Synthesis and Characterization of Leiurotoxin I Analogs Lacking One Disulfide Bridge: Evidence That Disulfide Pairing 3−21 Is Not Required for Full Toxin Activity

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ABSTRACT: Leiurotoxin I (Lei-NH_2), a toxin isolated from the venom of the scorpion *Leiurus quinquestriatus hebraeus*, is a blocker of the apamin-sensitive Ca^{2+}-activated K^+ channels. It is a 31-residue polypeptide cross-linked by three disulfide bridges which are presumably between Cys_3−Cys_{21}, Cys_8−Cys_{26}, and Cys_{12}−Cys_{28}. To investigate the role of these disulfides, analogs of Lei-NH_2 lacking one disulfide bridge (i.e., [$Abu_{3,21}$]Lei-NH_2, [$Abu_{8,26}$]Lei-NH_2, and [$Abu_{12,28}$]Lei-NH_2) were chemically synthesized by selective replacement of each pair of half-cystines forming a bridge by two α-aminobutyrate (Abu) residues. The two disulfide pairings of the main folded form of the synthetic analogs were established by enzymatic proteolysis. They were as expected between Cys_8−Cys_{26} and Cys_{12}−Cys_{28} for [$Abu_{3,21}$]Lei-NH_2 but were unexpectedly between Cys_3−Cys_{12} and Cys_{21}−Cys_{28} for [$Abu_{8,26}$]Lei-NH_2 and between Cys_3−Cys_8 and Cys_{21}−Cys_{26} for [$Abu_{12,28}$]Lei-NH_2. The synthetic peptides were tested in vitro for their capacity to compete with the binding of [^{125}I]apamin to rat brain synaptosomes and in vivo for their neurotoxicity in mice. In both assays, [$Abu_{3,21}$]Lei-NH_2 exhibited full Lei-NH_2-like activity whereas [$Abu_{8,26}$]Lei-NH_2 and [$Abu_{12,28}$]-Lei-NH_2 possessed only residual activities (<2% native toxin activity). This suggests that disulfide bridge Cys_3−Cys_{21} is not essential *per se* for high toxin activity. Circular dichroism (CD) spectroscopy of the three analogs showed that only [$Abu_{3,21}$]Lei-NH_2 exhibited a CD spectrum similar to that of Lei-NH_2, suggesting they both adopt closely related conformations, in agreement with the pharmacological data. Structural models of the analogs were constructed on the basis of the disulfide pairing assignment and compared with that of Lei-NH_2.

Polypeptide animal toxins are useful pharmacological probes to study ion-specific channel proteins because they alter channel function by interaction with and modulation of their activities (Strong, 1990; Garcia et al., 1991; Martin-Eauclaire & Couraud, 1995). In the last decade, toxins acting on various types of K^+ channels have been isolated from diverse scorpion venoms (Miller, 1995). One such toxin, leiurotoxin I (also called scyllatoxin), was purified from the venom of the Israeli scorpion *Leiurus quinquestriatus hebraeus* (0.02% of total protein in crude venom) and characterized. Leiurotoxin I (Lei-NH_2)[1] is a 31-residue toxin cross-linked by three disulfide bridges (Chicchi et al., 1988; Auguste et al., 1990). It is a potent blocker of small conductance Ca^{2+}-activated K^+ channels (SK channels) in various cell types (Abia et al., 1986; Chicchi et al., 1988; Moczydlowski et al., 1988; Castle et al., 1989). The binding and physiological properties of Lei-NH_2 are similar to those of the bee venom toxin, apamin (18 residues, two disulfide bridges). However, the sequences of the two toxins are dissimilar (Gauldie et al., 1976; Hugues et al., 1982; Blatz & Magleby, 1986; Castle & Strong, 1986; Auguste et al., 1990; Sabatier et al., 1994). In contrast, scorpion toxin P05 (31 residues, three disulfide bridges), which also possesses Lei-NH_2/apamin-like biological properties, is structurally related to Lei-NH_2 with 87% sequence identity (Zerrouk et al., 1993). The structure−activity relationships in this group of pharmacologically related toxins, Lei-NH_2, P05, and apamin, have been studied using synthetic analogs (Vincent et al., 1975; Granier et al., 1978; Labbé-Jullié et al., 1991; Sabatier et al., 1993, 1994). These studies suggest that particular positively charged residues (Arg_6 and Arg_{13} for Lei-NH_2, Arg_6 and Arg_7 for P05, Arg_{13} and Arg_{14} for apamin) are important for expression of the toxin biological activities. Notably, these residues are located within the α-helical core. Solution structures of several Lei-NH_2 and P05 analogs were recently solved by means of ^{1}H-NMR spectroscopy (Pagel et al., 1994; Inisan et al., 1995; Meunier et al., 1993) and indicate that the two groups of molecules adopt the same disulfide pairing arrangement (i.e., Cys_3−Cys_{21}, Cys_8−Cys_{26}, and Cys_{12}−Cys_{28}) with the classical "α/β scorpion fold" (Bontems et al., 1991). Although the disulfide pairings had not been formerly established for Lei-NH_2, they are likely to be organized accordingly.

