Takeuchi, M., Tahasaki, S., Miyazaki, H., Kato, T., Hoshi, S., Kochibe, N., & Kobata, A. (1988) *J. Biol. Chem. 263*, 3657-3663.
Townsend, R. R., Hardy, M. R., Hindsgaul, O., & Lee, Y. C. (1988) *Anal. Biochem. 174*, 459-470.
Townsend, R. R., Hardy, M. R., Cumming, D. A., Carver, J. P., & Bendiak, B. (1989) *Anal. Biochem. 182*, 1-8.
Whitefleet-Smith, J., Rosen, E., McLinden, J., Ploplis, V. A., Fraser, M. J., Tomlinson, J. E. McLean, J. W., & Castellino, F. J. (1989) *Arch. Biochem. Biophys. 271*, 390-399.
Wiman, B. (1973) *Eur. J. Biochem. 39*, 1-9.
Wiman, B. (1977) *Eur. J. Biochem. 76*, 129-137.
Wiman, B., Boman, L., & Collen, D. (1978) *Eur. J. Biochem. 87*, 143-146.
Yamashita, K., Ichishima, E., Arai, M., & Kobata, A. (1980) *Biochem. Biophys. Res. Commun. 96*, 1335-1342.
Yamashita, K., Ohkura, T., Yoshima, H., & Kobata, A. (1981) *Biochem. Biophys. Res. Commun. 100*, 226-232.
Yamashita, K., Kamerling, J. P., & Kobata, A. (1983) *J. Biol. Chem. 258*, 3099-3106.
Yamashita, K., Inui, K., Totani, K., Kochibe, N., Furukawa, M., & Okada, S. (1990) *Biochemisty 29*, 3030-3039.
Yet, M.-G., Shao, M.-C., & Wold, F. (1988) *FASEB J. 2*, 22-31.

An Anti-Insect Toxin Purified from the Scorpion *Androctonus australis* Hector Also Acts on the α- and β-Sites of the Mammalian Sodium Channel: Sequence and Circular Dichroism Study†

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Received July 26, 1990; Revised Manuscript Received October 9, 1990

ABSTRACT: A new anti-insect neurotoxin, AaH IT4, has been isolated from the venom of the North African scorpion *Androctonus australis* Hector. This polypeptide has a toxic effect on insects and mammals and is capable of competing with anti-insect scorpion toxins for binding to the sodium channel of insects; it also modulates the binding of α-type and β-type anti-mammal scorpion toxins to the mammal sodium channel. This is the first report of a scorpion toxin able to exhibit these three kinds of activity. The molecule is composed of 65 amino acid residues and lacks methionine and, more unexpectedly, proline, which until now has been considered to play a role in the folded structure of all scorpion neurotoxins. The primary structure showed a poor homology with the sequences of other scorpion toxins; however, it had features in common with β-type toxins. In fact, radioimmunoassays using antibodies directed to scorpion toxins representative of the main structural groups showed that there is a recognition of AaH IT4 via anti-β-type toxin antibodies only. A circular dichroism study revealed a low content of regular secondary structures, particularly in β-sheet structures, when compared to other scorpion toxins. This protein might be the first member of a new class of toxins to have ancestral structural features and a wide toxic range.

Scorpion venoms are well-known sources of neurotoxins, which are small basic polypeptides endowed with one of the highest toxicity encountered so far in peptidic macromolecules (Miranda et al., 1970). In the last 20 years, considerable data on these macromolecules have been gathered. Sequence data on more than 40 different toxins showed structural similarities among scorpion toxins (Rochat et al., 1979). The most apparent feature was that the eight half-cystine residues were located in same position, which made it possible to predict that the four disulfide bridges would be in homologous positions in all toxins (Kopeyan et al., 1974). The first pharmacological studies of scorpion neurotoxins were limited to the observation of their effect after injection on test animals, i.e., mice. Therefore, the only kind of activity encountered was the so-called anti-mammal activity. Zlotkin et al. (1971) showed the presence, in the venom of *Androctonus australis* Hector, of a toxin only active in insects, which induced a contractional paralysis. It was the first member of a new class of toxins, which now are known as anti-insect toxins. Electrophysiological and binding studies showed that the pharmacological target of anti-mammal toxins was the voltage-dependent sodium channel of excitable cells (Catterall, 1980). Moreover, it was found that anti-mammal toxins could be classified into α- and β-type toxins, according to their pharmacological and electrophysiological activity, each type possessing a distinct binding site on the sodium channel (Jover et al., 1980; Couraud et al., 1982). It was observed that toxins from the New World were mainly β-type toxins, whereas toxins from the Old World were exclusively α-type toxins. The anti-insect toxins were, in turn, divided into two different pharmacological categories: contraction-inducing toxins, which caused rapid excitatory contraction paralysis in *Sarcophaga argyrostoma* fly larvae, and depressant toxins, which induced a slow depressant flaccid paralysis (Zlotkin et al., 1985). Each of these two types of toxins showed distinct electrophysiological properties, but they were capable of binding to a common site on the sodium

† E.P.L. was supported by a grant from the IBRO-Mac Arthur Foundation.

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0006-2960/91/0430-633$02.50/0

channel of insects (Gordon et al., 1984). However, a new type of anti-insect toxin was recently described that bound to a different site on the sodium channel of insects (Eitan et al., 1990).

Up to now, toxins selectively active on insects have been purified only from venoms of Old World scorpions. Excitatory toxins have the highest toxicity and have an identical size of 70 amino acid residues; their sequences are very homologous (Darbon et al., 1982; Loret et al., 1990a; Kopeyan et al., 1990).

In the last few years, the classification of anti-insect or anti-mammal toxins has become somewhat more complex. For example, some *Centruroides* toxins (β-type anti-mammal toxins), isolated from South American (Brazil) or North American (Arizona) scorpion venoms, were found to also have a toxic effect on insects (Watt & Simard, 1984; Lima et al., 1986; Alagon et al., 1988), but which was 50 times lower than that of Old World anti-insect toxins. Moreover, the β-type Ts VII[1] toxin, from the Brazilian scorpion *Tityus serrulatus*, was found to compete with the labeled reference toxin on both insect and mammal synaptosomes of nervous tissues (Lima et al., 1989). However, this peptide showed no α-type toxin feature. Its bispecificity was attributed to a high molecular flexibility which would enable it to bind to both the mammal β-type and insect sodium channel receptors (Loret et al., 1990b). Since then, only a few toxins have been shown to possess these two kinds of activity, and they were from the New World.

