Review

Studies on sea snake venom

By Nobuo TAMIYA*1 and Tatsuhiko YAGI*2,†

(Communicated by Satoshi ŌMURA, M.J.A.)

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). 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 Wright 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. In the course of analyzing the primary structures of sea snake toxins, 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. 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). A minor toxic component from L. semifasciata, which accounted for 5% of the original venom lethality, was later isolated and...
named erabutoxin c (Ec). Ec was also a basic protein of 62 residues, and its LD<sub>50</sub> was 0.13 µg/g body weight of mice, a value similar to those of Ea and Eb.<sup>6)</sup> 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.<sup>7),8)</sup> A toxic component isolated from <i>L. laticaudata</i> was named laticutoxin 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<sub>50</sub> was 0.13 µg/g body weight of mice.<sup>9)</sup>

Toxicity of Ea and Eb was demonstrated with isolated frog muscle.<sup>4)</sup> In the aerated Ringer solution, the contracture of frog (<i>Rana nigromaculata</i>) 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.<sup>5),10)</sup> 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<sub>D</sub>) of a complex of erabutoxin with AChR was later estimated to be 10 × 10<sup>-11</sup> M for Ea, 4 × 10<sup>-11</sup> M for Eb and 6 × 10<sup>-11</sup> M for Ec<sup>5)</sup> whereas LD<sub>50</sub> values were similar (0.13–0.15 µg/g body weight of mice, <i>vide supra</i>). 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<sup>3),4)</sup> at the First International Symposium on Animal Toxins in 1966.<sup>11)</sup> 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 <i>Emydocephalus</i> (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.
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.\textsuperscript{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 \textit{L. laticaudata}, (about 80 cm long) and large \textit{L. semifasciata} (the largest one we observed was 1.8 m long). \textit{L. laticaudata} looked very different in appearance from those in Okinawa, Taiwan, Philippines, Papua New Guinea, New Caledonia, Vanuatu, Fiji and Australia, but \textit{L. semifasciata} looked very similar at these places.

**Amino acid sequences of snake toxins**

In 1967, Eaker\textsuperscript{13} in Sweden sequenced the amino acids of neurotoxin $\alpha$ from \textit{Naja nigricollis} (African cobra), which had been isolated by Boquet\textsuperscript{14} of Pasteur Institute. A toxin from \textit{Naja atra} (Formosan cobra), cobrotoxin was sequenced by Yang.\textsuperscript{15} The sea snakes in South Pacific and Indian Oceans are closely related. The authors worked on Australian snakes which have also similar toxins.\textsuperscript{16,17}

We found erabutoxins in 1964\textsuperscript{3} and sequenced them in 1971.\textsuperscript{5,18} We found several more homologous components from sea snakes and sequenced them.\textsuperscript{5,7,16,17} 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,\textsuperscript{19} namely short neurotoxins (around 60 amino acid residues), long neurotoxins (around 70 residues) and cytoxins

| Species               | Capture site       | Body weight | Venom | LD$_{50}$/g mouse |
|----------------------|--------------------|-------------|-------|------------------|
| \textit{Aipysurus laevis} | Scott Reef          | 200–840 g   | 0.05 mL | 0.96 mL          |
| \textit{Astrotia stokesi} | Darwin             | 2100–3050 g | 0.41 mL | 3.5 mL           |
| \textit{Hydrophis belcheri} | Ashmore Reef      | 360–520 g   | 0.01 mL | 1.0 mL           |
| \textit{Hydrophis elegans} | McCluer Gulf      | 330 g       | 0.02 mL | 0.38 mL          |
| \textit{Hydrophis ornatus} | McCluer Gulf      | 700 g       | 0.05 mL | 0.85 mL          |
| \textit{Lapemis hardwickii} | McCluer Gulf    | 400–870 g   | 0.03 mL | 0.98 mL          |

