

BBA 37962

THE STATUS OF TYROSYL RESIDUES IN A FORMOSAN COBRA CARDIOTOXIN

MIEN-CHIE HUNG, YUNG-HSIN PAN, KING-LAN CHENG and YEE-HSIUNG CHEN *

*Institute of Biochemical Sciences, College of Sciences, National Taiwan University, and
Institute of Biological Chemistry, Academia Sinica, Taipei (Taiwan)*

(Received January 25th, 1978)

Summary

Spectrophotometric titration of Formosan cobra cardiotoxin showed that two of the three tyrosyl residues were titrated freely with a normal apparent pK_a of 9.6 whereas the remaining one ionized at pH above 11.0. Nitration of cardiotoxin in Tris · HCl buffer with tetranitromethane resulted in the selective nitration of tyrosine 11 and tyrosine 22. It also revealed that tyrosine 51 was the abnormal one in the spectrophotometric titration. Complete nitration occurred in the presence of 6.0 M guanidine hydrochloride. Compared with the conformation of native cardiotoxin, the peptide conformation of the partially nitrated cardiotoxin did not change significantly but the conformation of the completely nitrated cardiotoxin changed remarkably. The biological activity of cardiotoxin was indeed affected by nitration, but the immunological activity was nearly intact even when all the tyrosine residues were nitrated.

Introduction

Cobra cardiotoxin in the venom of the snake (*Naja naja atra*) had been isolated and purified by Lo et al. [1]. Lee et al. used the purified toxin for their pharmacological studies [2]. It is now known that snake cardiotoxins are membrane-active polypeptides which cause irreversible depolarization of the cell membrane [3].

Cobra cardiotoxin is a single basic polypeptide comprising 60 amino acid residues [4,5]. There are four disulfide bonds and three tyrosyl residues in this relatively small protein molecule. Based on the results of optical studies [6], Hung and Chen suggested that at least one tyrosine in cardiotoxin would be buried.

* To whom correspondence should be addressed.

We report our studies in the status of the three tyrosyl residues of cobra cardiotoxin. We found: (i) Tyr-11 and Tyr-22 are exposed while Tyr-51 is buried in the protein molecule; (ii) nitration of all the tyrosyl residues disturbs the secondary and tertiary folding of the protein considerably, but this does not result in a complete loss of lethal effect by cardiotoxin.

Materials and Methods

Cobra cardiotoxin was isolated and purified from crude venom by repeated column chromatography on CM-Sephadex C-25 [1]. Its concentration was determined by measuring its light absorption at 280 nm and using $E_{1\text{cm}}^{1\%} = 6.82$ [6]. Reagent grade chemicals were used throughout.

Light absorption measured in a Cary 14 UV spectrophotometer which had been calibrated with a solution of alkaline dichromate [7].

A Radiometer pH meter 26 with a scale expander was used for all pH measurements. The meter had been standardized against Merck buffers.

Polyacrylamide disc gel electrophoresis was carried out in 15% acrylamide as described by Reisfield et al. [8]. The electrophoresis was performed by applying a constant current of 6 mA/tube for 40 min in 0.35 M β -alanine (pH 4.5) at room temperature. Gels were stained with amido black.

Amino acid analysis was performed according to the procedure of Spackmen et al. [9] with a Yanagimoto Amino Acid Analyzer Model LC5A. Protein was hydrolyzed in 0.5 ml of constant boiling HCl containing 0.2% phenol in an evacuated sealed tube at 110°C for 24 h.

Circular dichroism (CD) spectra were measured with a Jasco J-20 spectropolarimeter under constant nitrogen flush. Fused silica cylindrical cells of various path length were used so that the absorbance of solution was kept below two. The data were expressed in terms of mean residue ellipticity, $[\theta]$.

The mean residue weight was calculated from the primary structure of the toxin.

LD₅₀ was determined according to the 50% end-point method [10]. Toxin in normal saline was injected intraperitoneally into albino mice weighing 15–20 g. Eight animals were tested for each dosage.

Hemolytic activity of cardiotoxin toward guinea-pig erythrocytes was tested by the procedure described by Vogt et al. [11]. Incubation tubes were shaken gently at 37°C, and aliquots were removed at 1 h and 2 h for measurement of hemolysis. Hemoglobin was estimated by absorbance measurements at 540 nm to measure relative Hb release.

