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Solution structure of BmKαTx11, a toxin from the venom of the Chinese scorpion *Buthus martensii* Karsch

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ABSTRACT

The solution structure of BmKαTx11 presented by this paper is distinctive from any other structures of wide-type scorpion α-toxins reported so far, for its *trans*-9,10 peptide bond conformation is accompanied by 'protruding' topology of the 'NC-domain'. The orientation of the C-tail of BmKαTx11 is obviously different from that of classical α-toxins (e.g., AaH2, BmK-M8), despite the fact that they share common *trans* conformation of peptide bond between residues 9 and 10. Accordingly, there must be other structural factors dominating the orientation of the C-tail except the conformation of peptide bond 9–10. Our study reveals that residues at position 58 play an important role in it, and different type of residues at this position (e.g., Lys, Arg, Met, Ile) result in different spatial relationship between the C-terminus and the 'five-residue-turn' and then different topology of the 'NC-domain', therefore residues at position 58 are believed to function as structure and bioactivity switch for specificity of scorpion α-toxins. The mechanism for stabilizing the geometry of the 'NC-domain' in wide-type scorpion α-toxins is also discussed.

Introduction

Scorpion α-toxins are polypeptides which affect the inactivation process of Voltage-Gated Sodium Channels (VGSCs) in excitable membranes by binding to neurotoxin receptor Site-3 [1,2]. They have been used as pharmacological tools for probing the structures and the gating mechanism of the VGSCs for years [3–7].

According to their pharmacological differences in preference for sodium channels of insects and mammals, scorpion α-toxins are divided into three groups [6]: 'classical', 'anti-insect' and 'α-like'. The classical α-toxins, such as AaH2 from *Androctonus australis hector* [8], are highly active on mammals and show low toxicity to insects. The anti-insect α-toxins, such as LqhαIT [9] from *Leiurus quinquestriatus hebraeus*, are highly active on insects, and exhibit low toxicity to mammals when injected intracerebroventricularly (i.c.v.). The α-like toxins, such as BmK-M1 [10] from *Buthus martensii Karsch*, are active on both mammals and insects. Despite of the pharmacological diversity of scorpion α-toxins, they share a common structure motif of cysteine-stabilized βαββ [4,11].

The putative bioactive surface of scorpion α-toxins is composed of 'Core-domain' and 'NC-domain' [6,12]. Residues located on the loop preceding the α-helix and those connecting the β2 and β3 strands form the 'Core-domain'. The 'five-residue-turn' together with the C-terminal segment forms the 'NC-domain'. The 'Core-domain', highly conserved among α-toxins, is believed to play a role in the affinity to the receptor Site-3 of VGSCs [13,14]. The 'NC-domain' varies in amino acid sequence and spatial arrangement among α-toxins and is considered as molecular determinants of toxin specificity [10,15]. 'Flat' geometry of the 'NC-domain' implies activity on mammals, while 'protruding' conformation, usually accompanied by non-proline *cis*-9,10 peptide bond in the 'five-residue-turn', is associated with highly insecticidal potency [6,12,16].

In this paper, a long-chain scorpion toxin, BmKαTx11, isolated from the venom of *Buthus martensii Karsch*, is determined its solution structure by 2D-NMR spectroscopy and molecular modeling techniques. BmKαTx11 is found to be the first wild-type scorpion toxin whose 'NC-domain' is in 'protruding' conformation while no *cis*-9,10 peptide bond exists. Further study indicates the special Met58 of BmKαTx11 may be the key residue responsible for the distinct conformation of the 'NC-domain'. We suggest that Met58 in BmKαTx11 and corresponding position in all α-toxins function as structure switch of the 'NC-domain' and therefore the bioactivity switch of the toxin specificity.

Materials and methods

NMR data acquisition and analysis. The isolation and characterization of BmKαTx11 were reported previously as toxin "47-31" [17]. BmKαTx11 was dissolved at a concentration of 2.4 mM in 20 mM KH_2PO_4, pH 4.0, 10% (v/v) D_2O. The sample was then freeze-dried and dissolved in 100% D_2O for measurements of amide protons exchange rate. NMR data were acquired at 300 K employ-

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ing a Varian Inova spectrometer operating at 600 MHz proton frequency. The TOCSY spectra were recorded with mixing times of 80 and 120 ms, and the NOESY spectra were recorded with mixing times of 100, 200 and 250 ms. All 2D-NMR spectra were collected as 4096 × 512 data point matrices using 64-128 scans. NMR data were processed using vnmr or NMRPipe and a shifted sine window function and zero filling were applied prior to Fourier transformation. Data analysis was performed with XEASY.