In this paper we show that the venom of *A. australis* Hector, scorpion from the Old World, contains a new type of toxin, AaH IT4, which not only has an anti-insect activity but is also able to compete with both α- and β-type anti-mammal toxins. Such protcins could be new molecular tools to determine the structural basis of the specificity of scorpion toxins on either mammals or insects.

Experimental Procedures

Scorpion Venom. The venom of *A. australis* Hector was obtained by electrical stimulation of animals collected in the area of Tozeur (Tunisia). Two batches of venom were used. In the purification described in this paper, the venom was subjected to water extraction and dialysis prior to column chromatography, as described by Miranda et al. (1970).

Toxicity Tests. Toxicity on insects was monitored by assay of paralysis in *Sarcophaga argyrostoma* blowfly larvae (5–6 days old, 100–120 mg of body weight). Quantitative evaluation of toxicity was based on determination of the so-called contraction paralysis unit (CPU) according to the method of Zlotkin et al. (1971). In the CPU assay, samples (5 μL) of toxin solutions corresponding to four different concentrations were injected into the terminal segment of fly larvae. The larvae remained paralyzed for at least 5 s, which was due to the spastic contraction of their whole body, and this was considered a positive response. The amount of toxic material inducing 50% of positive responses within a group corresponded to the CPU. Three to eight larvae were used per dose. The CPU values were calculated according to the method of Reed and Muench (1938).

Toxicity on mammals was monitored by a lethality test after 24 h and carried out with 20 ± 2 g male C57/BL 6 mice. The toxic solution, supplemented with 1% serum albumin, was injected intracerebroventricularly (icv). The LD_{50} values were calculated according to the method of Reed and Muench (1938).

Low-Pressure Liquid Chromatography. The dialyzed and lyophilized water extract of the venom was then subjected to two successive fractionations. The first step consisted of gel filtration chromatography on Sephadex G-50 fine, according to the method of Martin and Rochat (1986), carried out on four columns (5 × 100 cm) in series, equilibrated and eluted with 0.1 M ammonium acetate buffer (pH 8.5, flow rate 180 mL/h). The second step consisted of DEAE-Sephadex anion exchange chromatography carried out on a 2 × 200 cm column with 0.1 M ammonium acetate buffer (pH 8.5, flow rate 20 mL/h). Each chromatographic step was performed at 25 °C.

High-Pressure Liquid Chromatography. The third and fourth purification steps were carried out with semipreparative high-pressure liquid chromatography performed with a Kratos HPLC system. Semipreparative C8 HPLC was carried out on a 10 × 250 mm column prepacked with Ultrasphere octyl, 5 μm (Beckman). Column temperature was regulated at 25 °C. The aqueous elution solvent (A) was 0.15 M ammonium formate (pH 2.75, conductivity 12 mS, at 25 °C), and the organic elution solvent (B) was pure acetonitrile (Carlo Erba), according to the method of Bougis et al. (1986). The flow rate was 4 mL/min.

Polyacrylamide Gel Electrophoreses. Electrophoreses in nondenaturing 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.

Binding Assay. Insect synaptosomal preparations were obtained from homogenates of nerve cords from the cockroach *Periplaneta americana*, according to the method of Lima et al. (1989). ^{125}I-Iodination of the toxin AaH IT1 and purification and characterization of the monoiodo derivative were performed according to the method of Lima et al. (1989). Mammal synaptosomal preparations were obtained from homogenates of rat brain according to the method of Jover et al. (1980). ^{125}I-Iodinations of toxins AaH II and Css II were according to the method of Rochat et al. (1977). Data points correspond to the average value of duplicates that differed by less than 10%.

Radioimmunoassays. Rabbit antisera against AaH IT1, AaH II, and Css II were obtained according to the method of Delori et al. (1981). Radioimmunoassay conditions were the same as those described by El Ayeb et al. (1984). Antiserum (100 μL, final dilution 2.5 × 10^5 times for anti-Css II and 1.25 × 10^6 times for anti-AaH II), IgG anti-AaH IT (7 × 10^{-10} M), ^{125}I-iodinated toxin (100 μL, 2.5 × 10^{-10} M for Css II and AaH II and 1.0 × 10^{-9} M for AaH IT1), and a variable concentration (50 μL, 1.0 × 10^{-6} to 1.0 × 10^{-12} M, initial concentration) of the reference toxin or AaH IT4 were mixed together to constitute a final volume of 500 μL of 50 mM phosphate buffer (pH 7.4, 0.1% bovine serum albumin). The mixture was incubated for 90 min at 37 °C and then

[1] Abbrevations: AaH IT1 and AaH IT2, anti-insect toxins purified from the venom of the scorpion *Androctonus australis* Hector; AaH II, α-type anti-mammal toxin from *Androctonus australis* Hector; Css II, β-type anti-mammal toxin from *Centruroides suffusus suffusus*; Ts VII, both β-type anti-mammal and anti-insect toxin from *Tityus serrulatus*; Var 3, protein related to β-type toxins from *Centruroides sculpturatus*; Lqq IT2, anti-insect toxin from *Leiurus quinquestriatus quinquestriatus*; Lqh αIT, anti-insect toxin from *Leiurus quinquestriatus hebraeus*; PAGE, polyacrylamide gel electrophoresis; C8 or C18 HPLC, reverse-phase high-performance liquid chromatography on C8 or C18 column; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; CD, circular dichroism; RIA, radioimmunoassay.

overnight at 4 °C. Immune complexes were precipitated by incubation with goat serum directed against rabbit IgG and then with 12.5% poly(ethylene glycol). After centrifugation, bound radioactivity in pellets was determined in a Packard Crystal II γ-counter. Data points correspond to the average value of duplicates that differed by less than 10%.

Amino Acid Analyses. Amino acid analyses were performed on a Model 6300 Beckman analyzer. Samples (1 nmol) were hydrolyzed in 6 M HCl for 20 or 70 h at 110 °C or for 1 h at 150 °C. Phenol was added to the acidic solution before hydrolysis to improve the recovery of tyrosine residues.