Antisera against purified cardiotoxin were prepared by the weekly injection of cardiotoxin emulsified in a complete Freund's adjuvant into the foot pads of rabbits. The dosage used was 0.5 mg of cardiotoxin per kg of body weight. The immunization was continued for six weeks. Antisera were collected from the immunized animals one week after the last injection. Double immunodiffusion was performed at 4°C in 1% agarose gel according to the technique of Ouchterlony [12]. A quantitative precipitin test was carried out according to the method of Roitt et al. [13].

Spectrophotometric titration of cardiotoxin was carried out in 0.15 M KCl solution. The concentration of NaOH used to adjust the pH value of the solu-

tion were: 0.1 N, pH 7.0–10.5; 1 N, pH 10.5–11.5; 6 N, above pH 11.5. The absorbance in alkaline solution was read against the reference solution at pH 7.1. The differential molar absorbance at 295 nm, $\Delta\epsilon_{295}$, could be calculated.

Nitration was performed according to the procedure described by Sokolovsky et al. [14]. Tetranitromethane was dissolved in 0.5 ml of 95% ethanol and added dropwise to 20 mg of cardiotoxin respectively in 5 ml Tris · HCl (pH 8.0) and in 6.0 M guanidine hydrochloride (pH 8.0) under vigorous stirring. The molar ratio of reagent to tyrosine was 50 : 1. The formation of nitrotyrosine was monitored by measuring the light absorption at 428 nm. When the absorbance approached a constant value, the reaction mixture was applied immediately to a Sephadex G-50 column (2.6 × 60 cm) pre-equilibrated with 0.05 M ammonium acetate (pH 6.8) and the nitrated products were collected.

5 mg each of cardiotoxin and its derivatives was dissolved in 0.5 ml of performic acid solution (4.5 ml of 99% formic acid and 0.5 ml of 30% hydrogen peroxide were mixed thoroughly and stored at room temperature for 1 h). The solution was incubated in an ice-water bath for 1.5 h, then 20 ml of distilled water was added and the solution was lyophilized right away. 5 mg of the oxidized protein was dissolved in 0.5 ml of 0.05 M Tris · HCl buffer (pH 8.0) and was digested by TPCK-trypsin (1 : 20, w/w) at 37°C for 24 h. The digested peptides were separated on a Whatman No. 3 MM paper (46 × 57 cm) in the order of (i) descending paper chromatography in *n*-butanol/acetic acid/water (4 : 1 : 5, by vol., upper phase); (ii) high-voltage electrophoresis in collidine/acetic acid/water (8.9 : 3.1 : 988, by vol.), pH 6.6 at 35 V/cm for 1 h. The nitrotyrosyl peptides, which showed yellow spots in the presence of ammonia vapor, were cut from the paper for amino acid analysis. The remaining peptides on the paper were sprayed by ninhydrin which rendered them visible as blue spots.

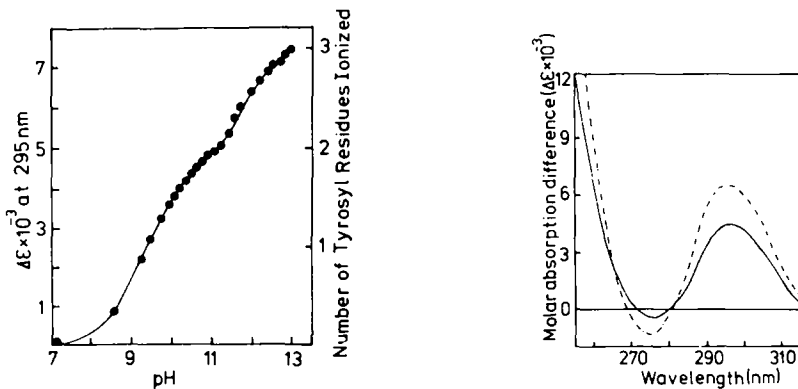
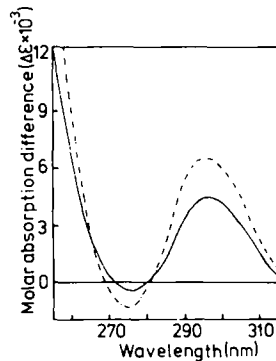


Fig. 1. Spectrophotometric titration curve of tyrosyl residues in cardiotoxin at 25°C.

