Characterization of Myotoxin a from the Venom of Prairie Rattlesnake (*Crotalus viridis viridis*)†

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ABSTRACT: A previously unknown polypeptide myotoxin, designated myotoxin a, was isolated for the first time from prairie rattlesnake (*Crotalus viridis viridis*) venom. Electrophoretic homogeneity of myotoxin a was shown in β-alanine disc gel polyacrylamide gel electrophoresis and in isoelectric focusing gel electrophoresis. Molecular weight and isoelectric point estimates of 4100 and 9.6 were obtained by gel filtration and isoelectric focusing gel electrophoresis, respectively. Amino acid composition showed a total of 39 amino acid residues, with 10 lysine residues and two disulfide bridges. When the two disulfide bridges were reduced and alkylated, the myotoxic activity was abolished, indicating that the disulfide bridges of myotoxin a are essential for its biological activity. The loss of the biological activity is probably due to a marked change in secondary structure. The circular dichroic spectrum indicates that the chemically modified, inactive myotoxin exhibits typical random-coil conformation.

The manifestations of local tissue damage, such as myonecrosis and hemorrhage, are among the best documented effects of certain types of snakebite (Stahnke, 1966; Emery and Russel, 1963). These effects are especially pronounced for the venoms of Crotalidae, which include rattlesnakes and copperheads in the United States. Myonecrosis and hemorrhage still represent a serious clinical problem in cases of snakebite, since the administration of antivenin may not prevent their occurrence unless given immediately following envenomation (Minton, 1954; Stahnke et al., 1957). The pathogenesis of myonecrosis (Ownby et al., 1974) and hemorrhage (Stringer et al., 1972) caused by crude snake venoms has been studied by electron microscopy. These studies have revealed that both myonecrosis and hemorrhage, as caused by crude snake venoms, are complex processes involving multiple sites of structural attack. Several venom components, including a protease, phospholipase A_2, and heat-stable factor in the case of *Timeresurus favoviridis* venom (Maeno, 1962), contribute to the overall, complex structural damage. Development of more effective means to minimize myonecrosis requires isolation and study of venom components responsible for discrete sites of initial structural attack. Despite the importance of myonecrosis in snake-venom poisoning, no fractionations of snake venom directed toward isolation of a specifically myotoxic component have been reported prior to the present study.

Toward this end, fractionation of crude prairie rattlesnake (*Crotalus viridis viridis*) venom based upon a specific histological assay for muscle damage was undertaken. A polypeptide myotoxin, designated myotoxin a, causing a specific type of initial structural lesion, was isolated from the crude venom. In a study reported elsewhere, the structural muscle damage caused by myotoxin a was described in detail by electron microscopy (Ownby et al., 1976). In this report, chemical and functional aspects of myotoxin a are described and their significance is discussed.

Materials and Methods

Lyophilized crude venom was purchased from Miami Serpentarium Laboratories. Phospholipase A_2 (from *Crotalus terrificus terrificus* venom), benzoylated dialysis tubing, polypeptide standards for molecular weight estimation, *p*-toluenesulfonic acid, and tryptamine hydrochloride were purchased from Sigma Chemical Co. Tryptamine hydrochloride was recrystallized as 3-(2-aminoethyl)indole as described by Liu and Chang (1971). Ampholytes over the pH ranges 3.5–10 and 9–11 were purchased from LKB.

Isolation Procedure. All fractionation steps were done at 2–4 °C. Crude prairie rattlesnake venom (500 mg) was dissolved in 3–4 mL of an elution buffer consisting of 0.05 M Tris,[1] pH 9.0, at 22 °C, containing 0.1 M KCl. This was applied to a Sephadex G-50 gel filtration column (2.5 × 45 cm, gel exclusion limit 10 000 daltons) equilibrated with the elution buffer. The column was developed at a flow rate of 30 mL/h, and 5-mL fractions were collected. Absorbance of each fraction at 280 nm was read on a Beckman DG-B spectrometer. Appropriate tubes were pooled, and samples were removed for protein determination by the Lowry method (Lowry et al., 1951). The Sephadex G-50 fractions were then desalted by dialysis or passage through a Sephadex G-10 column and lyophilized.

Desalted, lyophilized Sephadex G-50 fraction 3 was dissolved in about 2 mL of the Sephadex G-50 elution buffer, and was applied to a Sephadex C-25 (carboxymethyl-Sephadex) cation-exchange column (1 × 15 cm) equilibrated with the same elution buffer. The column was developed with a three-step KCl salt gradient in the 0.05 M Tris buffer, as shown in Figure 1B. Again, 5-mL fractions were collected, and the absorbance of each at 280 nm was measured. Appropriate tubes were pooled and samples were removed for Lowry protein determination, and then fractions were dialyzed and lyophilized.

Assay for Myotoxicity. Desalted, lyophilized fractions were tested for myotoxicity by injections into the medial aspect of the thigh muscles of Swiss Webster white mice. Approximately 50 μg of protein in 0.1 mL of 0.9% NaCl was used in each injection. Injected mice were sacrificed by cervical dislocation 72 h after injection, and an approximately cubical muscle

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[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; ACTH, adrenocorticotropin; CD, circular dichroism.

sample, 5 or 6 mm on a side, was cut from the back of the thigh immediately after sacrifice. The muscle sample was fixed for 16 h in Bouin's fixative at room temperature, washed, dehydrated in an autotechnicon, and embedded in paraffin. In every case, control injections of 0.9% NaCl were made, and the tissue was processed along with experimental tissue as a control for possible processing artifacts. Approximately 8–12-μm sections were cut and stained with hematoxylin and eosin for examination with the light microscope. Observations of hemorrhage were made on the slides used for assay of muscle damage.