---

Fig. 2. Effects of Ea and Eb on the contraction of isolated frog rectus abdominal muscles by acetylcholine and by KCl.\textsuperscript{4} An isolated frog rectus abdominal muscles (\textit{Rana nigromaculata}) was placed in 5 mL glucose-Ringer solution (6.43 g of NaCl, 0.30 g of KCl, 0.17 g of CaCl$_2$, 0.10 g of NaHCO$_3$ 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, A$_2$ and A$_{10}$, 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.
Cytotoxins, alternatively known as cardiotoxins, do not have a affinity to AChR, and cause fibrillation of the heart muscle. 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, 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 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/Cys24, Cys17/Cys41, Cys43/Cys54, and Cys55/Cys60 for the short neurotoxins (No. 1), Cys10/Cys34, Cys13/Cys21, Cys27/Cys53, Cys57/Cys68 and Cys69/Cys74 for the longN neurotoxins (No. 21), and Cys3/Cys20, Cys14/Cys41, Cys26/Cys30, Cys45/Cys56, and Cys57/Cys62 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.

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...
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 long N 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, Smith classified Laticauda (in subfamily Laticaudinae) and Hydrophis (in subfamily Hydrophiinae) in the same family Hydrophiidae, whereas Dowling classified them in family Elapidae. On the other hand, McDowell classified Laticauda in family Elapidae.
and **Hydropis** in family Hydrophiidae. Matsui’s classification\(^{20}\) 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. Differences 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 histories. 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 1985\(^{22}\) 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 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 \(^{131}\)I\(\text{KI}\) 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-diodohistidyl 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.\(^{33}\) \(\alpha\)-Toxin from *Naja nigricollis* was used as an affinity ligand 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 \(\alpha\)-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, \(\alpha\)-cobratoxin, a long neurotoxin, and demotoxin, 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 \(\beta\)-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
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.\textsuperscript{37} is very similar to that of Eb.

The solution structure of Eb in D\textsubscript{2}O (Fig. 7) elucidated by proton-NMR in collaboration with late Miyazawa and Inagaki\textsuperscript{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 D\textsubscript{2}O 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.\textsuperscript{40} From the pH (pH meter readings in D\textsubscript{2}O without calibration)-dependence of His26-protons, the pK\textsubscript{a} value for the imidazole ring was estimated to be 5.8, a value similar to that of free histidine (pK\textsubscript{a} = 6.04 in H\textsubscript{2}O). 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.\textsuperscript{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.\textsuperscript{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
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 amino acid residues, and are very similar in shape as the short neurotoxins shown in Fig. 6a.

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

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 8 M 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 diido-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 Menez, 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, 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.

Fig. 8. Schematic presentation of the microenvironments of some amino acid residues in Eb. Tentatively assigned residue numbers are in parentheses.
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 Å², a value expected for a contact area of protein–protein interactions. 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. 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.

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. 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
The facts that Ser8, Lys27 and Trp29 out of the ten functionally essential residues has only three (Ser8, Lys27 and Trp29) out of the ten functionally essential residues. 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.

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.

Acknowledgements

The authors wish to express their gratitude to collaborators, whose names appear as coauthors of the papers cited in this article. Thanks are due to Prof. Tamio Yamakawa, M.J.A., who encouraged us to write this review article, and Prof. Toshiya Endo for critical reading of the manuscript.