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[1] Abbreviations: Lei-NH_2, C-terminal carboxyl-amidated leiurotoxin I (natural toxin); [$Abu_{3,21}$]Lei-NH_2, Lei-NH_2 analog with Abu_3 and Abu_{21}; [$Abu_{8,26}$]Lei-NH_2, Lei-NH_2 analog with Abu_8 and Abu_{26}; [$Abu_{12,28}$]Lei-NH_2, Lei-NH_2 analog with Abu_{12} and Abu_{28}; Abu, α-aminobutyrate; LD_{50}, 50% lethal dose; CD, circular dichroism; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; MPLC, medium-pressure liquid chromatography.

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We report a study of the structure and function of the Lei-NH_2 disulfide bridges by chemical synthesis and testing of folded toxin analogs lacking one of the three disulfide bridges (i.e., [$Abu_{3,21}$]Lei-NH_2, [$Abu_{8,26}$]Lei-NH_2, and [$Abu_{12,28}$]Lei-NH_2). In these analogs, each pair of half-cystines forming a bridge was replaced by two L-α-aminobutyrate (Abu) residues. Abu was chosen because it is an isoster to half-cystine and possesses similar polarity. The disulfide pairings of the folded peptides were characterized by enzymatic cleavage and analysis of the proteolytic fragments. The synthetic analogs were tested in vitro for their capacity to compete with the binding of [^{125}I]apamin to rat brain synaptosomes and in vivo for their neurotoxicity following intracerebroventricular inoculation of mice. Circular dichroism (CD) spectroscopy was used to assess conformational properties of the analogs. Structural models were also constructed on the basis of the disulfide pairing assignment and compared with that of Lei-NH_2.

MATERIALS AND METHODS

Materials. N^{α}-Fmoc-L-amino acids, Fmoc-amide resin, and reagents used for peptide synthesis were from Perkin-Elmer. Solvents were analytical grade products from SDS. Synthetic Lei-NH_2 and enzymes (chymotrypsin, trypsin, and endoproteinase Glu-C) were obtained from Bachem and Boehringer Mannheim, respectively.

Chemical Synthesis and Physicochemical Characterization of Lei-NH_2 Analogs. The Lei-NH_2 analogs ([$Abu_{3,21}$]Lei-NH_2, [$Abu_{8,26}$]Lei-NH_2, and [$Abu_{12,28}$]Lei-NH_2) were synthesized by the solid-phase method (Merrifield, 1986) using an automated peptide synthesizer (Model 433A, Applied Biosystems Inc.). The peptide chains were assembled stepwise on 0.3 mequiv of Fmoc-amide resin (1% cross-linked; 0.64 mequiv of amino group/g) using 1 mmol of N^{α}-fluorenylmethyloxycarbonyl (Fmoc) amino acid derivatives (Carpino & Han, 1972). Side-chain protecting groups used for trifunctional residues were as follows: trityl (Trt) for Cys, His, Asn, and Gln; *tert*-butyl (*t*-Bu) for Ser, Asp, and Glu; pentamethylchroman (Pmc) for Arg, and *tert*-butyloxycarbonyl (Boc) for Lys. N^{α}-amino groups were deprotected by treatment with 18% and 20% (v/v) piperidine/*N*-methylpyrrolidone for 3 and 8 min, respectively. After repeated washing with *N*-methylpyrrolidone (5 × 1 min), the Fmoc-amino acids were double-coupled (2 × 20 min) as their hydroxybenzotriazole (HOBt) active esters in *N*-methylpyrrolidone (3-fold excess). After full assembly was completed and the N-terminal Fmoc group was removed, the peptidyl resins (about 1.5 g for each analog) were treated for 2 h at 25 °C with a mixture of trifluoroacetic acid/H_2O/thioanisole/ethanedithiol (88:5:5:2 v/v) in the presence of crystalline phenol (2.2 g) in a final volume of 20 mL/g of peptidyl resin. The peptide mixtures were then filtered to remove resin, and the filtrates were precipitated and washed twice by adding cold *tert*-butylmethyl ether. The resulting crude peptides were pelleted by centrifugation (2500*g*; 10 min) and the supernatants discarded. The peptides were then dissolved in H_2O and lyophilized. The reduced peptides were then solubilized in 0.2 M Tris-HCl buffer, pH 8, at a final concentration of 2 mM and stirred under air to allow folding (48 h, 25 °C). For full solubilization of the analogs, guanidine hydrochloride was added to the buffer to a final concentration of 2 M. The main oxidized products were purified to homogeneity by preparative reversed-phase medium-pressure liquid chromatography (Labomatic, C_{18} HD-SIL 15−25 μm, 26 × 313 mm) using a 90 min linear gradient of 0.08% (v/v) trifluoroacetic acid (TFA)/acetonitrile in 0.1% (v/v) TFA/H_2O from 0% to 45% at a flow rate of 8 mL/min (λ = 230 nm). The identities of the analogs were assessed by the following methods: (i) analytical reversed-phase HPLC (Merck, C_{18} Lichrospher 5 μm, 4 × 200 mm) using a 60-min linear gradient of 0.08% (v/v) TFA/acetonitrile in 0.1% (v/v) TFA/H_2O from 0% to 60% at a flow rate of 1 mL/min (λ = 230 nm); (ii) amino acid analysis after acid hydrolysis [6 N HCl/2% (w/v) phenol, 20 h, 120 °C, N_2 atmosphere]; and (iii) electrospray mass spectrometry (Neosystem Laboratoire).

