Unusual quaternary structure of a homodimeric synergistic-type toxin from mamba snake venom defines its molecular evolution

Narumi Aoki-Shioi\textsuperscript{a, c,*}, Chacko Jobichen\textsuperscript{b}, J. Sivaraman\textsuperscript{b}, R. Manjunatha Kini\textsuperscript{b, c,*}

\textsuperscript{a} Department of Chemistry, Faculty of Science, Fukuoka University, 19-1, 8-chome Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan, anarumi@fukuoka-u.ac.jp

\textsuperscript{b} Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, 117543, Singapore, jobichenc@nus.edu.sg, dbsjayar@nus.edu.sg, and dbskinim@nus.edu.sg

\textsuperscript{c} Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, MD3, 16 Medical Drive, Singapore 117600, Singapore, dbskinim@nus.edu.sg

*To whom correspondence should be addressed:

Narumi Shioi: Department of Chemistry, Faculty of Science, Fukuoka University, 19-1, 8-chome Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan; E-mail: anarumi@fukuoka-u.ac.jp; Tel.: +81-(0)92-870-6631 ext. 6215; Fax.: +81-(0)92-865-6030.

R. Manjunatha Kini (R.M.K.): Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, MD3, 16 Medical Drive, Singapore 117600, Singapore; E-mail: dbskinim@nus.edu.sg; Tel.: +65-6516-5235; Fax.: +65-6779-2486
Abstract

Snake venoms are complex mixtures of enzymes and non-enzymatic proteins that have evolved to immobilize and kill prey animals or deter predators. Among them, three-finger toxins (3FTxs) belong to the largest superfamily of non-enzymatic proteins. They share a common structure of three β-stranded loops extending like fingers from a central core containing all four conserved disulfide bonds. Most 3FTxs are monomers and through subtle changes in their amino acid sequences they interact with different receptors, ion channels and enzymes to exhibit a wide variety of biological effects. The 3FTxs have further expanded their pharmacological space through covalent or noncovalent dimerization. Synergistic-type toxins (SynTxs) isolated from the deadly mamba venoms, although nontoxic, have been known to enhance toxicity of other venom proteins. However, the details of three-dimensional structure and molecular mechanism of activity of this unusual class of 3FTxs are unclear. We determined the first three-dimensional structure of a SynTx isolated from Dendroaspis jamesoni jamesoni (Jameson's mamba) venom. The SynTx forms a unique homodimer that is held together by an interchain disulfide bond. The dimeric interface is elaborate and encompasses loops II and III. In addition to the inter-subunit disulfide bond, the hydrogen bonds and hydrophobic interactions between the monomers contribute to the dimer formation. Besides, two sulfate ions that mediate interactions between the monomers. This unique quaternary structure is evolved through noncovalent homodimers such as κ-bungarotoxins. This novel dimerization further enhances the diversity in structure and function of 3FTxs.

Keywords

Disulfide rich peptide, Dimerization, Molecular diversity, Molecular evolution, Three-finger toxin
Introduction

Snake venoms are cocktails of pharmacologically active toxins that have evolved to immobilize and/or kill the prey or to deter predators. These snake venom toxins belong to a small number of superfamilies of either enzymes or nonenzymatic proteins. Three-finger toxins (3FTxs) are a large superfamily of nonenzymatic proteins. Venoms from elapid snakes such as cobras, kraits, mambas, coral snakes and sea snakes, are rich sources of 3FTxs. 3FTxs are characterized by three β-stranded loops which stretch like fingers from a conserved core [1,2]. This canonical structure is maintained by four conserved disulfide bridges located in the hydrophobic core.

Some 3FTxs have an additional fifth disulfide bridge in either loop II (long-chain neurotoxins) or loop I (nonconventional toxins) [3]. Despite the structural conservations, snake venom 3FTxs exhibit markedly different biological properties [4,5]. Based on their functions, 3FTxs are classified as nicotinic acetylcholine receptor (nAChR) antagonists, muscarinic toxins, adrenergic toxins, GABA receptor antagonists, acetylcholinesterase inhibitors, cardiotoxins (or cytotoxins), L-type calcium channel blockers, anticoagulants and platelet aggregation inhibitors [4,5]. In addition, there are 20 classes of orphan toxins whose function and target receptors or ion channels are not yet known [6]. Most 3FTxs exist as monomers and the wide variety of their pharmacological effects is due to their subtle structural variations [4–6]. Less than 1% of 3FTxs occur as noncovalent and covalent dimers. The noncovalent dimers include the κ-neurotoxins [7,8], haditoxin [9] and Σ-neurotoxins [10]. The first two share similarity in their quaternary structures, while fulditoxin, the first member of Σ-neurotoxins, has a distinct quaternary structure [10]. The covalent dimers are held together by inter-subunit disulfide bonds. The disulfide-bonded homo- and hetero-dimers of 3FTxs were purified from Naja kaouthia (Monocled cobra) venom. α-cobratoxin, a long-chain α-neurotoxin, forms a homodimer with itself and heterodimers with different cardiotoxins [11,12]. We characterized a heterodimer, irditoxin from Boiga irregularis (Brown tree snake) in which the two subunits were held together by a single
disulfide bond [13]. Interestingly, all these dimers described above are antagonists of various nAChRs. Thus, the 3FTxs appears to have further expanded their pharmacological space through covalent or noncovalent dimerization.

