P05, a New Leiurotoxin I-like Scorpion Toxin: Synthesis and Structure–Activity Relationships of the α-Amidated Analog, a Ligand of Ca^{2+}-Activated K^+ Channels with Increased Affinity

J.-M. Sabatier,*,‡ H. Zerrouk,§ H. Darbon,∥ K. Mabrouk,‡ A. Benslimane,§ H. Rochat,‡ M.-F. Martin-Eauclaire,‡ and J. Van Rietschoten‡

Laboratoire de Biochimie, CNRS URA 1455, Faculté de Médecine Nord, Boulevard Pierre Dramard, 13326 Marseille Cédex 15, France, Laboratoire de Purification des Protéines, Institut Pasteur du Maroc, 1, Place Charles Nicolle, B.P. 120, Casablanca, Morocco, and Laboratoire de Cristallographie et Cristallisation des Macromolécules Biologiques, CNRS URA 1296, Faculté de Médecine Nord, Boulevard Pierre Dramard, 13326 Marseille Cédex 15, France

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ABSTRACT: The venom of the scorpion *Androctonus mauretanicus mauretanicus* contains a toxin, P05, which is structurally and functionally similar to scorpion leiurotoxin I (87% sequence identity), a blocker of the apamin-sensitive Ca^{2+}-activated K^+ channels. It is a 31-residue polypeptide cross-linked by three disulfide bridges. A C-terminal carboxyl-amidated analog of P05 (sP05-NH_2) was chemically synthesized by the solid-phase technique and fully characterized. Toxicity assays in vivo established that sP05-NH_2, like native P05, is a potent and lethal neurotoxic agent in mice (LD_{50} of 20 ng per mouse). Pharmacological assays in vitro however showed that, unlike P05 which has a binding affinity of 2×10^{-11} M, sP05-NH_2 apparently binds irreversibly to the apamin receptor. Iodination at the C-terminal His gave diiodo-sP05-NH_2, which had a binding affinity similar to that of native P05. The disulfide bridge pairings were chemically determined for sP05-NH_2 and thereby deduced for P05 and leiurotoxin I: linkages were between Cys_3 and Cys_{21}, Cys_8 and Cys_{26}, and Cys_{12} and Cys_{28}. Molecular dynamics refinement of P05 also using data from leiurotoxin I suggests that P05 is mainly composed of a double-stranded, antiparallel β-sheet (from Leu_{18} to Val_{29}) linked to an α-helix (from Arg_6 to Gly_{16}) by two disulfides (Cys_8–Cys_{26} and Cys_{12}–Cys_{28}) and to an extended fragment (from Thr_1 to Leu_5) by the third disulfide (Cys_3–Cys_{21}). In agreement with the model, circular dichroism analysis of sP05-NH_2 showed that the toxin structure is highly rigid. A common Arg-Arg-Cys-Gln sequence was identified in the helical region of both P05 and apamin. Two Arg-substituted analogs of sP05-NH_2 ([Lys_6,Lys_7] sP05-NH_2 and [Leu_6,Leu_7] sP05-NH_2) were synthesized and tested for bioactivity. The results indicate that, as for apamin, Arg residues of the motif are required in the binding and expression of the leiurotoxin I/apamin-like biological properties of P05.

Most polypeptide animal toxins are highly active ligands that interfere with the function of specific ion channels. They have been used as pharmacological probes for the study of channel proteins (Strong, 1990; Garcia et al., 1991). One such toxin, leiurotoxin I (also called scyllatoxin by Auguste et al. (1990)) from the scorpion *Leiurus quintestriatus hebraeus*, has been reported to act as a blocker of small-conductance, Ca^{2+}-activated K^+ channels in various cell types (Abia et al., 1986; Chicchi et al., 1988; Castle et al., 1989). Leiurotoxin I (31 residues, three disulfide bridges) possesses binding and physiological properties similar to those of the bee venom toxin, apamin (18 residues, two disulfides). However, the amino acid sequences of these two polypeptides are dissimilar (Gauldie et al., 1976; Hugues et al., 1982a,b; Blatz & Magleby, 1986; Castle & Strong, 1986; Auguste et al., 1990). Recently, a toxin—termed P05 (31 residues, three disulfides)—was isolated from the venom of the scorpion *Androctonus mauretanicus mauretanicus*. It is both structurally and functionally related to leiurotoxin I (87% sequence identity) (Zerrouk et al., submitted). The sequence differences between P05 and leiurotoxin I occur at positions 1 (Thr_1 for Ala_1), 2 (Val_2 for Phe_2), 7 (Arg_7 for Met_7), and 24 (Val_{24} for Asp_{24}). P05 makes up less than 0.05% of the protein in crude venom. We therefore undertook the chemical solid-phase synthesis of this toxin in order to facilitate the characterization of its biological properties. Whether or not the C-terminus of native P05 was free carboxylate or amidated had not been established. We presumed it to be amidated by analogy with leiurotoxin I, so that the amidated form of P05 was synthesized. The disulfide bridge pairings of leiurotoxin I had not been mapped. They were deduced by both enzymatic and partial acidolytic cleavages of the synthetic product sP05-NH_2.[1] The conformational properties of the toxin were assessed by circular dichroism analysis of sP05-NH_2 and molecular dynamics of P05 to improve our understanding of structure–activity relationships and to determine whether P05 may be a specific high-affinity ligand of the potassium channel. The binding properties of P05 were compared with those of several structural C-terminal carboxyl-amidated analogs and apamin.

* Author to whom correspondence should be addressed.
‡ Laboratoire de Biochimie, Faculté de Médecine Nord.
§ Institut Pasteur du Maroc.
∥ Laboratoire de Cristallographie et Cristallisation des Macromolécules Biologiques, Faculté de Médecine Nord.

[1] Abbreviations: sP05-NH_2, synthetic C-terminal carboxyl-amidated P05; [Lys_6,Lys_7] sP05-NH_2, sP05-NH_2 analog whose Arg are substituted by Lys; [Leu_6,Leu_7] sP05-NH_2, sP05-NH_2 analog whose Arg are substituted by Leu; AaH II, neurotoxin II from the scorpion *Androctonus australis* Hector; CsE V3, variant III from the scorpion *Centruroides sculpturatus* Ewing; AaH IT, insect toxin from the scorpion *Androctonus australis* Hector; CD, circular dichroism; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; MPLC, medium-pressure liquid chromatography.

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MATERIALS AND METHODS

Materials. *N*-α-Boc-L-amino acids and 4-methylbenzhydrylamine hydrochloride resin (MBHA) were from Applied Biosystems Inc., except for Boc-His(Z)-OH obtained from Bachem. Dichloromethane, from SDS, was freshly distilled over anhydrous potassium carbonate, and diisopropylethylamine (DIEA) from Merck was distilled over ninhydrin prior to use. Trifluoroacetic acid (TFA), from SDS, was refluxed overnight over chromoxide, filtered, and then distilled over free amino acid. *N*-Methylpyrrolidone, from SDS, was distilled under reduced pressure. Other solvents and reagents were analytical grade commercial products from Sigma and Merck. $Na^{125}I$ was obtained from Amersham Corp.

