CHEMICAL MODIFICATION OF TYROSINE RESIDUE IN "TOXIN B" FROM THE VENOM OF THE INDIAN COBRA, NAJA NAJA*

Mitsuhiro Ohta and Kyozo Hayashi**

Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

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SUMMARY

The single tyrosine residue in toxin B has been modified by the reaction with tetranitromethane. The amino acid analysis of the modified toxin shows the reaction to be specific for tyrosine with no effect on the other amino acids. The high toxicity of nitrated toxin B demonstrates that an intact tyrosine residue is not essential for full activity in contrast to the similarly modified tyrosine residue in cobratoxin. Antitoxin B sera differ immunologically from those of cobratoxin, indicative of the disparity of Tyr (21) and Trp (25), respectively.

Toxin B obtained from the venom of the Indian cobra, naja naja, consists of a single polypeptide chain of 71 amino acids (1), which are cross-linked by five disulfide bridges (2). The amino acid sequence of toxin B differs from toxin A (3) only by serine replacing isoleucine at position 32. There is only one tyrosine residue in toxin B located at position 21.

The environment of tyrosine residue in proteins has been the object of careful probing by techniques, such as spectrophotometric titration (4), iodination (5), and specific reagents, such as N-acetylimidazole (6, 7) and tetranitromethane (8, 9, 10). We modified the single tyrosine in toxin B which resides at the same position as in most other common neurotoxins in order to determine the degree of its exposure and reactivity and to assess its importance for full toxicity.

MATERIALS AND METHORDS

Toxin B was prepared from the venom of the Indian cobra, naja naja, as previously described (11).

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** To whom inquiries about this article should be directed.

Nitration of Toxin B with Tetranitromethane. Toxin B (10 mg) in 3 ml of 0.1 M sodium phosphate buffer at pH 8.0 at 20 °C was treated with a 10 fold molar excess of tetranitromethane (10). The reaction mixture was allowed to stand for 4 hours and then passed through a Sephadex G-25 column equilibrated with 1 % acetic acid. The ratio of nitrotyrosine formed per mole of protein was determined by amino acid analysis of the modified toxin B.

Amino Acid Analysis. Amino acid analyses were performed according to Spackman et al. (12) on a Hitachi model KLA-3B automatic amino acid analyzer. Samples were hydrolyzed in 1 ml of constant boiling HCl at 105 °C for 24 hours in an evacuated sealed tube.

Spectrophotometric Titration. Spectrophotometric titration of the tyrosine residue in toxin B was performed on a Hitachi model 214 type spectrophotometer with a 0.18 mg/ml protein solution in a 0.01 M phosphate buffer containing 0.1 M NaCl. The temperature was maintained at 20 °C in both compartments of the spectrophotometer. The pH of the solution in the reference cell was kept at pH 7.2, while the pH of the solution in the other cell was constantly varied by the addition of minute amounts of 1.0 N or 10 N alkali. PH values were measured after each NaOH addition and the spectra recorded between 240 - 320 nm.

Immunological Procedures. Antitoxin B sera were prepared by injecting into rabbits, weighing 2.0 to 2.5 Kg, increasing doses of toxin B emulsified with an equal volume of complete Freund's adjuvant. Ten μg to 1.5 mg per Kg body weight were injected subcutaneously into the right and left thigh alternating at weekly intervals during a period of three months and the rabbits were bled 10 days after the final injection. Double diffusion in agar gel was performed by Ouchterlony's technique (13). The quantitative precipitin reactions were carried out as described by Kabat (14). Increasing amounts of antigen in phosphate-buffered saline were added to a constant amount of antiserum in a total volume of 0.15 ml. The tubes were incubated for 30 min at 37 °C and then left overnight at 4 °C. The precipitates were washed three times with

2 ml of cold phosphate-buffered saline then dissolved in 1 ml of 1 % Na_2CO_3 solution and absorbances measured at 280 nm.

Toxicity. The toxicity of the modified toxin was measured by intraperitoneal injection (mice, 16 - 18 g) of a progressively diluted venom solution as described previously (15). Four mice of both sexces were used for each dilution and the LD_{50} was calculated according to the 50 % end point method of Reed and Muench (16).