Reduction and S-Carboxymethylation. A total of 30 nmol of AaH IT4 was reduced with dithioerythritol (15 μmol) in 250 μL of 0.25 M Tris–acetate, pH 8.6, 5 M guanidine, and 14 mM EDTA (sodium salt). Reduction was carried out in the dark under nitrogen, at 40 °C, for 24 h. Subsequently, the protein was S-alkylated with iodoacetic acid (30 μmol), for 30 min, at room temperature. The S-alkylated protein was desalted by dialysis in water and then solubilized in 12% ammonia solution and dialyzed in 0.2 M ammonium bicarbonate buffer, pH 8.5.

Enzymatic Cleavages. The S-alkylated protein was subjected to enzymatic cleavages. For each cleavage, 10 nmol of protein was used. Digestion with *Staphylococcus aureus* V8 protease (Miles Laboratories Inc.) was performed in 0.2 M ammonium bicarbonate buffer, pH 8.5, at 25 °C for 24 h, with 7.5% (w/w) enzyme. Digestion with endoproteinase Lys-C (Boehringer Mannheim Biochemica) was performed in 0.2 M ammonium bicarbonate buffer, pH 8.5, at 25 °C, for 24 h, with 6% (w/w) enzyme. Digestion with carboxypeptidase Y (Pierce) was performed in 50 mM sodium acetate buffer, pH 5.5, under stirring, with 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 on a Lichrospher 100 RP-18 (5 μm) 4.6 × 250 mm column (Merck). Column temperature was regulated at 25 °C. The A solvent was 0.1% TFA in water, and the B solvent was 0.1% TFA in acetonitrile.

Sequence Analyses. Automated Edman degradation was performed in a Beckman 890 M sequencer, and the phenylthiohydantoin derivatives were determined by C18 HPLC according to the method of Hawke et al. (1982) with a Beckman HPLC system.

Dendrogram. Scorpion toxins, including AaH IT4, were classified by taking into account sequence homologies and gaps with the Multalin programs, according to the method of Corpet (1988).

Circular Dichroism (CD) Measurements and Data Analyses. CD spectra were obtained on a Jobin-Yvon spectropolarimeter (Longjumeau, France). The instrument was calibrated with (+)-10-camphorsulfonic acid. 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 gathered after five recordings and averaged on 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 dichroic spectra (260–182 nm). Data were expressed as a variation of the molar amino acid residue absorption coefficient ($\Delta\epsilon$). CD data analyses were performed according to the method of Hennessey and Johnson (1981) with the singular value decomposition method (SVD) modified by Compton and Johnson (1986) and computed on a VAX system.

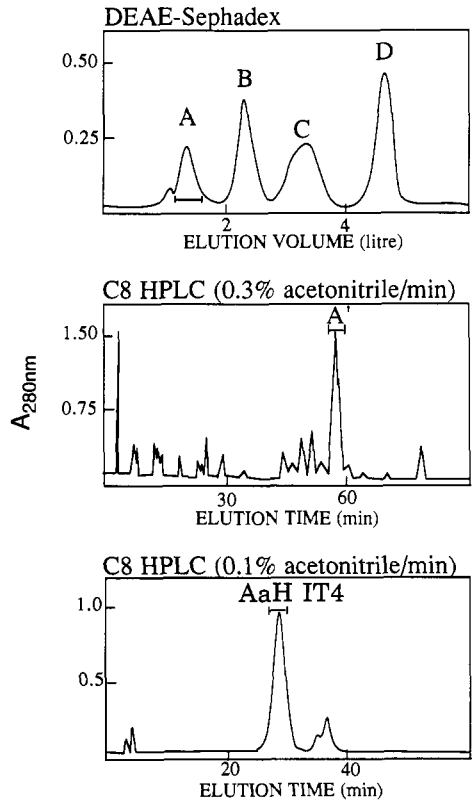

FIGURE 1: Elution profiles of chromatographic steps. Upper panel: DEAE chromatography of the fraction toxic to insects obtained from gel filtration on Sephadex G-50. The column (2 × 200 cm) was equilibrated then eluted at a flow rate of 20 mL/h with 0.1 M ammonium acetate, pH 8.5. Middle panel: C8 HPLC of fraction A on a semipreparative Ultrasphere RP-8 column eluted by a linear gradient from 15% to 40% acetonitrile in 80 min (slope 0.3% acetonitrile/min). Solvent A: 0.15 M ammonium formate, pH 2.75. Solvent B: acetonitrile. Flow rate: 4 mL/min at 25 °C. Lower panel: C8 HPLC of fraction A′ collected in the preceding step. Conditions were similar as above except that the gradient was 25%–30% acetonitrile in 5 min and 30%–40% acetonitrile in 100 min (slope 0.1% acetonitrile/min). Horizontal bars indicate the pooled fractions.

RESULTS

Purification of AaH IT4. The purification of AaH IT4 from the venom extract of scorpions collected in the area of Tozeur (Tunisia) was carried out in four successive chromatography steps. The first step involved recycling gel filtration on Sephadex G-50 carried out on four columns in series. The elution pattern [see supplementary material (see paragraph at end of paper regarding supplementary material)] was practically similar to the one observed during the purification of mammal toxins from scorpions collected in Chellala (Algeria) as described by Miranda et al. (1970). Further separation by DEAE-Sephadex chromatography of the IT fraction, the only fraction active on insects (see supplementary material), led to four main fractions (Figure 1, upper panel). In the third purification step, the A fraction, which contained AaH IT4, was subjected to C8 HPLC with a gradient slope of 0.3% acetonitrile/min (Figure 1, middle panel). In order to obtain pure proteins from the A′ fraction, a final purification step (Figure 1, lower panel) was performed as above but with a lesser gradient (0.1% acetonitrile/min). The main peak corresponded to AaH IT4 and produced a single peak in analytical C8 HPLC (results not shown). This purification procedure led to similar results for two batches of venom, and AaH IT4 corresponded to 0.06% of the proteic material from the crude venom.

