RAMAN SPECTRUM OF TOXIN B IN RELATION TO STRUCTURE AND TOXICITY

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1. Introduction

There are two types of neurotoxic proteins in the venoms of *Elapidae* and *Hydrophiidae* snakes. They are the short-chain neurotoxins (type I toxins) consisting of 60–62 amino acid residues with four disulfide bridges and the long-chain neurotoxins (type II toxins) consisting of 71–74 amino acid residues with five disulfide bridges [1]. Laticauda semifasciata III (Ls III) is the only one exceptional neurotoxin so far identified that does not belong to either type [2,3]. The short-chain and long-chain toxins have similar neurotoxicity [4]. However, the differences distinguished between them in the effects of chemical modifications on the toxicity [5,6] suggest some difference in the role of the essential amino acids for neurotoxicity.

In the preceding paper, Harada et al. reported the Raman spectra of the short-chain neurotoxins, erabutoxins a and b (Ea and Eb), the denatured toxins, and the weakly neurotoxic Ls III, and discussed on the structure-toxicity relationships [7].

Toxin B is a long-chain neurotoxin from the venom of the Indian cobra, *Naja naja*, and 71 amino acid sequence and the position of the five disulfide bridges have been determined [8].

In the present paper, the Raman spectrum of toxin B is reported and the structure-toxicity relationships are discussed in comparison with the former results on the short-chain toxins [7,9].

2. Materials and methods

Toxin B was prepared from the venom as previously reported [10]. The aqueous solution of toxin B was prepared at concentration of about 100 mg/ml. The sample handling and spectroscopic methods were similar to that described previously [7]. The Raman spectrum was recorded with a laser power at the sample of about 300 mW and a slit width of 7 cm^{-1}.

Original chart of Raman spectrum of toxin B is shown in fig.1.

3. Results and discussion

The primary structure [8] of toxin B is shown in fig.2 in the form of two-dimensional schematic diagram for the convenience of the following discussion.

The peaks of the amide I and III bands are observed at 1670 and 1241 cm^{-1}, respectively, which indicates the coexistence of random-coil and β-pleated-sheet

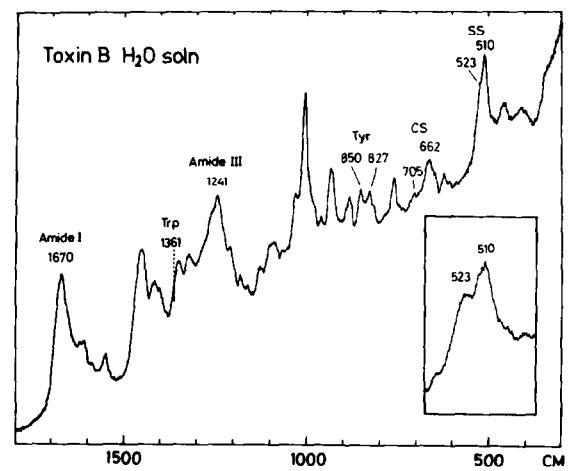

Fig.1. Raman spectrum of toxin B in water.

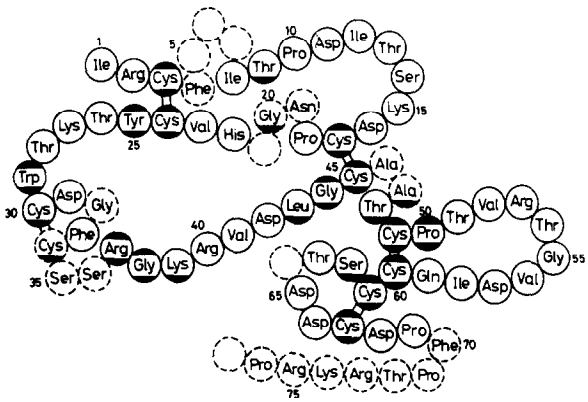

Fig.2. Two-dimensional schematic diagram of the primary structure of toxin B. The figure shows the general structure of snake neurotoxins as well. (⊖) invariant in 36 neurotoxins including Ls III. (◒) common to the 16 long-chain neurotoxins. (◌) lacking in some neurotoxins.

structures [11–13]. No distinct Raman scattering implying α-helical structure is observed in the amide I and III regions [11–13].