Pharmacological Characterization of Lei-NH_2 Analogs. (A) Neurotoxicity of the Peptides in Mice. The peptides were tested in vivo for toxicity by determining the 50% lethal dose (LD_{50}) after intracerebroventricular inoculation into 20 g of 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 solutions containing 0.1% (w/v) bovine serum albumin and 0.9% (w/v) sodium chloride.

(B) Competition Assays on 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}I]Apamin (2000 Ci/mmol) was obtained as described by Seagar et al. (1984). Aliquots of 50 μL of 10^{-10} M [^{125}I]apamin were added to 400 μL of synaptosome suspension (0.4 mg of protein/mL). Samples were incubated for 1 h at 0 °C together with 50 μL of one of a series of concentrations (10^{-13} to 10^{-3} M) of one of the Lei-NH_2 analogs in a final volume of 500 μL. The incubation buffer was 25 mM Tris-HCl and 10 mM KCl, pH 7.2. The samples were then centrifuged, and the resulting pellets were washed three times in 1 mL of the same buffer. Bound radioactivity was determined (Packard Crystal II). The values are the means of triplicate experiments. Nonspecific binding, less than 10% of the total binding, was determined in the presence of an excess (10^{-8} M) of unlabeled apamin.

Assignment of the Disulfide Pairings of Lei-NH_2 Analogs by Enzymatic Cleavage. The oxidized peptides (200−800 μg) were incubated with a mixture of trypsin/chymotrypsin/endoproteinase Glu-C at 10% (w/w) each in 50 mM sodium phosphate buffer, pH 7.1 or 7.8, for 20 h at 37 °C. The peptide fragments were purified by analytical reversed-phase HPLC (Merck, C_{18} Lichrospher 5 μm, 4 × 200 mm) with a 60 min linear gradient from 0% to 60% of 0.08% (v/v) TFA/acetonitrile in 0.1% (v/v) TFA/H_2O at a flow rate of 1 mL/min (λ = 230 nm). The fragments were lyophilized and hydrolyzed in 6 N HCl/phenol and their amino acid contents analyzed (Beckman, System 6300 amino acid analyzer). Most of the proteolytic fragments were further characterized by Edman degradation using a gas-phase microsequencer (Applied Biosystems 470A). In standard conditions of analysis by HPLC of the phenylthiohydantoin (PTH) amino acid derivatives, diPTH-cystine and PTH-Abu elute at retention times of 9.8 and 10.1 min, respectively. The cleavage experiments were performed twice for peptides [$Abu_{8,26}$]Lei-NH_2 and [$Abu_{12,28}$]Lei-NH_2.

Conformational Analysis. (A) Circular Dichroism Analysis of Lei-NH_2 and Its Analogs. Low-ultraviolet spectra were recorded on a Jobin-Yvon circular dichroism spectropho-

Table 1: Amino Acid Content (Uncorrected Values) of the Crude Reduced and Purified Oxidized Lei-NH_2 Analogs after Hydrolysis (120 °C, 20 h, N_2 Atmosphere) with 6 N HCl in the Presence of 2% (w/v) Phenol[a]

amino acid analysis	[$Abu_{3,21}$]Lei-NH_2 deduced M_r 3388.8 exptl M_r 3388.2 ± 0.4		[$Abu_{8,26}$]Lei-NH_2 deduced M_r 3388.8 exptl M_r 3388.7 ± 0.3		[$Abu_{12,28}$]Lei-NH_2 deduced M_r 3388.8 exptl M_r 3388.1 ± 0.7	
	crude	purified	crude	purified	crude	purified
Asx (2)	1.9	2.0	2.0	2.0	2.0	2.0
Ser(2)	1.2	1.7	1.4	1.8	1.9	1.8
Glx (2)	2.4	2.1	2.4	2.1	2.2	2.1
Gly (3)	3.2	3.0	3.1	3.1	3.3	3.0
Ala (1)	0.7	0.9	0.8	1.0	0.9	0.9
Cys/Abu (6)						
Val (1)	1.2	1.0	1.2	1.1	1.0	1.0
Met (1)	0.7	0.8	0.7	0.9	0.8	0.9
Ile (1)	1.2	1.1	1.1	1.0	0.9	1.0
Leu (5)	4.6	5.0	4.7	5.0	5.1	5.0
Phe (1)	0.8	0.9	0.7	0.9	0.8	0.9
His (1)	1.2	1.0	1.3	1.1	1.2	1.1
Lys (3)	3.2	2.9	3.2	3.0	3.2	3.0
Arg (2)	1.7	2.0	1.5	1.8	1.4	1.9

[a] The ratios of Cys and Abu were not determined, the amino acid analyzer being unable to discriminate between the two in routine use. The theoretical amino acid composition is given in parentheses. The deduced and experimental M_r's are indicated for each peptide.

