Role of Disulfide Bonds in Folding and Activity of Leiurotoxin I: Just Two Disulfides Suffice

Qi Zhu,[‡,§] Songping Liang,[‡] Loïc Martin,[§] Sylvaine Gasparini,[§] André Ménez,[§] and Claudio Vita*[,§]

College of Life Sciences, Peking University, Beijing, 100871, P. R. China, and Département d'Ingénierie et d'Etudes des Protéines, CEA Saclay, 91190 Gif-sur-Yvette, France

Received May 16, 2002; Revised Manuscript Received July 18, 2002

ABSTRACT: The aim of this study is to investigate the contribution of each disulfide bond in the folding and function of leiurotoxin I, a short scorpion toxin that blocks small conductance K^+ channels. The structure of leiurotoxin I contains a motif conserved in all scorpion toxins, formed by a helix and a double-stranded β-sheet and stabilized by three disulfide bridges. We synthesized three analogues, each presenting two α-aminobutyric acid (Abu) moieties replacing two bridged cysteine residues: LeTx1 ([Abu 3,21] Leiurotoxin I), LeTx2 ([Abu 8,26] Leiurotoxin I), and LeTx3 ([Abu 12,28] Leiurotoxin I). All three analogues fold into a major product containing two native disulfide bonds, while LeTx3 forms an additional isomer, containing non-native disulfides. In denaturing conditions, analogues LeTx2 and LeTx3 yield non-native isomers, while LeTx1 only forms the isomer with native disulfides. All isomers with native disulfides contain nativelike α-helical conformations and bind to synaptosomal membranes with affinities within a log of that shown by the native toxin. By contrast, the non-native LeTx3A analogue exhibits a disordered conformation and a decreased biological potency. Our results indicate that the "CxxxC, CxC" cysteine spacing, conserved in all scorpion toxins and preserved in LeTx1, may play an active role in folding, and that only two native disulfide bonds in leiurotoxin I are sufficient to preserve a nativelike and active conformation. Thus, in the scorpion toxin scaffold, modifications of conserved and interior cysteine residues may permit modulation of function, without significantly affecting folding efficiency and structure.

Leiurotoxin I (also called scyllatoxin) is a 31-residue toxin which was purified from the venom of the Israeli scorpion *Leiurus quinquestriatus hebraeus* (*1*, *2*). In various cell types, it blocks small-conductance Ca^{2+}-activated K^+ channels at 10^{-13}−10^{-11} M concentrations and shows binding specificity and physiological activity similar to the bee venom toxin apamin (*1*, *2*). Its structure, determined by two-dimensional NMR (*3*),[1] presents a helix and a short antiparallel β-sheet stabilized by three disulfide bonds. The two disulfides Cys8−Cys26 and Cys12−Cys28 link the helix to one strand of the β-sheet while the third disulfide Cys3−Cys21 links the other strand to an N-terminal segment preceding the helix (Figure 1). Mutational analysis of leiurotoxin I, the structurally homologous PO5 (87% sequence identity), and the biologically related apamin revealed that two positively charged residues play an important role in biological activity. These residues are located in the helical region in all three toxins and are Arg6 and Arg13 for leiurotoxin I (Figure 1), Arg6 and Arg7 for PO5, and Arg13 and Arg14 for apamin (*4*−*6*).

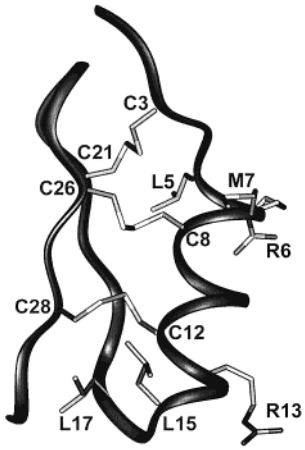

FIGURE 1: Three-dimensional structure of leiurotoxin I (PDB code 1scy) emphasizing the α/β structural motif. Indicated are also the two biologically important residues (R6, R13), the six cysteine residues (C3, C8, C12, C21, C26, C28) engaged in the three disulfide bonds, and other apolar side chains (L5, M7, L15, L17) contributing to form the interior core.

This conserved structural α/β motif stabilized by 3 interior disulfide bonds is typical of short scorpion toxins (*7*−*9*), which are 29−39 residue polypeptides that block different ion channels. Strikingly, a similar α/β motif is also found in insect defensins (*10*), in plant γ-thionins (*11*), in a sweet tasting protein (*12*), and in a family of protease inhibitors (*13*). Thus, this well-ordered motif has been naturally used

* To whom correspondence should be addressed. Tel: (+33) (0)1 69087133; Fax: (+33) (0)1 69089071; E-mail: claudio.vita@cea.fr.
‡ Peking University.
§ CEA Saclay.

[1] Abbreviations: Abu, L-α-aminobutyric acid; LeTx1, [Abu 3,21] Leiurotoxin I; LeTx2, [Abu 8,26] Leiurotoxin I; LeTx3, [Abu 12,28] Leiurotoxin I; CD, circular dichroism; ES-MS, electrospray mass spectrometry; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

10.1021/bi026136m CCC: $22.00
Published on Web 08/27/2002

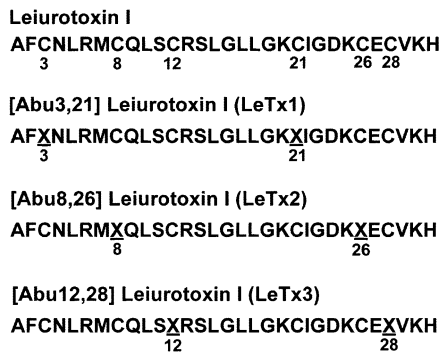

FIGURE 2: Amino acid sequences of leiurotoxin I and its analogues, [Abu 3,21] Leiurotoxin I (LeTx1), [Abu 8,26] Leiurotoxin I (LeTx2), and [Abu 12,28] Leiurotoxin I (LeTx3). The positions of half-cystine in leiurotoxin I and α-aminobutyric acid residues (X) in the analogues are labeled.

to express unrelated biological functions, emphasizing its great functional versatility but also suggesting that new artificial functions could be introduced on specific structural regions (*14*−*16*). Indeed, we have previously reported several examples of grafting new functions onto this structural scaffold using either charybdotoxin (*14*, *17*) or leiurotoxin I (scyllatoxin) (*18*).

