STUDIES ON THE STATUS OF DISULFIDE LINKAGES AND TYROSINE RESIDUES IN CARDIOTOXIN

W.M. Keung, W.W. Leung, Y.C. Kong

Department of Biochemistry
The Chinese University of Hong Kong
Shatin, N.T. Hong Kong

Received July 7,1975

Summary: Spectrophotometric titration of cardiotoxin showed that only one of the three tyrosine residues was titrated freely with a normal apparent pKa of 9.4 whereas the other two ionized at pH above 10.5 after an irreversible conformational change had occurred. Nitration of cardiotoxin in Tris-HCl buffer with tetranitromethane however, resulted in the nitration of two tyrosine residues. Complete nitration occurred in the presence of 5 M guanidine hydrochloride. Only the biological activity of cardiotoxin was affected by nitration. The immunological activity was intact even when all the tyrosine residues were nitrated. Reduction-alkylation of the disulfide bonds in cardiotoxin by acrylonitrile abolished both its immunological and biological activities completely.

INTRODUCTION

Cardiotoxin is the most basic and abundant constituent of cobra(Naja naja) venom. It has been reported to affect various kinds of tissue, both excitable and nonexcitable(1). Besides cardiotoxic effect, cardiotoxin also causes contracture of skeletal muscle, blockade of axonal conduction, vasoconstriction etc. (2). The nonspecificity of cardiotoxin actions makes it difficult to define its mode of action. Recently, the amino acid sequence as well as the positions of the four disulfide bridges in cardiotoxin have been reported(3,4,5). This permits a study of the structure-activity relationship of cardiotoxin and the results may contribute valuable information to the understanding of the action mechanism of cardiotoxin. In this communication, the effect of chemical modification of the disulfide bonds and the tyrosine residues on the immunological and biological activities of cardiotoxin are reported.

MATERIALS AND METHODS

Cardiotoxin was isolated from Chinese Cobra(Naja naja Linn.) venom according to the procedure previously described(6). Based on the amino acid content and the pharmacological properties, it was suggested that this cardiotoxin preparation was a close analogue of the Cytotoxin II isolated by Takechi et al.(5) from the venom of Indian Cobra.

Anti-cardiotoxin serum was prepared according to the procedure of Chang et al.(7) excepted that cardiotoxin was injected instead of cobrotoxin. Double diffusion test was carried out at 4° in 1 % agar gel according to the technique of Ouchterlony(8). Quantitative precipitin test was carried out according to the method of Roitt et al.(9).

EAT*** cell line was maintained by weekly intraperitoneal transplantation of 0.2 ml($5x10^7$ cells/ml) twice washed cells into mature albino mice of NMRI/BOM strain. Cells were harvested 6 days after the transplantation.

TNM was purchased from Aldrich Chemical Co. Inc. Milwaukee, Wisconsin. Agar-agar for immunodiffusion and Amberlite CG-50 I resin were obtained from Serva, Heidelberg. Sephadex gel was purchased from Pharmacia. 3-nitro-L-tyrosine was obtained from Sigma. All other chemicals used are of analytical grade.

Spectrophotometric titration The spectrophotometric titration was carried out in a Zeiss PMQ II spectrophotometer, using 1 cm quartz cell at 25°. 30 mg of cardiotoxin was dissolved in 30 ml of 0.15 M KCl solution and the pH was adjusted to 7.9 by the addition of 1.1 N HCl(volume change negligible). 2.5 ml of this solution was transferred to a cell and was served as reference. The solution was titrated by 5 N KOH. pH was measured at 25° on a Radiometer pH meter 25.

Nitration with TNM Nitration of tyrosine residues in cardiotoxin with TNM was performed exactly according to the procedure described by Riordan et al.(10). The nitration products were collected after desalting on a Sephadex G-15 column and was further purified on a Sephadex G-50 and an Amberlite CG-50 I resin columns. The purity of the nitrated-cardiotoxins was verified by disc gel electrophoresis.

Preparation of S-carboxyethylcysteine and S-cyanoethyl-cardiotoxin S-cyanoethyl-cardiotoxin and S-carboxyethylcysteine were prepared essentially according to the procedure of Weil et al.(11). The purity of the protein was verified by disc gel electrophoresis. S-carboxyethylcysteine was recrystallized from 70 % ethanol. The m.p. of the final product was 181-184°.

