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Complete Amino Acid Sequence of a Nonneurotoxic Hemolytic Protein from the Venom of *Haemachatus haemachates* (African Ringhals Cobra)†

Linda Fryklund* and David Eaker

ABSTRACT: The complete amino acid sequence of the nonneurotoxic, hemolytic, basic protein 12B from the venom of *Haemachatus haemachates* is described. The protein consists of 61 amino acids, cross-linked by four disulfide bridges, molecular weight 6841 from amino acid composition. The cyanogen bromide fragments and chymotryptic and tryptic peptides were separated by gel filtration on Sephadex G-50 or G-25 and zone electrophoresis on a cellulose column. The sequence was determined by Edman degradation using the direct phenylthiohydantoin method, and carboxypeptidase A, and is shown to be homologous to other types of proteins, cardiotoxins, and neurotoxins, in the same size range from Elapid venoms.

Elapid venoms contain neurotoxins of the post-synaptic curariform type and in most cases other proteins of similar molecular weight which have cardiotoxic or lytic activity. The purification of the hemolytic protein 12B from the venom of *Haemachatus haemachates* has already been described by Porath (1966) but the separation has been improved by the use of ammonium acetate buffers. The protein 12B, which is very basic, has been shown to directly lyse red blood cells and to have cardiotoxic and hypotensive activity, but is not neurotoxic (Cheymol, 1972[1]). The primary structure was determined in order to investigate possible homology between nonneurotoxic proteins and neurotoxins. Simultaneously with the completion of this, Narita and Lee (1970) described the sequence of a cardiotoxin from the venom of the Formosan cobra, *Naja naja atra*, which is shown to be homologous to the neurotoxin from the same venom, and is also very similar to the lytic protein 12B.

Experimental Procedure

Purification of the Protein 12B. Haemachatus haemachates venom (HHIF) was obtained from Miami Serpentarium, Miami, Fla. One gram of crude venom was dissolved in 10.0 ml of 0.2 M ammonium acetate, clarified by centrifugation for 10 min at 20,000*g*, and then run on a Sephadex G-75 column, 3.2 × 93 cm, in 0.2 M ammonium acetate. The gel-filtration fraction containing the lytic activity was further separated by

† From the Institute of Biochemistry, University of Uppsala, S-751 21 Uppsala, Sweden. *Received September 20, 1972.* The investigation was supported by the Swedish Natural Science Research Council (dnr 2859-5) and the Wallenberg Foundation (Beckman Spectrochrom Analyzer).

[1] J. Cheymol *et al.*, unpublished data.

ion-exchange chromatography on Bio-Rex 70 using a Beckman Spectrochrom Analyzer equipped with an automatic gradient device. (See Karlsson *et al.* (1971) for details of equilibration and packing of the resin and the chromatographic technique.) The column used was Bio-Rex 70 fine, 3.2 × 23 cm, equilibrated in 0.2 M ammonium acetate buffer, pH 7.3. The sample of 567 mg was applied in 10 ml of 0.1 M ammonium acetate, and the column eluted with 20 ml of the same medium before a double linear gradient, 0.15 M *vs.* 1.4 M ammonium acetate, was applied.

Ultraviolet Spectra and Determination of Molar Extinction Coefficient. This was done according to the procedure described by Karlsson *et al.* (1972).

Oxidation of the protein with preformed performic acid was carried out according to the method of Hirs (1956), and the oxidized protein was recovered by lyophilization.

Guanidination of the Protein. O-Methylisourea sulfate (0.625 g; 5 mequiv) was dissolved in 4 ml of water and the pH was adjusted to 10.4 by the addition of 6.0 ml of 1 N NaOH. The reagent solution was gassed thoroughly with nitrogen and 4 ml added to 5.0 μmol of protein (42.5 mg, 81% protein, 19% water) in a screw-cap vial, flushed with nitrogen, and rotated to dissolve the protein. The vial was then placed at 4°. At the end of each 24-hr period 25 μl was withdrawn (the vial was flushed with nitrogen after each opening) and added to 1 ml of 6 N HCl in a hydrolysis tube, which was sealed and hydrolyzed at 110° for 20 hr. The HCl was evaporated, and in order to remove any ammonia formed by hydrolysis of the *O*-methylisourea, 0.5 ml of 0.01 N NaOH was added to the dry residue and rotatory evaporated under reduced pressure for 5 min without heating. Citrate buffer (0.5 ml, pH 2.2) was added and the entire sample applied to the short column of a Beckman 120B amino acid analyzer. All the lysine had reacted after 24 hr, in fact, but the reaction was allowed to proceed for 3 days. The reaction was terminated by acidification, and a sample taken for alkaline hydrolysis to check for the presence of methionine sulfoxide. The protein was insoluble in acid solution but after dialysis against water for 1 hr and centrifugation a sparkling clear solution was formed.

