)
8	Val	Val (9.8)[d]	Ser	Ser (44)
9			Ser	Ser (36)
10			Thr	Thr (48)
11			Pro	Pro (72)
12			Gln	Gln (48)

[a] Sequenced from a PVDF blot. [b] Cys residues were not derivatized prior to sequence analysis. [c] nd, not detected. [d] Sequencer run was stopped at this point.

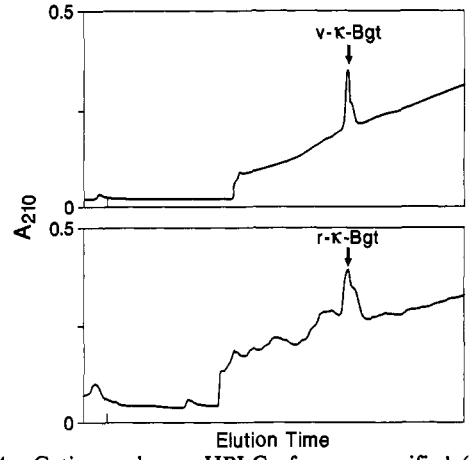

FIGURE 4: Cation-exchange HPLC of venom-purified (top) and recombinant (bottom) κ-bungarotoxin. Details are given in the text. Abbreviations: v, venom-derived; r, recombinant.

Cleavage of the Fusion Protein. The original fusion protein construct, as presented in Figure 2, contained a factor Xa cleavage site. Unfortunately, all attempts to cleave the pIK fusion protein with factor Xa were unsuccessful. Since κ-bungarotoxin is devoid of methionine residues, the factor Xa recognition sequence was replaced with a methionine residue for CNBr cleavage. The resultant construct was named pIKMET and was efficiently cleaved with CNBr to yield intact κ-bungarotoxin. The induction of pIKMET and identification of the induction products by reaction with anti-κ-bungarotoxin antibodies were identical to that shown in Figure 3. Sequence analysis of the unfractionated cyanogen bromide digest (Table III) indicates the presence, in approximately equivalent yield, of the four expected peptides, three from IFABP and the single, complete κ-bungarotoxin peptide. Sequence analysis and amino acid analysis of the isolated toxin are also presented in Tables I and II.

Isolation and Characterization of Recombinant κ-Bungarotoxin. Since κ-bungarotoxin does not possess enzymatic activity, there is no simple assay which will allow its accurate quantitation in crude mixtures or cell lysates. The two methods available for following the isolation of κ-bungarotoxin are Western blot analysis and the compound action potential assay (see below). Both of these techniques tend not to be quantitative in nature so it is very difficult to assess yields. However, Coomassie blue staining of SDS–PAGE-analyzed total cell lysate indicates that the fusion protein represents less than 5% of the total soluble protein. This is in contrast to the expression of IFABP alone (Figure 3) which may be as much as 10–20% of the total soluble protein.

When free κ-bungarotoxin is the desired final product, rather than the fusion protein itself, the gel filtration step can be omitted. After the QAE-Sepharose step, the pIKMET fusion protein was digested with CNBr, and the released toxin polypeptide was isolated by chromatography on a C-18 reverse-phase HPLC column eluted with a 0.5%/min linear gradient of acetonitrile in 0.1% trifluoroacetic acid. No special procedures are used for the folding of the recombinant toxin. Western blot analysis of unreduced toxin fractions indicates that in addition to monomeric toxin polypeptides, disulfide-linked dimeric, trimeric, and higher order aggregates are present. These are presumably formed upon exposure to air after lysis of the cells. Toxin polypeptides that are not linked through intermolecular disulfide bonds can be easily separated from those that are with reverse-phase HPLC. Automated Edman degradation of this non-cross-linked κ-bungarotoxin fraction indicates the presence of a single sequence which corresponds exactly to that expected for κ-bungarotoxin (Table II). Chromatography on cation-exchange HPLC (Figure 4) further separates biologically active toxin from inactive toxin which is presumably not folded correctly. Both recombinant and venom-purified κ-bungarotoxin elute at the same position, thus supporting their identity. Note that κ-bungarotoxin from both sources elutes with the same characteristic shoulder. The cause of this is unknown but is often observed on these columns.

