Conkunitzin-S1 is the first member of a new Kunitz-type neurotoxin family - structural and functional characterization

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Running Title: Conkunitzin-S1 Structure and Function

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Conkunitzin-S1 (Conk-S1) is a 60-residue neurotoxin from the venom of the cone snail *Conus striatus* that interacts with voltage gated potassium channels. Conk-S1 shares sequence homology with Kunitz type proteins but contains only two out of the three highly conserved cysteine bridges, which are typically found in these small, basic protein modules. In this study the three-dimensional structure of Conk-S1 has been solved by multidimensional NMR spectroscopy. The solution structure of recombinant Conk-S1 shows that a Kunitz fold is present, even though one of the highly conserved disulfide crosslinks is missing. Introduction of a third, homologous disulfide bond into Conk-S1 results in a functional toxin with similar affinity for Shaker K channels. The affinity of Conk-S1 can be enhanced by a pore mutation within the Shaker channel pore indicating an interaction of Conk-S1 with the vestibule of K channels.

Kunitz domain proteins, like the bovine pancreatic trypsin inhibitor (BPTI) (1) or the dendrotoxins (2), are small, basic proteins that contain three highly conserved disulfide bonds. The three disulfide crosslinks make these extracellular proteins extremely stable. Two different general functions are known for the different Kunitz proteins. One group, including BPTI consists of potent protease inhibitors. The complex of BPTI and Trypsin is exceptionally stable, with an association constant >10\(^{13}\) M\(^{-1}\) (3). The dendrotoxins belong to another group of Kunitz peptides found in the venom of the black mamba, that block different potassium channels with a high degree of specificity and selectivity (4). In snake and scorpion venoms a diverse set of different potassium channel blockers have been characterized (2).

Despite the great variety of toxins from the venoms of the predatory cone snails, relatively few have been identified so far that interact with K channels (5). Most conotoxins are small, peptidic toxins, that typically contain 10-30 amino acids and bind with high affinity and specificity to various ligand gated or voltage gated ion channels. One striking feature of these peptides is that they usually contain a diverse complement of posttranslational modifications, like C-terminal amidation, hydroxyprolines or glycosylation of serine or threonine (6). Conotoxins can be broadly divided into two groups, the non-disulfide-rich peptides and the disulfide-rich conotoxins. The latter conotoxins are further separated into several families based on cysteine bridge pattern and biological activities (5).

The K channel-targeted toxin Conkunitzin-S1 (Conk-S1) from the venom of *Conus striatus* is the first member of a new family of polypeptides. Recently it has been shown that Conk-S1 blocks Shaker K channels with an IC\(_{50}\) of less than 100 nM (Imperial et al., submitted). Compared to most toxins from Conus venoms, Conk-S1 is unusually long (60 amino acids). The only posttranslational modification of this peptide is

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the amidation of the C-terminal carboxylic acid. Conk-S1 shares 33% sequence identity with BPTI and 35% with Dendrotoxin I, indicating that it belongs to the Kunitz domain family of proteins (see Table 1). Therefore we do not use the term “conotoxin” for Conk-S1, which is restricted to smaller disulfide-rich peptide toxins from cone snails.

One striking difference between Conk-S1 and other native Kunitz-type proteins is that it contains only four cysteine residues, resulting in only two disulfide-bridges instead of the three found in all other native proteins that have been biochemically characterized in this group. The homologous cysteine residues of BPTI and Dendrotoxin I are replaced by Gly16 and Gln40 of Conk-S1. Therefore, Conk-S1 is a Kunitz domain protein, in which one of the highly conserved disulfide bridges is missing.

Here we present the NMR-derived solution structure of recombinant Conk-S1; these data demonstrate that structurally, Conk-S1 is a Kunitz domain protein. We show that the heterologously expressed peptide with a free carboxylic acid at the C-terminus is fully functional compared to the natural amidated form of Conk-S1. Conk-S1 represents the first 3D structure of a native Kunitz-type protein with only two disulfide bonds. To evaluate the functional importance of the "missing" third disulfide bond, these cysteine residues have been introduced into Conk-S1. The functional data obtained indicate, that this third disulfide bond does not affect the biological function of Conk-S1, i.e. blocking of its target K channel.