Fig. 2. Differential spectra of cardiotoxin. —, cardiotoxin in glycine/NaOH buffer (pH 10.7) against in phosphate buffer (pH 7.0); - · - · -, cardiotoxin in 6.0 M guanidine hydrochloride, at pH 10.7 against at pH 7.0.



Results

The result of spectrophotometric titration for the three tyrosine in cobra cardiotoxin is shown in Fig. 1. Two residues could be titrated normally with an apparent pK_a of 9.6. The third residue began to ionize at pH above 11.0. The differential molar extinction coefficient at 295 nm, $\Delta\epsilon_{295}$, for the ionized tyrosine was estimated to be 2500, which agrees well with the value reported by Tanford et al. [15]. Fig. 2 shows the differential spectra of cardiotoxin in the native and unfolded state in 6.0 M guanidine hydrochloride [6]. The differential molar extinction coefficient of native cardiotoxin is about two-thirds of that of unfolded cardiotoxin. The above results indicated that only one tyrosine in native cardiotoxin is inaccessible to the medium and all three tyrosine residues can be ionized near pH 11.0 (pH 10.7) in the presence of 6.0 M guanidine hydrochloride.

The nitration of cardiotoxin with tetranitromethane was carried out in 0.05 M Tris · HCl (pH 8.0) and in 6.0 M guanidine hydrochloride (pH 8.0), respectively. Fig. 3 shows the rates of nitration. The absorbance at 428 nm, expressed in terms of molar absorbance ($\epsilon_{428\text{nm}}$), indicated the amount of nitrotyrosine formed in the course of reaction. Apparently, the titration could be finished within 1 h. When the protein was unfolded, more residues were modified. The nitrated products were fractionated by a Sephadex G-50 column chromatography (Fig. 4). Three fractions, a main fraction (product I) and two

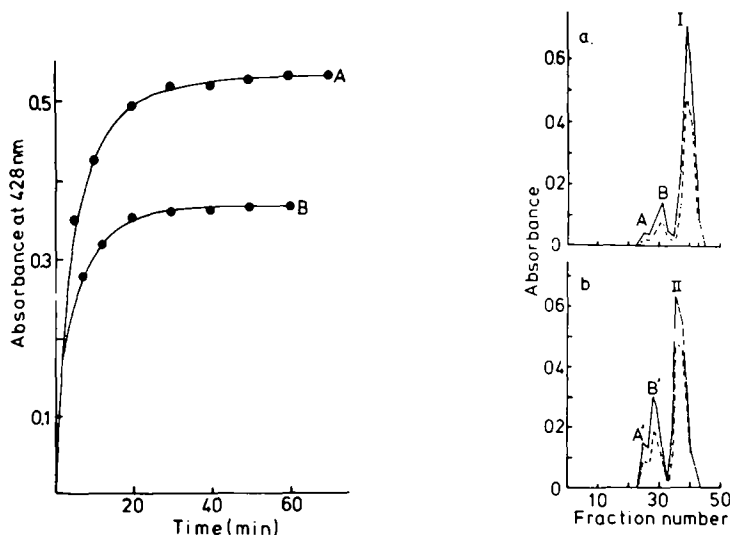


Fig. 3. The reaction of cardiotoxin with tetranitromethane as followed by the increase in absorbance at 428 nm. Curve A; the reaction was proceeded in 6.0 M guanidine hydrochloride (pH 8.0). Curve B; the reaction was proceeded in Tris · HCl (pH 8.0). Protein concentration was 4 mg/ml and the molar ratio of tetranitromethane to tyrosine in cardiotoxin was 50 : 1.

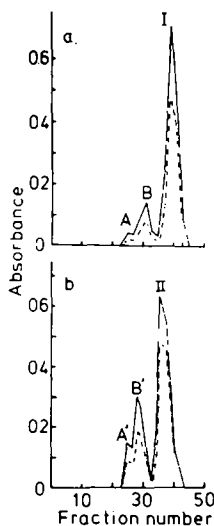


Fig. 4. The elution profiles of cardiotoxin nitrated in Tris · HCl (a) and cardiotoxin nitrated in guanidine hydrochloride (b) on a Sephadex G-50 column (2.6 × 60 cm). The column was equilibrated and eluted by 0.05 M ammonia acetate, pH 6.8. The elute was collected in 4.5 ml per tube at room temperature. The flow rate was adjusted to 24 ml/h. —, $A_{428\text{nm}}$; - · - · -, $A_{428\text{nm}}$.