Structure calculation. Distance and dihedral angle constraints (Table 1) were mainly derived from cross-peaks in NOESY (τ_{mix} = 200 ms). $^3J_{HN-H\alpha}$ and $^3J_{H\alpha-H\beta}$ coupling constants obtained from DQF-COSY spectra were converted into dihedral angle constraints. Structures were generated using CYANA, employing simulated annealing algorithms. A total of 200 structures were calculated, and a final ensemble of 20 structures was selected, on the basis of the CYANA target function, to represent the final solution structure. Structure evaluation was performed with PROCHECK-NMR. Three-dimensional conformations were inspected in MOLMOL. The coordinates file of the final ensemble of structures together with the distance constraints files was deposited in the Protein Data Bank as entry 2kbh.

Results

NMR assignments

The identification of amino acid spin systems and the sequential assignment were done using the standard strategy described by Wüthrich [18]. The spin systems were identified in the TOCSY and DQF-COSY spectra. Sequential assignments were obtained from analysis of HN–HN and HN–Hα connectivities in NOESY spectra. All ^{1}H atoms were assigned except HN of Val1, Gly34, Tyr42 and Hα of Ser40. Distance constraints were derived from the two-dimensional NOESY with a mixing time of 200 ms. A summary of the number of constraints per residue is given in Fig. 1A.

Secondary structure

Chemical Shift Index (CSI) was calculated according to the method of Wishart and Sykes [19] for each residue except Ser40 (Fig. 1B). The characteristic NOEs indicating helical, β-strand and Type-I turn structures were found in the NOE connectivity schema (Fig. 1C).

Table 1
Structural statistics for the ensemble of 20 solution structures of BmKαTx11.

Structural constraints	
Intra-residue	391
Sequential	162
Medium-range ($2 \leqslant \|i-j\| \leqslant 4$)	100
Long-range ($\|i-j\| > 4$)	175
Hydrogen bonds constraints	27
Dihedral constraints	261
Stereospecific assignments	26
Disulfide bonds constraints	4
Cyana result	
Cyana target function (Å^2)	0.56 ± 0.01
Maximum distance violation (Å)	0.18
Coordinate precision	
Backbone atom RMSD to the mean (residues 1–64) (Å)	0.47
Heavy atom RMSD to the mean (residues 1–64) (Å)	0.97
Ramachandran plot (% residues)	
Most favored regions	73.9
Additional allowed regions	24.7
Generously allowed regions	1.4
Disallowed regions	0.0

Solution structure

A final ensemble of 20 structures are selected to represent the solution structure (Supplementary Fig. 1A). These structures have low target function value of CYANA and slight distance violations (Table 1). Rmsd per residue is given in Supplementary Fig. 1B. The main secondary structure elements of BmKαTx11 are composed of one stretch of α-helix (Asn19-Lys28), three strands of an anti-parallel β-sheet (strand I, Lys2-Tyr5; strand II, Ser33-Trp38; strand III, Asn44-Leu51), a five-residue-turn (Asp8-Cys12) and two Type-I turn (Lys27-Arg30, Pro52-Ala55). The three-dimensional structure of BmKαTx11 shows a typical structural core of α-toxins with a βαββ topology, stabilized by four disulfide bridges and with flexible loops (Pro13-Arg18, Ala39-Gly43) connecting the main secondary elements (Supplementary Fig. 1C). The peptide bond between residues 9 and 10 definitely adopts the '*trans*' conformation which is supported by a series of NOE cross-peaks between backbone amide hydrogen atoms of residues 9, 10, 11 and 12. On the other hand, the NOE cross-peaks distinctive for the *cis*-9,10 conformation are not observed, especially the one between α-H atoms of residues 9 and 10 (Supplementary Fig. 2).