References

1) Toriba, M. (1994) Sea snakes of Japan. In Sea Snake Toxidology (ed. Gopalakrishnakone, P.), Singapore University Press, Singapore, pp. 206–211.
2) Carey, J.E. and Wright, E.A. (1960) Isolation of the neurotoxic component of the venom of the sea snake, Enhydrina schistosa. Nature 185, 103–104.
3) Arai, H., Tamiya, N., Toshioka, S., Shinonaga, S. and Kano, R. (1964) Studies on sea-snake venoms. I. Protein nature of the neurotoxic component. J. Biochem. 56, 568–571.
4) Tamiya, N. and Arai, H. (1966) Studies on sea-snake venoms. Crystallization of erabutoxins a and b from Laticauda semifasciata venom. Biochem. J. 99, 624–630.
5) Sato, S. and Tamiya, N. (1971) The amino acid sequences of erabutoxins, neurotoxic proteins of sea-snake (Laticauda semifasciata) venom. Biochem. J. 122, 453–461.
6) Tamiya, N. and Abe, H. (1972) The isolation, properties and amino acid sequence of erabutoxin c, a minor neurotoxic component of the venom of a sea snake Laticauda semifasciata. Biochem. J. 130, 547–555.
7) Maeda, N. and Tamiya, N. (1974) The primary structure of the toxin Laticauda semifasciata III, a weak and reversibly acting neurotoxin from the venom of a sea snake, Laticauda semifasciata. Biochem. J. 141, 389–400.
8) Ishikawa, Y., Menez, A., Hori, H., Yoshida, H. and Tamiya, N. (1977) Structure of snake toxins and their affinity to the acetylcholine receptor of fish electric organ. Toxicon 15, 477–488.
9) Sato, S., Yoshida, H., Abe, H. and Tamiya, N. (1969) Properties and biosynthesis of a neurotoxic protein of the venoms of sea snakes Laticauda laticaudata and Laticauda colubrina. Biochem. J. 115, 85–90.
10) Sato, S., Abe, H. and Tamiya, N. (1970) Binding of iodinated erabutoxin b, a sea snake toxin, to the endplates of the mouse diaphragm. Toxicon 8, 313–314.
11) Tamiya, N. (1967) Animal Toxins. Papers presented at the first international symposium, Atlantic City, N.J., April 9–11, 1966 (eds. Russell, F.E. and Saunders, P.R.), Pergamon, New York, pp. 249–258.
12) Tamiya, N. and Puﬀer, H. (1974) Lethality of sea snake venoms. Toxicon 12, 85–87.
13) Eaker, D.L. and Porath, J. (1967) The amino acid sequence of a neurotoxin from Naja nigericollis venom. Jpn. J. Microbiol. 11, 353–355.
14) Boquet, P., Izard, Y., Meaune, J., Jouannet, M., Ronseray, A.M., Dumarey, C., Ozenne, S. and Casseault, S. (1967) Biochemical and immunological studies on snake venom. II. Study of the enzymic and toxic properties of fractions separated by filtration of Naja nigericollis venom on Sephadex. Ann. Inst. Pasteur 112, 213–235 (cited from Chem. Abst. 67:52159m).
15) Yang, C.C., Yang, H.J. and Huang, J.S. (1969) The amino acid sequence of cobratoxin. Biochim. Biophys. Acta 188, 65–77.
16) Tamiya, N. and Maeda, N. (1979) Neurotoxins of Australian sea snakes. In Neurotoxins: Fundamental and Clinical Advances (eds. Chubb, I.W. and Greﬀen, L.B.), Adelaide University Union Press, Adelaide, pp. 95–101.
17) Takasaki, C. and Tamiya, N. (1985) Isolation and amino acid sequence of a short-chain neurotoxin from an Australian elapid snake, Pseudechis australis. Biochem. J. 232, 367–371.
18) Bourne, P.E., Sato, A., Corfield, P.W., Rosen, L.S., Birken, S. and Low, B.W. (1985) Erabutoxin b. Initial protein refinement and sequence analysis at 0.140-nm resolution. Eur. J. Biochem. 153, 521–527.
19) Dufton, M.J. (1984) Classification of elapid snake neurotoxins and cytotoxins according to chain length: evolutionary implications. J. Mol. Evol. 20, 128–134.
20) Fryklund, L. and Eaker, D. (1975) The complete
amino acid sequence of a cardiotoxin from the venom of \textit{Naja naja} (Cambodian Cobra). Biochemistry \textbf{14}, 2860–2865.