RESULTS AND DISCUSSION

Spectrophotometric Titration. Spectrophotometric titration curves of the tyrosyl group in toxin B at 244 nm - 295 nm are shown in Fig. 1. The titra-

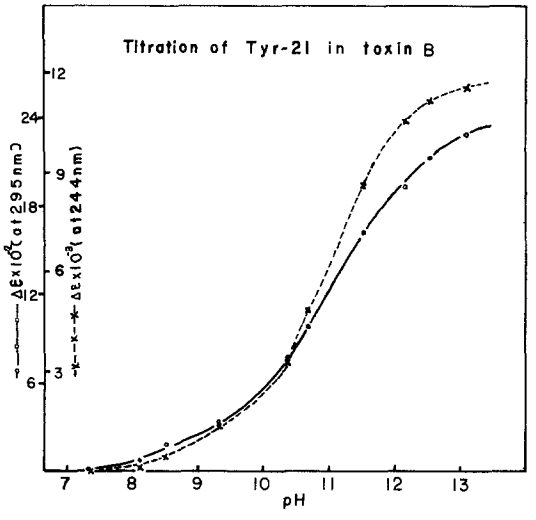

Fig. 1. Spectrophotometric titration of the single tyrosine group in toxin B.

tion curves neither at 244 nm nor at 295 nm changed after the solution had been left for 6 hours at pH 8.1 - 12.8. The apparent pK value for the stage of ionization was found to be 11.0 at 20 °C. This value is slightly higher than the normal pK value of 9.5 to 10.5. The single tyrosyl residue in toxin B, therefore, titrates freely and is not "buried" in contrast to cobratoxin

(17) and naja haje neurotoxin I (18) in which tyrosines 24 or 25 respectively, were found to be "buried" in the molecule.

Nitration of Toxin B. Amino acid analyses of nitrated toxin B (Table I) show

Table I

Amino Acid Composition of Nitrated Toxin B

Amino Acid	Native Toxin B	Nitrated Toxin B*
Aspartic acid	9	8.60
Threonine	9	8.36
Serine	4	3.90
Glutamic acid	1	1.21
Proline	6	6.13
Glycine	5	4.52
Alanine	2	2.17
Half-cystine	10	8.55
Valine	4	3.32
Methionine	0	0
Isoleucine	4	3.69
Leucine	1	1.00
Tyrosine	1	trace
Phenylalanine	3	2.82
NO_2-Tyrosine	0	0.90
Lysine	4	3.74
Histidine	1	0.80
Arginine	6	5.90

* All values are expressed as molar ratios on the basis of 1.0 for leucine as standard.

the disappearance of tyrosine after nitration in 0.1 M sodium phosphate buffer at pH 8.0 with a 10 fold excess of tetranitromethane. This disappearance of tyrosine is matched by the appearance of 3-nitrotyrosine. No other amino acids in toxin B were affected. On the other hand, the tyrosine residue at position 25 in cobratoxin, inaccessible to nitration even in the presence of 6 M urea, was modified in the presence of 5 M guanidine-HCl with a large molar excess of the reagent (17). Nitration of tyrosine 24 in naja haje neurotoxin I proceeded only to 50 % even in the presence of a 40 molar excess of tetranitromethane.

Effect of Nitration on Toxicity. The nitrated toxin B had a residual toxicity

of about 80 % of native toxin B. In the case of cobratoxin, nitration of tyrosines at positions 25 and 35 led to loss of toxicity (17).

Immunological Activity of the Modified Derivative. As illustrated in Fig. 2, nitrated toxin B gave a precipitin line identical with native toxin B as tested by immunodiffusion in agar with antitoxin B sera are shown in Fig. 3. The nitrated toxin B gave almost the same maximal precipitin

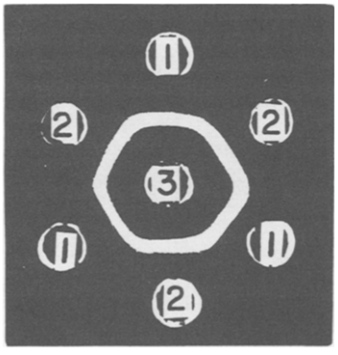

Fig. 2. Immunodiffusion in agar gel. Central well (3): antitoxin B sera. Surrounding wells: (1) toxin B ; (2) Tyr-21 nitrated toxin B.

