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The Complete Amino Acid Sequence of a Cardiotoxin from the Venom of *Naja naja* (Cambodian Cobra)†

Linda Fryklund* and David Eaker

ABSTRACT: The complete amino acid sequence of a small, basic protein with cardiotoxic activity is described. This toxin, designated *Naja naja* F8, was isolated from the venom of *Naja naja,* of Cambodian origin, by gel filtration on Sephadex G-75 followed by gradient ion exchange chromatography on Bio-Rex 70. The cardiotoxin F8, molecular weight 6727 from amino acid composition, consists of 60 amino acids in a single peptide chain cross-linked by four disulfide bridges and is devoid of histidine, tryptophan, and glutamic acid. The chymotryptic and tryptic peptides from the performic acid oxidized toxin were separated by gel filtration on Sephadex G-25 and zone electrophoresis in columns of cellulose powder. The sequence was established by Edman degradation, using the direct phenylthiohydantoin method, and with the aid of carboxypeptidase A, and is similar to the sequences reported for other cardiotoxins, cytotoxins, and/or lytic factors from cobra venoms, all of which show considerable homology with the functionally distinct neurotoxins.

Cobra venoms contain many small basic proteins representing several different pharmacological activities (Lee, 1971) and immunological classes (Boquet et al., 1972). The *Naja naja* cardiotoxin F8 described below is serologically related to the *Naja nigricollis* toxin γ and is distinct from all the curariform neurotoxins tested (Boquet et al., 1972). Cardiotoxin F8 is similar in sequence to the cardiotoxin from *Naja naja atra* venom (Narita and Lee, 1970), *Naja nigricollis* toxin γ (Fryklund and Eaker, 1975), the *Naja naja* cytotoxins I (Hayashi et al., 1971) and II (Takechi and Hayashi, 1972), the lytic protein 12B from *Haemachatus haemachates* venom (Fryklund and Eaker, 1973), the two cytotoxins from *Naje naje annulifera* (Weise et al., 1973), the major cytotoxin from *Naja melanoleuca* (Carlsson and Joubert, 1974), and *Naja mossambica mossambica* (Louw, 1974).

Materials and Methods

Isolation of the Cardiotoxin. The *Naja naja* venom of Cambodian origin was a gift from Dr. Paul Boquet, Pasteur Institute, Garches, France, and had been desiccated over silica gel; 1.0 g of dried crude venom was dissolved in 10.0 ml of 0.2 *M* ammonium acetate and the solution was centrifuged for 10 min at 20,000*g*. The clarified solution was separated on a 3.2 × 70 cm column of Sephadex G-75 in the same medium. The fraction containing the cardiotoxic and neurotoxic activities was further separated by ion exchange chromatography on Bio-Rex 70, minus 400 mesh, equilibrated with 0.20 *M* ammonium acetate at pH 7.3, using a 2-l. concave gradient of 0.11 vs. 1.5 *M* ammonium acetate. Details regarding the preparation and equilibration of the resin and the chromatographic technique have been described elsewhere (Karlsson et al., 1971).

Characterization and Sequence Analysis. Toxicity assays were done in triplicate at each dose level using female albino mice weighing ca. 20 g. The injections were done intravenously or intraperitoneally in 0.1 or 0.5 ml of 0.9% saline, respectively.

For sequence analysis the cardiotoxin was oxidized with preformed performic acid and recovered by lyophilization as described by Hirs (1956).

Amino acid analyses of the native and oxidized toxin were done with a Bio-Cal BC-200 analyzer equipped with an Infotronics CRS-110A integrator following hydrolysis at 110° in thoroughly evacuated tubes in 6 *N* HCl containing 10 mg/ml of reagent grade phenol. The molar absorptivity of the native cardiotoxin was determined in conjunction with the amino acid analyses as described by Karlsson et al. (1972).

Digestions with trypsin and chymotrypsin and the separation of peptides by gel filtration and column electrophoresis were done as described by Fryklund et al. (1972). Digestions with carboxypeptidases A and B and manual Edman degradation by the direct phenylthiohydantoin procedure were performed as described by Fryklund and Eaker (1973).