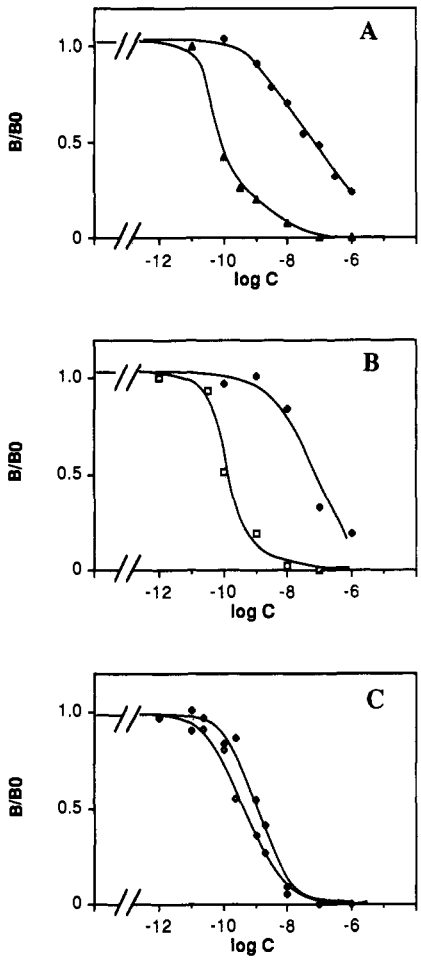

FIGURE 2: Competition experiments with AaH IT4 on synaptosomal fraction. (A) Competition for binding of ^{125}I-labeled Css II to rat brain synaptosomal fractions by increasing concentrations of Css II (▲) and AaH IT4 (◆). (B) Competition for binding of ^{125}I-labeled AaH II to rat brain synaptosomal fractions by increasing concentrations of AaH II (□) and AaH IT4 (◆). (C) Competition for binding of the mono-^{125}I-labeled AaH IT1 derivative to insect synaptosomal fractions by increasing concentrations of AaH IT1 (◇) and AaH IT4 (◆). B is bound radioactivity in the presence of competitor. B_0 is bound radioactivity in the absence of competitor.

Characterization. PAGE experiments showed a single band for AaH IT4 at pH 4.5 (results not shown). Amino acid composition (see supplementary material) gave values close to integers for all residues except acid-sensitive ones. It appeared that AaH IT4 was a protein of 65 amino acid residues with a calculated molecular mass of 8125 Da. At 280 nm, the experimental absorption coefficient was 31 680 $M^{-1}\cdot cm^{-1}$, in reasonable agreement with the theoretical absorption coefficient of 30 550 $M^{-1}\cdot cm^{-1}$.

Paralyzing contraction tests on fly larvae revealed a low toxicity for AaH IT4 (CPU = 50 ± 6 ng) compared to AaH IT1 or AaH IT2 (CP = 0.60 ± 0.08 ng; Loret et al., 1990a). Nevertheless, competition for binding of the mono-^{125}I derivative of AaH IT1 to cockroach nerve cord synaptosomes (Figure 2C) showed only minor differences between AaH IT4 and the reference toxin AaH IT1. Half-effects ($K_{0.5}$) were observed at concentrations of 0.55 ± 0.10 and 0.95 ± 0.18 nM for AaH IT1 and AaH IT4, respectively (n = 3).

When tested in the standard toxicity test on mice, AaH IT4 showed a LD_{50} of 1.0 ± 0.075 μg/kg, similar to the LD_{50} of α-type anti-mammal AaH IV toxin purified in the same venom (0.9 μg/kg) but 2 times lower than the LD_{50} of AaH I (0.5 μg/kg) and AaH III (0.35 μg/kg) and 40 times lower than the LD_{50} of AaH II (0.025 μg/kg). These constitute four

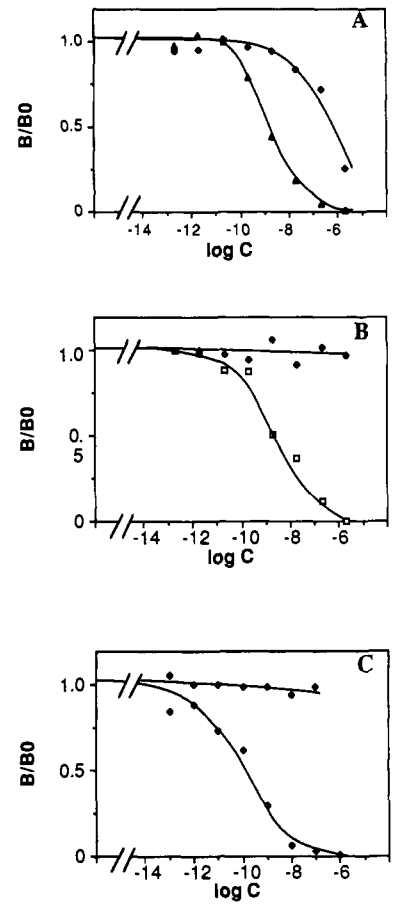

FIGURE 3: Antigenic cross-reactivity of AaH IT4 assessed by radioimmunoassay (see Experimental Procedures). (A) Inhibition of binding of ^{125}I-labeled Css II to anti-Css II serum by Css II (▲) and AaH IT4 (◆). (B) Inhibition of binding of ^{125}I-labeled AaH II to anti-AaH II serum by AaH II (□) and AaH IT4 (◆). (C) Inhibition of binding of ^{125}I-labeled AaH IT1 to anti-AaH IT IgG by AaH IT1 (◇) and AaH IT4 (◆).

α-type anti-mammal toxins present in AaH venom (Martin & Rochat, 1986). AaH IT4 was able to complete with ^{125}I AaH II (α-type toxin) and ^{125}I Css II (β-type toxin) for binding to the mammal sodium channel. For the α-type site, the half-effects were 60 ± 10 nM for AaH IT4 and 0.2 ± 0.05 nM for AaH II (n = 3). For the β-type site, the half-effects were 40 ± 20 nM for AaH IT4 and 0.1 ± 0.05 nM for Css II (n = 3).