Chemical Synthesis and Physicochemical Characterization of sP05-NH_2 and Its Analogs. sP05-NH_2 and the analogs ([Lys_6,Lys_7] sP05-NH_2 and [Leu_6,Leu_7] sP05-NH_2) were synthesized by the solid-phase method (Merrifield, 1986) using an automated peptide synthesizer (Model 430A, Applied Biosystems Inc.) (Sabatier et al., 1990). The peptide chains were assembled stepwise on either 0.5 (sP05-NH_2) or 0.25 mequiv (analogs) of MBHA (1% cross-linked; 0.77 mequiv of amino group/g) using the N^{α}-butyloxycarbonyl (Boc) amino acids (2 mmol). Side-chain protecting groups used for trifunctional residues were as follows: benzyl (Bzl) for Glu, Ser, and Thr; 2-chlorocarbobenzoxy (ClZ) for Lys; carbobenzoxy (Z) for His; *p*-tolylsulfonyl (Tos) for Arg; and 4-methylbenzyl (MeBzl) for Cys. N^{α}-Amino groups were deprotected by treatment with 33 and 50% (v/v) TFA/dichloromethane for 80 s and 18 min 30 s, respectively. After washings with dichloromethane (3 × 1 min), the free α-NH_2 was generated with 10% (v/v) DIEA/*N*-methylpyrrolidone (2 × 1 min). After further washings with *N*-methylpyrrolidone (3 × 1 min), the Boc-amino acids were double-coupled (48 min) as their hydroxybenzotriazole (HOBt) active esters in *N*-methylpyrrolidone (4-fold excess). A neutralization step with 10% (v/v) DIEA/*N*-methylpyrrolidone was performed before each recoupling. Coupling tests were not used for monitoring the condensation step.

After full assembly was completed and final cleavage of the N-terminal Boc group, the peptidyl resins (3.05 g for sP05-NH_2 and 1.55 g for the analogs) were treated for 1 h at 0 °C with anhydrous HF/*p*-cresol/ethanedithiol (85:10:5, v/v) in a volume of 15 mL/g of resin. The HF was then removed under reduced pressure, and the peptides were precipitated and washed with cold diethyl ether in the presence of 1% (v/v) β-mercaptoethanol. Finally, the crude peptides were extracted with 15–50 mL of degassed H_2O and partially purified by preparative reversed-phase medium-pressure liquid chromatography (MPLC) (Labomatic, C_{18} HD-SIL 15–25 μm, 26 × 313 mm). A 60-min linear gradient of acetonitrile in 0.1% (v/v) TFA/H_2O was used for elution from either 0 to 30% (sP05-NH_2 and [Lys_6,Lys_7] sP05-NH_2) or 0 to 45% ([Leu_6,Leu_7] sP05-NH_2) at a flow rate of 10 mL/min. The absorbance of the eluate was recorded at a wavelength of 206 nm. After lyophilization, the reduced peptide mixtures (1.15, 0.51, and 0.38 g for sP05-NH_2, [Lys_6,Lys_7] sP05-NH_2, and [Leu_6,Leu_7] sP05-NH_2, respectively) were solubilized in 0.2 M Tris-HCl buffer, pH 8, at a final concentration of 4.73 mM and stirred while exposed to air for folding (48 h, 25 °C). For full solubilization of [Leu_6,Leu_7] sP05-NH_2, guanidine hydrochloride was added to the buffer to a final concentration of 2 M. The target products were purified to homogeneity from injections of 10 mL of oxidized peptide solutions by semipreparative reversed-phase HPLC (Knauer, C_{18} Eurosil Bioselect 20 μm, 13 × 304 mm) using a 90-min linear gradient of acetonitrile in 0.1% (v/v) TFA/H_2O from 0 to 15% (sP05-NH_2 and [Lys_6,Lys_7] sP05-NH_2) and 0 to 30% ([Leu_6,Leu_7] sP05-NH_2) at a flow rate of 5 mL/min (λ = 230 nm).

Full oxidation of the synthetic products was verified by both S-carboxymethylation and 5,5′-dithiobis(2-nitrobenzoic acid) assays, as previously described (Sabatier et al., 1987). The identities of the products were confirmed by the following methods: (a) analytical reversed-phase HPLC (Merck, C_{18} Lichrospher 5 μm, 4 × 250 mm) using a 40-min linear gradient of acetonitrile in 0.1% (v/v) TFA/H_2O from 10 to 70% at a flow rate of 1 mL/min (λ = 230 nm); (b) amino acid analysis after acid hydrolysis (6 N HCl/1% (w/v) phenol, 20–48 h, 112 °C); (c) polyacrylamide gel electrophoresis; and (d) electrospray mass spectrometry (Neosystem Laboratoire).

Polyacrylamide Gel Electrophoresis. High-reticulation phast gels were used at pH 8.5 with migration toward cathode (phast system, Pharmacia). The peptides (100–200 pg) were silver stained according to the instructions of the manufacturer.

Determination of sP05-NH_2 Disulfide Pairings

Proteolytic Digestion. sP05-NH_2 (850 μg) was incubated with 6% (w/w) trypsin in 0.02 M Tris-HCl buffer, pH 8, for 20 h at 37 °C. The peptide fragments were purified by analytical reversed-phase HPLC (Merck, C_{18} Lichrospher 5 μm, 4 × 250 mm) with a 60-min linear gradient of acetonitrile in 0.1% (v/v) TFA/H_2O from 0 to 60% at a flow rate of 1 mL/min (λ = 230 nm). After lyophilization, the amino acid content of the peptides was analyzed after hydrolysis in 6 N HCl. Some of the peptides were further characterized by Edman degradation using a gas-phase microsequencer (Applied Biosystems 470A).

Partial Acid Hydrolysis. sP05-NH_2 (800 μg) was partially hydrolyzed by incubation in 6 N HCl for 10 min at 154 °C. The resulting peptide fragments were isolated by HPLC and analyzed as described above.

Pharmacological Characterization of the Peptides

Specific Neurotoxicity of the Peptides in Mice. The products were tested in vivo for toxicity by determining the 50% lethal dose (LD_{50}) after intracerebroventricular inoculation into 20-g C57/BL6 mice. The LD_{50} values were calculated according to the formula of Behrens and Karber (1935). Groups of eight mice per dose were injected with 5 μL of peptide solution containing 0.1% (w/v) bovine serum albumin and 0.9% (w/v) sodium chloride.