Results

Isolation and Characterization. Gel filtration on Sephadex G-75 resolved the crude venom into five protein fractions (Figure 1) which, together with the nonprotein fraction VI, accounted for all of the material applied to the column. Fraction IV containing the cardiotoxic and neurotoxic activity was further separated by ion exchange chromatography as illustrated in Figure 2. Peak 8 exhibited cardiotoxic activity and eluted in a single symmetrical peak upon rechromatography under the same conditions. Further evi-

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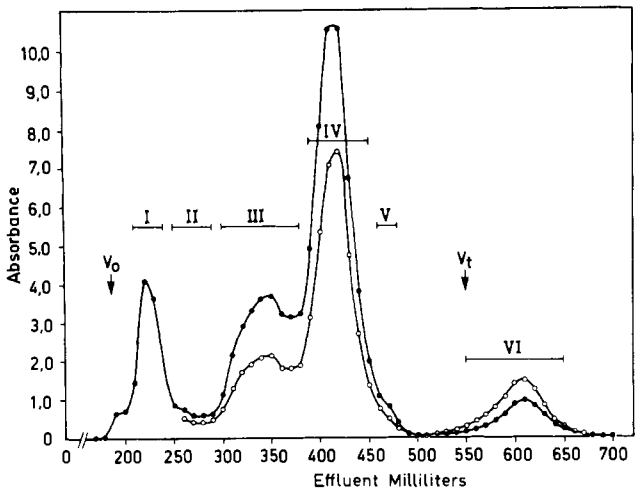

FIGURE 1: Gel filtration of crude venom on Sephadex G-75, column dimensions 3.2 × 70 cm, in 0.2 *M* ammonium acetate. V_0 and V_t indicate void volume and total bed volume, respectively. (●) $A_{279\ nm}$; (○) $A_{254\ nm}$.

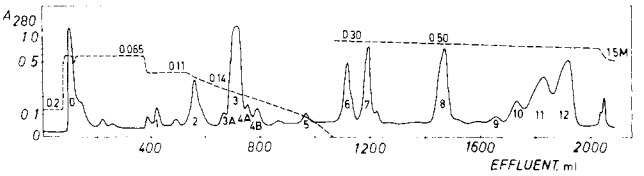

FIGURE 2: Ion exchange chromatography on Bio-Rex 70, column dimensions 3.2 × 25 cm, of fraction IV, Figure 1. The column was equilibrated in 0.2 *M* ammonium acetate buffer (pH 7.3), and eluted with 40 ml of 0.06 *M* ammonium acetate before application of the 363-mg sample in 10 ml of this buffer. The sample was followed by 250 ml of the 0.065 *M* buffer before applying the concave gradient, 0.11 *M* vs. 1.5 *M* ammonium acetate with a total volume of 2 l. The broken line (- - -) represents the conductivity trace, the figures on this indicate the molarity of the buffer.

dence for homogeneity is provided by the amino acid composition data given in Table I, where the values for all of the constituent amino acids are very close to integers and two acid-stable amino acids, histidine and glutamic acid, are *completely* absent.

The value of 4730 for the molar extinction coefficient of the native cardiotoxin at 279 nm is consistent with the presence of two residues of tyrosine, four disulfide bridges, and no tryptophan (molar absorptivities 1405, 150, and 5579, respectively). The difference of 1320 between the observed molar absorptivity of the cardiotoxin and the sum (3410) of the values for tyrosine and cystine is contributed by the tertiary structure (Wetlaufer, 1963) and is close to the corresponding values of 1500–1600 observed for several other related toxins studied in this laboratory (Karlsson et al., 1971, 1972).

The LD_{100} dose for 20-g mice was 15 μg for intravenous injection and 40–50 μg for intraperitoneal administration. Death was caused by ventricular fibrillation of the heart and usually occurred within 10 min following the injection of an LD_{100} dose. Early symptoms of intoxication were hyperexcitability and spasticity. These effects stand in marked contrast to those observed with the post-synaptic curarimimetic neurotoxins, which cause flaccid paralysis and death by respiratory failure, and where symptoms do not appear before 30 min following injection of the LD_{100} dose of approximately 2 μg (Karlsson et al., 1971).