About four decades ago, three unusual dimeric toxins were purified from the deadly Dendroaspis (mamba) venoms; a homodimer S2C4 from D. jamesoni kaimosae and two heterodimers C8S2 and C9S3 from D. angusticeps [14,15]. Interestingly, these proteins are nontoxic but enhance the lethality of some toxins. Therefore, these toxins were named as synergistic-type toxins (SynTxs) [15]. As the subunits are held together by disulfide bonds, the subunits were separated by reduction and alkylation and their amino acid sequences were determined by Edman degradation. There were minor errors in the sequences at the C-terminal ends of the subunits; these errors were corrected by the mRNA sequence of D. angusticeps [16] and recent transcriptomic studies [17]. Further, SynTxs as well as some novel 3FTxs and dendrotoxins were found in mamba venoms using high-throughput proteomic studies. Although mamba snakes are classified among the most dangerous venomous snake in the world, remarkably they lack the typical \( \alpha \)-neurotoxins found in other elapid snake venoms. In addition, toxicity studies of all reverse-phase-HPLC fractions showed the lack of significant lethal activity in any of the toxins [17,18]. Thus, the overall toxicity seems to depend on the synergistic action of various types of proteins. Thus, SynTxs probably play a key role in mechanism of toxicity of mamba snake venoms. Currently, the details of three-dimensional structure and molecular mechanism of action of SynTxs are unclear.

Herein we describe the first crystal structure of a SynTx isolated from D. jamesoni jamesoni venom. The homodimer is held together by a single disulfide bond resulting in unique quaternary structure. The structure will help in understanding of the evolution, structure-function relationships and mechanism of synergistic function of SynTxs.
Materials and Methods

Purification and isolation of synergistic type toxin from *Dendroaspis jamesoni jamesoni* crude venom (*Dj*-SynTx).

The crude *Dendroaspis jamesoni jamesoni* venom 150 mg dissolved in 1 mL ultra-pure water (Milli-Q, Merck, Darmstadt, Germany), filtered and loaded to a HiLoad™ 16/300 Superdex 30 prep grade size-exclusion chromatography column (GE Healthcare, Chicago, IL) with 10 mM ammonium bicarbonate buffer. Fractions containing the target protein were purified on two steps by reverse phase-high performance liquid chromatography (RP-HPLC) using two Jupiter C18 columns (5 μm, 300 Å) (Phenomenex, Torrence, CA); the first is 10 x 250 mm, and second is 4.6 x 250 mm. Each column was equilibrated with 0.1% (v/v) TFA. Proteins were eluted with a linear gradient of 80% (v/v) acetonitrile in 0.1% (v/v) TFA and monitored at 280 and 215 nm using a ÄKTA purifier system (GE Healthcare, Chicago, IL) and lyophilized. The molecular mass of purified proteins was determined by Electrospray ionization-mass spectrometry.

Protein alkylation and determination of amino acid sequence of *Dj*-SynTx. Following the ProteaseMAX™ surfactant digestion protocols (Promega, Madison, WI), purified *Dj*-SynTx (0.5 mg) in 50 mM NH₄HCO₃ (pH 8.5) incubated with DTT (final 5 mM) at 56 °C for 30 min. Subsequently, iodoacetamide (final 15 mM) was added and the mixture was incubated at room temperature for 15 min. The alkylated protein was separated from the reaction mixture by RP-HPLC on a Jupiter C18 column (5 μm, 300 Å, 4.6 x 150 mm) using a linear gradient of 80% acetonitrile in 0.1% TFA. To determine of amino acid sequence using mass spectrometry, we generated short peptides that are easily amenable for sequencing. The alkylated protein was digested by each enzyme such as trypsin, endoproteinases Asp-N or Lys-C (sequencing grade) (Promega, Madison, WI) in a 50:1 toxin:protease molar ratio at 37 °C for 16 h. The sequences of peptides from each toxin digest after protease cleavage was separated and sequenced by Liquid Chromatography- Mass Spectrometry (LC-MS/MS) on an LCQ Fleet Ion Trap Mass
Spectrometer (Thermo Fisher Scientific, United States) fitted with a 50 × 2.1 mm Hypersil Gold column (Phenomenex, Torrence, CA). Parent scans of multiply-charged ions were measured over a mass range of 500–2000 Da at 3 μscans/200 ms. Subsequently, MS/MS of the top 3 most intense ions from each parent scan were performed for peptide sequencing. Fragmentation of peptides was accomplished by collision-induced dissociation using helium gas. Product ions were scanned for 30 ms (3 μscans/25 ms), and dynamic exclusion was enabled in order to acquire sequences of all possible ions from the parent scan. The MS/MS data were analyzed using SEQUEST and Proteome Discoverer (Thermo Scientific) software. For more details, see [19].

**Data collection and structure determination of the covalent dimer of Dj-SynTx.**

The SynTx protein was concentrated to 8.0 mg/ml in phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) and the crystallization screens were setup with Hampton Research Crystal Screen I and II (Hampton Research, Aliso Viejo, CA) using the sitting-drop vapor-diffusion method by Mosquito robot (TTP Labtech, Royston, UK). Initial crystals were obtained from the following condition; 0.2 M potassium sodium tartrate tetrahydrate, 0.1 M sodium citrate tribasic dihydrate pH 5.6, 2.0 M ammonium sulfate. This condition was further optimized using grid screening and the diffraction quality crystals were obtained using 0.25 M potassium sodium tartrate tetrahydrate, 0.1 M sodium citrate tribasic dihydrate pH 5.4, 2.0 M ammonium sulfate. Prior to data collection the crystals were cryoprotected in the reservoir solution supplemented with 25% glycerol and flash cooled at 100°K. A complete data set was collected at beamline 19BM (Advanced Photon Source, Argonne National Laboratory, Chicago, USA) using a ADSC 315r detector. This crystal diffracted up to 2.4 Å resolution (Table 1). The data was processed with HKL2000 program [20]. There were two molecules of Dj-SynTx in the asymmetric unit. The Matthews coefficient was estimated to be 2.69 Å³/Da [21], corresponding to a solvent content of 54%. The structure was solved by Autorickshaw program [22] using
molecular replacement method. The coordinates of Muscarinic Toxin 2, which shows 61.5% identity and 75.4% similarity with Dj-SynTx (PDB Code: 1FF4) was used as the search model. The model was built using the AutoBuild program [23], followed by manual model building using COOT program [24]. The structure was refined using Phenix-refine program [25]. The model has good stereochemistry, with 99.0% residues within the allowed regions of the Ramachandran plot analyzed by PROCHECK [26] (Table 1).