Electrophoretic Homogeneity. Disc Gel Electrophoresis. Disc gel electrophoresis was done in the β-alanine disc gel system (Jovin, T. M., Dante, M. L., and Chrambach, A., unpublished). Compositions of the gel components and reservoir buffers were as follows. Stacking gel: 2.5% acrylamide monomer, 0.62% methylenebisacrylamide, and 0.0005% riboflavin, in 0.047 M NaOH containing 0.0027 mL of glacial acetic acid/4 mL of stacking gel solution (pH 4.15). Resolving gel: 7.5% acrylamide monomer, 0.2% methylenebisacrylamide, in 0.46 M NaOH containing 0.034 mL of glacial acetic acid, 0.002 mL of TEMED, and 0.002 g of ammonium persulfate/4 mL of resolving gel solution (pH 3.80). Upper reservoir buffer: 0.04 M β-alanine containing 2.37 mL of glacial acetic acid/L (pH 4.15). Lower reservoir buffer: 0.05 M NaOH containing 3.66 mL of glacial acetic acid/L. Gels were run at 0.5 mA/tube for 30 min, 1 mA/tube for 1 h, and 2 mA/tube for 2.5 h. Gels were stained immediately following electrophoresis with 0.05% Coomassie blue in 19% trichloroacetic acid, destained with 20% Cl_3AcOH, and stored in 7.5% acetic acid.

Isoelectric Focusing. Focusing was done over the pH range 3–10 in 9-cm-long, 7.5% polyacrylamide gels. The upper reservoir contained 2 mL of concentrated H_2SO_4/L, and the lower contained 8 mL of ethanolamine/L. Gels were focused at 100 V for 1 h and 200 V for 4 h, following which they were soaked for 48 h either in 20% Cl_3AcOH or in 20% Cl_3AcOH containing 10 mM $HgCl_2$. After soaking, gels were stained, destained, and stored as described for disc gel electrophoresis.

Estimate of Isoelectric Point. The p*I* of myotoxin a was estimated by isoelectric focusing gel electrophoresis over the pH range 9–11. The upper reservoir contained 0.1 M sodium phosphate buffer at pH 7, the lower contained 16 mL of ethanolamine/L. Gels were run at 100 V for 1 h and 200 V for 6 h, after which they were soaked in 20% Cl_3AcOH containing 10 mM $HgCl_2$. Staining, destaining, and storing were done as described above. An identical companion gel, focused in the same experiment, was cut into 3-mm slices, and the pH of each slice was measured in 1.0 mL of distilled water.

Estimate of Molecular Weight. Molecular weight was estimated by Sephadex G-50 gel filtration (column 1 × 30 cm), using the same elution buffer as described for the Sephadex G-50 fractionation step. Polypeptide standards were cytochrome *c* (12 400), salmine (7000); ACTH (4600); clupeine (4000); and glucagon (3500).

Amino Acid Composition. Amino acid compositions were determined with a JEOL Model JLC-6AH automatic amino acid analyzer. Hydrolysis of samples was done either with constant boiling HCl (Spackman et al., 1958), or with 3 N *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Liu and Chang, 1971). The presence of carbohydrate in the form of neutral hexose was tested for by the orcinol–sulfuric acid procedure described by Winzler (1955).

Reduction, Carboxyamidomethylation. Reaction. Modification of myotoxin a with mercaptoethanol and iodoacetamide was done as described by Gurd (1967). Reactions were done at pH 8.3 in 8 M recrystallized urea. Two microliters of mercaptoethanol per mg of myotoxin a were reacted at room temperature for 1 h, and then a slight molar excess of iodoacetamide was added and reacted at room temperature for 2.5 h to effect alkylation. Alkylation without reduction was done in the same way, but addition of mercaptoethanol was omitted.

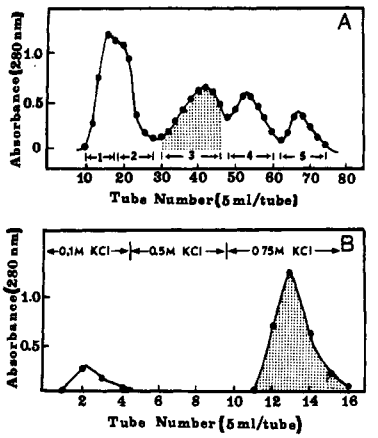

FIGURE 1: Fractionation of crude prairie rattlesnake venom. (A) Elution profile for Sephadex G-50 gel filtration column. Crude venom was eluted with 0.05 M Tris buffer (pH 9.0) containing 0.1 M KCl. The shaded peak, Sephadex G-50 fraction 3, was subjected to Sephadex C-25 cation-exchange chromatography. Absorbance points are shown for every other tube. (B) Elution profile for Sephadex C-25 cation-exchange column. Sephadex G-50 fraction 3 was dialyzed, lyophilized, and applied to the column in the Sephadex G-50 elution buffer. The column was developed with a three-step KCl salt gradient, as shown. Shaded area indicates fraction designated myotoxin a.