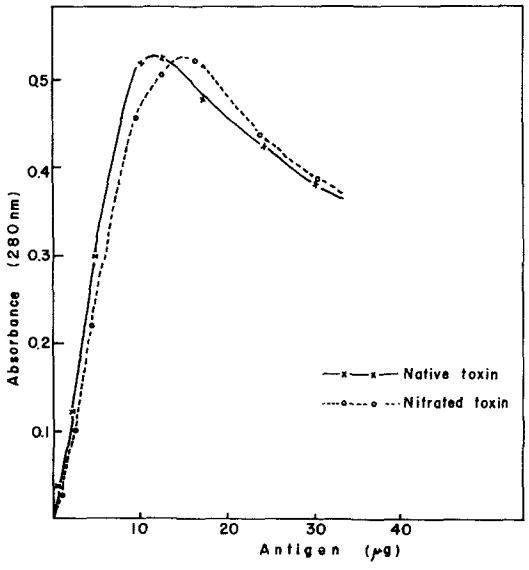

Fig. 3. Quantitative precipitin reactions of toxin B and its nitrated derivative with antitoxin B sera.

reaction as native toxin, but the precipitin curve was shifted toward the region of higher antigen concentration. However, anticobratoxin sera do not give any precipitin reaction with cobratoxin in which tyrosines 25 and 35 have been nitrated and which no longer shows toxicity. However, anticobratoxin sera give almost the same maximal precipitin reaction with fully active cobratoxin in which only tyrosine 35 has been nitrated. Since high toxicity and the normal pK value of the tyrosyl group were retained after selective nitration of the tyrosyl residue at position 21 it may be concluded that the integrity of the tyrosine residue is not as important for biological activity as it is for the tyrosine residue at position 25 in cobratoxin.

The neurotoxins can be subdivided into two groups: One group contains 61-62 and the second group 71-74 amino acid residues. The two groups differ from each other by the environment, function and reactivity of certain characteristic amino acid residues, not only of tyrosine but also of tryptophan which was recently also modified in toxin B (19).

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REFERENCES

1. M. Ohta, and K. Hayashi, to be published.
2. M. Ohta, and K. Hayashi, Biochem. Biophys. Res. Commun., in press.
3. K. Nakai, T. Sasaki, and K. Hayashi, Biochem. Biophys. Res. Commun., 44, 893 (1971).
4. Y. Inada, J. Biochem., 49, 217 (1961).
5. W. L. Jr. Hughes, and R. Stressle, J. Am. Chem. Soc., 72, 452 (1950).
6. J. F. Riordan, W. E. C. Wacker, and B. L. Vallee, Nature, 208, 1209 (1965).
7. J. F. Riordan, W. E. C. Wacker, and B. L. Vallee, Biochem., 4, 1758 (1965).
8. J. F. Riordan, M. Sokolovsky, and B. L. Vallee, J. Am. Chem. Soc., 88, 4104 (1966).
9. J. F. Riordan, M. Sokolovsky, and B. L. Vallee, Biochem., 6, 358 (1967).

10. J. F. Riordan, M. Sokolovsky, and B. L. Vallee, Biochem., 6, 3609 (1967).
11. K. Nakai, C. Nakai, T. Sasaki, K. Kakiuchi, and K. Hayashi, Naturwissenshaften, 57, 387 (1970).
12. D. H. Spackman, W. H. Stein, and S. J. Moore, J. Biol. Chem., 235, 648 (1960).
13. O. Ouchterlony, Progr. Allergy, 5, 1 (1958).
14. E. A. Kabat & Mayer's Experimental Immunochemistry, 2nd ed., p. 22, Charles, C. Thomas, Publisher, Springerfield, U. S. A., 1961.
15. C. C. Yang, J. Formosan Med. Assoc., 63, 325 (1964).
16. L. J. Reed, and H. Muench, Am. J. Hug., 27, 493 (1938).
17. C. C. Chang, C. C. Yang, K. Hamaguchi, K. Nakai, and K. Hayashi, Biochim. Biophys. Acta, 236, 164 (1971).
18. R. Chicheportiche, C. Rochat, F. Sampieri, and M. Lazdunski, Biochem. 9, 1681 (1972).
19. M. Ohta, and K. Hayashi, in preparation.