**Results and Discussion**

**Purification of a synergistic-type toxin from African mamba snake.**

We purified a synergistic-type toxin (Dj_SynTx) from *D. jamesoni jamesoni* venom by a two-step chromatography of size exclusion chromatography and reverse-phase chromatography (RP-HPLC) (Supplementary Figures S1A-B). The native Dj_SynTx eluted as a single peak on an analytical column with a molecular mass 14122.9±0.98 Da and upon reduction and alkylation with iodoacetamide eluted as a single peak with a molecular mass 7584.7±0.41 Da (Figure 1 and Supplementary Figures S1C-D) indicating that Dj_SynTx is a homodimer. The complete amino acid sequence was determined by LC-MS and LC-MS/MS of peptides obtained by digestion of alkylated Dj_SynTx by trypsin, Asp-N and Glu-C endoproteases (Figure 2A and 2B). The complete sequence matches with the deduced sequence (T3431) determined by transcriptome analysis of *D. jamesoni* [17]. Thus, Dj_SynTx shows high similarity with other SynTxs from mamba venom (Figure 1B and 2C).

**Three-dimensional structure of synergistic-type toxin.**

The crystal structure of the native Dj_SynTx was determined to 2.4 Å resolution (Table 1). The superposition the two subunits yielded an RMSD of 0.33 Å for 65 Ca atoms (Supplementary
Figure S2). Both subunits have typical 3FTx structure with four conserved disulfide bonds and five \( \beta \)-strands (Figures 1C-D). The comparison of topologically similar protein structures with \( Dj \text{--SynTx} \) selected from Protein Data Bank (PDB) database by DALI program [27] showed the high similarity with neurotoxins and cardiotoxins belonging to 3FTx family for \( Dj \text{--SynTx} \) on almost less than 3.0 Å of RMSD although amino acid sequence identity are less than 50 % (Supplementary Table S1).

Three structural features contribute to the formation of unique quaternary structure of \( Dj \text{--SynTx} \). The dimer is covalently held together by C54\(^A\)-C54\(^B\) interchain disulfide bond (Figure 3A), and dimeric interfaces formed by direct interaction between the two subunits (Figure 3B), and ionic and hydrogen bond interactions mediated through two sulfate ions (Figure 3C). The antiparallel \( \beta \)-sheet formed between loop III segments (Asp51-Lys56) brings the two monomers together through a series of hydrogen bonds, either directly between the \( \beta \)-strands or mediated through water molecules and two hydrogen bonds between \( \varepsilon \)-N of Lys56 with O of \( \alpha \)-carbonyl of Asp51 (Table 2), leading to the covalent C54\(^A\)-C54\(^B\) disulfide bond (Figure 3A). The dimeric interface is formed by hydrogen bonds and electrostatic interactions between Trp28 and Lys34 of one subunit with Tyr36 and Asp37 of the other subunit, respectively (Figure 3A, Table 2). There are two \( \pi \)-\( \pi \)-interactions (3.8 Å) between Trp28 from chain A and Tyr36 from chain B residues and vice versa. In addition, the dimer is held together by two sulfate ion-mediated interactions (Figure 3C, Table 3). Each sulfate ion is electrostatically caged by Lys30, Lys34, Leu35 and Tyr36 from one subunit and Lys7 and Arg40 from the other subunit. Sulfate ions exist in human blood (~0.5 mM), which are essential for the synthesis of many biomolecules such as glycosaminoglycans, choline sulfate, steroid sulfate, cerebroside sulfate and heparan sulfate [28]. Although crude venom contains inorganic salts, there is documented evidence for the presence of sulfate. It is unclear whether the sulfate ions are from \textit{Dendroaspis} venoms or are incorporated in the structure during crystallization. Of the 1164 Å\(^2\) of the buried surface area
during the dimer formation, 25% is formed by hydrophobic residues, 39% by polar residues, and 36% by charged residues. Most amino acid residues involved in dimerization are found on the concave surface of the monomers (Supplementary Figures S3 and S4). All these residues are conserved in mamba SynTx family of 3FTxs (Figure 1B).

Quaternary structures of dimeric 3FTxs.

The κ-neurotoxins, a family of 3FTxs all isolated from Bungarus (krait) venoms, are the first group of homodimers of 3FTxs [7] (Figure 4). They bind to a variety of neuronal nAChRs [8] but bind reversibly and with weak affinity to muscle-type peripheral nAChR [29]. κ-bungarotoxins show similarity to long-chain α-neurotoxins that have the fifth disulfide in loop II but without the C-terminal extension. In the dimer, the three-stranded β-sheet of the monomer is extended to a six-stranded β-sheet and the two monomers are antiparallel to each other [30]. Haditoxin from Ophiophagus hannah (king cobra) venom is a homodimer of short-chain type 3FTxs that does not have the fifth disulfide bond [9]. It interacts with neuronal α7 and muscle-type nAChRs. Haditoxin shares similar quaternary structure as the κ-neurotoxins. The antiparallel dimeric interface is maintained by six main chain-main chain hydrogen bonds (9, 20). κ-bungarotoxin and haditoxin have three and eight side chain hydrogen-bonding contacts between the monomers. Fulditoxin, the first member of Σ-neurotoxins from Micrurus (coral snake) venoms, is also a noncovalent homodimer of short-chain type 3FTxs [10]. It binds to chicken muscle-type receptor with high potency compared to cloned human receptor. Fulditoxin has a distinct quaternary structure; the two subunits intertwine each other through hydrophobic and hydrogen bond interactions. Thus, at least two distinct types of noncovalent dimers have evolved (Figure 4).