TABLE I

AMINO ACID COMPOSITIONS OF CARDIOTOXIN AND ITS NITRATED DERIVATIVES

Amino acid	Cardiotoxin	Product I	Product II
Lys	9	8.9	8.7
Arg	2	2.0	1.9
Asp	6	6.1	6.0
Thr	3	2.6	2.5
Ser	2	1.7	1.6
Pro	5	4.8	4.9
Gly	2	2.0	2.0
Ala	2	2.1	2.0
1/2 Cys	8	7.1	7.2
Val	7	6.9	7.1
Met	2	1.9	1.8
Ile	1	1.1	1.0
Leu	6	5.7	5.8
Tyr	3	0.9	0
Phe	2	1.8	1.7
3-NO ₂ -Tyr	0	1.9	2.9

minor fractions (products A and B), were obtained from the reaction in 0.05 M Tris · HCl (pH 8.0). The nitration in 6.0 M guanidine hydrochloride also gave three fractions, product II and products A' and B'. Both product I and II were eluted at the same elution volume of cobra cardiotoxin (not shown). Both of them showed a single band in the polyacrylamide disc electrophoresis, but the electromobility was indistinguishable from that of the native toxin. If 4100 was used as the molar absorbance of 3-nitrotyrosine at 428 nm [14], it was found that product I contained two nitrated tyrosines and product II contained three. This was further confirmed by the result of the amino acid analysis shown in Table I. Our results agree well with the previous study by Keung et al. on cardiotoxin of *Naja naja* Linn. [16]. Products, A, B, A' and B' were probably the polymerized substance formed by intermolecular crosslinking [17,18] between tyrosyl residues via phenoxide free radical intermediates.

Tryptic peptides of product I and product II are similar to that of native

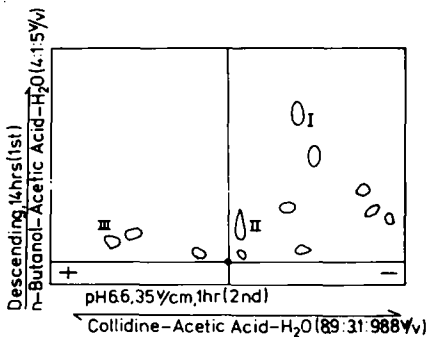


Fig. 5. Maps of tryptic peptides for cardiotoxin and its nitrated derivatives. Twelve spots of native cardiotoxin were detected by ninhydrin. Maps of products I and II are similar to that of cardiotoxin, except that product I and product II showed two (peptides I and II) and three (peptides I, II and III) deep yellow spots in the presence of ammonia vapor, respectively.

TABLE II

AMINO ACID COMPOSITION AND CORRESPONDING SEQUENCES OF TYROSINE-CONTAINING PEPTIDES

Peptide	Amino acid composition		Corresponding sequences of the peptides
	Product I	Product II	
I	Lys _{1.0} , Pro _{1.0} , Val _{0.9} , Leu _{1.8} , Phe _{0.8} , Tyr(3-NO ₂) _{0.9}	Lys _{1.0} , Pro _{0.8} , Val _{0.9} , Leu _{1.9} , Phe _{0.9} , Tyr(3-NO ₂) _{0.9}	Leu-Val-Pro-Leu-Phe- Tyr-Lys
II	Lys _{1.0} , Cys _{0.9} , Asp _{0.9} , Leu _{1.0} , Tyr(3-NO ₂) _{1.0}	Lys _{1.0} , Cys _{0.8} , Asp _{0.8} , Leu _{0.9} , Tyr(3-NO ₂) _{0.9}	Asn-Leu-Cys-Tyr-Lys
III	Arg _{0.9} , Cys _{2.2} , Asp _{2.0} , Thr _{0.7} , Val _{1.0} , Tyr _{0.7}	Arg _{1.0} , Cys _{2.1} , Asp _{2.0} , Thr _{0.7} , Val _{0.8} , Tyr(3-NO ₂) _{0.8}	Tyr-Val-Cys-Cys-Asn-Thr- Asp-Arg

