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Structure–Function Relationships of Scorpion Neurotoxins†

Catherine Habersetzer-Rochat* and François Sampieri

ABSTRACT: Chemical modification of some trifunctional amino acid residues in toxins I, II, and III of the scorpion *Androctonus australis* Hector have been performed. The results indicate: (1) Reduction and methylation of one disulfide bridge destroy toxic activity of toxin II. (2) The only tryptophan residue of toxin II (position 38) is not included in the active site of the molecule. (3) Modification of five carboxylates out of the seven contained in toxin II suppresses the toxic activity. (4) Acetylation of the lysine and tyrosine residues in toxin II leads to the loss of both toxic and antigenic activity. Treatment of the acetylated toxin by hydroxylamine restores partially the antigenic activity. In the case of toxin I, total acetylation abolishes only the toxic activity. It is concluded that at least one tyrosine residue must be involved in an antigenic site of toxin II. (5) Citraconylation of toxins II and III leads to complete loss of toxicity; decitraconylation restores full activity. (6) Guanidination of toxin II does not affect its toxicity significantly. (7) Alkylation of toxin II by iodoacetic acid affects both amino groups and histidine residues. The loss of toxicity is mainly due to the modification of the lysine residues. In the case of toxin I, the kinetics of toxicity loss closely parallel the covalent modification of one lysine residue.

Scorpion neurotoxins are made of single peptide chains of about 60 amino acid residues cross-linked by four disulfide bridges. The complete sequences of 7 neurotoxins and parts of the sequences of 14 other toxins have been established (Rochat, H., et al., 1970a, 1972, 1974a; Babin et al., 1974, 1975; Zlotkin et al., 1973). From the comparison of the N-terminal sequences, it was shown that they form a family of homologous proteins (Rochat, H., et al., 1970b, 1974a; Babin et al., 1975). The positions of the disulfide bridges in toxin II of *Androctonus australis* Hector have been determined (Kopeyan et al., 1974). They were found to link half-cystine residue numbers 12 and 63, 16 and 36, 22 and 46, and 26 and 48. In toxin I of *Androctonus australis* Hector, two disulfide bridges were located in identical positions (Rochat, H., et al., 1970c), suggesting that the positions of the disulfide bridges may be the same in all scorpion neurotoxins.

Some physicochemical properties of toxin II of *Androctonus australis* Hector have been studied by optical rotatory dispersion and ultraviolet difference spectrophotometry (Chicheportiche and Lazdunski, 1970). Depending upon pH and temperature, the protein takes at least four different molecular forms. The form which is predominant between pH 4 and 9 is very stable under heat denaturation and treatment with 9.5 M urea.

The relationships between iodinated tyrosine residues and toxic activity have been demonstrated for both toxins I and II in the course of experiments to obtain biologically active radioiodinated toxins (Rochat, C., et al., 1972; Rochat, H., et al., 1974b).

For this paper other modifications to trifunctional amino acids have been made, and the effects of these modifications on toxic activity have been studied (cf. also Sampieri and Habersetzer-Rochat, 1974). Some further conclusions about the relationships of structure and activity in scorpion neurotoxins have been drawn.

Materials and Methods

Three neurotoxins, I, II, and III, were purified from the venom of *Androctonus australis* Hector (Rochat, C., et al., 1967; Miranda et al., 1970). These neurotoxins have, respectively, molecular weights of 6808 (63 amino acid residues), 7249 (64 amino acid residues), and 6826 (64 amino acid residues), and contain, respectively, 6, 5, and 6 lysine residues and 1, 2, and 1 histidine residues. Only one tryptophan residue is present in each of the three molecules.

† From the Department of Biochemistry, Faculté de Médecine, Secteur Nord, 13326 Marseille, Cedex 3, France. *Received May 30, 1975.* This research was supported by the Centre National de la Recherche Scientifique (LA 178), the Institut National de la Santé et de la Recherche Médicale (U.38), the Direction des Recherches et Moyens d'Essais (authorization No. 164), and the Fondation pour la Recherche Médicale Française.

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was obtained from Pierce. Acetic anhydride was a Merck product. Citraconic anhydride (distilled under reduced pressure before use) and *O*-methylisourea came from K and K Laboratories. Iodoacetic acid and methyl iodide came from Fluka; iodoacetic acid was crystallized before use; and methyl iodide was distilled on a Widmer column from silver turnings. All other reagents were of analytical grade.