Such engineering studies require a better understanding of the structural determinants of this scaffold and in particular the role of evolutionary conserved half-cystine that forms the consensus motif C...CxxxC...C...CxC (where C is cysteine and x is any amino acid residue). A study on charybdotoxin analogues lacking one disulfide bond demonstrated that replacement of a covalent disulfide bond by a noncovalent hydrophobic interaction did not prevent formation of a nativelike and functional structure, even if some of these mutations affected structure stability and biological activity to some extent (*19*). A similar study carried out on leiurotoxin I analogues reached different conclusions, since the analogues lacking one disulfide bond (with only one exception) presented unfolded structures and non-native disulfide bridges (*20*).

In view of these different results and in order to evaluate the contribution of each disulfide bond in the folding and function of scorpion toxins and toxin mutants or chimerae, we reinvestigated the relationship between disulfide bond formation, folding, structure, and biological activity in leiurotoxin I. Three analogues were synthesized, LeTx1 ([Abu 3,21] Leiurotoxin I), LeTx2 ([Abu 8,26] Leiurotoxin I), and LeTx3 ([Abu 12,28] Leiurotoxin I) (Figure 2), each presenting four cysteines and two α-aminobutyric acid residues replacing a cystine residue. Each analogue was folded in the presence or absence of different ratios of reduced/oxidized glutathione or in the presence of 6 M guanidine hydrochloride. Disulfide bonds were assigned by peptide mapping, solution conformation studied by circular dichroism, and biological activity by binding experiments on rat brain synaptosomal membranes. Our results provide evidence for an active role of the conserved half-cystine spacing in the folding of leiurotoxin I and that two disulfide bonds is the minimal requirement in this toxin and, by extension, in the other members of the structural family to acquire a nativelike and bioactive conformation.

MATERIALS AND METHODS

Materials. Fluorenylmethyloxycarbonyl (Fmoc) amino acids were purchased from Nova Biochem (Laufelfingen, Switzerland). Dimethylformamide, methyl-*tert*-butyl ether, acetonitrile, acetic acid, trifluoroacetic acid, diisopropylethylamine (DIEA), and piperidine were from SDS (Peypin, France), thioanisole, ethanedithiol, phenol, and acetic anhydride were from Fluka (St. Quentin Fallavier, France), and *N*-methylpyrollidone and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from Applied Biosystems. Reduced and oxidized glutathione, trypsin, chymotrypsin, apamin, Tris, 2,4,6-collidine, and acetic anhydride were from Sigma-Aldrich, and guanidine hydrochloride was from Pierce. [^{125}I]Apamin (2200 Ci/mmol) was purchased from NEN.

Synthesis. Native leiurotoxin I as well as the analogues were synthesized starting from a poly(ethylene glycol)-polystyrene resin, equipped with a peptide amide linker (Fmoc-PAL-PEG-PS amide resin, Applied Biosystems, Les Ulis, France) on an automatic peptide synthesizer (Applied Biosystems 433A), using Fmoc/*tert*-butyl strategy and HBTU/DIEA coupling method. Amino acid side chain blocking groups were *tert*-butyl ether for Ser and Thr; *tert*-butyl ester for Asp and Glu; 2,2,5,7,8-pentamethylchromane-6-sulfonyl for Arg; trityl for Asn, Cys, His, and Gln; and *tert*-butyloxycarbonyl for Lys. Peptide synthesis was accomplished on a 0.10 mmol scale. Single 30 min coupling with 10-fold Fmoc-amino acid excess (1 mmol) was used, followed by capping with acetic anhydride. Double coupling was used within sequence 11−22. After completion of synthesis, the peptide was cleaved from the resin with simultaneous removal of side-chain protective groups by treatment with reagent K′ (81.5% trifluoroacetic acid, 5% water, 5% phenol, 5% thioanisole, 2.5% ethanedithiol, and 1% triisopropylsilane) for 2 h at room temperature. The resin was then filtered and the free peptide precipitated in methyl-*tert*-butyl ether at 4 °C. After centrifugation and washing 3 times with ether, the peptide was dissolved in 20% acetic acid and lyophilized. The reduced peptides were purified by preparative reversed-phase HPLC using a 80 min linear gradient of 5−50% eluent B (0.09% trifluoroacetic acid, 90% acetonitrile in water) in eluent A (0.1% trifluoroacetic acid in water) over 80 min, on a Vydac C18 column (2.2 × 25 cm) at 10 mL/min flow rate. Peaks were analyzed in analytical HPLC, and >95% pure fractions were pooled and lyophilized.