MATERIALS AND METHODS

Protein Preparation – Conk-S1 and Conk-S1 with an additional C-C bridge, Conk-S1<sup>13C</sup>, were expressed in <i>Escherichia coli</i> as insoluble intein fusion proteins. The inclusion bodies were dissolved in 6 M guanidinium hydrochloride and refolded by removal of the denaturant via dialysis. After intein cleavage triggered by a pH change (7) the released peptides were further purified by HPLC.

Mass Spectrometry – For Electrospray Quadrupole Mass spectrometry the source capillary was set to 2.94 kV. Scans were acquired in positive-ion mode at m/z 500-2500.

NMR Sample Preparation - NMR spectra were recorded from two samples, which contained either 0.5 mM <sup>15</sup>N-labeled, or 1 mM <sup>15</sup>N/<sup>13</sup>C-labeled Conk-S1 in 0.1 M sodium acetate buffer, pH 5.2, with 10% <sup>2</sup>H<sub>2</sub>O. Dipolar couplings were measured from an anisotropic sample, in which the peptide was partially aligned. contained 0.5 mM <sup>15</sup>N/<sup>13</sup>C-labeled Conk-S1 in 85.5% 0.1 M sodium acetate buffer, pH 5.2, 9.5% <sup>2</sup>H<sub>2</sub>O, 4.3% pentaethylene glycol monododecyl ether (C<sub>12</sub>E<sub>10</sub>) and 0.7% n-hexanol. The nematic phase was formed after vigorous mixing (8).

NMR Resonance Assignment and Structure Calculation - All spectra were recorded at 27°C on Bruker 600, 700, 800 or 900 MHz spectrometers equipped with shielded gradient triple resonance probes. For the backbone and side chain assignment the standard heteronuclear (H, <sup>15</sup>C, <sup>15</sup>N) strategy based on 3D HNCA, CBCA(CO)NH, HNCO, HN(CA)CO, HCCNH-TOCSY, CCONH-TOCSY, and HCCH-TOCSY experiments was used (9). The backbone resonance assignment was achieved automatically with the assignment program MARS (10). <sup>3</sup>H<sub>N</sub>-H<sub>A</sub> coupling constants were obtained using two-dimensional CT-HMQC-J spectra and were converted to Φ torsion angles with the empirical Karplus equation (11). χ<sub>i</sub> torsion angles were gained from two-dimensional HNCG spectra (12). The program TALOS was used to obtain the backbone dihedral angles (Φ and Ψ) on the basis of chemical shift information (13). Interproton distance restraints were derived from 3D <sup>15</sup>N-edited NOESY (120 ms mixing time) and <sup>13</sup>C-edited NOESY spectra (128 ms mixing time). The NMR data were processed and analyzed using NMRPipe, NMRDraw (14) and SPARKY (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco, CA).

The NOE spectra were automatically peak picked and integrated with SPARKY. NOE cross peaks were
assigned both manually and automatically by the programs ARIA2alpha (15) and CYANA (16). Unambiguous cross peak assignments with a range of 1.8 to 5 Å set by CYANA were used for the final structure calculations.

Residual $^{1}D_{N-H}$, $^{1}D_{CA-HA}$, $^{1}D_{N-C}$ and $^{1}D_{C-C}$ dipolar couplings were gained from the difference between the $J$ couplings measured in the aligned and isotropic media. $^{1}J_{N-H}$ and $^{1}J_{N-C}$ couplings were measured simultaneously from interleaved 3D TROSY-HNCO spectra and $^{1}J_{CA-HA}$ and $^{1}J_{CA-C}$ were obtained simultaneously from interleaved 3D CBCA(CO)NH spectra (Zweckstetter and Vijayan, submitted). The magnitude (normalized to $^{1}D_{N-H}$) and rhombicity of the alignment tensor were 13.0 Hz and 0.38, respectively, as determined from the histogram of dipolar couplings by the program PALES (17).