All three covalent dimers have distinct quaternary structures (Figure 4) and hence, they probably evolved independently. In α-cobratoxin homodimers from Naja kaouthia venom, the
subunits are covalently linked through two disulfide bonds; the loop I segments of both subunits stretch to form new antiparallel β-sheet with that of the other subunit (Figure 5) [12]. Normally, this long-chain α-neurotoxin occurs as a monomer (Figure 5-'a'). With the change from Cis to Trans isomerization of X-Pro7 peptide bond, the loop I opens and extends the N-terminal segment away [12] from the core of the protein (Figure 5-'b'). There is significant entropic cost for unfolding of loop I. The newly exposed surface is ‘buried’ through the second monomer with similar extended conformation forming two interchain disulfide bonds (C1A-C3B and C3A-C1B) (Figure 5-'c') and form this dimer. The high entropic cost of the extension of the N-terminal is probably the reason why this dimer is only 0.04% of the venom [9] compared to 15-20% of the monomer [31]. In the case of irditoxin isolated from Boiga irregularis (Brown tree snake) venom, the heterodimer is covalently held together by a single disulfide bond [13]. Both subunits show similarity to nonconventional toxins with the fifth disulfide bond in the loop I. Unlike other nonconventional toxins, the subunits of this colubrid toxin have the extraordinarily 7-residue long, unstructured NH2 terminal segments that are located above the core. The interchain disulfide bond linkage is between Cys in loop II of A subunit with Cys in loop I of B subunit and the subunits are placed in a diagonal geometry [13]. As described above, Dj_SynTx dimer has elaborate interactions in dimer formation. Interestingly, the disulfide linkage is driven by similar antiparallel β-sheet formation that is involved in κ-neurotoxins and haditoxin. We speculate that the interchain disulfide linkage followed by the evolution of other interactions between the subunits results in the unique quaternary structure of SynTxs (see below).

**Dimerization leads to diversification of pharmacological functions in 3FTxs.**

Monomeric long- and short-chain α-neurotoxins block peripheral muscle-type nAChRs with similar affinities [32] but only long-chain α-neurotoxins block neuronal α7, α9 and α9α10 nAChRs with high affinity [33]. The dimerization of long- and short-chain neurotoxins alters their
subtype selectivity. \(\kappa\)-bungarotoxins most potently block \(\alpha 3\)-containing nAChRs (IC\(_{50}\) <150 nM) but have lower or no affinity towards muscle-type (\(\alpha 1\)-), \(\alpha 7\), \(\alpha 8\), and \(\alpha 9\) nAChRs [29]. \(\alpha\)-cobratoxin homodimer acquires the ability to block \(\alpha 3\)\(\beta 2\) neuronal nAChR [9]. Its ability to muscle-type and \(\alpha 7\) is reduced. In the heterodimers, cardiotoxin subunits lose their cytolytic activity completely [9]. Similarly, the dimerization in haditoxin, a short-chain neurotoxin, results in its ability to bind to both muscle-type and neuronal \(\alpha 7\), \(\alpha 3\)\(\beta 2\) and \(\alpha 4\)\(\beta 2\) nAChRs [9]. Thus, the dimerization allows 3FTxs further diversify their pharmacological functions.

**Molecular evolution to unusual quaternary structure of SynTx dimers.**

A careful evaluation of dimeric interfaces indicates that SynTx quaternary structure evolved systematically from noncovalent dimers (Figure 6). Although most 3FTxs are monomers in solution (Figure 6-'a'), some 3FTxs crystallize as noncovalent dimers [34] (Figure 6-'b'). Such dimers have similar antiparallel \(\beta\)-sheet similar to \(\kappa\)-bungarotoxins. Two pairs of electrostatic interactions (Glu54\(^A\)-Lys56\(^B\) and Lys56\(^A\)-Glu54\(^B\) in \(\alpha\)-elapitoxin-Dpp2d) also contribute this 'transient' dimer formation (Figure 6A). With slightly increased salt concentration (isomotic), the dimer breaks down to monomers [34]. Thus, this group of 3FTxs are monomeric at physiological conditions. This dimeric interface is stabilized further with additional noncovalent (hydrophobic and electrostatic) interactions in \(\kappa\)-bungarotoxins and haditoxin [9,30] (Figures 6-'c', 7B). These 3FTxs remain as dimers even at high salt concentrations. However, when \(\kappa\)-bungarotoxin is mixed with \(\kappa\)-flavitoxin, due to dissociation and association kinetics, they form a mixture of homo- and bungarotoxin-flavitoxin hetero-dimers [35]. Thus, there is an equilibrium state in which the molecules remain in the dimeric forms but not in monomeric forms. In SynTxs, all the residues involved in the dimerization are fully conserved (Figure 1B). The cysteine in loop III segment is involved in interchain disulfide bond that covalently links the monomers (Figure 6-'d'). With further evolution of the interactions in the dimeric interface (described above), the
monomers twist and contort to evolve as the novel quaternary structure in SynTx (Figure 6-'e'). This contortion leads to a slight shift of the antiparallel β-sheet by two amino acid residues towards the N-terminal side (Figure 7C). Thus, we propose that the unique dimers of SynTxs have evolved from monomeric 3FTXs through noncovalent dimers like haditoxin and κ-bungarotoxins (Figure 6). To our knowledge, this is the first instance in which the evolution of a quaternary structure is determined through the three-dimensional structure.