Iodination of the C-Terminal Histidine Amide of sP05-NH_2. The peptide (83 nmol) solubilized in 10 mM Tris-HCl buffer, pH 8.6, was added to a tube precoated with iodogen (250 nmol). After addition of sodium iodine (83 nmol), the mixture (200 μL) was incubated for 1 h at 37 °C. The C-terminally iodinated sP05-NH_2 (diiodo-sP05-NH_2, as determined by electrospray mass spectrometry) was finally purified by HPLC, as described above, and characterized.

Inhibition of Binding of [^{125}I]Apamin to Rat Brain Synaptosomes. Rat brain synaptic nerve ending particles (synaptosomes, P2 fraction) were prepared according to Gray and Whittaker (1962). Protein content was assayed by a modified Lowry method. [^{125}IApamin was obtained as described by Seagar et al. (1984).

Competition Assay (Figures 3A and 4). [^{125}I]Apamin (50 μL) at 10^{-10} M and 400 μL of synaptosomes at 0.4 mg of protein/mL were incubated for 1 h at 0 °C together with 50 μL of one of a series of concentrations of competitor. The incubation buffer was 25 mM Tris-HCl and 10 mM KCl, pH 7.2. The incubation medium was centrifuged, and the resulting

pellets were washed three times in 1 mL of the same buffer. Bound radioactivity was determined (Packard Crystal II).

Displacement Assay (Figure 3A). [^{125}I]Apamin (50 μL) at 10^{-10} M was incubated for 1 h at 0 °C with 400 μL of synaptosomes (0.4 mg of protein/mL) before or after a 1-h incubation of the synaptosomes with 50 μL of a series of concentrations of sP05-NH_2 (10^{-13}–10^{-7} M). In Figure 3B, [^{125}I]apamin was first incubated for 30 min at 0 °C with synaptosomes before addition of a series of concentrations of sP05-NH_2 (10^{-14}–10^{-8} M), as described above. The reaction was stopped after 5, 10, 40, 60, or 90 min of incubation time with sP05-NH_2. The values are the means of quadruplicate assays. Nonspecific binding, less than 10% of the total binding, was determined in the presence of a large excess (10^{-8} M) of unlabeled apamin.

Conformational Analysis

Circular Dichroism Analysis of sP05-NH_2 and Its Analogs. Low ultraviolet spectra were recorded on a Jobin-Yvon spectrophotometer. The instrument was calibrated with (+)-10-camphorsulfonic acid. The spectra were determined at a temperature of 25 °C using a 0.05 cm path length cell, with a 2-s time constant and a scan rate of 0.25 nm/s. The synthetic peptide concentration in the solution, as determined by amino acid analysis, was 250 μg/mL. The spectra were cumulated 5-fold in water or water/trifluoroethanol (TFE) and automatically averaged.

Modeling of P05. The TURBO-FRODO and X-PLOR programs have been used for construction and molecular dynamics of the models, respectively (Roussel & Cambillau, 1989; Brünger, 1990). The P05 model was deduced from the sP05-NH_2 disulfide pairing location together with secondary structures of the very similar leiurotoxin I, previously determined by ^{1}H NMR (Martins et al., 1990). The starting model was refined by 10-ps molecular dynamics at 300 K, and energy was minimized to convergence using the Powell minimizer. The models of apamin from the bee *Apis mellifera mellifera* and of insect toxin from the scorpion *Androctonus australis* Hector (AaH IT; 70 residues, four disulfides) were constructed according to ^{1}H NMR data reported by Wemmer and Kallenbach (1983) and Darbon et al. (1991), respectively. The structures of variant III from the scorpion *Centruroides sculpturatus* Ewing (CsE V3; 65 residues, four disulfides) and of neurotoxin II from the scorpion *Androctonus australis* Hector (AaH II; 64 residues, four disulfides) were obtained by X-ray crystallography (Fontecilla-Camps et al., 1980, 1988). The toxin backbones were fitted with the fitting routine of the TURBO-FRODO software. For P05 and CsE V3, the best fit was obtained by using 23 α-carbons of P05, omitting four residues at each extremity, and gave a root mean square distance (RMSD) of 0.79 Å for backbone atoms. The fit between P05 and apamin was obtained by using seven α-carbons (i.e., residues 5–11 of P05), taking into account the sequence identity, and gave an RMSD value of 0.42 Å.

RESULTS

The primary structures of P05 (Zerrouk et al., submitted), leiurotoxin I, and apamin are presented in Figure 1. For the chemical synthesis of P05, the amidated form was chosen by analogy with leiurotoxin I, although the C-terminus of native P05 (free carboxylate or amide) was not established at this time because of the low quantity available. The comparison of the synthetic peptide (in its carboxyl-amidated form) with the natural toxin by electrospray mass spectrometry, HPLC, and electrophoresis has shown unambiguously that native P05 has a C-terminal carboxylate (Zerrouk et al., submitted).

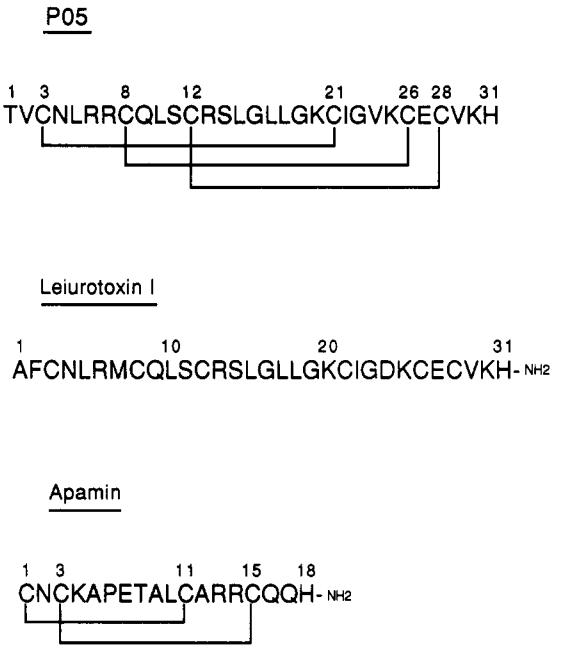

FIGURE 1: Primary structures (one-letter code) of P05, leiurotoxin I, and apamin. Established (apamin) or deduced (P05) disulfide bridge pairings are indicated by plain lines.

sP05-NH_2 was synthesized on 0.5 mmol of MBHA using optimized Boc/benzyl chemistry. After peptide chain assembly, the amount of final product linked to the resin was about 0.44 mmol, which represents an overall assembly yield of 88% (Δ resin weight = 2.4 g; M_r protected peptide = 5491 Da). The analytical HPLC profile of crude reduced peptide after HF cleavage (cleavage yield of 90%, estimated by weight) is shown in Figure 2A. The crude peptide was then partially purified by preparative MPLC, oxidized by exposure to air (Figure 2B), and purified to 98% homogeneity by semi-preparative HPLC (Figure 2C). As determined by amino acid analysis, the net peptide content of lyophilized sP05-NH_2 was 67%. The overall synthesis yield of sP05-NH_2, including peptide assembly, HF cleavage, MPLC, folding, and HPLC purification, was about 3% (63 mg). Amino acid ratios were as follows: Asp 1.0 (1), Thr 1.0 (1), Ser 1.8 (2), Glu 2.1 (2), Gly 3.1 (3), Cys 5.2 (6), Val 2.8 (3), Ile 1.0 (1), Leu 5.0 (5), His 1.0 (1), Lys 2.9 (3), and Arg 3.0 (3). The sP05-NH_2 appeared to be homogeneous by high-reticulation phast gel electrophoresis, which gave a single band (data not shown). Electrospray mass spectrometry of sP05-NH_2 gave an experimental mean M_r of 3414.6 ± 0.7 (three values), which is in perfect agreement with the deduced M_r of 3414.3 Da. The M_r of the native toxin was 3415.4 ± 0.6 (three values).