21) Pawlak, J., Mackessy, S.P., Fry, B.G., Bhatia, M., Mourier, G., Fruchart-Gailhard, C., Servent, D., Ménez, R., Stura, E., Ménez, A. and Kini, R.M. (2006) Denmotoxin, a three-toxin from the colubrid snake \textit{Boiga dendrophila} (Mangrove Catsnake) with bird-specific activity. J. Biol. Chem. \textbf{281}, 29030–29041.

22) Tamiya, N. and Yagi, T. (1985) Non-divergence theory of evolution: Sequence comparison of some proteins from snakes and bacteria. J. Biochem. \textbf{98}, 289–303.

23) Smith, M.A. (1926) Monograph of the sea snakes. Nakayama-Shoten, Tokyo, pp. 93.

24) Dowling, H.G. (1974) A provisional classification of snakes. In Yearbook of Herpetology (ed. Dowling, H.G.). HIS Publications, New York, pp. 167–170.

25) McDowell, S.B. (1967) \textit{Aspidomorphus}. London.

26) Matsui, M. (1992) Serpents (Vol. 9 IIIb). In Dobutsu Keito-Bunrui-Gaku (Systematic Zoology, in Japanese, eds. Uchida, T. and Yamada, M.). Nakayama-Shoten, Tokyo, pp. 93–146.

27) Nelson, K.E., Clayton, R.A., Gill, S.R., Gwinn, M.L., Dodson, R.J., Haft, D.H., Hickey, E.K., Peterson, J.D., Nelson, W.C., Ketchum, K.A., McDonald, L., Utterback, T.R., Malek, J.A., Linher, K.D., Garrett, M.M., Stewart, A.M., Cotton, M.D., Pratt, M.S., Phillips, C.A., Richardson, D., Heidelberg, J., Sutton, G.G., Fleischmann, R.D., Eisen, J.A., White, O., Salzberg, S.L., Smith, H.O., Venter, J.C. and Fraser, C.M. (1999) Evidence for lateral gene transfer between Archaea and bacteria from genome sequence of \textit{Thermotoga maritima}. Nature \textbf{399}, 323–329.

28) Tamiya, N. and Yagi, T. (2006) Evolution without divergence. IUBMB Life \textbf{58}, 309–311.

29) Yagi, T. and Tamiya, N. (2009) Molecular conformation and function of erabutoxins a and b as studied by nuclear magnetic resonance. Eur. J. Biochem. \textbf{109}, 129–138.

30) Hatanaka, H., Oka, M., Kohda, D., Tate, S., Suda, A., Tamiya, N. and Inagaki, F. (1994) Tertiary structure of erabutoxin b in aqueous solution as elucidated by two-dimensional nuclear magnetic resonance. J. Mol. Biol. \textbf{240}, 155–166.

31) Sato, S. and Tamiya, N. (1970) The iodination of erabutoxin b: diiodohistidine formation. J. Biol. Chem. \textbf{234}, 2050–2053.

32) Sato, S. and Tamiya, N. (1970) Iodination of erabutoxin b: diiodohistidine formation. J. Biochem. \textbf{68}, 867–872.

33) Ishikawa, Y., Kano, M., Tamiya, N. and Shimada, Y. (1985) Acetylcholine receptors of human skeletal muscle: a species difference detected by snake neurotoxins. Brain Res. \textbf{346}, 82–88.

34) Ringler, P., Kessler, P., Ménez, A. and Brisson, A. (1997) Purification of the nicotinic acetylcholine receptor protein by affinity chromatography using a regioselectively modified and reversibly immobi-

lized α-toxin from \textit{Naja nigricollis}. Biochim. Biophys. Acta \textbf{1324}, 37–46.

35) Low, B.W., Preston, H.S., Sato, A., Rosen, L.S., Searl, J.E., Rudko, A.D. and Richardson, J.S. (1976) Three dimensional structure of erabutoxin b neurotoxic protein: inhibitor of acetylcholine receptor. Proc. Natl. Acad. Sci. USA \textbf{73}, 2991–2994.

