Neurotoxins Active on Insects: Amino Acid Sequences, Chemical Modifications, and Secondary Structure Estimation by Circular Dichroism of Toxins from the Scorpion *Androctonus australis* Hector†

Erwann P. Loret,* Pascal Mansuelle, Hervé Rochat, and Claude Granier
Laboratoire de Biochimie, CNRS UA 1179, INSERM U 172, Faculté de Médecine secteur nord, Bd Pierre Dramard, 13326 Marseille Cedex 15, France

Received July 21, 1989; Revised Manuscript Received September 26, 1989

ABSTRACT: Two scorpion neurotoxins active only on insects, the insect toxins AaH IT1 and AaH IT2, were purified from the venom of scorpions *Androctonus australis* Hector collected in Tozeur (Tunisia) and characterized. AaH IT2 was sequenced and found to differ in four amino acid positions from AaH IT, the single previously sequenced insect toxin [Darbon, H., Zlotkin, E., Kopeyan, C., Van Rietschoten, J., & Rochat, H. (1982) *Int. J. Pept. Protein Res. 20*, 320–330] which possessed an equal potential for paralyzing fly larvae. The basic amino acid residues of AaH IT1, which differs from AaH IT by one amino acid residue, were selectively chemically modified. Six derivatives were characterized. Their toxicity toward fly larvae and cockroach was determined, and their affinity for the AaH IT1 synaptosomal receptor from cockroach nerve cord was measured. Modification of His-30, Lys-34, and Arg-60 showed no significant effect on biological activity. However, the modification of Lys-28 or Lys-51 demonstrated that these two amino acids are important for toxicity. Furthermore, simultaneous modifications of both Lys-28 and Lys-51 led to a cumulative decrease in biological activity. AaH IT1 and AaH IT2 show similar CD spectra. The secondary structures content of AaH IT2 was estimated from circular dichroism data. Results showed that this class of toxin should possess an additional α-helical region and a β-sheet strand, not found in toxins active on mammals. Attempts to localize these secondary structural features in the amino acid sequence of AaH IT2 indicated that these two regions would be located within the last 20 C-terminal amino acid residues. From these studies on secondary structures, it is possible to consider that toxins active on insects are more structurally constrained than those active on mammals; a decreased molecular flexibility may be, at least partially, responsible for the observed specificity of these toxins for the insect sodium channel. Furthermore, the two α-helices found in insect toxins enclosed the two conserved Lys-28 and Lys-51 and might thus be implicated in the toxic site of insect toxins.

Scorpion venoms are rich sources of polypeptide toxins that show various biological specificities. Although a hundred toxins have been isolated, much of the accumulated data concern the so-called mammal toxins, i.e., polypeptides lethal to mice (Rochat et al., 1979). Electrophysiological and binding studies revealed that the target of these toxins was the voltage-dependent sodium channel of excitable cells (Catterall, 1980). The mammal toxins have been divided into α- or β-type according to their pharmacological and electrophysiological effects (Jover et al., 1980; Couraud et al., 1982). Zlotkin et al. (1971a) showed the presence in scorpion venoms of peptides specifically toxic on insects. From the pharmacological viewpoint, two types of insect toxins have been isolated: an excitatory insect toxin inducing a fast excitatory contraction paralysis in *Sarcophaga argyrostoma* fly larvae and a depressant insect toxin inducing a slow depressant flacid paralysis to the same fly larvae (Zlotkin et al., 1985). Although these toxins exhibit distinct electrophysiological properties when tested in current and voltage-clamp conditions on an isolated axonal preparation of *Periplaneta americana* (Zlotkin et al., 1985), they share a common binding site on the sodium channel of insects (Gordon et al., 1984).

Up to now, insect toxins have been purified from scorpion venoms collected in North Africa and the Middle East (Zlotkin et al., 1971b, 1979, 1985; Lazarovici & Zlotkin, 1982; Lester et al., 1982; De Dianous et al., 1987a) as well as in China (Yong-Hua et al., 1988). However, only one amino acid sequence has been reported so far. This insect toxin, AaH IT,[1] from *Androctonus australis* Hector scorpions collected in Chellala (Algeria), was found to be 70 residues long. A peculiarity was the shift in the position of one half-cystine residue (Darbon et al., 1982), whereas the positions of the eight half-cystines were strictly conserved in mammal toxins. The singularity of AaH IT was also pointed out in the general classification of scorpion toxins (Dufton & Rochat, 1984). Homologies were found between AaH IT and *Centruroides* toxins groups, while the mammal toxins present in *A. australis* Hector venom belong to a group clearly distinct from AaH

† This work was supported by a grant from the Institut Scientifique Roussel.

* To whom correspondence should be addressed.

[1] Abbreviations: AaH IT, insect toxin purified from the venom of scorpions *A. australis* Hector collected in Chellala oasis (Algeria); AaH IT1 and AaH IT2, insect toxins purified from the venom of scorpions *A. australis* Hector collected in Tozeur oasis (Tunisia); Var 3, the variant 3 purified from the venom of scorpions *Centruroides sculpturatus* Ewing collected in Arizona; IT1 K1, derivative from AaH IT1 after modification of Lys-34; IT1 K2, derivative from AaH IT1 after modification of Lys-28 and -34; IT1 K3, derivative from AaH IT1 after modification of Lys-34 and -51; IT1 K4, derivative from AaH IT1 after modification of Lys-34, -28, and -51; IT1 R, derivative from AaH IT1 after modification of Arg-60; IT1 H derivative from AaH IT1 after modification of His-30; PAGE; polyacrylamide gel electrophoresis; C8 or C18 HPLC, reverse-phase high-performance liquid chromatography on C8 or C18 columns; TFA, trifluoroacetic acid; sulfo-NHS acetate, sulfo-*N*-hydroxysuccinimidyl acetate; HPG, *p*-(hydroxyphenyl)glyoxal; DEP, diethyl pyrocarbonate, Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

0006-2960/90/0429-1492$02.50/0

IT. From the functional viewpoint, some *Centruroides* toxins (β-type mammal toxins) isolated from South (Brazil) and North (Arizona) American scorpion venoms were found to exhibit a weak toxicity on insects (Watt & Simard, 1984; Lima et al., 1986; Alagon et al., 1988). Finally, short peptides called "insectotoxins", I5A and I2 (35 and 62 amino acid residues) from *Buthus eupeus* scorpion collected in the Caucasus area (USSR), were described as being specifically but weakly toxic on insects (Grishin, 1981).

Chemical modifications have been extensively carried out on toxins active on mammals (mammal toxins) in our laboratory to determine amino acid residues involved in the toxic and pharmacological activities (Habersetzer-Rochat & Sampieri, 1976; Sampieri & Habersetzer-Rochat, 1978; Darbon et al., 1983; El Ayeb et al., 1986; Kharrat et al., 1989). Except Lys-58 in toxin II and Lys-56 in toxin III of *A. australis* Hector, each residue involved in biological activity was not considered as "essential" but as contributing additively to toxin activity. For this reason, it was proposed that a multipoint interaction might occur in the recognition between a mammal toxin and its ion-channel receptor (Kharrat et al., 1989).

The main interest for the study of scorpion insect toxins is the high toxicity they display toward insects and their absence of toxicity toward mammals, which make them a good molecular model for a selective insecticide. Up to now, no chemical modification has been carried out on insect toxins, and the link between structure and toxicity of insect toxins remains to be established. There is a need to obtain further knowledge of these proteins from the structural and functional viewpoints. Furthermore, little is known about the conformation of insect toxins and how it could differ from that of mammal toxins. In the circular dichroism (CD) study of scorpion toxins by Dufton et al. (1986), AaH IT was considered to be the less structured protein of the set of 10 scorpion toxins investigated. The only detailed structural study was the NMR study of the short insectotoxin I5A from *B. eupeus* (Arseniev et al., 1984).

In our present study, the determination of the primary structures of AaH IT1 and AaH IT2, two homologous insect toxins purified and characterized from the venom of *A. australis* Hector, was achieved, and the basic amino acid residues of AaH IT1 were selectively modified. Furthermore, the secondary structure content was determined by circular dichroism data analysis, and these secondary structures were localized on the AaH IT2 sequence by amino acid residue homologies and secondary structure predictive method.

Experimental Procedures

Purification of Toxins. The venom of *A. australis* Hector was obtained by electrical stimulation of animals collected in the area of Tozeur (Tunisia). The venom was subjected to a water extraction, a dialysis procedure, and a recycling gel filtration as described by Miranda et al. (1970). The second step of purification was a DEAE-Sephadex chromatography as described by Zlotkin et al. (1971b). The two last steps of purification were carried out by semipreparative C8 HPLC (see supplementary material).

