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Multiplicity of Acidic Subunit Isoforms of Crotoxin, the Phospholipase A_2 Neurotoxin from *Crotalus durissus terrificus* Venom, Results from Posttranslational Modifications†

Grazyna Faure,‡ Jean-Luc Guillaume,§ Luc Camoin,§ Bernard Saliou,‡ and Cassian Bon*,‡

Unité des Venins, Unité Associée Institut Pasteur/INSERM 285, and Laboratoire de Microséquence, Unité de Biologie Moléculaire des Récepteurs URA 1336, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France

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ABSTRACT: Crotoxin, the major toxin of the venom of the South American rattlesnake, *Crotalus durissus terrificus*, is made of two subunits: component B, a basic and weakly toxic phospholipase A_2, and component A, an acidic and nontoxic protein that enhances the lethal potency of component B. Crotoxin is a mixture of isoforms that results from the association of several isoforms of its two subunits. In the present investigation, we have purified four component A isoforms that, when associated with the same purified component B isoform, produced different crotoxin isoforms, all having the same specific enzymatic activity and the same lethal potency. We further determined by Edman degradation the polypeptide sequences of these four component A isoforms. They are made of three disulfide-linked polypeptide chains (α, β, and γ) that correspond to three different regions of a phospholipase A_2 precursor. We observed that the polypeptide sequences of the various component A isoforms all agree with the sequence of an unique precursor. The differences between the isoforms result first by differences in the length of the various chains α and β, indicating that component A isoforms are generated from the proteolytic cleavage of the component A precursor at very close sites, possibly by the combined actions of endopeptidases and exopeptidases, and second by the possible cyclization of the α-NH_2 of the N-terminal glutamine residue of chains β and γ. These observations indicate that the component A isoforms are the consequence of different posttranslational events occurring on an unique precursor, rather than the expression of different genes.

Crotoxin, the major toxic component from the venom of the South American rattlesnake, *Crotalus durissus terrificus*, is a potent neurotoxin that possesses a phospholipase A_2 activity (Slotta & Fraenkel-Conrat, 1938) and exerts its lethal action by blocking neuromusclar transmission (Vital-Brazil & Excell, 1971). At this level, crotoxin acts on nerve terminals by altering the quantal release of acetylcholine (Hawgood & Smith, 1977; Chang & Lee, 1977; Hawgood & Santana de Sa, 1979). These effects are similar to those observed with other snake venom phospholipase A_2 neurotoxins such as β-bungarotoxin, taipoxin, notexin, ammodytoxin A, agkistrodotoxin, caudoxin, etc., which belong to the class of β-neurotoxins [for a review, see Hawgood & Bon (1991)]. Crotoxin has also been reported to act postsynaptically (Vital-Brazil, 1966; Bon et al., 1979; Lee & Ho, 1980), causing the pharmacological desensitization of the acetylcholine receptor (Bon et al., 1979) and possesses myotoxic properties when injected intramuscularly (Gopalakrishnakone et al., 1984; Kouyoumdjian et al., 1986).

Crotoxin is made up of two nonidentical subunits: a basic and weakly toxic phospholipase A_2 subunit, component B, and an acidic, nontoxic and nonenzymatic subunit, component A (Rübsamen et al., 1971; Hendon & Fraenkel-Conrat, 1971). When mixed together, components A and B spontaneously associate into a one-to-one complex that possesses all the properties of native crotoxin, in particular its high lethal potency [Horst et al., 1972; Breithaupt et al., 1975; for a review, see Breithaupt (1976)].

Binding experiments indicated that crotoxin dissociates when it interacts with biological membranes: component B binds

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* Address correspondence to this author.
‡ Unité des Venins, Unité Associée Institut Pasteur/INSERM 285.
§ Laboratoire de Microséquence, Unité de Biologie Moléculaire des Récepteurs URA 1336.

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to membranes and component A is released in solution (Jeng et al., 1978; Bon et al., 1979). The phospholipase component B alone binds in a nonsaturable (nonspecific) manner to many kinds of membranes, whereas the nontoxic component A, which never binds to membranes, prevents the nonspecific binding of the phospholipase component B but not its specific (saturable) binding. Specific binding is observed on synaptic membranes (Bon et al., 1979) but not on erythrocyte membranes (Jeng et al., 1978). In other words, the nontoxic component A behaves as a chaperon: by preventing the nonspecific adsorption of the weakly toxic component B, it enhances its ability to reach its target and therefore its lethal potency. Although the molecular target of crotoxin on nerve terminals has not yet been definitely identified, investigations performed with phospholipid vesicles of various compositions indicated that some negatively charged phospholipids, such as phosphoinositide phosphates, might be an important component of this target (Radvanyi et al., 1989a). Recently, photoaffinity cross-linking experiments indicated that crotoxin also interacts with proteins present on synaptosomal membranes (Lambeau et al., 1989; Hseu et al., 1990).

Crotoxin is in fact a nonhomogeneous protein, since its fractionation by fast-protein liquid chromatography (FPLC)[1] indicated that the venom from a single snake contains up to 15 different isoforms (Faure & Bon, 1987). Further, it has been shown that crotoxin isoforms result from the association of several isoforms of its two subunits. Fractionation of isolated crotoxin subunits allowed the purification of several component A and component B isoforms, which were found to differ slightly in their amino acid composition (Faure & Bon, 1988). Moreover, a quantitative comparison of enzymatic and pharmacological properties of crotoxin isoforms obtained by defined mixtures of purified component A and B isoforms indicated that component A isoforms have similar properties whereas component B isoforms might be classified in two groups, crotoxin complexes formed with component B isoforms of the first group being enzymatically more active and pharmacologically less potent than complexes formed with the second group of component B isoforms (Faure & Bon, 1988).

The polypeptide sequences of crotoxin components A and B have been established with a mixture of isoforms (Aird et al., 1985, 1986, 1990), and cDNAs encoding both crotoxin subunits have been cloned and their nucleotidic sequences determined (Bouchier et al., 1988, 1991). These results indicated that the enzymatic subunit, component B, is a single polypeptide of 122 amino acids, with a primary structure that is similar to that of other phospholipases A_2 from mammals and snakes venoms. The nonenzymatic component A is made up of three polypeptides linked by disulfide bridges (Breithaupt et al., 1974) and appears to result from the proteolytic cleavage of a larger polypeptide precursor, which also makes it homologous to phospholipases A_2 from mammals and snakes venoms (Bouchier et al., 1991).

The presence of several isoforms in the venom collected from a single snake could result from several mechanisms. It might result from the translation of different messenger RNAs or from the posttranslational modification of an unique precursor. In the present investigation, we have purified four isoforms of the acidic subunit of crotoxin, component A, and have determined their polypeptide sequence. The results indicate that all isoforms are the consequence of different posttranslational events occurring on the same component A precursor.

Experimental Procedures

Animals. Male Swiss mice, 3 weeks old (18–20 g), were obtained from Charles River (Saint-Aubin-lès-Elbeuf, France).

