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Amino Acid Sequence of κ-Flavitoxin: Establishment of a New Family of Snake Venom Neurotoxins†

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ABSTRACT: The complete amino acid sequence of κ-flavitoxin, a neurotoxin isolated from the venom of *Bungarus flaviceps*, has been determined by automated Edman analysis of the intact protein and of peptides derived from digests with trypsin and chymotrypsin. κ-Flavitoxin consists of a single 66-residue polypeptide chain which is completely devoid of methionine. The amino acid sequence of κ-flavitoxin demonstrates that although the toxin is related to the α-neurotoxin family, it displays a much higher degree of homology with κ-bungarotoxin. The conserved structural features of the κ-neurotoxins and their pharmacological profiles, which are distinct from those of all known α-neurotoxins, provide evidence for a new, structurally and functionally unique family of snake venom neurotoxins.

Poisonous snakes produce a number of toxic proteins, many of which are directed against sites in nervous tissue. Several different families of such neurotoxins have been identified in the venom of elapid snakes. β-Neurotoxins interfere with neurotransmitter release by binding to sites on presynaptic nerve terminals (Othman et al., 1982). α-Neurotoxins bind with high affinity to postsynaptic nicotinic acetylcholine receptors found in vertebrate skeletal muscle and in the muscle-derived electric tissue of electric fish (Changeux et al., 1984).

We have previously reported the complete amino acid sequence of κ-bungarotoxin, purified from the venom of *Bungarus multicinctus* (Grant & Chiappinelli, 1985). This toxin (molecular weight 7313) displays an average of 47% sequence identity with the long α-neurotoxins, but its physiological effects are quite different. κ-Bungarotoxin is a potent antagonist at a variety of neuronal nicotinic receptors which are insensitive to blockade by α-neurotoxins (Chiappinelli, 1983; Chiappinelli & Dryer, 1984). Conversely, κ-bungarotoxin binds to muscle nicotinic receptors with much lower affinity than do the α-neurotoxins (Wolf et al., 1987).

κ-Flavitoxin, a protein recently purified from the venom of *Bungarus flaviceps* (Chiappinelli et al., 1987), exhibits a number of properties which are similar to those of κ-bungarotoxin. Both toxins produce a complete blockade of nicotinic transmission in chick autonomic ganglia at 50–75 nM. This blockade is only slowly reversible and can be protected against by short-acting competitive nicotinic antagonists such as D-tubocurarine, but not by α-bungarotoxin or noncompetitive nicotinic antagonists (Chiappinelli, 1983; Sorenson et al., 1987). Radiolabeled κ-bungarotoxin and κ-flavitoxin bind with high affinity ($K_d \sim 5$ nM) to a nicotinic site in chick

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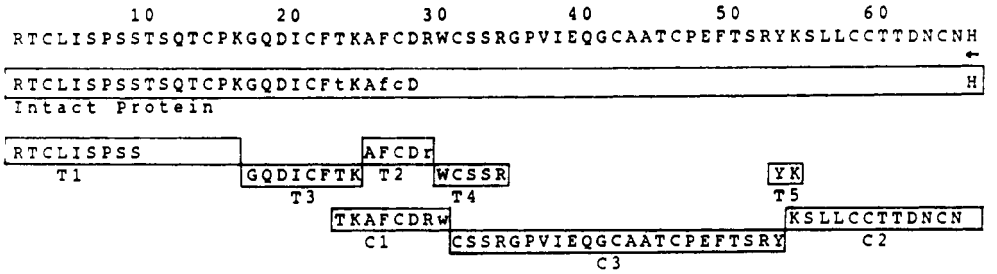

FIGURE 1: Summary proof of the primary structure of κ-flavitoxin. Each enclosed bar designates an isolated peptide. Only those peptides on which sequence analysis was performed are included. Upper case letters denote positive identification, and lower case letters denote tentative identification from automated Edman degradation. Reverse arrows designate positive identification by carboxypeptidase analysis. Letters below the bars designate tryptic (T) and chymotryptic (C) peptides.

autonomic ganglia which is not recognized by α-bungarotoxin, and which appears to be the physiologically detected neuronal nicotinic receptor (Chiappinelli, 1983; Chiappinelli et al., 1987). Both toxins exhibit a single band of protein (apparent molecular weight 6500) following sodium dodecyl sulfate–polyacrylamide gel electrophoresis and have similar, but not identical, isoelectric points (pI = 9.1 for κ-bungarotoxin; pI = 8.8 for κ-flavitoxin).