Synthesis of the two Arg-subtituted analogs yielded about 20 mg of purified products. Amino acid analyses after hydrolysis were as follows: [Lys_6,Lys_7] sP05-NH_2, Asp 1.0 (1), Thr 0.9 (1), Ser 1.7 (2), Glu 2.1 (2), Gly 3.3 (3), Cys 5.1 (6), Val 2.8 (3), Ile 1.0 (1), Leu 4.7 (5), His 1.0 (1), Lys 5.0 (5), Arg 0.9 (1); [Leu_6,Leu_7] sP05-NH_2, Asp 0.9 (1), Thr 0.9 (1), Ser 1.8 (2), Glu 2.1 (2), Gly 3.3 (3), Cys 4.9 (6), Val 2.7 (3), Ile 1.0 (1), Leu 6.8 (7), His 1.0 (1), Lys 3.1 (3), and Arg 1.0 (1). Electrospray mass spectrometry gave experimental means M_r of 3357.3 ± 0.3 for [Lys_6,Lys_7] sP05-NH_2 (deduced M_r of 3358.2 Da) and 3327.8 ± 0.6 for [Leu_6,Leu_7] sP05-NH_2 (deduced M_r of 3328.2 Da). sP05-NH_2 was iodinated and the diiodo derivative was purified (experimental M_r of 3666.0 ± 0.6, deduced M_r of 3666.1 Da).

All of the synthetic peptides, native P05, and apamin were tested for inhibition of [^{125}I]apamin binding to rat brain

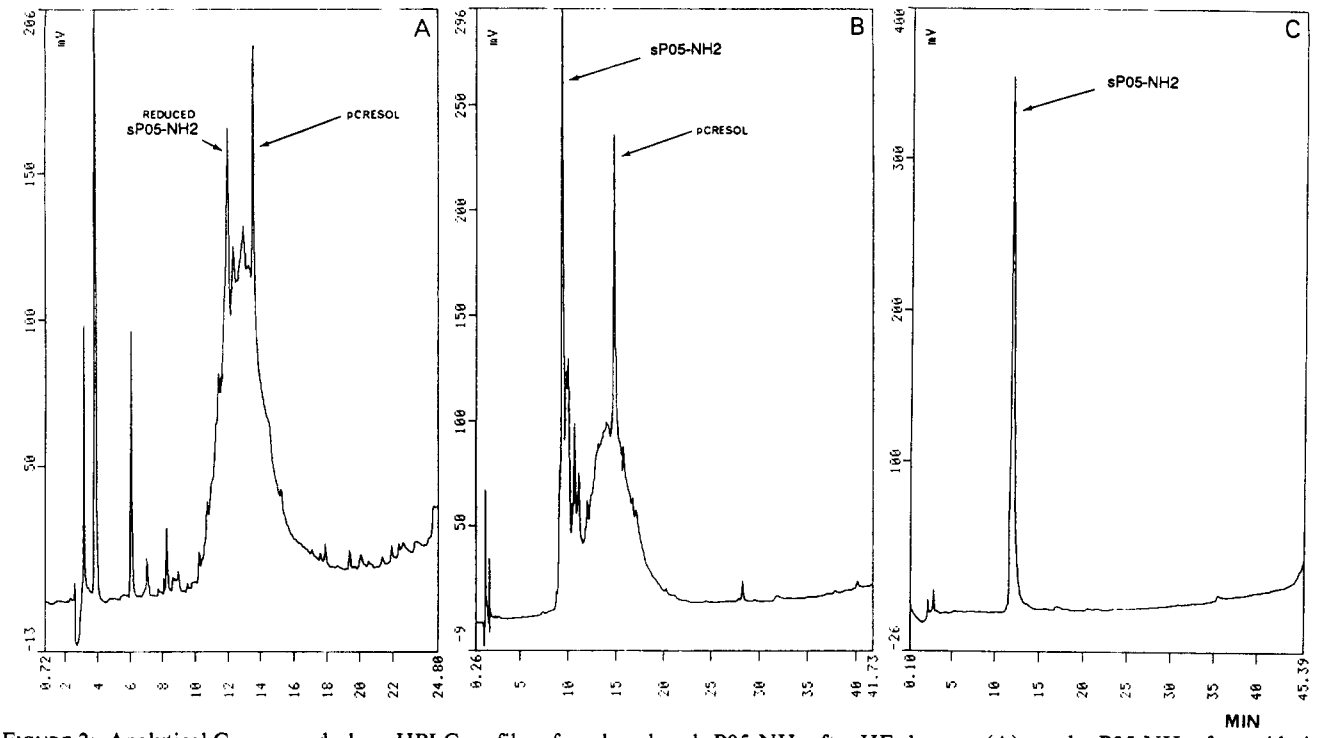

FIGURE 2: Analytical C_{18} reversed-phase HPLC profiles of crude reduced sP05-NH_2 after HF cleavage (A), crude sP05-NH_2 after oxidation (B), and purified sP05-NH_2 (C). Experimental conditions are described in the Materials and Methods section.

synaptosomes. sP05-NH_2 competed with [^{125}I]apamin and had a much higher affinity for the binding site (Figure 3A). The binding of [^{125}I]apamin was totally abolished at a 10^{-13} M concentration of sP05-NH_2 when the competing molecules were added together or when sP05-NH_2 was preincubated with the synaptosomes before addition of [^{125}I]apamin. However, when sP05-NH_2 was added 1 h after [^{125}I]apamin, about 10% of the [^{125}I]apamin remained bound at a concentration of 10^{-7} M. Figure 3B shows binding experiments at different incubation times with sP05-NH_2. The longer the period of incubation, the more efficient the displacement of [^{125}I]apamin from its receptor by sP05-NH_2. These experiments suggest that sP05-NH_2 either binds irreversibly or dissociates much more slowly than apamin. Native P05 and analogs of sP05-NH_2 also competed with [^{125}I]apamin for binding to synaptosomes (Figure 4), with half-effects ($K_{0.5}$) obtained at concentrations of 6×10^{-12} (apamin), 2×10^{-11} (P05), 2×10^{-10} (diiodo-sP05-NH_2), 2×10^{-9} ([Lys_6,Lys_7] sP05-NH_2), and 10^{-7} M ([Leu_6,Leu_7] sP05-NH_2). In vivo, the synthetic peptides—except [Leu_6,Leu_7] sP05-NH_2—caused toxic and lethal effects in mice, with clinical symptoms identical to those induced by native P05 or apamin. The LD_{50} values per mouse were 12 (apamin), 20 (sP05-NH_2), 80 (diiodo-sP05-NH_2), and 200 ng ([Lys_6,Lys_7] sP05-NH_2). Peptide [Leu_6,Leu_7] sP05-NH_2 was inactive at the dose of 10 μg.