Materials. The venom from *C. durissus terrificus* was purchased from Pentapharm (Basel, Switzerland). Sephadex G-25 (medium) and G-75 (superfine) beads, prepacked Mono-Q HR 5/5, Mono-Q HR 16/10, and the equipment for FPLC (P-500 pumps, GP-250 programmer, UV-1 detector, REC-492 recorder and FRAC-100 fraction collector) were from Pharmacia LKB Biotechnology (Uppsala, Sweden). DEAE-cellulose DE-52 was purchased from Whatman Biochemicals Ltd. (Springfield Mill, U.K.). The high-performance liquid chromatography (HPLC) C_{18} μBondapak column was purchased from Millipore-Waters (Milford, MA). Nucleosil C_8 (5 μm, 300 Å) column was purchased from Société Francaise de Chromato Colonne. The C_8 columns (Aquapore RP-300, 220 × 2.1 mm or 30 × 2.1 mm) used for peptide separations were from Brownlee Labs. Acetonitrile (HPLC grade) was obtained from Baker Chemical Co. (Phillipsburg, NJ). Trifluoroacetic acid (TFA) was from Pierce Chemical Co. (Rockford, Il.). *N*-Tosyl-L-phenylalanine chloromethyl ketone treated (TPCK-treated) trypsin, *Staphylococcus aureus* V_8 proteinase, pyroglutamate aminopeptidase, and carboxypeptidase Y were from Boehringer (Mannheim, FRG). Bovine serum albumin (BSA), fatty acid free (A-7511), and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and ethylenediaminetetraacetic acid (EDTA) were from Sigma Chemical Co. (St. Louis, MO). 1-Palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-monomethylphosphatidic acid (PA-monomethyl ester) was from KSV Chemicals (Helsinki, Finland). The reagents used in the gas-phase protein sequencer were obtained from Applied Biosystems (Foster City, CA).

Purification of Crotoxin and Isolation of Its Subunits. Crotoxin was purified from *C. durissus terrificus* venom by gel filtration on a Sephadex G-75 column, as previously described (Faure & Bon, 1988). Crotoxin subunits were isolated according to the procedure of Hendon and Fraenkel-Conrat (1971). Briefly, crotoxin was dissolved in 50 mM sodium phosphate buffer, pH 6.5, containing 6 M urea (freshly deionized by acid treatment with HCl, 1 N) and applied to a DEAE-cellulose column previously equilibrated with the same buffer. The basic component B did not adsorb to the column and was eluted with the washing buffer. The acidic component A was eluted by increasing the ionic strength of the equilibration buffer with 0.4 M NaCl. Crotoxin components A and B were carefully dialyzed to remove salts and urea, then lyophilized, and could be kept at −20 °C for years without any detectable loss of activity.

Purification of Component A and Component B Isoforms. Component A isoforms were purified by preparative FPLC with use of an anionic exchange column (Mono-Q HR 16/10). The crude component A preparation containing the various isoforms was dissolved in 50 mM sodium phosphate buffer, pH 6.5, and applied to the Mono-Q column. Elution was performed in 35 min at a flow rate of 8 mL/min with a linear NaCl gradient from 0.1 to 0.4 M. The various component A isoforms were collected, dialyzed against water, and lyophilized. The homogeneity of each isoform was checked by analytical FPLC in the Mono-Q column (HR 5/5) and by

[1] Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast-protein liquid chromatography; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PA-monomethyl ester, 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-monomethylphosphatidic acid; PTH-amino acid, phenylthiohydantoin derivative of an amino acid; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

polyacrylamide gel electrophoresis (PAGE) carried out at pH 6.5 in 20% acrylamide, in the absence of sodium dodecyl sulfate (SDS) and reducing agent, electrophoresis being carried out with a PhastSystem (Pharmacia, Uppsala, Sweden).

Component B isoforms were purified according to the procedure described by Faure and Bon (1988), by preparative FPLC with a cationic exchange column Mono-S (HR 10/10). Briefly, the crude component B preparation, containing various isoforms, was dissolved in 50 mM sodium phosphate buffer, pH 6.5, and applied to the Mono-S column. Elution was achieved with a gradient from 0 to 1 M NaCl and from 0 to 6 M urea in 50 mM sodium phosphate buffer, pH 6.5. The various major component B isoforms were dialyzed against water and lyophilized. Their homogeneity was checked by analytical FPLC in the Mono-S column (HR 5/5), according to the procedure of Faure and Bon (1988).

Carboxymethylation. Each component A isoform (0.1 μmol of component A; 1.4 μmol of half-cysteine) was dissolved in 0.3 mL of 1 M Tris-HCl buffer, pH 8.25, containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 6 M guanidine hydrochloride. Reduced dithiothreitol (25 μmol) was added, and the solution was allowed to stand for 75 min at 20 °C. Free sulfhydryl groups were then alkylated with iodoacetic acid (130 μmol). After an additional incubation of 15 min at 20 °C in the dark, the reaction was stopped by adding glacial acetic acid. The protein was immediately desalted by gel filtration through a Sephadex G-25 column equilibrated with 10% acetic acid and lyophilized.

Isolation of the Component A Polypeptide Chains. The three polypeptide chains (α, β, and γ) of the various carboxymethylated component A isoforms (CA_1 to CA_4) were isolated by reverse-phase HPLC in a Nucleosil C_8 (5 μm, 300 Å) column or in a μBondapak C_{18} column. The initial buffer A contained 0.1% TFA in 10% acetonitrile and the final buffer B, 0.1% TFA in 60% acetonitrile. Elution was carried out at a flow rate of 1 mL/min with two linear gradients, which increased the proportion of buffer B from 7 to 25% in 22 min and then from 25 to 40% in 68 min. Elution was monitored by recording the absorbance at 280 and 226 nm simultaneously. The polypeptides were collected and identified by their amino acid composition.

Amino Acid Analysis and Peptide Sequencing. Hydrolyses were performed with 6 N HCl containing 0.1% phenol and carried out in vacuo at 110 °C for 24 h (Moore & Stein, 1948). The amino acid compositions were determined with a Beckman 7300 amino acid analyzer.

The sequence determination of peptides and proteins was performed by Edman degradation with a 470 A gas-phase sequencer. Phenylthiohydantoin- (PTH-) amino acids were separated and identified by on-line reverse-phase HPLC with a 120-A PTH analyzer in an RP_{18} column. The purified peptides were subjected directly to automated Edman degradation (Edman & Begg, 1967). Biobrene (Applied Biosystems) was added to reduce peptide wash-out and to improve initial yields (Tarr et al., 1978). In general a standard program for Edman degradation (03RPTH) was used; however, specific cycles (03CPRO or 03CSER) were occasionally inserted for peptides containing proline or serine residues, in order to increase their yields.

Occasionally, when sequences determined by Edman degradation were not in complete agreement with the amino acid composition, enzymatic C-terminus sequencing was carried out with carboxypeptidase Y (Hayashi et al., 1973; Hayashi, 1977). Peptides were incubated with carboxypeptidase at 37 °C in 400 mL of 0.1 M pyridine acetic acid buffer, pH 5.5, with an enzyme to substrate ratio of 1:10 (mole/mole). Aliquots were removed at various time intervals and acidified with acetic acid to stop enzymatic digestion. Their amino acid composition was determined. An enzyme blank was performed with the same procedure, omitting carboxypeptidase Y.