Removal of Reagents and Isolation of Modified Myotoxin a. Following the 2.5 h incubation with iodoacetamide, the reaction mixture was cooled in ice, and then applied to a Sephadex C-25 cation-exchange column in a Pasteur pipet (bed volume ~1.5 mL) equilibrated with the Sephadex G-50 elution buffer. About 5 column volumes of buffer were washed through the column, and then a three-step KCl salt gradient was passed through the column. Fractions of 0.8 mL were collected, and the absorbance of each at 280 nm was measured. Appropriate tubes were pooled and lyophilized. Lyophilizates were taken up in dilute NaOH (pH 9.6) and desalted by passage through a Sephadex G-10 column developed with the dilute NaOH.

CD Spectroscopy. The circular dichroic spectra of native and reduced carboxyamidomethylmyotoxin a were taken on a JASCO Model J-41C spectropolarimeter at 25 °C. The solvent used was 1 mM sodium phosphate buffer, pH 7. Protein concentrations were 0.1 and 1.0 mg/mL, in 1.0- and 0.01-cm cells, to obtain the CD spectra over the range 190–350 nm.

Assay for Hemolytic Activity. The hemolytic activity of myotoxin a toward dog erythrocytes was tested by the procedure described by Lankisch et al. (1971). Incubation tubes were shaken gently at 40 °C, and aliquots were removed at 90 and 150 min for measurement of hemolysis. Hemoglobin was estimated by absorbance measurements at 541 and 577 nm to measure relative Hb release.

Results

Isolation and Myotoxin a. The elution profiles for the Sephadex G-50 and C-25 columns used in fractionation of the crude venom are shown in Figure 1A,B, respectively. Protein recovered from the Sephadex G-50 column was distributed among the fractions as shown in Table I. Each Sephadex G-50

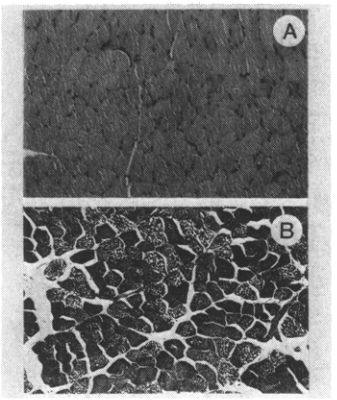

FIGURE 2: Histological results of injection of 50 μg of myotoxin a in 0.1 mL of 0.9% NaCl. (A) Control sample from muscle injected with 0.9% NaCl only (×40). (B) Test sample from muscle injected with myotoxin a (×40).

TABLE I: Distribution of Protein and Local Tissue Effects of Fractions from Sephadex G-50 and C-25 Fractionation of *Crotalus viridis viridis* Venom.

Fractional step	Fraction	% of total protein recovered from step	Ca. % of protein in crude venom	Local tissue[a] effects: Myonecrosis	Local tissue[a] effects: Hemorrhage
G-50 Gel Filtration	1	20	20	+++	+++
	2	44	44	+	+[b]
	3	21	21	++	−
	4	9	9	−	−
	5	6	6	−	−
C-25 Ion Exchange	1	13	2.7	−	−
	2	87	18.3	++	−

[a] Positive myonecrotic or hemorrhagic effects in skeletal muscle indicated by +; negative effects indicated by −. Approximate relative intensities of effects are indicated by numbers of symbols used. [b] The weak myonecrotic and hemorrhagic effects of Sephadex G-50 fraction 2 may be due to partial overlap of Sephadex G-50 fraction 1 into 2.

fraction was desalted, lyophilized, and tested for myotoxicity. In addition to being myotoxic, Sephadex G-50 fraction 1 was observed to be hemorrhagic. Sephadex G-50 fraction 3 was myotoxic, but not hemorrhagic.

Fraction 3 was subjected to cation-exchange chromatography, giving Sephadex C-25 fractions 1 and 2. In later fractionations, Sephadex G-50 fraction 3 was applied directly to the Sephadex C-25 column without desalting and lyophilization, since the Sephadex C-25 column was equilibrated with the Sephadex G-50 elution buffer. The Sephadex C-25 fractions were dialyzed, lyophilized, and tested for myotoxicity. Sephadex C-25 fraction 1 caused no apparent muscle damage, as revealed by the light-microscope assay, while Sephadex C-25 fraction 2 caused the same type of damage as that caused by Sephadex G-50 fraction 3. Sephadex C-25 fraction 2, having strong myotoxic activity, was designated myotoxin a. Myotoxin a caused pronounced vacuolization of muscle fibers as the initial structural lesion, the effect becoming apparent with the light microscope about 24 h after injection and reaching a peak at about 72 h after injection. Typical results of myotoxin a injection are shown in Figure 2B. In the study done with the electron microscope (Ownby et al., 1976), the vacuolization caused by myotoxin a was ascribed to dilation