Cyanogen Bromide Cleavage and Oxidation of the Guanidinated Protein. The protein was treated with a 30-fold excess of CNBr according to the method of Gross and Witkop (1962). Five micromoles was dissolved in 5 ml of 0.1 N HCl and 450 μmol of CNBr was added in 5 ml of the same medium. The solution was allowed to stand at room temperature for 20 hr and then frozen directly and lyophilized using a NaOH–pumice tower to adsorb the cyanogen bromide. The derivative was then oxidized as described for the native protein. After 20 hr of lyophilization, a little water was added and the material was brought into solution by the addition of small amounts of HCOOH and pyridine. The solution was then gel filtered on a Sephadex G-50 column, 2 × 102 cm, in 1 M pyridine acetate, pH 6.0.

Chymotryptic Digest of the Oxidized Protein. Three micromoles of protein was dissolved in 2.0 ml of 0.1 M *N*-ethylmorpholine acetate buffer, pH 7.5. Chymotrypsin, 0.82 mg, to give a molar ratio of enzyme:substrate of 1:100, was added in 100 μl of the same buffer at 0 time. After incubation at 37° for 17 min, the digestion was terminated by acidification, and the solution lyophilized. Group fractionation of the peptides was performed by gel filtration on a Sephadex G-50 column, 2 × 102 cm, in 0.02 N HCl.

Zone Electrophoretic Separation of the Guanidinated and Chymotryptic Peptides. All gel-filtration fractions were routinely examined by low voltage paper electrophoresis (440 V) in the following buffers, 0.05 M pyridine acetate, pH 5.0, 0.03 M pyridine formate, pH 3.2, and 0.5 M pyridine acetate, pH 6.2 (guanidinated peptides), to determine the best conditions for column separation. Gel-filtration fractions were separated preparatively by zone electrophoresis in a 1 × 86 cm bed of pyridine-washed cellulose (Porath, 1964) in an all-glass column as described by Porath (1956). Details regarding the packing, evaluation, and operation of the column shall be shortly published elsewhere (Arnberg *et al.*[2]). After electrophoresis the column was eluted in the same buffer at a flow rate of 12 ml/hr, and 1-ml fractions were collected. Appropriate aliquots were analyzed by ninhydrin following alkaline hydrolysis at 110°, to detect peptide zones. Further gel filtration of peptides was done on a Sephadex G-25 column, 1 × 143 cm, equilibrated in 0.02 N HCl.

Carboxypeptidase A digestions were carried out on the whole protein and on chymotryptic peptides. A digestion mixture was made up as follows: 0.2 μmol of peptide, 240 μg of carboxypeptidase A (Worthington DFP treated), 0.2 ml of 2.0 M ammonium bicarbonate, 1.0 μmol of norleucine, and water were added to give a total volume of 1 ml. Incubation was done at room temperature and 100-μl aliquots were removed at regular intervals, mixed with 100 μl of 1 M HCOOH, and lyophilized before being run on an amino acid analyzer.

Amino acid analyses were performed on an automatic Biocal 200 or a Beckman 120B amino acid analyzer. Carboxypeptidase A digests of the whole protein were run in the lithium buffer system to permit direct identification of asparagine (Benson *et al.*, 1967).

Tryptic Digestion of the Whole Protein. Oxidized protein (2.0 μmol) was dissolved in 2.0 ml of 0.1 M *N*-ethylmorpholine acetate, pH 7.5, and digested with trypsin (Worthington 3× crystallized) at an enzyme:substrate molar ratio of 1:50 for 1 hr at 37°. The reaction was stopped by acidification. Separation of the peptides was done in a similar manner as described for the chymotryptic peptides.

Edman Degradation. Edman degradation of the whole oxidized protein, the guanidinated fragment, and the chymotryptic peptides was done according to the direct PTH[3] method as described by Iwanaga *et al.* (1969). After the conversion step the PTH amino acid concentrations of the organic and aqueous phases were estimated from ultraviolet spectra, using a provisional value of 16,000 for the molar absorbance coefficient at 269 nm. Aliquots corresponding to 5 nmol were then applied to silica gel plates (Merck Fertigplatten F-254) for the identification of the PTH amino acid by thin layer chromatography in the following solvent systems: solvents D and E of Edman and Sjöquist (1956) and solvents II and III of Brenner *et al.* (1962). The spots were located by visual scanning under ultraviolet (254 nm) illumination.

