Binding of Myotoxin *a* to Sarcoplasmic Reticulum Ca^{2+}-ATPase: A Structural Study†

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ABSTRACT: The interaction of myotoxin *a* with intact sarcoplasmic reticulum (SR) components was investigated, and two SR proteins were identified that associated with myotoxin *a*. One of the proteins has an apparent molecular weight similar to the Ca^{2+}-ATPase, the major SR protein responsible for calcium loading. Ca^{2+}-ATPase was purified, and its interaction with myotoxin *a* was studied. Evidence for specific binding of myotoxin *a* to Ca^{2+}-ATPase was established by isolating chemically cross-linked myotoxin *a*–Ca^{2+}-ATPase complexes and further proving their association with anti-myotoxin *a* antibodies. The binding region of myotoxin *a* was further delineated by cleaving the protein with cyanogen bromide (CNBr) into two fragments, a larger N-terminal fragment of 28 residues and a smaller *C*-terminal fragment of 14 residues. Competition experiments with ^{125}I-myotoxin *a* showed that the C-terminal fragment competed better against ^{125}I-myotoxin *a* than the N-terminal fragment for SR protein binding. Two overlapping peptides covering the sequence of the N-terminal fragment were synthesized to clarify the interaction of the N-terminal fragment of myotoxin *a* with SR proteins. A 16-residue peptide corresponding to residues 1–16 competed strongly with ^{125}I-myotoxin *a*, while a second peptide (residues 13–28) did not.

Myonecrosis, or muscle damage, is one of the common pathologic symptoms induced by snake envenomation, especially from snakes in the families Crotalidae and Viperidae. Among the many muscle-necrotising proteins, myotoxin *a* is the one most extensively studied. Myotoxin *a* is a relatively small polypeptide of 42 amino acid residues (M_r 4620), containing 3 disulfide bonds with an isoelectric point of 9.6 (Fox et al., 1979). It was first isolated from the venom of *Crotalus viridis viridis* (prairie rattlesnake) (Cameron & Tu, 1977). Myotoxin *a* shares 78% sequence homology with four other myotoxins: crotamine (Laure, 1975), myotoxins I and II (Pool & Bieber, 1981), and peptide C (Maeda & Tamiya, 1978). The N-terminal residues 1–14 are the most conserved among the five myotoxins. Myotoxin *a* causes vacuolization and eventual destruction of skeletal muscle (Ownby et al., 1976). Crotamine has also been shown to cause vacuolization of skeletal muscle (Cameron & Tu, 1978) similar to that caused by myotoxin *a*, but the other myotoxins have not been studied for their effect on the skeletal muscle in vivo. Electron microscopy studies using horseradish peroxidase conjugated to myotoxin *a* showed that the toxin is first localized on the sarcoplasmic reticulum (SR)[1] membrane (Tu & Morita, 1983). The SR is a calcium storage reservoir within the skeletal muscle and strictly regulates calcium concentration within the muscle cell. Calcium influx and efflux from the SR are controlled by different proteins. Calcium efflux from the SR is via the calcium channel, while calcium influx into the SR is via the Ca^{2+}-ATPase, or the calcium pump. The calcium pump is the major protein (50–70%) of longitudinal SR. Biochemical investigations revealed that myotoxin *a* is an uncoupler of Ca^{2+} loading activity in SR (calcium pump inhibitor) without inhibiting calcium ion efflux activity in the calcium channel (Volpe et al., 1986).

Two methods were used to elucidate which components in the SR are responsible for binding myotoxin *a*. The first method utilized a photoaffinity cross-linking agent to covalently link myotoxin *a* with receptor proteins in intact SR. Two proteins were found to bind myotoxin *a*. One of these proteins was identified as Ca^{2+}-ATPase. The second method used anti-myotoxin *a* antibodies from rabbit sera to assess the degree of myotoxin *a* binding to purified Ca^{2+}-ATPase by ELISA.

The region of the primary sequence binding to Ca^{2+}-ATPase was further delineated through binding experiments using myotoxin *a* fragments obtained by cleavage of the native toxin with cyanogen bromide and chemically synthesized peptides. The C-terminal cleavaged fragment and a synthetic peptide corresponding to residues 1–16 of the N-terminal of myotoxin *a* were found to compete with ^{125}I-myotoxin *a* for binding to two SR proteins.

Materials and Methods

Materials. Crude *Crotalus viridis viridis* venom was purchased from the Miami Serpentarium, Salt Lake City, UT. HSAB was purchased from Pierce, Rockford, IL. Anti-horse (anti-rabbit IgG) conjugated with alkaline phosphatase and cobra neurotoxin I (from *Naja naja atra*) were purchased from Sigma, St. Lous, MO. Affi-gel agarose gel bead support (Affi-gel 10) was purchased from Bio-Rad, Richmond, CA. $Na^{125}I$ was from Amersham, Arlington Heights, IL. Problott membrane was purchased from Applied Biosystems, Foster City, CA. Precast 16% Tricine gels were purchase from NOVEX, Encinitas, CA. Phenylacetamidomethyl (PAM) 2% cross-linked divinylbenzene resin substituted at approximately

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[1] Abbreviations: HSAB, *N*-hydroxysuccinimidyl 4-azidobenzoate; LSR, longitudinal sarcoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; RP HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; PITC, phenyl isothiocyanate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; p*I*, isoelectric point.