Amino Acid Analyses. Amino acid analyses were performed on a Model 6300 Beckman analyzer. Sample (1 nmol) was hydrolyzed in 6 N HCl for 20 or 72 h at 110 °C or 1 h at 150 °C. Phenol (1% w/v) was added to the acidic solution before hydrolysis to improve the recovery of tyrosine residues.

Polyacrylamide Gel Electrophoreses. Electrophoreses in nondenaturating conditions were carried out in 15% (w/v) acrylamide gel according to the method of Reisfeld et al. (1962). Electrophoresis buffers were 0.35 M β-alanine at pH 4.5 and 0.1 M Tris–glycine at pH 9.5. Runs were performed on a small vertical slab gel unit (Hoefer Scientific Instrument). Gels were fixed with acetic acid and stained with Coomassie blue.

Reduction and S-Carboxymethylation. Toxins or their derivatives were reduced with dithioerythritol (250-fold molar excess) in 250 μL of 0.25 M Tris-acetate, pH 8.6, 5 M guanidine, and 14 mM Na_2EDTA. Reduction was carried out in the dark at 40 °C under nitrogen for 24 h. Subsequently, the proteins were S-alkylated with iodoacetic acid (500-fold molar excess) for 30 min at room temperature. The S-alkylated proteins were desalted by dialysis against 50 mM ammonium bicarbonate buffer, pH 8.0.

Enzymatic Cleavages. The S-alkylated proteins were subjected to enzymatic cleavages. For each cleavage, 5–10 nmol of protein was used. Digestion with *Staphylococcus aureus* V8 protease (Miles Laboratories Inc.) was performed in 50 mM ammonium bicarbonate buffer, pH 8.0, at 37 °C for 24 h using 10% (w/w) enzyme. Digestion with endoproteinase Lys-C (Boehringer Mannheim Biochemica) was performed in 25 mM Tris-HCl buffer, pH 8.5, and 1 mM EDTA at 37 °C for 18 h by using 1% (w/w) enzyme. Digestion with carboxypeptidase A (Worthington) was performed in 0.2 M *N*-ethylmorpholine at 37 °C under stirring by using 10% (w/w) enzyme. Aliquots (1 nmol) were removed at the appropriate times, acidified with acetic acid, and freeze-dried, and the free amino acid content was assessed on an amino acid analyzer. Peptides from endoproteinase cleavages were separated by C18 HPLC (Lichrospher 100, 5 μm, 4.6 × 250 mm column; Merck). Column temperature was regulated at 25 °C. The A solvent was 0.1% TFA (v/v) in water, and the B solvent was 0.1% TFA (v/v) in acetonitrile.

Amino Acid Sequence Analyses. Automated Edman degradations were performed in a Beckman 890 MM sequencer, and the characterization of phenylthiohydantoin derivatives was carried out by C18-HPLC according to the method of Hawke et al. (1982).

Reaction of AaH IT1 with Sulfo-NHS Acetate (Klotz, 1967). Sulfo-NHS acetate (Pierce) was stored dried at 4 °C until mixed in solution with AaH IT1. The reaction was carried out in the dark at 37 °C for 30 min at pH 7.9 in 25 mM phosphate buffer and stopped by addition of 10% (v/v) acetic acid. Two hundred nanomoles of AaH IT1 was reacted with 1 μmol of sulfo-NHS acetate. The reaction mixture was then purified by ion-exchange HPLC on a TSK sulfopropyl column (7.5 × 75 mm; LKB). Elution was performed at a flow rate of 1.0 mL/min, where solvents A and B were, respectively, 10 and 500 mM ammonium acetate buffers at pH 4.5. Gradient was in percent of solvent B: 0–10 in 50 min, 10–100 in 2 min, and 100 for 5 min. A second step of purification was carried out by C8 HPLC (Lichrospher 100, 5 μm, 4.6 × 250 mm column; Merck). Elution was performed at a flow rate of 1.5 mL/min. According to the method of Bougis et al. (1986), solvent A was 150 mM ammonium formate buffer, 12 mS, at pH 2.75, and solvent B was pure acetonitrile. Gradient was in percent of solvent B: 20–25 in 5 min and 25–31.5 in 65 min.

The localization of the acetylated lysines was assessed by sequence analysis of peptides from the endoproteinase Lys-C digest as described above.

Reaction of AaH IT1 with HPG (Yamasaki et al., 1980). HPG (Pierce) was stored dried at 4 °C prior to use. The reaction was carried out in the dark at 25 °C for 24 h at pH 6.0 in 50 mM phosphate buffer, and the reaction mixture (60 nmol of AaH IT1 reacting with 3 μmol of HPG) was directly

purified by C8 HPLC (Lichrospher 100, 5 μm, 4.6 × 250 mm column; Merck). Elution was performed at 25 °C at a flow rate of 1.5 mL/min, where solvent A was 150 mM ammonium formate buffer at pH 2.75 and solvent B was pure acetonitrile. Gradient was in percent of solvent B: 20–25 in 5 min and 25–32 in 35 min.

Reaction of AaH IT1 with DEP (Miles, 1977). A 5% (v/v) solution of DEP (Aldrich) was prepared in cold anhydrous ethanol, and the concentration of DEP was determined by the increase in absorption at 230 nm ($\Delta\epsilon$ = 3000 M^{-1} cm^{-1}) when an aliquot was added to 3 mL of 10 mM imidazole, pH 7.5 (Miles, 1977). Forty nanomoles of AaH IT1 was incubated at 25 °C with 1.2 μmol of DEP in 50 mM phosphate buffer at pH 6.0. The time course of N-carbethoxylation of histidyl residues was monitored by differential spectra with native AaH IT1 between 340 and 230 nm at different times. The number of molecules with their modified histidyl residue was calculated from the molar absorption difference at 242 nm ($\Delta\epsilon$ = 3800 M^{-1} cm^{-1}). After 30 min, the reaction mixture was stored at −20 °C.

Toxicity Tests. AaH IT1, AaH IT2, and AaH IT1 derivatives were tested by assay of paralysis on *S. argyrostoma* blowfly larvae and *P. americana* cockroach. Quantitative estimation of toxicity on fly larvae (5–6 days old, 100–120 mg body weight) was based on the determination of the so-called contraction paralysis unit (CPU) as described by Zlotkin et al. (1971b). In the CPU assay, aliquots (5 μL) of four increasing concentrations were injected in the terminal segment of fly larvae; a positive response was an immobility for at least 5 s due to the spastic contraction of larvae, a CPU being the amount of toxic material that induces 50% positive response. Quantitative estimation of the toxicity on cockroaches was based on the determination of the paralysis unit (PU) as described by De Dianous et al. (1987a). In the PU assay, aliquots (20 μL) of four increasing concentrations were injected in the abdominal segment of male cockroaches (males of 700–800 mg body weight); a positive response was paralysis of the cockroach, for at least 1 h, a PU being the amount of toxic material that induces 50% positive response. The CPU and PU were calculated according to the method of Reed and Muench (1938). Three to eight insects were used per concentration in both tests.

Binding Assays. A crude synaptosomal fraction was prepared by the method of Lummis and Sattelle (1985) as modified by Lima et al. (1989) from homogenates of thoracic and abdominal nerve cords of male cockroaches. ^{125}I-Iodination of AaH IT1, purification, and characterization of the monoiodinated derivative were performed according to the method of Lima et al. (1989). Increasing concentrations of modified derivatives or native AaH IT1 were tested for their ability to inhibit the binding of ^{125}I-labeled AaH IT1 to membranes.

Circular Dichroism (CD) Measurements and Data Analysis. CD spectra were obtained on a Jobin-Yvon spectropolarimeter (Longjumeau, France). The instrument was calibrated with (+)-10-camphorsulfonic acid, and a ratio of 1.94 was found between the positive CD band at 290.5 nm and the negative band at 192.5 nm. Spectra were measured at 1-nm intervals with a time constant of 20 s at 25 °C. Data were collected from five separate recordings and averaged by using a microcomputer. A quartz cell of 0.5-mm path length with a sample concentration of 0.2–0.4 mg/mL, determined by amino acid analysis, was used in far ultraviolet circular spectra (260–182 nm). Data were expressed in variation of molar amino acid residue absorption coefficient ($\Delta\epsilon$). CD data analyses were performed according to the method of Hennessey and Johnson (1981) using the singular value decomposition method (SVD) modified by Compton and Johnson (1986) and computed on a VAX system.

Secondary Structures Prediction from Amino Acid Sequence. The method of Chou and Fasman (1978) modified by Dufton and Hider (1977) and Fontecilla-Camps et al. (1982) has been used in this study.