Finally, AaH IT4 was tested for its ability to complete with ^{125}I-labeled Css II, AaH II, and AaH IT1, to bind to their respective antisera (Figure 3). AaH IT4 inhibited binding of ^{125}I-labeled Css II to anti-Css II antibodies ($K_{0.5}$ = 500 nM, as compared to 1.6 nM for the relevant reference toxin). Conversely, no significant displacement of ^{125}I-labeled AaH II from anti-AaH II antibodies or of ^{125}I-labeled AaH IT1 from anti-AaH IT1 antibodies was observed. Taken as a whole, these results indicated that AaH IT4 shared antigenic homologies with Css II but did not cross-react either with AaH II or AaH IT1.

Amino Acid Sequence Determination (Figure 4). Automated Edman degradation of 3 nmol of the intact S-alkylated AaH IT4 enabled assignment of the first 21 amino-terminal residues. Carboxypeptidase Y digestion of the intact S-alkylated AaH IT4 provided the C-terminus sequence Cys-Asp-Leu-COOH. Peptide fragments (10 nmol) from endoproteinase Lys-C cleavage of the S-alkylated protein were isolated by C18 HPLC, and their amino acid composition was established (results not shown). The sequencing of the peptide

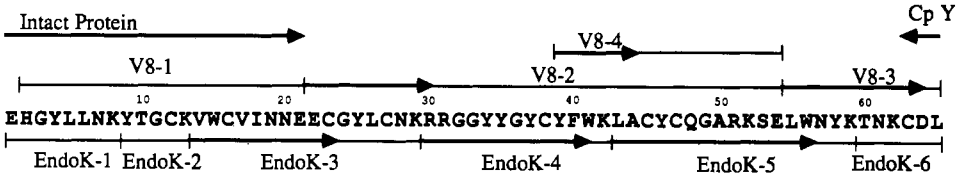

FIGURE 4: Amino acid sequence determination of AaH IT4. Sequence determined by automatic Edman degradation of the intact S-carboxymethylated protein and of proteolytic peptides. The four V8 peptides were obtained by proteolytic cleavage by *S. aureus* protease, and the six Endo-K peptides were obtained by proteolytic cleavage by endoproteinase Lys-C protease. Cp Y corresponds to amino acids identified by carboxypeptidase Y digestion. Arrows indicate either residues identified by Edman degradation (→) or amino acids released by Cp Y digestion (←).

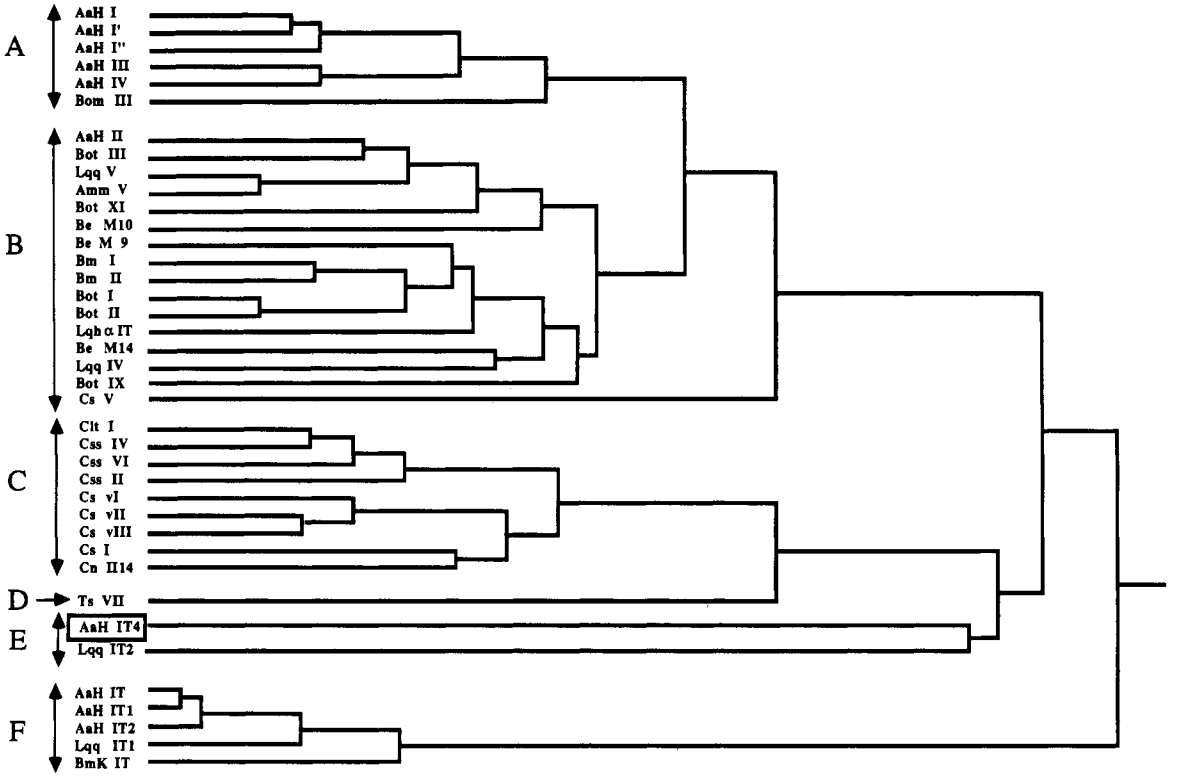

FIGURE 5: Dendrogram of scorpion toxins including AaH IT4 (enclosed in a box) constructed from sequence homology and gap events. A–F indicate groups of scorpion toxins corresponding, in most cases, to specific pharmacological properties (see Results). AaH, *Androctonus australis* Hector; Bom, *Buthus occitanus mardochei*; Bot, *Buthus occitanus tunetanus*; Lqq, *Leiurus quinquestriatus quinquestriatus*; Amm, *Androctonus mauretanicus mauretanicus*; Be, *Buthus eupeus*; Bm, *Buthus martensii*; Lqh, *Leiurus quinquestriatus hebraeus*; Cs, *Centruroides sculpturatus*; Clt, *Centruroides limpidus tecomanus*; Css, *Centruroides suffusus suffusus*; Cn, *Centruroides noxius*; Ts, *Tityus serrulatus*; BmK, *Buthus martensii* Karsch.