The bands at 510 and 523 cm^{-1} are assigned to the SS stretching vibrations of the gauche–gauche (G–G) and gauche–trans (G–T) structures with respect to the $C_\alpha C_\beta$–SS–$C_\beta C_\alpha$ linkage [14,15]. The intensity ratio of the peaks at 510 and 523 cm^{-1} is roughly 10:8, which indicates that three out of five disulfide bridges take the G–G structure and the remaining two take the G–T structure. The bands at 662 (strong) and 705 cm^{-1} (weak) are assigned to the CS stretching vibrations of the trans and gauche forms, respectively, about the HC_α–$C_\beta S$ bond [14,16].

The intensity ratio of the tyrosine doublet at 827 and 850 cm^{-1} is about 8:10. This is interpreted to indicate that the phenolic hydroxyl group of the single tyrosine residue at the 25th position is in the state with moderate-to-weak hydrogen bond but not forming the strong hydrogen bond with a proton-acceptor such as CO_2^- or NH_2 [17].

The lack of a distinct peak at 1361 cm^{-1} due to tryptophan residue suggests that the single tryptophan at the 29th position is exposed to the solvent [18,19].

3.1. *Comparison with the short-chain neurotoxins*

The Raman spectra of the short-chain neurotoxins, toxins isolated from the venoms of *Lapemis hardwickii* and *Enhydrina schistosa* [9], Ea and Eb [7] have been investigated. The peak positions of the amide I and III bands of these neurotoxins indicate the predominant coexistence of random-coil and β-pleated-sheet structures similarly to the case of toxin B. However, the amide I bands of the short-chain neurotoxins are sharper than that of toxin B and observed at higher frequencies (1672–1674 cm^{-1}). This fact suggests that toxin B contains more fraction of random-coil than the short-chain neurotoxins.

With respect to the structure of the disulfide bridges, the number of the bridges with the G–T structure about $C_\alpha C_\beta$–SS–$C_\beta C_\alpha$ linkage is more in toxin B than that in each of the short-chain neurotoxins. Each Ea and Eb contains one disulfide bridge with the G–T structure and three with the G–G structure [7], while all of the disulfide bridges in the toxins from the venoms of *L. hardwickii* and *E. schistosa* take the G–G structure [9]. The peak due to the gauche form about HC_α–$C_\beta S$ bond is observed at 705 cm^{-1} for toxin B, but not for the short-chain neurotoxins.

The most remarkable spectral difference between toxin B and the short-chain neurotoxins is found in the intensity ratio of tyrosine peaks around 830 and 850 cm^{-1}. Toxin B and all of the short-chain neurotoxins discussed in this study have commonly single tyrosine residue at the 25th position which is one of the 14 common amino acid residues in neurotoxins. The tyrosine doublets of the short-chain neurotoxins are observed in common at 834 (strong) and about 847 cm^{-1} (shoulder) [7,9], which indicates that the phenolic OH group of the tyrosine molecule is forming a hydrogen bond with a strong proton-acceptor such as CO_2^- or NH_2. On the other hand, the intensity ratio of the doublet of toxin B shows that the tyrosine is not forming a strong hydrogen bond but a moderate-to-weak hydrogen bond.

The tryptophan at the 29th position which is also one of the 14 common amino acid residues in neurotoxins is exposed to the solvent in every case.

It is concluded from the above comparison that the structural similarity among the short-chain neurotoxins is much higher than that between toxin B and the short-chain neurotoxins. This seems to indicate the difference of the essential structure of neurotoxicity between the short-chain and long-chain neurotoxins. This was also suggested by the studies on chemical modification [1,5,6].

It has been shown that Tyr25 in the short-chain

neurotoxins is not modified in the mild condition and that once Tyr25 is modified the toxin loses its biological activity and conformational change occurs concurrently [20–22]. It is titrated only at pH values above 11.3 [20,21]. The Tyr25 in toxin B is easily modified without loss of toxicity nor conformational change and it is titrated at pH 11.0 [5]. These facts are consistent with the conclusion from the Raman studies that the state of Tyr25 is quite different between the short-chain and long-chain neurotoxins.

A similar phenomenon is also found in the relationship between modification of Trp29 and toxicity. The Trp-modified derivatives of the short-chain neurotoxins have no toxicity [23–27], but those of the long-chain neurotoxins retain half the toxicity of native toxin at least [6,28].