The other 3FTx dimers all exhibit postsynaptic neurotoxicity by binding to acetylcholine-binding pocket of nicotinic acetylcholine receptors. Dimerization changes their subtype selectivity (discussed above). Unlike other dimers of 3FTxs, SynTxs have acquired ability to synergistically enhance the toxicity of other snake venom toxins. Interestingly, the electrostatic charge distribution in Dj_SynTx (Supplementary Figure S5) indicates that the positive and negative charges cluster on both surfaces of the molecule. This structure will help in understanding the structure-function relationships and mechanism of action of this unique class of 3FTxs including mechanism of synergistic function.

Data Availability
Coordinates and structure factors of the Synergistic-type toxin are deposited in PDB (Protein Data Bank) with accession code 7C28.

Competing Interests
Authors declare that there are no competing interests with the data described in this manuscript.

Funding
This work was supported by Discovery Grants by JSPS KAKENHI Grant Number 17KK0179 and JBC31025 from Japanese government. Central Research Institute of Fukuoka University (181042 and 197104) supported by the researcher support programme of Fukuoka University to N.S. and Academic Research Grants to R.M.K.
Author Contributions

N.A.S. performed purification, sequence determination and crystallization, as well as the writing of the first draft. C.J. performed X-ray diffraction, data analysis and structure determination. J.S. supervised the structure determination and evaluation. R.M.K. contributed to the concept, evaluation of structure, writing and administration. N.A.S. and R.M.K. contributed to funding acquisition. All authors contributed to preparations of figures and tables, provided critical feedbacks and read all versions of the manuscript.

Acknowledgments

We thank Febby Anne for her assistance of preliminary purification of Synergistic toxins.

Abbreviations

3FTxs, three-finger toxins; SynTxs, Synergistic-type toxins; nAChR, nicotinic acetylcholine receptor; Dj_SynTx, a synergistic-type toxin from *D. jamesoni jamesoni* venom.

References

[1] Ménez, A. (1998) Functional architectures of animal toxins: a clue to drug design? *Toxicon* 36, 1557–1572.

[2] Dufton, M. J. and Hider, R. C. (1988) Structure and pharmacology of elapid cytotoxins. *Pharmacol. Ther.* 36, 1–40.

[3] Nirthanan, S., Gopalakrishnakone, P., Gwee, M. C. E., Khoo, H. E. and Kini, R. M. (2003) Non-conventional toxins from Elapid venoms. *Toxicon* 41, 397–407.

[4] Kini, R. M. (2002) Molecular moulds with multiple missions: Functional sites in three-finger toxins. *Clin. Exp. Pharmacol. Physiol.* 29, 815–822.

[5] Kini, R. M. and Doley, R. (2010) Structure, function and evolution of three-finger toxins: Mini proteins with multiple targets. *Toxicon* 56, 855–867.
[6] Fry, B. G., Wüster, W., Kini, R. M., Brusic, V., Khan, A., Venkataraman, D. and Rooney, A. P. (2003) Molecular Evolution and Phylogeny of Elapid Snake Venom Three-Finger Toxins. *J. Mol. Evol.* 57, 110–129.

[7] Grant, G. A. and Chiappinelli, V. A. (1985) κ-Bungarotoxin: complete amino acid sequence of a neuronal nicotinic receptor probe. *Biochemistry* 24, 1532–1537.

[8] Chiappinelli, V. A., Weaver, W. R., McLane, K. E., Conti-Fine, B. M., Fiordalisi, J. J. and Grant, G. A. (1996) Binding of native κ-neurotoxins and site-directed mutants to nicotinic acetylcholine receptors. *Toxicon* 34, 1243–1256.

[9] Roy, A., Zhou, X., Chong, M. Z., D’hoedt, D., Foo, C. S., Rajagopalan, N., Nirthanan, S., Bertrand, D., Sivaraman, J. and Kini, R. M. (2010) Structural and Functional Characterization of a Novel Homodimeric Three-finger Neurotoxin from the Venom of *Ophiophagus hannah* (King Cobra). *J. Biol. Chem.* 285, 8302–8315.

[10] Foo, C. S., Jobichen, C., Hassan - Puttaswamy, V., Dekan, Z., Tae, H., Bertrand, D., Adams, D. J., Alewood, P. F., Sivaraman, J., Nirthanan, S., et al. (2020) Fulditoxin, representing a new class of dimeric snake toxins, defines novel pharmacology at nicotinic ACh receptors. *Br. J. Pharmacol.* 177, 1822–1840.

[11] Osipov, A. V., Kasheverov, I. E., Makarova, Y. V., Starkov, V. G., Vorontsova, O. V., Ziganshin, R. Kh., Andreeva, T. V., Serebryakova, M. V., Benoit, A., Hogg, R. C., et al. (2008) Naturally Occurring Disulfide-bound Dimers of Three-fingered Toxins: A Paradigm for Biological Activity Diversification. *J. Biol. Chem.* 283, 14571–14580.

[12] Osipov, A. V., Rucktooa, P., Kasheverov, I. E., Filkin, S. Yu., Starkov, V. G., Andreeva, T. V., Sixma, T. K., Bertrand, D., Utkin, Y. N. and Tsetlin, V. I. (2012) Dimeric α-Cobratoxin X-ray Structure: Localization of Intermolecular Disulfides and Possible Mode of Binding to Nicotinic Acetylcholine Receptors. *J. Biol. Chem.* 287, 6725–6734.
[13] Pawlak, J., Mackessy, S. P., Sixberry, N. M., Stura, E. A., Le Du, M. H., Ménez, R., Foo, C. S., Ménez, A., Nirthanan, S. and Kini, R. M. (2009) Irditoxin, a novel covalently linked heterodimeric three-finger toxin with high taxon-specific neurotoxicity. *FASEB J.* 23, 534–545.

[14] Joubert, F. J. and Viljoen, C. C. (1979) Snake Venom. The Amino-Acid Sequence of the Subunits of two Reduced and S- Carboxymethylated Proteins (C₈ S₂ and C₉ S₃) from *Dendroaspis angusticeps* Venom. *Hoppe-Seyler’s Z. Für Physiol. Chem.* 360, 1075–1090.