The water-soluble derivatives of arginine, cysteic acid, and histidine were identified after paper electrophoresis at 440 V for 2 hr at pH 6.5 in sodium phosphate buffer containing 0.015 M Na_2HPO_4, 0.03 M NaH_2PO_4, 1 g of disodium EDTA, and 5 g of soluble starch per liter. The PTH derivatives were detected as white spots on a coffee-colored background by means of the iodine azide reagent (Edman, 1970). Isoleucine and leucine residues were distinguished by amino acid analysis after alkaline hydrolysis of the PTH amino acids.

[2] Arnberg, H., Eaker, D., and Karlsson, E. (1973), manuscript in preparation.

[3] Abbreviation used: PTH amino acid, phenylthiohydantoyl amino acid.

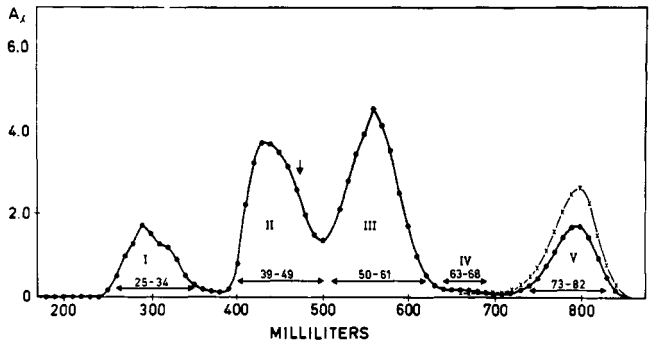

FIGURE 1: Gel filtration of crude venom on Sephadex G-75, column dimensions 3.2 × 93 cm, in 0.2 M ammonium acetate. The arrow indicates the elution position of cytochrome *c*, mol wt 12,270; (●) $A_{280\ nm}$; (×) $A_{260\ nm}$. Arrows under peaks indicate pooling of numbered fractions.

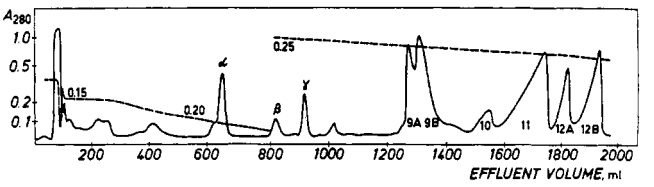

FIGURE 2: Ion-exchange chromatography on Bio-Rex 70, column dimensions 3.2 × 23 cm, of peak III, Figure 1. Column equilibrated in 0.2 M ammonium acetate, pH 7.3. Double linear gradient 0.15 M *vs.* 1.4 M ammonium acetate (total gradient volume = 2 l.). The numbers on the conductivity trace (—) indicate the approximate molarity.

Results

Purification of Protein 12B. The gel filtration on Sephadex G-75, Figure 1, resolved the venom into five peaks, together accounting for all of the material applied. The material distribution is shown in Table I. Fraction III, Figure 1, was then separated by ion-exchange chromatography (Figure 2) into three main peaks, α, β, and γ, with neurotoxic activity and a double peak at 12A and 12B, where the protein 12B had lytic activity. The material distribution of the peaks is shown in Table I. The amino acid composition of the peaks 12A and 12B are shown in Table II. The lytic activity has been determined by Cheymol *et al.*[1] Neurotoxin α has the same composition as toxin 5 (Porath, 1966) and toxin IV isolated and sequenced by Strydom and Botes (1971). Neurotoxin γ is identical in composition with neurotoxin α, except for one more valine and one less glutamic acid. Neurotoxin β is similar to toxin 3 (Porath, 1966) and toxin II (Strydom and Botes, 1971) with regard to basic and hydrophobic amino acids, but has one less aspartic acid, proline, and leucine, and one more threonine, serine, and glutamic acid. Peak 12B is identical with peak 12 (Porath, 1966). However, the change of buffer from sodium phosphate to ammonium acetate has resulted in a change in elution position for the other peaks. Peak 12A is similar to 12B in amino acid composition, but has only 60 amino acids, one more threonine and valine but one less methionine, leucine, and lysine than 12B. The molar extinction coefficient was determined to be 2340 for the native protein 12B; this is consistent with a content of one tyrosine residue and four disulfide bridges but no tryptophan. Aloof-Hirsch *et al.* (1968) also describe the purification of a direct lytic factor from *Haemachatus haemachates;* this has only 57 amino acid residues but resembles 12A in composition.