To investigate conformational properties of sP05-NH2, disulfide pairings were established after enzymatic digestion and partial acid hydrolysis. Tryptic cleavage of sP05-NH_2 yielded two free amino acids, three proteolytic fragments, and uncleaved sP05-NH_2 (Figure 5). Unexpectedly, chymotrypsin did not cleave sP05-NH_2 at Leu residues. Analysis of the tryptic digest components (see the Materials and Methods section and Figure 5) indicated disulfide bridging between Cys_3 and Cys_{21} and either Cys_8 and Cys_{26} and Cys_{12} and Cys_{28} or Cys_8 and Cys_{28} and Cys_{12} and Cys_{26}. A peptide was isolated from the partial acid hydrolysis of sP05-NH_2 by analytical HPLC (retention time of 28 min). It was fragment Leu_{10}–Gly_{16} linked to Cys_{28}–His_{31}-NH_2. This clearly indicates the presence of disulfide Cys_{12}–Cys_{28}. This chemically defined disulfide arrangement was confirmed by ^{1}H NMR structural analysis of sP05-NH_2 (Manuscript in preparation), thus eliminating the possibility that the disulfides had been scrambled during hydrolysis with a halogen acid. From NMR data, nuclear Overhauser effects (NOE) were detected between β-protons of Cys_8–Cys_{26} and Cys_{12}–Cys_{28}, while no NOE was observed between Cys_8 and Cys_{28}. Thus, the disulfides were mapped between Cys_3 and Cys_{21}, Cys_8 and Cys_{26}, and Cys_{12} and Cys_{28}.

A model for P05 was obtained by molecular dynamics (Figure 6) using the positions of the disulfide bridges of sP05-NH_2 and the secondary structure of leiurotoxin I (i.e., α-helix from Arg_6 to Gly_{16}, β-sheet from Leu_{18} to Val_{29}, and β-turn at Gly_{23}–Asp_{24}) (Martins et al., 1990). The resulting minimized model has a total energy of −810 kcal/mol. P05 appears to be mainly composed of a double-stranded, antiparallel β-sheet (from Leu_{18} to Val_{29}) linked to an α-helix (from Arg_6 to Gly_{16}) by disulfides (Cys_8–Cys_{26} and Cys_{12}–Cys_{28}) and to an extended fragment (from Thr_1 to Leu_5) by disulfide (Cys_3–Cys_{21}). Circular dichroism analyses of sP05-NH_2, [Lys_6,Lys_7] sP05-NH_2, and [Leu_6,Leu_7] sP05-NH_2 were performed at various pH values or percentages of TFE. The spectra obtained for each peptide under different conditions were superimposable, confirming the rigidity of their structures which were insensitive to their environments (Figure 7). The spectra exhibited a double minimum at 207–220 nm and a positive band at 190 nm, indicating the presence of partial α-helical structure. Remarkably, the P05 polypeptide backbone from the model was found to be very similar to that of particular stretches of CsE V3 (Figure 8), AaH II, and AaH IT (data not shown). Leu_5, Arg_6, Arg_7, Cys_8, and Gln_9 of P05 could be superimposed in three dimensions on Leu_{10}, Arg_{13}, Arg_{14}, Cys_{15}, and Gln_{16} of apamin, respectively (Figure 9A). This similar spatial arrangement of particular residues in the helical region of the toxins was associated with a common Arg-Arg-Cys-Gln sequence (Figure 9B).

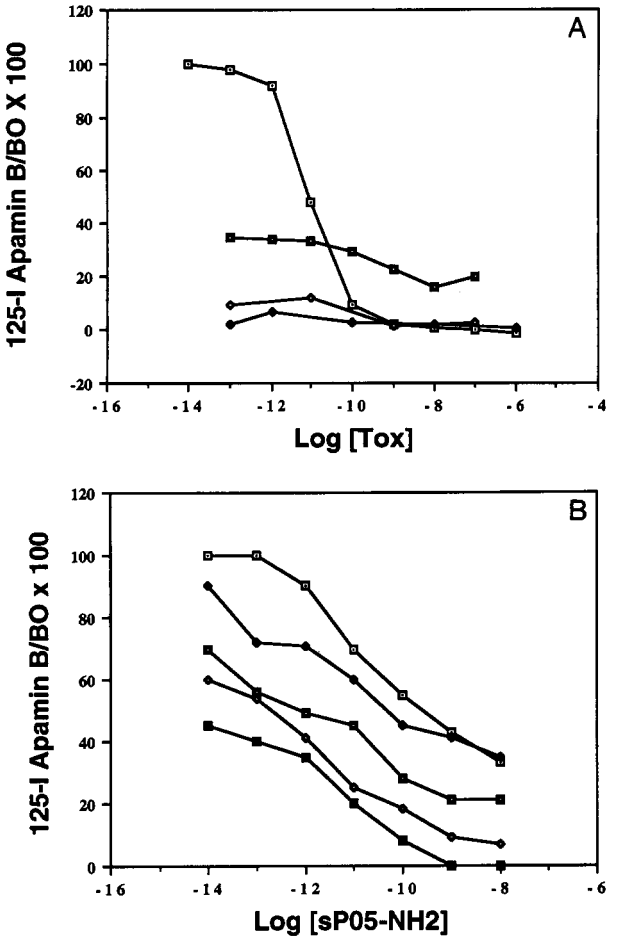

FIGURE 3: (A) Inhibition of binding of [^{125}I]apamin to rat brain synaptosomes by competition and displacement assays. In the competition assay, [^{125}I]apamin and synaptosomes were incubated together with a series of concentrations of either unlabeled apamin (⧈) or sP05-NH_2 (◆). In the displacement assay, sP05-NH_2 was added and incubated with the synaptosomes before (◇) or after (□) incubation of the synaptosomes with [^{125}I]apamin. (B) Inhibition of binding of [^{125}I]apamin to rat brain synaptosomes by unlabeled sP05-NH_2 as a function of incubation times. In this displacement assay, [^{125}I]apamin was preincubated with synaptosomes before addition of a series of concentrations of sP05-NH_2. After addition of sP05-NH_2, the incubation was stopped at the times of 5 (⧈), 10 (◆), 40 (□), 60 (◇), and 90 min (■). B_0 and B are the binding of [^{125}I]-apamin in the absence or in the presence of sP05-NH_2, respectively. Experimental conditions for A and B are described in the Materials and Methods section.