Sequence Determination of Polypeptide Chains α. We first performed polypeptide sequencing on native component A isoforms. In most cases, the N-terminal residues of polypeptide chains β and γ were blocked, so that only polypeptide chains α were sequenced. After carboxymethylation and purification, isolated polypeptide chains α were submitted to enzymatic digestion by trypsin or *S. aureus* V_8 proteinase.

Digestion with *S. aureus* V_8 proteinase (Houmard & Drapeau, 1972) was performed at 37 °C in 50 mM NH_4HCO_3, pH 7.8, with an enzyme to substrate ratio of 1:40 (w/w). The digests were immediately lyophilized and chromatographed by reverse-phase HPLC in a C_{18} μBondapak column. The initial buffer A contained 0.1% TFA in 10% acetonitrile and the final buffer B, 0.1% TFA in 60% acetonitrile. Elutions were carried out at a flow rate of 1 mL/min with two linear gradients, which increased the proportion of buffer B from 7 to 30% in 45 min and then from 30 to 40%, again in 45 min. The elution of the peptides was followed by recording absorbance at 226 nm. The peptides were identified by amino acid composition.

Trypsin digestions were performed for 6 h at 37 °C in 0.1 M sodium phosphate buffer, pH 8.0, with an enzyme to protein ratio of 1:50 (w/w). The tryptic digests were chromatographed in a C_{18} μBondapak column. The initial buffer A contained 0.1% TFA in water and the final buffer B, 0.1% TFA in 60% acetonitrile. Elutions were carried out at a flow rate of 1 mL/min with a single linear gradient, which increased the proportion of buffer B from 3 to 80% in 120 min. Elution was followed by recording absorbance at 226 nm, and each peptide was identified by amino acid composition.

Polypeptide Sequence of Chains β and γ. Except polypeptide chain β of isoform CA_1, all polypeptide chains β and γ have blocked N-terminal residues. They were therefore submitted to pyroglutamate aminopeptidase digestion (Podell & Abraham, 1978) to remove a possible pyrrolidone carboxylyl residue at the N-terminus. This treatment proved to be efficient after 48 h of digestion at 37 °C in the case of the four polypeptide chains γ, or after 9 h at 4 °C and 16 h at 37 °C in the case of polypeptide chains β. All attempts were, however, unsuccessful in the case of polypeptide chain β of CA_3. In that case, partial acidic cleavages were obtained by incubation at 45 °C for 2 h in 100% TFA (Inglis, 1980). The resulting peptides were purified by HPLC in an Aquapore RP 300 column with a linear gradient of acetonitrile from 5 to 40% in 0.1% TFA.

Phospholipase Assay. Phospholipase A_2 activity was measured spectrofluorometrically with a fluorescent substrate, PA-monomethyl ester, according to the method described by Radvanyi et al. (1989b). Briefly, the reaction medium was prepared by sequential addition in a disposal plastic cuvette of 1 mL of 50 mM Tris-HCl, pH 7.5, containing 500 mM NaCl and 1 mM EGTA; 10 μL of 200 μM PA-monomethyl ester in ethanol; and 10 μL of 10% fatty acid free BSA. The sample to be tested (10 μL) was introduced, and the enzymatic reaction was initiated by adding 6 μL of 1 M $CaCl_2$. Excitation and emission wavelengths were 345 and 398 nm, respectively.

Lethality Assays. Lethality assays were performed with male Swiss mice by intravenous injection of 0.2 mL of the tested solution per 20 g of body weight. The LD_{50} values were

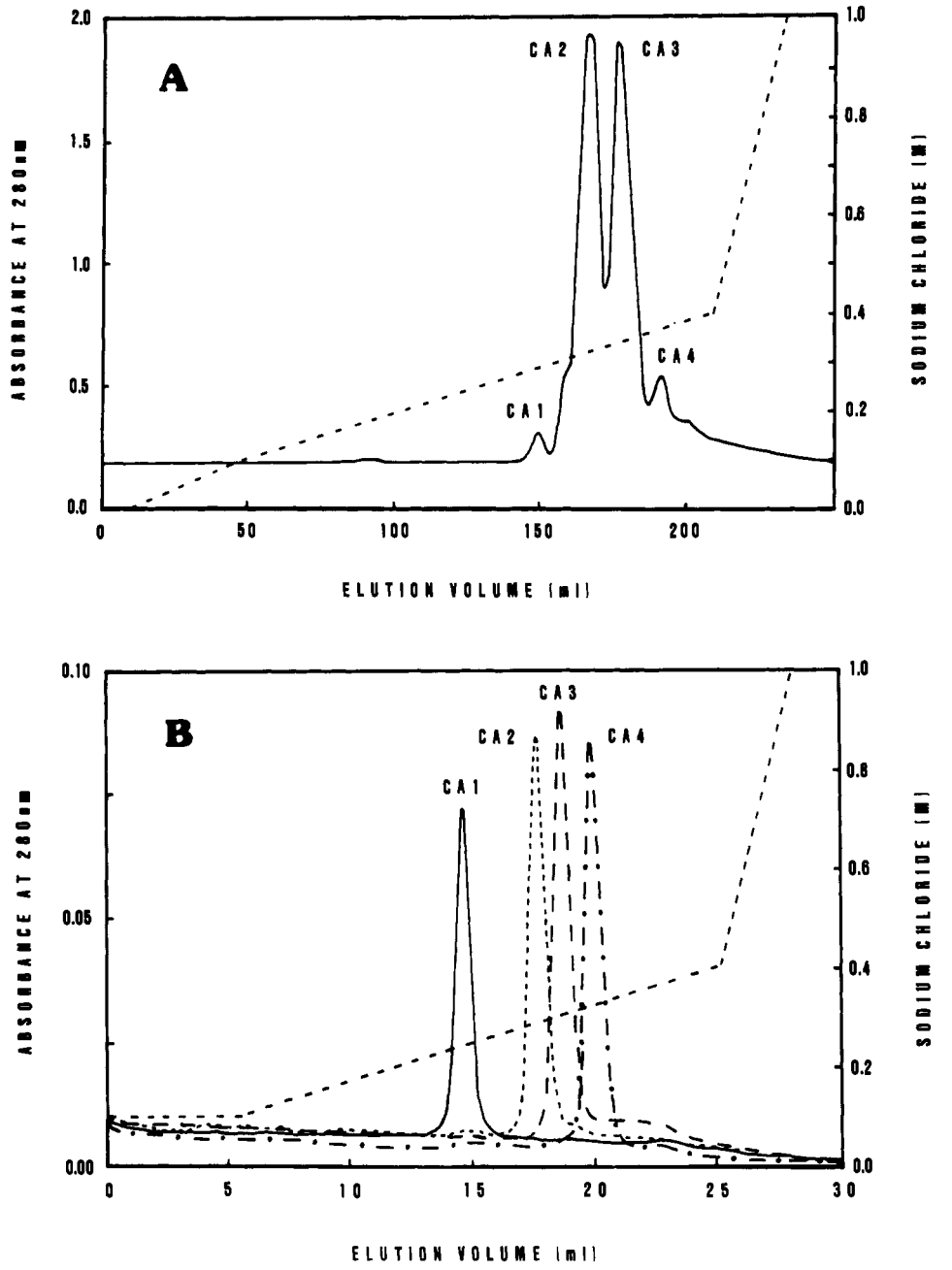

FIGURE 1: FPLC chromatography of component A isoforms on Mono-Q columns. (A) Preparative chromatography of a mixture of component A isoforms on the Mono-Q HR 16/10 column. (B) Analytical chromatography of each purified component A isoform on the Mono-Q HR 5/5 column.

calculated by the method of Spearman-Kärber, three mice being injected with each dose, the doses differing by a factor of 1.414 ($\sqrt{2}$).