Amino acid analysis Amino acid analysis was performed according to the method described by Spackman et al.(12) with a Beckman model 120-C automatic amino acid analyser. Samples of 300-500 ug protein was hydrolysed in constant boiling hydrochloric acid at 110° for 24 hr in evacuated sealed tubes. Tryptophane content was determined by hydrolysing samples in 2 N NaOH at 110° for 24 hr.

***Abbreviations used in this paper: EAT, Ehrlich ascites tumor; Tris, tris(hydroxymethyl)aminomethane; TNM, tetranitromethane.

Measurement of cytolytic activity Cytolysis is determined quantitatively according to the methods employed by Petel et al. (13) as modified by Leung et al.(14). The cytosol materials of EAT cell showed an absorption maximum at 260 nm. Thus the increase in optical density at 260 nm in the incubation medium served as a measurement of the degree of cytolysis.

RESULTS AND DISCUSSION

Spectrophotometric titration The spectrophotometric titration curves of tyrosine residues in cardiotoxin at 295 nm (Fig. 1) show that only one of the three tyrosine residues can be titrated freely with a normal apparent pKa of 9.4. The other two tyrosine residues begin to ionize at pH above 10.5 where a time-dependent ionization is observed. These results suggest that the two "buried" tyrosine residues can be titrated only after irreversible conformational change has occurred. The molar extinction coefficient of tyrosine residue obtained in this experiment is 2300 in 0.15 M KCl at 295 nm which is in good agreement with the value reported by Bewley et al.(15)

Identification of the nitration products Nitration of

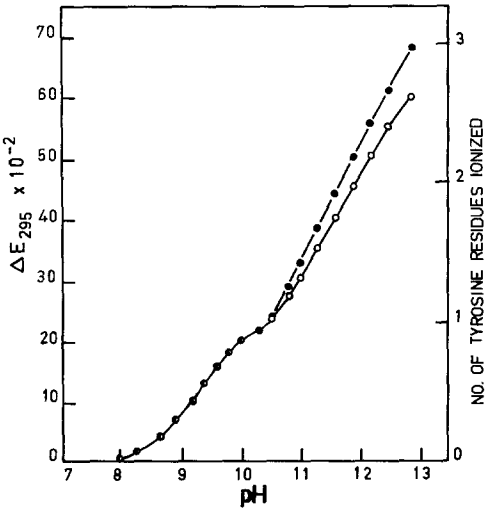

Fig. 1 Spectrophotometric titration of tyrosine residues in cardiotoxin. The open marks denote the initial reading(within 5 min after mixing) and the solid marks the final reading(2 hr after mixing).

tyrosine residues in cardiotoxin was carried out in two media, i.e. 0.05 M Tris-HCl buffer pH 8.0 and 5 M guanidine hydrochloride solution. The gel chromatography elution profiles of the nitrated cardiotoxin in both media on Sephadex G-50 column are shown in Fig. 2a and 2b respectively. Comparing with the elution profile of calibration substances(Fig. 2c), it is clearly shown that during the nitration with TNM, polymerized cardiotoxin products were produced. In Tris-HCl, only dimer (peak A) was formed whereas in guanidine hydrochloride solution, both dimer and trimer(peak A' and B') were formed. According to Robert and Frederick(16) and Thomas et al.(17), the polymerized products obtained during the nitration of proteins with TNM are formed by intermolecular crosslinking between tyrosine residues via phenoxide free-radical intermediates. Thus, based on the result of spectrophotometric titration, it is obvious that while higher cardiotoxin polymers can be formed in the presence