cardiotoxin (Fig. 5). Before sprayed with ninhydrin, peptides I, II and III of product II showed a deep yellow color in the presence of ammonia vapor, whereas only peptide I and II showed a yellow color in product I. Table II lists the amino acid composition and the inferred sequences for peptide I-III. Peptide I and II included residues 6-12 and residues 19-23, respectively. Peptide III represented residues 51-58. It turned out that Tyr-11 and Tyr-22 were the two exposed residues and Tyr-51 was the abnormal one in the spectrophotometric titration.

The results of the CD study provided the information to what extent nitration could effect the structure integrity of the toxin. Fig. 6 shows the CD spectra of product I and II and their parent toxin. All the CD were measured in water at 25°C. According to the study of Hung and Chen [6], the CD of cobra cardiotoxin is divided into three parts: (i) the fine structure in the region of 250-300 nm arise from non-peptide chromophores and the peak at 287 nm is

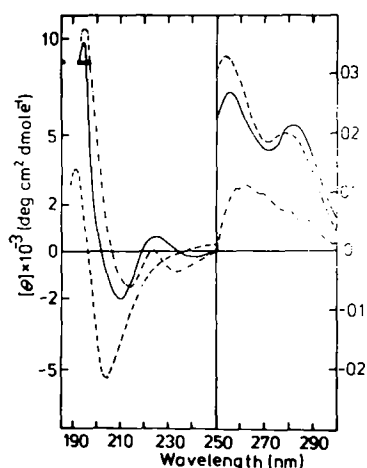


Fig. 6. CD spectra of cardiotoxin and nitrated derivatives in 0.05 M phosphate buffer (pH 6.8). - · - · -, native cardiotoxin; —, dinitro-cardiotoxin; - - - -, trinitro-cardiotoxin.

the result of O-O transition of the buried tyrosine; (ii) the two Cotton effects in the region of 220–250 nm result from the interaction among peptide chromophores and the side chain of asymmetric tyrosine; (iii) a trough and a peak in the region of 190–220 nm correspond to the activity due to the β -structure including β -sheets and β -turns in the protein molecule.

The nitration of the two exposed tyrosyl residues does not change the polypeptide folding of cardiotoxin significantly, noting that product I and cardiotoxin gave similar CD spectra in the region of 190–220 nm. This may suggest that no major peptide conformational change occurs in product I. There appeared two Cotton effects in the CD of product I at 220–250 nm. Their profiles were similar to those of cardiotoxin, but their intensities and specific position of the extrema were modified. In the region 250–300 nm, instead of the fine structure in the spectrum of cardiotoxin, two large positive bands appeared in the CD spectra of product I. The spectral difference between cardiotoxin and product I, Tyr-11 and Tyr-22 nitrated cardiotoxin, was perhaps caused by the chromophoric change from the phenolic group in cardiotoxin to *O*-nitrophenyl group in product I (see below).

In the region 190–220 nm, the CD spectrum of the product II changed remarkably from the spectrum of cardiotoxin. The spectral characteristic did not suggest that product II was a completely unfolded form when compared with the CD spectra of unfolded toxin at an elevated temperature or in concentrated guanidine hydrochloride [6]. In the region 250–300 nm, the CD spectrum of product II was virtually the same as the spectrum of product I, except that the extrema of the former were shifted to a shorter wavelength. Since the *pK* value of *O*-nitrophenyl group is near neutral, it should be almost completely ionized at pH 9.0. At this pH, the CD profiles of nitrated derivatives were similar to those at neutral pH, but a red shift can be observed for these two positive bands in the region 250–300 nm. The red shift is in accord with the similar change in absorption spectra of *N*-acetyl-3-nitrotyrosine [19]. The results suggest that these two positive bands may mainly result from *O*-nitrophenyl side chains in nitrated derivatives. Since the unfolded conformation of cardiotoxin in 6.0 M guanidine hydrochloride can be refolded completely after the removal of guanidine hydrochloride [6], it is unlikely that guanidine hydrochloride in product II during the reaction with tetranitro-

TABLE III
HEMOLYTIC ACTIVITY OF CARDIOTOXIN AND ITS NITRATED DERIVATIVES

Test substance *	% Hemolysis ** time of incubation	
	1 h	2 h
Cardiotoxin	32	50
Product I	22	35
Product II	9	16

* Test substances were incubated in phosphate-buffer saline (pH 7.4) at 37°C and their final concentrations were 200 μ g/ml.