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0.7 mmol/g with *t*-Boc amino acids was obtained from Applied Biosystems Inc.

Isolation of Myotoxin a. Myotoxin *a* was isolated from the venom of *Crotalus viridis viridis* by gel filtration (Sephadex G-50) and cation exchange (CM-Sephadex) according to the method of Cameron and Tu (1977), and homogeneity was established by HPLC and SDS–PAGE.

Isolation of SR Vesicles and Ca^{2+}-ATPase. SR vesicles were isolated and purified from rabbit fast-twitch skeletal muscle by differential and sucrose density centrifugation as previously described (Saito et al., 1984). Light sarcoplasmic reticulum (LSR) was suspended in 0.3 M sucrose/5 mM imidazole, pH 7.4, and stored at −70 °C until used. The purified Ca^{2+}-ATPase was obtained from LSR by deoxycholate treatment as described in Kashima et al. (1990). The purified Ca^{2+}-ATPase was stored in 0.3 M sucrose, 10 mM $MgCl_2$, and 5 mM imidazole, pH 7.4, at −70 °C until used. The purity of Ca^{2+}-ATPase is >90% as judged by SDS–PAGE.

Radioiodination of Myotoxin a. Five to ten micrograms of myotoxin *a* was dissolved in 50 μL of 0.5 M phosphate buffer, pH 7.5. To the solution was added 2 mg/mL chloramine T. The reaction was initiated by adding 1 mCi of sodium [^{125}I]iodide; 2 min later, iodination was quenched by addition of 2 mg/mL sodium metabisulfite. The radioiodinated myotoxin *a* was immediately fractionated on a 0.6 × 15 cm Sephadex G-10 column.

Cross-Linking. Cross-linking was carried out as previously described (Massague et al., 1981). SR or purified Ca^{2+}-ATPase (100 μg of protein) was pelleted at 14000*g* for 10 min in an Eppendorf microfuge. The pellet was resuspended in incubation buffer (10 mM HEPES, pH 7.5, 0.1 M KCl, and 10 mM $MgCl_2$) at a protein concentration of 3 mg/mL. The SR pellets were incubated with 0.1 μM of ^{125}I-myotoxin *a* or ^{125}I-neurotoxin at room temperature for 1 h. In competition studies, 1 μM nonradioactive myotoxin *a*, neurotoxin, CNBR fragments, or synthetic peptides was incubated with SR pellets at room temperature for 30 min; then 0.1 μM ^{125}I-myotoxin *a* was added for a further 30 min of incubation. The incubation solutions were then centrifuged at 14000*g* for 15 min. The pellets were washed 2 times in incubation buffer and brought to a final concentration of 3 mg/mL. To initiate the cross-linking reaction, 350 nmol of HSAB (dissolved in 5 μL of DMSO at 0 °C) was added to the protein solution. The samples were photolyzed by exposure to UV light from a Zeiss 100-W mercury lamp at a distance of approximately 10 cm. The cross-linking reaction was quenched with a final concentration of 50 mM Tris-HCl, pH 7. The SR was pelleted (14000*g* for 15 min) and washed 3 times with 10 mM Tris-HCl, pH 7, and the pellet was retained for electrophoresis and autoradiography.

Antibody Purification. Anti-myotoxin *a* antibodies were raised by subcutaneous injection of myotoxin *a* (0.25 mg/kg) in saline diluted 1:1 with Freund's complete adjuvant into New Zealand White rabbits (7 kg). Subsequent booster injections of 0.25 mg/kg myotoxin *a* in saline diluted 1:1 with Fruend's incomplete adjuvant were given biweekly, and animals were bled at 1–2-week intervals. Serum from the rabbits was passed through an Affi-Gel column conjugated with myotoxin *a* as described (Bober & Ownby, 1988). The column was washed with phosphate-buffered saline at a flow rate of 5 mL/min until the absorbance at 280 nm returned to base line. The column was eluted with distilled water, and fractions were collected and assayed for antimyotoxin *a* antibody using ELISA.