Leiurotoxin I-NH_2
AFCNLRMCQLSCRSLGLLGKCIGDKCECVKH-NH_2
3 8 12 21 26 28

[Abu $_{3,21}$] Leiurotoxin I-NH_2
AFAbuNLRMCQLSCRSLGLLGKAbuIGDKCECVKH-NH_2
8 12 26 28

[Abu $_{8,26}$] Leiurotoxin I-NH_2
AFCNLRMAbuQLSCRSLGLLGKCIGDKAbuECVKH-NH_2
3 12 21 28

[Abu $_{12,28}$] Leiurotoxin I-NH_2
AFCNLRMCQLSAbuRSLGLLGKCIGDKCEAbuVKH-NH_2
3 8 21 26

FIGURE 1: Primary structures (one-letter code) of Lei-NH_2 and its analogs lacking one disulfide bridge. The positions of half-cystines are indicated. Abu indicates α-aminobutyrate.

tometer (Longjumeau, France). The apparatus was calibrated with (+)-10-camphorsulfonic acid, and a ratio of 2.20 was found between the positive and the negative CD bands at 290.5 and 192.5 nm, respectively. Spectra were obtained in 20 mM sodium phosphate buffer, pH 7.2, and recorded from 260 to 178 nm at 25 °C using a 0.5 mm path-length cell. As determined by amino acid analysis, the peptide concentration in the solution was 500 μg/mL. Data were collected at 0.5 nm intervals with a scan rate of 1 nm/min. They are expressed as the variation of the molar amino acid residue absorption coefficient ($\Delta\epsilon$) per amide and analyzed according to the method of Manavalan and Johnson (1987).

(B) Modeling of Lei-NH_2 Analogs. The TURBO-FRODO and X-PLOR programs were used for construction and molecular dynamic analysis of the models, respectively (Roussel & Cambillau, 1989; Brünger, 1990). The models were constructed according to the experimentally determined disulfide pairings of the Lei-NH_2 analogs and the three-dimensional structures of Lei-NH_2/P05 analogs (Inisan et al., 1995; Meunier et al., 1993). The starting models were refined by 10 ps molecular dynamics at 300 K using the Amber force field, and energies were minimized to convergence using the Powell minimizer.

RESULTS

The primary structures of Lei-NH_2 and its analogs are presented in Figure 1. The peptides were synthesized on 0.3 mmol of Fmoc-amide resin using optimized Fmoc/*tert*-Butyl chemistry. After peptide chain assembly, the amount of final product linked to the resin was between 0.25 and 0.28 mmol, which represents an overall assembly yield of 83−93%. The analytical HPLC profiles of crude reduced peptides after final acidolytic cleavage are shown in Figure 2 (left panel). The crude peptides were then oxidized by exposure to air (Figure 2, middle panel) and purified to 98% homogeneity by preparative MPLC (Figure 2, right panel). The net peptide contents of lyophilized products, as determined by amino acid analysis, were 76% ([$Abu_{3,21}$]Lei-NH_2), 88% ([$Abu_{8,26}$]Lei-NH_2), and 80% ([$Abu_{12,28}$]Lei-NH_2). The overall synthesis yields of the analogs, including peptide assembly, final cleavage, folding, and MPLC purification were about 2.5% ([$Abu_{3,21}$]Lei-NH_2), 0.2% ([$Abu_{8,26}$]Lei-NH_2), and 1.2% ([$Abu_{12,28}$]Lei-NH_2). Amino acid ratios for the crude reduced and purified oxidized analogs are given in Table 1. Electrospray mass spectrometry of the oxidized peptides gave experimental M_r in agreement with the deduced M_r of 3388.8 (see Table 1). Further to characterize the folded peptides, disulfide pairings were established by enzymatic digestion, purification, and analysis of the proteolytic fragments (see Materials and Methods). Under our experimental conditions, the yield of enzymatic cleavage was over 80% for each analog, which corresponds to a protease sensitivity similar to that of Lei-NH_2 (J.-M. Sabatier, unpublished data). The effects of proteolysis by a mixture of enzymes (see Materials and Methods) are summarized in Figure 3. The HPLC-purified proteolytic fragments were characterized using both amino acid analysis and Edman sequencing techniques. The disulfide pairings were thereby mapped unambiguously as Cys_8−Cys_{26} and Cys_{12}−Cys_{28} for [$Abu_{3,21}$]-Lei-NH_2, Cys_3−Cys_{12} and Cys_{21}−Cys_{28} for [$Abu_{8,26}$]Lei-NH_2, and Cys_3−Cys_8 and Cys_{21}−Cys_{26} for [$Abu_{12,28}$]Lei-NH_2 (Figure 4). The analogs and Lei-NH_2 were tested in competition experiments with [^{125}I]apamin for binding to rat brain synaptosomes (Figure 5). The peptides competed with radiolabeled apamin with half-effects ($K_{0.5}$) at concentrations of 8×10^{-11} M ([$Abu_{3,21}$]Lei-NH_2), 10^{-10} M (Lei-NH_2), 8×10^{-9} M ([$Abu_{12,28}$]Lei-NH_2), and 10^{-8} M ([$Abu_{8,26}$]Lei-NH_2). In vivo, all the peptides were lethal to mice, with clinical symptoms indistinguishable to those induced by natural Lei-NH_2 and by apamin. The neurotoxicity of