Although we have never been able to obtain any precise information regarding the particular subspecies of *Naja naja* from which the venom of Cambodian origin used in the present investigation was taken, we have recently isolated the same cardiotoxin from a commercial preparation of authentic *Naja naja siamensis* venom (Miami Serpentarium, lot no. NS1EL-1), where it accounts for 4.5% of the total protein. Furthermore, the principal neurotoxin of the Cambodian *Naja naja* venom, which corresponds to peak 3 in Figure 2, is identical with the neurotoxin *siamensis* 3 described earlier (Karlsson et al., 1971) as the principal neurotoxin of *Naja naja siamensis* venom from Miami Serpentarium lot no. NS1S, which contained only a fraction of a percent of the cardiotoxin F8. Despite the quantitative variation in the content of the cardiotoxin F8, the presence of neurotoxin *siamensis* 3 as the principal toxic constituent of

Table I: Amino Acid Composition of the Native and Oxidized Cardiotoxin.[a]

Amino Acid	Native[b]		Oxidized	
Tryptophan				
Lysine	7.75	8	7.97	8
Histidine				
Arginine	2.00	2	2.00	2
Cysteic acid			7.79	8
Aspartic acid	8.08	8	8.16	8
Methionine sulfone			2.98	3
Threonine	3.08	3	2.98	3
Serine	3.16	3	3.03	3
Glutamic acid				
Proline	4.20	4	3.82	4
Glycine	2.18	2	2.06	2
Alanine	1.99	2	2.06	2
Half-cystine[c]		8		
Valine	3.77	4	3.73	4
Methionine	2.86	3		
Isoleucine	3.91	4	3.76	4
Leucine	5.92	6	6.01	6
Tyrosine	1.65	2	1.85	2
Phenylalanine	0.88	1	0.98	1
Total		60		60

[a] $E'_{279\ nm}$ 4730; $A_{279}^{1\ cm}$ (1 mg/ml) 0.702. [b] Average of 24- and 72-hr determinations. [c] As cysteic acid.

all three venom preparations strongly indicates that the Cambodian cobras were *Naja naja siamensis*. The important point is that the cardiotoxin F8 can be obtained from a commercially available venom.

Determination of the Sequence. The sequence determination was done with the performic acid oxidized derivative of the cardiotoxin, and in all of the sequences written below Met and Cys represent residues of methionine sulfone and cysteic acid, respectively.

Edman degradation of the intact oxidized protein gave unambiguous data for the first 20 stages as follows:

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                5                  10
Leu-Lys-Cys-Asn-Lys-Leu-Ile-Pro-Ile-Ala-Ser-Lys-
                                  15                  20
                Thr-Cys-Pro-Ala-Gly-Lys-Asn-Leu-
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No amino acids were released from the intact oxidized protein by either carboxypeptidase A or B, even after overnight digestion.

Chymotryptic Peptides. Gel filtration on Sephadex G-25 separated the chymotryptic digest of the oxidized toxin into five peptide zones, as shown in Figure 3. Fractions A and B required further separation, but fractions D, E, and F were