EndoK-3 confirmed a portion of the NH_2-terminal sequence of the intact protein and extended it to residue 23. The sequencing of EndoK-4 produced sequence 30–41, and the sequencing of EndoK-5 produced sequence 43–56. Subsequently, *S. aureus* protease peptides (10 nmol) were isolated by C18 HPLC and analyzed in order to be identified (results not shown). Three of them were sequenced to overlap the peptides obtained from the action of endoproteinase Lys-C. Peptides V8-2 and V8-4 overlapped residue Arg-30 and the sequence Leu-43–Ala-44, respectively. Peptide V8-3 overlapped Leu-55 and Trp-56 and made it possible to extend the sequence to Asp-64, which overlapped the sequence obtained by carboxypeptidase Y. These results made it possible to deduce the complete amino acid sequence of AaH IT4 (Figure 4). It was observed that peptide V8-4 was generated by an usual cleavage with *S. aureus* protease at the carboxyl side of the carboxymethylated Cys-38. Peptides expected to be produced from proteolytic cleavage of the S-alkylated protein with endoproteinase Lys-C after Lys-52 and Lys-62 were not obtained.

Dendrogram. A classification taking into account sequence homologies and gaps made it possible to separate the scorpion toxins, mainly, into six different groups corresponding also, for most of the cases, to specific pharmacological properties (Figure 5). Groups A and B corresponded to α-type anti-mammal toxins purified from venoms of Old World scorpions, except for Cs V which comes from the venom of a New World scorpion and Lqh αIT which is a new anti-insect toxin (Eitan et al., 1990). Group C corresponded to β-type anti-mammal toxins purified essentially from venoms of New World scorpions. Group D was represented only by Ts VII which acted as a β-type anti-mammal toxin and also as an anti-insect toxin. AaH IT4 and Lqq IT2 made up group E. The only pharmacological property these two toxins had in common was that they both acted on insects. Lastly, group F corresponded to anti-insect toxins inducing a contractional paralysis in fly larvae. These toxins were found only in venoms from Old World scorpions.

Circular Dichroism (CD) Spectrum. The CD spectrum of AaH IT4 between 182 and 260 nm was characterized by three minima at 182, 205, and 215 nm and by a maximum at 187 nm (Figure 6). The negative band at 205 nm was nearly as intense as the positive band at 187 nm. An inflection was also observed, which could be attributed to a weak positive CD band at 196 nm. By use of CD data, the secondary structure

Table I: Secondary Structure Analyses[a]

proteins		H	A	P	T	O	tot
AaH IT4	CD	0.13 (13%)	0.23 (23%)	0.06 (6%)	0.20 (20%)	0.35 (35%)	0.98 (100%)
Ts VII	CD	0.12 (12%)	0.26 (27%)	0	0.22 (23%)	0.35 (37%)	0.96 (100%)
Css II	CD	0.16 (16%)	0.35 (35%)	−0.01	0.26 (26%)	0.24 (24%)	1.01 (100%)
Var 3	CD	0.14 (15%)	0.30 (32%)	0.04 (4%)	0.21 (23%)	0.26 (28%)	0.93 (100%)
Var 3	X-ray	14%	22%	0	24%	40%	100%
AaH IT2	CD	0.24 (22%)	0.34 (31%)	−0.02	0.25 (23%)	0.29 (26%)	1.10 (100%)
AaH IT1	2D NMR	21%	27%		23%	29%	100%
AaH II	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; tot, total; 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, 1988; Almassy et al., 1983); 2D NMR, structure from two-dimensional NMR (Darbon, personal communication).

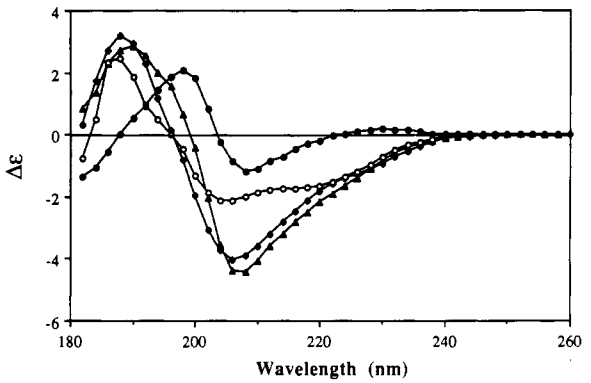

FIGURE 6: Circular dichroism spectra of AaH IT4 (○), AaH IT2 (▲), Css II (◆), and AaH II (●) from 182 to 260 nm. $\Delta\epsilon$ corresponds to the variation of molar amino acid residue absorption coefficient expressed in $M^{-1}\cdot cm^{-1}$.

content of AaH IT4 (Table I) was calculated according to the method of Hennessey and Johnson (1981). The sum of all the 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 I). These two criteria must be met for a valid analysis (Johnson, 1988). In Table I, the CD data analysis of AaH IT4 was compared to the CD data analyses of other scorpion toxins. For two scorpion toxins, the CD data analysis was compared to X-ray data (Var 3) or 2D NMR data (AaH IT1 had a CD spectrum identical with that of AaH IT2). Analysis of the AaH II CD spectrum (results not published) with the Johnson method gave no valid results since the sum of all secondary structures was close to 0.3. The CD spectrum of AaH II differed greatly from the other scorpion toxin spectra (Figure 6) and therefore deserves further study, since the absence of CD bands characteristic of an α-helix, particularly the positive band at 190 nm (Yang et al., 1986), conflicted with the fact that 14% α-helix was described in the crystal structure (Fontecilla-Camps et al., 1988).