Folding and Purification. Reduced peptides (0.1 mg) were dissolved in 0.1 M Tris·HCl buffer, containing 0.1 M NaCl and different ratios of reduced/oxidized glutathione, at various pH values, in the absence or in the presence of 6.0 M guanidine·HCl at room temperature. The reduced peptides were also folded in 0.1 M Tris·HCl, 0.1 M NaCl, pH 8.0, with stirring under air at room temperature. In each case, folding was monitored by reverse-phase HPLC. Then 100 μL aliquots of each peptide in different folding buffers were withdrawn at various times, acidified with 20 μL of 50% trifluoroacetic acid, and injected onto a Vydac C18 (0.46 × 15 cm) column. Separation was achieved using a 40 min linear gradient from 5 to 45% eluent B in eluent A, at a flow rate of 1 mL/min. Materials eluted from the peaks were collected, lyophilized, and analyzed by ES-MS. For prepara-

tive purposes, 10 mg of purified reduced product was dissolved (0.1 mg/mL final concentration) in 50 mL of folding buffer, consisting of 0.1 M Tris·HCl, 0.1 M NaCl, 0.5 mM oxidized glutathione, and 5 mM reduced glutathione, pH 8.0, and incubated 2 h at room temperature. The solution was then acidified with 5 mL of 50% trifluoroacetic acid and loaded directly on a Vydac C18 (1.0 × 25 cm) column at 3 mL/min flow rate. Elution was performed by using a 60 min linear gradient of 5−45% eluent B in eluent A. Fractions containing the folded peptide were analyzed on a Vydac C18 (0.46 × 15 cm) column and eluted at 1 mL/min with a 40 min linear gradient 5−45% eluent B in eluent A. Homogeneous fractions (>95% purity) were pooled and lyophilized. The oxidized peptides were weighted and dissolved in water at 1×10^{-3} M as peptide stock solutions, and concentrations were verified on the basis of quantitative amino acid analysis.

Amino Acid Analyses. Samples corresponding to 1 nmol of peptide were hydrolyzed in 6 N HCl at 120 °C for 17 h and then analyzed on an Applied Biosystems model 130A automatic analyzer, equipped with an on-line model 420-A derivatizer.

Mass Analysis. Ion electrospray mass spectra were determined on a Micromass Platform II (Micromass, Altrincham, U.K.) in the *m*/*z* range 300−1500. Samples were dissolved in 50−100 μL of water/acetonitrile/formic acid (49.9/49.9/0.2) and injected at a speed of 10 μL/min at 60 °C. Myoglobin (16 951.48 Da) was used as calibration standard.

Disulfide Bridge Assignment. The oxidized peptides LeTx2, LeTx3, and LeTx3A (100 μg) were digested with trypsin (10% w/w) in buffer 0.1 M Tris·HCl, 1 mM $CaC1_2$, pH 7.8, at 37 °C for 3 h. The oxidized peptide LeTx1 (120 μg) was digested with chymotrypsin (10% w/w) and Glu-C protease (10% w/w) in buffer 0.1 M NH_4HCO_3, 1 mM $CaC1_2$, pH 7.8, at 37 °C for 3 h. Then, 100 μL of 50% trifluoroacetic acid was added to stop the proteolysis, and the digest was loaded onto a Vydac C18 column (0.46 × 15 cm). Separation was achieved using a 50 min linear gradient of 0−50% eluent B in eluent A, at 1 mL/min flow rate. The same protease solution, but without the peptide, was used as a negative control, and no apparent autolytic fragments were detected in the HPLC profile after 3 h of incubation (data not shown). Peak materials were collected, lyophilized, and identified by mass analysis.

Conformational Analysis. Circular dichroism spectra were recorded on a Jobin Yvon CD6 dichrograph, equipped with a thermostatically controlled cell holder and a PC operating with a CDMax data acquisition and manipulation program. The samples were prepared at 1×10^{-5} M in 2 mM Tris·HCl buffer, pH 7.4. Spectra were recorded at 20 °C with a 0.1 cm path length quartz cell by accumulating 4 scans, from 180 to 250 nm, obtained with an integration time of 0.5 s every 0.2 nm.

Binding Assays. Rat brain synaptosomal membranes were prepared as described (*21*), and protein concentration was determined by the Lowry assay, using bovine serum albumin as a standard. All competition experiments were carried out at room temperature in a binding buffer consisting of 10 mM Tris·HCl, 5 mM KCl, 0.1% bovine serum albumin, pH 7.4. Various concentrations of peptides were incubated for 2 h with 20 pM [^{125}I]apamin and rat brain synaptosomal membranes (10 μg of proteins) in 500 μL of binding buffer. At the end of the incubation period, samples were filtered through Whatman GF/C glass-fiber filters (Aldrich) presoaked with 0.5% (w/v) polyethylenimine (Sigma), filters were rinsed with 3 mL of the binding buffer, and the radioactivity associated with filters was counted in a gamma counter. Duplicate samples were run for each experimental point, and experiments were repeated 3 times separately. Data from specific binding were analyzed according to the Hill equation: $B = B_0/[1 + (I/IC_{50})^{n_H}]$, where B is the amount of ligand specifically bound at equilibrium, B_0 the amount of radioligand specifically bound in the absence of inhibitor, I the inhibitor concentration, IC_{50} the inhibitor concentration at 50% inhibition, and n_H the Hill coefficient.

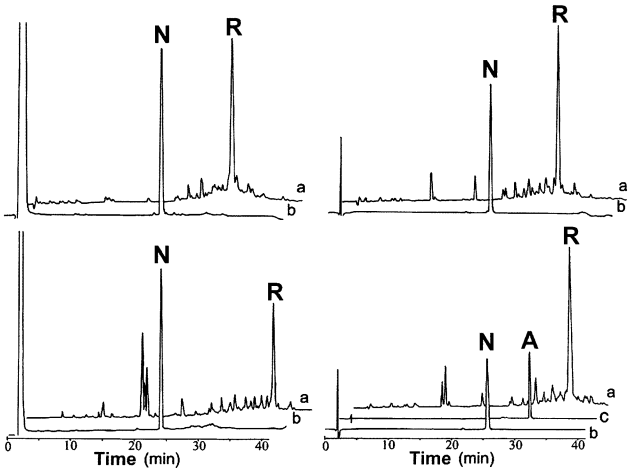

FIGURE 3: Analytical reverse-phase HPLC profiles of the crude reduced (trace a) and oxidized (traces b, c) leiurotoxin I (left upper), LeTx1 (right upper), LeTx2 (left lower), and LeTx3 (right lower). N, A, and R indicate isomers with two native, two non-native, and fully reduced disulfides, respectively. An analytical Vydac C18 column (0.46 × 15 cm) was used and eluted with a linear 5−45% acetonitrile gradient over 40 min at a 1 mL/min flow rate, with detection at 214 nm.