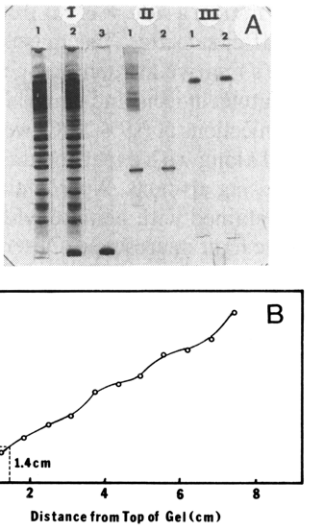

FIGURE 3: (A) I. Isoelectric focusing polyacrylamide pH 3.5–10, anode at top. Gels 1 and 2 loaded with 150 μg of crude venom, gel 3 with 25 μg of myotoxin a. (A) II. β-Alanine disc gels. Gel 1 loaded with 100 μg of crude venom, gel 2 with 25 μg of myotoxin a. (A) III. Isoelectric focusing gels, pH 9–11, anode at top. Gel 1 loaded with 100 μg of crude venom, gel 2 with 25 μg of myotoxin a. (B) pH gradient through pH 9–11 focusing gel. Points are plotted for measurements from every other gel slice.

of the sarcoplasmic reticulum, and severe secondary structural changes were observed. Similar initial effects were observed with the electron microscope following injection of crude prairie rattlesnake venom (Stringer et al., 1972). This indicates that the myotoxic effects of myotoxin a are a major part of the biological effects observed with the crude venom. This is not too surprising in that myotoxin a comprises a major part of the crude venom protein, about 18%, as shown in Table I.

Homogeneity. The purity of myotoxin a was checked by β-alanine disc gel electrophoresis and isoelectric focusing gel electrophoresis. As shown in Figure 3A, myotoxin a was electrophoretically homogeneous in each type of gel electrophoresis. Some difficulty was encountered in fixation of myotoxin a during ampholyte removal from focusing gels by 20% Cl_3AcOH (gel I.1), but this was overcome by addition of 10 mM $HgCl_2$ to the 20% Cl_3AcOH (gels I.2 and I.3).

Chemical Properties. As shown in Figure 3, A-III and B, the isoelectric point of myotoxin a was estimated to be 9.6 by isoelectric focusing gel electrophoresis over the pH range 9–11. That myotoxin a is the most basic polypeptide in crude prairie rattlesnake venom is indicated by the absence of bands at pHs higher than 9.6 in the focusing gel loaded with the crude venom. The conclusion must be qualified somewhat, however, in that some of the smallest polypeptides in the crude venom may not be fixed in the gel and stained by the procedures used.

Standard gel filtration in Sephadex G-50 (stated gel exclusion limit of 10 000 daltons) was done to estimate the molecular weight of myotoxin a. The results are shown in Figure 4. Of the polypeptide standards used, only clupeine deviated from the standardization curve. Clupeine was selected for use because of its low molecular weight and high solubility in the elution buffer. Its unusual composition, 21 arginines out of 31 total residues, may be the cause of its deviant chromatographic behavior on sephadex. Using the line determined by the other four standards, the molecular weight estimate obtained for myotoxin a was 4100.

The amino acid composition of myotoxin a is given in Table II. The most striking aspect of the amino acid composition is

TABLE II: Amino Acid Composition of Myotoxin a.

Amino acid[a]	Time of HCl hydrolysis (h) 24	48	72	Corrected[b] value	Nearest integer	Wt
Lys	9.84	9.72	9.61	9.72	10	1280
His	1.88	2.01	1.66	1.85	2	274
Arg[d]	0.95	0.95	0.93	0.94	1	156
Asx	2.25	1.99	1.65	1.97	2	230
Ser	2.17	1.68	1.56	2.56	3	261
Glx	1.83	1.86	1.87	1.85	2	258
Pro	3.15	2.99	2.92	3.02	3	291
Gly	4.82	4.70	4.20	4.57	5	285
½-Cystine	4.08	3.51	2.86	3.48	4[c]	408
Met	0.82	0.98	0.96	0.92	1	131
Ile	1.78	1.81	1.73	1.77	2	226
Leu[d]	1.09	1.10	0.99	1.06	1	113
Tyr[d]	1.07	1.10	1.01	1.05	1	163
Phe[d]	1.20	1.14	1.01	1.12	1	147
Trp[e]	1.37	1.24	1.35	1.32	1	186
Total					39	4427

[a] The amino acids threonine, alanine, and valine were detected in trace amounts only. [b] The value for serine was corrected by extrapolation to zero time; average values are given for all other amino acids. [c] The nearest integral value for half-cystine was confirmed by analysis of reduced, alkylated and alkylated myotoxin a. [d] Recoveries for arginine, leucine, tyrosine, and phenylalanine were averaged to obtain normalized integration values for each hydrolysis. [e] Tryptophan was determined following hydrolysis with *p*-toluenesulfonic acid. Recoveries of all other amino acids were comparable to their recoveries following hydrolysis with constant-boiling HCl.