Activity of Recombinant κ-Bungarotoxin. Bioassay of the recombinant κ-bungarotoxin using chick ciliary ganglia indicated (Figure 5) that the preparation was biologically active as judged by its ability to produce a complete block of nicotinic transmission in ciliary and choroid nerves. Furthermore, the response to the recombinant κ-bungarotoxin was approximately equivalent to that of venom-purified κ-bungarotoxin in concentration and duration of effect. Complete blockade of

Table III: Automated Sequence Analysis of the Unfractionated CNBr Digest of the Isolated Fusion Protein (p-IKMET)

	expected sequence		sequence found residue (pmol)			
cycle	from IFABP	from κ-BgT				
1	AGE	R	A (3708)	G (3030)	E (3470)	R (490)
2	FIG	T	F (3007)	I (3700)	G (4333)	T (2882)
3	DNN	C	D (1858)	N (3692)[a]	*a*	C (–)[b]
4	GVK	L	G (927)	V (1350)	K (518)	L (1235)
5	TVL	I	T (1123)	V (1985)[c]	L (2330)[c]	I (1096)
6	WKV	S	W (188)	K (2017)	V (1911)	S (452)
7	KRG	P	K (2402)[c]	R (671)	G (1660)	P (956)
8	VKK	S	V (1608)	K (4590)[a]	*a*	S (528)
9	DLF	S	D (986)	L (1893)	F (1717)	S (553)[c]
10	RGK	T	R (552)	G (1262)	K (2460)	T (727)

[a] Identical residue found in this position in two polypeptides. [b] Cys residues were not derivatized prior to sequence analysis. [c] Includes carryover from the previous cycle.

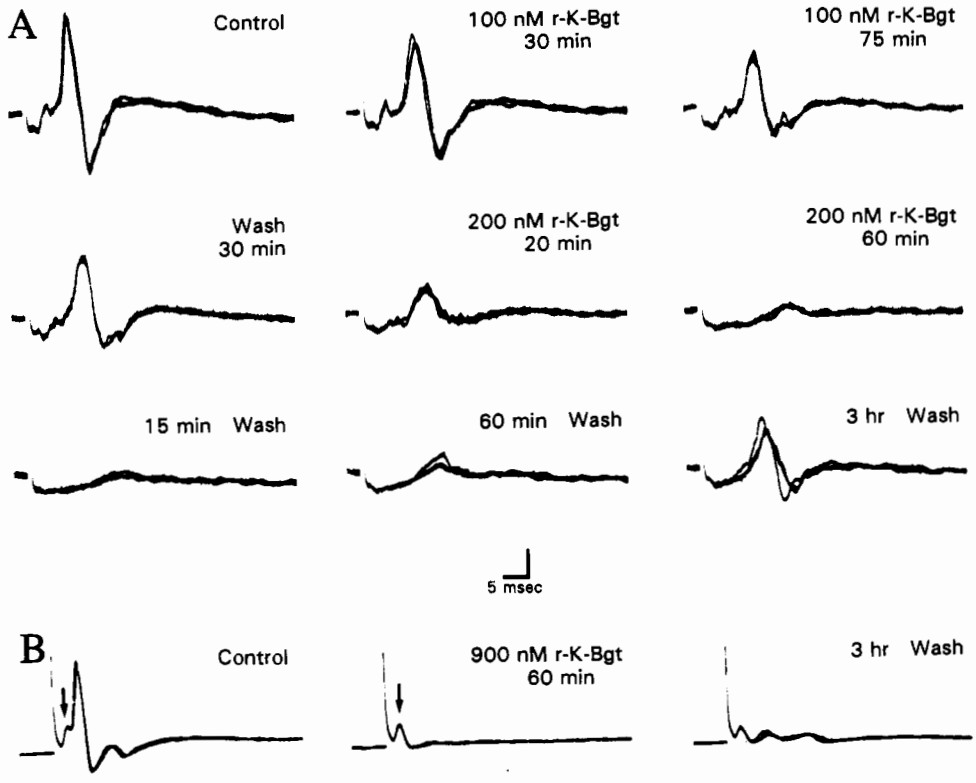

FIGURE 5: Effect of recombinant κ-bungarotoxin on the function of neuronal nicotinic receptors in the chick ciliary ganglia. (A) Electrical stimulation of the preganglionic nerve (initial break in record) results in a compound action potential recorded from the postsynaptic ciliary and choroid nerves. In this ganglion, the largest response is from the longer latency choroid neurons, while the smaller upward peak just prior to the choroid response is from the ciliary neurons. Recombinant κ-bungarotoxin (100 nM) blocked 40% of the nicotinic response after 75 min (top row). No recovery from this blockade was observed after washing out the toxin for 30 min (middle row, left). Subsequently, 200 nM recombinant κ-bungarotoxin blocked virtually all of the nicotinic response in 60 min (middle row, right). Recovery from the blockade was 40% complete after washing out the toxin for 3 h (bottom row, right). (B) In another ganglion, the prominent peak is due to nicotinic excitation of ciliary neurons, with a small choroid response also present. Ahead of these nicotinic responses, a very short latency electrically mediated response is present (arrow) which is not nicotinic in nature (Chiappinelli et al., 1990). Recombinant κ-bungarotoxin (900 nM) completely blocked all nicotinic responses in this ganglion, while not affecting the electrically mediated response (center, arrow). Recovery from this concentration of recombinant κ-bungarotoxin was slower (right) than recovery from lower concentrations, which was also the case for native, venom-purified κ-bungarotoxin. Vertical scale bar: (A) 0.1 mV; (B) 0.5 mV.

nicotinic transmission, but not the electrical component of transmission, was produced by a 100–200 nM concentration of recombinant toxin in 1 h. Below these concentrations, only partial blockade was observed. After blockade, the effect was slowly reversible with complete recovery of nicotinic transmission taking in excess of 3–5 h. Similar responses are observed with native κ-bungarotoxin at these concentrations (Chiappinelli, 1983; Loring et al., 1984), and thus, this indicates that the purified recombinant toxin is approximately equipotent with venom-purified toxin.