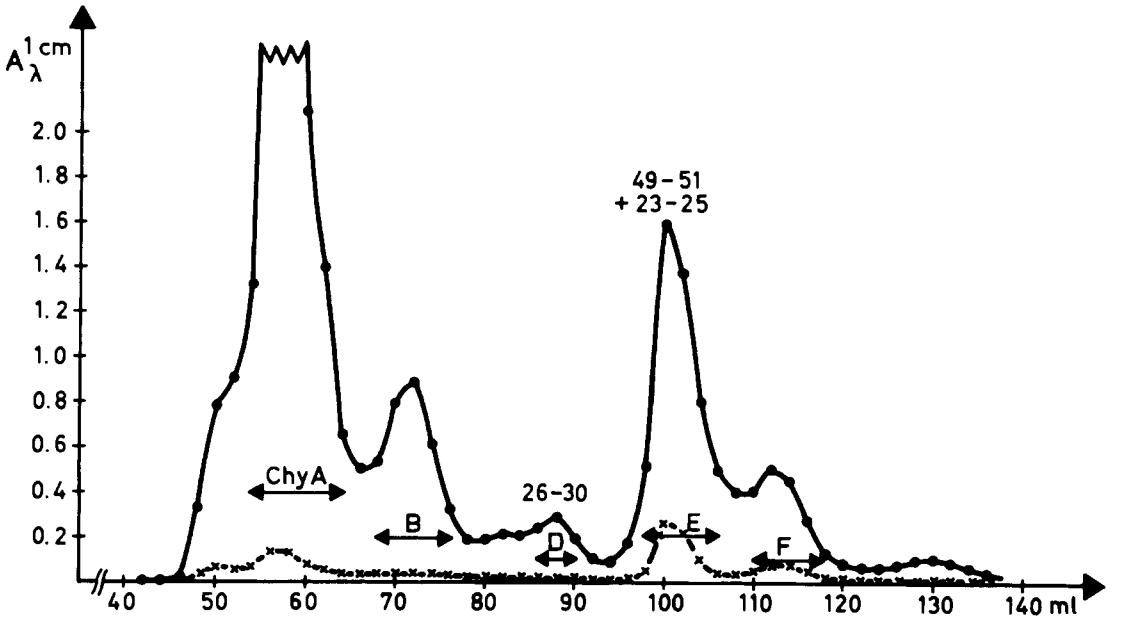

FIGURE 3: Gel filtration of the chymotryptic digest on Sephadex G-25, 1 × 145 cm, in 0.02 *N* HCl. (●) $A_{230\,nm}$; (x) $A_{275\,nm}$.

Table II: Amino Acid Composition of the Chymotryptic Peptides.

	Fragment[a]															
	Chy A-1[b]		Chy A-2[b]		Chy F		Chy E[c]				Chy D[c]		Chy A-3[b]		Chy B[d]	
Amino Acid	1–20		1–22		21–25		23–25		49–51		26–30		31–48		52–60	
Lysine	4.59	4	3.75	4	1.00	1	1.0	1	1.2	1			2.24	2		
Arginine													0.88	1	1.01	1
Cysteic acid	2.05	2	2.59	3	1.00	1							1.83	2	3.13	3
Aspartic acid	2.10	2	2.08	2							1.16	1	2.04	2	3.15	3
Methionine sulfone					1.01	1	1.0	1			1.95	2				
Threonine	0.98	1	1.00	1									0.95	1	1.00	1
Serine	1.02	1	1.11	1							1.00	1	1.00	1		
Proline	2.03	2	2.05	2									1.98	2		
Glycine	1.01	1	1.00	1									1.00	1		
Alanine	2.01	2	1.81	2												
Valine									0.99	1			1.73	2	1.00	1
Isoleucine	1.91	2	2.02	2									1.90	2		
Leucine	2.88	3	2.78	3							1.05	1	2.09	2		
Tyrosine			0.63	1	1.00	1			1.01	1						
Phenylalanine					0.99	1	0.96	1								
Total	20		22		5		3		3		5		18		9	

[a] See text for identification and Figure 8. [b] See Figures 3 and 4. [c] See Figure 3. [d] See Figures 3 and 5.

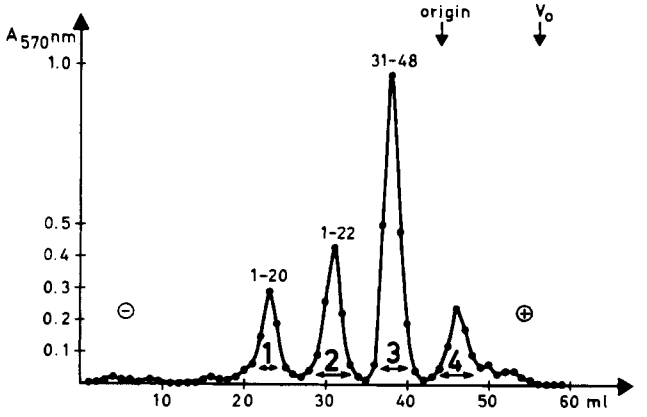

FIGURE 4: Zone electrophoresis of fraction Chy A in pyridine acetate buffer, 0.05 *M*, pH 5.0, for 18 hr at 1000 V and 8 mA. The arrow at origin indicates the position of the starting zone, V_0 indicates the void volume of the column. Fractions analyzed by ninhydrin after alkaline hydrolysis.

suitable for direct analysis. The amino acid compositions of purified chymotryptic peptides are given in Table II.