RESULTS

Synthesis. Solid-phase synthesis of Leiurotoxin I and the analogues LeTx1, LeTx2, LeTx3, lacking one disulfide bond, using Fmoc-protected amino acids and HBTU coupling, yielded a major product, as revealed by reverse-phase HPLC analysis (Figure 3, trace a). For folding studies, peptides were purified in the reduced state by preparative HPLC. Masses of purified analogues, determined by electrospray mass spectrometry (ES-MS), were in the range 3392.1−3393.3 Da, in agreement with the 3393.15 Da theoretical mass.

Folding Studies. Preliminary experiments were performed in buffer 0.1 M Tris·HCl, 0.1 M NaCl, pH 7.8, containing different ratios of reduced (GSH)/oxidized (GSSG) glutathione (5 mM/0.5 mM, 2 mM/2 mM, or 0.5 mM/5 mM), and also in buffer containing a fixed ratio of 5 mM/0.5 mM GSH/GSSG, but at different pH values, 7.4, 7.8, 8.0, 8.5 (not shown). Samples were withdrawn at different times, acidified to stop folding, and then injected in reverse-phase HPLC to separate and identify the species formed. With the exception of LeTx3 (vide infra), a predominant earlier eluting peak, named species N, was observed and supposed to represent a folded molecule with native disulfides. This supposition is based on the argument that a native disulfide pairing would better bury apolar amino acid side chains in

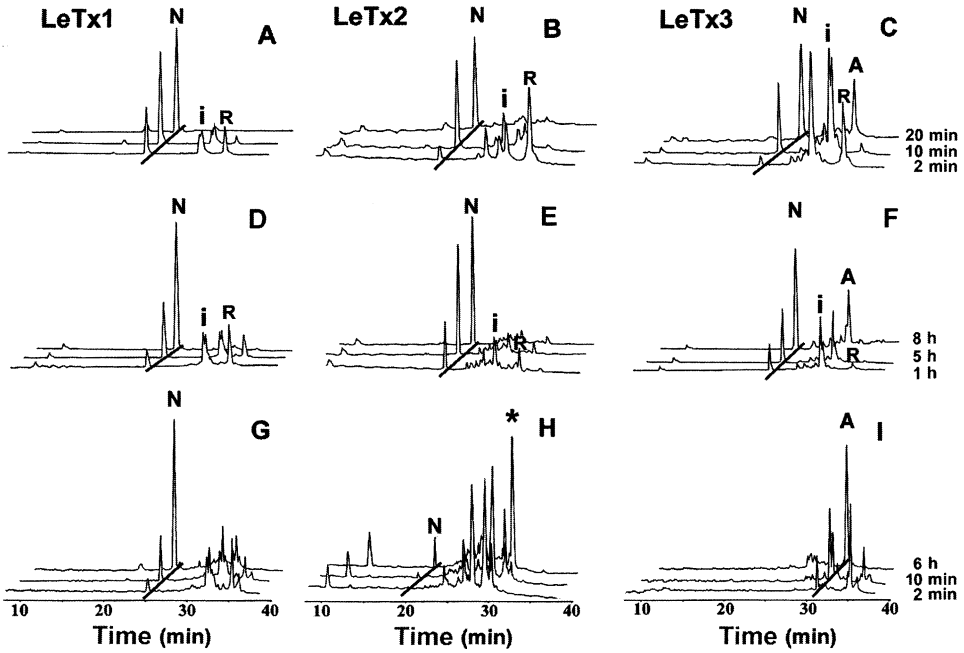

FIGURE 4: Analytical reverse-phase HPLC profiles of the oxidation products of LeTx1 (left column), LeTx2 (middle column), and LeTx3 (right column) in different experimental conditions. Oxidation was performed in 5 mM/0.5 mM GSH/GSSG, 0.1 M NaCl, 0.1 M Tris·HCl buffer, pH 8.0, in the absence (upper row, A−C) and in the presence (lower row, G−I) of 6 M guanidine hydrochloride, or in 0.1 M NaCl, 0.1 M Tris·HCl buffer, pH 8.0, in the absence of glutathione (middle row, D−F). N, A, and R indicate isomers with two native, two non-native, and fully reduced disulfides, respectively. Label i indicates folding intermediates. The column was eluted as in Figure 3.

the interior of the molecule, protected from the solvent and reverse-phase matrixes (*19*). Oxidized species N were purified by reverse-phase HPLC, and their masses, determined by ES-MS, were in the range 3388.6−3389.4 Da, in good agreement with the 3389.1 Da theoretical mass for all oxidized analogues. This confirms the presence of two disulfide bonds in these species. The condition with a low ratio of GSH/GSSG or higher pH values makes the folding process faster, but yields in earlier eluted species N are lower. In the conditions with higher ratios of GSH/GSSH and pH closer to neutral, the species N are more abundant, suggesting that non-native species accumulate in more oxidizing conditions and that, in more reducing conditions, these forms are then converted to native ones by reduced glutathione.