[15] Joubert, F. J. and Taljaard, N. (1979) Snake venoms. The amino-acid sequence of protein S2C4 from *Dendroaspis jamesoni kaimosae* (Jameson’s mamba) venom. *Hoppe. Seylers Z. Physiol. Chem.* 360, 571–580.

[16] Rowan, E. G., Ducancel, F., Doljansky, Y., Harvey, A. L., Boulain, J.‐C. and Ménez, A. (1990) Nucleotide sequence encoding a ‘synergistic-like’ protein from the venom glands of *Dendroaspis angusticeps*. *Nucleic Acids Res.* 18, 1639–1639.

[17] Ainsworth, S., Petras, D., Engmark, M., Süßmuth, R. D., Whiteley, G., Albulescu, L.O., Kazandjian, T. D., Wagstaff, S. C., Rowley, P., Wüster, W., et al. (2018) The medical threat of mamba envenoming in sub-Saharan Africa revealed by genus-wide analysis of venom composition, toxicity and antivenomics profiling of available antivenoms. *J. Proteomics* 172, 173–189.

[18] Lauridsen, L. P., Laustsen, A. H., Lomonte, B. and Gutiérrez, J. M. (2016) Toxicovenomics and antivenom profiling of the Eastern green mamba snake (*Dendroaspis angusticeps*). *J. Proteomics* 136, 248–261.

[19] McCleary, R. J. R., Sridharan, S., Dunstan, N. L., Mirtschin, P. J. and Kini, R. M. (2016) Proteomic comparisons of venoms of long-term captive and recently wild-caught Eastern brown snakes (*Pseudonaja textilis*) indicate venom does not change due to captivity. *J. Proteomics* 144, 51–62.
[20] Otwinowski, Z. and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* 276, 307–326.

[21] Matthews, B. W. (1968) Solvent content of protein crystals. *J. Mol. Biol.* 33, 491–497.

[22] Panjikar, S., Parthasarathy, V., Lamzin, V. S., Weiss, M. S. and Tucker, P. A. (2009) On the combination of molecular replacement and single-wavelength anomalous diffraction phasing for automated structure determination. *Acta Crystallogr. D Biol. Crystallogr.* 65, 1089–1097.

[23] Terwilliger, T. C., Grosse-Kunstleve, R. W., Afonine, P. V., Moriarty, N. W., Zwart, P. H., Hung, L.-W., Read, R. J. and Adams, P. D. (2008) Iterative model building, structure refinement and density modification with the PHENIX AutoBuild wizard. *Acta Crystallogr. D Biol. Crystallogr.* 64, 61–69.

[24] Emsley, P. and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* 60, 2126–2132.

[25] Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. J., Moriarty, N. W., Mustyakimov, M., Terwilliger, T. C., Urzhumtsev, A., Zwart, P. H. and Adams, P. D. (2012) Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallogr. D Biol. Crystallogr.* 68, 352–367.

[26] Laskowski, R. A., MacArthur, M. W., Moss, D. S. and Thornton, J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* 26, 283–291.

[27] Holm, L. (1998) Touring protein fold space with Dali/FSSP. *Nucleic Acids Res.* 26, 316–319.

[28] Chattaraj, S. and Das, A. K. (1992) Indirect atomic absorption spectrometric determination of sulfate in human blood serum. *The Analyst* 117, 413.
[29] Roger, L.P., Robert, M.D. and Stephen, F.H. (1993) The amino terminal half of the nicotinic β-subunit extracellular domain regulates the kinetics of inhibition by neuronal bungarotoxin. Proc. R. Soc. Lond. B Biol. Sci. 252, 141–148.

[30] Dewan, J. C., Grant, G. A. and Sacchettini, J. C. (1994) Crystal structure of kappa-bungarotoxin at 2.3-A resolution. Biochemistry 33, 13147–13154.

[31] Deka, A., Gogoi, A., Das, D., Purkayastha, J. and Doley, R. (2019) Proteomics of Naja kaouthia venom from North East India and assessment of Indian polyvalent antivenom by third generation antivenomics. J. Proteomics 207, 103463.

[32] Servent, D., Antil-Delbeke, S., Gaillard, C., Corringer, P.J., Changeux, J. P. and Ménez, A. (2000) Molecular characterization of the specificity of interactions of various neurotoxins on two distinct nicotinic acetylcholine receptors. Eur. J. Pharmacol. 393, 197–204.

[33] Tsetlin, V. I. (2015) Three-finger snake neurotoxins and Ly6 proteins targeting nicotinic acetylcholine receptors: pharmacological tools and endogenous modulators. Trends Pharmacol. Sci. 36, 109–123.

[34] Wang, C.I. A., Reeks, T., Vetter, I., Vergara, I., Kvtun, O., Lewis, R. J., Alewood, P. F. and Durek, T. (2014) Isolation and Structural and Pharmacological Characterization of α-Elapitoxin-Dpp2d, an Amidated Three Finger Toxin from Black Mamba Venom. Biochemistry 53, 3758–3766.