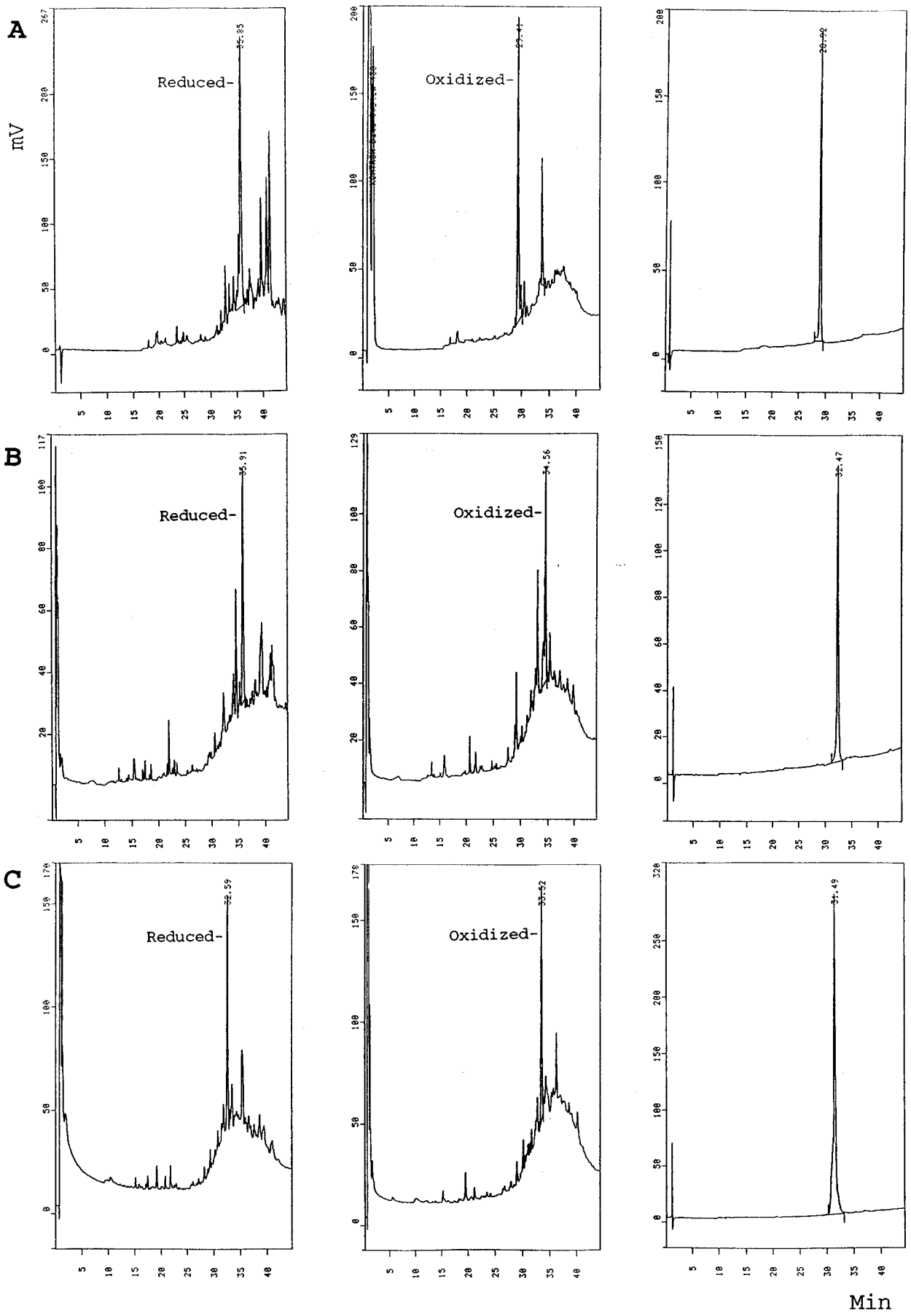

FIGURE 2: Analytical C_{18} reversed-phase HPLC profiles of [$Abu_{3,21}$]Lei-NH_2 (A), [$Abu_{8,26}$]Lei-NH_2 (B), and [$Abu_{12,28}$]Lei-NH_2 (C) at different steps of the synthesis. The crude reduced peptides after trifluoroacetic acid cleavage (left panel) and after oxidation (middle panel) and the purified peptide (right panel) are shown. Experimental conditions are described in Materials and Methods.

Retention Time (min)	Proteolytic Fragments	Disulfide Pairing Determination
[Abu $_{3,21}$] Lei-NH_2		
16.0	SCR CVK	Cys_{12}-Cys_{28}
20.8	AF	
22.7	AbuNL	
28.0*	CQL GKAbuIGDKCE	Cys_8-Cys_{26}
31.2	GL	
31.6	MCQL LGKAbuIGDKCE	Cys_8-Cys_{26}
[Abu $_{8,26}$] Lei-NH_2		
20.8*	AF	
22.8*	CIGDK CVK	Cys_{21}-Cys_{28}
26.1*	CIGDKAbuECVK	Cys_{21}-Cys_{28}
28.1*	AFCNLR MAbuQLSCR	Cys_3-Cys_{12}
28.8*	GLLGK	
45.6*	C KAbuECVK	
46.4*	C AbuECVK	
[Abu $_{12,28}$] Lei-NH_2		
5.3	AbuVK	
14.5	SL	
19.7*	CIGDKCE	Cys_{21}-Cys_{26}
20.9	AF	
25.0	LSAbu	
27.0	CNLRMCQL	Cys_3-Cys_8
28.9*	GLLGK	
29.2*	CNLR CQL	Cys_3-Cys_8
36.2*	CNL MCQL	Cys_3-Cys_8
38.9*	SLGLL	

FIGURE 3: Analysis of the peptide fragments obtained by enzymatic cleavage of [$Abu_{3,21}$]Lei-NH_2 (top), [$Abu_{8,26}$]Lei-NH_2 (middle), and [$Abu_{12,28}$]Lei-NH_2 (bottom) using a mixture of trypsin, chymotrypsin, and endoproteinase Glu-C. The peptides were purified by analytical C_{18} reversed-phase HPLC and characterized by amino acid analysis. Asterisks indicate that the peptides were characterized by both amino acid analysis and Edman sequencing. From these analyses, the deduced peptide sequences are shown. Retention time in HPLC (left column) and established disulfide bridge pairings (right column) are indicated.