Results

(1) Isolation of Component A Isoforms. Crotoxin is an heterogeneous molecule with numerous forms resulting from the combination of different isoforms of the noncatalytic acidic subunit, component A, with different isoforms of the phospholipase A_2 subunit, component B (Faure & Bon, 1988). In the present investigation we examined the various isoforms of component A in a sample of *C. durissus terrificus* venom collected from a large number of Brazilian snakes.

We slightly modified the previously described purification protocol (Faure & Bon, 1988). The mixture of crotoxin isoforms present in the venom was first purified by gel filtration in a Sephadex G-75 column. Crotoxin subunits A and B, which were mixtures of isoforms in both cases, were then isolated by ion-exchange chromatography in DEAE-cellulose in the presence of 6 M urea (Hendon & Fraenkel-Conrat, 1971). Finally, two major (CA_2 and CA_3) and two minor (CA_1 and CA_4) component A isoforms were isolated by preparative FPLC in a Mono-Q column (Figure 1A). Following secondary chromatography purification of each fraction under the same conditions, the CA isoforms proved to be homogeneous by analytical FPLC on Mono-Q (Figure 1B). They were named CA_1–CA_4, according to their order of elution from the Mono-Q column. Elution was obtained with 250 ± 10 mM (CA_1), 290 ± 10 mM (CA_2), 300 ± 10 mM (CA_3), and 325 ± 10 mM (CA_4) NaCl when chromatography was carried out with the analytical Mono-Q column (Figure 1B). The purified component A isoforms were separately reassociated with several purified component B isoforms (CB_a, CB_b, and CB_d) at a 1.5:1 molar ratio of component A/mol of component B. The 12 resulting crotoxin isoforms proved to be homogeneous when analyzed by PAGE (20%) in the ab-

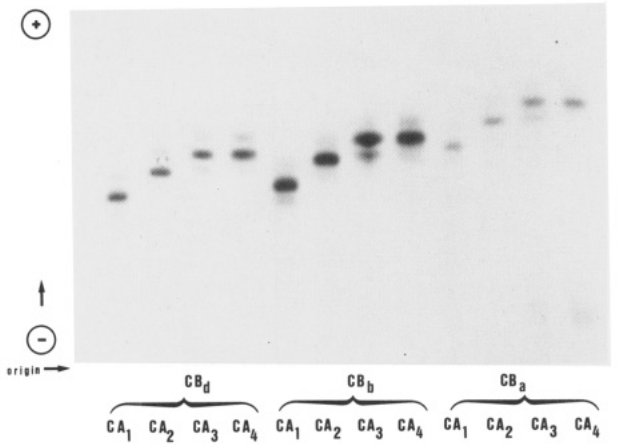

FIGURE 2: PAGE of crotoxin isoforms obtained by reassociation of purified subunits. PAGE was carried out in 20% acrylamide gels, in the absence of SDS and reducing agent. Crotoxin isoforms were prepared by mixing stoichiometric amounts of purified isoforms CA_1, CA_2, CA_3, and CA_4 with purified component B isoforms CB_a, CB_b, and CB_d. Proteins were stained with Coomassie Brilliant Blue.

Table I: Phospholipase A_2 Activity and Lethal Potency of Crotoxin Isoforms[a]

component A isoform	CA_1	CA_2	CA_3	CA_4
phospholipase A_2 activity (μmol·min^{-1}·mg^{-1})	16 ± 2	15 ± 2	13 ± 2	13 ± 2
lethal potency (LD_{50}) (μg·kg^{-1})	55 ± 10	65 ± 10	70 ± 10	75 ± 10

[a] Crotoxin isoforms have been made by the reassociation of component B isoform CB_b and of the various component A isoforms.

sence of SDS and reducing agent (Figure 2).

The various crotoxin isoforms obtained by reassociation of the component A isoforms with the purified component B isoform CB_b all possess the same lethal potency (LD_{50} = 65 ± 10 μg/kg) by intravenous injection into mice and present the same phospholipase activity of 14 ± 2 μmol/min·mg (values given in Table I being not significantly different).

It should be noted that the two major component A isoforms, CA_2 and CA_3, are respectively identical with the isoforms CAII and CAIV, which have been isolated with a different procedure (Faure & Bon, 1988). In particular, (i) they elute from the analytical Mono-Q column at the same ionic strength (285 ± 10 mM for CAII and 300 ± 10 mM for CAIV compared to 290 ± 10 mM and 300 ± 10 mM for CA_2 and CA_3, respectively), (ii) they migrate at the same position when analyzed by PAGE, and (iii) they have the same amino acid compositions.

(*2*) *Separation of the Three Polypeptide Chains* α, β, *and* γ *of the Component A Isoforms*. The component A isoforms are made of three different polypeptide chains, α, β, and γ, cross-linked by disulfide bridges (Breithaupt et al., 1974). In previous investigations (Breithaupt et al., 1974; Aird et al., 1985) polypeptide chains α, β, and γ were named A, B, and C, respectively. This nomenclature has been changed in the present investigation in order to prevent confusion between the polypeptide chains of component A and the A and B subunits themselves. The reduced and carboxymethylated polypeptide chains α, β, and γ were purified by reverse-phase HPLC (Figure 3). The amino acid composition of each peak was determined and used to identify the different polypeptide chains.