[35] Chiappinelli, V. A. and Wolf, K. M. (1989) κ-Neurotoxins: heterodimer formation between different neuronal nicotinic receptor antagonists. Biochemistry 28, 8543–8547.
Table 1. Crystallographic Data and refinement statistics.

| Data collection | Value                                      |
|-----------------|--------------------------------------------|
| Unit cell (Å)   | a=52.42; b=55.97, c= 102.79               |
| Space group     | I222                                       |
| Resolution range (Å): | 50-2.4 (2.49-2.40)                         |
| Wavelength(Å):  | 0.98                                       |
| Unique reflections: | 6015                                     |
| Completeness (%): | 99.0 (99.7)                              |
| Overall (I/σI): | 7.2                                        |
| Redundancy:     | 8.0 (4.3)                                  |
| R Sym\[^a\]:    | 0.05 (0.82)                                |

Refinement and quality

| Resolution range (Å): | 38-2.4                                     |
| R work\[^b\]:         | 0.241                                      |
| R free\[^c\]:         | 0.286                                      |
| RMSD bond length (Å)  | 0.008                                      |
| RMSD bond angles (Degree) | 1.106                                    |

Average B factors

| Main Chain: | 37.0 |
| Ligands    | 34.5 |
| Waters:    | 37.8 |

Ramachandran Plot

| Favoured regions (%) | 94.9 |
| Allowed regions (%)  | 5.1  |
| Disallowed regions (%) | 0   |

Statistics from the current model.

\[^a\]R_{sym} = \Sigma |I_i - <I>|/\Sigma |I_i| where I_i is the intensity of the i^{th} measurement, and <I> is the mean intensity for that reflection. \[^b\]R_{work} = \Sigma |F_{obs} - F_{calc}|/\Sigma |F_{obs}| where F_{calc} and F_{obs} are the calculated and observed structure factor amplitudes, respectively. \[^c\]R_{free} = as for R_{work}, but for 10.0% of the total reflections chosen at random and omitted from refinement. *Values in the parenthesis are the highest resolution bin values.
Table 2. Hydrogen bonding interactions between chain A and chain B.

| Chain A (residue/atom) | Chain B (residue/atom) | Distance |
|------------------------|------------------------|----------|
| 36 (TYR) / OH [O]      | 28 (TRP) / NE1 [N]     | 3.81     |
| 34 (LYS) / O [O]       | 34 (LYS) / O [O]       | 3.57     |
| 36 (TYR) / N [N]       | 34 (LYS) / O [O]       | 2.96     |
| 36 (TYR) / O [O]       | 34 (LYS) / O [O]       | 3.41     |
| 37 (ASP) / OD2 [O]     | 34 (LYS) / NZ [N]      | 3.65     |
| 34 (LYS) / O [O]       | 36 (TYR) / N [N]       | 2.94     |
| 34 (LYS) / O [O]       | 36 (TYR) / O [O]       | 3.33     |
| 28 (TRP) / NE1 [N]     | 36 (TYR) / OH [O]      | 3.87     |
| 34 (LYS) / NZ [N]      | 37 (ASP) / OD2 [O]     | 3.77     |
| 56 (LYS) / NZ [N]      | 51 (ASP) / O [O]       | 3.13 AB![^a] |
| 54 (CYS) / SG [S]      | 54 (CYS) / SG [S]      | 2.04 ABI (disulfide) |
| 55 (VAL) / N [N]       | 54 (CYS) / SG [S]      | 3.48 ABI |
| 55 (VAL) / O [O]       | 54 (CYS) / SG [S]      | 3.56 ABI |
| 54 (CYS) / SG [S]      | 55 (VAL) / N [N]       | 3.45 ABI |
| 54 (CYS) / SG [S]      | 55 (VAL) / O [O]       | 3.52 ABI |
| 51 (ASP) / O [O]       | 56 (LYS) / NZ [N]      | 3.17 ABI |
| 50 (ASP) / O [O]       | 63 (Cys) / O [O]       | Water mediated |
| 50 (ASP) / O [O]       | 56 (Lys) / NZ [N]      | Water mediated |

[^a] ABI: Antiparallel beta-sheet interactions
Table 3. Ionic interactions between chain A and SO4.

| Chain A (residue/atom) | SO4       | Distance |
|------------------------|-----------|----------|
| 7(LYS) / NZ [N]        | 2(SO4) / O1 [O] | 3.9      |
| 7(LYS) / NZ [N]        | 2(SO4) / O3 [O] | 3.5      |
| 34(LYS) / N [N]        | 1(SO4) / O1 [O] | 3.64     |
| 30(LYS) / NZ [N]       | 1(SO4) / O1 [O] | 3.53     |
| 30(LYS) / NZ [N]       | 1(SO4) / O2 [O] | 2.69     |
| 36(TYR) / OH [O]       | 1(SO4) / O2 [O] | 3.48     |
| 34(LYS) / N [N]        | 1(SO4) / O4 [O] | 3.25     |
| 30(LYS) / NZ [N]       | 1(SO4) / O4 [O] | 3.97     |
| 36(TYR) / OH [O]       | 1(SO4) / O4 [O] | 2.9      |
| 30(LYS) / NZ [N]       | 1(SO4) / S [S]  | 3.56     |
| 36(TYR) / OH [O]       | 1(SO4) / S [S]  | 3.78     |
| 40(ARG) / NH2 [N]      | 2(SO4) / O3 [O] | 2.71     |
| 40(ARG) / NH1 [N]      | 2(SO4) / O3 [O] | 3.6      |
| 40(ARG) / NH2 [N]      | 2(SO4) / O4 [O] | 3.65     |
| 40(ARG) / NH1 [N]      | 2(SO4) / O4 [O] | 2.88     |
| 40(ARG) / NH2 [N]      | 2(SO4) / S [S]  | 3.77     |
| 40(ARG) / NH1 [N]      | 2(SO4) / S [S]  | 3.83     |
Figure 1. Purification and structure determination of a synergistic type toxin from Dendroaspis jamesoni jamesoni (Dj_SynTx).

(A) Analytical chromatography of native and reduced and alkylated Dj_SynTx. Dj_SynTx samples were chromatographed on a Jupiter C18 column (5 μm, 300 Å, 4.6 x 250 mm) using a gradient (dotted line) of 80% acetonitrile in 0.1% TFA at a flow rate of 1 mL/min. Protein elution was monitored at 215 nm. Molecular weight of each protein peak was determined by Electrospray ionization (ESI)-Mass spectrometry. 