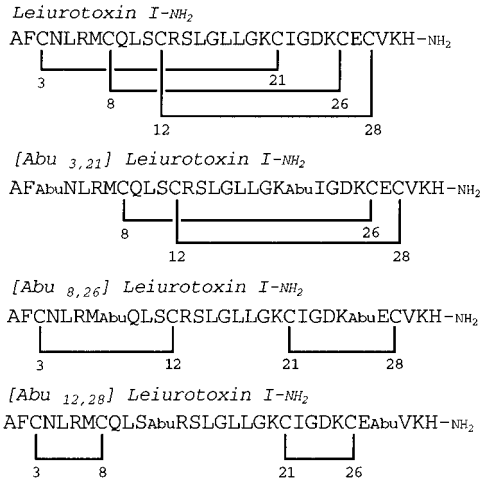

FIGURE 4: Disulfide pairings (plain lines) of Lei-NH_2 and its analogs lacking one disulfide bridge. The disulfide pairings were either deduced from the solution structures of several Lei-NH_2 derivatives (Lei-NH_2) or formerly established (Lei-NH_2 analogs lacking one disulfide bridge). The positions of half-cystines are indicated. Abu corresponds to α-aminobutyrate.

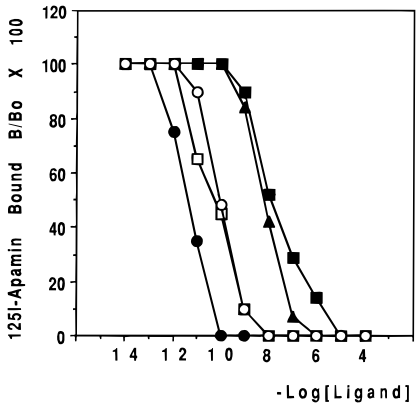

FIGURE 5: Inhibition of binding of [^{125}I]apamin to rat brain synaptosomes by unlabeled apamin (black circles), Lei-NH_2 (open circles), [$Abu_{3,21}$]Lei-NH_2 (open squares), [$Abu_{8,26}$]Lei-NH_2 (black squares), and [$Abu_{12,28}$]Lei-NH_2 (black triangles) in a competition assay. B_0 is the binding of [^{125}I]apamin in the absence of ligand, and B is the binding in the presence of the indicated concentrations of competitors. Nonspecific binding, less than 10% of total binding, was subtracted for the calculation of the ratios. Experimental conditions are described in Materials and Methods.

[$Abu_{3,21}$]Lei-NH_2 was identical to that of the native Lei-NH_2 (LD_{50} = 30 ng per mouse). The analogs [$Abu_{8,26}$]Lei-NH_2 and [$Abu_{12,28}$]Lei-NH_2 were less active with LD_{50} values per mouse of 2.0 μg (1.5% Lei-NH_2 activity) and 2.5 μg (1.2% Lei-NH_2 activity), respectively. A CD analysis of the peptides was performed in 20 mM sodium phosphate buffer, pH 7.2, and compared with that of Lei-NH_2 in identical experimental conditions (Figure 6). The CD spectra of Lei-NH_2 and [$Abu_{3,21}$]Lei-NH_2 exhibited a double minimum at 207−220 nm and a positive band at 190 nm, indicating the presence of partial α-helical structure. For [$Abu_{8,26}$]Lei-NH_2 and [$Abu_{12,28}$]Lei-NH_2, the shape of CD spectrum was typical of a random coil, with a negative band around 200 nm. The CD data were analyzed for secondary structures according to the method of Manavalan and Johnson (1987) and indicated the presence of α-helix (35%, 14%, 5%), β-sheet (34%, 15%, 31%), β-turn (17%, 33%, 30%), and other structures (14%, 38%, 34%) for [$Abu_{3,21}$]Lei-NH_2, [$Abu_{8,26}$]-Lei-NH_2, and [$Abu_{12,28}$]Lei-NH_2, respectively. The secondary structures of [$Abu_{3,21}$]Lei-NH_2 are close to those of Lei-NH_2, i.e., 32% α-helix, 35% β-sheet, 14% β-turn, and 19% other structures (Inisan et al., 1995). A model of [$Abu_{3,21}$]Lei-NH_2 was obtained by molecular dynamics (Figure 7, top right) according to the experimental disulfide pairings and the three-dimensional structures of Lei-NH_2 and its analogs (Martins et al., 1990; Inisan et al., 1995). The resulting minimized model has a total energy of −591 kcal/mol. The structure is mainly proposed as an N-terminal extended fragment (from Ala_1 to Asn_4), followed by an α-helix (from Arg_6 to Gly_{16}) linked to a double-stranded, antiparallel β-sheet (from Leu_{18} to Val_{29}) by disulfides Cys_8−Cys_{26} and Cys_{12}−Cys_{28}. The resulting [$Abu_{3,21}$]Lei-NH_2 conformation superimposed well on that of Lei-NH_2, in agreement with CD data. The two disulfide bridges of [$Abu_{3,21}$]Lei-NH_2 that connect the α-helix to one strand of the β-sheet in the models appeared to be "homologous" to those of apamin that connect the α-helix to its N-terminus (Figure 7, bottom right). This suggests that they participate in the stabilization of the α-helical structure, which is