Conformational heterogeneity

Two stable conformations of BmKαTx11 in solution were noticed at the stage of NMR assignments. Two sets of resonances for residues in the 'five-residue-turn' (Asp8-Cys12) and C-terminal segment (Met58-Asn64) were observed in two-dimensional TOCSY, DQF-COSY and NOESY spectra (Supplementary Fig. 3A and B). The population ratio of two conformers is about 3:1 at 300k according to relevant NMR signal intensity. Two sets of sequential NOE connectivity among residues in the 'five-residue-turn' are similar to a great extent. The difference between major and minor conformations exists in the C-terminal segment. The long-range NOEs between Arg10 and Asn64, observed in the major conformation, disappear in the minor conformation (Supplementary Fig. 3C). Backbone superposition of major and minor conformations reveals the difference in the topology of C-terminal (Supplementary Fig. 3D). The conformation of BmKαTx11 discussed in this paper refers generally to 'major conformation', unless 'minor conformation' is noted.

Discussion

Multiple sequence alignment suggests that BmKαTx11 shares more than 70% sequence identity with classical α-toxins (Fig. 2A). Phylogenetic tree results in three clusters corresponding respectively to three subtypes of α-toxins, and BmKαTx11 is found to cluster with classical α-toxins (AaH2, Lqq5, Bot3, etc.) (Fig. 2B).

A backbone comparison of BmKαTx11 with some representative α-toxins reveals their structural commonality and distinction (Supplementary Fig. 4). The conformation of 'five-residue-turn' in BmKαTx11 is very similar to that in AaH2 [20] with a *trans*-9,10 peptide bond, but different from those in BmK-M1 [21] and LqhαIT [22] where an unusual non-proline *cis* peptide bond between residue 9 and 10 occurs [17,23]. On the other hand, the stretch of C-terminal residues 61–64 in BmKαTx11 same as those in BmK-M1 and LqhαIT is spatially close to the five-residue-turn, while counterpart residues in AaH2 approach to the loop connecting β2 and β3 strands.

Bioinformatics analysis and backbone alignment exhibit the apparent similarity between BmKαTx11 and AaH2, except for the orientation of C-terminus, which is close to the 'five-residue-turn' in BmKαTx11 but far from the 'five-residue-turn' in the latter. Reasonable explanation for such difference is therefore under consid-