Initially, a medium resolution structure was calculated with the program Rosetta NMR (18) based on dipolar couplings and chemical shift information, and applying the ITAS approach (19). This structure was used as a starting point for calculation of a high-resolution structure using either CNS Solve 1.1 (20), interfaced with ARIA 2alpha or CYANA 2.0.32. Finally a simulated annealing protocol with 7 Iterations were performed with CYANA. In the last a total of 20 structures were calculated. The 20 structures were further refined in explicit water (21) and were used for the statistics. A total of 551 NOE’s, 365 of them short range ($|i-j| \leq 1$), 73 medium range ($1 < |i-j| < 5$) and 113 long range ($|i-j| \geq 5$) NOE’s, 201 dipolar couplings, 126 dihedral angles and two disulfide bridge constraints were used in structure calculations.

Electrophysiological Measurements - To study the effects of the wild type and mutants of Conk-S1 with the Shaker K channel the Xenopus oocyte expression system was used. Oocyte preparation and Shaker RNA injection were performed as described by Jacobson et al. (22). Whole-cell currents were recorded under two-electrode voltage-clamp control using a Turbo-Tec amplifier (npi electronic, Tamm, Germany). The intracellular electrodes were filled with 2 M KCl and had a resistance between 0.4 and 1 MΩ. Current records were low-pass filtered at 1 KHz (-3 db) and sampled at 4 kHz. The bath solution was normal frog Ringer’s (NFR) containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl$_2$ and 10 mM Hepes (pH 7.2) (NaOH). All electrophysiological experiments were performed at room temperature (19-22 °C). Toxin-containing solutions were applied continuously during the whole experiment from a reservoir through a silicon tube directly into the bath chamber. Whole-cell currents at a test potential of 0 mV were measured every 15 s during toxin application.

The IC$_{50}$ values of Conk-S1 and of Conk-S1$^{13}$C were calculated from the reduction of whole-cell currents at a test potential of 0 mV obtained from oocytes expressing Shaker K according to the relationship $IC_{50} = fc/(1-fc)[Tx]$, where fc is the fractional current and [Tx] is the toxin concentration. Data are given as means ± the standard deviation (rmsd).

The $k_{on}$ and $k_{off}$ values were obtained by an exponential fit [$y = y_0 + A*exp(-k*x)$] to the experimental data during decrease and increase of the current, obtained during the wash-in and wash-out of the peptide. This is possible because the “on” reaction is pseudo first order, due to the constant toxin concentration, and the “off” reaction is first order.

RESULTS

Resonance Assignment and Tertiary Structure - The $^{15}$N HSQC of Conk-S1 displayed an excellent chemical shift dispersion indicative of a well-folded, rigid protein (data not shown). Backbone resonances for all residues except Gly39 and Tyr51 could be identified in the $^{15}$N HSQC spectrum. In total 96.97 % of the backbone resonances and 91.63 % of the side chain chemical shifts have been assigned. The superposition of the 20 structures with the lowest total energy is shown in Figure 1. The quality of the solution structures is summarized in Supplemental Table 1. In the Ramachandran plot 88.2% of the dihedral angles appear in the most favorable region.
and 9.8% in the additionally allowed region. For all heavy atoms the root mean square deviation to the mean structure is 1.4 Å and for the backbone atoms it is 0.7 Å. Side chains are well defined, as can be seen from Fig. 1b.

Steady state heteronuclear \(^{15}\text{N}\{^{1}\text{H}\}\)-NOEs for most of the Conk-S1 backbone amides are above 0.6 indicating that the backbone of Conk-S1 is well ordered in solution (data not shown). Only Thr 60 and Ala19 are flexible with \(^{15}\text{N}\{^{1}\text{H}\}\)-NOE values of 0.22 and 0.15, respectively, and the three N-terminal residues are affected by motion. Ala19 is located in the region homolog to the antiprotease loop of BPTI and is close to the “missing” third disulfide bond, which would be between the position Gly16 and Gln40.