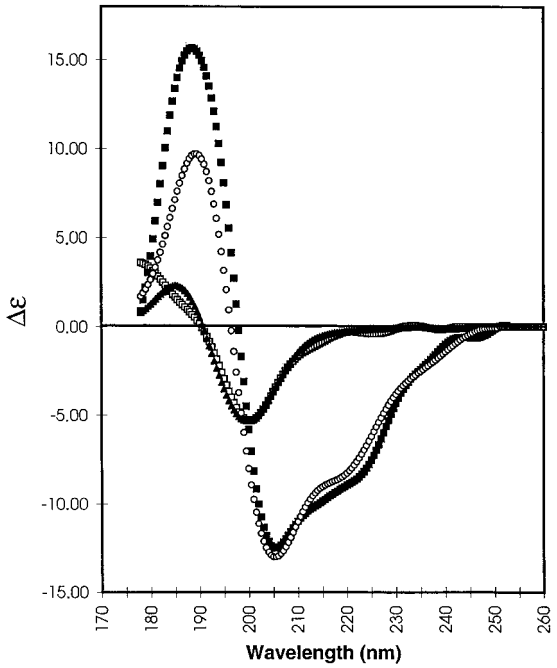

FIGURE 6: Circular dichroism spectra of Lei-NH_2 (open circles), [$Abu_{3,21}$]Lei-NH_2 (black squares), [$Abu_{8,26}$]Lei-NH_2 (open squares), and [$Abu_{12,28}$]Lei-NH_2 (black triangles) in 20 mM sodium phosphate buffer, pH 7.2. $\Delta\epsilon$ is the variation of the molar amino acid residue absorption coefficient expressed in M^{-1} cm^{-1}.

important for Lei-NH_2/apamin-like biological activity. Models of [$Abu_{8,26}$]Lei-NH_2 and [$Abu_{12,28}$]Lei-NH_2 were tentatively constructed and possessed total energies of −412 and −390 kcal/mol, respectively. In agreement with CD analyses, the disulfide pairings of these analogs did not allow Lei-NH_2-like structuration of the peptide backbones, as shown for [$Abu_{8,26}$]Lei-NH_2 (Figure 7, bottom left).

DISCUSSION

We report the design, chemical synthesis, and biological activity of Lei-NH_2 analogs, each lacking one of the three disulfide bridges. A similar approach has been used to study apamin (Huyghues-Despointes & Nelson, 1992; Xu & Nelson, 1994), charybdotoxin (Vita et al., 1994), and iberiotoxin (Flinn et al., 1995). The analogs were obtained by the solid-phase technique using optimized Fmoc/*tert*-butyl chemistry. After final cleavages, the reduced peptides were air-oxidized in the presence of a high concentration of a chaotropic salt (guanidine hydrochloride) which was necessary for full solubilization of the products, as reported for other synthesized Lei-NH_2 analogs (Sabatier et al., 1994). The kinetics of the folding process were similar to those of synthetic Lei-NH_2 and P05 (Sabatier et al., 1993, 1994) with full oxidation in less than 4 h. The folding process was independent of the peptide concentration (data not shown) and appeared to be thermodynamically favored as a major oxidized form was obtained for each analog. Therefore, other oxidation procedures were not tested for the reduced peptides. The disulfide pairings were studied by proteolysis of the main folded forms of the synthetic analogs. The disulfide pairing pattern of [$Abu_{3,21}$]Lei-NH_2 was, as expected, similar to that of the parent toxin, as reported for a two-disulfide core peptide derived from Lei-NH_2 (Pagel & Wemmer, 1994). In contrast, peptides [$Abu_{8,26}$]Lei-NH_2 and [$Abu_{12,28}$]Lei-NH_2 folded differently, probably because of the sequence context of the cysteine causing different structural constraints. Therefore, the position in the sequence of the cysteine may favor a particular pairing and disfavor all the others, at least in the experimental conditions of oxidation used. In the case of charybdotoxin (37 residues, three disulfides), the three synthetic peptides lacking one disulfide bridge were reported to possess a "native-like" disulfide organization (Vita et al., 1994). The Lei-NH_2 analogs were tested in vitro for their capacity to compete with [^{125}I]apamin for binding to rat brain synaptosomes and in vivo for their neurotoxicity in mice. In both assays, [$Abu_{3,21}$]Lei-NH_2 exhibited full Lei-NH_2-like activity whereas [$Abu_{8,26}$]Lei-NH_2 and [$Abu_{12,28}$]Lei-NH_2 possessed only residual activities (<2% native toxin activity). This implies that disulfide