Results

Purification and Characterization. Insect toxins AaH IT1 and AaH IT2 were purified from the venom of scorpions *A. australis* Hector collected in the area of Tozeur, Tunisia (see Experimental Procedures and Supplementary Material). The purity of AaH IT1 and AaH IT2 was assessed by several criteria: (a) a single peak in analytical C8 and C18 HPLC; (b) a single band at basic and acidic pH in electrophoretical analysis (see Supplementary Material); (c) a single amino acid phenylthiohydantoin released during Edman sequencing. A supplementary criterion for AaH IT2 was a crystal formation in orthorhombic and quadratic space groups (Fontecilla-Camps, personal communication). PAGE experiments showed that both toxins possessed an identical mobility at pH 4.5, but AaH IT2 was more acidic than AaH IT1 at pH 9.5 (see Supplementary Material). It appeared from amino acid compositions that AaH IT1 and AaH IT2 were closely related proteins of 70 amino acid residues. Paralyzing contraction tests on fly larvae revealed a CPU of 0.60 ± 0.08 ng for AaH IT1 and of 0.55 ± 0.08 ng for AaH IT2, similar to the CPU measured for AaH IT (Zlotkin et al., 1971a). Competition for the binding of the monoiodo ^{125}I-labeled derivative of AaH IT1 to cockroach nerve cord synaptosomes (see Supplementary Material) showed only minor differences between AaH IT1 and AaH IT2. Half-effects ($K_{0.5}$) were observed at concentrations of 0.55 ± 0.18 and 0.25 ± 0.18 nM for AaH IT1 and AaH IT2, respectively (n = 5).

Amino Acid Sequence of AaH IT1 and AaH IT2 (Figure 1). Automated Edman degradation of 3 nmol of the two intact S-alkylated proteins made possible the assignment of the first 23 N-terminal residues. Carboxypeptidase A digestion of the intact S-alkylated AaH IT2 provided C-terminal sequence Ile-Ile-Asn. Peptide fragments from proteolytic cleavage of the S-alkylated AaH IT2 with *S. aureus* protease (peptides V8-1–5) were isolated by C18 HPLC (see Supplementary Material) and their amino acid compositions established. The sequencing of the peptide V8-2 confirmed a portion of the N-terminal sequence of the intact protein and extended it through residue 24. The sequencing of V8-3 gave the sequence 26–39, V8-4 the sequence 42–53, and V8-5 the sequence 56–69. Subsequently, seven endoproteinase Lys-C peptides were isolated by C18 HPLC from S-alkylated AaH IT1 (see Figure 3) and AaH IT2 and their amino acid compositions determined. Three peptides were sequenced (EndoK-2, EndoK-4, and EndoK-5) to overlap peptides obtained from *S. aureus* protease cleavage. Thus, these results made it possible to deduce the complete amino acid sequence of AaH IT2 (Figure 1). It was observed that the peptide V8-4 was generated by a nonselective cleavage with *S. aureus* protease at the carboxyl side of Ser-41. The amino acid sequence of AaH IT1 was found to be similar to the amino acid sequence of AaH IT (Darbon et al., 1982) except that a Glu instead of a Gln occurred at position 25 of the sequence (Figure 1).

Modification of Lysines of AaH IT1 by Sulfo-NHS Acetate. The eight lysines and the N-terminal group of AaH IT1 were potentially able to react with the amino-directed reagent sulfo-NHS acetate. Experiments with different ratios of

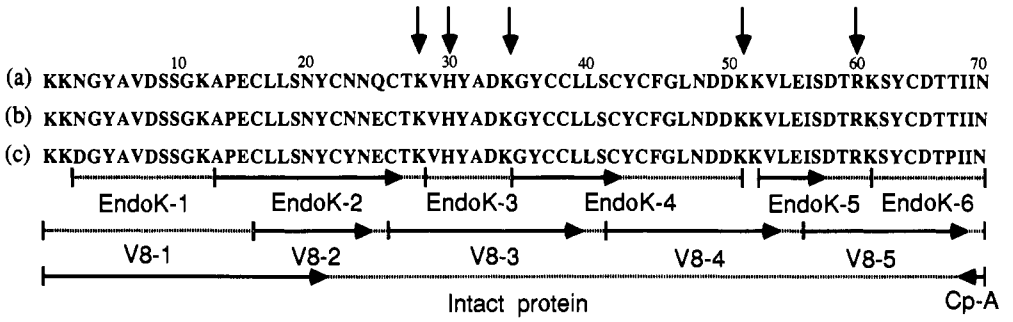

FIGURE 1: Comparison of the primary structures of (a) AaH IT (Darbon et al., 1982), (b) AaH IT1, and (c) AaH IT2. Amino acid sequences were determined by automatic Edman degradation of the intact S-carboxymethylated proteins and proteolytic peptides. The five V8 peptides were obtained by cleavage of AaH IT2 with *S. aureus* protease, and the six EndoK peptides were obtained by cleavage of AaH IT1 and AaH IT2 with endoproteinase Lys-C protease. Cp-A correspond to amino acids identified by carboxypeptidase A digestion of AaH IT2. Horizontal arrows indicate either residues identified by Edman degradation (→) or amino acid released by Cp-A digestion (←). Vertical arrows indicate the position of basic amino acid modified in this study.

reagent over toxin made it possible to select a 5-fold molar excess as the best compromise between the yield of modification and the complexity of the mixture obtained in these experimental conditions. After modification, a first step of purification was carried out by anion-exchange HPLC on a sulfopropyl column to take advantage of the loss of positive charge due to acetylation (Klotz, 1967). The five major peaks arising between 20 and 70 min (Figure 2, upper panel) were analyzed by PAGE. Peak IT1 K4 was considered a trimodified derivative due to its electrophoretic mobility in PAGE (see Supplementary Material). Peak B was considered a dimodified derivative and peak C a mixture of di- and monomodified derivatives (see Supplementary Material). The fourth peak was native AaH IT1, and the fifth (released when the gradient reached 500 mM ammonium acetate) was considered a denatured mixture of di- and monomodified derivatives. Peaks arising between 0 and 20 min were not proteic. A second step of purification on peaks B and C was carried out by C8 HPLC. Peaks IT1 K2 and IT1 K3 obtained from C8 HPLC of fraction B (Figure 2, middle panel) were two dimodified derivatives as judged by electrophoretic mobility (results not shown). Peak IT1 K1 from C8 HPLC of fraction C (Figure 2, middle panel) was a monomodified derivative as assessed by PAGE analysis (results not shown). The two peaks eluting after IT1 K1 were identified as being similar to IT1 K2 and IT1 K3 but were not used to avoid contamination of IT1 K1. From 200 nmol of native AaH IT1 subjected to derivatization, 18 nmol of IT1 K1, 9 nmol of IT1 K2, 14 nmol of IT1 K3, and 13 nmol of IT1 K4 were obtained. Each derivative gave a single peak in analytical C8 HPLC analysis (results not shown).

To identify the modified lysine residues, IT1 K1, IT1 K3, and IT1 K4 were digested with endoproteinase Lys-C; the resulting peptides were separated by C18 HPLC, and the elution profile was compared to that of native AaH IT1 (Figure 3). From the C18 HPLC experiment reported in Figure 3, the pattern of IT1 K1 digest revealed the disappearance of peptides EndoK-3 and EndoK-4 and the emergence of a new peak at 42 min. As peptide EndoK-3 was due to enzyme splitting after Lys-28 and Lys-34 and peptide EndoK-4 from a cleavage after Lys-34 and Lys-51 (or -52), only modification of Lys-34 could produce this C18 HPLC profile. The C18 HPLC pattern of IT1 K3 digest showed the disappearance of peptides EndoK-3, EndoK-4, and EndoK-5′ and the occurrence of a new peak at 42 min. These results were consistent with modification of Lys-34 and Lys-51 since peptide EndoK-5′ originated from the enzymatic cleavage after Lys-51 and Lys-61. The C18 HPLC pattern of IT1 K4 digest showed the disappearance of peptides EndoK-2, EndoK-3,