Fraction Chy D was a pure pentapeptide with the sequence Met-Met-Ser-Asp-Leu, as deduced from the amino acid composition (Table II) and the results of four stages of degradation.

Fraction Chy E was a 1:2 mixture of the two tripeptides Lys-Met-Phe and Val-Lys-Tyr, as determined from the amino acid composition data and two stages of degradation on the mixture. The C-terminal positions of Phe and Tyr are also inferred from chymotryptic specificity.

Fraction Chy F was a pure pentapeptide with the composition (Cys, Tyr, Lys, Met, Phe), but no sequencing was done on this fragment.

Fraction Chy A was separated into four peaks by column zone electrophoresis, as shown in Figure 4. The amino acid composition of peak 1 (Chy A-1, Table II) corresponds to the sequence 1–20 given above for the intact oxidized toxin. Peak 2 (Chy A-2, Table II) contains a third residue of cys-

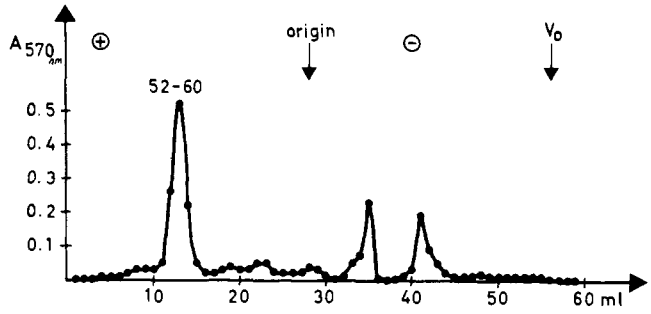

FIGURE 5: Zone electrophoresis of fraction Chy B Figure 3, with the same column and conditions as in Figure 4, for 10 hr at 1000 V and 7.8 mA. Arrows indicate the same as in Figure 4. Analysis by ninhydrin after alkaline hydrolysis.

teic acid and a tyrosine residue, and evidently corresponds to residues 1–22, the tyrosine being assigned to position 22 to account for the chymotryptic cleavage.

Peak 3 (Chy A-3, Table II) was a pure octadecapeptide with the amino-terminal sequence Thr-Ile-Pro-Val-, as obtained by four stages of degradation. Digestion of the peptide with carboxypeptidase A released two residues of leucine and one residue each of asparagine and serine. Leucine levelled off at two residues within 30 min, while serine and asparagine rose to only 0.47 residue each after 4 hr of digestion.

Since the very similar rates of release of these latter two amino acids allowed no clear decision regarding their order, a tryptic digest of peptide Chy A-3 was separated by column electrophoresis at pH 5.0, and 0.18 μmol of the neutral tetrapeptide Chy A-3 (Trp-4) with the amino acid composition Asx, Ser, Leu_2 was subjected to two cycles of Edman degradation, thereby establishing the sequence Asn-Ser-Leu-Leu.

Fraction Chy B yielded a pure anodic nonapeptide (Table II) upon column electrophoresis, as shown in Figure 5. Seven stages of degradation established the sequence Val-Cys-Cys-Asn-Thr-Asp-Arg-(Cys, Asx).

Tryptic Peptides. Gel filtration of the tryptic digest of the oxidized toxin on Sephadex G-25 gave the pattern shown in Figure 6. Fraction Trp A was a complex mixture, whereas fraction Trp B was a pure pentapeptide with the composition (Asx, Leu, Cys, Tyr)-Lys (Table III), the carboxyl-terminal location of the lysine residue being inferred from tryptic specificity.

Fraction Trp A separated into four cathodic peaks, a neutral peak, and an anodic peak upon column electrophoresis, as shown in Figure 7. Peak 2 (Trp A-2, Table III) was a pure dodecapeptide with the amino-terminal sequence Met-Phe-Met-, as determined by three stages of degradation.

Peak 3 in Figure 7 (Trp A-3) was a mixture of two peptides which were separated readily by gel filtration on a 1 × 145 cm column of Sephadex G-25 in 0.2 *M* acetic acid (pattern not shown). The earlier-eluting peak was a pure nonapeptide with the composition Arg-(Cys_2, Asx, Pro, Gly, Val, Ile)-Lys (Table III). The amino-terminal arginine was determined by one stage of degradation, and the lysine residue was assigned to the carboxyl terminus by tryptic specificity. The second peptide in Trp A-3 was a hexapeptide identifiable as residues 13–18 in the protein from its amino acid composition (Table III).