The polypeptide γ-chains of the various component A isoforms have the same retention time (Figure 3). Furthermore, their amino acid compositions are identical, suggesting that they do not differ in their polypeptide sequence. On the other

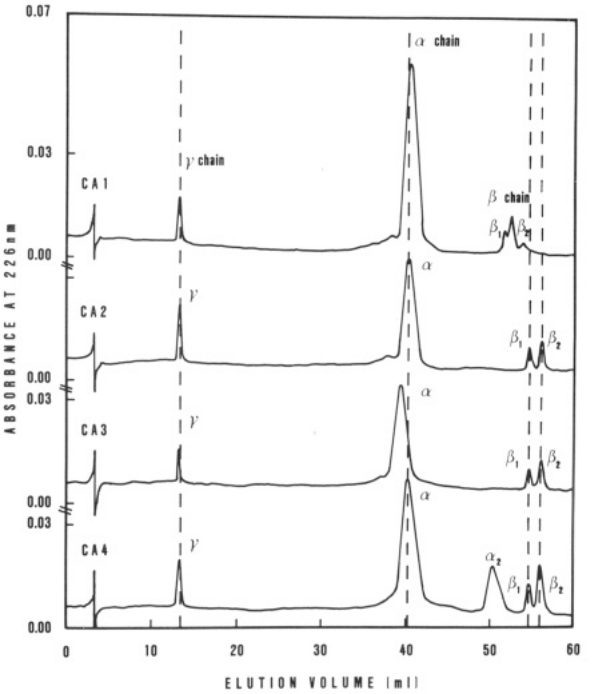

FIGURE 3: Reverse-phase HPLC of reduced and carboxymethylated component A isoforms CA_1, CA_2, CA_3, and CA_4 on a Nucleosil C_8 column.

hand, the various polypeptide α-chains, which are easily identified by their absorbance at 280 nm, eluted with slightly different proportions of acetonitrile: 56% for α-chains of CA_1, CA_2, and CA_4 and 55% for the α-chain of CA_3 (Figure 3), suggesting that the polypeptide structure of the α-chain of the CA_3 isoform differs from that of the other CA isoforms. Surprisingly, the β-chains of each isoform eluted as two peaks, β1 and β2 (Figure 3), which do not significantly differ in their amino acid compositions. The amino acid compositions and the relative elution positions of the β-chains suggest a possible difference between the CA_1 isoform and other CA isoforms. Furthermore, in the case of CA_4, an additional peak (α2) having an amino acid composition similar to that of polypeptide α-chain, eluted between the normal α-chain and the β1- and β2-chains (Figure 3).

(*3*) *Polypeptide Sequences of* α*-Chains*. Breithaupt et al. (1974) and Aird et al. (1985, 1990), who studied mixtures of component A isoforms, reported that the N-terminus residues of the β- and γ-chains are blocked and therefore insensitive to Edman degradation. With the exception of isoform CA_1, the sequence determination carried out with native purified CA isoforms (CA_2, CA_3, and CA_4) was identical with that obtained with their α-chain, purified after reduction of disulfide bridges and carboxymethylation of SH groups (Figure 4). In the case of isoform CA_1, two PTH-amino acids were observed at each degradation step. They corresponded to the simultaneous degradation of chains α and β, indicating that, unlike that of other isoforms, the β-chain of CA_1 is not blocked.

Proteolytic cleavages of the polypeptide chains α with *S. aureus* V_8 proteinase led in each case to the production of two polypeptides corresponding to the hydrolysis of the peptidic bond between Glu_{25} and His_{26}. The two fragments were isolated by reverse-phase HPLC (not shown) and sequenced by Edman degradation (Figure 4). The sequences determined with native CA isoforms, isolated polypeptide chains α, and polypeptides resulting from proteolytic digestion with *S. aureus* V_8 proteinase allowed us to identify the amino acid sequences of the α-chain of CA_1 and CA_4 isoforms. The polypeptide

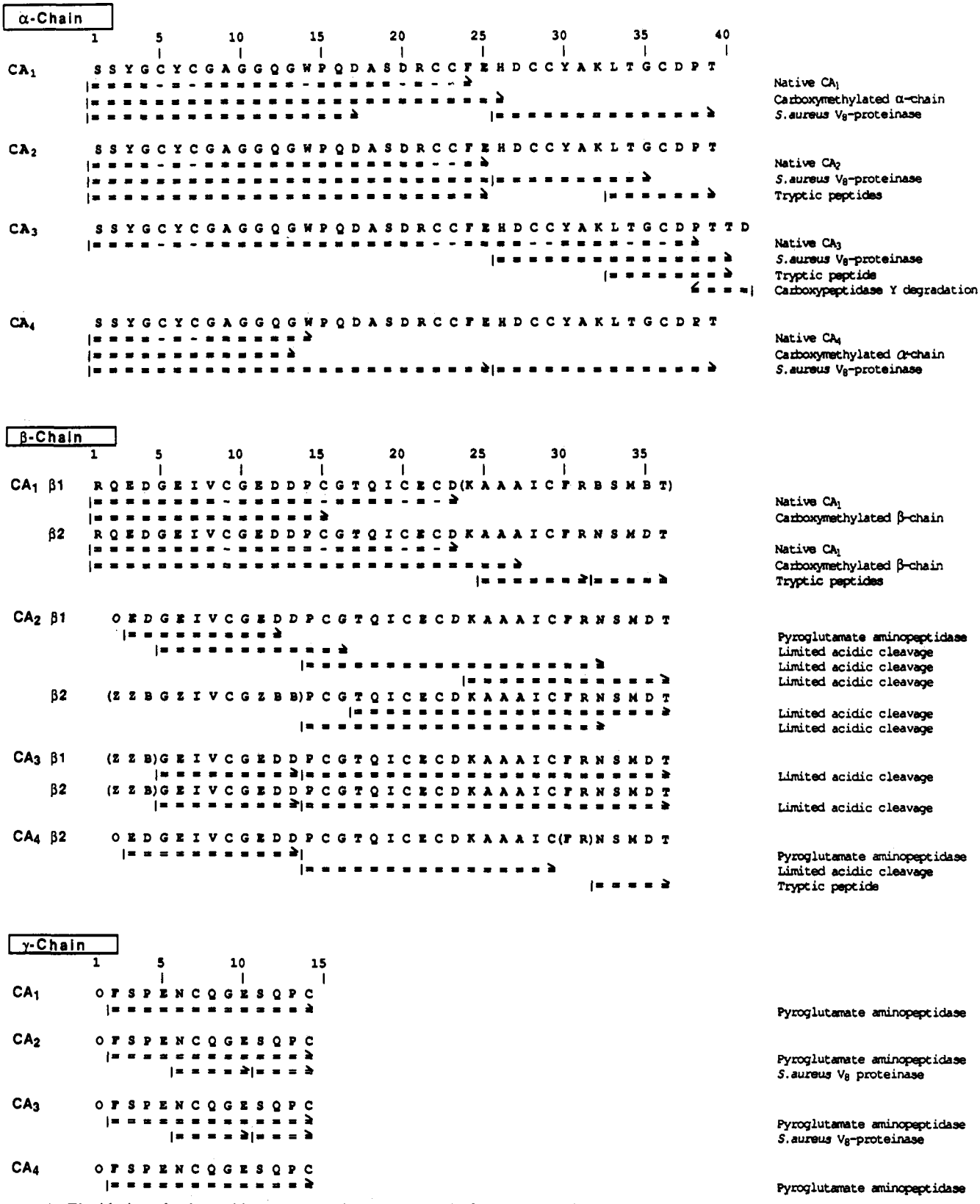

FIGURE 4: Elucidation of polypeptide sequences of component A isoforms CA_1, CA_2, CA_3, and CA_4. The amino acid residues identified by automated Edman degradation are indicated by = whereas – represents ambiguous determinations. Residues in parentheses were determined by amino acid composition but not sequenced. Z means Q or E, B means N or D, and O indicates cyclization of N-terminal glutaminyl residues to pyrrolidone carboxylyl (pyroglutamyl) residues.

sequences of the α-chains of CA_2 and CA_3 isoforms were determined by submitting them to trypsin digestion. In both cases, cleavages efficiently occur between Lys_{32} and Leu_{33} but not between Arg_{21} and the tandem Cys_{22}-Cys_{23}, leading to the formation of only two polypeptides, which were purified and sequenced by Edman degradation (Figure 4).