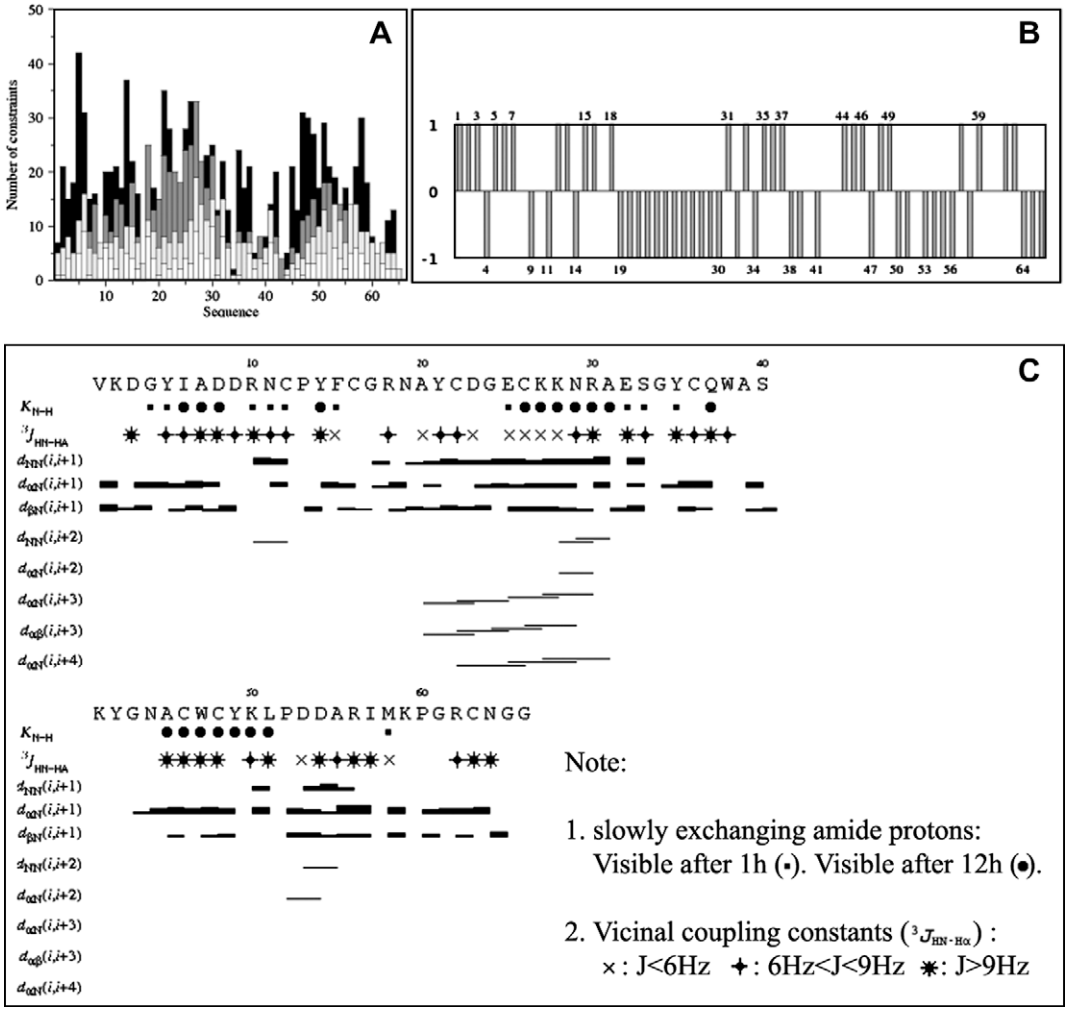

Fig. 1. Structure informations derived from NMR spectra of BmKαTx11. (A) Summary of the number of restraints for each amino acid residue: white for intra-residue, light gray for sequential, dark gray for medium-range, and black for long-range NOEs. (B) Chemical Shift Index (CSI) for α-H of each residue except S40. (C) Sequential and medium-range NOE connectivities are shown as lines according to NOE intensity.

eration. In this study, the distinct residue Met58 in BmKαTx11 or the relevant positions in all α-toxins is assigned as one of structural determinants for the C-tail orientation of α-toxins.

The influences of residue 58 on the C-terminal conformation

Position 58 in most α-toxins is usually occupied by a positively charged residue, Lys (for classical α-toxins) or Arg (for anti-insect α-toxins and part of α-like toxins). The side-chain of Lys or Arg is capable of formation hydrogen bond with backbone carbonyl of Gly61 [12,24]. Notably, the side-chain stretch of Lys is shorter than that of Arg in length, and affords fewer alternative hydrogen atoms than guanidinium group of Arg to form hydrogen bond with Gly61. These structural differences bring about important influences on the geometry of the C-tail. Firstly, due to its shorter side-chain, Lys58 proves to draw up residue 61 much closer to itself than Arg58 does. The d_{58-61} (the distance between Cα of residue 58 and carbonyl oxygen atom of residue 61 is designated as d_{58-61} for convenience) is 7.19 Å in AaH2 and 7.03 Å in BmK-M8 (classical