More detailed folding experiments were then performed with LeTx1, LeTx2, LeTx3 (0.1 mg/mL) in buffer 0.1 M Tris·HCl, 0.1 M NaCl, 5 mM/0.5 mM GSH/GSSG, pH 8.0, at room temperature. As shown in Figure 4 A−C, all three analogues fold efficiently in these conditions. In fact, the area under the earlier eluted peak N represents ∼90%, ∼85%, and ∼60% of the total eluting material for LeTx1, LeTx2, and LeTx3, respectively. After 20 min, the height of the peaks is unchanged, indicating that the folding process has reached an apparent equilibrium. LeTx1 forms two folding intermediates, partially overlapping in HPLC (forms "i" in Figure 4A) and eluting in positions intermediate between fully reduced LeTx1 (species R) and the earlier eluted isomer (species N). As revealed by mass analysis, these two intermediates (3390.3 and 3390.4 Da) both possess one disulfide bond. HPLC monitoring of LeTx2 folding (Figure 4B) shows three major one-disulfide intermediates (forms "i") exhibiting masses of 3390.6, 3390.6, 3390.8 Da. Two partially overlapping peaks between species R and species N are also apparent in the HPLC profiles of LeTx3 (Figure 4C, forms "i"): one peak has a mass of 3390.4 Da, corresponding to a one-disulfide intermediate, while the second has a mass of 3388.9 Da, corresponding to a two-disulfide intermediate. Furthermore, a species A, eluting later than the species N, is also observed. Its mass is 3388.9 Da, indicating that this species represents an additional two-disulfide isomer.

Folding of all three analogues was also examined in buffer 0.1 M Tris·HCl, 0.1 M NaCl, pH 8.0, in the absence of glutathione ("air oxidation"). The intermediates and final oxidized products formed in this condition from each analogue are very similar to those formed in GSH/GSSG buffer, with the notable difference that the corresponding folding is much slower, taking 8 h to reach full oxidation (Figure 4D−F).

Finally, folding studies of these three analogues were also performed in denaturing conditions, in buffer 0.1 M Tris·HCl, 0.1 M NaCl, pH 8.0, containing 5 mM/0.5 mM GSH/GSSG and 6 M guanidine·HCl (Figure 4G−I). Remarkably, LeTx1 still forms the species N, as in the conditions without denaturant. LeTx2 also forms the species N with much lower yields, while a non-native disulfide isomer (labeled with an asterisk in Figure 4H), eluted at 31.70 min, dominates. In these denaturing conditions, LeTx3 only forms the isomer A, but no isomer N. Thus, the presence of guanidine·HCl minimally affects the folding process of LeTx1, while strongly affects that of LeTx2 and LeTx3.

Purification. For preparative purposes, the purified reduced products were folded in buffer 0.1 M Tris·HCl buffer, 0.1 M NaCl, pH 8.0, containing 5 mM/0.5 mM reduced/oxidized glutathione (GSH/GSSG) for 2 h. Purified products were homogeneous in analytical reverse-phase HPLC (Figure 3, traces b and c), and their masses (3388.7, 3388.5, 3388.7, and 3388.9 Da for LeTx1, LeTx2, LeTx3, and LeTx3A, respectively, and 3322.7 Da for leiurotoxin I) were in good agreement with the presence of two disulfide bonds in the

Table 1: Assignment of Disulfide Bonds of Leiurotoxin I Analogues

peak retention time (min)	determined mass (Da)	deduced sequence of fragments[a]	cysteine pairing
		LeTx1	
9.39	1046.4	(11−15) − (28−31)	Cys_{12}−Cys_{28}
15.58	1581.1	(6−10) − (19−27)	Cys 8−Cys26
16.03	1395.7	(6−10) − (21−27)	Cys 8−Cys26
17.33	2610.4	(3−17) − (19−27)	
17.85	2424.3	(3−17) − (21−27)	
20.09	548.4	(1−5)	
		LeTx2	
12.27	924.6	(11−13) − (26−31)	Cys_{12}−Cys_{28}
20.45	1382.3	(7−13) − (26−30)	Cys_{12}−Cys_{28}
21.56	475.2	(7−10)	
22.63	1255.3	(1−6) − (21−25)	Cys_3−Cys_{21}
26.09	686.4	(14−20)	
		LeTx3	
21.47	1382.3	(7−13) − (26−30)	Cys_8−Cys_{26}
22.73	1053.7	(7−10) − (26−30)	Cys_8−Cys_{26}
23.39	1255.0	(1−6) − (21−25)	Cys_3−Cys_{21}
26.66	686.5	(14−20)	
27.29	2619.7	(1−13) − (21−30)	
28.94	2273.4	(1−10) − (21−30)	
		LeTx3A	
10.54	316.4	(18−20)	
17.34	1076.4	(21−30)	Cys21−Cys26
27.26	686.4	(14−20)	
29.48	1214.9	(1−6) − (7−10)	Cys_3−Cys_8

[a] Sequences were deduced using the mass searching tool Masslynx (Micromass, UK).

analogues LeTx1, LeTx2, LeTx3, and LeTx3A, and three disulfide bonds in the native form of leiurotoxin I.

Assignment of the Disulfide Bonds. The cysteine pairings of each purified oxidized analogue were examined by peptide mapping. Suitable proteases, i.e., trypsin for LeTx2 and LeTx3, chymotrypsin and Glu-C protease for LeTx1, were chosen to produce fragments containing only one cysteine residue. The digestion was monitored by analytical reversed-phase HPLC. This analysis allowed stopping the proteolysis after 3 h, thus avoiding possible disulfide exchange, which could occur in the produced fragments during unnecessary longer times. Fragments were purified by HPLC and analyzed by electrospray mass spectrometry (Table 1). This analysis demonstrates that the native isomer N of each leiurotoxin analogue contains two native disulfide bonds: Cys8−Cys26 and Cys12−Cys28 for LeTx1, Cys3−Cys21 and Cys12−Cys28 for LeTx2, and Cys3−Cys21 and Cys8−Cys26 for LeTx3. However, the isomer LeTx3A contains two non-native disulfide bonds: Cys3−Cys8 and Cys21−Cys26. Disulfide bond patterns of all analogues are graphically summarized in Figure 5.