The solution structure of Conk-S1 resembles the typical Kunitz type fold. It consists of a \(3_{10}\)-helix of residues 6 to 8, a twisted \(\beta\)-hairpin of residues 20 to 36 and an \(\alpha\)-helix of residues 50 to 56. The two helices are connected by the disulfide bridge between Cys7 and Cys57. The C-terminal helix is also connected to the \(\beta\) sheet by the disulfide bridge between Cys32 and Cys53. The disulfide bridge 7-57 was confirmed by 4 direct NOE contacts between these residues. For the other S-S pairing no direct contact could be observed. In this case NOEs between Cys32 and Gln54 and other long-range NOEs between residues close to the cysteines were used to confirm the cysteine bridge.

To verify that the disulfide bonds were actually formed, Electrospray Quadrupole mass spectra were recorded. The theoretical masses of Conk-S1 and Conk-S1\(^{CC}\) without disulfide bridge formation would be 6933.6 Da and 6954.7 Da, respectively. The masses measured for Conk-S1 and Conk-S1\(^{CC}\) were 6929.3 \(\pm\) 0.8 Da and 6948.2 \(\pm\) 0.9 Da, consistent with the formation of two disulfide bonds in the wildtype and three disulfide bonds in the mutant Conk-S1.

Functional characterization with voltage clamp experiments - Since the recombinant Conk-S1 does not contain the amidated C-terminus observed in the native peptide, the affinity of the recombinant peptide on Shaker K channels expressed in Xenopus oocytes was measured. The functionality of the Conk-S1 with the free acid at the C-terminus was assayed by two-electrode voltage clamp measurements. For these experiments the Shaker K channel with removed N-terminal inactivation (Shaker-\(\Delta\)6-46) was used. Fig. 2 shows that this channel was blocked by Conk-S1 with an IC\(_{50}\) value of 502 \(\pm\) 140 nM (n = 3). Since it has been reported that \(\kappa\)-conotoxin PVIIA and \(\kappa\)M-conotoxin RIIK, which are both also blocking Shaker K channels, showed an increase in affinity when tested on a pore mutant of this channel (K427D), the functional effects of Conk-S1 on this mutated Shaker channel was also investigated. Most interestingly the affinity of Conk-S1 for this mutant was 0.22 \(\pm\) 0.08 nM (n = 4), which is more than 2000-fold higher than for the wild type channel (see Fig. 2). This result indicates that Conk-S1 blocks K channels by interacting with the ion channel pore. Furthermore it underscores the importance of residue 427, located in the outer vestibule of the ion permeation pathway, for the binding of different conotoxins to K channels.

All Kunitz domain proteins functionally analysed so far contain three disulfide bridges. To evaluate the importance of the "missing" disulfide bridge in Conk-S1 a double cysteine mutant (Conk-S1\(^{CC}\)) carrying an additional cysteine bridge between the positions 16 and 40 was constructed and the activity of this peptide on K channels was measured. Most interestingly the affinity of Conk-S1\(^{CC}\) to Shaker-\(\Delta\)6-46 was 385 \(\pm\) 58 nM (n = 3) showing that within the accuracy of measurements wild type and mutant Conk-S1 displayed the same inhibitory potential for Shaker channels. In addition, the affinity of Conk-S1\(^{CC}\) to Shaker-\(\Delta\)6-46 K427D channels is 0.22 \(\pm\) 0.05 nM (n = 3), which is also identical to the binding affinity of Conk-S1 to this channel mutant. Therefore, for both the wild type sequence and Conk-S1\(^{CC}\) an increase in affinity by a factor of approximately 2000 is observed. For Conk-S1 and Conk-S1\(^{CC}\) dose
response measurements with the wild type and K427D K channels were performed as well, resulting in almost identical IC₅₀ values (see Fig. 2). The Hill coefficients for the block of both channels by both peptides were all about 1, indicating no cooperativity for the binding of both peptides to the channels.

The kinetic analysis of the block of Shaker channels by Conk-S1 and Conk-S1⁴⁴ resulted in kₜ values (for the forth reaction) of 17 ± 5 s⁻¹µM⁻¹ for Conk-S1 and 7 ± 3 s⁻¹µM⁻¹ for Conk-S1⁴⁴. The kₜ values (for the back reaction) were 0.0041 ± 0.0006 s⁻¹ for Conk-S1 and 0.0029 ± 0.0023 s⁻¹ for Conk-S1⁴⁴. This analysis revealed that although the steady state affinity of Conk-S1 and Conk-S1⁴⁴ is virtually identical there are some differences in the kinetics of binding of both peptides to the ion channel.