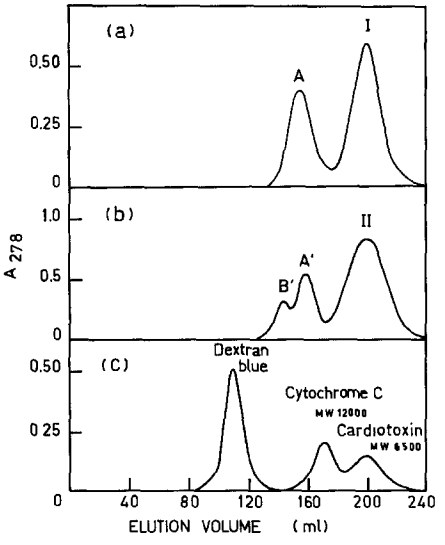

Fig. 2 The elution profiles of (a) cardiotoxin nitrated in Tris-HCl (b)cardiotoxin nitrated in guanidine hydrochloride (c)calibrating substances, on a 2x95 cm Sephadex G-50 column. The column was prepared as recommended by the manufacturer and was equilibrated and eluted by 0.05 M ammonium bicarbonate. The eluate was collected in 5 ml fractions at room temperature. Flow rate was adjusted to 10 ml per hr.

of denaturant, e.g. guanidine hydrochloride solution, only dimers are produced in Tris-buffer.

The monomeric nitrated products I and II(Fig. 2) were further purified on an Amberlite CG-50 column. The final products were homogeneous when analysed by disc gel electrophoresis. The number of tyrosine residues nitrated was determined according to the method of Sokolovsky et al.(18) using $\Delta E=4100$ at 428 nm. It was shown that product I contains two nitrated tyrosine groups whereas product II contains three. This result was further confirmed by amino acid composition analysis(Table 1). This result obviously indicates that although in an intact cardiotoxin molecule, the phenolic groups of the two "buried" tyrosine residues are not free for titration but the 3 position of the phenolic group of one of them is still succeptible to TNM nitration even in the absence of denaturant.

Identification of S-cyanoethyl-cardiotoxin The S-cyanoethyl-cardiotoxin prepared was identified by amino acid analysis. The result obtained(Table 2) shows that the reaction between acrylonitrile and the reduced cardiotoxin was confined specifically and quantitatively to the conversion of cysteine within the protein chain to a new amino acid derivative, which was eluted as a distinct peak between the serine and glutamic acid peaks, while the cystine peak was completely eliminated. The rest of the amino acids, as compared with the analysis of the parent protein, were not affected.

Effect of chemical modification on immunological activity Both the di-nitrated(product I) and the tri-nitrated(product II) cardiotoxin gave identical precipitin line as intact cardiotoxin

	moles/mole of cardiotoxin		
	Native cardiotoxin	Nitrated derivatives of cardiotoxin	
Amino acid		in Tris-HCl	in guanidine hydrochloride
Tyrosine	3.04	0.87	0
3-nitro-tyrosine	0	1.86	3.12

Table 1 Tyrosine content of native and nitrated cardiotoxin.

	moles/mole of cardiotoxin	
Amino acid	cardiotoxin	S-cyanoethyl-cardiotoxin
Lysine	9.00	9.00
Histidine	0	0
Arginine	2.05	1.93
Aspartic acid	6.90	7.06
Threonine	2.58	2.39
Serine	1.53	1.46
S-carboxyethylcysteine	0	7.72
Glutamic acid	0	0
Proline	5.02	4.87
Glycine	2.13	1.94
Alanine	2.70	2.68
Cystine	3.84	0
Valine	6.47	6.24
Methionine	1.84	1.68
Isoleucine	1.72	1.48
Leucine	6.53	6.34
Tyrosine	2.76	2.65
Phenylalanine	1.86	1.78
Tryptophane	0	0

Table 2 Amino acid composition of native cardiotoxin and S-cyanoethyl-cardiotoxin after 24 hr of acid hydrolysis.

when tested on immunodiffusion in agar gel with anti-cardiotoxin serum(Fig. 3). The S-cyanoethyl-cardiotoxin did not interact with anti-cardiotoxin serum.

Quantitative precipitin test(Fig. 4) showed that the di-nitrated and the tri-nitrated cardiotoxin gave almost the same maximal precipitation as cardiotoxin whereas S-cyanoethyl-cardiotoxin gave very little, if any precipitate even though the range of concentration tested was expanded 3-fold.