DISCUSSION

On the basis of the criterion of toxicity for insects, a screening of the venom of *A. australis* Hector made it possible to purify a new anti-insect toxin: AaH IT4. After dialysis, the venom was submitted to gel filtration on Sephadex G-50, which resulted in five fractions. Toxicity on insects was found in only one fraction (see Results). After a further purification on DEAE-Sephadex, this fraction was in turn divided into four fractions, A–D (Figure 1), and the toxic effect on insects was mainly found in fraction B, from which AaH IT1 and AaH IT2 were purified (Loret et al., 1990a). Nevertheless, fractions A, C, and D exhibited consistent toxic effects on insects and subsequently underwent C8 HPLC purification, as described for fraction A under Results. Consequently, 65 peaks were isolated from the initial toxic fraction, and their toxicity was measured on insects. Nine peaks showed a paralyzing action on fly larvae, including the peak containing AaH IT1 and AaH IT2. Of the other eight peaks showing a low toxic effect on larvae, one demonstrated in binding experiments, a great ability to compete with AaH IT1. Moreover, this peak was the only one to show a toxic activity on mammals. The purity of that fraction, called AaH IT4, was assessed by several criteria: (a) it produced a single peak in analytical C8 HPLC, (b) it produced a single band in electrophoresis, and (c) it released only one amino acid phenylthiohydantoin after one step of Edman degradation. Supplementary criteria came from immunological experiments, since RIA clearly showed that AaH IT4 was not contaminated by traces of other known toxins, i.e., AaH IT1, AaH II, or antigenically related toxins. Furthermore, to check for any possible contamination by known anti-mammal toxins (AaH I, AaH III, or AaH IV), AaH IT4 was incubated with antibodies directed to AaH I and AaH III. Toxicity of AaH IT4 was not modified after contact with anti-AaH I and anti-AaH III antibodies (results not shown).

Amino acid analyses of AaH IT4 revealed a peculiarity in this toxin: the absence of the amino acid proline, which has, until now, always been found in scorpion toxins. The sequence, as established by automated Edman degradation of the protein and of derived proteolytic peptides, confirmed this point. The amino acid sequence showed no obvious homology with other toxins. However, the position of the eight half-cysteine residues was similar to that of anti-mammal toxins. The computer program Multalin (Figure 5) showed that this protein was clearly different from any toxic protein, with the exception of Lqq IT2.

Information regarding the secondary structure contents of AaH IT4 was drawn from the CD data analysis (Table I). In a previously study, the reliability of the Johnson method for analyzing CD spectra of scorpion toxins was tested on Var 3 (Loret et al., 1990a). A good agreement was observed between X-ray data (Fontecilla-Camps et al., 1980) and the α-helix and β-turn contents deduced from CD data analysis. In contrast, the CD data analysis apparently overestimated the β-sheet structures (Table I). A 2D NMR study of AaH IT1 was recently performed (Darbon, personal communication). This protein was homologous to AaH IT2 and showed a similar CD spectrum (Loret et al., 1990a). A good agreement was observed between 2D NMR data and CD data values (Table I) although the β-sheet structures seemed to have been slightly overestimated in the CD data analysis (31% instead of 27% antiparallel β-sheets).

The CD spectrum of AaH IT4 in water (Figure 6) was different from those of AaH IT2 and Css II. The negative band at 205 nm had a lower intensity in the case of AaH IT4 when compared to that in the spectra of AaH IT2 and Css II. Moreover, the negative band at 215 nm, present in the CD spectrum of AaH IT4, was not observed in the CD spectra

of AaH IT2 and Css II, which could be due to the high intensity of the negative bands visible in the two latter spectra at 206–207 nm. The negative band at 215 nm is commonly related to the n–π^* transition characteristic of α-helix structures (Woody, 1985). The CD spectrum of the α-helix is also characterized by a negative band at 205–207 nm and a positive band at 190 nm due to π–π^* transition (Johnson, 1985). The AaH IT4 CD spectrum resembled the CD spectrum of a pure α-helix, but there were two major restrictions: a shift in the positive band from 190 nm to 187 nm and an intensity of the dichroic signal that was six times less than the spectrum of a protein where all residues are in α-helix. This was probably why the CD data analysis of AaH IT4 resulted only 13% α-helix. In the AaH IT4 spectrum, there was no strong negative band near 207–208 nm when compared to Css II and AaH IT2 spectra (Figure 6). Furthermore, the intensity of the positive band near 190 nm was close to that of bands in the AaH IT2 and Css II spectra, which could be due to a similar content in α-helix structure for these three proteins, as confirmed by the CD spectra analyses (Table I). Thus, in the Css II and AaH IT2 spectra, the strong negative band near 206–208 nm was probably the result of the additive effect of the n–π^* transition, due to β-sheet structures, and the parallel π–π^* transition of the α-helix. Therefore, for AaH IT4, the low intensity of that band may indicate a weaker content in β-sheet structures when compared to the contents in AaH IT2 and Css II. The data analysis of the AaH IT4 spectrum resulted in 35% of structures differing from α-helix, β-sheet, or β-turn. Such a high percentage was also found for Ts VII (Table I), although in that case the 37% of "other structures" were associated to random coil structures, because of the presence of a negative band at 200 nm (Loret et al., 1990b). Such was not the case with AaH IT4, and the nature of the 35% of other structures remains undefined.

AaH IT4 was not only capable of binding to the sodium channel of insects, but it also modulated the specific binding of both α- and β-type anti-mammal neurotoxins to the mammal sodium channel (Figure 2). However, it showed a higher affinity for the insect sodium channel than for the α- or β-site on the mammal sodium channel. The in vivo toxicity of AaH IT4 on insects was not related to its binding affinity for the insect sodium channel since it was 50 times less toxic than AaH IT1, but it competed with the same binding affinity in the in vitro test. The discrepancy could be explained by different hypotheses: either there were two kinds of sodium channels (peripheral vs central) in insect nervous system, or AaH IT4 may act as an antagonist of the very potent anti-insect toxins.

Immunological experiments showed that AaH IT4, purified from the venom of a North African scorpion, cross-reacted with Css II purified from a New World venom. This agreed with the classification of scorpion toxins obtained by means of sequence computation (Figure 5), since AaH IT4 belonged to group E which was closely related to the group of β-type toxins (group C). Furthermore, it was amazing to observe that there was no cross-reaction of AaH IT4 with α-type toxins such as AaH II (group B) or with anti-insect toxins such as AaH IT1 (group F), although all these toxins were purified from the venom of *A. australis* Hector. Nevertheless, this fact can be better understood if we consider the position of AaH IT4 in the dendrogram.

AaH IT4 and Lqq IT2 made up group E; however, Lqq IT2 showed no effects on mammals (Kopeyan, 1990) and induced a flaccid paralysis in fly larvae (Zlotkin et al., 1985) while AaH IT4 induced a contractional paralysis in fly larvae. According to the dendrogram in Figure 5, it is clear that these two toxins diverged at a very early stage. This early separation means that a low homology exists between these two proteins, which explains the differences in their pharmacological properties.