We now report the complete amino acid sequence of κ-flavitoxin. On the basis of this sequence, it is possible to define a new family of snake venom proteins, the κ-neurotoxins. Sequence homology between the κ-neurotoxins and the long α-neurotoxins is considerable; however, the two known κ-neurotoxins deviate significantly from all known α-neurotoxins at a number of positions, several of which have a demonstrated functional requirement in the α-neurotoxins. These nonconservative substitutions presumably account for the selectivity of κ-neurotoxins for neuronal nicotinic receptors and the selectivity of α-neurotoxins for muscle nicotinic receptors. The distinguishing structural features of the κ-neurotoxins are discussed.

Materials and Methods

Protein Preparation. κ-Flavitoxin was purified from crude lyophilized *Bungarus flaviceps* venom (Miami Serpentarium, Salt Lake City, UT, lot BY3S) as previously described (Chiappinelli et al., 1987).

Peptide Preparation. κ-Flavitoxin was S-carboxymethylated and desalted as previously described (Grant & Chiappinelli, 1985). All enzymatic digests were also conducted as previously described (Grant & Chiappinelli, 1985). All peptides were purified by reverse-phase high-pressure liquid chromatography (HPLC)[1] after acidification of the proteolytic digests. Tryptic peptides were loaded directly onto a 4.6 mm × 25 cm Waters C-18 Microbondapak column which had been equilibrated in 0.05% TFA. After sample injection, the column was washed with 0.05% TFA for 5 min and then developed with a linear gradient of acetonitrile which was made 0.05% in TFA. Chymotryptic peptides were loaded directly onto a 2.1 mm × 30 mm Applied Biosystems C-8 Aquapore RP-300 column mounted on an Applied Biosystems Model 130A microbore HPLC. The column was equilibrated in 0.1% TFA. After sample injection, the column was washed with 0.1% TFA for 5 min and then developed with a linear gradient of 70% acetonitrile in water which was made 0.08% in TFA. All other parameters are as described in the figure legends. Peptides were recovered by pooling appropriate fractions and drying under a stream of nitrogen. They were dissolved in 50% TFA for sequencing.

[1] Abbreviations: HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin.

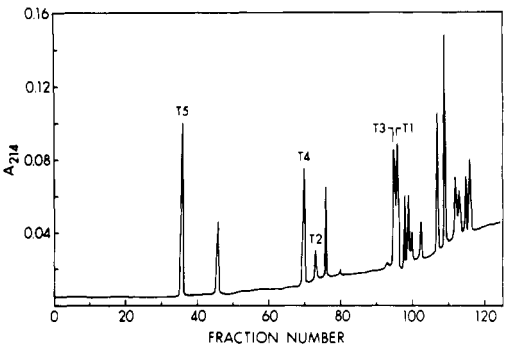

FIGURE 2: HPLC purification of tryptic peptides. The column was eluted with a 0.5%/min linear gradient. The flow rate was 1 mL/min, and 0.5-min fractions were collected.

Amino Acid Analysis. Samples were hydrolyzed in 6 N HCl in evacuated sealed glass tubes at 110 °C for the indicated times. Hydrolysates were dried by rotary evaporation and redissolved in 0.2 M sodium citrate, pH 2.0. Amino acid analyses were performed on a Waters HPLC amino acid analyzer equipped for o-phthalaldehyde detection and continuous infusion of hypochlorite for the detection of proline. Data were collected and processed electronically on a Waters 840 data system.

Sequence Analysis. All polypeptides were sequenced by automated Edman degradation on an Applied Biosystems Model 470A gas-phase protein sequencer equipped for "on-line" PTH-amino acid identification using an Applied Biosystems Model 120A HPLC. Standard columns and conditions were employed.

