

Review

Studies on sea snake venom

By Nobuo TAMIYA*¹ and Tatsuhiko YAGI*^{2,†}

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Abstract: Erabutoxins a and b are neurotoxins isolated from venom of a sea snake *Laticauda semifasciata* (erabu-umihebi). Amino acid sequences of the toxins indicated that the toxins are members of a superfamily consisting of short and long neurotoxins and cytotoxins found in sea snakes and terrestrial snakes. The short neurotoxins to which erabutoxins belong act by blocking the nicotinic acetylcholine receptor on the post synaptic membrane in a manner similar to that of curare. X-ray crystallography and NMR analyses showed that the toxins have a three-finger structure, in which three fingers made of three loops emerging from a dense core make a gently concave surface of the protein. The sequence comparison and the location of essential residues on the protein suggested the mechanism of binding of the toxin to the acetylcholine receptor. Classification of snakes by means of sequence comparison and that based on different morphological features were inconsistent, which led the authors to propose a hypothesis “Evolution without divergence.”

Keywords: sea snake, erabutoxin, neurotoxin, three-finger structure, acetylcholine receptor, evolution without divergence

Introduction

There are two main species of sea snakes in Okinawa. One is called “Erabu (name of an island in Okinawa)” (*Laticauda semifasciata*) and the other is “Hiroo (wide tail)” (*Laticauda laticaudata*).¹⁾ The snakes are about 1 m long. The toxicity of the sea snake venom is known among local people by the fatal bites, but its systematic investigations were rarely found. Carey and Wright²⁾ isolated a neurotoxic component from closely related sea snake *Enhydrina schistosa*, but the studies were not aimed at characterizing the toxic component in view point of protein chemistry. The toxic components from *L. semifasciata* and *L. laticaudata* were isolated and suggested to be small basic proteins.^{3),4)} In the course of analyzing the primary structures of sea snake toxins,^{5),6)} the authors recognized that the toxins constitute a large superfamily of neurotoxic proteins including not only those from sea snakes but those from variety of terrestrial snakes. This review focuses on the structure, mechanism of action, and evolutionary relationships of snake venom toxins.

Isolation of toxins and demonstration of their toxicity

The sea snakes were provided from local collectors. In Okinawa, the snake meat and skin are commercially useful for food and handicrafts, but snake heads were useless. The heads were cut off at the capture site, and given to us. The venoms from both species, *L. semifasciata* and *L. laticaudata*, were colorless viscous solution. The venom glands were cut off from the heads and freeze-dried. The venom components were extracted with 0.01 M acetic acid from the dried glands, and were separated by column chromatography on CM-cellulose. Two toxic components obtained from *L. semifasciata* venom were named erabutoxin a (Ea) and erabutoxin b (Eb). Ea and Eb fractions were desalted and freeze-dried. Both Ea and Eb showed the LD₅₀ values of 0.15 µg/g body weight of mice.⁴⁾

The two toxins explained 95% of the original venom lethality. Each of the toxins showed a single band on disc electrophoresis and on ultracentrifugation. The amino acid analyses showed that each toxin consisted of 61 amino acid residues (later corrected to be 62 residues).⁴⁾ A minor toxic component from *L. semifasciata*, which accounted for 5% of the original venom lethality, was later isolated and

*¹ Tohoku University, Miyagi, Japan.

*² Shizuoka University, Shizuoka, Japan.

† Correspondence should be addressed: T. Yagi, 1664-33 Kita, Aoi-ku, Shizuoka 420-0961, Japan (e-mail: asarlun@kitty.jp).

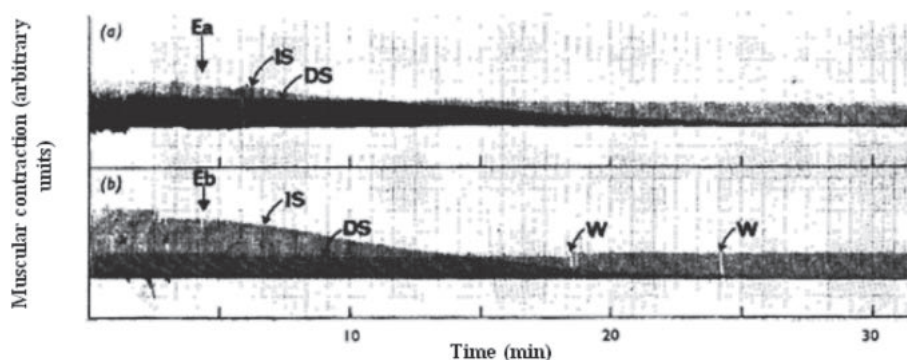


Fig. 1. Effects of Ea and Eb on the contraction of sciatic-nerve-sartorius-muscle preparation of frogs (*Rana nigromaculata*).⁴⁾ An isolated sciatic-nerve-sartorius muscle preparation was placed in 4.3 mL of glucose Ringer solution (9.0 g of NaCl, 0.42 g of KCl, 0.24 g of CaCl_2 , 0.5 g of NaHCO_3 and 1.0 g of glucose in 1.4 L of water), which was continuously bubbled with air. The electrical stimulations were given to the preparation either directly to the muscle (DS) or indirectly through the nerve (IS) every 5 s, and the contractions were recorded by a strain-gauze transducer. The solution of Ea (0.1 mL) was added to the medium at the point marked Ea (final concn. $0.11 \mu\text{g}$ of Kjeldahl nitrogen/mL of Ringer solution) in (a). The recrystallized Eb solution (0.1 mL) was added at the point marked Eb (final concn. $0.12 \mu\text{g}$ of Kjeldahl nitrogen/mL of Ringer solution) in (b). The muscle was washed with the Ringer solution at the points marked W in (b).

named erabutoxin c (Ec). Ec was also a basic protein of 62 residues, and its LD_{50} was $0.13 \mu\text{g/g}$ body weight of mice, a value similar to those of Ea and Eb.⁶⁾ Still another toxic component, laticauda semifasciata III (component LsIII), was isolated, which was composed of 66 residues, and had only 15% of the lethality as those of erabutoxins.^{7,8)} A toxic component isolated from *L. laticaudata* was named laticotoxin a (La, deposited in NCBI database under the name, short neurotoxin OKI-01/OKI-19). This toxin was also a basic protein of 62 residues, and its LD_{50} was $0.13 \mu\text{g/g}$ body weight of mice.⁹⁾

Toxicity of Ea and Eb was demonstrated with isolated frog muscle.⁴⁾ In the aerated Ringer solution, the contracture of frog (*Rana nigromaculata*) sciatic-nerve-sartorius-muscle preparation by indirect electric stimuli through the nerve was inhibited, but the contracture by direct stimuli of the muscle was not inhibited (Fig. 1). Acetylcholine-induced contracture of frog rectus abdominal muscle was inhibited by the toxins (Fig. 2). These results clearly demonstrated that the toxins interfered with the binding of acetylcholine to the acetylcholine receptor (AChR) on the post synaptic membrane in a manner similar to that of curare, because they blocked neuromuscular transmission, and inhibited the muscle contraction induced by acetylcholine.^{4,10)} The toxin was suggested to exert toxicity by blocking respiration, because mice injected with lethal amount of the toxin were rescued by artificial respiration.