Moreover, a few residues around the C-terminal of the long-chain neurotoxins which are absent in the short-chain neurotoxins have been found to contribute to toxicity to some extent. The fragments without four residues at the C-terminal of toxin B and *Naja naja siamensis* toxin 3 retains 70% and 50% toxicity, respectively [1,28]. There is no common amino acid residue in the C-terminal region but there is at least one basic amino acid residue such as lysine or arginine [1].

3.2. *Structure-toxicity relationships of the short-chain and long-chain neurotoxins*

Both the short-chain and long-chain neurotoxins so far studied are known to block the acetylcholine receptor sites on the post-synaptic membrane [1,29]. However, it is evident from the above discussion that the essential structure for neurotoxicity is not the same between them. For the clarification of the structure-toxicity relationships of neurotoxins, accordingly, it is necessary to investigate the essential structures of the two types of toxins separately.

The Raman spectral study on Eb and Trp-modified Eb revealed that Trp29 plays the role of a part of the key to the key-hole of acetylcholine receptor in the lethal activity [7]. The preservation of the higher structural location of Trp29 is essential for the full neurotoxicity of the short-chain neurotoxins and the strong hydrogen bond of Tyr25 is considered to be important in the maintenance of active conformation. The complete loss of toxicity caused by the modification of Lys53 is reported in the case of cobrotoxin [30]. Hence, there are at least two key parts which are both indispensable for the toxicity of the short-chain neurotoxins.

The contribution of Trp29 moiety to toxicity is about 50% in the long-chain neurotoxins. The less significance of Trp29 in this case may be partly due to the lack of strong hydrogen bond of Tyr25 and the addition of the disulfide bridge between Cys30 and Cys34. They may distort the optimum structure around Trp29 for full toxicity as suggested previously in the discussion about the less toxicity of Ls III (about one-eighth toxicity of erabutoxins [31]) [7]. It is probable that the decrease of toxicity of the Trp29 moiety is compensated by the addition of some residues at the C-terminal in the long-chain neurotoxins and that this is not the case in Ls III because of the lack of such residues. The amino acid at 53rd position of the long-chain neurotoxins is lysine or arginine [1]. This part may contribute to the toxicity as well. If it is so, the long-chain neurotoxins have at least three key parts which cooperate to block the acetylcholine receptor sites. Ls III has no plus charge at 53rd position. This also may be responsible for its very weak neurotoxicity.

It is plausible that the neurotoxicity of either type toxin is presented by the complementary action of more than two key parts.

3.3. *Search for the proton-acceptor of the hydrogen bond of Tyr25 in the short-chain neurotoxins*

In the preceding paper Harada et al. discussed the protonacceptor of hydrogen bond of Tyr25 on the basis of the assumption that Tyr25 of each of all the neurotoxins were forming a strong hydrogen bond with CO_2^- or NH_2 at a common position [7]. However, present study reveals that Tyr25 of toxin B is not forming such a strong hydrogen bond. It is now necessary to search for the candidate of the proton-acceptor of Tyr25 in accordance with the above discussion.

We assume that Tyr25 of each of all the short-chain neurotoxins is hydrogen-bonded to a strong-acceptor amino acid with CO_2^- or NH_2 in a common position which is not common to toxin B. Arg43 is the only residue that satisfies the above assumption. Next we extend the definition of common position to include the adjacent amino acids. In this case, Glu21 and Glu22 can be the possible proton-acceptors. On the other

hand, Arg43 is eliminated because toxin B has aspartic acid at a next (42nd) position. The results [30,32] of the chemical modification of the carboxyl groups in cobrotoxin that among the seven carboxyl groups only Glu21 is buried and that the modification of Glu21 causes the loss of toxicity are indicative that the CO_2^- group of Glu21 or Glu22 is hydrogen-bonded with Tyr25 in the short-chain neurotoxins.

In conclusion, the comparison of the Raman spectra of short-chain and long-chain neurotoxins has revealed the difference of higher structure between them which is now believed to cause the different contribution of Trp29 moiety to neurotoxicity in both type neurotoxins. Structural studies by the analysis of Raman spectra of other neurotoxins and cobra basic proteins with no neurotoxicity will give more detailed information on the problem.