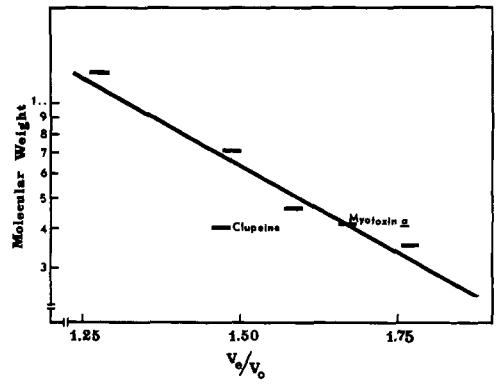

FIGURE 4: Estimation of the molecular weight of myotoxin a by gel filtration. V_e is elution volume for each polypeptide, and V_0 is the column void volume measured with blue dextran 2000. Lengths of data bars indicate uncertainty in V_e/V_0.

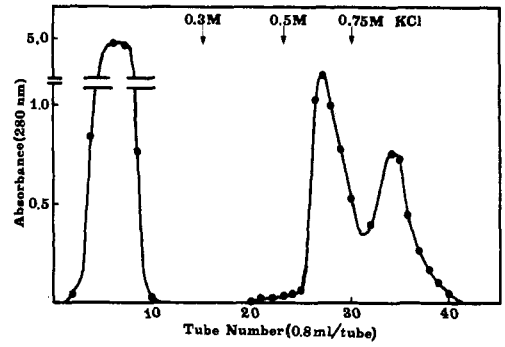

FIGURE 5: Elution profile for Sephadex C-25 cation-exchange column in isolation of reduced, carboxyamidomethylated myotoxin a. Column was equilibrated with 0.05 M Tris buffer (pH 9.0) containing 0.1 M KCl, and was developed with a stepwise KCl salt gradient, the beginning point of each step indicated by an arrow.

the very great preponderance of basic amino acids. Of the 15 amino acids present in a total of 39 residues, 10 are lysine and 1 is arginine, making myotoxin a 28% basic amino acids by numbers of residues. This result is in good agreement with the 9.6 isoelectric point estimate obtained by isoelectric focusing gel electrophoresis. The composition indicates the possibility of two disulfide bridges. The presence of two disulfide bridges, with no free sulfhydryls, was confirmed by amino acid analysis of alkylated and reduced alkylated myotoxin a (results of analyses in following paragraph). The minimum molecular weight calculated on the basis of the amino acid composition is 4427. The molecular weight estimate of 4100 from gel filtration indicates that the minimum molecular weight represents the most accurate value for true molecular weight. The orcinol–sulfuric acid test for neutral hexose in myotoxin a was negative. The recovery following 24-h hydrolysis with constant boiling HCl was 94%. The recoveries of amino acids other than tryptophan following hydrolysis with *p*-toluenesulfonic acid were comparable to their recoveries following hydrolysis with HCl.

Disulfide Bridges and Myotoxic Activity. The presence of disulfide bridges has been demonstrated in many biologically active polypeptides from snake venoms (Tu, 1973; Jimenez-Porras, 1968). In most cases, disruption of disulfide bridges destroys the biological activities of these polypeptides (Yang, 1967; Tu et al., 1975); however, retention of biological activity following breakage of a disulfide bridge has also been observed (Botes, 1974). The functional significance of the disulfide bridges in myotoxin a was, therefore, investigated by reduction, carboxyamidomethylation. The first step after chemical modification, Sephadex C-25 cation-exchange chromatography, accomplished both the removal of reagents and the separation of modified from unmodified myotoxin a. Fractionation of the reduction, carboxyamidomethylation reaction mixture by the Sephadex C-25 column gave the elution profile shown in Figure 5. The large absorbance at 280 nm for tubes 3–9 was taken to be due to elution of reactants and side products from the reaction. Tubes 26–30 and 33–40 were pooled to give two protein fractions, which were desalted by passage through a Sephadex G-10 column as described under Methods. Their amino acid compositions were determined following 24 h hydrolysis with constant boiling HCl. The first protein fraction, eluting from the Sephadex C-25 column primarily at 0.5 M KCl concentration, gave a composition comparable to that of native myotoxin a (Table II), except that only a trace of half-cystine and 3.86 residues of carboxymethylcysteine were recovered. The composition of this fraction showed essentially complete reduction and alkylation of the disulfides, with no apparent modification of other residues. The second protein fraction, eluting from the Sephadex C-25 column primarily at 0.75 M KCl concentration, gave the composition of unmodified myotoxin a. The reduced, carboxyamidomethylated polypeptide (the first fraction) represented about 60–70% of the protein in the reaction mixture. The reaction mixture from which mercaptoethanol was omitted gave a large side-product peak and a single protein peak, which eluted primarily at 0.75 M KCl concentration. The amino acid composition of the protein peak was that of native myotoxin a, with no apparent chemical modification.