From an evolutionary viewpoint, the presence in *A. australis* Hector venom of anti-mammal toxins next to anti-insect toxins can be seen as a progress in the adaptation of the venomous secretion to putative preys of scorpions. In fact, the presence of highly toxic anti-insect toxins in a venom can be considered to be an advantage in the evolution of scorpions since their prey consists mainly of insects (Zlotkin, 1983). Moreover, the efficiency of scorpion toxins on insects seems to be accompanied by a specificity for their target since these toxins have no toxic effect on mammals. Conversely, selective anti-insect toxins have not yet been found in New World scorpion venoms. There are few toxins able to act on both insects and mammals (Lima et al., 1986), and their toxic effect on insects is very weak when compared to anti-insect toxins from Old World scorpion venoms. Consequently, scorpion venoms from the New World can be considered to be less evolved than those from the Old World. Due to its wide toxic range, AaH IT4 might be an "ancestral scorpion toxin" present in the venom of *A. australis* Hector, which shows both anti-insect and anti-mammal activity and antigenic properties shared by New World scorpion toxins.

The molecular basis for the ability of some toxins to bind to either insect or mammal sodium channel receptor is unknown. In an earlier paper (Loret et al., 1990b), it was shown after a CD study that Ts VII possesses a backbone flexibility which might result in a more pronounced tendency for this toxin molecule to undergo conformational changes and thus might explain the fact that it competes with both anti-insect and β-type anti-mammal toxins for binding to the sodium channel. One particular region of the Ts VII molecule appeared capable of folding into either an α-helix or a β-sheet. In Ts VII, during contact with the insect sodium channel, the formation of an additional α-helix similar to the one described for the anti-insect toxin AaH IT2 (Loret et al., 1990a) might endow the toxin with insect sodium channel binding properties. Conversely, the formation of a supplementary antiparallel β-sheet in the same region might give the toxin its ability to bind to mammal-type sodium channels, as do the β-type toxins (Loret et al., 1990b).

One argument for the ability of the C-terminal region of Ts VII to adopt different conformations consisted in the fact that there was a lack of proline in this region (Loret et al., 1990b). Similarly, in the case of AaH IT4, the percentage of "other structures" was found to be similar to that of Ts VII (Table I), and the amino acid proline was absent throughout the sequence. Thus, the flexibility of AaH IT4, due to the absence of proline, may enable this molecule to adopt different conformations required for binding to the insect sodium channel or to the α- or β-site of the mammal sodium channel.

Acknowledgments

We are greatly indebted to Mme. Maryse Alvitre and Mme. Thérèse Brando for technical assistance. Drs. Pascale Marchot, Jean-Marc Sabatier, Halim Zerrouk, and François Couraud are acknowledged for fruitful discussions. We gratefully acknowledge Drs. Hervé Darbon and Ji Yong Hua for providing us with results before their publication.

Supplementary Material Available

Table presenting amino acid analysis of AaH IT4 and two figures showing the first step of purification of AaH IT4 by recycling gel filtration on Sephadex G-50 and data of Edman

degradation (3 pages). Ordering information is given on any current masthead page.

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.

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

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

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

Corpet, F. (1988) *Nucleic Acids Res. 16*, 10881–10890.

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.

Delori, P., Van Rietschoten, J., & Rochat, H. (1981) *Toxicon 19*, 393–407.

Eitan, M., Fowler, E., Herrmann, R., Duval, A., Pelhate, M., & Zlotkin, E. (1990) *Biochemistry 29*, 5941–5947.

El Ayeb, M., & Delori, P. (1984) in *Handbook of Natural Toxins* (Tu, A. T., Ed.) Vol. 2, p 607, Dekker, New York.

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. L., Habersetzer-Rochat, C., & Rochat, H. (1988) *Proc. Nat. Acad. Sci. U.S.A. 85*, 7443–7447.

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

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.

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.

Kopeyan, C., Martinez, G., Lissitzky, S., Miranda, F., & Rochat, H. (1974) *Eur. J. Biochem. 47*, 483–489.

Kopeyan, C., Mansuelle, P., Sampieri, F., Brando, T., Bahraouri, E. M., Rochat, H., & Granier, C. (1990) *FEBS Lett. 261*, 423–426.

Lima, M. E., Martin, M. F., Diniz, C. R., & Rochat, H. (1986) *Biochem. Biophys. Res. Commun. 139*, 296–302.

Lima, M. E., Martin, M. F., Hue, B., Loret, E. P., Dinitz, C. R., & Rochat, H. (1989) *Insect Biochem. 19*, 413–422.

Loret, E. P., Mansuelle, P., Rochat, H., & Granier, C. (1990a) *Biochemistry 29*, 1492–1501.

Loret, E. P., Sampieri, F., Roussel, A., Granier, C., & Rochat, H. (1990b) *Proteins 8*, 164–172.

Martin, M. F., & Rochat, H. (1986) *Toxicon 24*, 1131–1139.

Miranda, F., Kopeyan, C., Rochat, H., Rochat, C., & Lissitzky, S. (1970) *Eur. J. Biochem. 16*, 314–323.

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., Tessier, M., Miranda, F., & Lissitzky, S. (1977) *Anal. Biochem. 20*, 532–548.

Rochat, H., Bernard, P., & Couraud, F. (1979) *Adv. Cytopharmacol. 3*, 325, 334.

Watt, D. D., & Simard, J. M. (1984) *J. Toxicol. 3*, 181–221.

Woody, R. W. (1985) in *The Peptides* (Hruby, V., Ed.) Vol. 7, pp 15–114, Academic Press, New York.

Yang, J. T., Wu, C. S. C., & Martinez, H. M. (1986) *Methods Enzymol. 130*, 208–269.

Zlotkin, E. (1983) *Insect Biochem. 13*, 219–236.

Zlotkin, E., Rochat, H., Kopeyan, G., Miranda, F., & Lissitzky, S. (1971) *Biochimie 53*, 1073–1078.

Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M. F., & Rochat, H. (1985) *Arch. Biochem. Biophys. 240*, 877–887.