The dissociation constant (K_D) of a complex of erabutoxin with AChR was later estimated to be

$10 \times 10^{-11} \text{ M}$ for Ea, $4 \times 10^{-11} \text{ M}$ for Eb and $6 \times 10^{-11} \text{ M}$ for Ec,⁸⁾ whereas LD_{50} values were similar ($0.13\text{--}0.15 \mu\text{g/g}$ body weight of mice, *vide supra*). For a toxin of extreme affinity to AChR, the fatal dose may be related to the number of AChRs necessary to keep the victim's respiration.

Expedition to collect sea snakes

One of the authors (N.T.) presented the results obtained in early days of the research^{3,4)} at the First International Symposium on Animal Toxins in 1966.¹¹⁾ Many proposals for collaborations were made and the author was invited to join the sea snake expeditions to be held by Scripps Institution of Oceanography, University of California, San Diego on Research Vessel Alpha Helix in Dec. 1972–Jan. 1973 and Dec. 1975–Jan. 1976 (Chief Scientist: Dunson, W.A.). In the expedition, the author learned the way to organize similar expeditions for more snake collections later. The author also could make many biologist friends who helped him in later snake collections.

In the expedition 453 sea snakes of 15 species (identified by Dr. H.G. Cogger of the Australian Museum) including nonvenomous snake *Emydocephalus* (fish egg eater) were collected in the Pacific and Indian Oceans. In case when snake bodies were not needed for other studies, the venom was ejected by pressing the venom glands of the upper jaw from outside through the grooves on the upper front fangs, and the snakes were released to the sea. The venom was immediately extracted with 0.01 M acetic acid.

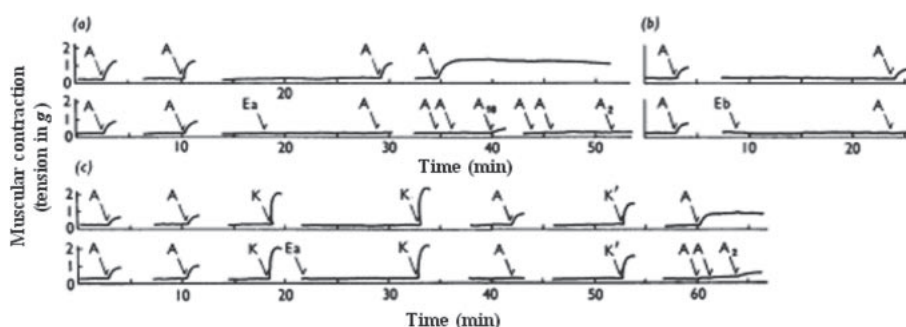


Fig. 2. Effects of Ea and Eb on the contraction of isolated frog rectus abdominal muscles by acetylcholine and by KCl.⁴⁾ An isolated frog rectus abdominal muscles (*Rana nigromaculata*) was placed in 5 mL glucose-Ringer solution (6.43 g of NaCl, 0.30 g of KCl, 0.17 g of CaCl₂, 0.10 g of NaHCO₃ and 0.71 g of glucose in 1.0 L of water), which was continuously bubbled with air. The contraction of the muscle was recorded by a strain-gauze transducer. At the points marked A, A₂ and A₁₀, acetylcholine solution (0.1 mL) was added to the medium Ringer solution to give final concentrations 0.05 M and 0.02 M, respectively. At every interval between the curves, the muscle was washed three times with 5 mL of the Ringer solution and placed in 5 mL of fresh Ringer solution. The right and left muscles of a single individual were used as a pair. Ea (0.12 µg of Kjeldahl nitrogen/mL of Ringer solution) and Eb (the same final concentration as Ea) were added at the points marked Ea and Eb, respectively.

Table 1. The lethality of sea snake venoms

Species	Capture site	Body weight	Venom	LD ₅₀ /g mouse
<i>Aipysurus laevis</i>	Scott Reef	200–840 g	0.05 mL	0.96 nL
<i>Astrotia stokesii</i>	Darwin	2100–3050 g	0.41 mL	3.5 nL
<i>Hydrophis belcheri</i>	Ashmore Reef	360–520 g	0.01 mL	1.0 nL
<i>Hydrophis elegans</i>	McCluer Gulf	330 g	0.02 mL	0.38 nL
<i>Hydrophis ornatus</i>	McCluer Gulf	700 g	0.05 mL	0.85 nL
<i>Lapemis hardwicki</i>	McCluer Gulf	400–870 g	0.03 mL	0.98 nL

The fact that the sea snake toxins are stable in 0.01 M acetic acid was very helpful for the venom collection at the remote places. Some of the venoms were chromatographed on a CM-cellulose column on the Vessel. Lethality of sea snake venoms collected in the expeditions is shown in Table 1 together with other data.¹²⁾ The lethal activities were found in basic protein fractions, suggesting the presence of components similar to erabutoxins.

There is a report that an expedition group visited Rennell Island, which is located to the south of Gadalkanal Island of Solomon Islands. They found sea snakes there and gave tentative name, after the name of the expedition group “Crocker’s sea snake.” We visited Tengano Lake of the island and found sea snakes there in 1980 and 1983. The lake is separated from the sea but the sea water comes in and goes out on tide through a small groove in the Tengano village. Local people say that there are sea snakes called “Tugihono” and “Ungveruna.” We found small *L. laticaudata*. (about 80 cm long) and large *L. semifasciata* (the largest one we observed was 1.8 m long). *L. laticaudata* looked very different in appearance from those in

Okinawa, Taiwan, Philippines, Papua New Guinea, New Caledonia, Vanuatu, Fiji and Australia, but *L. semifasciata* looked very similar at these places.