**DISCUSSION**

In this work, we solved the structure of a Kunitz domain polypeptide toxin from the venom of the fish-hunting cone snail, *Conus striatus*. Proteins with Kunitz domains can be divided into two general classes: the “heterogeneous Kunitz-domain-containing proteins”, with one or more Kunitz domains, but in combination with other structural motifs, and the “homogeneous Kunitz-domain polypeptides” (which we have referred to above simply as “Kunitz domain proteins”) that are exclusively composed of Kunitz domains. Such polypeptides may contain one or more Kunitz domains, but have no other domain motifs. Conkunitzin-S1 clearly belongs to the latter class.

Conkunitzin-S1 is the first natural Kunitz-domain protein with only two disulfide bonds (all other natural Kunitz-domain proteins such as BPTI or the dendrotoxins have three disulfide crosslinks). Only among the heterogeneous Kunitz-domain-containing proteins Kunitz domains with only two disulfides have been reported (the trophoblast Kunitz-domain proteins (TKDPs) (23)). These appear to function as protease inhibitors, and their structure has not been solved.

We have demonstrated that, despite containing only two cysteine bridges, the structure of Conk-S1 is very similar to that of other Kunitz domain peptides like BPTI (backbone Cα r.m.s.d. = 1.3 Å) and the dendrotoxins (backbone Cα r.m.s.d. to Dendrotoxin I = 2.2 Å).

**Comparison of Conk-S1 with BPTI and Dendrotoxins structures -** Native BPTI with three disulphide bonds is extremely stable. It has been demonstrated that removing any one of these cysteine bridges still results in the native conformation, which is stable under normal conditions (24). The most productive BPTI folding pathway includes a cysteine bridge rearrangement of non-native disulfide intermediates. This rearrangement facilitates the folding process under most experimental conditions. The most important of these intermediates of BPTI are (Cys30-Cys51, Cys5-Cys14) and (Cys30-Cys51, Cys5-Cys38) (25). This folding pathway is not possible for Conk-S1 because the cysteines 14 and 38 (corresponding to 16 and 40 in Conk-S1) are replaced by glycine and glutamine, respectively. The folding of Dendrotoxin I and Dendrotoxin K uses similar folding pathways as BPTI, but with important energetic and kinetic differences. In particular, a direct pathway, without disulfide rearrangements, is significantly more populated than in BPTI folding (24). The existence of a native Kunitz domain with two replaced cysteines, as determined in this study, confirms that non-native disulfide intermediates are not necessary for the folding of Kunitz domains. This is also consistent with other studies, where mutants of BPTI, which lack Cys14 and Cys38, still folded properly (26). Another mutant of BPTI with only two disulfide bridges (Cys30-Cys51, Cys14-Cys38) also shows a native fold while the corresponding mutant in Dendrotoxin K is only partly folded. This is due to the generally lower stability of dendrotoxins compared to BPTI (24). Conk-S1 is structurally and functionally similar to the dendrotoxins. Considering the compromised folding of cysteine mutants...
in dendrotoxins, it is surprising that Conk-S1 still attains the typical Kunitz fold.

**Functionality of Conk-S1**

Recently it has been shown that the native, C-terminally amidated Conk-S1 blocks Shaker K channels with an IC$_{50}$ of about 60 nM (Imperial et al., submitted). In our study a recombinant Conk-S1 has been used, which is lacking the C-terminal amidation. The functional consequence of this difference was studied by measuring the affinity of the recombinant Conk-S1 on Shaker-Δ6-46 channels expressed in Xenopus oocytes. The IC$_{50}$ value of the recombinant Conk-S1 used in this study is 6 to 7 times higher than that of the native Conk-S1, indicating a reduced inhibitory potential of the recombinant toxin. Despite this contribution of C-terminal amidation of Conk-S1 for K channel inhibition, our results demonstrate that recombinant Conk-S1 is functional. This provided the opportunity to use this recombinant neurotoxin for investigating structure, dynamics, electrophysiological properties and the effects of mutations.