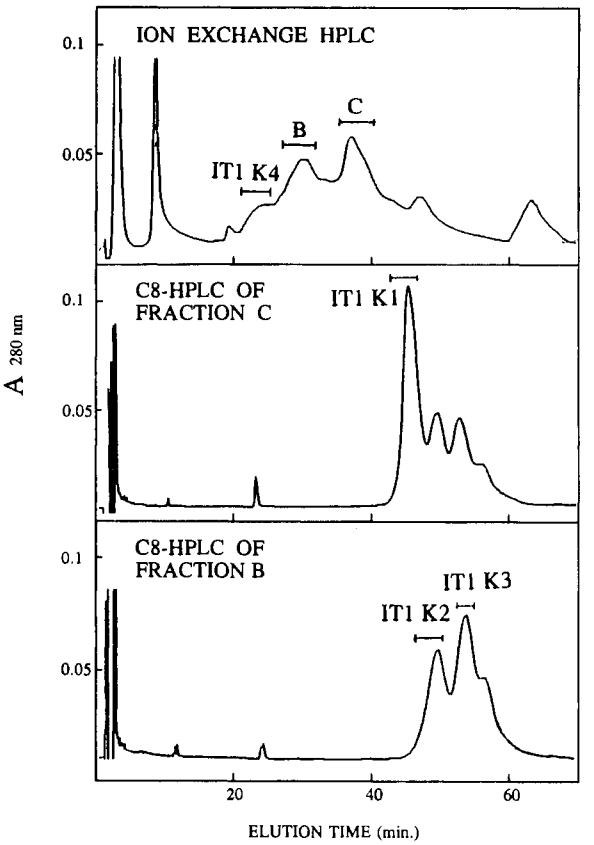

FIGURE 2: Purification of acetylated derivatives of AaH IT1. Purification was carried out by ion-exchange HPLC in the first step (upper panel) and by C8 HPLC in the second step (middle and lower panels). Two hundred nanomoles of AaH IT1 was reacted with 1 μmol of sulfo-NHS acetate, and the reaction mixture was directly fractionated by ion-exchange HPLC on a TSK sulfopropyl column (7.5 × 75 mm). Peak IT1 K4 was a pure derivative, and peaks B and C were further purified by C8 HPLC (4.5 × 250 mm column) in conditions described under Experimental Procedures.

EndoK-4, and EndoK-5′ and the occurrence of a new peak at 45 min. Since peptide EndoK-2 was due to the cleavage after Lys-12 and Lys-28, only modification of Lys-28, Lys-34, and Lys-51 may explain this result.

Amino acid sequence of the peak eluting at 42 min (2.3 nmol) from IT1 K3 digest made it possible to identify a peptide corresponding to the sequence 29–52 where Lys-34 and Lys-51 were found to be a phenylthiohydantoin acetyllysine. Sequencing of peak eluting at 45 min (1.5 nmol) from IT1 K4 digest made it possible to identify a peptide corresponding to the sequence 13–51 (or 52) where Lys-28 and Lys-34 were acetylated. It was not possible to reach the C-terminal ex-

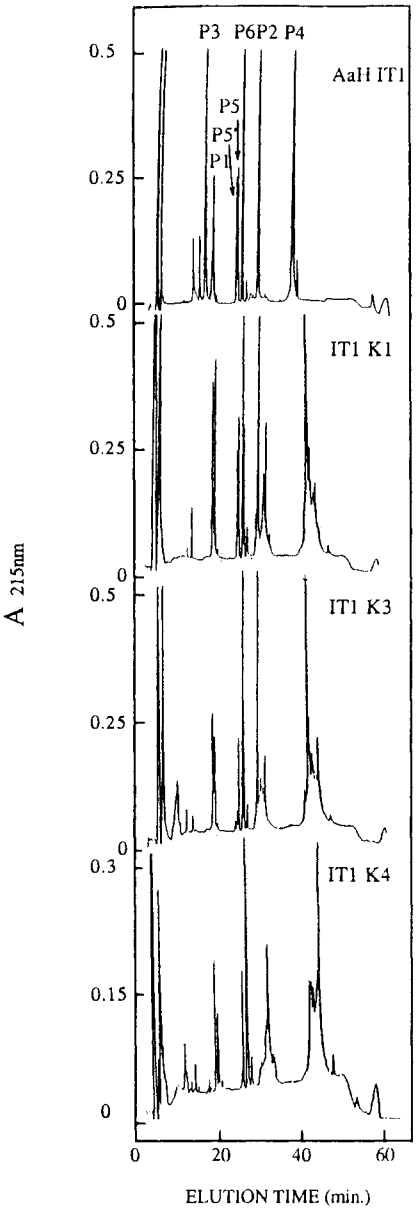

FIGURE 3: Purification of endoproteinase Lys-C digests of native AaH IT1, monoacetylated derivative (IT1 K1), diacetylated derivative (IT1 K3), and triacetylated derivative (IT1 K4). Purification was carried out by C18 HPLC (4.5 × 250 mm column) with a gradient of 10–60% solvent B for 50 min and then 75% solvent B for 5 min (see Experimental Procedures). Identification of peptides was obtained by amino acid analysis after acid hydrolysis (see supplementary Material). P1, P2, P3, P4, P5, P5′, and P6 correspond to peptides EndoK-1, -2, -3, -4, -5, -5′, and -6 in Figure 1, respectively.

tremity to check the acetylation of Lys-51. Direct identification of modified lysines in derivative IT1 K2 was not possible due to the low amount of material remaining after analysis and activity tests. However, it was reasoned that (i) there was a single monoacetylated derivative (IT1 K1, acetylated on Lys-34), (ii) there was one single trimodified derivative (IT1 K4, acetylated on Lys-28, -34, and -51), and (iii) there were two dimodified derivatives, one of which (IT1 K3) was identified as being acetylated on Lys-34 and Lys-51, the other being IT1 K2. Thus, this last dimodified derivative was very likely AaH IT1 modified on Lys-34 and Lys-28.

Modification of Arginine by HPG. The single arginine residue at position 60 in the AaH IT1 amino acid sequence was modified by a 50-fold molar excess of HPG. After modification, two major peaks were observed in C8 HPLC (Figure 4). The first one had the same retention time as native

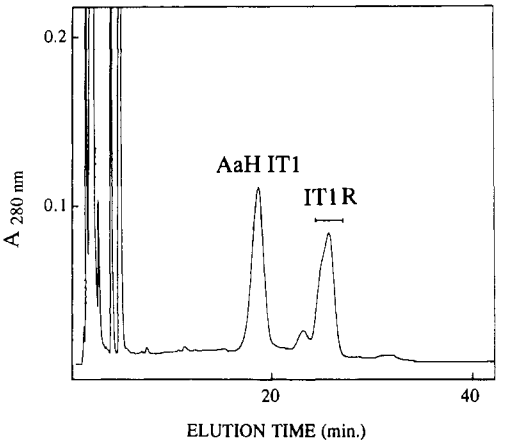

FIGURE 4: Purification of the arginine-modified derivative of AaH IT1 by C8 HPLC. The reaction mixture (60 nmol of AaH IT1 reacting with 3 μmol of HPG) was directly purified by C8 HPLC (4.5 × 250 mm column). Elution was performed at a flow rate of 1.5 mL/min. Solvent A was 150 mM ammonium formate buffer at pH 2.75, and solvent B was pure acetonitrile. Gradient was in percent of solvent B: 20–25 in 5 min and 25–32 in 35 min. Peak IT1 R was identified as a modified derivative of the single arginine residue of AaH IT1 (see Results).

AaH IT1. The second peak showed the electrophoretic behavior of a toxin where one positive charge was lacking and exhibited the specific absorbance of HPG (Yamasaki et al., 1980) at 340 nm at pH 8.0 (results not shown). Furthermore, its amino acid analysis revealed a decrease in the arginine amount as previously described after modification of arginine by HPG (Yamasaki et al., 1980). Therefore, this derivative (IT1 R) most probably corresponds to the Arg-modified form of AaH IT1.

Modification of Histidine by DEP. The single histidyl residue in AaH IT1 (position 30) was modified by a 30-fold molar excess of DEP and the reaction monitored by the specific increase in UV absorbance (Figure 5) that follows the N-carbethoxylation of imidazolyl ring. The results were consistent with the modification of 70% of the available His residue. Minor modification of tyrosyl residues cannot be excluded since a decrease in absorbance at 278 nm, ascribable to O-carbethoxylation of the phenolic ring (Miles, 1977), was observed (Figure 5). The resultant mixture (IT1 H) of native and histidine-modified AaH IT1 was not purified.