Amino acid sequences of snake toxins

In 1967, Eaker¹³⁾ in Sweden sequenced the amino acids of neurotoxin α from *Naja nigricollis* (African cobra), which had been isolated by Boquet¹⁴⁾ of Pasteur Institute. A toxin from *Naja atra* (Formosan cobra), cobrotoxin was sequenced by Yang.¹⁵⁾ The sea snakes in South Pacific and Indian Oceans are closely related. The authors worked on Australian snakes which have also similar toxins.^{16),17)}

We found erabutoxins in 1964³⁾ and sequenced them in 1971.^{5),18)} We found several more homologous components from sea snakes and sequenced them.^{6),7),16),17)} There are now 475 homologous proteins, isolated from sea snakes and terrestrial snakes, registered in Pfam database (<http://pfam.janelia.org/family/PF00087>). They were classified into three groups by the sequence,¹⁹⁾ namely short neurotoxins (around 60 amino acid residues), long neurotoxins (around 70 residues) and cytotoxins

Residue numbers for (1)	1	10	20	30	40	50	60	Residues	Accession										
(1) <i>Laticauda semifasciata</i> (Ec)	RIC--FNHQSSQPQT--KTCS	PGESSCYHKQWSD---FRGTII	ERGCG--CPTVKP--GINKL	CC	EESEV	CNN	62	Q7T2I5											
(2) <i>Laticauda semifasciata</i> (Eb)	RIC--FNHQSSQPQT--KTCS	PGESSCYHKQWSD---FRGTII	ERGCG--CPTVKP--GIKLS	CC	EESEV	CNN	62	Q90VW1 3EBX											
(3) <i>Laticauda semifasciata</i> (Ea)	RIC--FNHQSSQPQT--KTCS	PGESSCYNKQWSD---FRGTII	ERGCG--CPTVKP--GIKLS	CC	EESEV	CNN	62	1QKD											
(4) <i>Laticauda laticaudata</i> (La)	RRC--FNHPSSQPQT--KSCPP	GENSCYNKQWSD---HRGTII	ERGCG--CPTVKP--GIKLT	CC	QSED	CNN	62	Q9YGC4											
(5) <i>Laticauda laticaudata</i> (La')	RRC--FNHPSSQPQT--KSCPP	GENSCYNKQWSD---HRGTII	ERGCG--CPQVKS--GIKLT	CC	QSD	CNN	62	P10459											
(6*) <i>Naja sputatrix</i> (toxin α)	LEC--HNQSSQPPTT--KTCS	PGETNCYKKVWRD---HRGTII	ERGCG--CPTVKP--GIKLN	CC	TTDR	CNN	62	3NDS											
(7*) <i>Naja atra</i> (cobrotoxin)	LEC--HNQSSQPPTT--TGCS	GGETNCYKKRWRD---HRGYRTE	RGCG--CPSVKP--GIEIN	CC	TTDR	CNN	62	P60770											
(8*) <i>Naja nigricollis</i> (neurotoxin α)	LEC--HNQSSQPPTT--KTC--	PGETNCYKKVWRD---HRGTII	ERGCG--CPTVKP--GIKLN	CC	TTDR	CNN	61	P01426											
(9*) <i>Naja melanoleuca</i> (neurotoxin D)	MEC--HNQSSQPPTT--KTC--	PGETNCYKKQWSD---HRGTII	ERGCG--CPSVKK--GVKIN	CC	TTDR	CNN	61	P01424											
(10*) <i>Naja haje annulifera</i> (toxin CM-10)	MIC--YKQSLQFPIT--TVCP	GEKNCYKKQWSD---HRGTII	ERGCG--CPSVKK--GIEIN	CC	TTDR	CNN	61	751415A											
(11*) <i>Dendroaspis jamesoni kaimosae</i> (toxin 1)	RIC--YNHQSTTPATT--KSC--	GENSCYKKTWSD---HRGTII	ERGCG--CPKVQ--GIHL	CC	QSD	CNN	60	P01417											
(12) <i>Astrotia stokesii</i> (toxin A)	MTC--CNQSSSQPKTT--TNC--	AGNSCYKKTWSD---HRGTII	ERGCG--CPQVKS--GIKLE	CC	HTNE	CNN	60	P68412											
(13) <i>Hydrophis ornatus</i> (toxin '73a)	MTC--CNQSSSQPKTT--TNC--	AGNSCYKKTWSD---HRGTII	ERGCG--CPQVKS--GIKLE	CC	HTNE	CNN	60	P68413											
(14) <i>Hydrophis ornatus</i> (toxin '75a)	MTC--CNQSSSQPKTT--TNC--	AESSCYKKTWSD---FRGTIER	RGCG--CPQVKP--GIKLE	CC	HTNE	CNN	60	P01437											
(15) <i>Aipysurus laevis</i> (short neurotoxin A)	LTC--CNQSSSQPKTT--TDC--	ADNSCYKKTWSD---HRGTIER	RGCG--CPQVKP--GIKLE	CC	HTNE	CNN	60	P32879											
(16) <i>Aipysurus laevis</i> (short neurotoxin C)	LTC--CNQSSSQPKTT--TDC--	ADNSCYKKTWSD---HRGTIER	RGCG--CPQVKP--GIKLE	CC	HTNE	CNN	60	P19958											
(17) <i>Aipysurus laevis</i> (short neurotoxin B)	LTC--CNQSSSQPKTT--TDC--	ADNSCYKKTWSD---HRGTIER	RGCG--CPQVKP--GIKLE	CC	HTNE	CNN	60	P19959											
(18*) <i>Naja haje haje</i> (toxin CM-2)	FTC--FTTFS---DTS--ET	CPDQNICYEKRWNS---HQGVEI--	KGCVASCE	FESEFR	RYLL	CC	RRID	CNN	61	P01415									
(19*) <i>Ophiophagus hannah</i> (neurotoxin)	TKC--YNHQSTTPETT--ET	CPDSGYEYKKTWSD---GREGRIER	GC	FTTFTCP	ELTPNG	KYVY	CC	RRID	CNN	65	3HH7								
(20*) <i>Boiga irregularis</i> (iridotoxin chain A)	QAVGPPYTLCEFCNRMSSDCSTALRC--	YRGS	CYTLRPDE--NCE	LKWA	VGC	AACT	CP	TAQ	P--NERV	CC	RRSP	CND	75	2H7Z					
(21*) <i>Boiga irregularis</i> (iridotoxin chain B)	QAKGPPYTLCEFCNRETSCNCFKDNRCPPYHRT	CYTLRPDG--NGEMKWA	VGC	AACT	CP	TAQ	P--GESV	CC	NTPK	CND	77	2H7Z							
(22*) <i>Boiga dendrophila</i> (denmotoxin)	QAVGLPHGFCIQNRKTSNCSIGHRC	LPYHMT	CYTLRPDE--NGEMKWA	VGC	AACT	CP	TAQ	P--GERV	CC	NTPK	CND	77	2H5F						
(23) <i>Laticauda semifasciata</i> (LsIII)	REC--YLN---PHDT--QT	CPSGQE	ICVYKSWC	NAWSS	RGKVLE	FG	CAAT	CP	SVNT	--GTE	IK	CC	SADK	CNT	YYP	66	P01379		
(24) <i>Astrotia stokesii</i> (toxin B)	LSC--YLG---YKHS--QT	CPPGENV	CFVKTW	CDGFC	NTGER	IIM	GC	AACT	CP	TAQ	P--GVH	IA	CC	STDN	CNI	YK	WGS	70	P01380
(25) <i>Astrotia stokesii</i> (toxin C)	LSC--YLG---YKHS--QT	CPPGENV	CFVKTW	CDGFC	NTGER	IIM	GC	AACT	CP	TAQ	P--GVH	IA	CC	STDN	CNI	YK	WGS	72	P01381
(26*) <i>Bungarus multicinctus</i> (α-bungarotoxin)	IVC--HTTATS--PISA--VT	CPPGEN	ICYRKMW	CDAF	CSRG	KVLE	FG	CAAT	CP	SKKP	--YEEV	CC	STDK	CNP	HPK	QRP	74	1KPH	
(27*) <i>Dendroaspis polylepis</i> (toxin γ)	RTC--NKTFF---SDQS--KI	CPPGEN	ICYRKMW	CDAF	CSRG	KVLE	FG	CAAT	CP	SKKP	--YEEV	CC	STDK	CNP	HPK	QRP	72	P01396	
(28*) <i>Naja naja siamensis</i> (α-cobrotoxin)	IRC--FITP---DITS--KD	CPNG--HV	CYTKW	CDAF	CSRG	KVLE	FG	CAAT	CP	TKVT	--GVD	IC	CC	STDN	CNP	FP	TRK	71	1YI5 2CTX
Residue numbers for (28)	1	10	20	30	40	50	60	Residues	Accession										