In the case of isoform CA_3, the amino acid composition of the α-chain was not in agreement with the composition deduced from the sequence identified by Edman degradation. It indicated the existence of an additional aspartic acid or an asparagine residue (Table II). The C-terminal polypeptide sequence of the reduced and carboxymethylated α-chain was

Table II: Amino Acid Compositions of Peptides Generated by Trypsin (T1 and T2) or *S. aureus* V_8 Proteinase (P1 and P2) on the α-Chain of Isoforms CA_2 and CA_3[a]

isoforms	CA2				CA3			
peptides	T1	T2	P1	P2	T1	T2	P1	P2
amino acid								
Asx	1.1 (1)	2.7 (3)	2.2 (2)	2.0 (2)	1.8 (1)*	2.7 (3)	3.0 (2)*	2.0 (2)
Thr	1.9 (2)		2.2 (2)		2.8 (3)		3.0 (3)	
Ser		2.7 (3)		2.6 (3)		2.8 (3)		2.7 (3)
Glx		2.7 (3)		3.1 (3)		2.7 (3)		3.0 (3)
Pro	1.1 (1)	0.9 (1)	1.1 (1)	0.9 (1)	1.0 (1)	1.0 (1)	1.0 (1)	0.9 (1)
Gly	1.2 (1)	4.7 (5)	1.1 (1)	5.1 (5)	1.3 (1)	4.8 (5)	1.1 (1)	5.0 (5)
Ala		2.8 (3)	1.1 (1)	2.0 (2)		2.8 (3)	1.1 (1)	2.0 (2)
CM-Cys	1.0 (1)	5.7 (6)	2.9 (3)	3.9 (4)	1.0 (1)	5.8 (6)	2.8 (3)	3.8 (4)
Val								
Met								
Iso								
Leu	1[b] (1)		1.0 (1)		1[b] (1)		1.1 (1)	
Tyr		2.7 (3)	1.0 (1)	2.0 (2)		2.8 (3)	1.0 (1)	1.9 (2)
Phe		0.8 (1)		1.1 (1)		0.9 (1)		0.9 (1)
His		1.1 (1)	1[b] (1)			1.1 (1)	1[b] (1)	
Lys		1.1 (1)	1.0 (1)			1.1 (1)	1.0 (1)	
Arg		1[b] (1)		1[b] (1)		1[b] (1)		1[b] (1)
Trp		+		+		+		+
peptides (Figure 4)	33–39	1.32	26–39	1–25	33–41	1–32	26–41	1–25

[a] Peptides were purified by HPLC, and their amino acid compositions were determined as described in the Experimental Procedures section. [b] Values are expressed as the molar ratio of each amino acid assuming one residue per peptide of this reference amino acid. Numbers in parentheses refer to amino acid compositions deduced from Edman degradation (Figure 4). An asterisk indicates that the value determined by amino acid composition significantly differs from that deduced from Edman determination. The presence of Trp was determined by absorption and indicated by a plus sign.

therefore determined with carboxypeptidase Y, by measuring the kinetics of amino acid liberation. They appeared in the following order: aspartic acid, threonine, threonine, and proline. This confirmed the existence of an additional aspartic acid residue at the C-terminal position of the CA_3 α-chain (Figure 4).

(*4*) *Polypeptide Sequences of β-Chains.* With the exception of isoform CA_1, the polypeptide β-chains of other isoforms were blocked. Treatment of reduced and carboxymethylated β-chains with pyroglutamate aminopeptidase successfully hydrolyzed the blocked amino acid residues of the CA_2 and CA_4 β-chains. In these two cases the N-terminal sequence was determined by Edman degradation (Figure 4). All attempts to remove the blocked N-terminal residue of the β-chain of the isoform CA_3 were unsuccessful.

Reduced and carboxymethylated β-chains were also hydrolyzed by limited acidic cleavage (2 h at 37 °C in 100% TFA). This treatment led to the partial hydrolysis of polypeptide bonds at several positions: Asp_4–Gly_5, Asp_{13}–Pro_{14}, Gly_{16}–Thr_{17}, Asp_{23}–Lys_{24}, and Asn_{32}–Ser_{33}, generating numerous peptides. Some of them, purified by reverse-phase HPLC, were sequenced. A trypsin digestion of reduced and carboxymethylated β-chains was also performed, in order to generate overlapping peptides that were further purified and sequenced. Figure 4 summarizes the polypeptide sequences that have been obtained.

As reported above, the β-chains of the CA isoforms eluted from the reverse-phase column as two peaks (β1 and β2), which do not differ in their amino acid compositions. In fact, when partial sequences of the two β-chains from the same isoform were determined (for example, in the case of CA_3), they were found to be identical (Figure 4). This indicates that differences in the chromatographic properties of β1- and β2-chains do not result from differences in their primary structures. These differences could, however, result from different conformational structures and/or from artifacts such as incomplete carboxymethylation, although the sequence determinations failed to detect them. Posttranslational modifications such as glycosylations and artifactual desamidations might be responsible for the differences between β1- and β2-chains. No consensus N-glycosylation sequence Asn-X-Ser/Thr (Gavel & von Heijne 1990) was present in any component A isoform. Potential phosphorylation sites are found at various positions (Figure 5).

(*5*) *Polypeptide Sequences of γ-Chains.* The blocked N-terminal residues of the γ-chains of the four CA isoforms were all hydrolyzed by pyroglutamate aminopeptidase. The complete polypeptide sequences of the four γ-chains were determined by subsequent Edman degradation (Figure 4). The sequences of CA_2 and CA_3 γ-chains were confirmed by sequencing the peptides obtained after enzymatic digestion with *S. aureus* V_8 proteinase.

Discussion

It is well established that crotoxin is a mixture of numerous variants that result from the association of several isoforms of its two subunits (Faure & Bon, 1988). In the present investigation, we purified four isoforms of the acidic subunit, component A, from the same venom batch and established their polypeptide sequence. The chromatographic properties in the Mono-Q column and the mobility in PAGE of each CA isoform are in agreement with their primary structure. (i) The less acidic isoform, CA_1, which elutes first from the Mono-Q column, possesses, at the first position of β-chain, an Arg instead of the pyro-Glu in all other isoforms. This is also consistent with the different retention times of the β-chain of CA_1 observed by reverse-phase HPLC. (ii) The isoform CA_3, which is more acidic than CA_1 and CA_2, is characterized by two additional C-terminal residues, Thr and Asp, on the α-chain. Indeed, the α-chain of isoform CA_3 elutes with a retention time different from those of other CA isoforms, when analyzed by reverse-phase HPLC. On the other hand, it is of interest to note that the sequence differences noted between CA_2 and CA_3 were expected on the basis of their amino acid compositions determined in a preceding investigation (Faure & Bon, 1988). However, no difference in the polypeptide sequence of CA_4 may explain that it elutes at a higher ionic strength from a Mono-Q column.