ELISA Assay. Purified Ca^{2+}-ATPase was diluted at a concentration of 12.5 μg/mL with 0.1 M sodium carbonate buffer, pH 9.6. Aliquots (0.2 mL) were incubated at 4 °C overnight in the wells of microtiter plates. The microtiter plates were washed 2 times with washing buffer (0.9% NaCl/0.05% Tween 20), and 0.2 mL/well of increasing concentrations of myotoxin *a* (diluted in PBS, 0.01% NaN_3, 0.05% Tween 20, and 1% BSA) was incubated with the Ca^{2+}-ATPase at room tempeature for 1 h. After the plates were washed as described previously, 0.2 mL/well of anti-myotoxin *a* antibody (titer 1:1) was added, and the plate was incubated at room temperature for 2 h. The samples were washed as above before addition of 0.2 mL of anti-rabbit IgG (alkaline phosphatase conjugated, diluted 1:1000 with PBS, 0.01% NaN_3, 0.05% Tween 20, and 1% BSA) and incubated for 2 h at room temperature. After washing 3 times as above, 0.2 mL of *p*-nitrophenyl phosphate (1 mg/mL) in 0.94 M diethanolamine buffer (pH 9.8, 1 mM $MgCl_2$) was added. The reaction was quenched after 20 min with 50 μL/ well of 3 N NaOH, and alkaline phosphatase activity was measured at 405 nm. Control wells were not coated with Ca^{2+}-ATPase, but treated identically otherwise.

Chemical Cleavage of Myotoxin a by Cyanogen Bromide. Myotoxin *a* was cleaved at methionine residues with cyanogen bromide (CNBr) according to of Mori and Tu (1988). Twenty milligrams of myotoxin *a* was dissolved in 5 mL of 70% formic acid; 100 molar excess CNBr to methionine was added. The reaction mixture was sealed and reacted for 24 h at 37 °C. The reaction was stopped by adding 10 volumes of water and lyophilized from water 3 times to remove CNBr and formic acid.

S-Sulfonation of Myotoxin a. Disulfide bonds were reduced following the method of Bailey and Cole (1959). Twenty milligrams of protein was dissolved at 38 °C in 0.5 mL of 8 M urea buffered at pH 7.4 with 0.2 M Tris. The disulfide bonds were reduced with 0.062 mL of 1.0 M Na_2SO_3 at pH 7.4 for 10 min and then reoxidized with 0.125 mL of 0.5 M iodosobenzoate in 8 M urea for another 10 min. This reduction–oxidation cycle was repeated 2 more times to facilitate complete formation of the 8-sulfonated protein. The final mixture was dialyzed against three changes of distilled water to remove salt, and the protein was recovered by lyophilization. Efficiency of cleavage and reduction was determined by resolving the cleavage mixture on a Tricine/SDS–PAGE system.

Isolation of Cyanogen Bromide Cleavage Products. CNBr-generated fragments of myotoxin *a* were separated and isolated by RP HPLC. The CNBr cleavage products were loaded onto a 10 mm × 30 cm Vydac C_{18} RP column equilibrated with 0.1% TFA/water. The column was eluted with a linear gradient of 0.6% change of 0.1% TFA/acetonitrile per minute. Homogeneity of the fractions were assessed by N-terminal sequencing or by amino acid analysis.

PTC Amino Acid Analysis. Dried samples were hydrolyzed under nitrogen by exposure to gaseous vapor of 6 N constant-boiling HCl at 116 °C in sealed ampules for 24 h. Samples were dried under vacuum, resuspended in 20 μL of neutralization buffer (0.7 mL of absolute ethanol, 0.1 mL of triethylamine, and 0.1 mL of distilled water), vortexed, and redried. Twenty microliters of coupling buffer (0.7 mL of absolute ethanol, 0.1 mL of triethylamine, 0.1 mL of water, and 0.1 mL of PITC) was added, and the mixture was allowed to react at room temperature for 20 min and taken to dryness. The resulting phenylthiocarbamyl amino acids were dried and analyzed on a Waters chromatography Picotag HPLC (Waters Chromatography Division).

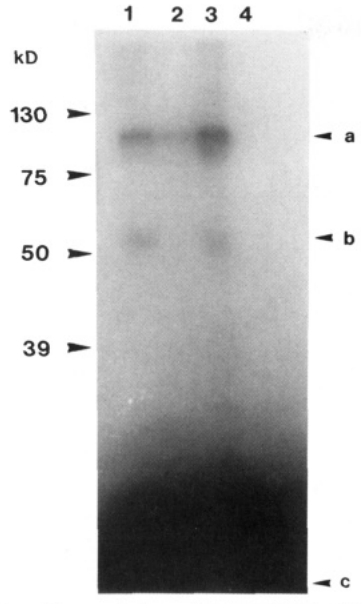

FIGURE 1: Autoradiograph from SDS–PAGE of ^{125}I-myotoxin *a* cross-linked to SR proteins with HSAB: (a) 110 kD; (b) 57 kD; (c) unbound ^{125}I-myotoxin *a*. Lane 1, ^{125}I-myotoxin *a* was incubated with SR proteins for 1 h at room temperature before addition of HSAB and exposure to UV light for 8 min as described under Materials and Methods. Lane 2, nonradiolabeled myotoxin *a* was incubated with SR for 30 min before the addition of ^{125}I-myotoxin *a* for a further incubation of 30 min before addition of HSAB. Lane 3, cobra neurotoxin was incubated with SR for 30 min prior to the addition of ^{125}I-myotoxin *a* for a further incubation of 30 min before addition of HSAB. Lane 4, same conditions as lane 1 but with ^{125}I-neurotoxin instead of ^{125}I-myotoxin *a*.