α-toxin) [25], closer than that in LqhαIT and BmK-M1, where the d_{58-61} is 7.71 Å and 7.74 Å, respectively (Fig. 3). As residue Met58 of BmKαTx11 is taken into account, no hydrogen bonds will exist between its hydrophobic side-chain and carbonyl group of Gly61, so that Met58 could hardly restrain the C-terminal loop as Arg58 or Lys58 does. It is reported that the C-tail of Lqh3 (Ile59, corresponding to Met58 in BmKαTx11) is more flexible than that of LqhαIT (Arg58) [26]). The d_{58-61} is 9.27 Å, longer than any other distances mentioned above (Fig. 3C). Secondly, the alteration of the d_{58-61} accompanies the change in orientation of the carbonyl group and main chain conformation of residue 61. The backbone superposition of C-terminal residues reveals that different distance constraints induced by different residue at position 58 result in different orientation of carbonyl group of residue 61: the carbonyl group of residue Gly61 in AaH2 and BmK-M8 points to the inside of the 'NC-domain', while the carbonyl group of residue Gly61 in BmK-M1 and LqhαIT points to out-side of the 'NC-domain' due to the longer distance constraint by Arg58. Accordingly, the different distance constraints lead to alteration in backbone torsion angles of residue Gly61 (Fig. 3D). The angle phi of residue Gly61 is calculated using MOLMOL for BmK-M8 (98.8°), AaH2 (112.1°), LqhαIT (131.0°), BmK-M1 (135.7°) and BmKαTx11 (150.7°). It means that the main chain torsion angle phi in LqhαIT and BmK-M1 is turned over about 30° as compared with those in BmK-M8 and AaH2.