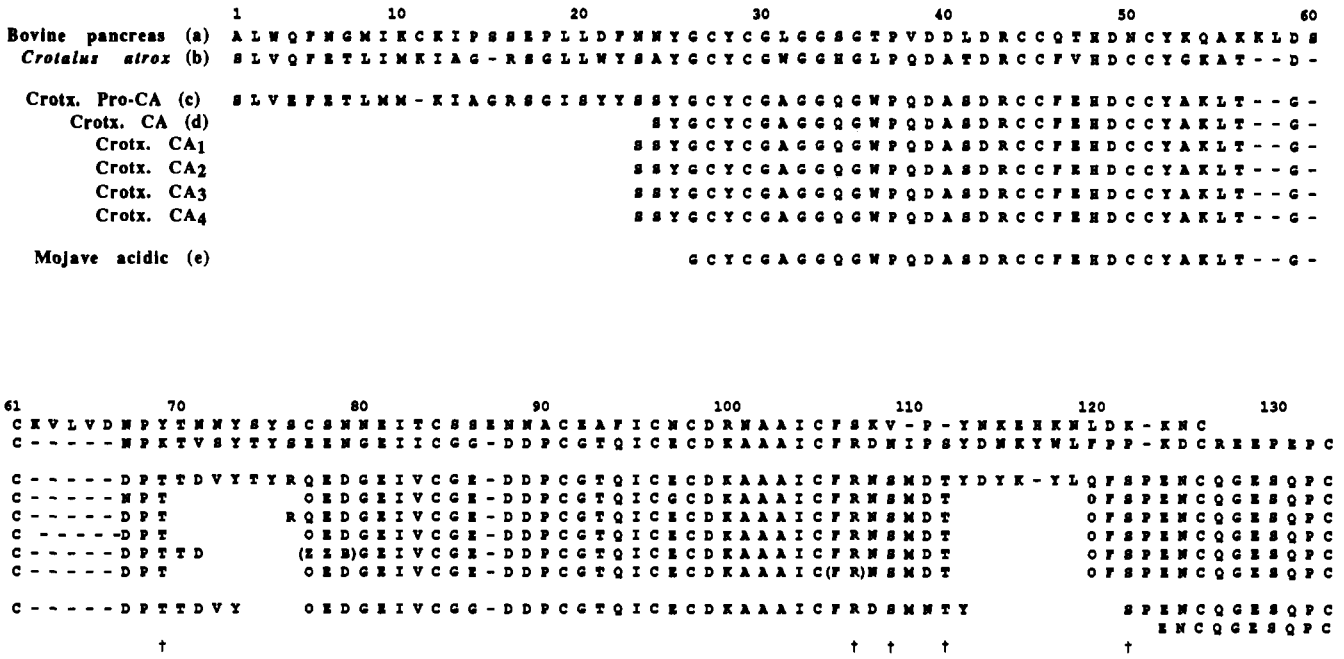

FIGURE 5: Comparison of the polypeptide sequences of component A isoforms with that of other phospholipases A_2. The numbering system is taken from Renetseder et al. (1985). Residues in parentheses were determined by amino acid composition but not sequenced. O indicates cyclization of N-terminal glutaminyl residues to pyrrolidone carboxylyl (pyroglutamyl) residues. ↑ indicates the position of possible phosphorylation sites. (a) PLA_2 of bovine pancreas (Fleer et al., 1978); (b) PLA_2 from *C. atrox* (Randolph et al., 1982); (c) Pro-CA *C. durissus terrificus* (Bouchier et al., 1988); (d) crotoxin CA *C. durissus terrificus* (Aird et al., 1985); (e) mojave toxin, acidic subunit *C. scutulatus scutulatus* (Bieber et al., 1990).

The complete polypeptide sequences of the four CA isoforms have been aligned with the sequence deduced from the nucleotidic sequence of the cDNA encoding the precursor of component A, described by Bouchier et al. (1988). The common numbering system described by Renetseder et al. (1985) is used to specify the positions of amino acid residues in the primary sequence (Figure 5). Gaps in the sequence, indicated by dashes, have been introduced in order to obtain optimal sequence alignments.

The precursor of component A, as reported by Bouchier et al. (1991), is homologous to that of component B and therefore to phospholipases A_2. Figure 5 shows clearly that the three polypeptide chains α, β, and γ of each component A isoform correspond to various regions of the sequence of the component A precursor. It is interesting to note that the polypeptide sequences determined for the four isoforms are all contained in the sequence deduced from the cDNA encoding the component A precursor. They differ, however, from the sequence determined by Aird et al. (1985) at positions 67 (where Asn was found instead of Asp) and 97 (Gly instead of Glu). Aird et al., (1990) confirmed the existence of a Glu residue at position 97 in their more recent sequence determination, carried out by tandem mass spectrometry. These authors interpreted the difference at position 97 from the existence of different component A isoforms in the various venom batches used by different investigators. However, the existence of multiple messenger RNAs has never been proved. In the present investigation, we purified almost all component A isoforms present in a venom batch, including the isoforms present in very small proportions (CA_1 and CA_4). We observed that their sequences all agree with the deduced sequence of the component A precursor determined by Bouchier et al. (1988, 1991) from the unique cDNA found to encode component A.

On the other hand, it is obvious from Figure 5 that the various component A isoforms result from the proteolytic cleavage of the component A precursor at close but nonidentical sites. In this line, it is of interest to note that the first N-terminal Ser residue of the α-chain was absent in the sequence determined by Aird et al. (1985), while it is present in the four isoforms that we isolated. Additional differences are introduced by the possible pyroglutamylation of the glutamine α-NH_2 group, leading to the formation of a pyroglutamate residue. These observations prove, at least in the case of the venom sample that we examined, that the various component A isoforms result from different posttranslational events acting on the same precursor and not from the expression of different mRNAs.

The posttranslational maturation of component A results in the elimination of three peptides: a large one (22 or 23 amino acid residues) corresponding to the N-terminal part of phospholipase A_2 toxins and two smaller ones (4–7 amino acid residues). Proteolytic cleavages of component A precursor occur in the various component A isoforms between Tyr/Ser, Thr/Thr, Asp/Val, Tyr/Arg, Arg/Gln, Thr/Tyr, and Leu/Gln (Figure 5). This does not allow us to define the specificity of the enzyme (or enzymes) involved in these maturation processes. More likely, the cleavages might be due to the combined actions of endo- and exopeptidases. Venom proteases also might be artifactually responsible for the formation of additional component A isoforms. It should, however, be noticed that incubation of crude *C. durissus terrificus* venom, for more than 3 days at room temperature, did not significantly cause a change in the relative proportions of the various component A isoforms of the venom (G. Faure, unpublished results). Further, crotoxin isoforms have also been found in freshly collected venoms from individual snakes (Faure & Bon, 1987). These observations indicate that component A isoforms are generated in vivo, in the venom gland, and not during venom storage. On the other hand, it should also be noticed that the venom used in this investigation has been collected from a large number of snakes, so that the different posttranslational events may occur in different animals. Also, it is interesting to note that the peptide bonds hydrolyzed during