Determination of the Sequence. Residues 1–26 were determined by Edman degradation of the intact oxidized protein, which gave unambiguous data for the first 26 stages as follows

```
1                5                  10                  15
Leu-Lys-Cys-His-Asn-Lys-Leu-Val-Pro-Phe-Leu-Ser-Lys-Thr-Cys-

                                   20                  25
                    Pro-Glu-Gly-Lys-Asn-Leu-Cys-Tyr-Lys-Met-Thr-
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The yields of the PTH amino acids, where 100% corresponds to 0.7 μmol of protein, determined spectrophotometrically at each stage, are shown in Figure 3. The solid line represents the overall repetitive yield, which, assuming there is no loss of

TABLE I: Material Distribution.

Fraction	Gel Filtration (Crude Venom) (mg)	Peak	Ion Exchange (mg)
I	166.7	α	9.7
II	214.6	β	2.4
III	566.7	γ	5.1
IV	48.8	12A	41.1
V	22.5	12B	94.0
	1019.3		

TABLE II: Amino Acid Compositions of Proteins 12A and 12B and the Peptide 31–61 from 12B.

	12A		12B		Oxidized 12B		31–61[a]	
Lysine	11.10	11	12.90	12	12.0	12		
Histidine	1.00	1	0.92	1	0.99	1		
Arginine	1.05	1	1.00	1	0.99	1	1.00	1
Homoarginine							5.36	6
Cysteic acid					7.73	8	4.97	5
Aspartic acid	5.72	6	5.95	6	6.04	6	3.97	4
Methionine sulfone					2.95	3		
Threonine[b]	3.77	4	2.99	3	3.16	3	1.22	1
Serine[b]	2.98	3	2.89	3	2.96	3	2.00	2
Glutamic acid	0.98	1	0.99	1	1.05	1		
Proline	4.70	5	4.95	5	4.90	5	3.21	3
Glycine	2.06	2	1.97	2	2.00	2	1.09	1
Alanine	0.99	1	1.00	1	1.00	1	0.89	1
Half-cystine[c]	8.03	8	7.62	8				
Valine[d]	4.85	5	3.70	4	3.45	4	2.27	2–3[e]
Methionine	1.93	2	2.46	3				
Isoleucine[d]	1.88	2	2.19	2	1.91	2	1.78	2
Leucine	5.90	6	7.00	7	6.87	7	2.22	2
Tyrosine	0.96	1	0.72	1	0.86	1		
Phenylalanine	1.00	1	0.86	1	0.99	1		
Total[f]		60		61		61		31

[a] 24-hr hydrolysis only. [b] Extrapolated to 0 time. [c] As cysteic acid. [d] 72-hr value only. [e] Val-Val sequence gives only 60% value in 24 hr. [f] All values as average of 24- and 72-hr hydrolyses.

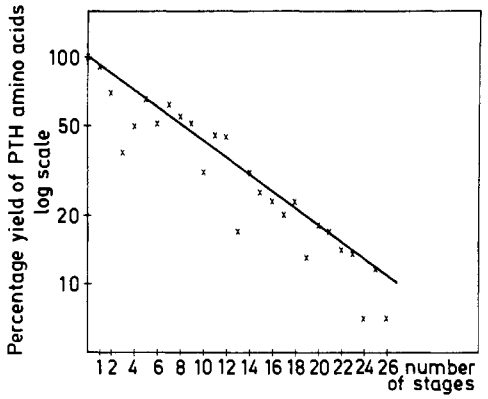

FIGURE 3: The percentage yield of PTH amino acids plotted as log *vs.* the number of stages.

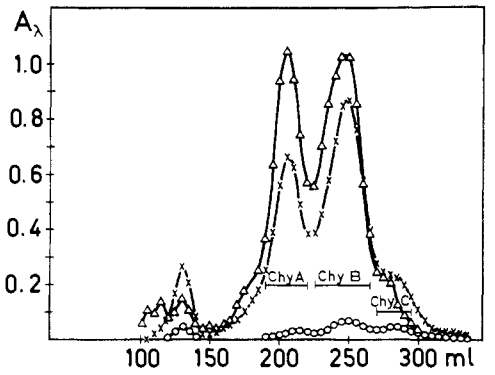

FIGURE 6: Gel filtration of chymotryptic digest of oxidized 12B on a Sephadex G-50 column, 102 × 2 cm in 0.02 N HCl; flow rate 20 ml/hr. Ninhydrin analysis after alkaline hydrolysis: (△) $A_{230\ nm}$; (×) $A_{570\ nm}$; (○) $A_{275\ nm}$.

protein due to extraction, is 92%. Yields of lysine and cysteic acid are significantly lower. Further confirmation of the sequence is shown by the isolation of the tryptic peptides (Table IV) 1–2, 3–6, 7–10, 11–13, 14–19, 20–24, 25–29. (The parasitic split at Phe residue 10 could be a result of ψ-trypsin.)

Residues 31–44. Figure 4 shows the separation of the guanidinated CNBr cleaved oxidized protein on Sephadex G-50. Fraction II was then subjected to zone electrophoresis to obtain a peptide of 31 amino acids, the main peak in Figure 5. This was identified as the C-terminal peptide 31–61, since it lacked homoserine and because of the degradation of the whole protein. The amino acid compositions of the whole oxidized protein and this peptide are shown in Table II. Fourteen stages of degradation on the peptide 31–61 gave the sequence 31–44 as follows