** Each value is the average of four independent experiments.

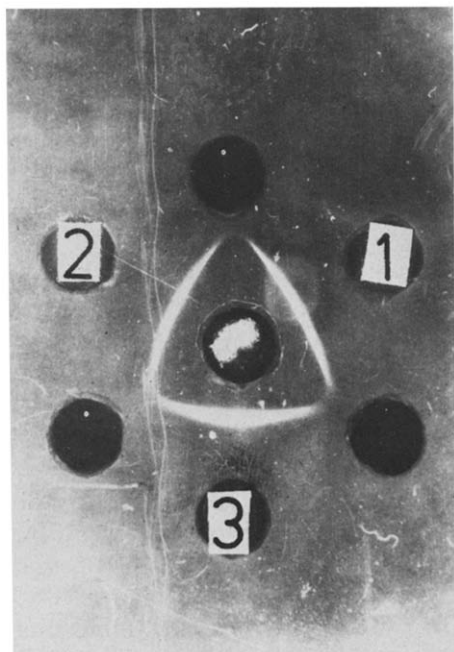


Fig. 7. Immunodiffusion pattern in 1% agarose gel equilibrated with phosphate buffer, pH 6.0. Centre well: anti-cardiotoxin serum. Surrounding wells: 1, native cardiotoxin; 2, dinitro-cardiotoxin; 3, trinitro-cardiotoxin.

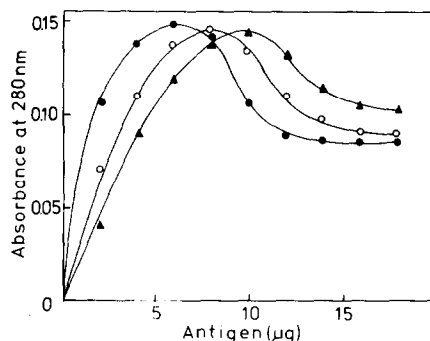


Fig. 8. Quantitative precipitin reactions of cardiotoxin and its nitrated derivatives with anti-cardiotoxin serum. ●—●, native cardiotoxin; ○—○, dinitro-cardiotoxin; ▲—▲, trinitro-cardiotoxin.

methane would cause the difference in CD spectra between product I and product II. In 6.0 M guanidine hydrochloride, product II did undergo a further change in conformation. The two positive bands in the region 250–300 nm disappeared and there emerged a large negative band below 210 nm, which indicated the presence of an unordered form. Apparently, product II was a partially unfolded state of cardiotoxin. Since the two Cotton effects of cardiotoxin in the region 220–250 nm disappeared from the CD spectrum of product II, the buried Tyr-51 should be mainly responsible for the activity of cardiotoxin in the spectral region. It is likely that the nitration of Tyr-51 would destroy the native format around the residue, thereby the asymmetric environment for producing the optical activity no longer remained.

Product I and II were toxic but they had a lower lethality than the parent toxin. LD₅₀ for cardiotoxin, product I and II were determined in mice as 2.5, 4.1 and 7.2 µg per gram body weight, respectively.

The hemolytic activity of cardiotoxin and its nitrated derivatives is shown in Table III. Product I and III retained about 70 and 30% hemolytic activity of native cardiotoxin, respectively. Apparently, effect of nitration on hemolytic activity correlates with that on toxicity.

Product I and II gave identical precipitin line as intact cardiotoxin when tested on a double diffusion plate with anti-cardiotoxin serum (Fig. 7). Quanti-

tative precipitin test (Fig. 8) showed that both product I and II could reach the same maximal precipitation at slightly higher concentration. This result that there is no alteration in immunological property upon modification of the tyrosine residues in cardiotoxin is similar to those of neurotoxins [20,21].