Results

A summary of the sequencing strategy as well as the complete sequence of κ-flavitoxin is presented in Figure 1. Automated Edman degradation of the intact carboxymethylated protein yielded the sequence of the first 28 amino acids with tentative identification at positions 23, 26, and 27. The remaining sequence was determined from partial sets of tryptic and chymotryptic peptides. The carboxy terminus was verified by carboxypeptidase A analysis.

Tryptic Peptides. Approximately 8 nmol (60 μg) of S-carboxymethylated κ-flavitoxin was digested with bovine trypsin, and the resulting peptides were separated by reverse-phase HPLC as shown in Figure 2. The peptides labeled T1–T5 yielded single unambiguous sequences which were used in the solution of the κ-flavitoxin sequence as shown in Figure 1. The peaks at fractions 46 and 76 also produced single sequences that represented the same peptides as T5 and T4, respectively. In the case of T4 and the fraction 76 peptide, this difference in elution position resulted from a chemical difference in the cysteinyl residue of the peptide. T4 contained S-carboxymethylated cysteine which was clearly identified as

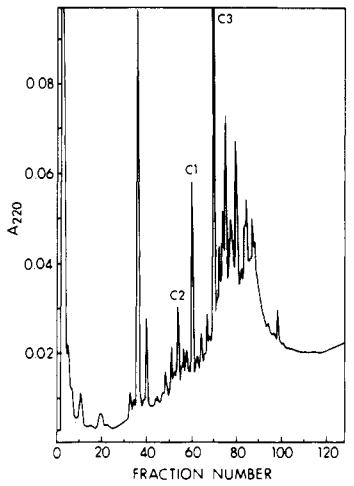

FIGURE 3: HPLC purification of chymotryptic peptides. The column was eluted with a 1%/min linear gradient. The flow rate was 0.1 mL/min, and 0.5-min fractions were collected.

PTH-S-(carboxymethyl)-Cys. The fraction 76 peptide contained an underivatized cysteinyl residue that was identified as the dithiothreitol derivative of dehydroalanine on the HPLC tracing of the sequencer fraction. This undoubtedly resulted from incomplete derivatization of Cys during the carboxymethylation. Furthermore, their relative elution positions on the reversed-phase column are consistent with the identification of these chemical forms. Direct chemical evidence explaining the appearance of the Tyr-Lys dipeptide in two separate HPLC peaks (T5 and fraction 46) is not available. However, this is probably due to heterologous salt formation or ion pairing with the positively charged lysyl residue. The remaining peaks either produced mixed sequences or were not subjected to analysis.

Chymotryptic Peptides. Approximately 4 nmol (30 μg) of S-carboxymethylated κ-flavitoxin was digested with bovine chymotrypsin, and the separation of the resulting peptides is shown in Figure 3. The peptides labeled C1–C3 produced single sequences which were used, as shown in Figure 1, to solve the complete sequence. The peaks at fractions 36 and 40 failed to give any sequence data. The remaining major peaks either produced mixed sequences or were not subjected to analysis.

Assignment of the Complete Sequence. With the exception of residue 23, the first 25 residues of κ-flavitoxin were unambiguously identified by automated sequence analysis of the intact protein. The identity of residue 23 was verified with peptide T3, and the sequence was extended to position 29 with peptides T2 and C1. Peptide C1 verified the identity of Arg-29, and peptide T4 verified Trp-30. The sequence is extended to residue 65 with the overlap of peptides T4, C3, T5, and C2. The carboxy-terminal histidine was verified with carboxypeptidase A analysis of the intact protein.

Table I: Amino Acid Composition of κ-Flavitoxin

amino acid	composition calcd[a]	composition sequence	amino acid	composition calcd	composition sequence
Asp	5.1	5	Met	0	0
Thr	7.8	8	Ile	3.2	3
Ser	8.3	8	Leu	2.6	3
Glu	4.6	5	Tyr	0.9	1
Pro	3.6	4	Phe	2.8	3
Gly	3.3	3	His	1.0	1
Ala	3.0	3	Lys	3.1	3
$^1/_2$-Cys	ND[b]	10	Arg	3.8	4
Val	0.8	1	Trp	ND	1
					total 66

[a] Amino acid composition was determined after 24-, 48-, and 72-h hydrolyses. Serine and threonine were extrapolated to zero-time hydrolysis. Valine and isoleucine were estimated from the 72-h value. [b] ND, not determined.