36) Walkinshaw, M.D., Saenger, W. and Maelicke, A. (1980) Three-dimensional structure of the long neurotoxin from cobra venom. Proc. Natl. Acad. Sci. USA \textbf{77}, 2400–2404.

37) Nastopoulos, V., Kanellopoulos, P.N. and Tsernoglou, D. (1998) Structure of dimeric and monomeric erabutoxin a refined at 1.5 Å resolution. Acta Crystallogr. D Biol. Crystallogr. \textbf{54}, 964–974.

38) Inagaki, F., Tamiya, N. and Miyazawa, T. (1980) Molecular conformation and function of erabutoxins as studied by nuclear magnetic resonance. Eur. J. Biochem. \textbf{109}, 129–138.

39) Hatanaka, H., Oka, M., Kohda, D., Tate, S., Suda, A., Tamiya, N. and Inagaki, F. (1994) Tertiary structure of erabutoxin b in aqueous solution as elucidated by two-dimensional nuclear magnetic resonance. J. Mol. Biol. \textbf{240}, 155–166.

40) Inagaki, F., Miyazawa, T., Hori, H. and Tamiya, N. (1978) Conformation of erabutoxins a and b in aqueous solution as studied by nuclear magnetic resonance and circular dichroism. Eur. J. Biochem. \textbf{89}, 433–442.

41) Hori, H. and Tamiya, N. (1976) Preparation and activity of guanidinated or acetylated erabutoxins. Biochem. J. \textbf{153}, 217–222.

42) Seto, A., Sato, S. and Tamiya, N. (1970) The properties and modification of tryptophan in a sea snake toxin, erabutoxin a. Biochim. Biophys. Acta \textbf{214}, 483–489.

43) Horton, H.R. and Koshland, D.E. Jr. (1965) A highly reactive colored reagent with selectivity for the tryptophan residue in proteins. 2-Hydroxy-5-nitrobenzyl bromide. J. Am. Chem. Soc. \textbf{87}, 1126–1132.

44) Pillet, L., Trémeau, O., Ducancel, F., Drevet, P., Zimm-Justin, S., Pinkasfeld, S., Boulain, J.C. and Ménez, A. (1993) Genetic engineering of snake toxins. Role of invariant residues in the structural and functional properties of a curaremimetic toxin, as probed by site-directed mutagenesis. J. Biol. Chem. \textbf{268}, 909–916.

45) Trémeau, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Ducancel, F., Boulain, J.C. and Ménez, A. (1995) Genetic engineering of snake toxins. The functional site of erabutoxin a, as delineated by site-directed mutagenesis, includes variant residues. J. Biol. Chem. \textbf{270}, 9362–9369.

46) Janin, J. and Chothia, C. (1990) The structure of protein-protein recognition sites. J. Biol. Chem. \textbf{265}, 16027–16030.

47) Inagaki, F., Clayden, N.J., Tamiya, N. and Williams, R.J. (1981) A proton-magnetic-resonance study on the molecular conformation and structure-function
relationship of a long neurotoxin, laticauda semifasciata III from *Laticauda semifasciata*. Eur. J. Biochem. **120**, 313–322.

48) Bourne, Y., Talley, T.T., Hansen, S.B., Taylor, P. and Marchot, P. (2005) Crystal structure of a Cbtx-AChBP complex reveals essential interactions between snake α-neurotoxins and nicotinic receptors. EMBO J. **24**, 1512–1522 (Erratum: EMBO J. 2006, **25**, 266).

49) Joubert, F.J. and Taljaard, N. (1978) *Naja haje haje* (Egyptian cobra) venom. Some properties and the complete primary structure of three toxins (CM-2, CM-11 and CM-12). Eur. J. Biochem. **90**, 359–367.

50) Joubert, F.J. (1975) The amino acid sequences of three toxins (CM-10, CM-12 and CM-14) from *Naja haje annulifera* (Egyptian cobra) venom. Hoppe Seylers Z. Physiol. Chem. **356**, 53–72.

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 c₅₅, cytochrome c-553, forredoxins 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.