31 35 40
Pro-X-Ile-Pro-Ile-X-X-Gly-Cys-Thr-Asp-Ala-Cys-Pro-

where X represents homoarginine or arginine.

Residues 29–30 and Arginine Residue 37. Figure 6 shows the gel-filtration pattern obtained with the chymotryptic digest of the whole protein. This gave three main peaks. Fraction Chy A was further separated by zone electrophoresis as shown in Figure 7a. Peak 2 was a pure peptide of 21 amino acids and identified as residues 29–49, on the basis of amino

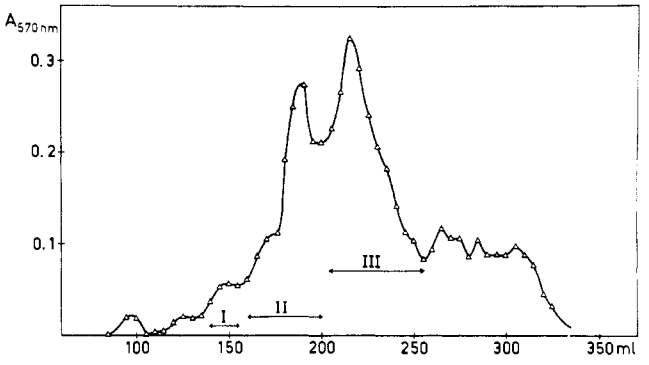

FIGURE 4: Gel filtration of the guanidinated, cyanogen bromide cleaved, oxidized 12B on Sephadex G-50, 102 × 2 cm, in 1.0 M pyridine acetate buffer, pH 6.0; flow rate 20 ml/hr.

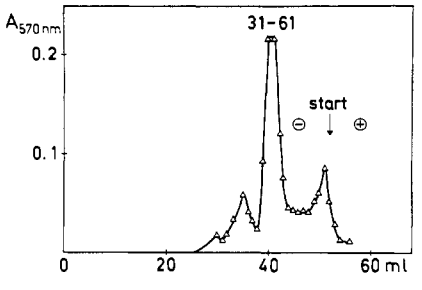

FIGURE 5: Zone electrophoresis on a cellulose column 86 × 1 cm in 0.5 M pyridine acetate buffer, pH 6.2, of peak II from Figure 4. Electrophoresis for 24 hr at 1000 V and 8 mA, migration to cathode. Ninhydrin analysis after alkaline hydrolysis. The arrow at 56 ml indicates the position of the starting zone.

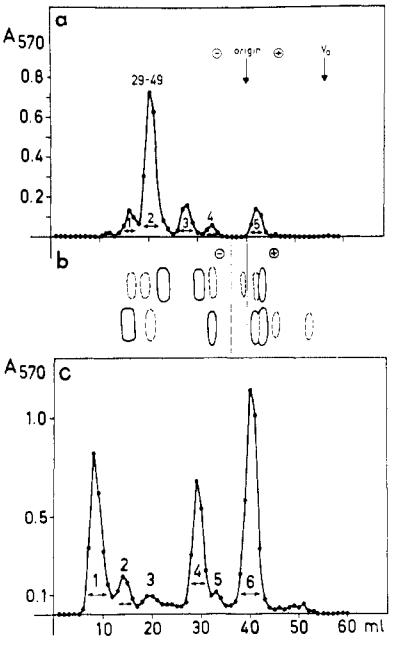

FIGURE 7: (a) Zone electrophoresis of Chy A (Figure 6) on the same column as in Figure 5: pyridine acetate buffer 0.05 M, pH 5.0, for 15 hr at 1000 V and 8 mA; the arrow indicates the start of the run. (b) Tracing of the paper electrophoresis run (2 hr at 440 V, ninhydrin stain) obtained with the fractions Chy A and Chy B, respectively. The solid and dashed vertical lines indicate the site of application and electroosmotic origin, respectively. (c) Electrophoresis of Chy B in the same conditions as for Figure 7a for 18 hr at 1000 V and 8 mA.

TABLE III: Amino Acid Compositions of Chymotryptic Peptides.

	11–23		22–28		29–49		50–61	
Lysine	2.06	2	1.10	1	4.12	4	3.03	3
Histidine								
Arginine					0.98	1		
Cysteic acid	1.92	2	0.80	1	2.10	2	2.95	3
Aspartic acid	1.06	1			1.06	1	3.03	3
Methionine sulfone			1.80	2	1.25	1		
Threonine	0.94	1	0.97	1	1.01	1		
Serine	0.98	1			1.99	2		
Glutamic acid	0.99	1						
Proline	0.88	1			3.21	3		
Glycine	0.99	1			1.01	1		
Alanine					0.99	1		
Valine							2.48	3
Isoleucine					1.84	2		
Leucine	2.02	2	1.10	1	2.00	2		
Tyrosine	0.88	1	0.76	1				
Phenylalanine								
Total		13		7		21		12

acid composition as compared to the peptide 31–61. The amino acid compositions of the chymotryptic peptides are shown in Table III. Nine stages of degradation were done on this peptide 29–49, to determine lysine residue 29, methionine residue 30, and the overlap 30–31. Homoarginine and arginine were not distinguishable as PTH amino acids, and the degradation was therefore continued for nine stages until the unique arginine at residue 37 was encountered. The sequence was as follows

29 30 35 37
Lys-Met-Pro-Lys-Ile-Pro-Ile-Lys-Arg-

Figure 7b shows a typical example of the preliminary paper electrophoresis done to determine the best conditions for separation on the column.

Residues 50–61. Fraction Chy B (Figure 6) was also separated by zone electrophoresis and gave the pattern shown in Figure 7c. Peaks 1 and 4 were identified as peptides 1–10 and 11–21, respectively, from the amino acid composition of the amino terminal half, while peak 6 was a mixture of two peptides,