A typical yield of biologically active κ-bungarotoxin is approximately 200–400 μg from a 50-L fermentation. While this number is low compared to the total amount of recombinant protein produced by the *E. coli*, it must be kept in mind that this represents only that portion of the recombinant κ-bungarotoxin which spontaneously folds to active species. Significant losses result from improperly folded species and disulfide-linked aggregates. We estimate that less than 5% of the κ-bungarotoxin produced is isolated as a biologically active species. Refolding studies to attempt to increase the yields of active material are presently being conducted. Most importantly, separation methods have been developed that allow the isolation of relatively pure biologically active recombinant κ-bungarotoxin at levels more than sufficient to pursue mutagenesis studies of the structure–function relationships in this class of toxins.

The genetic constructs described in these studies represent the first nonvenom source of a κ-neurotoxin polypeptide. The ability to produce this neurotoxin in a bacterial expression system should eventually supply enough material for a vast array of neuropharmacological, structure–function, crystallographic, and mutagenesis studies. Furthermore, the recombinant κ-bungarotoxin has another important advantage over venom-derived toxin. The best available venom-derived κ-bungarotoxin preparations contain small amounts (<2%) of α-bungarotoxin and other snake venom neurotoxins that can confound the interpretation of neurophysiological experiments (Luetje et al., 1990; Couturier et al., 1990). Recombinant κ-bungarotoxins, which cannot be contaminated by other venom-derived neurotoxins, should greatly enhance the usefulness of this toxin for the pharmacological characterization of neuronal nicotinic receptor subtypes.

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Abortive Products as Initiating Nucleotides during Transcription by T7 RNA Polymerase†

Simon E. Moroney* and Joseph A. Piccirilli‡
Laboratory for Organic Chemistry, Swiss Federal Institute of Technology, 8092 Zurich, Switzerland
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ABSTRACT: The kinetics of formation of abortive initiation products during transcription of a synthetic template (encoding the transcript GAUGGC) by T7 RNA polymerase have been determined. This study revealed that while total RNA was formed in the reaction as expected, the levels of the dinucleoside tetraphosphate guanylyl-3′,5′-adenosine-5′-triphosphate (pppGpA) and trinucleoside pentaphosphate guanylyl-3′,5′-adenosine-3′,5′-uridine-5′-triphosphate (pppGpApU) formed by premature termination of transcription reached a maximum after 10 min, and then decreased. Transcription of the same template, in the presence of either [γ-^{32}P]GTP and ATP, or GTP and [α-^{32}P]ATP, gave the ^{32}P-labeled dinucleotides *pppGpA and pppG*pA. Incorporation of each of these substrates into longer RNA transcripts in the same enzyme–template system was demonstrated. The incorporation was shown to require the presence of template in the reaction mixture. The requirement for base complementarity restricts the position of incorporation to that of initiating (5′) nucleotide. Transcription of a second template, which encodes an RNA transcript having the partial sequence GpA at two internal positions, in the presence of each of the labeled dinucleoside tetraphosphates, failed to bring about the synthesis of significant yields of any longer radiolabeled transcripts. It is concluded that dinucleoside tetraphosphate (and perhaps trinucleoside pentaphosphate) can function as initiating nucleotides when complementary to the nucleotide sequence at promoter regions. However, a dinucleotide is not used as substrate for subsequent chain elongation in T7 RNA polymerase catalyzed transcription reactions.

T7 RNA polymerase is now the enzyme of choice for the in vitro synthesis of RNA (Milligan et al., 1987), and it provides a convenient system for studying the individual steps in transcription (Martin & Coleman, 1987). As with other RNA polymerases, that encoded by the phage T7 utilizes a DNA template, variation of which allows the synthesis of virtually any sequence of RNA. The interactions between enzyme, template, and the nucleoside triphosphates have been the subject of footprinting (Ikeda & Richardson, 1986; Basu & Maitra, 1986; Muller et al., 1989) and kinetic studies (Martin

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* Address correspondence to this author at the Department of Pharmacology, Cambridge University, Cambridge, England CB21QJ.
‡ Present address: Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80303.

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