Discussion

The complete sequence of κ-flavitoxin has been determined from peptides generated with trypsin and chymotrypsin in conjunction with automated Edman degradation and carboxypeptidase analysis of the intact protein. The complete κ-flavitoxin structure contains 66 amino acid residues, is devoid of methionine, and has a calculated molecular weight of 7242. Although the overlaps at residues 30 and 53–54 are minimal, the proteolytic patterns and the excellent agreement between the amino acid composition (Table I) and that determined from the sequence analysis of the individual peptides strongly support the sequence presented. In the case of the overlap of C3 and C2 with T5, the protein contains only a single residue of tyrosine, and it is clearly identified as the C-terminus of C3 and the N-terminus of T5. Two of the three residues of lysine are unequivocally identified in regions of convincing overlap (positions 16 and 24 of Figure 1), and the third lysyl residue is clearly identified at the C-terminus of T5 and the N-terminus of C2. The only possible interpretation is that C3

```
                    10        20        30        40        50        60        70        80
                    .         .         .         .         .         .         .         .
1   66-10  RTCL---ISPSSTSQT---CPKGQDICFTKAFCDRWCSSRGPVIEQGCAATCPEFTSRYKSLLCCTTDNCNH
2   66-10  RTCL---ISPSSTPQT---CPNGQDICFLKAQCDKFCSIRGPVIEQGCVATCPQFRSNYRSLLCCTTDNCNH

3   74-10  IVCHTTATIPSS-AVT---CPPGENLCYRKMWCDAFCSSRGKVVELGCAATCPSKKPYEEVT-CCSTDKCNHPPKRQPG
4   73-10  LICY---MGPKT-PRT---CPRGQNLCYTKTWCDAFCSSRGKVVELGCAATCPIAKSYEDVT-CCSTDNCNPFPVRPRH
5   73-10  TKCY---VTPDATSQT---CPDGQDICYTKTWCDGFCSSRGKRIDLGCAATCPKVKPGVDIK-CCSTDNCNPFPTWKRKH
6   72-10  IRCF---ITPDVTSQA---CPDGQNICYTKTWCDNFCGMRGKRVDLGCAATCPTVKPGVDIK-CCSTDNCNPFPTRERS
7   72-10  RTCY---KTPSVKPET---CPHGENICYTETWCDAWCSQRGKREELGCAATCPKVKAGVGIK-CCSTDNCDPFPVKNPR
8   71-10  IRCF---ITPDVTSQI---CADG-HVCYTKTWCDNFCASRGKRVDLGCAATCPTVKPGVNIK-CCSTDNCNPFPTRNRP
9   70-10  LSCY---LGYKH-SQT---CPPGENVCFVKTWCDGFCNTRGERIIMGCAATCPTAKSGVHIA-CCSTDNCNIYA-KWGS
10  66-10  RECY---LNPHD-TQT---CPSGQEICYVKSWCNAWCSSRGKVLEFGCAATCPSVNTGTEIK-CCSADKCNTYP

11  62-8   RRCF---NHPSSQPQTNKSCPPGENSCYNKQWRDH----RGTITERGC--GCPTVKPGIKLT-CCQSEDCNN
```

FIGURE 4: Comparison of κ-flavitoxin with κ-bungarotoxin and selected α-neurotoxins. Sequence 1 is that of κ-flavitoxin determined in this study, and sequence 2 is that of κ-bungarotoxin. Sequences 3–10, which are long α-neurotoxins, are (3) *Bungarus multicinctus* (α-bungarotoxin), (4) *Notechis scutatus* (toxin III-4), (5) *Ophiophagus hannah* (toxin b), (6) *Naja haje anchietae* (toxin III), *Dendroaspis viridis* (toxin 4.7.3), (8) *Naja melanoleuca* (toxin b), (9) *Astrotia stokesii* (toxin b), and (10) *Laticauda semifasciata* (toxin LS III). Sequence 11 is a representative short α-neurotoxin, *Laticauda laticauda*: laticotoxin A. The numbers separated by a dash which appear at the beginning of the sequences indicate the number of amino acids and the number of half-cystinyl residues, respectively. Gaps (indicated by dashes) have been placed in the sequences to optimize homology. Boxes show conserved residues between long α-neurotoxins and κ-neurotoxins. Asterisks denote unusual features in the κ-neurotoxins as compared to the α-neurotoxins. The numbering at the top designates total positions rather than the number of actual residues. All sequences were taken from the PIR–NBRF data base and references therein.