Conformational Analysis. The circular dichroism (CD) spectra (Figure 6) of isomers containing native disulfide isomers, such as LeTx1, LeTx2, and LeTx3, exhibit a minimum at 207 nm with a shoulder at 220 nm and a positive band around 190 nm, indicating the presence of an α-helical conformation. Their CD spectra are very similar to that shown by native leiurotoxin I. Thus, the isomers containing native disulfides and the native toxin share similar overall secondary structures. LeTx3A shows a significantly different CD spectrum, with a minimum at 202 nm, a shoulder at 220 nm, and an ill-defined maximum around 188 nm, suggesting the presence of a greater proportion of disordered conformation (Figure 6).

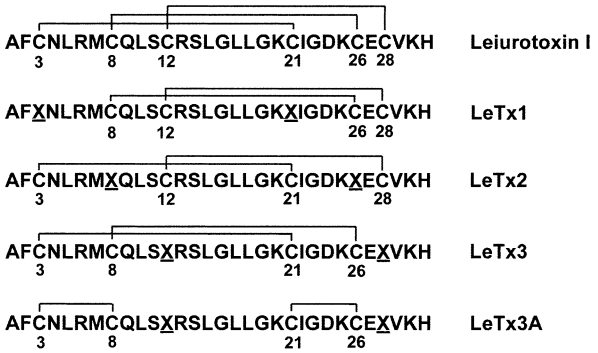

FIGURE 5: Schematic representation of cysteine pairings in native leiurotoxin I (*20*) and LeTx1, LeTx2, LeTx3, LeTx3A analogues, as deduced from the present study. X stands for α-aminobutyric acid.

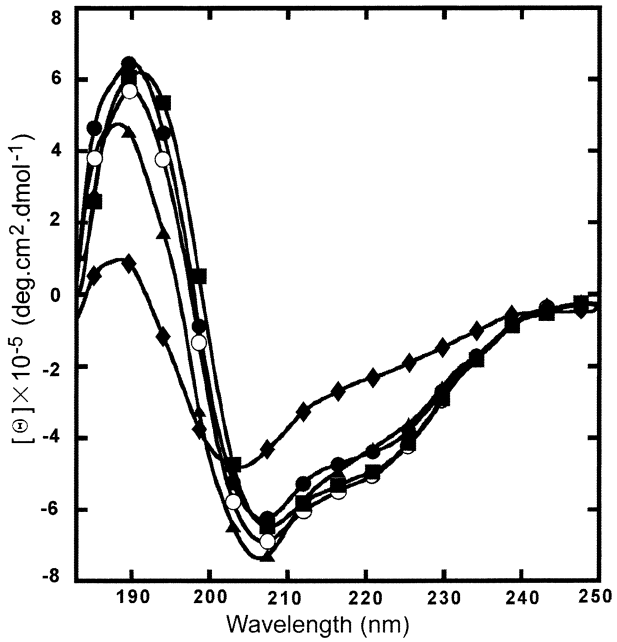

FIGURE 6: Circular dichroism spectra of leiurotoxin I (○), LeTx1 (●), LeTx2 (■), LeTx3 (▲), and LeTx3A (♦). Spectra were recorded at 20 °C in 2 mM Tris·HCl buffer, pH 7.4. [Θ] is molar ellipticity.

Biological Activity. The biological activity of leiurotoxin I and its analogues was studied by testing their capacity to inhibit the binding of [^{125}I]apamin to rat brain synaptosomal membranes. All analogues as well as native leiurotoxin I totally inhibit the binding of [^{125}I]apamin (Figure 7, the IC_{50} values are reported in Table 2). LeTx2 is as potent as native leiurotoxin I, while analogues LeTx1 and LeTx3 have 1 log decreased binding affinities. The analogue LeTx3A that possesses non-native disulfides has a 100-fold lower affinity for apamin receptors as compared to native leiurotoxin I. These results indicate that removal of one disulfide bond in leiurotoxin I, yielding LeTx1, LeTx2, and LeTx3, which still have two native disulfide bonds, caused a limited decrease in biological activity. In contrast, formation of non-native disulfide bridges, as in LeTx3A, produces a much larger effect.

DISCUSSION

To investigate the structural role of disulfide bond formation in leiurotoxin I, we synthesized three leiurotoxin I analogues, LeTx1, LeTx2, LeTx3, each presenting two

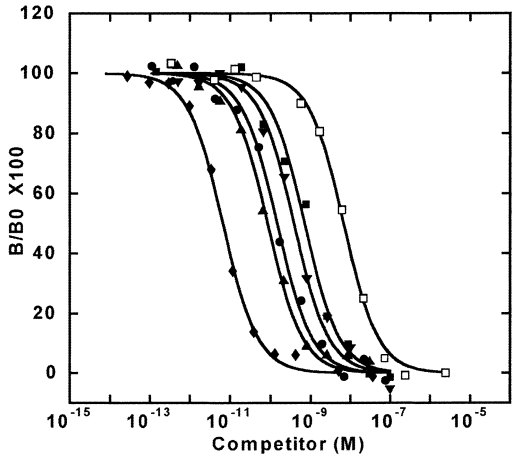

FIGURE 7: Inhibition of [^{125}I]apamin binding to rat brain synaptosomal membranes by unlabeled apamin (♦) and native leiurotoxin I (▲) and its analogues, LeTx1 (▼), LeTx2 (●), LeTx3 (■), and LeTx3A (□). Data from specific binding are displayed and fitted as described under Materials and Methods.

Table 2: Inhibition Constants (IC_{50}) and Relative Affinities of Analogue Competing with [^{125}I]Apamin for Binding to Rat Brain Synaptosomal Membranes

peptide IC_{50}[a]	pM	IC_{50}(analogue)/IC_{50}(leiurotoxin I)
apamin	5.9 ± 1.5	
leiurotoxin I	73.7 ± 14	1
LeTx1	500 ± 280	6.7 ± 3.2
LeTx2	126.5 ± 64	1.6 ± 0.7
LeTx3	872 ± 168	12.5 ± 5
LeTx3A	7261 ± 512	102 ± 29

[a] IC_{50} ± SD values were calculated from competition experiments as described under Materials and Methods.