(B) Alignment of amino acid sequences of synergistic-type toxins from Dendroaspis venoms. The protein sequences with accession numbers of National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/) are indicated with asterisks. Deduced sequences obtained by transcriptome analysis are shown in italics [17]. All conserved cysteine residues are shown in red. The cysteine residues involved in intrachain disulfide bridges are identified by a number below. The cysteine residue involved in interchain disulfide bridge is boxed and identified as C*.
The segment involved in interchain β-sheet formation is highlighted in yellow. (C) Disulfide pairings, secondary structures and loops of Dj_SynTx. Three conserved loops in 3FTxs are shown in solid blue line. Red arrowhead identifies the cysteine involved in interchain disulfide linkage. (D) Crystal structure of dimeric Dj_SynTx. Cartoon representation of dimeric Dj_SynTx structure of Chain A (green) and Chain B (blue) are shown. Two sulfate ions are shown in space filling model. The front overview (top) of the tertiary structure is rotated by 90° around the vertical axis (bottom).
Figure 2. Determination of complete amino acid sequence of Dj-SynTx.

(A) The complete amino acid sequence of Dj-SynTx was assembled using the peptide sequences determined by LC-MS and LC-MS/MS of peptides obtained by digestion of alkylated Dj_SynTx by trypsin, Asp-N and Glu-C endoproteases. Purified Dj-SynTx (0.5 mg) in 50 mM NH₄HCO₃ (pH 8.5) was incubated with DTT (final 5 mM) at 56 °C for 30 min. IAA (final 15 mM) was subsequently added and incubated with at room temperature for 15 min. The alkylated protein was separated from the reaction mixture by RP-HPLC on a Jupiter C18 column (5 μm, 300 Å, 4.6 x 150 mm) using a linear gradient of 80% acetonitrile in 0.1% TFA. Following the ProteaseMAX™ surfactant digestion protocols (Promega, USA), the alkylated protein was digested by each enzyme such as trypsin, endoproteinases Asp-N or Lys-C (Promega, sequencing grade) in a 50:1 toxin:protease molar ratio at 37 °C for 16 h. Each protease digest was loaded on a Hypersil Gold column (Phenomenex, United States) and subjected into the Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) on an LCQ Fleet Ion Trap.
Mass Spectrometer (Thermo Fisher Scientific, United States) to obtain peptide sequence data. Based on the peptide sequences the entire amino acid sequence of Dj_SynTx was assembled. (B) The calculated masses match estimated masses of each peptide fragment and the full-length protein. (C) Amino acid sequence of Dj_SynTx matches 100% with the deduced amino acid sequence of J-T343, a SynTx from D. jamesoni jamesoni [17]. The conserved cysteine residues are highlighted in gray.
Figure 3. Interactions between two subunits of *Dj_SynTx*.

(A) Representative 2Fo-Fc map (contoured at 1σ) showing the inter-molecular disulfide bond and two water molecules (red ball) between the antiparallel β-sheet between two subunits. (B) The dimeric interface is held together by several hydrogen bonds and electrostatic interactions. (C) Dimeric interaction mediated through SO₄²⁻ ions. The view from chain A is shown.
Figure 4. Quaternary structures of three-finger toxin dimers.

The quaternary structures of noncovalent (top row) and covalent (bottom row) dimers of 3FTxs are shown. All figures were prepared using the PDB files in Pymol (https://pymol.org/2/).
Figure 5. Formation of α-cobrotoxin homodimer.

In the α-cobrotoxin monomer ‘a’, Pro7 has Cis peptide bond resulting turn leading to loop I formation. When the this Pro7 has Trans peptide bond, the loop I opens and extends the N-terminal segment away [12] from the core of the protein ‘b’. This will double the exposed surface area. The newly exposed surface is ‘buried’ through the second monomer with similar extended conformation. Two interchain disulfide bonds (C1^A-C3^B and C3^A-C1^B) are formed ‘c’, instead of intrachain disulfide bond (C1-C3) within each monomer. We propose that higher entropic cost in such extended N-terminal limits its occurrence to very low levels (0.04% of the venom) [9].
Figure 6. Evolution of novel quaternary structure in synergistic-type toxins.

Most 3FTxs exist as monomers ‘a’, Some of monomers evolved to form transient ‘b’ or stable ‘c’ noncovalent dimers through the formation of antiparallel $\beta$-sheet between loop III segments of two monomers. They are held together by hydrogen bonds between the $\beta$-strands and a small number of electrostatic and/or hydrophobic interactions (Figure 6). Even in the stable noncovalent dimers, the subunits can interchange to form homo- or hetero-dimers [27]. Some of these dimers have evolved to have a disulfide bond to covalently hold the dimeric structure ‘d’. With further dimeric interactions, the monomers twist and contort across the disulfide bond to form a stable quaternary structure of SynTxs ‘e’.
Figure 7. Evolution of dimeric interface in three-finger toxins.

(A) Some 3FTxs, such as α-elapitoxin-Dpp2d, form dimers through antiparallel β-sheet between two monomers during crystallization [26]. In addition to hydrogen bonds, sometimes electrostatic interactions (highlighted in blue) also contribute the noncovalent interaction. At isosmotic conditions, the transient dimer breaks into stable monomers [26]. (B) Stable dimers are fortified by additional interactions between these β-strand and elsewhere (for details, see [9, 22]). Because of the noncovalent nature, the subunits interchange forming homo- or heterodimers [27]. (C) A newly formed interchain disulfide in this segment covalently holds the monomers together. The Cys residue involved in this disulfide is identified by a red triangle. Because of the contortions (Figure 5), the antiparallel β-sheet is ‘shifted’ two residues towards the N-terminal (indicated by blue arrow). Thus, monomeric 3FTxs have evolved into noncovalent dimers and subsequently into covalent dimeric organization of SynTx.