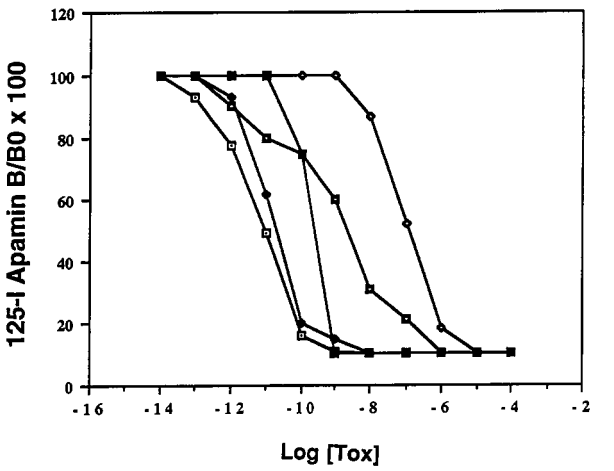

FIGURE 4: Inhibition of binding of [^{125}I]apamin to rat brain synaptosomes by apamin (⧈), native P05 (◆), [Lys_6,Lys_7] sP05-NH_2 (□), [Leu_6,Leu_7] sP05-NH_2 (◇), and diiodo-sP05-NH_2 (■) in a competition assay. B_0 is the binding of [^{125}I]apamin in the absence of ligand, and B is the binding in the presence of the indicated concentrations of competitors. Experimental conditions are described in the Materials and Methods section.

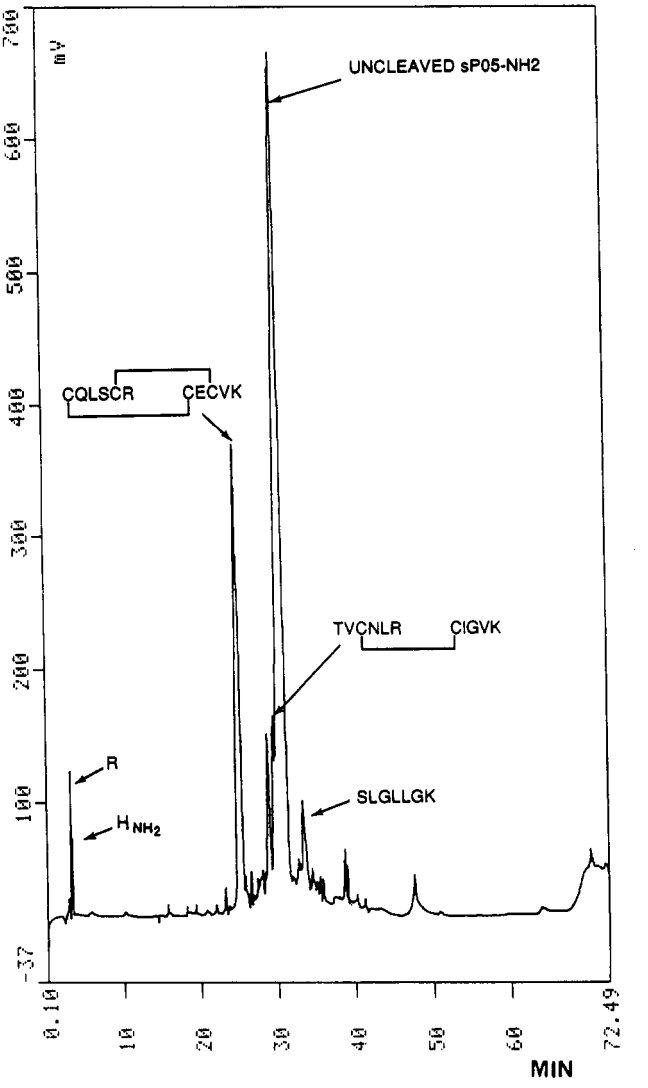

FIGURE 5: Analytical C_{18} reversed-phase HPLC profile of sP05-NH_2 tryptic digestion. The peptides corresponding to the peaks are indicated.

DISCUSSION

P05-NH_2 and its analogs were synthesized by the solid-phase method using the Boc/benzyl scheme and a double-coupling strategy. This optimized protocol permits efficient assembly with high step yields and percentage of target peptide in the crude product. After final cleavage, the reduced peptide could be air-oxidized in a concentration-independent manner, from 0.01 to 5 mM, without any significant modification of the folding process (data not shown). The folding was rapid: air oxidation was completed in less than 4 h. The dialysis oxidation system with a pH gradient from 2.2 to 8, as described (Sabatier et al., 1987), also caused the synthetic peptide to adopt the native folding conformation. This is unusual and indicates that the spontaneous intrachain folding is a thermodynamically favored process. Leiurotoxin I probably adopts this folding pattern as P05, on the basis of sequence similarity (Martins et al., 1990). In contrast, refolding of reduced AaH II (64 residues, four disulfides) was previously found to be very difficult (Sabatier et al., 1987), in spite of the existence of a structural motif common to P05. As the P05 sequence

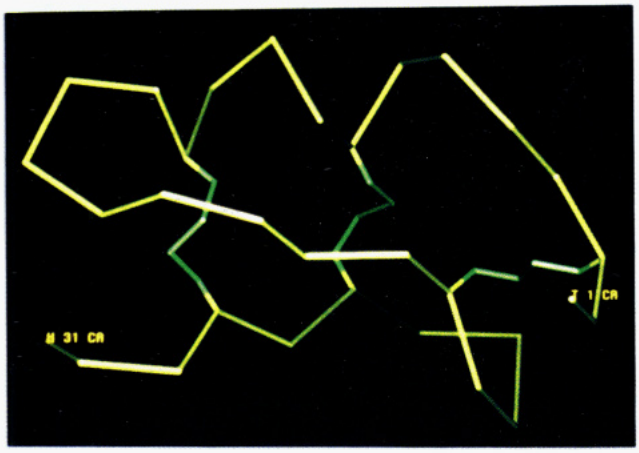

FIGURE 6: Modeling of P05 by molecular dynamics. The polypeptide backbone is in yellow, and the three disulfide bridges are in green. The N- and C-terminal residues of P05 are indicated.

is similar to that of leiurotoxin I, their three-dimensional organizations are presumably very close.