Biological Activity of Derivatives. The six different derivatives obtained, IT1 K1 acetylated on Lys-34, IT1 K2 acetylated on Lys-28 and Lys-34, IT1 K3 acetylated on Lys-34 and Lys-51, IT1 K4 acetylated on Lys-28, Lys-34, and Lys-51, IT1 R modified on Arg-60, and IT1 H modified on His-30, were tested for biological activity in three different assays (see Experimental Procedures). The first two assays (CPU and PU) measured the paralyzing properties on two different insects. The results are summarized in Table I. No significant loss of toxicity was observed after modification of His-30, Lys-34, or Arg-60. However, the acetylation of Lys-28 or Lys-51 led to a derivative possessing a residual toxicity corresponding to 15–20% of that of AaH IT1. Furthermore, the simultaneous modification of these two Lys yielded the derivative IT1 K4 with a residual activity corresponding to 2–6% of the AaH IT1 toxicity. In the third assay, the ability of derivatives to inhibit the binding of monoiodinated ^{125}I-labeled AaH IT1 to insect nervous system was assessed. The half-inhibition effect ($K_{0.5}$) was determined for the competition curve of each derivative (Table I). There were minor differences in the inhibiting capacity of IT1 R and IT1 K1 with respect to native AaH IT1, while 43%, 46%, and 33% of

Table I: Biological Activity of AaH IT1 and Its Derivatives

protein tested	site of modification	CPU[a] (ng)	PU[a] (ng)	$K_{0.5}$ from binding assay[a] (nM)
AaH IT1		0.60 ± 0.08 (1.00)	70 ± 10 (1.00)	0.58 ± 0.18 (1.00)
IT1 H	His-30	0.64 ± 0.08 (0.93)	80 ± 11 (0.88)	not determined
IT1 R	Arg-60	0.66 ± 0.08 (0.91)	90 ± 13 (0.77)	0.95 ± 0.18 (0.61)
IT1 K1	Lys-34	0.66 ± 0.08 (0.91)	80 ± 11 (0.88)	0.72 ± 0.18 (0.80)
IT1 K2	Lys-34, -28	4.00 ± 0.50 (0.15)	320 ± 45 (0.21)	1.33 ± 0.18 (0.43)
IT1 K3	Lys-34, -51	4.00 ± 0.50 (0.15)	315 ± 45 (0.22)	1.24 ± 0.18 (0.46)
IT1 K4	Lys-28, -34, -51	25.00 ± 3.50 (0.02)	1050 ± 150 (0.06)	1.74 ± 0.18 (0.33)

[a] Numbers in parentheses represent the relative activity corresponding to ratio of the CPU, PU, or $K_{0.5}$ of the proteins tested to the CPU, PU, or $K_{0.5}$ of the native AaH IT1.

Table II: Secondary Structure Analysis of AaH IT2 and Var 3[a]

protein	method	H	A	P	T	O	total
AaH IT2	CD	0.24 (22%)	0.34 (31%)	−0.02	0.25 (23%)	0.29 (26%)	1.10 (100%)
Var 3	CD	0.14 (15%)	0.30 (32%)	0.04 (4%)	0.21 (23%)	0.26 (28%)	0.93 (100%)
	X-ray	14%	22%	0	24%	40%	100%

[a] Abbreviations: H, α-helix, A, antiparallel β-sheet; P, parallel β-sheet, T, β-turn; O, other structures; CD, secondary structures from analysis by the method of Hennessey and Johnson (1981); X-ray, structure from X-ray data (Fontecilla-Camps et al., 1980; Almassy et al., 1983).

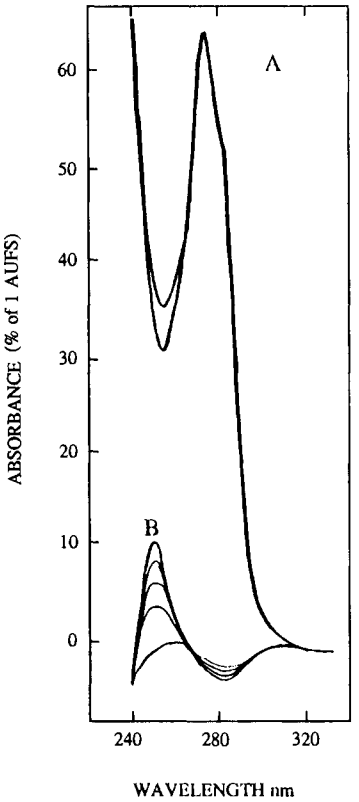

FIGURE 5: Modification of AaH IT1 by DEP. Forty nanomoles of AaH IT1 was incubated at 25 °C with 1.2 μmol of DEP in 50 mM phosphate buffer at pH 6.0. The time course of N-carbethoxylation of histidyl residues was monitored by difference spectra with native AaH IT1 between 0 and 30 min (A). The spectrum after modification (30 min) was compared to the native AaH IT1 spectrum (B).

capacity to inhibit the binding of the labeled AaH IT1 was observed for IT1 K2, IT1 K3, and IT1 K4, respectively.

The loss of biological activities observed for IT1 K2, IT1 K3, and IT1 K4 was likely to be a direct consequence of the acetylation of ε-amino groups since the derivative IT1 K1, which was subjected to the same workup procedure, showed no significant difference in the biological activity with respect to native AaH IT1. Furthermore, the CD spectra of AaH IT1, IT1 K1, IT1 K2, IT1 K3, and IT1 K4 were measured from 190 to 250 nm and found to be closely related to that of native AaH IT1, indicating that the modified toxins had not undergone any extensive changes in their secondary structures (see Supplementary Material).

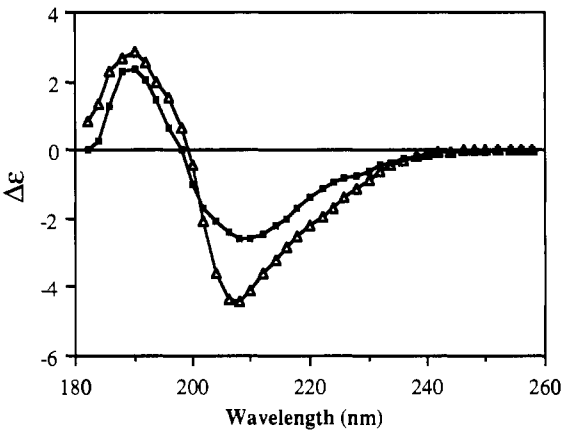

FIGURE 6: Circular dichroism spectra of AaH IT2 (△) and Var 3 (■) from 182 to 260 nm. Δε correspond to the variation of molar amino acid residue absorption coefficient expressed in M^{-1} cm^{-1}.

Circular Dichroism (CD) Spectra. The CD spectra between 182 and 260 nm of AaH IT1 (not shown) and AaH IT2 were found to be similar (Figure 6). They were characterized by two minima at 182 and 207 nm and by a maximum at 190 nm. The negative band at 207 nm had an intensity approximately twice that of the positive band at 190 nm. An inflection attributed to a weak positive CD band at 196 nm was also observed. The CD spectrum of Var 3 was also recorded. This protein from *Centruroides sculpturatus* Ewing venom is not toxic on mammals but has a weak insecticidal effect. A three-dimensional structure has been determined by X-ray crystallography (Fontecilla-Camps et al., 1980; Almassy et al.,1983), and detailed NMR data are also available (Nettesheim et al. 1989; Krishna et al., 1989). The Var 3 CD spectrum resembled the AaH IT2 spectrum and was characterized by two minima at 182 and 209 nm and by a maximum at 190 nm. The positive CD band at 190 nm had an almost equal intensity for AaH IT2 and Var 3, while the negative band at 207 nm of AaH IT2 was characterized by an intensity approximately twice that of the negative band at 209 nm of Var 3. Furthermore, the negative band at 209 nm of Var 3 was broader than the band at 207 nm of AaH IT2.

Secondary Structure Content of AaH IT2 from CD Data Analysis. Starting from CD data, the secondary structure content of AaH IT2 was calculated by singular value decomposition according to the method of Hennessey and Johnson (1981). The analysis gave 22% of α-helix, 31% of the antiparallel β-sheet, 23% of β-turns, and 26% of other structures

(Table II). Secondary structure analysis from CD data was also carried out on Var 3. As for AaH IT2, the sum of all secondary structures obtained by CD analysis fell between 0.90 and 1.10, and the values for contents in secondary structures were positive or never lower than −0.05 (Table II). These two criteria must be met for a valuable analysis (Johnson, 1988). When the predicted secondary structure content of Var 3 was compared to the experimental values from X-ray analysis, a good agreement between the two sets of values was observed (Table II), except for antiparallel β-sheets and unordered structures. This point will be discussed later. The difference appearing between AaH IT2 secondary structures from CD analysis and Var 3 secondary structures from X-ray analysis concerned the α-helix content of 22% (corresponding to 16 amino acid residues) instead of 14% (9 residues) in Var 3, antiparallel β-sheets content of 31% (22 residues) instead of 22% (14 residues), and "other structures" content of 28% (20 residues) instead of 40% (26 residues). Therefore, the CD data analysis indicated that AaH IT2 seemed to contain more secondary structural features than Var 3.