References

[1] Hayashi, K. and Ohta, M. (1975) Tampakushitsu Kakusan Koso 20, 53–69.
[2] Maeda, N., Takagi, K., Tamiya, N., Chen, Y. M. and Lee, C. Y. (1974) Biochem. J. 141, 383–387.
[3] Maeda, N. and Tamiya, N. (1974) Biochem. J. 141, 389–400.
[4] Lee, C. Y. and Chen, Y. M. (1974) Abstr 4th Intern. Symp. on Animal, Plant and Microbial Toxins (Tokyo) 85–86.
[5] Ohta, M. and Hayashi, K. (1974) Biochem. Biophys. Res. Commun. 56, 981–987.
[6] Ohta, M. and Hayashi, K. (1974) Biochem. Biophys. Res. Commun. 57, 973–979.
[7] Harada, I., Takamatsu, T., Shimanouchi, T., Miyazawa, T. Tamiya, N. (1976) J. Phys. Chem. 80, 1153–1156.
[8] Ohta, M. and Hayashi, K. (1973) Biochem. Biophys. Res. Commun. 55, 431–438.
[9] Yu, N. T., Lin, T. S. and Tu, A. T. (1975) J. Biol. Chem. 250, 1782–1785.
[10] Nakai, K., Nakai, C., Sasaki, T., Kakiuchi, K. and Hayashi, K. (1970) Naturwissenschaften 57, 387–388.
[11] Yu, N. T. and Liu, C. S. (1972) J. Am. Chem. Soc. 94, 5127–5128.
[12] Yu, T. J., Lippert, J. L. and Peticolas, W. L. (1973) Biopolymers 12, 2161–2176.
[13] Chen, M. C. and Lord, R. C. (1974) J. Am. Chem. Soc. 96, 4750–4752.
[14] (a) Sugeta, H., Go, A. and Miyazawa, T. (1972) Chem. Lett. 83–86 (b) Sugeta, H., Go, A. and Miyazawa, T. (1973) Bull. Chem. Soc. Jpn. 46, 2752–2755.
[15] Martin, R. B. (1974) J. Phys. Chem. 78, 855–856.
[16] Bastian, Jr., E. J. and Martin, R. B. (1973) J. Phys. Chem. 77, 1129–1133.
[17] Siamwiza, M. N., Lord, R. C., Chen, M. C., Takamatsu, T., Harada, I., Matsuura, H. and Shimanouchi, T. (1975) Biochemistry 14, 4870–4876.
[18] Chen, M. C., Lord, R. C. and Mendelsohn, R. (1973) Biochim. Biophys. Acta 328, 252–260.
[19] Yu, N. T. (1974) J. Am. Chem. Soc. 96, 4664–4668.
[20] Chan, C. C., Yang, C. C., Hamaguchi, K., Nakai, K. and Hayashi, K. (1971) Biochim. Biophys. Acta 236, 164–173.
[21] Chicheportiche, R., Rochart, C., Sampieri, F. and Lazdunski, M. (1972) Biochemistry 11, 1681–1691.
[22] Sato, S. and Tamiya, N. (1970) J. Biochem. (Tokyo) 68, 867–872.
[23] (a) Chang, C. C. and Hayashi, K. (1969) Biochem. Biophys. Res. Commun. 37, 841–846: (b) Chang, C. C. and Yang, C. C. (1973) Biochim. Biophys. Acta 295, 595–604.
[24] Tu, A. T. and Hong, B. (1971) J. Biol. Chem. 246, 2772–2779.
[25] Tu, A. T. and Toom, P. M. (1971) J. Biol. Chem. 246, 1012–1016.
[26] Hong, B. S. and Tu, A. T. (1970) Federation Proc. 29, 888.
[27] Seto, A., Sato, S. and Tamiya, N, (1970) Biochim. Biophys. Acta 214, 483–489.
[28] Karlsson, E., Eaker, D. and Ponterius, G. (1972) Biochim. Biophys. Acta 257, 235–248.
[29] Tamiya, N. (1975) in: The Biology of Sea Snakes (Dunson, W. A. ed), pp. 385–415, University Park Press, Baltimore, Md.
[30] Chang, C. C., Yang, C. C., Nakai, K. and Hayashi, K. (1972) Biochim. Biophys. Acta 251, 334–344.
[31] Maeda, N. and Tamiya, N. (1974) Biochem. J. 141, 389–400.
[32] Chang, C. C., Yang, C. C., Kurobe, M., Nakai, K. and Hayashi, K. (1971) Biochem. Biophys. Res. Commun. 43, 429–434.