As discussed above, Lys58 generally results in shorter d_{58-61} and smaller phi angle of residue 61 than Arg58 does in wide-type scorpion α-toxins. Besides, such geometric difference induced by Lys58 and Arg58 is also proved by R58K and K58R mutants where the mutation of Arg58 to Lys always leads to shorter d_{58-61} and smaller

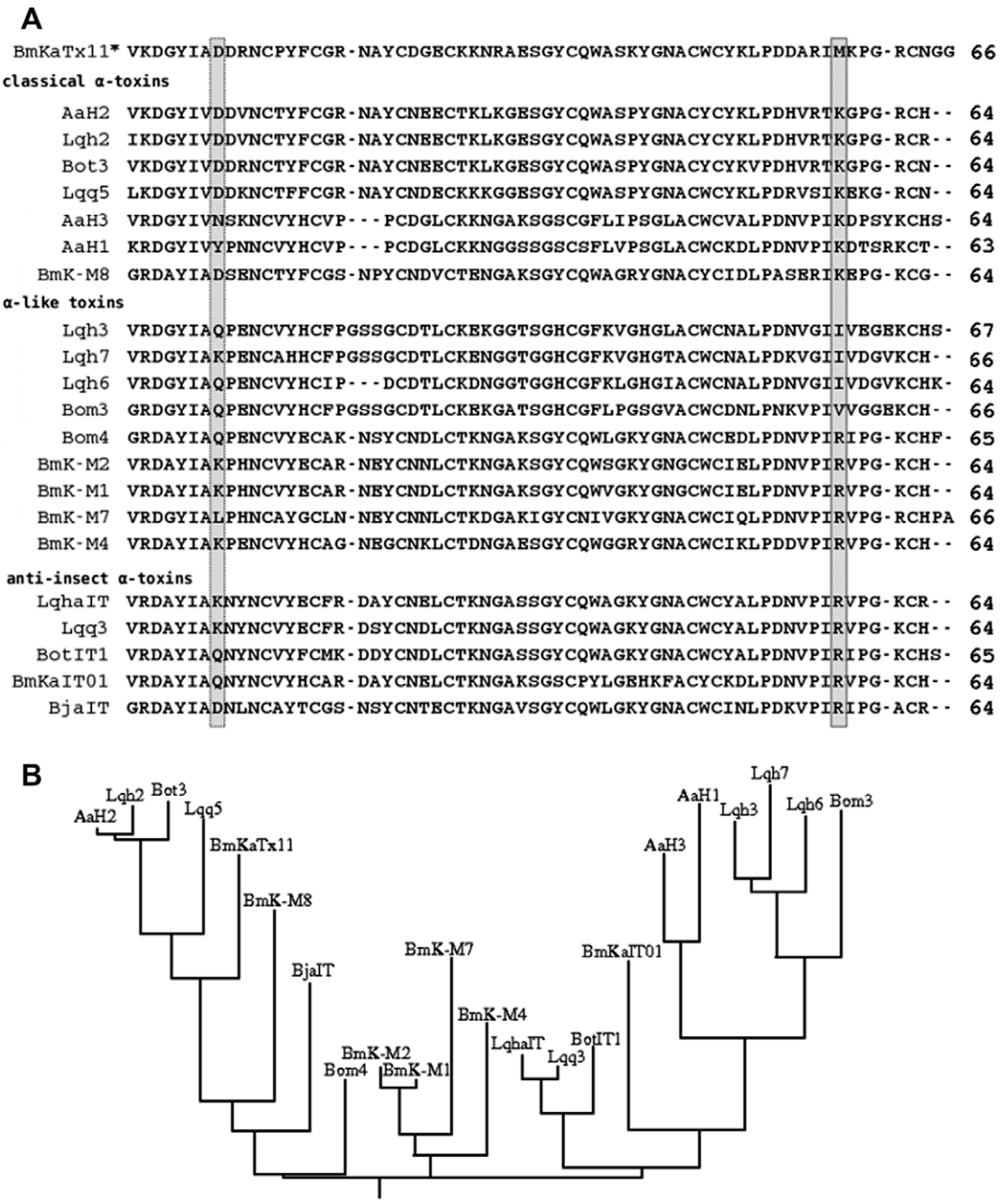

Fig. 2. Bioinformatics analysis of BmKαTx11. Residues at position 8 and 58 in BmKαTx11 and counterpart residues in other α-toxins are shaded. AaH1, AaH2, AaH3 are from *Androctonus australis hector*; Lqh2, Lqh3, Lqh6, Lqh7 and LqhaIT are from *Leiurus quinquestriatus hebraeus*; BotIT1 and Bot3 are from *Bothus occitanus tunetanus*; Lqq3 and Lqq5 are from *Leiurus quinquestriatus quinquestriatus*; BmK-M1, BmK-M2, BmK-M4, BmK-M7, BmKαIT01 and BmK-M8 are from *Buthus martensii* Karsch; Bom3 and Bom4 are from *Buthus occitanus mardochei*; BjaIT is from *Hottentotta judaica*. (A) Multiple sequence alignment using ClustalX (version 2.0.9). (B) Phylogenetic tree is generated using ClustalX and drawn using phylip (version 3.68). (*) BmKαTx11 is supposed to be α-toxin by sequence similarity.

phi angle of residue 61, and vice versa (see BmK-M1 and its R58K mutants, pdb entry: 1sn1, 1zut, 1zyv and 1zyw; AaH2 and its K58R mutant, pdb entry: 1ptx and 1seg).

Notably, the bulky residue at position 58 also acts as a support to stabilize the local conformation through the side-chain hydrophobic interactions with residues 5, 13 and 42 (Supplementary Fig. 5). The mutation of Arg58 to Ala [10] in BmK-M1 greatly reduces the volume of residue 58 and makes it impossible to sustain the original hydrophobic cluster, which inevitably alters at least the spatial arrangement of two highly conserved aromatic residues Tyr5 and Tyr42 that are important for toxicity [13]. Therefore, the dramatic loss of toxin activity is not surprising.

The hydrogen bond network in the 'NC-domain'

The different influences on the C-terminal conformation exerted by Lys58, Arg58 and Met58 are correlated with different pattern of hydrogen bond networks which connect the C-tail with the 'five-residue-turn'. Structurally, the C-terminal segment is connected to the 'five-residue-turn' by Cys12–Cys63 disulfide linkage. The side-chain of residue Asn11 from the 'five-residue-turn' extends to the C-terminal segment and its terminal amide group forms complex hydrogen bond network with residues wherein. One pair of hydrogen bonds between the side-chain of Asn11 and backbone of residue 59 usually exist in α-toxins (11OD1...59HN and 11HD22...59O, Fig. 4). The disulfide bond