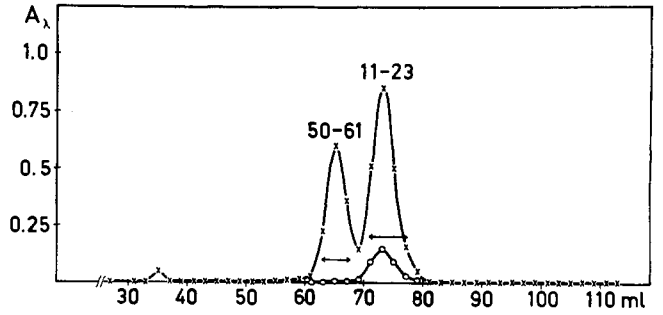

FIGURE 8: Gel filtration of peak 6, Figure 7c, on Sephadex G-25, 143 × 1 cm, in 0.02 N HCl; flow rate 10 ml/hr: (×) $A_{370\ nm}$; (○) $A_{275\ nm}$.

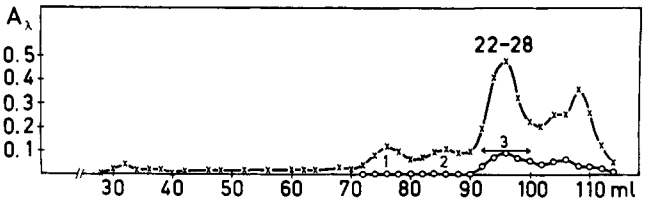

FIGURE 9: Gel filtration of Chy C on the same column and under the same conditions as described in Figure 8: (×) $A_{370\ nm}$; (○) $A_{275\ nm}$.

which was resolved by gel filtration on Sephadex G-25, Figure 8. This gave two peptides, one of 12 amino acid residues which eluted first, and was identified as residues 50–61 from the amino acid compositions of peptides 29–49 and 31–61. The other was a tyrosine-containing peptide, residues 11–23. Ten stages of degradation on the peptide 50–61 gave the sequence as follows

50 55
Val-Lys-Val-Val-Cys-Cys-Asn-Lys-Asp-Lys-(Cys,Asn)

Residues 22–28. Fraction Chy C, Figure 6, was gel filtered on Sephadex G-25 and gave three peptide peaks. The fourth peak is *N*-ethylmorpholine acetate, which gives ninhydrin color after alkaline hydrolysis. Peak 3 in Figure 9 was identified as residues 22–28 from the tyrosine and methionine content. Carboxypeptidase A digestion of this peptide indicated the carboxy-terminal sequence Thr-Met-Leu-OH.

Residues 46–49 and Residue 61. With carboxypeptidase A, the whole molecule gave one residue of asparagine which establishes this as residue 61. The chymotryptic peptide 29–49 gave two residues of leucine and two residues of serine per molecule. From the time course of the release of these amino acids shown in Figure 10, the sequence Ser-Ser-Leu-Leu-OH is deduced for residues 46–49. The complete amino acid sequence is shown in Figure 11.

Discussion

Since the protein is small, an accurate amino acid analysis is a good criterion of purity. The amino acid composition shown in Table II gives values close to integers for all amino acids. The alignment of the molecule (Figure 11) is obvious from the Edman degradation of the N terminal of the whole molecule and the peptide 31–61 obtained after cyanogen bromide cleavage and oxidation. The degradation of 31–61, the chymotryptic peptide 29–49, and also the carboxypeptidase A digestion of the whole molecule show that the chymotryptic peptide from Chy B6 (see Figure 8) must be C terminal,

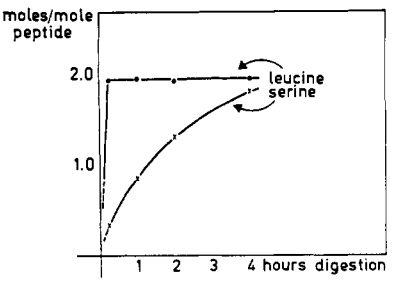

FIGURE 10: Carboxypeptidase A digestion of chymotryptic peptide 29–49.

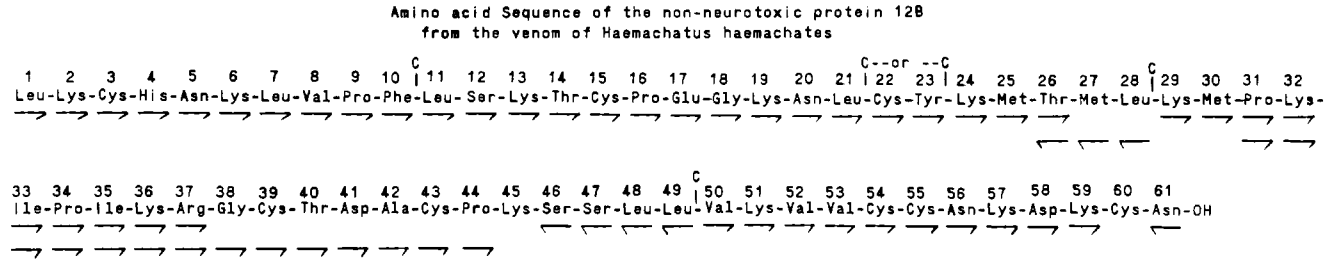

FIGURE 11: Complete amino acid sequence of the protein 12B. Chymotryptic cleavages are indicated by C. Cleavages of 21–22 and 23–24 were mutually exclusive: no Cys-Tyr was observed. Edman degradation is indicated by →; carboxypeptidase A digestion is indicated by ←. Edman degradation stages 1–26 were done on the whole protein, 29–37 on the chymotryptic peptide 29–49, 31–44 on the guanidinated peptide 31–61, and stages 50–59 on the chymotryptic peptide 50–61. Residue 61 was obtained by carboxypeptidase A digestion on the whole protein, residues 26–28 from digestion of the chymotryptic peptide 22–28, and residues 46–49 from the digestion of the chymotryptic peptide 29–49.