Most interestingly the electrophysiological measurements revealed that there is no difference in the affinity between Conk-S1 with two disulfide bonds and the three-disulfide bonded mutant Conk-S1$^{CC}$. Furthermore, both peptides exhibit an approximately 2000 fold higher affinity to the K427D mutant of the Shaker K channel than to the wild type channel. This demonstrates clearly the importance of the region around residue K427 in Shaker K channel for the binding to Conk-S1 and indicates that Conk-S1 is interacting with the pore region of the ion channel. In addition, it suggests that the "missing" cysteine bridge is not of critical functional importance for the block of the K channel by Conk-S1.

Despite the identical steady state values, the kinetic analysis revealed some differences in the binding of both toxin analogues. The $k_{on}$ and the $k_{off}$ values for Conk-S1 were approximately two times higher than for Conk-S1$^{CC}$. Thus, Conk-S1 binds twice as fast to the channel as Conk-S1$^{CC}$, but it is also released twice as fast from its binding site. Therefore, the two effects compensate, resulting in identical IC$_{50}$ values of Conk-S1 and Conk-S1$^{CC}$.

From the kinetic point of view, this indicates that for Conk-S1$^{CC}$ the formation of the complex with the channel as well as the dissociation of this complex might have a higher energy barrier. This is likely related to the number of disulfide bonds: the additional cysteine bridge makes Conk-S1$^{CC}$ more rigid. For binding of the toxin to the channel, flexibility within Conk-S1 may enhance the rate of binding to the channel by allowing the toxin to adjust more easily into the binding pocket within the ion channel pore.

Interestingly, experiments with dendrotoxins resulted in the reverse situation (27). When the disulfide bond homologous to the missing third disulfide bond of Conk-S1$^{CC}$ was selectively removed from Dendrotoxins I and K with iodoacetamide, derivatized dendrotoxins showed a 5 to 10 times lower affinity than the unmodified toxins. One possible explanation for this effect could be steric hindrance by the acetamide group. On the other hand, the different results obtained for dendrotoxins and Conk-S1 may indicate a different binding mode of Conk-S1 and dendrotoxins, even though these peptides both target voltage gated K channels. In another report it was shown, that by selectively reducing the cysteine bridge in BPTI, which is homologous to the one introduced into Conk-S1$^{CC}$, with borohydride, no change in activity was observed (28). Therefore, Conk-S1 more closely resembles BPTI than the dendrotoxins with respect to the importance of this disulfide bond for the functional activity of the protein.

In conclusion, we have determined the structure of Conk-S1, an unusual K channel-targeted toxin that has a consensus Kunitz-domain AA sequence, but which lacks one of the three disulfide bonds that are conserved in all natural Kunitz domain peptides characterized to date. The structure does not strongly diverge from those of standard Kunitz domains with three disulfide bonds. A Conk-S1 analog with the third disulfide bond had similar K channel blocking activity, indistinguishable from that of the native peptide. Both the two- and three-disulfide crosslinked toxins had much higher affinity for the K427D Shaker K-
channel mutant, suggesting that they interacted equally avidly with the vestibule of the ion channel pore.

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# The coordinates of Conk-S1 were deposited in the Protein Data Bank (PDB ID 1YL2, RCSB ID RCSB031638). The NMR assignments were deposited in the BioMagResBank (accession no. 6505).

The Abbreviations used are: Conk-S1, Conkunitzin-S1; Conk-S1CC, Conkunitzin-S1 G16C Q40C; BPTI, Bovine Pancreatic Trypsin Inhibitor; NOE, nuclear overhauser effect; NOESY, nuclear overhauser effect spectroscopy; NFR, normal frog ringer; Shaker-Δ6-46, Shaker K channel with removed N-terminal inactivation domain.

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Fig. 1. NMR solution structures of Conk-S1. (a) Stereo view of the backbone atoms (N, Cα, C, O). Represented is the mean structure of the 20 structures with lowest total energy. Cysteine bridges are marked in orange. (b) Superposition of the 20 structures with lowest total energy. Ribbon presentation of the backbone including side chains, represented as lines. Helices are marked in red and β strands in cyan. All figures were created with the program MOLMOL (29).