Fig. 3. Sequence alignment of some short, long and longN neurotoxins of sea and terrestrial snakes. The sequences above the dotted line are those of the short neurotoxins, the sequences Nos. 20, 21 and 22, the longN neurotoxins, and the others, the long neurotoxins. The structurally essential residues are boldfaced. The disulfide bonds are Cys3/24, Cys43/54, and Cys55/60 for the short neurotoxins (No. 1), Cys10/34, Cys13/21, Cys27/53, Cys57/68 and Cys69/74 for the longN neurotoxins (No. 21), and Cys3/20, Cys14/41, Cys26/30, Cys45/56, and Cys57/62 for the long neurotoxins (No. 28). *Astrotia* toxins (Nos. 24 and 25) are unique among neurotoxins in having C-terminal amide. The toxins from terrestrial snakes have sequence numbers asterisked. In the column, Accession (for NCBI Protein sequence database), those for Protein Data Bank (PDB), which deposits three-dimensional structures, are boldfaced. One-letter codes for amino acids: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

(around 60 residues). Cytotoxins, alternatively known as cardiotoxins, do not have affinity to AChR, and cause fibrillation of the heart muscle.²⁰⁾ Recently new variants of the long neurotoxins were found. Whereas the canonical long toxin has a C-terminal extension to the short toxin, the new variant has an N-terminal extension, instead,²¹⁾ and will be called longN neurotoxin, in this account. We had short and long neurotoxins but no cytotoxins or longN neurotoxins in sea snakes.

Sequence alignment of selected snake toxins are shown in Fig. 3. Four disulfide bridges are found in common in short, long and longN neurotoxins and cytotoxins. An additional disulfide bridge is present in long and longN neurotoxins, whose positions are different in the two groups. General features of protein folding and disulfide bridges of representative

short and long neurotoxins are schematically illustrated in Fig. 4. There are medium-sized neurotoxins (around 65 residues), one of which (No. 19 in Fig. 3) belongs to the short toxins having four disulfide bridges, and the other (No. 23) belongs to the long toxins having five disulfide bridges. Sequence alignments composed of more short and long neurotoxins, as well as cytotoxins, can be found elsewhere.^{7),22)}

Evolutional aspects of snakes based on the sequence comparison of neurotoxins

From the nomina of snakes recorded in NCBI sequence database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein>), the phylogenetic tree in superfamily Colubroidea can be constructed as shown in Fig. 5a. From the comparison of the sequences of short toxins, snakes in families Elapidae

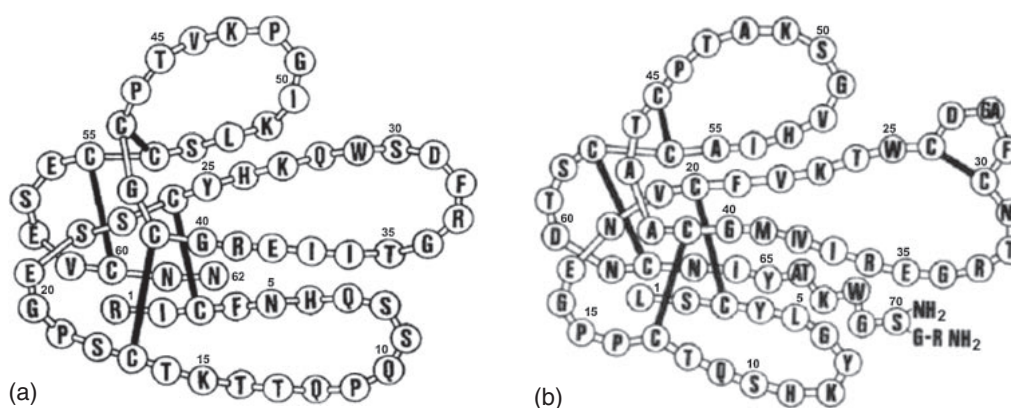


Fig. 4. Schematic representation of typical short and long neurotoxins from snake venoms. (a) Eb from *L. semifasciata*. (b) Toxins B and C from *Astrotia stokesii*, which differ from each other at residues 28, 31, 38, and 66 (the residues in toxin B, at the left in circles), and a C-terminal extension of two residues in toxin C. The shape of the protein folding was based on the three-dimensional structure of Eb^{18),35)} (*vide infra*).