TABLE IV: Amino Acid Composition of Tryptic Peptides.

	1–2	3–6	7–10	11–13	14–19	20–24	25–29	46–51
Lysine	1.19 1	1.00 1		1.10 1	1.05 1	0.99 1	1.01 1	1.00 1
Histidine		0.97 1						
Arginine								
Cysteic acid		1.00 1			1.02 1	0.95 1		
Aspartic acid		1.17 1				1.05 1		
Methionine sulfone							1.89 2	
Threonine					1.01 1		0.99 1	
Serine				1.01 1				2.00 2
Glutamic acid					0.99 1			
Proline			1.00 1		0.95 1			
Glycine					0.97 1			
Alanine								
Valine			0.97 1					0.92 1
Isoleucine								
Leucine	0.80 1		1.05 1	1.00 1		1.01 1	0.96 1	2.00 2
Tyrosine						1.00 1		
Phenylalanine			1.04 1					
Total	2	4	4	3	6	5	5	6

residues 50–61. The chymotryptic peptide 22–28 is unambiguously identified on the basis of its amino acid composition, from the initial degradation of the molecule through residue 26, which placed the unique tyrosine residue at 23. The overlap 28–29 is shown by the isolation of the tryptic peptide 25–29 (Table IV). The overlap 49–50 is shown both from the composition of 31–61 and also the tryptic peptide 46–51 (Table IV).

The guanidinated peptide, 31–61, had very good properties as regards the degradation, but solubility problems and visible aggregation caused serious losses of material during the purification. The fragment corresponding to 31–61 was isolated in a preliminary CNBr experiment, but the use of the guanidinated peptide was prompted by the anticipation of solubility problems in the degradation of the rather hydrophobic fragment with six lysine residues. The corresponding peptide from the guanidinated protein gave clear and colorless solutions throughout degradation and extractive losses were also minimal. Unfortunately PTH arginine and PTH homoarginine are not distinguishable by paper electrophoresis, and the PTH fractions were inadvertently discarded before the issue could be settled by alkaline hydrolysis, which gives ornithine from PTH arginine and lysine from PTH homoarginine. Therefore, the degradation of the peptide 29–49 was continued until the unique arginine residue was encountered. No cyanogen bromide cleavage between Met-25 and Thr-26 was observed.

The 12 lysine residues are distributed evenly throughout the molecule, occurring at approximately every fifth residue. Hydrophobic amino acids predominate in the N-terminal half. Nothing is known about the secondary and tertiary structures, but the lytic activity could be a direct result of the high charge density, combined with the high content of nonpolar amino acids giving the molecule a tensid character. The protein exhibits somewhat unusual solubility properties: a 1% solution in 1 M ammonium sulfate precipitates at room temperature, but on cooling below 10° forms a sparkling clear solution; this process can be repeated indefinitely. The sequence shows that there is homology between this protein and other nonneurotoxic venom components as well as neurotoxins; this will be discussed further in a subsequent paper (see also Dayhoff (1972), Strydom and Botes (1971), Fryklund *et al.* (1972)).