Discussion

As compared with the primary sequences [22], cardiotoxins are basically similar to type I neurotoxins, post-synaptic neurotoxins containing 61–63 amino acid residues. The location of disulfide bridges had been determined and reported to be identical [23–26]. Although the similarity in disulfide linkage backbone is remarkable, there is considerable difference in amino acid composition between cardiotoxins and neurotoxins. The primary difference is that cardiotoxins have high lysine contents. There are 7–10 lysine residues in most cardiotoxins as compared to 2–7 residues in neurotoxins. The three dimensional structures of the two toxins seemed not the same as suggested by the following evidence. On analytical gel filtration, the neurotoxin of *Naja naja atra* showed a more compact and smaller Stoke's radius than cardiotoxin. We found that the relative elution volume, V_e/V_0 , of cardiotoxin on a Sephadex G-50 column is 1.85 but 2.00 for neurotoxin in spite of their similar molecular weights. By using the procedure of Chou and Fasman [27] with some modifications, Chen et al. [28] reported that the region of secondary structure along the polypeptide chains of the two toxins were not identical. Later on, Hseu et al. [29] confirmed this conclusion. By comparing the CD spectra of cardiotoxin and neurotoxin [6,30], it was revealed that unequal surroundings exist around the non-peptide groups in the two toxins. Apparently, the polypeptide folding in the two toxins can not be identical. The status of Tyr-22 in cardiotoxin and the corresponding Tyr-25 (or 24) in neurotoxin provides more evidence for the justification because Cys-Tyr in both residues 21 and 22 of cardiotoxin and residues 24 and 25 (or 23 and 24) of neurotoxin is considered an invariant fragment in the toxins of snake venom [3,31]. The present work proves that Tyr-11 and Tyr-22 are exposed and that Tyr-51 is buried in cardiotoxin molecule. The results of both chemical modification [20,32–34] and X-ray study [35,36] ascertain that Tyr-25 is inaccessible in neurotoxins.

Since Tyr-22 has long been thought to be equivalent to Tyr-25 (or 24) in neurotoxins as they are all adjacent to Cys (3rd cysteine residue from the N-terminal), it seems difficult to believe that Tyr-22 is exposed in the present study. In order to clarify this argument, we repeated the modification procedure described in Materials and Methods, but the molar ratio of tetranitromethane to tyrosine of cardiotoxin was decreased to 1 : 1. This product contains about 0.9 of a 3-nitrotyrosine residue and 2.0 tyrosine residues. Its tryptic peptide map was similar to that of product I as shown in Fig. 5. Before spraying with ninhydrin, two yellow spots also appeared (I and II in Fig. 5) in the presence of ammonia vapor. These two yellow spots contain similar tyrosine and 3-nitrotyrosine contents as judged from amino acid analysis (Tyr_{0.4} and Tyr(3-NO₂)_{0.5} for spot I; Tyr_{0.5} and Tyr(3-NO₂)_{0.4} for spot II). There is no significant difference between the reactivity of Tyr-11 and Tyr-22. This also gives strong support to Tyr-11 and Tyr-22 being exposed. Since

Tyr-25 (or 24) in neurotoxins can be modified only under much more drastic conditions, e.g. in 5 M guanidine hydrochloride [20,32], the status of Tyr-22 in cardiotoxins is different from that of Tyr-25 (or 24) in neurotoxins.

The shapes of quantitative precipitin curves of products I and II are similar to that of cardiotoxin except that they reach the maximal precipitation at a slightly higher concentration. The results suggest that most of antigenic determinant sites of cardiotoxin still keep the immunologically active conformation after cardiotoxin was nitrated with tetranitromethane.

Acknowledgement

The authors wish to thank Dr. T.B. Lo and Dr. W.C. Chang for valuable discussion. This investigation was financially supported by National Science Council, Taiwan.