Effect of chemical modification on cytolytic activity The cytolytic activity of cardiotoxins were determined by measuring the absorption of EAT cell-free supernates obtained after incubation with 10 μg/ml cardiotoxins for various time intervals at 260 nm. Fig. 5 shows that in all cases, the percent cytolysis increases as a function of incubation time. While 10 μg/ml of intact cardiotoxin produces 50 % cytolysis after 80 min incubation, the same amount of di-nitrated and tri-nitrated derivatives produce 37 % and 25 % cytolysis respectively. 10 μg/ml of S-cyanoethyl-cardiotoxin however, does not induce cytolysis

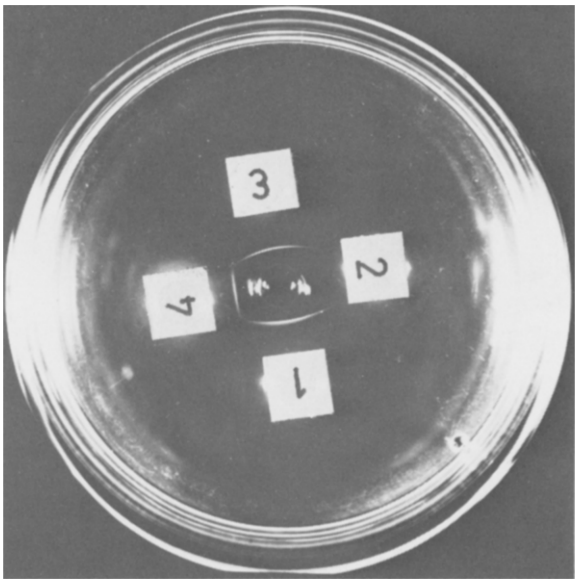

Fig. 3 Immunodiffusion in agar gel. Central well, anti-cardiotoxin serum. Surrounding wells, (1)cardiotoxin (2)S-cyanoethyl-cardiotoxin (3)di-nitrated cardiotoxin (4)tri-nitrated cardiotoxin.

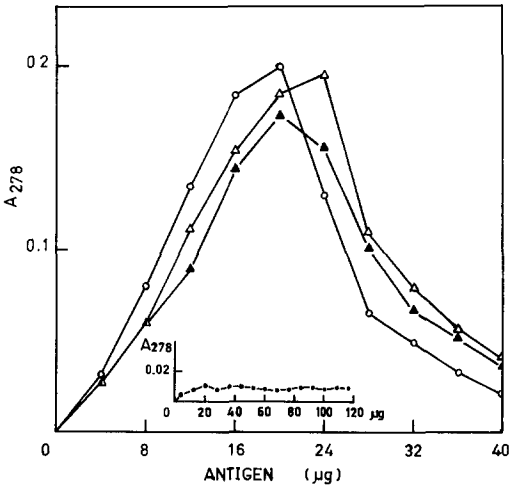

Fig. 4 Quantitative precipitin reactions of cardiotoxin and its chemically modified derivatives with anti-cardiotoxin serum. O——O, cardiotoxin; ●——● S-cyanoethyl-cardiotoxin; Δ——Δ di-nitrated cardiotoxin; ▲——▲ tri-nitrated cardiotoxin.

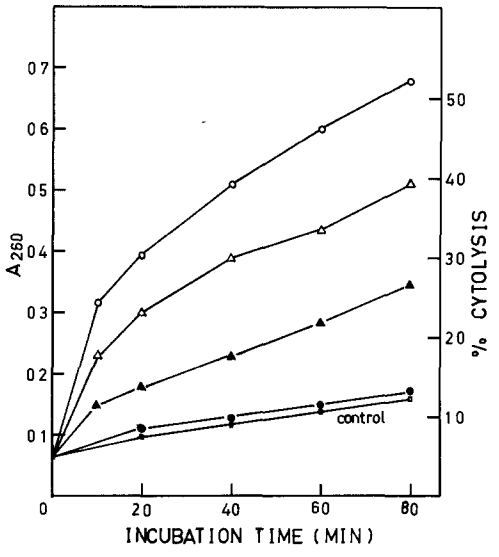

Fig. 5 Cytolysis of EAT cells under 10 μg/ml of cardiotoxin, O———O ; S-cyanoethyl-cardiotoxin, ●———● ; di-nitrated cardiotoxin, Δ———Δ ; tri-nitrated cardiotoxin, ▲———▲ . Calculation of percent cytolysis was based on the optical density at 260 nm of the tube with all its cells being lysed. Complete cytolysis was obtained by repeated freeze-thawing in a dry-ice acetone bath until they were just disintegrated as detected under microscope.