Structural data from leiurotoxin I were used together with the positions of disulfide bridges in sP05-NH_2 (Cys_3–Cys_{21}, Cys_8–Cys_{26}, and Cys_{12}–Cys_{28}) to model P05. The resulting P05 conformation superimposed well on that of particular stretches of CsE V3, AaH II, and AaH IT, although these toxins share neither sequence nor activity similarities. CsE V3 belongs to the β-type toxin structural group, while AaH II and AaH IT are α-type and insect-directed toxins, respectively (Jover et al., 1980; Couraud et al., 1982). All of these molecules possess a triple-stranded, antiparallel β-sheet linked to an α-helix. The main differences are the orientation and length of the loop connecting two strands of the β-sheet (Fontecilla-Camps, 1989). This length varies from a tight turn in both CsE V3 and AaH IT to a large loop in AaH II (Fontecilla-Camps et al., 1980, 1988; Darbon et al., 1991). This observation agrees with a recent report claiming the existence of a common structural motif in all known scorpion toxins also found in insect defensins, irrespective of their size, primary structure, and biological activity (Bontems et al., 1991; Bonmatin et al., 1992).

Pharmacological experiments on rat brain synaptosomes indicated that the C-terminal carboxyl-amidated analog of P05 (sP05-NH_2) binds irreversibly, a novel property for scorpion toxins. It could therefore serve as a tool for the purification of the receptor protein. In agreement with structure–activity relationship studies on leiurotoxin I, this result indicates that the C-terminal His is implicated in the toxin to receptor interaction. Interestingly, the specific binding properties of sP05-NH_2 need the integrity of the His imidazole ring, as shown by the different activity of the iodinated product.

From the models, the N-terminal region of P05 appears to be structurally similar to the C-terminus of apamin: both contain an α-helical core with a pair of Arg residues protruding from the surface of the molecule (Wemmer & Kallenbach, 1983). The pair of Arg residues (Arg_{13}–Arg_{14}) was reported to be crucial for high apamin pharmacological activity (Vincent et al., 1975). These residues have been shown to interact with the receptor mainly by the two adjacent positive charges (Granier et al., 1978; Cook & Haylett, 1985; Seagar et al., 1987) but also by their side chains (Labbé-Jullié et al., 1991). These observations suggest that Arg_6 and Arg_7 of P05 may be essential, or at least contribute, to the apamin-like biological properties of P05. Some authors (Martins et al., 1990) have speculated on the importance of Arg_6 and Arg_{13} of leiurotoxin I, both located in the α-helix, for expression of biological

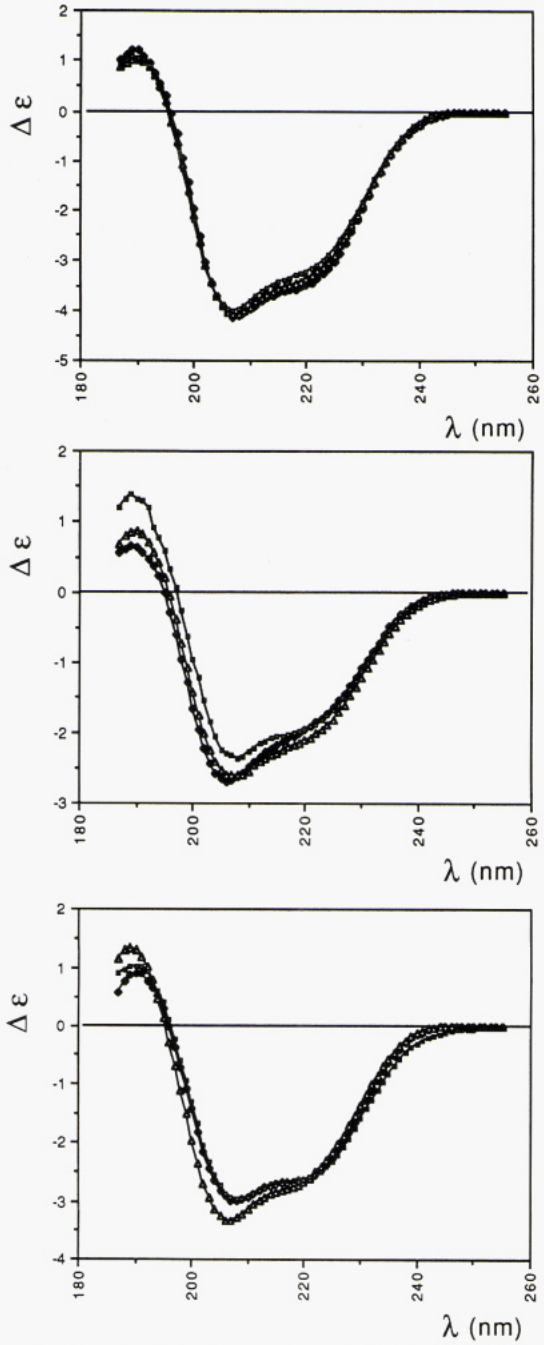

FIGURE 7: Circular dichroism spectra of sP05-NH_2 (top), [Leu_6, Leu_7] sP05-NH_2 (middle), and [Lys_6,Lys_7] sP05-NH_2 (bottom) solubilized in water (◆), water/trifluoroethanol (v/v) (△), and trifluoroethanol (□). $\Delta\epsilon$ corresponds to the variation of molar amino acid residue absorption coefficient expressed in M^{-1} cm^{-1}.

activity, although these Arg residues are not sequential. To investigate their importance in the bioactivity of sP05-NH_2, two Arg-substituted sP05-NH_2 analogs were designed and synthesized. Pharmacological assays clearly showed that these residues, or at least positive charges, are required for the biological activity of sP05-NH_2. Analogs whose Arg residues are substituted by Lys ([Lys_6,Lys_7] sP05-NH_2) had lower activities than sP05-NH_2 (10% toxicity; $K_{0.5} = 2 \times 10^{-9}$ M), while those whose Arg residues are substituted by Leu ([Leu_6,-Leu_7] sP05-NH_2) exhibited only residual activity (toxicity <0.2%; $K_{0.5} = 10^{-7}$ M). These results agree with structure–activity analyses of apamin. Circular dichroism spectra of these analogs solubilized in different solvents are very similar to those obtained with sP05-NH_2. Thus, the overall secondary structures of the analogs are similar to that of sP05-NH_2 and

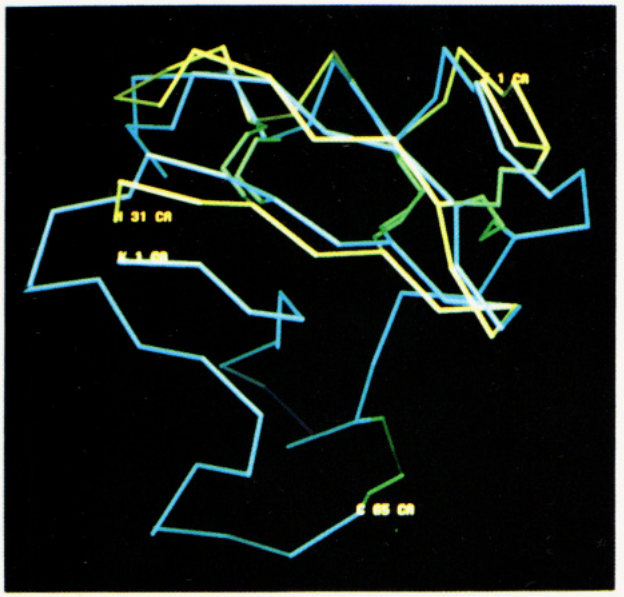

FIGURE 8: Superimposition of the P05 model on the corresponding domain of $CsEV_3$. Backbones of P05 and CsE V3 are shown in yellow and blue, respectively. The disulfide bridges are in green. The N- and C-termini of the toxins are indicated.

presumably, therefore, not directly responsible for the differences in bioactivities.