Peak 4 in Figure 7 (Trp A-4, Table III) was an octapeptide which differed in amino acid composition from Trp A-3 only by the absence of arginine. Seven stages of degradation established the sequence Gly-Cys-Ile-Asp-Val-Cys-Pro-Lys.

The anodic peak 6 in Figure 7 was a pure decapeptide (Trp A-6, Table III) corresponding in amino acid composition to peptide Chy B plus a residue of tyrosine.

Alignment of the Molecule. The alignment shown in Figure 8 can be deduced from the results presented above as follows. The sequence of residues 1–22 is based on a 20-cycle degradation of the intact oxidized protein and the amino acid compositions of peptides Chy A-1 (1–20) and Chy A-2 (1–20 + Cys-Tyr). The peptide Trp B corresponds in amino acid composition to residues 19–22 plus lysine, placing lysine at position 23. Since the molecule contains only one residue of phenylalanine, the Chy E peptide Lys-Met-Phe is assigned to positions 23–25 from the amino acid composition of Chy F (Table II, not indicated in Figure 8), which contains residues 21–25. The dodecapeptide Trp A-2 with the amino-terminal sequence Met-Phe-Met must then correspond to residues 24–35, providing the overlap be-

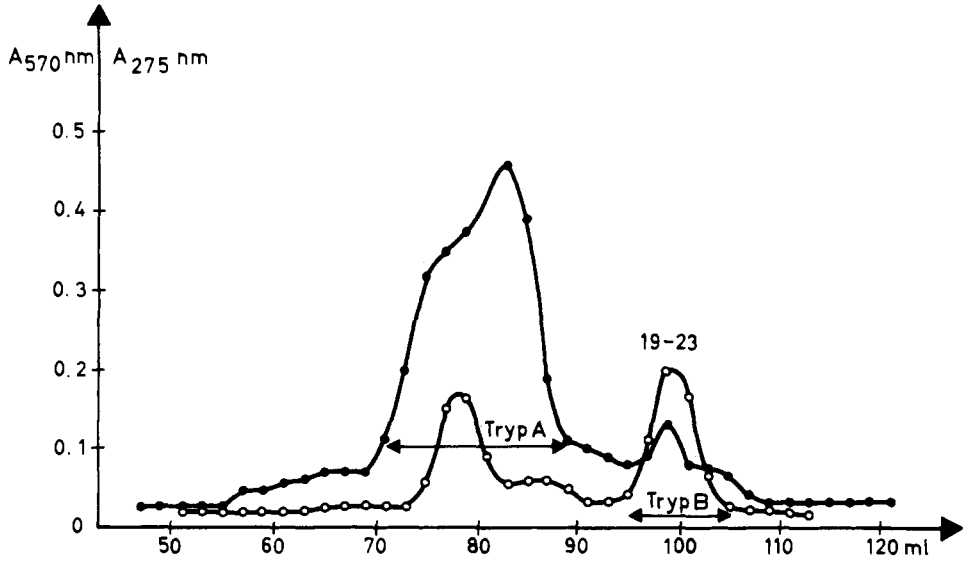

FIGURE 6: Gel filtration of the tryptic digest on Sephadex G-25, 1 × 145 cm, in 0.2 *M* acetic acid. (●) $A_{570\ nm}$; (○) $A_{275\ nm}$.

Table III: Amino Acid Composition of Tryptic Peptides.