---

as compared to control. The complete abolishment of cytolytic activity by reduction-alkylation is expectable since the toxicity of cardiotoxin as well as neurotoxin have been reported to depend on intact disulfide bridges(19,20).

Based on the result of spectrophotometric titration, it is expected that under normal condition, only one tyrosine residue should be nitrated. However, in this experiment, mono-nitrated cardiotoxin was not obtained. Result from cytolytic activity studies strongly suggested that conformational change did occur during the formation of di-nitrated derivative even though the nitration was carried out under very mild condition, i.e. in Tris-buffer for 1 hr. This conformational change may be induced by the nitration of the first tyrosine group.

According to klibansky(21), the mode of cytolytic action of cardiotoxin involves two steps: 1. The polypeptides become attached to the membrane owing to their electropositive surface

charge; 2. The polypeptides penetrate the membrane structure through their lipophilic residues. If this is true, nitration of cardiotoxin may change its electric property or the spatial arrangement of its lipophilic constituents. Change in electric property may decrease its binding affinity to the cell membrane whereas rearrangement of lipophilic moiety may decrease its penetration power. Preliminary studies on the isoelectric point of the nitration products showed that nitration, both partial or complete, did not change its pI value. Therefore, it is suggested that the deactivation of cardiotoxin by nitration may probably be a result of spatial rearrangement of its lipophilic groups. Further studies of this problem is still in progress.

ACKNOWLEDGEMENT

The authors wish to thank sincerely Professor L. Ma for his encouragement and many helpful suggestions.

REFERENCES

1. Lee, C.Y.(1972) Ann. Rev. Pharmacol. 12:265-286.
2. Lee, C.Y., Chang, C.C., Chiu, T.H., Chiu, P.J.S., Tseng, T.C. and Lee, S.Y.(1968) Naumyn-schiedebergs Arch. Pharmak. u. exp. Path. 259:360-374.
3. Narita, K. and Lee, C.Y.(1970) Biochem. Biophys. Res. Comm. 41:339-343.
4. Lee, C.Y.(1972) J. Chinese Biochem. Soc. 1:47-56.
5. Takechi, M. and Hayashi, K.(1972) Biochem. Biophys. Res. Comm. 49:584-590.
6. Keung, W.M., Yip, T.T. and Kong, Y.C.(1975) Toxicon(in press)
7. Chang, C.C. and Yang, C.C.(1969) J. Immunol. 102:1437-1440.
8. Ouchterlony, O.(1958) Progr. in Allergy 5:1-78.
9. Roitt, J.M., Campbell, P.N. and Doniach, D.(1958) Biochem. J. 69:248-297.
10. Riordan, J.F., Sokolovsky, M. and Vallee, B.L.(1967) Biochemistry 6:358-364.
11. Spackman, D.H., Stein, W.H. and Moore, S.(1958) Anal. Chem. 30:1190-1205.
12. Weil, L. and Seibles, T.S.(1961) Arch. Biochem. Biophys. 95:470-473.
13. Patel, T.N., Braganca, B.M. and Bellare, R.A.(1969) Exptl. Cell Res. 57:289-297.
14. Leung, W.W., Keung, W.M. and Kong, Y.C.(manuscript submitted to Naunyn-scheidebergs Arch. Pharmak. u. exp. Path.)
15. Bewley, T.A., Brovetto, C.J. and Li, C.H.(1969) Biochemistry 8:4701-4708.
16. Robert, W.B., and Frederick, H.C.(1970) Biochem. Biophys. Res. Comm. 38:678-682.

17. Thomas, C.B., Maurice, J.G. and Sandra, L.W.(1968) J. Amer. Chem. Soc. 96:1612-1619.
18. Sokolovsky, M., Riordan, J.F. and Vallee, B.L.(1966) Biochemistry 5:3582-3589.
19. Vogt, W., Patzer, P., Lege, L., Oldings, H.D. and Wille, G. (1970) Naunyn-schiedebergs Arch. Pharmak. u. exp. Path. 265:442-454.
20. Yang, C.C. and Chang, C.C.(1972) J. Chinese Biochem. Soc. 1:21-31.
21. Klibansky, C., London, Y., Frenkel, A. and de Vries, A. (1968) Biochim. Biophys. Acta 150:15-23.