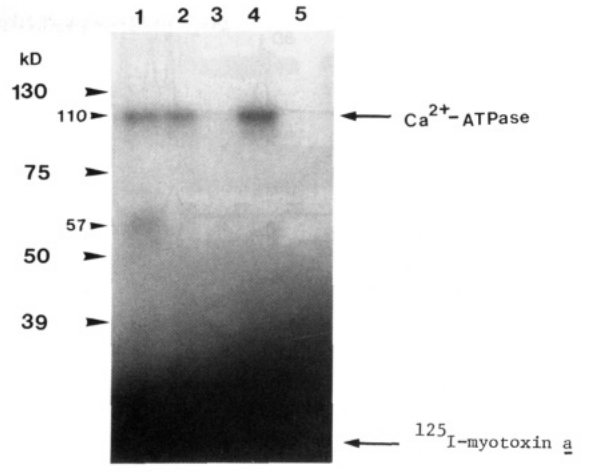

FIGURE 2: Autoradiograph from SDS–PAGE of ^{125}I-myotoxin *a* cross-linked to SR proteins (lane 1) or purified Ca^{2+}-ATPase (100 μg) (lanes 2–4). 0.1 μM ^{125}I-myotoxin *a* was incubated with SR (lane 1) and purified Ca^{2+}-ATPase (lane 2) for 1 h at room temperature before addition of the cross-linker as described under Materials and Methods. Lane 3, 1 μM myotoxin *a* was incubated with SR for 30 min before the addition of ^{125}I-myotoxin *a* for a further 30 min before adding HSAB. Lane 4, same as lane 3 except 1 μM cobra neurotoxin was used instead of myotoxin *a*. Lane 5, 1 μM ^{125}I-neurotoxin incubated with Ca^{2+}-ATPase for 1 h at room temperature.

N-Terminal Analysis. N-Terminal sequencing was accomplished by using an Applied Biosystems 473 A with standard cycles provided by Applied Biosystems (Applied Biosystems, Inc.).

Solid-Phase Peptide Synthesis. Peptides were synthesized manually using *N-tert*-butyloxycarbonyl (*t*-Boc)-protected amino acids with the following side chain protecting groups: serine, *o*-benzyl; histidine, benzyloxymethyl; tyrosine, bromobenzyloxycarbonyl; lysine, chlorobenzyloxycarbonyl; cysteine, 4-methylbenzyl. The *t*-Boc group was removed by a 30-min exposure to 50% trifluoroacetic acid in dichloromethane. The resin was then washed twice with dichloromethane, twice with 2-propanol, and twice again with dichloromethane. The residual acid was then neutralized by three washes of 5% diisopropylethylamine in dichloromethane followed by two additional washes of dichloromethane. A 10-fold excess of *t*-Boc-amino acid ester of diisopropylcarbodiimide [hydroxybenzotriazole esters were formed for asparagine, glutamine, arginine (tos), and histidine (BOM)] was stirred into the resin and reacted for 2–4 h. The coupling was continued until the resin tested negative for amine by ninhydrin assay (Stewart & Young, 1984).

Peptide Purification. Peptides were purified by RP HPLC based on a method developed by Rivier et al. (1984). Up to 10 mg of crude peptide was solubilized in deionized water (<5 mL). If cysteine residues were present, a 4:1 molar amount of dithiothreitol (DTT) was added and incubated at room temperature for several hours to reduce disulfide bonds. Aliquots of the peptide solution were loaded onto a 10 mm × 30 cm C_{18} RP Vydac column equilibrated with 0.1% TFA/water. The column was eluted with a linear gradient at 0–50% of 0.1% TFA/acetonitrile over 30 min at a flow rate of 3 mL/min, and fractions were collected. Fractions were assessed for homogeneity on a C_{18} RP analytical Vydak column eluted isocratically. Fractions 90% chromatographically homogeneous were pooled and lyophilized.

Results

Binding of ^{125}I-Myotoxin a to SR Proteins. Figure 1 shows the autoradiograph from SDS–PAGE of cross-linking experiments with SR proteins. In lane 1, ^{125}I-myotoxin *a* was cross-linked to proteins in whole SR vesicles. Two radiolabeled bands can be seen with apparent molecular weights of 110K and 57K (Figure 1a,b). Unbound ^{125}I-myotoxin *a* can be seen in Figure 1c.