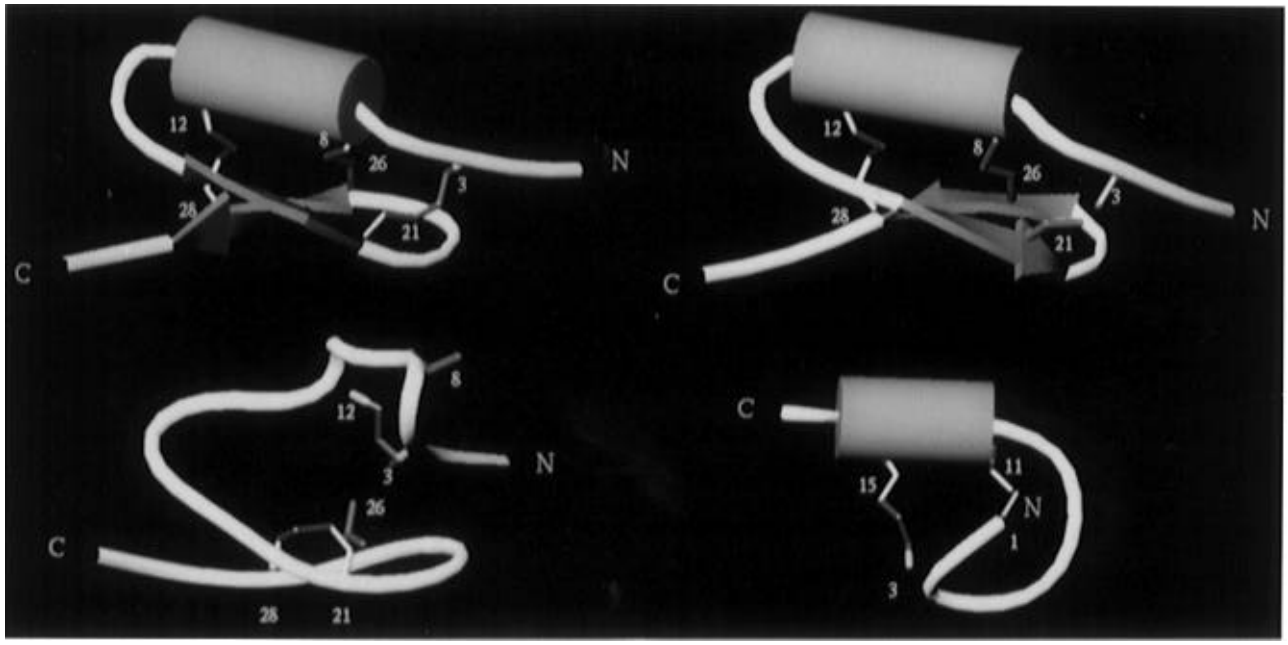

FIGURE 7: Comparison of the models of Lei-NH_2 (top left), [$Abu_{3,21}$]Lei-NH_2 (top right), [$Abu_{8,26}$]Lei-NH_2 (bottom left), and apamin (bottom right). The polypeptide backbones are colored according to secondary structures: α-helix (red), β-sheet (blue), and others (yellow). The disulfide bridges (green) and Abu residues (purple) are numbered. The N- and C-termini are indicated.

bridge Cys_3-Cys_{21} is not essential *per se* for toxin activity. Therefore, this disulfide bridge did not appear to possess any functional role, but it might participate in the relative rigidity of the final toxin structure. However, it is possible that the side chains of Abu_3 and Abu_{21} residues interacted by hydrophobic interactions leading to disulfide bridge mimickry and associated stabilization of the peptide backbone. The data also suggest that the overall conformation of [$Abu_{3,21}$]Lei-NH_2 could be close to that of Lei-NH_2 but different from those of [$Abu_{8,26}$]Lei-NH_2 and [$Abu_{12,28}$]Lei-NH_2. In agreement, preliminary structural analysis of the analogs by CD spectroscopy showed that only [$Abu_{3,21}$]Lei-NH_2 exhibited a CD spectrum grossly similar to that of Lei-NH_2, indicating that they are likely to adopt similar conformations. The peptides [$Abu_{8,26}$]Lei-NH_2 and [$Abu_{12,28}$]Lei-NH_2 did not possess significant native-like structural elements as expected from their particular disulfide pairings. Notably, the CD analysis of these peptides indicated a significant increase in β-turn and other structures when compared with either [$Abu_{3,21}$]Lei-NH_2 or Lei-NH_2. However, they retained residual toxin activity whereas their reduced forms were inactive (data not shown). Structural models of the analogs were constructed on the basis of the disulfide pairing assignment and were consistent with CD data.

Finally, the conformational and functional characterization of synthetic Lei-NH_2 analogs lacking one disulfide bridge suggests that incorporation of a pair of Abu residues in the positions of half-cystines 3 and 21 did not affect the structure and activity of this fold, whereas this substitution in positions 8 and 26, or 12 and 28, had large effects on folding, altering both peptide structure (e.g., α-helix) and activity. In the case of charybdotoxin (Vita et al., 1994), the most active toxin analog (Chab II) lacks disulfide bridge $Cys_{13}-Cys_{33}$ (i.e., the intermediate disulfide), which corresponds by analogy to disulfide Cys_8-Cys_{26} of Lei-NH_2 according to the consensus disulfide organization of short scorpion toxins. The CD analysis of Chab II indicates that it retained a grossly charybdotoxin-like conformation whereas other analogs exhibited significantly different structures. Therefore, it appears that the activity of toxin analogs lacking one disulfide bridge could solely rely on the relative contribution of that particular disulfide to the maintenance of specific structural domain conformation(s). To investigate this, the solution structure of Lei-NH_2 analogs is currently being determined by ^{1}H-NMR.

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