α-aminobutyric acid (Abu) moieties replacing two bridged cysteine residues. These analogues formed native disulfide bonds, in contrast to a previous study in which leiurotoxin analogues lacking two half-cystine residues folded to form non-native disulfides (*20*). This difference may be attributed to different folding conditions. Indeed, we show that folding in the presence of 6 M guanidine hydrochloride (Figure 4G−I) occurred with formation of intermediates that are different from those observed in nondenaturing conditions (Figure 4A−F). The denaturant thus significantly perturbs folding pathways. Furthermore, the denaturant also perturbs the formation of final products. In denaturing conditions, removal of Cys3, Cys21 in LeTx1 yields only one isomer with native disulfides, identical to that observed in the absence of denaturant. However, removal of Cys8, Cys26 in LeTx2 yields an isomer (marked by an asterisk in Figure 4H) presenting a longer retention time and probably representing a non-native structure that exposes hydrophobic side chains to solvent (and to HPLC column matrixes). In the same denaturing conditions, removal of Cys12, Cys28 in LeTx3 yields only one product, isomer A, presenting non-native disulfide bridges. These non-native leiurotoxin I isomers, formed in 6 M guanidine·HCl, correspond to those previously described in a study (*20*) where the authors used 2 M guanidine·HCl for folding, a condition that favored non-native isomers (with one exception, vide infra). Four of the six cysteine residues of the α/β structural motif found in the scorpion toxin family (Cys8, Cys12, Cys26, and Cys28 in leiurotoxin I) present a conserved spacing, constituting the consensus motif CxxxC...CxC (*7*, *8*). As already discussed (*19*), a study on short synthetic peptides containing two cysteine residues revealed that formation of a disulfide bond between two cysteine residues separated either by one or by three residues leads to a highly constrained and thermodynamically disfavored loop conformation (*22*). Indeed, such bridges are not formed in any scorpion toxins, nor in other short natural peptides, such as sarafotoxin, apamin, or endothelin which contain four cysteine residues and the CxxxC...CxC spacing. In addition, while sarafotoxins and edothelins have the disulfide 1−4, 2−3 topology and apamin has the 1−3, 2−4 topology, the 1−2, 3−4 topology, corresponding to the shortest and unfavorable loops with one or three residues intervening between the cysteine residues, has not been found in any of these molecules (*23*). LeTx1, the only analogue preserving the CxxxC...CxC spacing, forms the isomer with native disulfides both in denaturing and nondenaturing conditions. This has also been observed for the corresponding analogue of charybdotoxin ChabI. In contrast, the two other leiurotoxin I analogues, which lack two of the four cysteines from the consensus motif, yielded non-native isomers in denaturing conditions. This confirms conclusions from a previous study on charybdotoxin (*19*), that this conserved spacing may play an active role in folding by disfavoring one of the two non-native cysteine pairings. However, other factors may also contribute to specify the native 1−3, 2−4 topology, since solely by the constrained loop criterion the 1−4, 2−3 topology is also possible but is not actually observed. Furthermore, removal of Cys12, Cys28 in LeTx3 leads to the A isomer, which is formed in both denaturing and nondenaturing conditions and presents the non-native Cys21−Cys26 bridge. This bond joins two cysteine residues that are spaced by four residues, which represents a spacing leading to a thermodynamically favored loop (*22*). This may explain the fact that LeTx3A is the major isomer formed in denaturing conditions, where native interactions that can stabilize the native LeTx3 isomer are disrupted. This emphasizes the important role of bridge Cys12−Cys28 in structure stabilization and folding, since its removal favors alternative folding pathways and formation of alternative isomers.

CD analysis demonstrates that the three analogues LeTx1, LeTx2, and LeTx3 possess a helical content similar to leiurotoxin I. Biologically relevant residues Arg6 and Arg13 in leiurotoxin I are located on the helical region, as in the biologically related toxins PO5 and apamin (*4*−*6*). Interestingly, the three leiurotoxin I analogues displayed biological activity similar to that of the native toxin. Taken together, these results indicate that both the Cys/Abu substitutions and the lack of one disulfide do not destabilize the helical region in LeTx1, LeTx2, and LeTx3. In the case of charybdotoxin, the corresponding three analogues lacking one disulfide bond, named ChabI, ChabII, ChabIII, also presented native disulfides and overall structures (*19*). However, in charybdotoxin the Cys/Abu mutations affected the biological potency to a greater extent (up to 500-fold). Since the functional residues of charybdotoxin are located on the β-sheet, this suggests that removing one disulfide has a more drastic effect on the β-sheet than on the helix. Analysis of the three-dimensional structure of ChabII (*24*), which is analogous to LeTx2, shows that replacement of cystine moieties by α-aminobutyric acid residues preserved the formation of the hydrophobic core into which the disulfide bridges are implicated. Furthermore,

this preservation contributed to stabilize the helix and two β-strands, while the N-terminal segment is poorly defined and does not form any H-bond with the adjacent C-terminal β-strand (*24*). This may thus explain the somewhat reduced biological activity of ChabII. Inspection of the leiurotoxin I three-dimensional structure (Figure 1) shows that the three disulfide bridges are in the interior of the molecule, protected from solvent by the hydrophobic side chains Leu5, Met7, Leu15, and Leu17. In leiurotoxin I, replacement of each pair of bridged cysteine residues by two α-aminobutyric acid moieties, which are isosteric to half-cysteine and hydrophobic, may preserve an interior hydrophobic core (like in ChabII structure), thus minimally perturbing the α-helix, which is the biologically active region, and explaining the preserved biological activity of the three "native" analogues LeTx1, LeTx2, LeTx3.