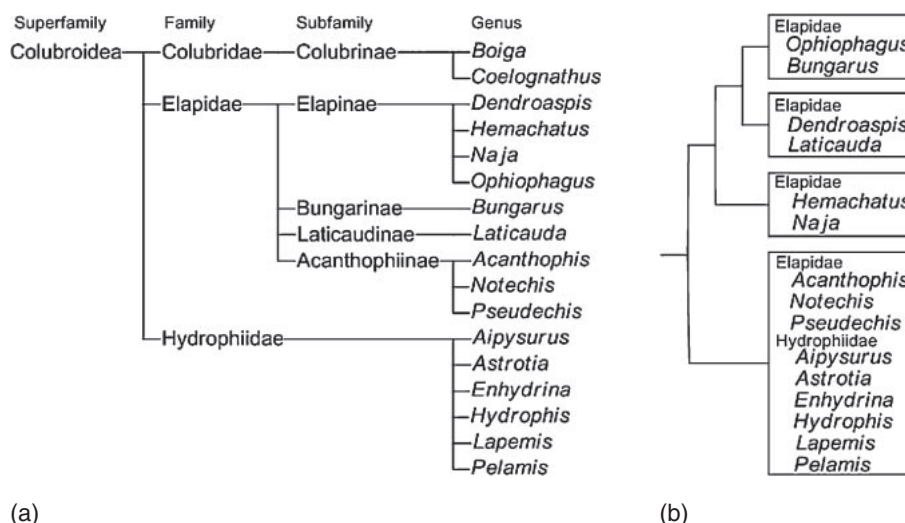


Fig. 5. Classification of snakes in superfamily Colubroidea (snakes). Selected families and genera were shown. (a) Classification of snakes based on the nomina appeared in NCBI sequence database. (b) The phylogenetic tree based on the sequences of short toxins. The sequence alignment, on which the tree is based are not shown, instead accessions for the venom sequences are given: Q2VBN9, 3HH7 (*Ophiophagus*), Q9YGI8 (*Bungarus*), P01416, P01417, P01418, P01419 (*Dendroaspis*), P10459, P10460, 1QKD, Q7T2I5, Q90VW1, Q9YGC4, BAA75754 (*Laticauda*), P01425, P01433 (*Hemachatus*), P01422, P01423, P01424, P01426, P01427, P25675, P60770, P60772, P68417, P68418, P68419, P80958, 3NDS, AAA66025, AAA66026, ABY61977 (*Naja*), P01434 (*Acanthophis*), A8HDK0 (*Notechis*), P19958, P19959, P32879 (*Aipysurus*), P68412 (*Astrotia*), P62389, P68415 (*Enhydrina*), P01437, P25494, P62376, P68413 (*Hydrophis*), P68416, Q8UW26, Q8UW27 (*Lapemis*) and P62388 (*Pelamis*). Branches to individual genera in each box are inconsistent and not shown.

and Hydrophiidae may be classified into four groups as shown in Fig. 5b. Snakes in family Colubridae are not included in this figure, because they have no short neurotoxins deposited in the NCBI database. It seems they have only longN neurotoxins, which are lacking in snakes of the other families. This grouping (Fig. 5b) is only in part conform to the phylogenetic tree shown in Fig. 5a.

Classification of snakes based on morphology is more controversial, because different taxonomists classified snakes differently. For example, Smith²³⁾ classified *Laticauda* (in subfamily Laticaudinae) and *Hydrophis* (in subfamily Hydrophiinae) in the same family Hydrophiidae, whereas Dowling²⁴⁾ classified them in family Elapidae. On the other hand, McDowell²⁵⁾ classified *Laticauda* in family Elapidae

and *Hydrophis* in family Hydrophiidae. Matsui's classification²⁶⁾ is similar to that of Dowling, but division into subfamilies in family Elapidae is different. Differences in the classification might have resulted from the fact that different taxonomists attach importance to different morphological features.

Different morphological features, as well as sequences of different proteins, of an organism are a consequence of the expression of different parts of the genome. Difference in the phylogenetic trees based on different morphological features and protein sequences may mean that different parts of the genome in a single organism have their own history of evolution, *i.e.*, the genome is a mosaic of genes of different evolutionary history. This conclusion conflicts with the generally accepted idea that all the living things diverged by the accumulation of mutations from a single ancestor, which emerged only once on our planet. A hypothesis "evolution without divergence" proposed by the authors in 1985²²⁾ may be one of hypotheses which would solve the problems of discrepant phylogeny among different parts of the genome. This hypothesis states that the major driving force for evolution is interspecies gene exchanges, rather than divergence of species from a single ancestor by the accumulation of mutations, and life might have been originated from multiple ancestors instead of a single ancestor. Efforts have been made to collect pieces of evidence to support this hypothesis. The authors were able to collect many examples that many crucial features and processes are by no means in common in living things in spite of the general belief that most living things have essential biochemical features in common, and that interspecies gene transfer, now called "horizontal gene transfer" or "lateral gene transfer" is more prevailing than what was thought to be in 1985. For example, as much as 24% of genes of thermophilic bacteria, *Thermotoga maritima*, has been suggested to be brought from archaea by horizontal gene transfer.²⁷⁾ The reinforced and improved versions of "Evolution without divergence" were published recently.^{28),29)}

Use of erabutoxins and related neurotoxins in the study of AChR

High affinity of erabutoxins to AChR makes the toxins useful tools in the study of AChR. When Ea and Eb were treated with iodinating reagent containing radioactive [¹³¹I]KI at pH 9.6 and 273 K,³⁰⁾ only Eb was iodinated.³¹⁾ Of two histidyl residues,

His6 and His26 of Eb, only His26 was converted to 2,4-diiodohistidyl residue. Ea lacks His26. The other residues including His6, Tyr25 and Trp29, in both proteins, were found unmodified. Diiodo-Eb had the same toxicity and crystallizability as the native Eb, and formed a precipitation line with anti-Eb serum of rabbit.³¹⁾ Crystals of Ea, Eb and diiodo-Eb showed similar habits and grew in orthorhombic plates or prisms. The crystallographic parameters were similar to each other.³²⁾ The radioactive diiodo-Eb may be used in studying the mechanism of action of the neurotoxins, and in radioimmunoassay experiments. For example, the radioautogram of the diaphragm of a mouse injected with radioactive diiodo-Eb showed the concentration of the toxin at the endplates.¹⁰⁾

Eb labeled with rhodamine and bungarotoxin labeled with fluorescein isothiocyanate were used to distinguish between properties of human and mouse AChRs.³³⁾ α -Toxin from *Naja nigricollis* was used as affinity ligands for the purification of detergent-solubilized nicotinic AChR protein.³⁴⁾

Three-dimensional structures of venom toxins

For the X-ray crystallographic analysis, one of the authors (N.T.) tried to grow the crystals of neurotoxins. Erabutoxins were crystallized without difficulty,⁴⁾ but for unknown reasons, the toxins from other sources were difficult to be crystallized. The author himself tried to crystallize several neurotoxins from sea snakes, but never succeeded. The author sent the crystals of Eb with a PhD student Sato to Dr. Low of Columbia University in New York. The X-ray structure of Eb was reported by Low *et al.* in 1976,³⁵⁾ and the refined structure thereof in 1985.¹⁸⁾ Walkinshaw grew the crystals of α -cobratoxin, a long neurotoxin from *Naja naja siamensis*, in a mixture of 2-methylpentane-2,4-diol and pH 2-buffer solution, and reported its X-ray structure in 1980.³⁶⁾