Addition of nonradiolabeled myotoxin *a* to the incubation mixture containing ^{125}I-myotoxin *a* with SR proteins resulted in a decrease in the intensity of the two corresponding bands (Figure 1, lane 2). The addition of cobra neurotoxin as a negative control to the reaction mixture containing ^{125}I-myotoxin *a* and SR proteins did not decrease the intensity of the two bands (Figure 1, lane 3). When ^{125}I-labeled cobra neurotoxin was incubated with SR proteins, no radioactive bands were seen on the gel (Figure 1, lane 4).

Binding of ^{125}I-Myotoxin a to Isolated Ca^{2+}-ATPase. The 110K band is similar to the known molecular weight of Ca^{2+}-ATPase. Therefore, the enzyme was isolated, and its affinity for myotoxin *a* was investigated. Figure 2 shows an autoradiograph of an SDS–PAGE of cross-linking experiments with purified Ca^{2+}-ATPase. Lane 1 shows the results of cross-linking the SR with ^{125}I-myotoxin *a*. Two protein bands are visible on the gel at 110 and at 57 kDa. Lane 2 shows the results of incubating ^{125}I-myotoxin *a* with Ca^{2+}-ATPase. One radioactive band is seen on the gel. Lane 3 show the results of incubating ^{125}I-myotoxin *a* with native myotoxin *a* and Ca^{2+}-ATPase. The relative density of the one band is greatly decreased as compared to lane 2. Lane 4 shows the results of the addition of cobra neurotoxin to the incubation mixture of ^{125}I-myotoxin *a* and Ca^{2+}-ATPase. One radioactive band is seen. Lane 5 shows the results of incubating ^{125}I-labeled cobra neurotoxin with Ca^{2+}-ATPase. No radioactive bands can be seen on the gel.

ELISA. Further evidence of a myotoxin *a*–Ca^{2+}-ATPase complex was obtained by assessing the complex affinity for

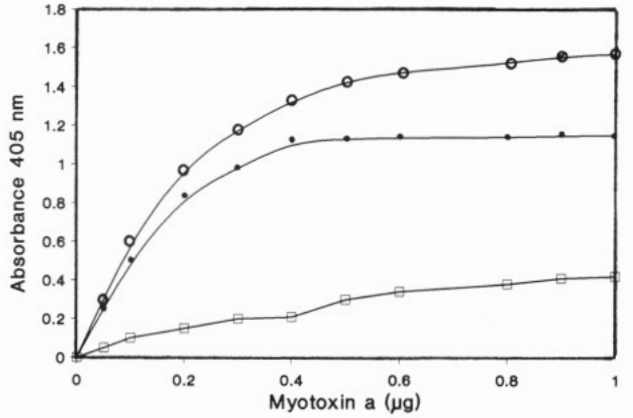

FIGURE 3: Binding of myotoxin *a* to purified Ca^{2+}-ATPase: ELISA assay. Twenty-five picomoles of purified Ca^{2+}-ATPase was coated onto microtiter plates for overnight. Myotoxin *a* was added at different concentrations before being subjected to anti-myotoxin *a* and anti-rabbit IgG (conjugated with alkaline phosphatase) and developed as described under Materials and Methods.

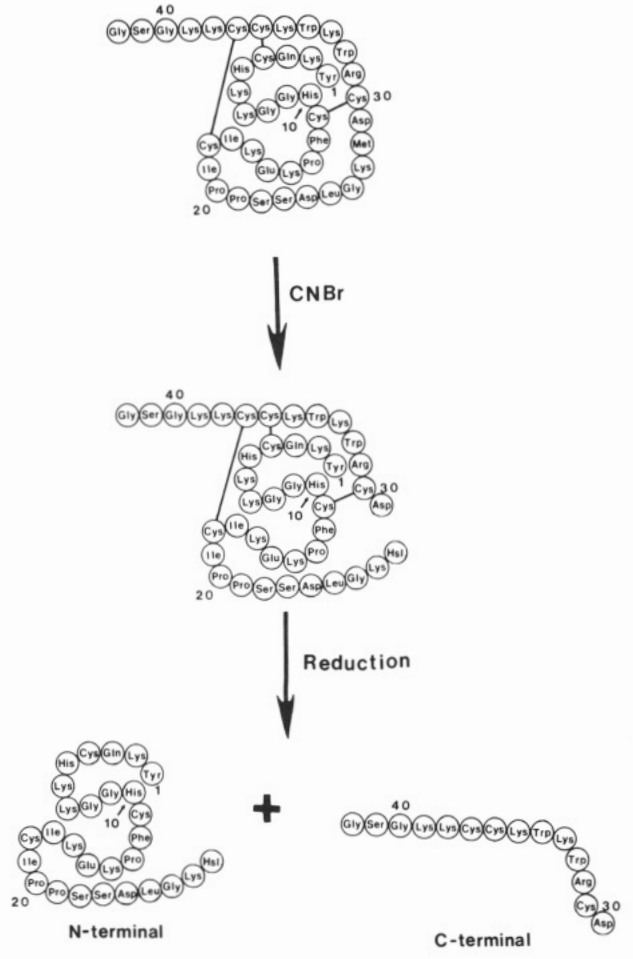

FIGURE 4: Illustration of myotoxin *a* CNBr cleavage and reduction yielding two fragments: N-terminal and C-terminal.

an anti-myotoxin *a* antibody. Figure 3 shows the results of incubating increasing concentrations of myotoxin *a* with Ca^{2+}-ATPase. The lower limit of detection was at 0.05 μg/well of myotoxin *a* and eventually reaches a saturation point at 0.6 μg/well of myotoxin *a*. The binding, defined as the difference between the total binding (Figure 3, open circles) and the nonspecific binding (open squares), is a saturable function of myotoxin *a* concentration (closed circles).