References

- 1 Lo, T.B., Chen, Y.H. and Lee, C.Y. (1966) *J. Chinese Chem. Soc.* 13, 25–37
- 2 Lee, C.Y., Chang, C.C., Chiu, T.H., Chiu, P.J.S., Tseng, T.C. and Lee, S.Y. (1968) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 259, 360–374
- 3 Condrea, E. (1974) *Separatum Experientia* 30, 121–129
- 4 Narita, K. and Lee, C.Y. (1970) *Biochem. Biophys. Res. Commun.* 41, 339–343
- 5 Hayashi, K., Takechi, M., Kandeda, N. and Sasaki, T. (1976) *FEBS Lett.* 66, 210–214
- 6 Hung, M.C. and Chen, Y.H. (1977) *Int. J. Peptide Protein Res.* 10, 277–285
- 7 Haupt, G.W. (1952) *BS J. Res.* 48, 414–423
- 8 Reisfield, R.A., Lewis, U.J. and Williams, D.E. (1962) *Nature* 195, 281–283
- 9 Spackman, D.H., Stein, W.H. and Moore, S. (1958) *Anal. Chem.* 30, 1190–1205
- 10 Litchfield, J.T. and Wilcoxon, F. (1949) *J. Pharmacol. Exp. Ther.* 96, 99–113
- 11 Vogt, W., Patzer, P., Lege, L., Oldigs, H.D. and Wille, G. (1970) *Naunyn-Schiedeborgs Arch. Pharmacol.* 265, 442–454
- 12 Ouchterlony, O. (1958) *Progr. Allergy* 5, 1–78
- 13 Roitt, J.M., Campbell, P.N. and Doniach, D. (1958) *Biochem. J.* 69, 248–297
- 14 Sokolovsky, M., Riordan, J.F. and Vallee, B.L. (1966) *Biochemistry* 5, 3582–3589
- 15 Tanford, C., Hauenstein, J.D. and Rands, D.G. (1955) *J. Am. Chem. Soc.* 77, 6409–6413
- 16 Keung, W.M., Leung, W.W. and Kong, Y.C. (1975) *Biochem. Biophys. Res. Commun.* 66, 383–392
- 17 Boesel, R.W. and Carpenter, F.H. (1970) *Biochem. Biophys. Res. Commun.* 38, 678–682
- 18 Thomas, C.B., Maurice, J.G. and Sandra, L.W. (1968) *J. Am. Chem. Soc.* 96, 1612–1619
- 19 Riordan, J.F., Sokolovsky, M. and Vallee, B.L. (1967) *Biochemistry* 6, 358–361
- 20 Huang, J.S., Liu, S.S., Ling, K., Chang, C.C. and Yang, C.C. (1973) *Toxicol.* 11, 39–45
- 21 Ohta, M. and Hayashi, K. (1974) *Biochem. Biophys. Res. Commun.* 56, 981–987
- 22 Tu, A.T. (1977) *Venoms: Chemistry and Molecular Biology*, pp. 257–320, John Wiley and Sons, New York
- 23 Yang, C.C., Yang, H.J. and Chiu, R.H.C. (1970) *Biochim. Biophys. Acta* 214, 355–363
- 24 Endo, Y., Sato, S., Ishii, S. and Tamiya, N. (1971) *Biochem. J.* 122, 463–469
- 25 Takechi, M. and Hayashi, K. (1972) *Biochem. Biophys. Res. Commun.* 49, 584–589
- 26 Narita, K., Cheng, K.L., Chang, W.C. and Lo, T.B. (1978) *Int. J. Peptide Protein Res.* in press
- 27 Chou, P.Y. and Fasman, G.D. (1974) *Biochemistry* 13, 222–245
- 28 Chen, Y.H., Lu, H.S. and Lo, T.B. (1975) *J. Chinese Biochem. Soc.* 4, 69–82
- 29 Hseu, T.H., Liu, Y.C., Wang, C., Chang, H., Hwang, D.M. and Yang, C.C. (1977) *Biochemistry* 16, 2999–3006
- 30 Chen, Y.H., Lo, T.B. and Yang, J.T. (1977) *Biochemistry* 16, 1826–1830
- 31 Yang, C.C. (1974) *Toxicol.* 12, 1–43
- 32 Chang, C.C., Yang, C.C., Hamaguchi, K., Nakai, K. and Hayashi, K. (1971) *Biochim. Biophys. Acta* 236, 164–173
- 33 Chicheportiche, R., Rochat, C., Sampieri, F. and Lazdunski, M. (1972) *Biochemistry* 11, 1681–1690
- 34 Raymond, M.L. and Tu, A.T. (1972) *Biochim. Biophys. Acta* 285, 498–502
- 35 Tsernoglou, D. and Petsko, G.A. (1976) *FEBS Lett.* 68, 1–4
- 36 Low, B.W., Preston, H.S., Sato, A., Rosen, L.S., Searl, J.E., Rudko, A.D. and Richardson, J.S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* 73, 2991–2994