The X-ray structures of Eb, a short neurotoxin, α -cobratoxin, a long neurotoxin, and denmotoxin, a longN neurotoxin, are illustrated in Fig. 6. All of these toxins share the common features, *i.e.*, three fingers emerging from a dense core make a gently concave surface. The loops forming the three fingers are called loop I, loop II and loop III from the N-terminus, and consist of five antiparallel β -strands and a single unfeatured main chain. These structures, as well as many other reported structures (see Fig. 3 for the PDBids) have the similar folding pattern as are predicted from the presence of many common residues such as Cys residues to form disulfide bridges at corresponding positions. It is thus assumed that all

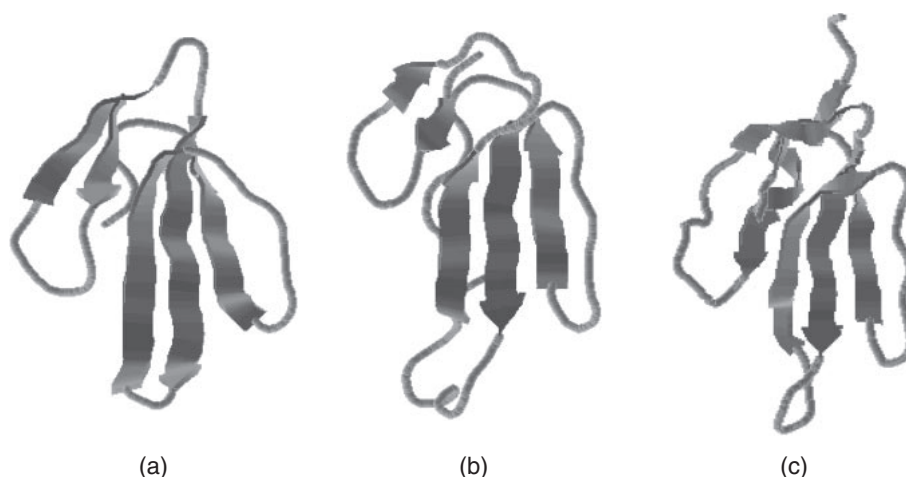


Fig. 6. The polypeptide backbones are shown such that the concave surfaces face to the viewer. (a) Eb, a short neurotoxin from *L. semifasciata* [PDBid 3EBX], (b) α -cobratoxin, a long neurotoxin from *Naja naja siamensis* [PDBid 2CTX], and (c) denmotoxin, a longN neurotoxin from *Boiga dendrophila* [PDBid 2H5F]. Loops I, II and III arrange from left to right.

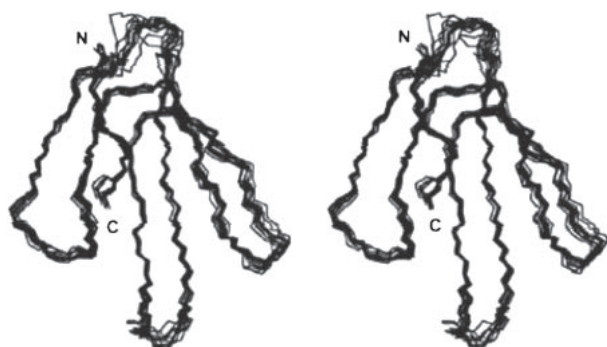


Fig. 7. Stereopairs of the best fit superposition of 14 final converged structures of Eb [PDBid: 1FRA] viewed similarly to that in Fig. 6a.

the toxins have the similar three-finger structure shown in Fig. 6. The refined structure of Ea at 1.5 Å resolution reported by Nastopoulos *et al.*³⁷⁾ is very similar to that of Eb.

The solution structure of Eb in D₂O (Fig. 7) elucidated by proton-NMR in collaboration with late Miyazawa and Inagaki^{38),39)} was in good agreement with that observed by X-ray crystallography (Fig. 6a).

Comparison of proton-NMR spectra of Ea and Eb in D₂O unequivocally identified the signals of C2 and C4 protons of His26 in Eb, because Eb has two histidyl residues, His6 and His26, whereas Ea has only one, His6, and both gave almost identical NMR spectra except for those of C2 and C4 protons of His26 residue in Eb.⁴⁰⁾ From the pH (pH meter readings in D₂O without calibration)-dependence of

His26-protons, the pK_a value for the imidazole ring was estimated to be 5.8, a value similar to that of free histidine ($pK_a = 6.04$ in H₂O). Both of C2-proton signals at 8.86 ppm of His26 and 6.87 ppm of His6 were replaced by a single broader signal at 8.60 ppm by lowering the pH from 3.96 to 1.80, at which Eb became denatured. These spectral changes of C2-proton signals indicated that His6 was buried in the interior of the molecule, whose protonation occurred only on the onset of the acid denaturation of Eb. The behaviors of signals in the aromatic region and methyl groups on pH and temperature changes, as well as by other techniques like decoupling, double resonance methods, convolution difference technique, *etc.*, allowed Inagaki *et al.*⁴⁰⁾ to assign the signals of Tyr25, Trp29, and some hydrophobic side chains. From the pH-dependence of the fraction of NMR signal intensity of native Eb, the acid denaturation was found to be reversible with a mid point pH of 2.84, the conclusion being supported by the pH dependence of circular dichroism spectra. The microenvironments of some side chains elucidated by these experiments are schematically illustrated in Fig. 8.⁴⁰⁾

Structurally essential residues of snake toxins

The amino acid sequences of the venom toxins share four disulfide bridges (Cys3/24, Cys17/41, Cys43/54, and Cys55/60), and Gly40 and Pro44 (numbering of erabutoxins), regardless of the size of the toxin (Fig. 3). These ten residues are, thus, structurally invariant which are essential to keep the protein in the three-finger structure common to all

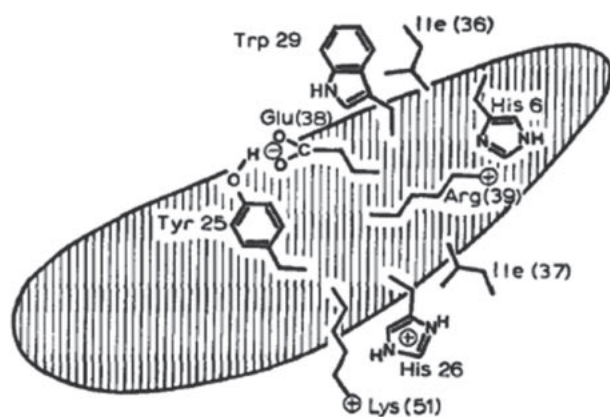


Fig. 8. Schematic presentation of the microenvironments of some amino acid residues in Eb. Tentatively assigned residue numbers are in parentheses.