Localization of Secondary Structures on AaH IT2 Amino Acid Sequence. Taking into account the secondary structure content from the CD analysis, the localization of secondary structure patterns was first determined according to sequence homologies between AaH IT2, mammal toxins, and Var 3. The secondary structures for the residues 1–11 and 22–47 in AaH IT2 sequence were considered to be similar to those found in Var 3, i.e., antiparallel β-sheet structure for residues 2–5, 33–37, and 42–46, an α-helix structure for residues 20–28, and β-turn structure for residues 8–11. Residues 29–32 and 38–41 were predicted as β-turns in homology with the secondary structure of mammal toxins, although no sequence homology was found for these residues. No sequence homology with other mammal toxin was observed either between residues 11 and 20 or after residue 47 in AaH IT2 sequence. According to AaH IT2 CD data analysis, eight residues in β-sheet, seven residues in α-helix, and one β-turn remained to be positioned. The Chou–Fasman method (see Experimental Procedures) predicted the occurrence of antiparallel β-sheet structures for residues 15–18 and 59–62, an α-helix structure for residues 51–57, and a β-turn structure for residues 48–51. Figure 7 shows a conventional representation of the secondary structures in the amino acid sequence of AaH IT2.

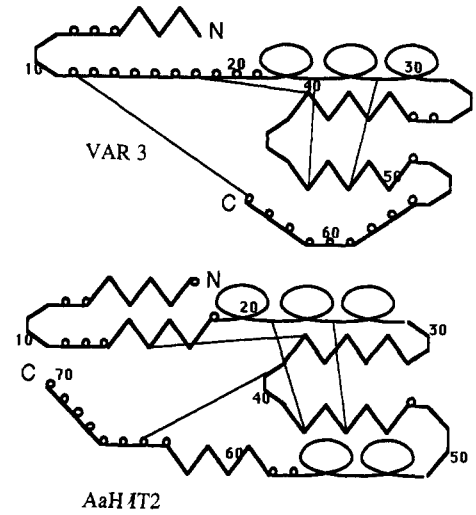

FIGURE 7: Secondary structural representation of AaH IT2 and Var 3. AaH IT2 secondary structures were estimated as described under Results, while Var 3 secondary structures were issued from X-ray data. α-Helices are indicated as large loops, β-sheets as jagged lines, β-turns as angular brackets, and other structures as small loops. The disulfide bridges are indicated by a light line cross-linking two amino acids not adjacent in the amino acid sequence.

Discussion

The amino acid sequence of AaH IT1 differs from the previously published sequence of AaH IT (Darbon et al., 1982) by a Glu instead of a Gln in position 25. The amino acid sequence of AaH IT2 also reveals a high homology with AaH IT, since only four amino acid residues are different: Asp instead of Asn in position 3, Tyr replacing Asn in 23, Glu instead of Gln in position 25, and Pro replacing Thr in position 67. These modifications, in both cases, involve no effect on biological activity of insect toxins since the toxicity and the affinity for the insect sodium channel are almost identical for AaH IT1 and AaH IT2. Recent results (Bougis et al., 1989) show that there is a mRNA encoding a protein sequence identical with that of AaH IT1 in the venomous gland of the scorpion *A. australis* Hector from Tozeur. There is also a mRNA encoding a protein sequence close to AaH IT2, but with a Asn instead of Asp in position 3 in the amino acid sequence of AaH IT2. Thus, a family of closely related insect toxins exists altogether with the known family of differing mammal toxins in *A. australis* Hector venom. Stinging activity of scorpions, in the field, is mainly directed to arthropods and especially soft-bodied insects serving as prey food (Zlotkin, 1983). So the selective pressure in scorpion venom may lead to the selection of efficient insect toxins rather than toxins active on mammals. This natural selection might explain the sequence homogeneity of insect toxins compared to mammal toxins in *A. australis* Hector venom.

The chemical basis for toxicity of insect toxins has been approached in this study by means of selective chemical modifications. Chemical reagents used in this study were chosen with regard to their known specificity for lysine, arginine, or histidine side chains; nevertheless attention was paid to the location of each modified amino acid residue. In the experimental conditions used in this study, the possibility of tyrosine modification by sulfo-NHS acetate was negligible due to the small molar excess of reagent. In preliminary assays N-hydroxysuccinimidylbiotin was used as a modifying reagent for lysine residues of AaH IT1. Loss of activity, but also changes in the secondary structures, were observed after modification of Lys-34 by *N*-hydroxysuccinimidylbiotin (unpublished experiments). In contrast, no change in the secondary structures is induced after acetylation by means of sulfo-NHS acetate. Interestingly, with both acylating reagents, Lys-34 is demonstrated to be the most reactive lysine in AaH IT1. In order of reactivity, Lys-28 and Lys-51 ranked after Lys-34.

Modification of arginine residue by 1,2-cyclohexanedione in 0.2 M NaOH (Toi et al., 1967) was unsuccessful due to the denaturation of AaH IT1 at pH 12.0 (results not shown). Reaction of arginine with HPG was carried out at pH 6.0 to avoid lysine modification. Deprotonation is not necessary for arginine to react with HPG, and the reversibility of the reaction is negligible (Yamasaki et al., 1980).

During the modification of the single histidine of AaH IT1 by DEP, it was not possible to quantitatively convert the single histidine residue into its carbethoxy derivative, since a 100-fold molar excess resulted in total denaturation of AaH IT1. About 70% of histidine-modified toxin was obtained as quantified by spectroscopy (see Experimental Procedures), and the testing of the crude reaction mixture showed no detectable loss of activity. Since the blank, reaction mixture without toxin, is not toxic after injection on fly larvae or cockroach, the purification of the histidine-modified derivative was not at-

tempted. Since the stability of *N*-carbethoxyimidazole at 25 °C in water has been shown to be about 55 h at pH 7.0 (Miles, 1977), the possibility of decarbethoxylation of histidine in the insect body has been neglected. Because of the presence of Tris in the binding buffer, IT1 H was not tested in the competition assay. By acting as a nucleophile, the Tris buffer accelerates the reversal of DEP on histidine, the half-life of DEP on histidine in Tris-HCl at 25 °C being 1.25 min at pH 7.5 and 0.37 min at pH 8.2 (Miles, 1977).

From the results of the toxicity tests (CPU and PU), it becomes clearly apparent that the chemical modification of His-30 or Arg-60 has no major effect on the toxic properties of AaH IT1. This is also true for Lys-34; however, further acetylation of either Lys-28 or Lys-51 involved the formation of two derivatives exhibiting a weak toxicity (15–20% of the AaH IT1 toxicity). This result is in favor of a similar role of these two lysine residues in the toxicity of AaH IT1. Acetylation of both Lys-28 and Lys-51 induces a greater loss of activity since only 2–6% of the AaH IT1 toxicity was displayed by the triacetylated toxin. Therefore, there is a cumulative effect of both modifications of Lys-28 and Lys-51 in the toxicity of AaH IT1. This result suggests that the toxic effect of AaH IT1 on insects necessitates, at least, the presence of these two lysines. The fact that the triacetylated toxin still displays a weak toxicity probably means that one (or more) other amino acid residue(s) is (are) involved in the toxicity of AaH IT1 on insects. The biological activity of mammal toxins also requires the contribution of multiple residues (Kharrat et al. 1989).

When tested for their ability to compete with ^{125}I-labeled AaH IT1 on insect sodium channels, the different modified derivatives displayed reduced potencies with regard to native toxin (Table I). However, their relative activities in this test are not strictly comparable with their relative toxicities. For example, the modification of Arg-60 is not without influence on binding, and modifications of Lys-28 and Lys-51 have only moderate effects on binding. This fact led to the formulation of two hypotheses: either there are two different sodium channels in the insect nervous system, the first one in the peripheral nervous system, reacting during the toxicity test, and another one in the central nervous system used for synaptosomal preparation; or the peripheral and central sodium channels are identical, and the toxicity of the insect toxins is not strictly related to their binding capacity. An argument in favor of the second hypothesis is that an excitatory insect toxin inducing a fast excitatory contraction paralysis in fly larvae and a depressant insect toxin inducing a slow depressant flacid paralysis in fly larvae share a common binding site on insect sodium channel. However, they exhibit distinct electrophysiological properties when tested on an isolated axonal preparation of the *P. americana* in current and voltage-clamp conditions (Zlotkin et al., 1985). An argument in favor of the first hypothesis can be found in electrophysiological assays with the toxin Ts VII from the South American scorpion *Tityus serrulatus* (Lima et al., 1989). Both Ts VII and AaH IT1 are competitors in the binding experiment on insect nerve preparations, although AaH IT1 only exhibits high toxicity in the CPU test. However, electrophysiological assays on the insect central nervous system demonstrate that AaH IT1 induces the same effects as does Ts VII but with a 10-fold less potency. These results are in agreement with the higher affinity observed for Ts VII in binding assay, but contrast with the high toxicity of AaH IT1.