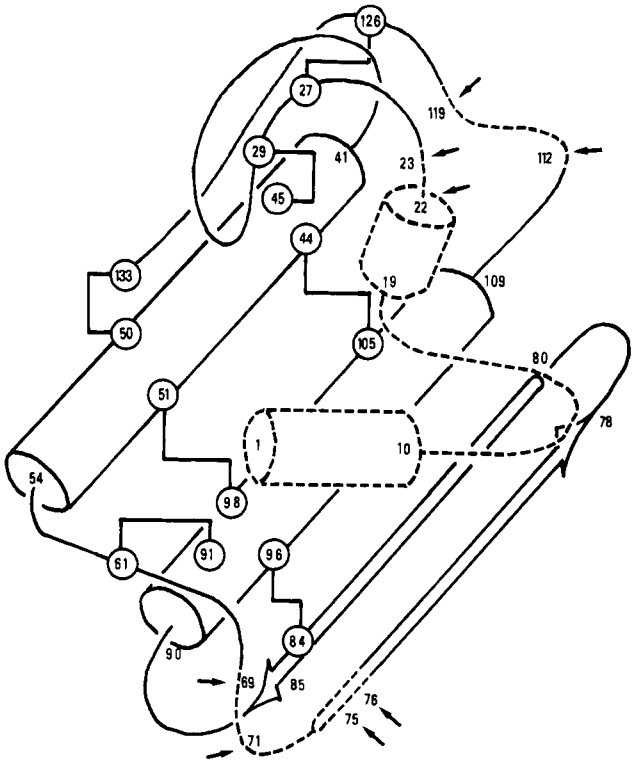

FIGURE 6: Hypothetical three-dimensional structure of component A precursor showing the polypeptides proteolyzed during posttranslational maturation of component A isoforms. The arrows indicate the cleavage sites on the component A precursor. The positions of disulfide bridges are those indicated by Aird et al. (1985).

posttranslational maturation of the component A precursor are localized in easily accessible regions of the molecule (Figure 6), assuming that its three-dimensional structure is similar to that of phospholipases A_2 from bovine pancreas (Dijkstra et al., 1981) and from *Crotalus atrox* venom (Brunie et al., 1985). The sites of cleavage may thus be defined by the conformation rather than the primary structure.

The four component A isoforms, when associated with the same purified component B isoform, produce four crotoxin complexes, which possess identical phospholipase A_2 specific activity and lethal potency. This indicates that the differences in the primary structures noticed between the various component A isoforms are not responsible for significant changes in the three-dimensional structure of component A and are not involved in its pharmacological function, in particular, its ability to associate with component B.

The polypeptide sequence of the acidic nonenzymatic subunit of a crotoxin-like molecule, mojave toxin from *Crotalus scutulatus scutulatus* venom, has recently been reported (Bieber et al., 1990). As shown on Figure 5, it is in excellent agreement with the sequence of component A precursor from *C. durissus terrificus*. Only three differences may be noticed: at position 86 Gly replaces Glu, at position 108 Asp replaces Asn, and at position 111 Asn replaces Asp. It also appears that, as in the case of crotoxin component A, the acidic subunit of mojave toxin results from the proteolytic cleavage of a precursor that seems to be very similar to that of crotoxin component A. Furthermore, the posttranslational proteolytic cleavages occur at very similar positions, supporting the hypothesis of a nonspecific enzymatic cleavage of the precursor. The existence of isoforms has also been reported in the case of the acidic subunit of mojave toxin (Bieber et al., 1990) but they have not yet been purified and sequenced.

Does the proteolytic cleavage of the component A precursor occur before or after its association with component B? Although we are presently unable to answer this question, it is interesting to note that the precursor of component A has a high degree of similarity with *C. atrox* phospholipase A_2 (Figure 5), a dimeric phospholipase A_2 (Brunie et al., 1985), and that component B tends to dimerize when separated from component A (Radvanyi & Bon, 1982). It is thus possible to imagine that the precursor of component A associates with component B before undergoing its posttranslational maturation. In this case, it would be interesting to define the role of this maturation process in the mechanism of action of crotoxin, particularly the dissociation of its two subunits in the presence of its specific target. On the other hand, it is conceivable that posttranslational maturation is a prerequisite for the association of the two subunits forming the crotoxin complex.

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C-Terminal Structure and Mobility of Rabbit Skeletal Muscle Light Meromyosin As Studied by One- and Two-Dimensional ^{1}H NMR Spectroscopy and X-ray Small-Angle Scattering

Hans Robert Kalbitzer,* Kayo Maeda, Andrea Rösch, Yuichiro Maéda,‡ Matthias Geyer, Wolfgang Beneicke, Klaus-Peter Neidig, and Alfred Wittinghofer

Max-Planck-Institute for Medical Research, Department of Biophysics, Jahnstrasse 29, D-6900 Heidelberg, Germany, and European Molecular Biology Laboratory at the German Electron Synchrotron (DESY), Notkestrasse 85, D-2000 Hamburg 52, Germany

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ABSTRACT: Intact rabbit myosin and two different C-terminal fragments of rabbit muscle light meromyosin (LMM) expressed in *Escherichia coli*, LMM-30, and LMM-30C′, were studied by ^{1}H NMR spectroscopy. X-ray small-angle scattering shows that at high ionic strength two polypeptide chains of LMM-30 (which consists of the C-terminal 262 amino acids of myosin heavy chain) or LMM-30C′ (which corresponds to LMM-30 but lacks the last 17 residues) assemble to form an α-helical coiled-coil as it is found also in myosin. The last 12 C-terminal residues of one polypeptide chain of LMM-30 and the last 9 C-terminal residues of the other chain are very mobile. The last 8 residues of the two strands are equivalent from the NMR point of view and unfolded; the valine residues in position 255 in the two strands are not equivalent, suggesting an interaction between the two strands, Ser-252, Arg-253, and Asp-254 are completely immobilized in one of the polypeptide strands and partly mobile in the other. Essentially the same pattern is observed in intact myosin. In spite of the large molecular weights of LMM-30 and LMM-30C′, it is possible to resolve almost all aromatic residues and to determine the pK values of all the 4 tyrosine and of 9 (out of 10) histidine residues. The tyrosine residues in the two strands are equivalent in the two polypeptide chains and both have a pK of 10.5. The pK values of the histidine residues vary between 5.7 and 7.0.

A deeper understanding of muscle contraction can only be obtained if the molecular structure of its contractile elements is known [for reviews, see e.g., McLachlan and Karn (1982), Emerson and Bernstein (1987), and Levine et al. (1990)]. Recently, the structure of G-actin complexed with DNase I has been solved by X-ray crystallography (Kabsch et al., 1990) and a model of F-actin has been derived from X-ray fiber diffraction studies (Holmes et al., 1990). Only a low-resolution X-ray structure of myosin subfragment S1 (Winkelmann et

‡European Molecular Biology Laboratory at DESY.
*To whom correspondence should be addressed.

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