the known venom neurotoxins. The role of the four disulfide bridges is obvious in keeping the structure. Gly40 may be necessary to snugly fill in the crowded region near the disulfide bridges at the base of loops II and III. Pro44 may be responsible for sharply changing the direction of the peptide backbone just after the disulfide bridge to keep loop III in shape. The additional disulfide bridge in the long toxin binds up the tip of loop II to make a little protrusion (Fig. 6b), whereas that in the longN toxin binds the loop I (Fig. 6c), either of which does not make much influence on the shape of the three-finger structure. Cytotoxins have amino acid sequences homologous to those of the short neurotoxins, keep all the ten structurally essential amino acid residues, and are very similar in shape as the short neurotoxins shown in Fig. 6a.

Functionally essential residues of snake toxins

From a simple comparison of the amino acid sequences (Fig. 3), one can say that Ea is a His26Asn mutant of Eb, and Ec is a Lys51Asn mutant of Eb. As there is little difference in the lethality among these toxins, positions 26 and 51 are silent on their activity. Both of these replacements are explained by a single base replacement. In addition to the ten structurally essential residues mentioned above, residues conserved in most (not all) of short and long neurotoxins include Tyr25, Lys27, Trp29, Asp31, Arg33, Gly34, Lys47 and Leu/Ile/Val52, as well as Ser8 in short toxins, and Ala28-Phe29 (numbering of α -cobratoxin) in long toxins. These residues are candidates for the functionally essential residues of neurotoxins.

Eb has five amino groups, the N-terminus and four lysyl residues, Lys15, Lys27, Lys47 and Lys51. Of five *N*-acetylated Ebs, α -*N*-acetyl, *N*-acetylLys15 and *N*-acetylLys51 toxins had full lethality, indicating that these amino groups are not necessary for the toxicity (Lys15 and Lys51 are variable residues in neurotoxins), but the toxicity of *N*-acetylLys27 and *N*-acetylLys47 toxins were 17% and 8% of the unmodified Eb, respectively. The overall profiles of circular dichroism spectra of the acetylated derivatives of Eb were similar to that of the unmodified Eb.⁴¹⁾

The only tryptophanyl residue, Trp29 of Ea, which was found not to be buried by NMR and fluorescence intensity measurements,^{38),40)} was destroyed with *N*-bromosuccinimide in the presence of 8M urea without modification of Tyr25, and without cleavage of the peptide bond following the modified Trp29.⁴²⁾ The modified protein was only 3% as lethal as the unmodified Ea. Ea was treated with 2-hydroxy-5-nitrobenzyl bromide, which was known to modify only tryptophanyl residues in proteins.⁴³⁾ Parallel decrease of tryptophanyl residues and toxicity was observed, but the modified protein showed similar precipitation lines to those of the native Ea in the immunodiffusion experiment. The treated Ea inhibited the binding of radioactive diiodo-Eb to the antibody to the same extent as the native Ea.⁴²⁾ These results showed that Ea bearing Trp29 modified with 2-hydroxy-5-nitrobenzyl bromide maintained the immunological properties of Ea, but its toxicity was significantly reduced.

Genetic engineering technique was extensively practiced by Ménez, a collaborator of one of the authors (N.T.) to produce various kinds of site-directed mutants of Ea.^{44),45)} Ten residues, Gln7, Ser8, Gln10, Lys27, Trp29, Asp31, Arg33, Ile36, Glu38, and Lys47, were suggested to be crucial for binding with the nicotinic AChR, of which Ser8 in loop I appeared to be involved in keeping the structure, because its hydroxy group forms a hydrogen bond with main-chain NH of Ile37, a residue sandwiched with the essential Ile36 and Glu38 in loop II. Three residues, Gln10, Lys27 and Arg33, appeared to be the most important. The essential residues except Ser8 and Lys47 are located on the gently concave surface made of the three loops, and Lys47 on loop III locates above one of the edges of the concave surface as shown in Fig. 9. Mutations of the residues His6, Ser9, Tyr25, Phe32 and Gly34 surrounding the important residues caused moderate affinity decrease. The functionally essential residues

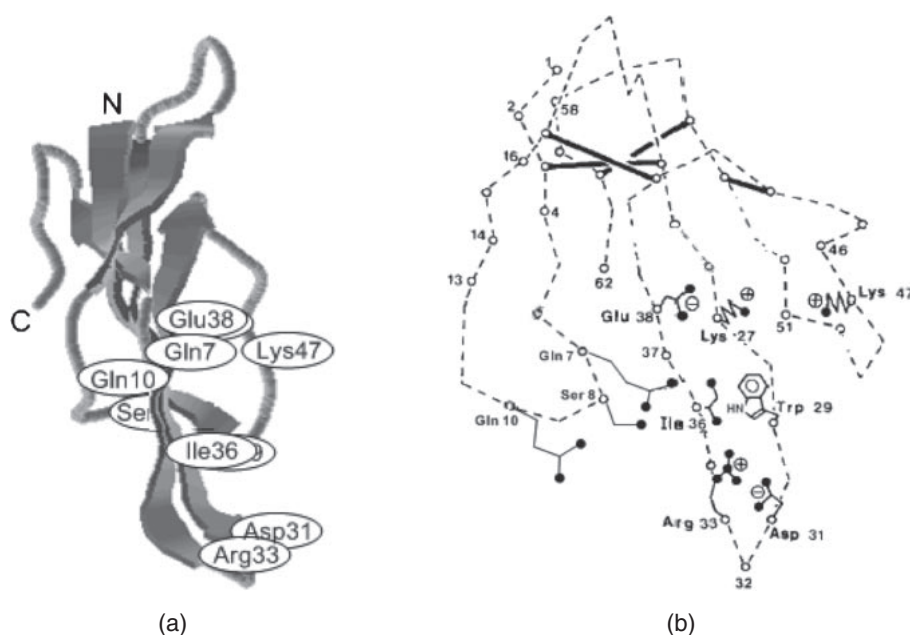


Fig. 9. Distribution of the functionally essential residues of Ea. (a) Side view to show that most of the functionally essential residues reside on the concave surface, Lys27 and Trp29 being hidden behind Glu38 and Ile36, respectively, from the viewer. (b) Front view (the concave surface to the viewer) to show side chains of the functionally essential residues.

are oriented in a manner in which the hydrophobic core (Ile36 + Trp29) is surrounded with two ionic patches (Glu38 + Lys27 and Arg33 + Asp31), one hydrophilic patch (Gln10 + Gln7) and a cation (Lys47), and occupy an area of approximately 680 \AA^2 , a value expected for a contact area of protein-protein interactions.⁴⁶⁾ Cytotoxins, which are structural analogues of short neurotoxins (*vide supra*) lack most of the ten functionally essential residues, and do not have affinity to AChR.