Both the charybdotoxin and leiurotoxin I structures have been utilized as scaffolds, and new functions, unrelated to toxic activity, have been introduced (*14*−*18*). The conserved cysteine residues and their positions were preserved in these engineering studies, as they substantially contribute to forming a hydrophobic interior core, which is important for structure stability. The present work contributes to a better knowledge on the role of the evolutionary conserved cysteine residues of these scaffolds and suggests that each disulfide bond can be substituted by a noncovalent interaction, provided by the α-aminobutyric acid side chains, without a significant loss in folding efficiency and structure integrity.

In conclusion, our study clearly shows that two disulfide bonds are the minimal requirement needed to produce a nativelike and bio-active conformation in leiurotoxin I and, by extension, in the α/β motif common to scorpion toxins. The third disulfide provides an additional contribution to structure stabilization and can modulate biological potency depending on its position and the structural regions involved in biological activity. Thus, modifications of conserved and interior cysteine residues can be used to modulate function in the protein design of scorpion toxins and chimerae, without significantly affecting folding efficiency and structure attainment.

REFERENCES

1. Chicchi, G. G., Gimenez-Gallego, G., Ber, E., Garcia, M. L., Winquist, R., and Cascieri, M. A. (1988) *J. Biol. Chem. 263*, 10192−10197.
2. Auguste, P., Hugues, M., Grave, B., Gesquiere, J.-C., Maes, P., Tartar, A., Romey, G., Schweitz, H., and Lazdunski, M. (1990) *J. Biol. Chem. 265*, 4753−4759.
3. Martins, J.-C., Zhang, W., Tartar, A., Lazdunski, M., and Borremans, F. (1990) *FEBS Lett. 260*, 249−253.
4. Granier, C., Pedroso-Muller, E., and Van Rietschoten, J. (1978) *Eur. J. Biochem. 82*, 293−299.
5. Zerrouk, H., Mansuelle, P., Benslimane, A., Rochat, H., and Martin-Eauclaire, M.-F. (1993) *FEBS Lett. 320*, 189−192.
6. Sabatier, J.-M., Fremont, V., Mabrouk, K., Crest, M., Darbon, H., Rochat, H., Van Rietschoten, J., and Martin-Eauclaire, M.-F. (1994) *Int. J. Pept. Protein Res. 43*, 486−495.
7. Bontems, F., Roumestand, C., Gilquin, B., Ménez, A., and Toma, F. (1991) *Science 254*, 1521−1523.
8. Ménez, A., Bontems, F., Roumestand, C., Gilquin, B., and Toma, F. (1992) *Proc. R. Soc. Edinburgh 99B*, 83−103.
9. Tytgat, J., Chandy, K. G., Garcia, M. L., Gutman, G. A., Martin-Eauclaire, M.-F., Van der Walt, J. J., and Possani, L. D. (1999) *Trends Pharmacol. Sci. 20*, 444−447.
10. Bonmatin, J. M., Bonnat, J. L., Gallet, X., Vovelle, F., Ptak, M., Reichhart, J. M., Hoffmann, J. A., Keppi, E., Legrain, M., and Achstetter, T. (1992) *J. Biomol. NMR 2*, 235−256.
11. Bruix, M., Jiménez, M. A., Santoro, J., Gonzàlez, C., Collila, F..J., Méndez, E., and Rico, M. (1993) *Biochemistry 32*, 715−724.
12. Caldwell, J. E., Abildgaad, F., Dzakula, Z., Ming, D., Hellekant, G., and Markley, J. L. (1998) *Nat. Struct. Biol. 5*, 427−431.
13. Ceciliani, F., Bortolotti, F., Menegatti, E., Ronchi, S., Ascenzi, P., and Palmieri, S. (1994) *FEBS Lett. 342*, 221−224.
14. Vita, C., Roumestand, C., Toma, F., and Ménez, A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 6404−6408.
15. Vita, C. (1997) *Curr. Opin. Biotechnol. 8*, 429−434.
16. Martin, L., Barthe, P., Combes, O., Roumestand, C., and Vita, C. (2000) *Tetrahedron 56*, 9451−9460.
17. Drakopoulou, E., Zinn-Justin, S., Guenneugues, M., Gilquin, B., Ménez, A., and Vita, C. (1996) *J. Biol. Chem. 271*, 11979−11987.
18. Vita, C., Drakopoulou, E., Vizzavona, J., Rochette, S., Martin, L., Ménez, A., Roumestand, C., Yang, Y. S., Ylistagui, L., Benjouad, A., and Gluckman, J. C. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 13091−13096.
19. Drakopoulou, E., Vizzavona, J., Neyton, J., Aniort, V., Bouet, F., Virelizier, H., Ménez, A., and Vita, C. (1998) *Biochemistry 37*, 1292−1301.
20. Sabatier, J. M., Lecomte, C., Mabrouk, K., Darbon, H., Oughideni, R., Canarelli, S., Rochat, H., Martin-Eauclaire, M.-F., and Van Rietschoten, J. (1996) *Biochemistry 35*, 10641−10647.
21. Gasparini, S., Danse, J., Lecoq, A., Pinkasfeld, S., Zinn-Justin, S., Young, L. C., Medeiros, C. C. L., Rowan, E. G., Harvey, A. L., and Ménez, A. (1998) *J. Biol. Chem. 273*, 25393−25403.
22. Zhang, R., and Snyder, G. H. (1989) *J. Biol. Chem. 264*, 18472−18479.
23. Ramalingam, K., and Snyder, G. H. (1993) *Biochemistry 32*, 11555−11561.
24. Zinn-Justin, S., Guenneugues, M., Drakopoulou, E., Gilquin, B., Vita, C., and Ménez, A. (1996) *Biochemistry 35*, 8535−8543.

BI026136M