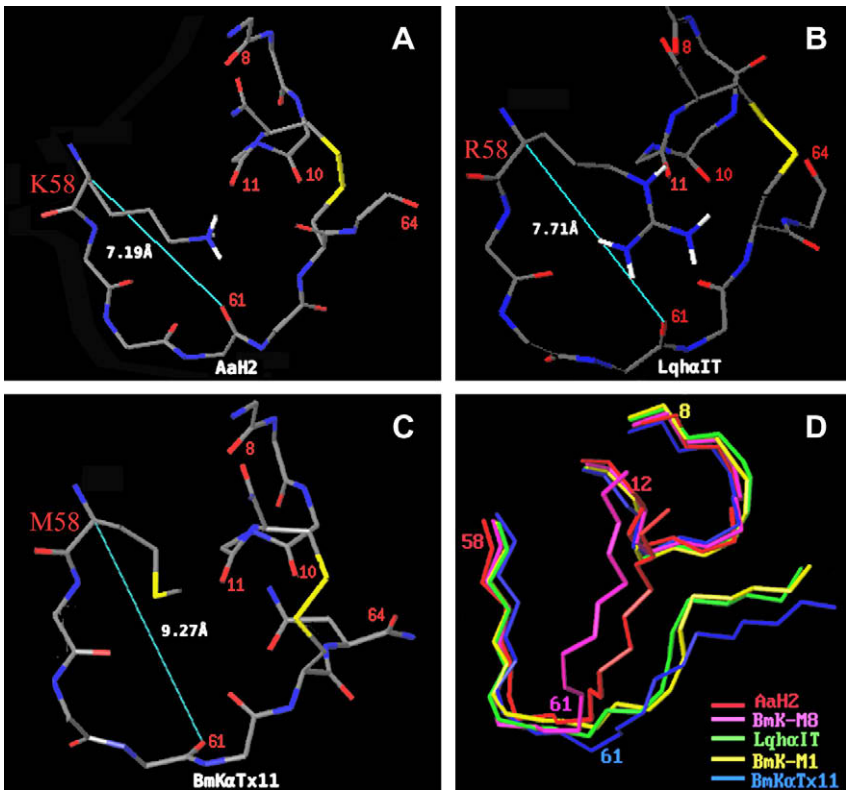

Fig. 3. Different influences on the geometry of C-tail exerted by Lys58, Arg58 and Met58. The distances between Cα of residue 58 and O of residue 61 in (A) AaH2 (pdb entry: 1ptx), (B) LqhαIT (pdb entry: 2asc), (C) BmKαTx11 (pdb entry: 2kbh) are calculated. (D) Lys58 results in distinct backbone torsion angle Phi of Gly61.

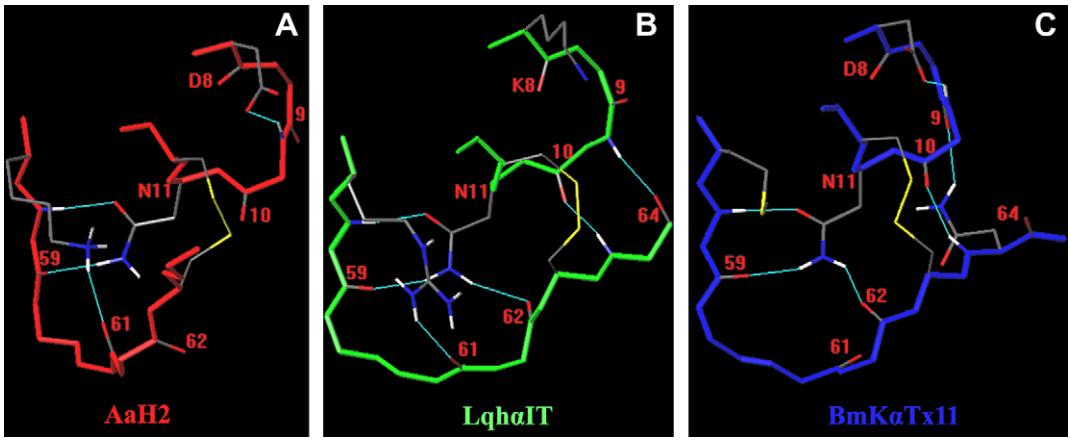

Fig. 4. Hydrogen bond networks of the 'NC-domain' of scorpion α-toxins. One pair of hydrogen bonds between side-chain of residue 11 and backbone of residue 59 are generally present. (A) AaH2 represents the classical α-toxins. (B) LqhαIT represents insecticidal α-toxins with Arg58. (C) BmKαTx11 represents the α-toxins with hydrophobic residues at position 58.

Cys12–Cys63 together with this pair of hydrogen bonds acts as common structural basis for tertiary arrangement of the 'five-residue-turn' and C-tail (the 'NC-domain') in scorpion α-toxins. While the 'protruding' or 'flat' topology of the 'NC-domain' for α-toxins active on insects or mammals is accomplished by distinct hydrogen bond pattern, respectively.

For α-toxins active on insects, i.e., anti-insect α-toxins or α-like toxins, the residue type at position 58 is usually Arg or hydrophobic residues (Fig. 2A) when residue 62 naturally occupies the convenient location near the extended line from the side-chain atom ND2 to atom HD21 of Asn11 to form strong hydrogen bond (11HD21...62O) which makes the C-terminus spatially close to the 'five-residue-turn' (Supplementary Fig. 5) and then facilitates the formation of hydrogen bond between backbone atoms of residues 10 and 64 (10O...64HN). In this situation, the 'NC-domain' features 'protruding' topology which is usually reinforced by another hydrogen bond, e.g., 10HN...64O in LqhαIT (Fig. 4B).