Fig. 2. a) Whole cell currents upon depolarizing from −100 to 0 mV are shown before and after toxin application. Currents were recorded from oocytes expressing either wild type or K427D Shaker-Δ6-46 channel. The corresponding channel is indicated beside, the corresponding toxin above the curves, respectively. The toxin concentration is shown next to the curve, which was recorded after toxin application. The vertical bars represent 2 µA. b) Dose-response curves of wild type (filled symbols) and K427D (open symbols) Shaker-Δ6-46 channels. Currents were blocked by Conk-S1 (circles) and Conk-S1CC (squares). The symbols are also shown in section a) at the corresponding toxin/channel combination. The test potential was 0 mV and n was between 2 and 5 for the different toxin concentrations.
Table 1. Amino acid sequence of Conk-S1 and alignment with selected Kunitz domain proteins

|        | 1  | 10 | 20 | 30 | 40 | 50 | 60 |
|--------|----|----|----|----|----|----|----|
| Conk-S1| KDRPSLCDLPADSGSGTKAEEKRYNSARKQCLRFDYTQGGGNENNFRRTYDCQRTCLYT |
| BPTI   | RPDFCLEPPYTGPCARKIIRFYNAVAGLCQTGVVGGCRAKRRNFSADCMRRTCGGA |
| DTI    | QPLRKLGHNPGCQKIPAFYNQKQCKGEFTWSSGGGMNRFKTEEECRRTIRK |
| DTK    | AAKYCKLPLRIGPCRRKIPSFYYKWKAKQCLPFDYGCGGNARFTIEECRRTCVG |

BPTI is the bovine pancreatic trypsin inhibitor (30), DTI and DTK are dendrotoxin I and K from *Dendroaspis polylepis polylepis* (30).

Figure 1

![Figure 1](http://www.jbc.org/Downloaded from)
Figure 2

(a) 

Conk-S1  

Shaker-Δ  

200 nM  
50 ms  

Conk-S1\textsuperscript{CG}  

Shaker-Δ K427D  

0.5 nM  

(b)  

Toxin concentration [nM]  

Toxin/Control  

IC\textsubscript{50} [nM]:  

- 0.27 ± 0.03  
- 0.29 ± 0.03  
- 370 ± 30  
- 530 ± 70  

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**Supplemental Data**

**Table 1. Structural statistics**

| Parameter                                                                 | Value  |
|---------------------------------------------------------------------------|--------|
| R.m.s.d.s from experimental restraints                                   |        |
| NOEs (551) [Å]                                                           | 0.024 ± 0.002 |
| Dihedral angles (126) [°]                                                | 1.3 ± 0.2 |
| Correlation coefficients of experimental RDCs                           |        |
| $^1$D$_{NH}$                                                              | 0.985  |
| $^1$D$_{CN}$                                                              | 0.996  |
| $^1$D$_{CAC'}$                                                            | 0.979  |
| $^1$D$_{CAHA}$                                                            | 0.999  |
| R.m.s.d.s to the averaged coordinates [Å]                                |        |
| Backbone heavy atoms                                                     | 0.7    |
| All heavy atoms                                                           | 1.4    |
| Distance restraint violations                                            |        |
| Number > 0.1 Å                                                           | 3 ± 1  |
| Maximum [Å]                                                              | 0.13 ± 0.01 |
| Torsion angle violations                                                 |        |
| Number > 5°                                                              | 3 ± 1  |
| Maximum [°]                                                              | 5.23 ± 0.5 |
| Energies [kcal/mol]                                                      |        |
| $E_{all}$                                                                | -1267 ± 79 |
| $E_{dihedral}$                                                           | 13.5 ± 3.9 |
| $E_{NOE}$                                                                | 14.5 ± 2.9 |
| Ramachandran statistics                                                  |        |
| Most favouable region (%)                                                | 88.2   |
| Additional allowed region (%)                                            | 9.8    |
| Generally allowed region (%)                                             | 0.0    |
| Disallowed region (%)                                                    | 2.0    |
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