Several samples of reduced, carboxyamidomethylmyotoxin a were injected for assay of myotoxicity by the light-microscope

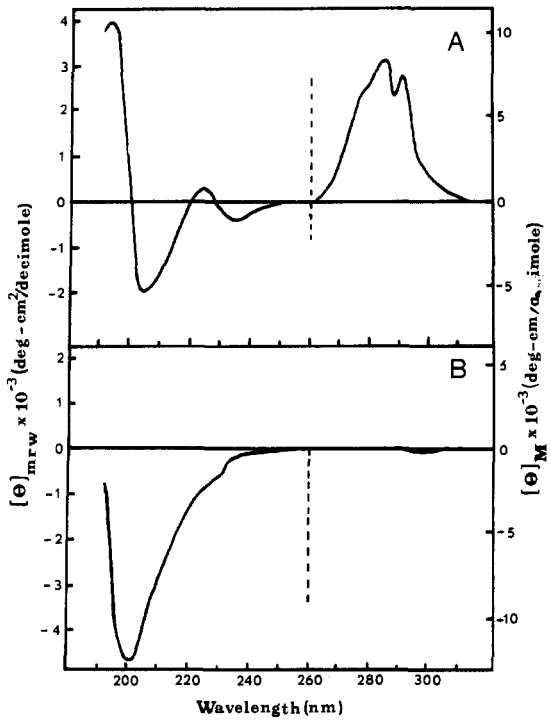

FIGURE 6: Circular dichroic spectra of myotoxin a. Molar ellipticities ($[\theta]_M$) for the 260–320-nm region based on molecular weight 4427; mean residue ellipticities ($[\theta]_{mrw}$) for the 190–260-nm region based on mean residue weight of 113. (A) Native myotoxin a; (B) reduced, carboxyamidomethyl myotoxin a.

TABLE III: Hemolytic Activity of Myotoxin a.

Additions to isotonic medium[a]	% Hemolysis time of incubation (min)	
	90	180
$CaCl_2$	0	0
Phospholipase A_2	0	80
Myotoxin a		
0.1 mg/mL	6	11
0.25 mg/mL	9	20
0.50 mg/mL	30	98
1.0 mg/mL	28	98
$CaCl_2$ + Phospholipase A_2	28	94
$CaCl_2$ + Myotoxin a	0	12
Phospholipase A_2 + Myotoxin a	0	64
$CaCl_2$ + Phospholipase A_2 + Myotoxin a	30	94

[a] Final concentrations of $CaCl_2$ and phospholipase A_2 were 1 mM and 2 μg/mL, respectively. In media containing $CaCl_2$ or phospholipase A_2, the concentration of myotoxin a was 1.0 mg/mL. Erythrocytes from dog blood were suspended at a 1:20 dilution of the original whole blood.

assay. None of the muscle samples examined showed structural damage. The disulfide bridges of myotoxin a are, therefore, apparently essential for its myotoxic activity.

CD Spectra. The circular dichroic (CD) spectra of native and reduced, carboxyamidomethylmyotoxin a are shown in Figure 6. A comparison of the spectra shows a striking change from an apparently ordered conformation to a characteristic random coil. For the native polypeptide, the spectrum over the 190–260-nm region does not clearly resemble any of the standard model spectra for known polypeptide conformations. A major source of deviation from the model spectra is the apparently non-peptide-bond contributions in the 220–250-nm region, giving rise to a positive band at 225 nm ($[\theta]_{mrw}$ = +320), and to a negative band at 234 nm ($[\theta]_{mrw}$ = −390). The peptide-bond maximum appears at about 195 nm ($[\theta]_{mrw}$ = +4000), and the minimum appears at 206 nm ($[\theta]_{mrw}$ = −2000). The relatively low intensities of these bands, and the low wavelength of the minimum, do not correlate well with CD spectra from model compounds of known structure. Our intention in measuring the CD spectra of the native and chemically modified myotoxin a was to obtain a comparison of the polypeptide conformations before and after modification. The CD spectrum of the native myotoxin a may become more meaningful in itself as additional CD model spectra become available from compounds of known structure. The strong aromatic CD in the 260–320-nm region is primarily suggestive of tryptophan contributions. The presence of only one tryptophan in myotoxin a, avoiding partial cancelling of CD bands that can occur when many aromatic residues are present, may contribute to the relatively high intensity in the aromatic region.

In sharp contrast, the CD spectrum of the reduced, carboxyamidomethylmyotoxin a is characteristic of denatured proteins; the broad negative over the 190–240-nm region suggests the random-coil conformation, and the loss of aromatic bands in the 260–320-nm region suggests loss of defined orientation of aromatic residues. Although the conformation of the native polypeptide cannot be determined from its CD spectrum alone, a drastic change to the random-coil conformation upon reduction, carboxyamidomethylation is confirmed by the CD spectra.

It is appealing to correlate the loss of myotoxicity upon reduction, carboxyamidomethylation with the striking change in conformation confirmed by the CD spectra. Large changes in secondary structure can be the cause of protein inactivation, and the altered secondary structure in the modified myotoxin a is probably responsible for its lack of myotoxicity. The possibility exists, however, that the disulfide bridges per se, rather than the conformational restraints they impose on the polypeptide, are required for myotoxicity.

Hemolytic Activity. Direct lytic factors (DLF), basic polypeptides that have been isolated from various snake venoms, exert a hemolytic action on red blood cells in isotonic suspension (Condrea, 1974). Because of some chemical similarity of myotoxin a to the direct lytic factors, the hemolytic activity of myotoxin a was tested. Myotoxin a was found to be relatively weakly hemolytic, as shown in Table III, requiring between 2.5 and 5 times more myotoxin a than cobra (*Naja naja*) venom DLF to cause about 40% hemolysis at 180 min of incubation (Lankisch et al., 1971).