Structure–activity analysis of the new natural analog of leiurotoxin I, P05, has shown the following: (i) the C-terminal carboxyl amidation of His induces a considerable strengthening of the toxin to receptor interaction, giving to sP05-NH_2 the properties of an irreversible, apamin-sensitive, Ca^{2+}-activated K^+ channel blocker; (ii) the two Arg residues, presumably located in an α-helix (like apamin), and the C-terminal His are involved in the interaction with the receptor, and any modification of these residues considerably lowers the affinity for the binding site. Modeling of P05 suggests that the three residues are located on the same side of the molecule. For the three common pharmacologically related toxins, apamin, leiurotoxin I, and P05, it seems that the α-helix holds the positive charges of Arg residues in the appropriate positions for activity. Because the interaction of apamin, P05, and leiurotoxin I is already of high affinity with the Ca^{2+}-activated K^+ channel ($K_{0.5} \approx 10^{-11}$ M), any modification which favors interaction of the ligand with its receptor may cause binding to appear to be irreversible. This seems to be the case for the C-terminal amidation of P05. The overall data strongly suggest the importance of the common α-helical core for

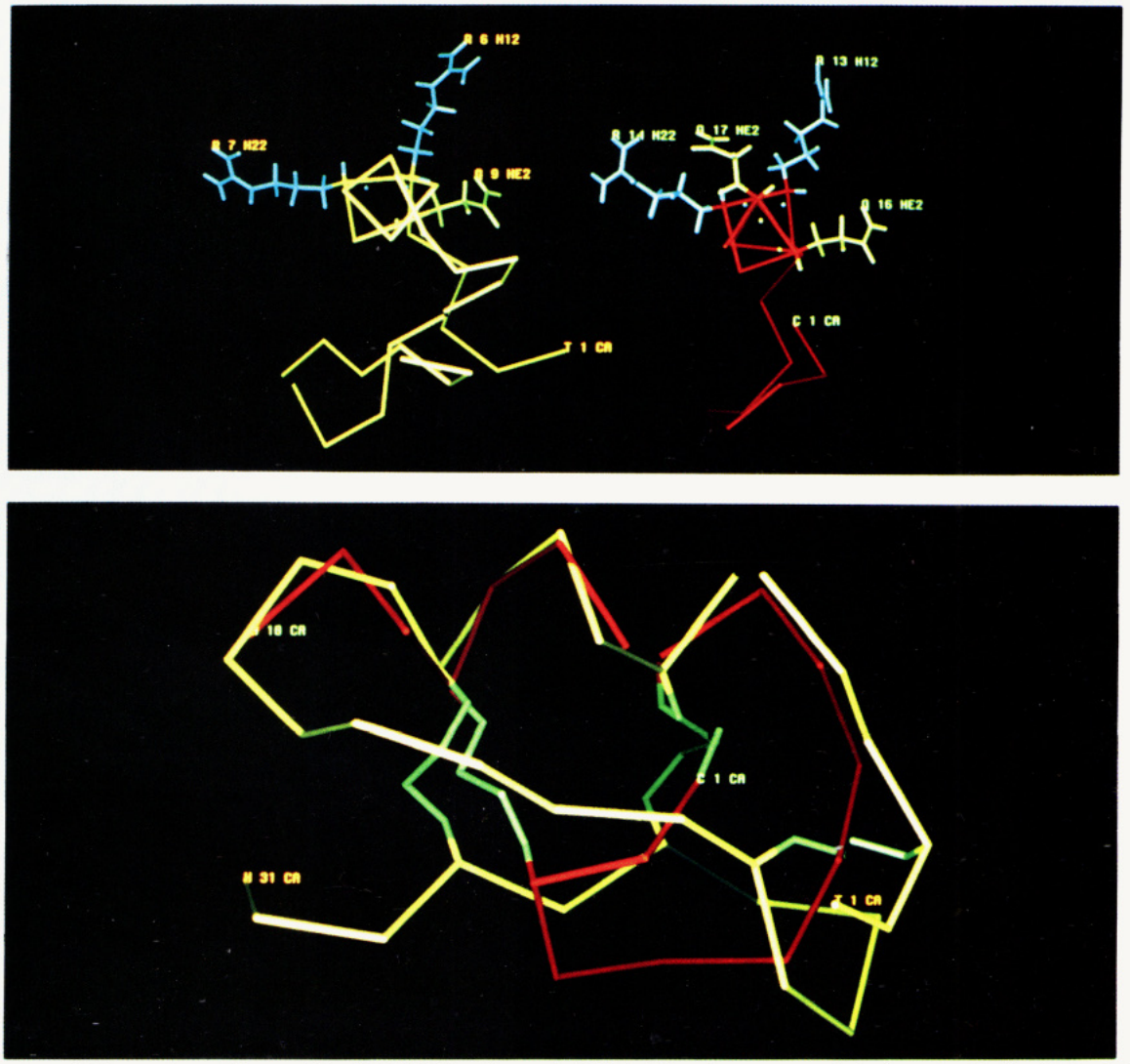

FIGURE 9: (A, top) Comparison of the models of P05 (left) and apamin (right). The view corresponds to the axis of both α-helical structures. Backbones of P05 and apamin are shown in yellow and red, respectively. The side chains of the Arg and Gln residues are represented in blue and green, respectively. The N-termini and some target residues are indicated for both toxins. The disulfide bridges have been omitted for clarity. (B, bottom) Superimposition of the models of P05 and apamin. In this front view, the backbones of P05 and apamin are shown in yellow and red, respectively. The disulfide bridges are in green. The N- and C-termini of toxins are indicated.

activity of this group of pharmacologically related toxins. Interestingly, extensive structure–activity relationship studies of long scorpion toxins (60–70 residues, four disulfide bridges) indicate that, in this case, the α-helical structure is not involved in pharmacological activity, but more probably in stabilizing an active conformation of a solvent-exposed hydrophobic region (Darbon et al., 1983; Kharrat et al., 1989, 1990): the α-helices of the long toxins do not contain an Arg residue. This raises the question of the biological significance of the other domains of the toxins besides maintenance of a bioactive conformation. Further studies on scorpion toxins are needed to understand the precise molecular basis of the toxin–receptor recognition.

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