and C2 are contiguous. The overlap at position 30 is supported in the same way. The positions of all four arginines are clearly identified in their respective peptides, and therefore only one solution is possible. Furthermore, this sequence displays excellent overall homology with κ-bungarotoxin. Figure 4 compares the sequence of κ-flavitoxin with κ-bungarotoxin and demonstrates that they display 82% identity.

On the basis of the these two protein sequences, it is possible to define a new family of snake venom proteins, the κ-neurotoxins. In addition to their unique structural features, the κ-neurotoxins exhibit a distinctive pharmacological profile; they are potent, competitive antagonists at a variety of neuronal nicotinic receptors which are unaffected by the α-neurotoxins, and they are much weaker ligands at muscle nicotinic receptors than are the α-neurotoxins.

κ-Bungarotoxin was the first κ-neurotoxin to be completely sequenced (Grant & Chiappinelli, 1985). Several other proteins with properties similar to those of κ-bungarotoxin have been isolated from *Bungarus multicinctus* venom by different purification schemes. Toxin F (Loring et al., 1984, 1986) has an amino acid sequence identical with that previously published for κ-bungarotoxin. A partial amino acid sequence for Bgt 3.1 (Ravdin & Berg, 1979; Halvorsen & Berg, 1986) has been reported (Loring et al., 1986) along with other evidence which makes it likely that the complete amino acid sequence of Bgt 3.1 is also identical with that of κ-bungarotoxin. Thus, to date only a single κ-neurotoxin sequence has been obtained from *Bungarus multicinctus* venom. κ-Flavitoxin is the second unique κ-neurotoxin to be sequenced and thus demonstrates that members of this snake neurotoxin family are produced by at least two different species of *Bungarus*.

κ-Neurotoxins are closely related to the long α-neurotoxins in structure (Figure 4). Both families of proteins share the following invariant residues: 10 cysteinyl residues forming 5 disulfide bonds, Gly-23, Asp-34, Trp (or Phe)-36, Arg-40, Gly-41, Ala-50, Thr-51, Pro-53, and Asp-68. In addition, Asn-71 occurs in all but one α-neurotoxin (where an Asp residue has been reported), and Tyr-28 is conservatively replaced by Phe-28 in the κ-neurotoxins. This latter substitution is also present in one α-neurotoxin (toxin B from *Astrotia stokesii*) which shows undiminished α-neurotoxin activity in physiological assays (Maeda & Tamiya, 1978). All of the invariant cysteinyl residues have been determined to be involved in disulfide bonds, five in the long α-neurotoxins and four in the short α-neurotoxins, in several of the toxins studied (Ohta & Hayash, 1973; Bates, 1971; see PIR–NBRF data base[2] and references cited therein). It is assumed by homology that this is the case with all of these related neurotoxins, but this has not been directly determined in many instances or in this study.

It should be remembered when comparing these two families of neurotoxins that they both bind to nicotinic acetylcholine receptors. Thus, the differences between them are likely to reflect the differences between the acetylcholine recognition site regions of muscle and neuronal nicotinic receptors. The key features which distinguish the κ-neurotoxins from all known α-neurotoxins are described below. κ-Neurotoxins lack the Trp-32 which is invariant in both the long and short α-neurotoxins and which has a demonstrated functional role in binding to muscle nicotinic receptors (Karlsson et al., 1973; Ohta & Hayashi, 1974; Seto et al., 1970). While κ-neurotoxins and α-neurotoxins share the invariant Arg-40 and Gly-41 which have also been strongly implicated in functional studies, these residues are immediately followed in the κ-neurotoxins by a proline residue. This Pro-42 may interfere with the formation of a β-pleated-sheet structure which has been observed in this region in several long α-neurotoxins (Walkinshaw et al., 1980; Inagaki et al., 1982, 1985). The κ-neurotoxins also have a positively charged residue at position 35, within a five-membered loop located near the presumed active site of the α-neurotoxins.