Discussion

Neurotoxins from the venoms of cobras and sea snakes have been extensively studied by many investigators (reviewed by Tu, 1973). In contrast, the toxic components of rattlesnake venoms, especially from rattlesnakes indigenous to North America, have been studied very little. A cardiotoxin was recently isolated from the venom of Mojave rattlesnake (*Crotalus scutulatus*), indigenous to Arizona (Bieber et al., 1975). Myonecrosis is one of the serious toxic effects of rattlesnake bite. The isolation of a muscle toxin from tarantula venom has been recently reported (Lee et al., 1974), but no isolations of myotoxins from rattlesnake venoms have been reported prior to the current investigation. The complexity of crude venom

TABLE IV: Comparative Amino Acid Compositions of Basic Polypeptides from Various Snake Venoms.[a]

Family:	Elapidae					Hydrophiidae	Viperidae	Crotalidae
Genus:	*Hemachatus*	*Naja*	*N.*	*N.*	*N.*	*Pelamis*	*Vipera*	*Crotalus*
Species:	*hemachatus*	*naja*	*naja*	*naja*	*naja*	*platurus*	*palestinae*	*viridis*
Subspecies:			*atra*	*atra*	*oxiana*			*viridis*
Name	DLF	Cobramin B	Cobrotoxin	Cardiotoxin	Cytotoxin	Toxin a	Viperotoxin	Myotoxin a
Biol Act.	Hemolytic	Transport Inhibition	Neurotoxic	Cytotoxic	Neurotoxic	Neurotoxic	Neurotoxic	Myotoxic
Amino Acid								
Lys	10	8	3	9	10	5	9	10
His	1	0	2	0	1	2	3	2
Arg	1	2	6	2	1	3	6	1
Asx	6	5	8	6	5	6	10	2
Thr	3	3	8	3	2	7	4	0
Ser	3	2	4	2	3	5	6	3
Glx	1	0	7	0	0	7	10	2
Pro	5	4	2	5	5	1	12	3
Gly	2	2	7	2	2	3	10	5
Ala	1	2	0	2	2	1	6	0
½-Cystine	8	6	8	8	8	8	6	4
Val	4	6	1	7	7	1	4	0
Met	2	2	0	2	2	1	1	1
Ile	2	1	2	1	1	2	4	2
Leu	6	5	1	6	6	1	4	1
Tyr	1	3	2	3	2	1	4	1
Phe	1	1	0	2	2	0	5	1
Trp	0	0	1	0	0	1	4	1
Total Res	57	52	62	60	60	55	108	39
Ref	Aloof-Hirsch et al. (1968)	Larsen and Wolff (1968)	Chang and Hayashi (1969)	Lee et al. (1971)	Grishin et al. (1974)	Tu et al. (1975)	Moroz et al. (1966)	Present Invest.

[a] Representative polypeptides are included in the table. Many more chemically similar polypeptides are known, but cannot be included due to space limitations.

action and the need for using a biological assay specific for muscle damage have tended to limit progress toward a comprehensive understanding of snake-venom-induced myonecrosis. Isolation of muscle damaging components of crude venom is an essential step toward gaining this understanding. Use of a reliable, specific assay for muscle damage is most appropriate for the systematic isolation of the myotoxic components of crude venoms. Light microscope histology is the most reliable and specific assay available for detection of structural muscle damage. In the present study, use of the light microscope as an assay tool enabled isolation of a polypeptide myotoxin which might well have gone unnoticed by another assay procedure during venom fractionation. This isolation represents the first reported purification of a snake venom polypeptide solely on the basis of its ability to cause structural damage in skeletal muscle.

Disulfide bridges, when present, are important in maintaining the conformation of a polypeptide. Cobrotoxin and *Pelamis* toxin a have been shown to lose biological activity upon reduction of disulfide bridges (Yang, 1967; Tu et al., 1975). However, *Naja naja nivea* toxin α has been shown to retain its neurotoxicity following reduction of one disulfide bridge (Botes, 1974). The amino acid compositions of alkylated and reduced, alkylated myotoxin a show that it has two disulfide bridges, with no free sulfhydryls. Both disulfides are broken by the reduction, alkylation procedure used. Since fractions were obtained which were effectively totally modified and nonmodified, but no material could be detected which was chromatographically homogeneous and only half modified, cleavage of one of the disulfides in 8 M urea may lead to facilitated cleavage of the second. Several samples of reduced, carboxyamidomethylated myotoxin a (up to 200 μg in 0.1 mL of 0.9% NaCl) were injected without producing muscle damage visible under the light microscope. The CD spectrum of the modified myotoxin a was typical of denatured, primarily random-coil proteins. Therefore, the reduction, carboxyamidomethylation of myotoxin a apparently produced a nonmyotoxic polypeptide by changing the conformation to a typical random coil.