Myotoxin a Fragments Compete with ^{125}I-Myotoxin a. Myotoxin *a* was subjected to CNBr cleavage and reduction

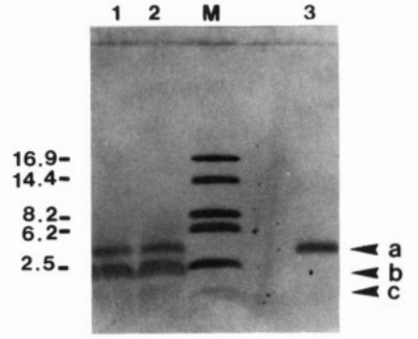

FIGURE 5: SDS–PAGE of CNBr cleavage products: (a) myotoxin *a*; (b) 2.8 kD; (c) 1.6 kD. Lanes 1 and 2, CNBr cleavage products of myotoxin *a*. Lane 3, native myotoxin *a*. Lane M, molecular mass markers indicated in kilodaltons.

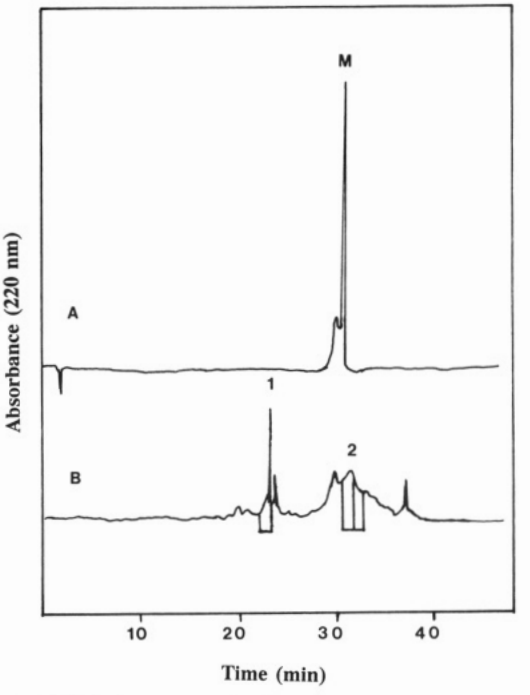

FIGURE 6: HPLC chromatograph of native myotoxin *a* (A) and CNBr cleavage products (B). Samples were loaded onto a 4.5 mm × 25 cm Vydac C_{18} RP column equilibrated with 0.1% TFA/water. The column was eluted with a linear gradient of 0.6% change of 0.1% TFA/acetonitrile per minute.

as described under Materials and Methods. Figure 4 illustrates the expected results of CNBr cleavage and subsequent reduction of myotoxin *a* to yield two fragments: a larger N-terminal fragment and a smaller C-terminal fragment. The reaction mixture was assessed for completeness by two methods, electrophoresis or a Tricine gel system and RP HPLC. Figure 5 shows the results of this reaction on a 16% Tricine gel system. Lane 3 shows one band for myotoxin *a*. Lanes 1 and 2 show three bands; one band resolves the same as native myotoxin *a*, and one band resolves at an apparent molecular weight of 2.8K. The third band was barely visible at 1.6K. However, the formation of this fragment was clearly proven by HPLC and by amino acid analysis. The proteins were electroblotted onto a Problott membrane, and the 2.8- and 1.6-kDa fragments were excised for analysis. Figure 6A shows the HPLC chromatograph of native myotoxin *a*. Figure 6B shows the HPLC chromatograph of cleaved and reduced myotoxin *a*. Fractions from peaks 1 and 2 were collected, and samples were sent for amino acid analysis. The amino acid analysis of the fraction corresponding to peak 1 identified it as the C-terminal fragment. Fractions corresponding to peak