Amino Acid	Fragments[a] Trp A-3[b] 13–18	Trp B[c] 19–23	Trp A-2[b] 24–35	Trp A-3[b] 36–44	Trp A-4[b] 37–44	Trp A-6[b] 51–60	Chy A-3 Trp 4 45–48
Lysine	1.03 1	1.16 1	1.00 1	1.25 1	1.10 1		
Arginine				0.86 1		0.93 1	
Cysteic acid	1.00 1	1.15 1		1.72 2	2.20 2	3.01 3	
Aspartic acid		1.00 1	1.00 1	1.36 1	1.25 1	2.98 3	1.01 1
Methionine sulfone			2.21 3				
Threonine	0.95 1		0.78 1			0.95 1	
Serine			1.13 1				1.10 1
Proline	0.98 1		0.92 1	0.97 1	1.06 1		
Glycine	1.00 1			1.08 1	1.10 1		
Alanine	1.04 1						
Valine			0.85 1	1.00 1	1.10 1	1.06 1	
Isoleucine			0.98 1	1.00 1	1.00 1		
Leucine		1.11 1	1.10 1				1.90 2
Phenylalanine			0.71 1				
Tyrosine		1.00 1				0.89 1	
Total	6	5	12	9	8	10	4

[a] For identification in sequence see text and Figure 8. [b] See Figures 6 and 7. [c] See Figure 6.

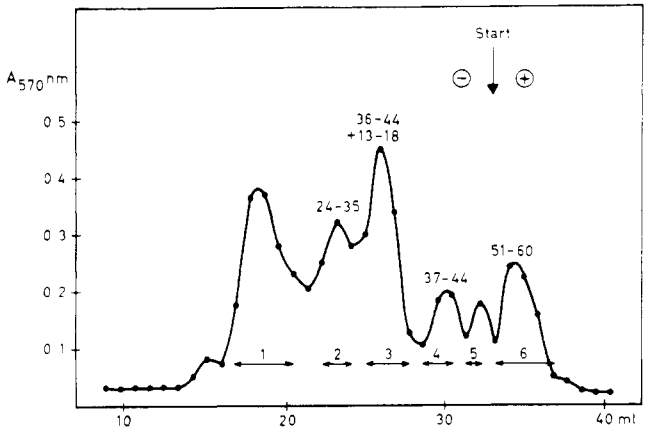

FIGURE 7: Zone electrophoresis of fraction Trp A from Figure 6 in 0.03 *M* pyridine formate buffer (pH 3.2) for 7 hr at 1000 V and 9 mA. The arrow indicates the position of the starting zone. Fractions analyzed by ninhydrin after alkaline hydrolysis.

tween the pentapeptide Chy D (26–30) and the octa decapeptide Chy A-3, which then represents residues 31–48.

The sequences 31–34 and 47–48 were established by Edman degradation and carboxypeptidase A digestion, respectively, of Chy A-3. The lysine residue in Trp A-2 can then be assigned to position 35. The peptides Trp A-3, Trp A-4, and Chy A-3 (Trp 4) can then be assigned to positions 36–44, 37–44, and 45–48, respectively.

The Chy E peptide Val-Lys-Tyr and peptide Chy B together account for the remaining 12 residues, and can therefore be assigned to positions 49–51 and 52–60, respectively, from the composition of Trp A-6, which corresponds to Chy B plus Tyr-51.

Discussion

The designation cardiotoxin has been given to this protein because the direct cause of death is fibrillation of the heart muscle. However, in vitro experiments show that cardiotoxins of this type affect other membranes as well, leading to irreversible depolarization (Chang et al., 1972, Lai et al., 1972). This can also explain the large difference in lethal dose depending on the site of injection. The cardiotoxin is not specific for heart muscle, but the heart is obviously most sensitive to any activity which results in loss of synchronization.

Since the protein contains three methionine residues, it would seem most reasonable to cleave the molecule with cyanogen bromide for sequence analysis. However, our experience with the *H. haemachates* lytic factor, which gave incomplete splitting and also solubility problems in separation (Fryklund and Eaker, 1973), prompted the use of enzymatic techniques.