Several unique features are also seen near the C-terminal region of the κ-neurotoxins. Most obvious is the shortened C-terminal tail, with only 2 amino acids following the last cysteinyl residue in the κ-neurotoxins compared with between 4 and 11 such residues in all long α-neurotoxins. The lack of an extended C-terminal tail is also a feature of the short α-neurotoxins (Figure 4, sequence 11) which are potent acetylcholine receptor antagonists but which are structurally distinct from the long α-neurotoxins in several other areas as well. In addition, the κ-neurotoxins have a positively charged residue at position 60, a single amino acid residue insertion at Leu-63, and a threonine residue in place of an invariant serine residue at position 66. The sequences of the two κ-neurotoxins are in fact identical in this region (positions 59–72) with the exception of a conservative substitution (Arg or Lys) at position 60.

Thus, the structural features which distinguish the κ-neurotoxins from the long α-neurotoxins are all contained within two distinct regions. The first region (positions 32–42) has been strongly implicated in function in the α-neurotoxins and contains the presumed active site which interacts directly with the nicotinic receptor. The second region (positions 59–72) is likely to be of structural importance in the κ-neurotoxins, even if it does not participate directly in the recognition of neuronal nicotinic receptors. It may determine the overall degree of β-pleating of the molecules, as this region does appear to participate in β-pleating in the long α-neurotoxins (Inagaki et al., 1982).

The postsynaptic neurotoxins produced by *Bungarus* snakes include the long and short α-neurotoxins and the κ-neurotoxins. These toxins probably evolved from a common ancestral digestive enzyme, such as an RNase. Strydom (1973) has presented evidence that the short α-neurotoxins evolved first and then the long α-neurotoxins evolved from the short neurotoxins. If this is indeed the case, then the κ-neurotoxins most likely evolved last, from the long α-neurotoxins, since the long α-neurotoxins are more closely related to the short α-neurotoxins than are the κ-neurotoxins.

The evolutionary pressure leading to the development of the κ-neurotoxins may have its origins in the dietary habits of *Bungarus* snakes. Kraits, including *B. multicinctus* and *B. flaviceps*, feed almost entirely on other snakes (Tweedie, 1957; Campden-Main, 1970). While short and long α-neurotoxins are extremely potent toxins in mammals and birds, they exhibit little or no affinity for the nicotinic receptors found in the skeletal muscle of snakes and advanced lizards (Burden et al., 1975). Thus, the α-neurotoxins present in *Bungarus* venom would seem ill-suited for incapacitating the snakes' prey. The κ-neurotoxins, on the other hand, recognize a number of nicotinic receptors which are undetected by α-neurotoxins, and thus they may contribute directly to the lethality of *Bungarus* venom toward snakes and lizards.

The close homology between the κ-neurotoxins and the α-neurotoxins implies that there is a considerable degree of homology between neuronal nicotinic receptors and vertebrate muscle nicotinic receptors. The complete amino acid sequences

[2] Protein Identification Resource, National Biomedical Research Foundation, Georgetown University Medical Center, 3900 Reservoir Rd., N.W., Washington, DC 20007.

and subunit structures of several muscle nicotinic receptors are now known (Noda et al., 1982; Takai et al., 1985). Although no functional neuronal nicotinic receptor has as yet been sequenced, several sequences expressed in nervous tissue exhibit considerably homology with muscle nicotinic receptors (Barnard et al., 1986; Goldman et al., 1987). When the complete structures of neuronal nicotinic receptors are finally elucidated, the κ-neurotoxins should be of value in determining the regions of the receptors which contain the acetylcholine recognition sites, just as α-neurotoxins have been used to identify this region in muscle nicotinic receptors (Neumann et al., 1986).

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