The cysteines and one tyrosine residue occur in almost identical positions, assuming one or two deletions, in both neurotoxins and this lytic factor. The carboxyl ends of the molecules, from residue 38 (lytic factor), are also very similar. The most apparent difference lies in the amino terminal half, where the lytic factor has predominantly hydrophobic amino acids and the typical neurotoxin hydrophilic. The invariant sequence found so far in all neurotoxins, Lys-X-Trp-X-Asp-X-Arg-Gly-, occurring between residues 24 and 32, is also missing in the lytic factor.

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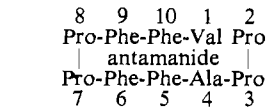
Antamanide Conformations in Nonaqueous Media. Dependence on Hydrogen-Bond Acceptor Properties of Solvent†,‡

Dinshaw J. Patel

ABSTRACT: Proton and carbon nmr studies and model building were combined to generate conformations for antamanide in nonaqueous solution. The conformation in weak hydrogen-bond acceptor nonaqueous solvent (designated I) contains all intramolecularly hydrogen-bonded peptide protons. This conformation occurs in rapid equilibrium with a conformation with all peptide protons exposed to solvent (designated II) in strong hydrogen-bond acceptor nonaqueous media. An analysis of the multiplicity of the C^{α} proline proton resonances and the chemical shifts of the C^{β} and C^{γ} proline carbon resonances for the nmr spectra of antamanide in solution suggests the presence of two cis and two trans X-Pro peptide bonds. Conformations were generated with cis peptide bonds either at Val_1-Pro_2 and Phe_6-Pro_7 or at Pro_2-Pro_3 and Pro_7-Pro_8 using Corey–Pauling–Koltun models which were in agreement with the nmr data and energy maps. The experimental evidence appears to be more consistent with the conformations 1,6-*cis*-I $\rightleftarrows$ 1,6-*cis*-II, containing cis peptide bonds at Val_1-Pro_2 and Phe_6-Pro_7, for antamanide in nonaqueous solution.

Nuclear magnetic resonance (nmr) spectroscopy and circular dichroism (CD) measurements were coupled with an approximate theoretical treatment to determine the conformational characteristics of the cyclic *all*-L-decapeptide antamanide (Tonelli *et al.*, 1971). Proton nmr decoupling and exchange studies for antamanide in $CDCl_3$ solution suggested that the observable peptide N protons were neither hydrogen bonded nor in solvent-shielded environments. The CD spectrum of antamanide (in methanol, dioxane, trimethyl phosphate, hexafluoroisopropyl alcohol) exhibited $[\theta] > -100{,}000°$ in the 190–205-nm region consistent with all X-Pro peptide bonds in the trans conformation. The solvent-dependent CD data exhibited no isosbestic point and were assigned to medium effects rather than conformational changes. The lowest energy cyclic conformation with all-trans peptide bonds generated in the theoretical portion of the study was not stabilized by intramolecular hydrogen bonding and possessed a pseudo-twofold center of symmetry (Tonelli *et al.*, 1971). The suggestion of all-trans X-Pro peptide bonds from the CD data needs reevaluation and is considered in detail later in the manuscript.



† From Bell Laboratories, Murray Hill, New Jersey 07974. *Received July 24, 1972.*

‡ This paper and the following paper both contain color plates. Plates 1 and 2 of this paper appear on p 685.