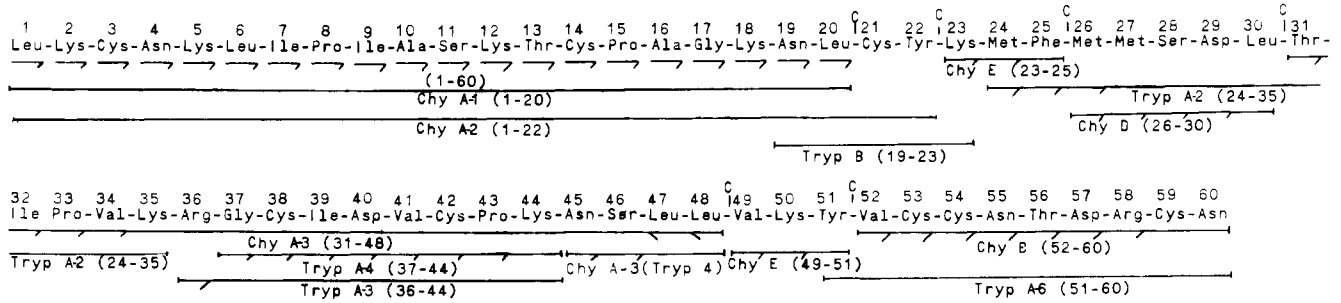

FIGURE 8: The complete amino acid sequence of the cardiotoxin. (→) Edman degradation; (⇀) carboxypeptidase A digestion; chymotryptic splits indicated by C. Stages 1–20 on the intact oxidized protein; 23–24 on chymotryptic peptide 23–25 Chy E; 24–26 on tryptic peptide 24–35 Trp A-2; 26–29 on chymotryptic peptide 26–30 Chy D; 31–34 on chymotryptic peptide 31–48 Chy A-3; 37–43 on tryptic peptide 37–44 Trp A-4; 49–50 on chymotryptic peptide 49–51 Chy E; 52–58 on chymotryptic peptide 52–60 Chy B.

The complete sequence is shown in Figure 8. The alignment has already been shown in the Results section. The sequence Cys-Asn for residues 59–60 is assumed from homology with the lytic factor (Fryklund and Eaker, 1973). For some reason carboxypeptidase A will not digest this molecule; it could be the cysteic acid residue at 59, or perhaps the presence of arginine as opposed to lysine in the lytic factor at residue 58. Neurotoxins with two residues of asparagine at the carboxyl terminal digest readily. The sequence shows the features also present in the *H. haemachates* lytic factor and the *Naja naja atra* cardiotoxin (Narita and Lee, 1971), namely the hydrophobic amino terminal and the cluster of methionines in the center of the molecule. The cysteine residues are also found in homologous positions.

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The Complete Covalent Structure of a Cardiotoxin from the Venom of *Naja nigricollis* (African Black-Necked Spitting Cobra)†

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ABSTRACT: The complete covalent structure of a small, basic protein with cardiotoxic activity is described. This has been isolated from the venom of *Naja nigricollis* by gel filtration on Sephadex G-75 and gradient ion exchange chromatography on Bio-Rex 70. The cardiotoxin, molecular weight 6806 from amino acid composition, consists of 60 amino acids, cross-linked by four disulfide bridges, connecting 3–21, 14–38, 42–53, and 54–59. The protein contains one residue of tryptophan, phenylalanine, and glutamic acid, two residues of arginine and tyrosine, four residues of methionine, and nine residues of lysine. Histidine is absent. The chymotryptic peptides of the oxidized and S-carboxymethylated protein were isolated by gel filtration on Sephadex G-25 and zone electrophoresis on a cellulose column. The sequence was determined by Edman degradation, using the (manual) direct phenylthiohydantoin method and with the use of carboxypeptidase A. Disulfide pairing was determined on thermolysin cleaved peptides from the native protein. The sequence is shown to be homologous to other cardiotoxins and a lytic factor from snake venoms and also shows homology, both in sequence and disulfide pairing to neurotoxins. A partial reduction experiment in the absence of denaturing agent using ^{14}C-labeled iodoacetic acid as S-carboxymethylating agent shows that disulfide bonds 14–38 and 42–53 were reduced fastest followed marginally by 54–59, and then bond 3–21.

This cardiotoxin has the same pharmacological properties as that described in the accompanying paper (Fryklund and Eaker, 1975). The sequence of this cardiotoxin completes the series on small basic proteins and their homology relationships with neurotoxins in Elapid and Hydrophid venoms. The disulfide pairing was also established to see whether homology was apparent even at this structural level (see also Fryklund et al. (1972), Fryklund and Eaker (1973), and accompanying paper). This cardiotoxin has also been characterized immunologically by Boquet et al. (1972) and is denoted Toxin γ by them.

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Experimental Procedure

Isolation of the Cardiotoxin. The *Naja nigricollis* venom