For the purposes of this study, the most important thing to emphasize is the role of Lys-28 and Lys-51 in activity of the insect toxin AaH IT1 in every biological test and especially toxicity tests. The amino acid sequences of four insect toxins have been determined: AaH IT (Darbon et al., 1982), AaH IT1, and AaH IT2 (this work) from *A. australis* Hector venom and BmK IT (Yong-Hua, personal communication) from *Buthus martensi* Karsh venom. It is worth noting that in BmK IT (which differs from AaH IT by 16 amino acid residues), AaH IT, AaH IT1, and AaH IT2 amino acid sequences, Lys-28, and Lys-51 are conserved, while His-30, Lys-34, and Arg-60 were substituted by Tyr-30, Ser-34, and Thr-60, respectively, in BmK IT. These results are in accordance with the maintaining of biological activity of AaH IT1 after modification of His-30, Lys-34, and Arg-60, and the functional role of Lys-28 and Lys-51.

The CD spectra of AaH IT2 and Var 3 (Figure 6) showed many similarities in band positions and magnitudes. These results are in accordance with the previous CD analysis performed by Dufton et al. (1986), where the spectrum of the β-type mammal toxin Css II from *Centruroides suffusus suffusus* venom, with a sequence close to that of Var 3 (Rochat et al., 1979), was considered as similar to the AaH IT spectrum. Both had the most intense spectra and were considered close to the random coil archetype, although the resemblance was not considered completely exact. Furthermore, the CD spectra of 10 scorpion toxins of different venoms revealed a wide range of conformational variation, with AaH II (α-type mammal toxin) and AaH IT from *A. australis* Hector representing the two extremes (Dufton et al., 1986). Between 180 and 260 nm the most prominent CD bands arise from $n-\pi^*$ and $\pi-\pi^*$ transitions due to chromophores in the polypeptide backbone of the protein (Johnson, 1985; Yang et al., 1986). The wavelengths associated with these transitions vary with the secondary structure of the polypeptide backbone. The AaH IT2 spectrum shows a positive and a negative band at 192 and 208 nm characteristic of the splitting of amide $\pi-\pi^*$ transition into two bands in α-helix CD spectra (Woody, 1985). However, the broad negative band at 220–225 nm due to $n-\pi^*$ transition in α-helix CD spectrum is not observed for AaH IT2. This result might be due to the presence of a broad positive band at 228 nm as observed in the AaH II and Bot III scorpion toxins spectra (Dufton et al., 1986) counterbalancing the effect of the negative band at 220–225 nm. This positive band at 228 nm was also observed in *Elapidae* snake toxins and was attributed essentially to the disulfide chromophore (Hider et al., 1988). This assignment may also be valid for scorpion toxins since they have the same size and are also cross-linked by four disulfide bridges. The availability of the refined three-dimensional structure of Var 3 allows us to assess the reliability for scorpion toxins of the structural information obtained by the analysis of the CD spectra by the method of Hennessey and Johnson. A good agreement is observed between experimental (i.e., from X-ray) and predicted (i.e., from CD) values for the α-helix and β-turns. In contrast, the CD data analysis apparently overestimated the β-sheet structures since 32% antiparallel and 4% parallel β-sheets were predicted instead of 22% observed in the crystal. However, an extended structure (residues 12–16) is observed in the three-dimensional structure of Var 3, but no hydrogen bonds have been found with another β-sheet strand (Fontecilla-Camps, personal communication). As the CD spectrum depends on the structural organization of the peptidic backbone, this extended structure might explain the overestimation of β-sheets by CD data analysis.

The most prominent secondary structural features observed in mammal toxins are an α-helix and a three-strand stretch

of antiparallel β-sheet (Fontecilla-Camps et al., 1980, 1988; Almassy et al., 1983; Pashkov et al., 1988; Nettesheim et al., 1989). These two structural elements, described as being approximately parallel in the three-dimensional structure and cross-linked by two disulfide bridges (see Var 3 in Figure 7), correspond to conserved or semiconserved sequences in mammal toxins as well as in insect toxins. Recently the three-dimensional structure of AaH IT has been modeled by using computer graphics and energy-minimization techniques (Fontecilla-Camps et al., 1989). The model-building procedure was based on the X-ray structure of Var 3 and AaH II. In spite of their atypical disulfide bridge, it appears that the general structural features of scorpion toxin family are likely to occur in insect toxins. However, it appears from CD data analysis (Table II) that the insect toxin possesses at least two distinguishing features. The first one is a high content in α-helical structures. Up to now, the three-dimensional structures of mammal toxins have shown the occurrence of two and a half turns of α-helix in similar positions for α- and β-types toxins. In the attempt to localize the predicted secondary structure in the amino acid sequence of AaH IT2, a second helical motif might be located between residues 51 and 57 (Figure 7). If the overall architecture of scorpion toxins active on mammals is conserved in insect toxins, this region might be adjacent to the "conserved hydrophobic surface" thought to play a role in the binding of mammal toxins to the sodium channel (Fontecilla-Camps et al., 1980). As the chemical modification of the Lys-51 has detrimental effects on the pharmacological properties of AaH IT1, this might implicate the supplementary helical region in the specific bioactivities of insect toxins. The second striking difference with mammal toxins, appearing from secondary structure predictions, is a supplementary β-pleated region, localized in the C-terminal part of AaH IT2.

Taken together, these results seem to indicate that the three-dimensional structure of insect toxins is more constrained than that of mammal toxins, particularly in the C-terminal region. This higher content in ordered structures is likely to be accompanied by a decreased molecular flexibility. Thus, the high specificity displayed by the insect toxin for the insect sodium channel might be related to the inability to adapt to a different sodium channel. However, some β-type toxins are able to bind either to the mammal type or insect type of receptor (Lima et al., 1988), and there is evidence for high structural homologies between insect and mammal sodium channels (Salkoff et al., 1987; Ramaswami & Tanouye, 1989; Gordon et al., 1988); the molecular bases for the discrimination of the type of receptor by scorpion toxins may at least partially stem from differences in their molecular flexibility.

ACKNOWLEDGMENTS

We are greatly indebted to Dr. Marie-France Martin for help during purification and sequencing, to Thérèse Brando for technical assistance in amino acid analysis, to Colette Roussarie and Peter McCavana for English reviewing, to Dr. Alain Roussel for his expert assistance in computation, and to Drs. Jean-Marc Sabatier, Pierre Bougis, and Hervé Darbon for fruitful discussion. We gratefully acknowledge Drs. Chantal Abergel, Juan Fontecilla-Camps, and Ji Yong Hua for providing us with results before publication. We thank Dr. Dean D. Watt for providing Var 3.

SUPPLEMENTARY MATERIAL AVAILABLE

Purification and characterization of AaH IT1 and AaH IT2 and PAGE analysis and CD spectra of the acetylated derivatives of AaH IT1 (8 pages). Ordering information is given on any current masthead page.

Registry No. AaH IT1, 124354-85-4; AaH IT2, 124354-86-5.

REFERENCES

Alagon, A. C., Guzman, H. S., Martin, B. M., Ramirez, A. N., Carbone, E., & Possani, L. D. (1988) *Comp. Biochem. Physiol. 1*, 153–161.

Almassy, R. J., Fontecilla-Camps, J. C., Suddath, F. L., & Bugg, C. E. (1983) *J. Mol. Biol. 170*, 497–527.

Arseniev, A. S., Kondakov, V. I., Maiorov, V. N., & Bystrov, V. F. (1984) *FEBS Lett. 165*, 57–61.

Bougis, P. E., Marchot, P., & Rochat, H. (1986) *Biochemistry 25*, 7235–7243.

Bougis, P. E., Rochat, H., & Smith, L. A. (1989) *J. Biol. Chem. 264*, 19259–19265.

Catterall, W. A. (1980) *Annu. Rev. Pharmacol. Toxicol. 20*, 15–43.

Chou, P. Y., & Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251–276.

Compton, L. A., & Johnson, W. C., Jr. (1986) *Anal. Biochem. 155*, 155–167.

Couraud, F., Jover, E., Dubois, J. N., & Rochat, H. (1982) *Toxicon 20*, 9–13.

Darbon, H., Zlotkin, E., Kopeyan, C., Van Rietschoten, J., & Rochat, H. (1982) *Int. J. Pept. Protein Res. 20*, 320–330.

Darbon, H., Jover, E., Couraud, F., & Rochat, H. (1983) *Int. J. Pept. Protein Res. 22*, 179–186.

De Dianous, S., Hoaro, F., & Rochat, H. (1987a) *Toxicon 25*, 411–417.