For α-toxins specific for mammals, i.e., classical α-toxins, Lys58 is thoroughly conserved (Fig. 2A). The hydrogen bond between 58HZ and 61O transforms the backbone of the C-tail into a distinct orientation (Fig. 3D), and then locates residue 62 in a position unfavorable to form hydrogen bond between 11HD21 and 62O (Fig. 4A), which makes the C-terminus spatially far from the 'five-residue-turn' (Supplementary Fig. 5) and close to the loop

connecting β2 and β3 strands (part of the 'Core-domain'). In this situation, the 'NC-domain' features 'flat' topology which is further stabilized by a hydrogen bond between backbone carbonyl of residue 42 and side-chain of residue 64 (e.g., 42O...64HD1 in AaH2 [12]).

The conformation of the 'five-residue-turn'

The topology of 'NC-domain' is vital for the function and specificity of α-toxins. The 'protruding' or 'flat' topology of 'NC-domain' usually correlates with *cis* or *trans*-9,10 conformation in the 'five-residue-turn' of wide-type scorpion α-toxins specific for insects or mammals, respectively. But exceptions have been found in BmKαTx11 and some K8D mutants of LqhαIT (pdb entry: 2atb) and BmK-M1 (pdb entry: 1t7a and 1zvg) where *trans*-9,10 still results in 'protruding' topology of 'NC-domain'. It is believed that the formation of hydrogen bond between Oδ of Asp8 and HN of residue 10 inhibits the conversion of *trans*/*cis*-9,10 peptide bond (Fig. 4A and C). The anti-insect α-toxin BjαIT [6,27] same as BmKαTx11 is supposed to adopt *trans*-9,10 conformation in the 'five-residue-turn' for its Asp8 (Fig. 2A), and its 'NC-domain' is supposed to be in 'protruding' topology according to its obvious anti-insect specificity [12]. For toxins possessing 'protruding' topology of the 'NC-domain' and *trans*-9,10 conformation in the 'five-residue-turn', such as BmKαTx11 and those K8D mutants mentioned above, their side-chains of residue 64 (N64, R64 and H64) extend to residue 9, which facilitates the formation of hydrogen bond between side-chain amide hydrogen of residue 64 and backbone carbonyl oxygen atom of residue 9 (e.g., 9O...64HD21 in BmKαTx11, Fig. 4C). We consider this hydrogen bond as an alternative of the hydrogen bond 10HN...64O which is typical for *cis*-9,10 conformation (Fig. 4B). Therefore, the topology of 'NC-domain' is clearly independent of the *trans*/*cis*-9,10 conformation in the 'five-residue-turn' and *cis*-9,10 conformation [27,28] is one kind of means for wide-type scorpion α-toxins to stabilize their 'protruding' topology of the 'NC-domain'. The conversion of *trans*/*cis*-9,10 conformation will be not necessary if the sequence composition of the C-terminus and 'five-residue-turn' affords an alternative hydrogen bond combination to stabilize its 'protruding' topology of the 'NC-domain'.

According to our earlier study, BmKαTx11 is lack of obvious toxicity to mammals and insects [17]. Its 'protruding' topology of the 'NC-domain' seems reasonable for the weak toxicity to mammals, while whether the combination of its hydrophobic residue at position 58 and *trans*-9,10 conformation in the 'five-residue-turn' decreases the toxicity to insects requires further investigation. It is also possible for this kind of wide-type scorpion toxins to be active on animals other than insects or mammals, e.g., animals from class Aves. However, the corresponding research remains vacant.

Conclusion

BmKαTx11, as the first wide-type α-toxin with 'protruding' topology of the 'NC-domain' where no *cis*-9,10 peptide bond exists, provides valuable clues about the structural determinants of C-tail orientation. Our study highlights that the residue at position 58 plays an important role as structure switch of the 'NC-domain' geometry and then bioactivity switch of the toxin specificity. The distinct topology of 'NC-domain' is stabilized by two types of hydrogen bond networks for α-toxins to perform anti-mammal or anti-insect activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.110.

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