By comparison of the proton-NMR spectra of LsIII, a long neurotoxin from *L. semifasciata* with α -cobratoxin from *Naja naja siamensis*, all the aromatic and methyl proton signals have been assigned to specific amino acid residues.⁴⁷⁾ It was suggested that the backbone structure of a long neurotoxin was more rigid than that of short neurotoxins. The positively charged surface of LsIII, which is responsible for binding to AChR, was confirmed as the concave surface, and the arrangement of the amino acid residues on this surface was similar to that of all other neurotoxins. Accordingly, the slow on-off rate of association of long neurotoxins with AChR was considered to arise from the rigid backbone structure. A small conformational change was thought to be associated with binding to the receptor protein.⁴⁷⁾

The structure of a complex of a long toxin, α -cobratoxin from *Naja naja siamensis* (Indian cobra)

complexed with an acetylcholine binding protein (AChBP, a structural homolog of the extracellular ligand-binding domain of muscle-type and neuronal nicotinic AChRs) was elucidated.⁴⁸⁾ In α -cobratoxin, ten residues, Trp25, Asp27, Ala28, Phe29, Arg33, Lys35, Arg36, Phe65 and Cys26/30, which were implicated as important for interaction with AChBP by mutagenesis analysis, were found located within contact distance in the complex. These essential residues except Lys35 and Cys26/30 disulfide bridge are located on the gently concave surface, as in the case of Ea shown in Fig. 9.

Conclusion

None of a single residue is responsible for the binding of erabutoxins to AChR, the target protein of the toxins, but the collaboration of charged side chains (Lys27, Asp31, Arg33, Glu38, Lys47), hydrophilic side chains (Gln7, Gln10), and hydrophobic side chains (Trp29, Ile36) located in appropriate orientation (Fig. 9) are required for the tight binding of the toxin to AChR. Supporting evidence for this conclusion can be found by examining the sequences of toxins of lower toxicity. For example, a short toxin CM-10 from *Naja haje annulifera* (banded Egyptian cobra), which lacks an essential residue, Asp31, and has bulky Leu9 instead of Ser9, which locates between the essential Ser8 and Gln10 residues, is

3% as toxic as erabutoxins.⁴⁹⁾ Another weak toxin CM-2 from *Naja haje haje* (Egyptian cobra), which is less than 1% as toxic as erabutoxins has only three (Ser8, Lys27 and Trp29) out of the ten functionally essential residues.⁵⁰⁾ The facts that *N*-acetylation of either of essential lysyl residues, Lys27 and Lys47, destruction of essential Trp29 residue, or single replacement of an essential residue by site-directed mutagenesis, did not result in complete loss of toxicity are considered compatible with this conclusion. This conclusion seems to be valid for the long toxin, too, because a toxic component LsIII from *L. semifasciata*, which has four of the ten functionally essential residues in α -cobratoxin substituted (Asp27Asn, Phe29Trp, Arg36Val and Phe65Tyr) still retains 18% lethality of α -cobratoxin.⁸⁾

The present studies on erabutoxins and related neurotoxins contributed to establish a new superfamily of proteins composed of short and long neurotoxins and cytotoxins, and helped elucidate the possible mechanism of the action of the short and long neurotoxins. At the same time, use of neurotoxins as tools for isolating and studying AChR was practiced. A hypothesis "Evolution without divergence" is another outcome emerged from the studies on the snake venom neurotoxins.

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Profile

Nobuo Tamiya was born in 1922. He graduated from the Department of Chemistry, Faculty of Science, Tokyo Imperial University in 1944, and started his research carrier, which was interrupted by military service. He restarted his research in 1945, first on the bacterial metabolism of sulfur-containing amino acids, then on hydrogenase as advised by his supervisor, Prof. Shiro Akabori. His first achievement was to device a reliable assay method of hydrogenase. During his overseas studies (1955–1957), he learned to work on stable isotopes from Prof. David Rittenberg. In 1958, he moved to the Institute for Hard Tissue Research, Tokyo Medical and Dental University, as a professor. Here he made use of his isotopic technique to prove that atmospheric oxygen was incorporated to hydroxyprolyl residues in collagen of hard tissues. At the same time he was invited to join a research group on sea snake venom. In 1966, he moved to the Department of Chemistry, Faculty of Science, Tohoku University, where his studies on snake toxins opened the door to a large superfamily of neurotoxic and cytotoxic proteins, and he was inspired to propose a new hypothesis of evolution. He is also a good educator and published translations of distinguished biochemical textbooks and monographs. He received the Chemical Society of Japan Award in 1970, l'Ordre des Palmes Académiques (France) in 1980, Redi Award (the International Society on Toxinology) in 1982, the Medal with Purple Ribbon (Japan) in 1987, and the Order of the Sacred Treasure (Japan) in 1993. He was a member of the board of administration of the Chemical Society of Japan and an honorary member of the Japanese Biochemical Society. He passed away on 19 January 2011.



Profile

Tatsuhiko Yagi was born in 1933. He graduated from the Department of Chemistry, Faculty of Science, University of Tokyo in 1955, and started his biochemical research carrier. His first achievement was discovery of carbon monoxide dehydrogenase. He moved to the Institute for Hard Tissue Research, Tokyo Medical and Dental University, as an assistant of Prof. Nobuo Tamiya in 1958. He went as a Fulbrighter to the laboratory of Prof. Andrew A. Benson at the Pennsylvania State University, where he contributed to determine the structure of sulfolipid, which had been erroneously described. On returning to Japan in 1962, he started to study hydrogenase with Prof. Tamiya, and established its electron carrier specificity, which had been unknown since the discovery of hydrogenase in 1931. He moved to Shizuoka University in 1966, and promoted to professor in 1972. His accomplishments there include elucidation of structure and function of cytochrome c_3 , cytochrome c -553, ferredoxins and hydrogenase, discovery of multiheme high-molecular-weight cytochrome c , practice of an enzymatic electric cell for the activity assay, discovery of 1-methoxyPMS as a versatile photostable electron mediator, etc. He helped Prof. Tamiya to reinforce his hypothesis of evolution. He published many biochemical textbooks with him, and authored an encyclopedia of compounds to search for enzymes. He is a councilor of the Japanese Biochemical Society, an emeritus member of the American Chemical Society and a member of the Chemical Society of Japan.