De Dianous, S., Kopeyan, C., Bahraoui, E. M., & Rochat, H. (1987b) *Toxicon 25*, 731–741.

Dufton, M. J., & Hider, R. C. (1977) *J. Mol. Biol. 115*, 177–193.

Dufton, M. J., & Rochat, H. (1984) *J. Mol. Evol. 20*, 120–127.

Dufton, M. J., Drake, A. F., & Rochat, H. (1986) *Biochim. Biophys. Acta 869*, 16–22.

El Ayeb, M., Darbon, H., Bahraoui, E. M., Vargas, O., & Rochat, H. (1986) *Eur. J. Biochem. 155*, 289–294.

Fontecilla-Camps, J. (1989) *J. Mol. Evol. 29*, 63–67.

Fontecilla-Camps, J., Almassy, R. J., Suddath, F. Z., Watt, D. D., & Bugg, C. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6496–6500.

Fontecilla-Camps, J., Almassy, R. J., Suddath, F. Z., & Bugg, C. E. (1982) *Toxicon 20*, 1–7.

Fontecilla-Camps, J., Haberzetzer-Rochat, C., & Rochat, H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7443–7447.

Gordon, D., Jover, E., Couraud, F., & Zlotkin, E. (1984) *Biochim. Biophys. Acta 778*, 349–358.

Gordon, D., Merrick, D., Wollner, D. A., & Catterall, W. A. (1988) *Biochemistry 27*, 7032–7038.

Grishin, E. V. (1981) *Int. J. Quantum Chem. 19*, 291–298.

Habersetzer-Rochat, C., & Sampieri, F. (1976) *Biochemistry 15*, 2254–2261.

Hawke, D., Yuan, P. M., & Shively, J. E. (1982) *Anal. Biochem. 120*, 302–311.

Hennessey, J. P., Jr., & Johnson, W. C., Jr. (1981) *Biochemistry 20*, 1085–1094.

Hider, R. C., Kupryszewski, G., Rekowski, P., & Lammek, B. (1988) *Biophys. Chem. 30*, 45–51.

Johnson, W. C., Jr. (1985) *Methods Biochem. Anal. 31*, 61–163.

Johnson, W. C., Jr. (1988) *Annu. Rev. Biophys. Chem. 17*, 145–166.

Jover, E., Couraud, F., & Rochat, H. (1980) *Biochem. Biophys. Res. Commun. 95*, 1607–1614.

Kharrat, R., Darbon, H., Rochat, H., & Granier, C. (1989) *Eur. J. Biochem. 181*, 381–390.
Klotz, I. M. (1967) *Methods Enzymol. 11*, 576–585.
Krishna, N. R., Nettesheim, D. G., Klevit, R. E., Drobny, G., Watt, D. D., & Bugg, C. E. (1989) *Biochemistry 28*, 1556–1562.
Lazarovici, P., & Zlotkin, E. (1982) *J. Biol. Chem. 257*, 8397–8404.
Lester, D., Lazarovici, P., Pelhate, M., & Zlotkin, E. (1982) *Biochim. Biophys. Acta 701*, 370–381.
Lima, M. E., Martin, M. F., Diniz, C. R., & Rochat, H. (1986) *Biochem. Biophys. Res. Commun. 139*, 296–302.
Lima, M. E., Couraud, F., Lapied, B., Pelhate, M., Diniz, C. R., & Rochat, H. (1988) *Biochem. Biophys. Res. Commun. 151*, 187–192.
Lima, M. E., Martin, M. F., Hue, B., Loret, E. P., Dinitz, C. R., & Rochat, H. (1989) *Insect Biochem. 19*, 413–422.
Lummis, S. C. R., & Sattelle, D. B. (1985) *Neurosci. Lett. 60*, 13–18.
Miles, E. W. (1977) *Methods Enzymol. 47*, 431–442.
Miranda, F., Kopeyan, C., Rochat, H., Rochat, C., & Lissitzky, S. (1970) *Eur. J. Biochem. 16*, 314–323.
Nettesheim, D. G., Klevit, R. E., Dorbny, G., Watt, D. D., & Krishna, N. R. (1989) *Biochemistry 28*, 1548–1555.
Pashkov, V. S., Maiorov, V. N., Bystrov, V. F., Hoang, A. N., Volkova, T. M., & Grishin, E. V. (1988) *Biophys. Chem. 31*, 121–131.
Ramaswami, M., & Tanouye, M. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2079–2082.
Reed, L. J., & Muench, S. (1938) *Am. J. Hyg. 27*, 493–497.
Reisfeld, R. A., Williams, D. E., & Lewis, V. J. (1962) *Nature 195*, 291–293.
Rochat, H., Bernard, P., & Couraud, F. (1979) *Adv. Cytopharmacol. 3*, 325–334.
Salkoff, L., Butler, A., Wei, A., Scavarda, N., Giffen, K., Ifune, C., Goodman, R., & Mandel, G. (1987) *Science 237*, 744–749.
Sampieri, F., & Habersetzer-Rochat, C. (1978) *Biochim. Biophys. Acta 535*, 100–109.
Toi, K., Bynum, E., Novis, T., & Itano, H. A. (1967) *J. Biol. Chem. 242*, 1036–1043.
Watt, D. D., & Simard, J. M. (1984) *J. Toxicol. 3*, 181–221.
Woody, R. W. (1985) *Peptides (N.Y.) 7*, 15–114.
Yamasaki, R. B., Vega, A., & Feeney, R. E. (1980) *Anal. Biochem. 109*, 32–40.
Yang, J. T., Wu, C. S. C., & Martinez, H. M. (1986) *Methods Enzymol. 130*, 208–269.
Yong-Hua, J., Kimura, Y., Hsu, K., & Terakawa, S. (1988) *Comp. Biochem. Physiol. 90C*, 237–240.
Zlotkin, E. (1983) *Insect Biochem. 13*, 219–236.
Zlotkin, E., Miranda, F., Kopeyan, G., & Lissitzky, S. (1971a) *Toxicon 9*, 9–13.
Zlotkin, E., Rochat, H., Kopeyan, G., Miranda, F., & Lissitzky, S. (1971b) *Biochimie 53*, 1073–1078.
Zlotkin, E., Teitelbaum, Z., Rochat, H., & Miranda, F. (1979) *Insect Biochem. 9*, 347–354.
Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M. F., & Rochat, H. (1985) *Arch. Biochem. Biophys. 240*, 877–887.

Disulfide Bond Reduction in Fibrinogen: Calcium Protection and Effect on Clottability†

Roman Procyk* and Birger Blombäck
Blood Coagulation Biochemistry, The New York Blood Center, 310 East 67th Street, New York, New York 10021
Received August 14, 1989; Revised Manuscript Received October 10, 1989

ABSTRACT: Fibrinogen contains 29 disulfide bonds. Limited reduction in buffers containing calcium led to cleavage of three of them: the two Aα442Cys–Aα472Cys intrapeptide disulfide bonds and the symmetrical Aα28Cys–Aα28Cys bond. The limited reduction did not affect clotting by thrombin. However, a prolongation of the thrombin clotting time occurred when the limited reduction took place in the absence of calcium. The bonds reduced under this condition included the three already mentioned and also the two γ326Cys–γ339Cys intrapeptide disulfide bonds located in the C-terminal ends of the γ-chain. N-Terminal analysis of thrombin-treated samples showed that thrombin cleavage occurred at the normal Aα16–Aα17 site in fibrinogen that was partially reduced in the presence of calcium. By contrast, thrombin cleaved at the Aα19–Aα20 site in fibrinogen that was partially reduced in the absence of calcium, rendering the protein unclottable by removing the Aα17Gly–18Pro–19Arg peptide. The loss of thrombin clottability may have also come from γ326Cys–γ339Cys disulfide bond reduction since the structure supported by this bond may be important for the function of the C-terminal polymerization site. In samples of the partially reduced fibrinogen lacking the Aα17–19 residues, gel formation occurred through an oligomerization mechanism catalyzed by factor XIII.

Fibrinogen, the clotting protein in blood, has a molecular weight of approximately 340000 and a dimeric chemical structure. Many details about human fibrinogen are known, including the amino acid sequence of its three polypeptide chains [for a review, see Furlan (1988) and Doolittle (1984)]. Each half of the molecule contains a set of Aα-, Bβ-, and γ-chains, which are linked together by seven interchain disulfide bridges. The disulfide bridges are located in clusters in the amino-terminal and middle regions of the three chains. There are also six intrachain disulfide bonds in each half-molecule: two in the middle region of the Bβ-chain, one in

†Supported by grants from the National Institutes of Health (HL-27279-09) and the Swedish Medical Research Council (13X-02475).

0006-2960/90/0429-1501$02.50/0