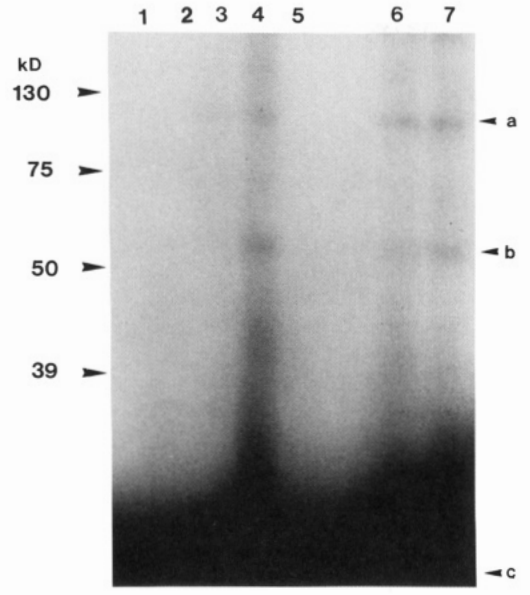

FIGURE 7: Autoradiograph of SDS–PAGE of HSAB cross-linking competition study of ^{125}I-myotoxin *a* with CNBr cleavage products and synthetic peptides. All lanes were competed with ^{125}I-myotoxin *a* as described under Materials and Methods. Lane 1, nonradiolabeled myotoxin *a*. Lane 2, C-terminal fragment. Lane 3, N-terminal fragment. Lane 4, peptide$_{13-28}$. Lane 5, peptide$_{1-16}$. Lane 6, cobra neurotoxin. Lane 7, alphabet peptide.

2 were identified as containing the N-terminal fragment. Peaks 1 and 2 were also N-terminally sequenced to assess homogeneity of the protein fragments in these fractions.

Two peptides were synthesized to correspond to the partial primary sequence of myotoxin *a*: peptide$_{1-16}$ and peptide$_{13-28}$. The sequence for peptide$_{1-16}$ is YKQCHKKGGHCFPKEK, and peptide$_{13-28}$ is PKEKICIPPSSDLGKM. An alphabet peptide, containing all the amino acids in alphabetical order according to their one-letter code (except for an N-terminal glutamic acid), was synthesized as a negative control. Its sequence is EACDEFGHIKLMNPQRSTVWY.

Figure 7 shows an autoradiograph of an SDS–PAGE of products generated when myotoxin *a* fragments and synthetic peptides are competed with ^{125}I-myotoxin *a* for SR protein binding. Lane 1 shows the competition of nonradioactive myotoxin *a* with ^{125}I-myotoxin *a*. No bands can be seen. Lanes 2 and 3 show the competition of the CNBr cleavage products, C-terminal and N-terminal, respectively, with ^{125}I-myotoxin *a*. Lane 2 has no detectable bands, while lane 3 has two barely detectable bands. Competition of peptide$_{13-28}$ and peptide$_{1-16}$ with ^{125}I-myotoxin *a* is shown in lanes 4 and 5. Lane 4 has two bands, while lane 5 has no bands. Lanes 6 and 7 show the controls, cobra neurotoxin and the alphabet peptide, competing with ^{125}I-myotoxin *a*. In each of these lanes, two bands resolved without any decrease in band density.

Discussion

Myotoxin *a* causes the vacuolization of skeletal muscle which is thought to be the first step of myonecrosis (Ownby et al., 1976). The mechanism of myonecrosis by myotoxin *a* is still unknown. Tu amd Morita (1983) have shown that myotoxin *a* is first localized in the SR membrane. The inhibition of calcium ion influx into SR by myotoxin *a* may be due to the interaction of myotoxin *a* with SR components. In order to prove this assumption, SR vesicles were incubated with ^{125}I-myotoxin *a* in the presence of the cross-linking agent HSAB. ^{125}I-Myotoxin *a* cross-linked to two SR proteins with apparent molecular weights of 110K and 57K.

The major protein in the SR is Ca^{2+}-ATPase (M_r 100-K) which plays an essential role in calcium transport into the SR. Recently, Volpe et al. (1986) obtained indirect evidence indicating the binding of myotoxin *a* to Ca^{2+}-ATPase by showing that myotoxin *a* inhibited binding of anti-(rabbit SR Ca^{2+}-ATPase) antibodies to SR membranes. Our ability to cross-link ^{125}I-myotoxin *a* to purified Ca^{2+}-ATPase is further evidence that the 110-kDa protein myotoxin *a* cross-linked complex isolated from intact SR is Ca^{2+}-ATPase. Furthermore, anti-myotoxin *a* antibodies are shown to bind this complex.

Cobra neurotoxin (M_r 6990, pI = 9.2), which has a slightly higher molecular weight and an isoelectric point similar to myotoxin *a*, was used as a control for nonspecific binding to SR proteins. Cobra neurotoxin did not interfere with the binding of ^{125}I-myotoxin *a* to SR protein nor did it associate with any SR proteins in our cross-linking studies. These results indicate that myotoxin *a* specifically binds two SR proteins.