As noted under Results, the CD spectrum of native myotoxin a apparently has a non-peptide-bond maximum at about 225 nm, and a minimum at about 235 nm. Aromatic side chains and disulfide bridges have been shown to contribute CD bands near 230 nm (Green and Melamed, 1966; Ludescher and Schwyzer, 1971). Some snake venom polypeptides, including cobrotoxin, α-bungarotoxin, and erabutoxin a, have been reported to have strong positive CD bands near 228 nm (Yang et al., 1968). Native myotoxin a seems to have a similar band in the 220-230-nm region, partially overlapping with negative bands on either side. This overlap may at least partially account for the appearance of the peptide-bond minimum at about 206 nm ($[\theta]_{mrw} = -2000$) rather than at higher wavelengths. Partial overlap of a negative band with a positive band (at a higher wavelength) would result in lower apparent wavelength for the minimum and higher wavelength for the maximum, with decreased intensity for both. Native myotoxin a apparently contains no significant proportion of α helix, as shown by its CD spectrum. This would be expected, since native

TABLE V: Comparative Amino Acid Compositions of Biologically Active Polypeptides Isolated from the Venoms of Rattlesnakes (Family: Crotalidae; Genus: *Crotalus*).

Genus:	*Crotalus*	*C.*	*C.*	*C.*
Species:	*adamanteus*	*durrissus terrificus*	*durrissus terrificus*	*viridis viridis*
Subspecies:				
Name of polypeptide	Basic protein	Crotoxin basic protein	Crotamine	Myotoxin a
Biol Act.	Neurotoxic	Neurotoxic[a]	Paralytic	Myotoxic
Amino Acid				
Lys	5	9	9	10
His	2	2	2	2
Arg	2	8	2	1
Asx	2	9	2	2
Thr	1	6	0	0
Ser	2	6	3	3
Glx	1	8	2	2
Pro	2	5	3	3
Gly	4	10	5	5
Ala	1	6	0	0
½-Cystine	3	10	6	4
Val	1	2	0	0
Met	1	1	1	1
Ile	1	4	1	2
Leu	1	6	1	1
Tyr	1	9	1	1
Phe	2	6	2	1
Trp	0	2	2	1
Total Res	32	110	42	39
Ref	Bonilla et al. (1971)	Hendon and Fraenkel-Conrat (1972)	Laure (1975)	Present Invest.

[a] Crotoxin basic protein, a basic phospholipase A_2, becomes neurotoxic in combination with crotoxin acidic protein.

myotoxin a has two disulfides, three prolines, and five glycines in a total of 39 amino acid residues. The possible presence of β structure and perhaps β turn is not excluded, but cannot be assigned with confidence without additional conformational model spectra. The conformation of native myotoxin a cannot be determined unambiguously at this time from its CD spectrum.

Although isolation of myotoxin a has not been previously reported, many snake venom polypeptides with different biological activities have been isolated which have some gross chemical similarity to each other and to myotoxin a. The amino acid compositions of some of these polypeptides from various snake venoms are shown in Table IV. Each of the polypeptides is markedly basic, with correspondingly high content of lysine and/or arginine, and each is relatively rich in disulfide bridges per number of amino acid residues. Hydrophilic residues tend to be more abundant than hydrophobic residues, but both types are present in significant proportions. Relatively high amounts of proline and glycine, especially pronounced in viperotoxin, may be important for the various known biological activities of these polypeptides, in that these amino acids may influence conformation. Myotoxin a was found to be hemolytic, but only weakly so relative to the activity of DLF (Lankisch et al., 1971). This result emphasizes the probability that, although there may be partially overlapping biological activities between some of the basic snake venom polypeptides, even slight chemical differences may account for marked differences in abilities to produce given specific biological effects. Induction of myovacuolization in skeletal muscle is the biological effect most characteristic of myotoxin a.

Amino acid compositions of some biologically active polypeptides isolated specifically from rattlesnake venoms are shown in Table V. These rattlesnake polypeptides also are high in lysine and/or arginine, and they possess at least one disulfide bridge, both hydrophilic and hydrophobic residues, and relatively high amounts of proline and glycine. Crotoxin basic protein, by far the largest of the polypeptides included in the table, is the only one known to have enzymatic activity, being a phospholipase A_2. Considerable chemical similarity is shown between crotamine and myotoxin a, the compositional differences being confined to six amino acids. Crotamine has 42 total residues and three disulfide bridges, as compared to 39 residues and two disulfides in myotoxin a. Despite the chemical similarity and possible overlap of biological activities, there is apparently no identity of the biological activities of crotamine and myotoxin a, since crotamine has never been reported to cause structural damage in muscle.

Some early work with snake venoms led to the speculation that venom enzymes, especially proteases, might account totally for the myonecrotic effects of crude snake venoms (Houssay, 1930; Porges, 1953). More recently, myonecrotic effects of *Habu* (*Timeresurus flavoviridis*) venom have been ascribed to the combined actions of a protease, phospholipase A_2, and a heat-stable factor (Maeno, 1962). The isolation of myotoxin a extends the observation beyond *Habu* venom that snake venom components other than known enzymes are important in myonecrosis. As cited previously, myonecrosis may not be prevented by use of antivenin to crude venom. This suggests that significant muscle damage may be due to a venom component with low antigenicity, having a very low titer of antibody in the antivenin. Our findings that myotoxin a is a small polypeptide with a molecular weight of only 4400 makes it a likely cause of antivenin-resistant muscle damage. Further studies of such myotoxins are needed, since development of maximally effective treatment for snake-venom-induced myonecrosis requires that treatment be directed at least partially toward eliminating their antivenin-resistant muscle-damaging effects. Previous authors have suggested (Stringer et al., 1972) that venom studies might also help provide useful insight in treatment of myodegeneration in afflictions such as dystrophia musculorum progressiva (DMP).

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