To determine which residues of myotoxin *a* are involved in binding to SR proteins, myotoxin *a* was cleaved by CNBr. Since myotoxin *a* only has one methionine, two fragments can be expected from complete cleavage and reduction (Figure 4). These fragments were isolated and were shown to compete with ^{125}I-myotoxin *a* for binding to SR proteins. Both the N-terminal and the C-terminal fragments bound to the same SR proteins, although the C-terminal fragment competed better than the N-terminal fragment. The myotoxin *a* binding domain may include the ends of both the N-terminal fragment and the C-terminal fragment. Two peptides were synthesized corresponding to the primary sequence of the N-terminal fragment, peptide$_{1-16}$ and peptide$_{13-28}$. Peptide$_{1-16}$ competed as well as nonradiolabeled myotoxin *a* against ^{125}I-myotoxin *a*, but peptide$_{13-28}$ did not compete at all. The alphabet peptide and cobra neurotoxin, used as negative controls, did not compete against ^{125}I-myotoxin *a*. These results suggests that the myotoxin *a* binding region may in part reside within residues 1–16 in addition to a region at the C-terminal.

Since myotoxin *a* is a highly basic protein with a pI = 9.6, it has been suggested that the toxin may bind to the acidic proteins of the SR by electrostatic interaction such as calsequestrin (MacLennan & Wong, 1971), the high-affinity calcium binding protein (Ostwald & MacLennan, 1974), and the 53-kDa glycoprotein (Campbell & MacLennan, 1981). However, our results indicate that myotoxin *a* bound to only one other protein other than the Ca^{2+}-ATPase, indicating that binding is more than just an electrostatic interaction.

Myotoxin *a* also associated with a protein, which has a relative molecular weight of 57K, that could be the 53-kDa glycoprotein bound to myotoxin *a* (M_r 4600). The 53-kDa glycoprotein is thought to be a modulator of Ca^{2+}-ATPase in the SR membrane. Kutchai and Campbell (1989) found that monoclonal antibodies against 53-kDa glycoprotein decrease the rate of calcium uptake into the SR, suggesting that this glycoprotein may regulate the function of Ca^{2+}-ATPase. It has also been observed that reconstituted Ca^{2+}-ATPase vesicles containing this glycoprotein take up calcium more efficiently than vesicles deprived of it (MacLennan et al., 1979). However, Martin (1990) found that Ca^{2+}-ATPase vesicles reconstituted with or without the 53-kDa glycoprotein still transported calcium into the SR with the same high efficiency.

While it is unclear what role, if any, the 53-kDa glycoprotein has in calcium uptake in the SR, it is interesting that myotoxin *a*, which has been shown to inhibit calcium uptake, not only binds Ca^{2+}-ATPase but also may bind to a 53-kDa glycoprotein. Myotoxin *a* may be a useful tool to study the role

of the regulation of calcium in the SR.

Although the specific binding of myotoxin *a* to two SR proteins is conclusively shown in this investigation, it is important to emphasize that the experiment was done with an isolated cell component in vitro. Since the path of myotoxin *a*'s entry into the muscle cell has not been investigated either in vivo or in tissue culture, it is premature to say that the binding of myotoxin *a* to SR protein may also take place in the intact cell system.

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Registry No. ATPase, 9000-83-3; YKQCHKKGGHCFPKEK, 134882-41-0; PKEKICIPPSSDLGKM, 134882-42-1.

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Intramolecular Semiquinone Disproportionation in DNA. Pulse Radiolysis Study of the One-Electron Reduction of Daunorubicin Intercalated in DNA†

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ABSTRACT: The one-electron reduction of daunorubicin, a quinonic antitumor antibiotic, intercalated in DNA was studied by pulse radiolysis using carboxyl radicals as reductants. The reaction's first stage is the daunorubicin semiquinone formation ($k = 1.9 \times 10^8$ mol^{-1}·dm^3·s^{-1}) in a way entirely consistent with a simple competition between $^{\bullet}COO^-$ disproportionation and the drug reduction. The semiquinone drug disappears by a first-order reaction ($k = 1340$ s^{-1}) producing the hydroquinone form. This reaction leads to an equilibrium similar to the one without DNA and the equilibrium constant is very close to its value free in water ($K_c \sim 25$). In addition, the stoichiometry of the first-order reaction is the one of a dismutation process. Therefore, it appears that the disproportionation occurs along an intramolecular path across DNA. This migration takes place under our experimental conditions, over a distance of ca. 100 base pairs, with a mobility of ca. 4.4×10^{-11} m^2·V^{-1}·s^{-1}, similar in magnitude to an excess electron mobility in doped organic polymers.

There is a great interest in long-range electron transfer across biological macromolecules (Isied, 1984; Marcus & Sutin, 1985; Gray & Malstrom, 1989). More generally speaking, the conductivity of organic polymers is being investigated and a considerable amount of literature has been accumulated on the subject of electronic properties at the molecular level (Frommer, 1986; Hopfield et al., 1989). In all kinds of polymer assemblies, electron transfer can occur over large distances (>10-20 Å) (Isied, 1984). Theories indicate that donor-acceptor distance, thermodynamic driving force, and the nature of the intervening medium are critical in determining rates of electron transfer (Marcus & Sutin, 1985; Closs & Miller, 1988).

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