Amino acid compositions were obtained after protein hydrolysis in 6 N HCl at 110 °C for 20 h in a Beckman 120C amino acid analyzer.

Toxicity of native and modified neurotoxins was determined by subcutaneous injection into mice (Miranda et al., 1970).

Antisera were prepared by subcutaneous injection into rabbits of progressive amounts (50–500 μg) of neurotoxins, the total amount injected being 2.5 mg for a period of about 3 months (Delori et al., 1974). Double diffusion in gels was done at 25 °C in 2% agarose made in 0.15 M NaCl.

Partial Cleavage of Disulfide Bridges. Toxin II (3.2 mg) was dissolved in 0.5 M sodium phosphate, pH 6.8, at a concentration of 0.13 mM in the presence of 1.3 mM dithiothreitol (final volume, 2.5 ml). The glass-stoppered reaction tube was flushed with nitrogen and left at room temperature for 18 h in the dark. To avoid reoxidation of the partially reduced protein, alkylation by methyl iodide was performed essentially according to Rochat, C., et al. (1970), but in a simpler way: because the bottom of the tube was flat, it was possible to use a small round magnetic disk allowing a vigorous agitation of the whole solution. Methyl iodide was added in excess under nitrogen to saturate both solution and atmosphere above the solution (0.1 ml for 2.5 ml of the mixture). The tube was tightly closed by a glass-stopper held by two springs (the pressure due to methyl iodide at the temperature of the reaction is significant). Temperature was maintained at 40 °C using a heating magnetic stirrer. The reaction was allowed to proceed in the dark for 5 min and stopped by cooling. The mixture was then filtered on a Sephadex G-15 column (3 × 20 cm) equilibrated in 1 M acetic acid. The extent of reduction was deduced after acid hydrolysis from the number of methylcysteine residues. We have verified that methylation of the fully reduced neurotoxin performed in these conditions gave the same amount of *S*-methylcysteine as found in the previously described experiments (Rochat, C., et al., 1970). In the case of complete reduction and methylation, the dilution of the solution (which contains also a denaturing agent: 5 M guanidine) with an equal volume of 2 M acetic acid before filtration is advised. This avoids a possible precipitation of the S-methylated protein on the column.

Formylation of Tryptophan. Formylation was carried out according to Previero et al. (1967). Toxin (2 μmol) was dissolved in 0.5 ml of anhydrous formic acid; then 1 ml of formic acid saturated with gaseous HCl was added. At suitable intervals 25-μl aliquots were taken and the reaction was stopped by dilution with 2.5 ml of 1 M sodium acetate, pH 4.5, to get a final pH of 4.0. The extent of tryptophan transformation into *N*-formyltryptophan was estimated spectrophotometrically at 298 nm ($\epsilon_M = 4.88 \times 10^3$) in a Cary 14 spectrophotometer.

Chemical Modification of Carboxylates. This modification was performed essentially according to the method of Hoare and Koshland (1967). The reaction was carried out on 4.2 mg of toxin at pH 4.7 with 1 M glycine ethyl ester and 0.065 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Protein concentration was 0.13 mM. The pH was kept constant by addition of 0.1 M HCl in a pH-stat (Radiometer); the reaction was allowed to proceed at room temperature for 3 h. Reagent excess was then removed by passage through a Bio-Gel P_2 column (3.2 × 18 cm) equilibrated in 0.1 M acetic acid. The protein fraction was lyophilized and reacted with hydroxylamine (0.5 M) at pH 7.7 and 25 °C to reverse a possible modification of the tyrosine residues (Carraway and Koshland, 1968). The mixture was rechromatographed on the same column and the number of glycine ethyl ester residues incorporated was measured after acid hydrolysis of the modified protein. It corresponds to the difference between the total number of glycine residues found in the analysis and the theoretical number of glycine residues in the molecule.

Acetylation. Acetylation was carried out on 6.4 mg of toxin II. Protein concentration was 0.3 mM. [^{14}C]Acetic anhydride (6×10^4 cpm/μequiv of acetyl group) was added in fractions of: 20 μl, then 5 × 5 μl every 10 min. The reaction was performed at 4 °C in 0.1 M Tris-HCl buffer, pH 8.0. The pH was maintained constant in a Radiometer pH-stat. After 100 min the mixture was acidified with 0.1 ml of acetic acid and filtered on a Sephadex G-15 column (3 × 20 cm) equilibrated in 0.2 M acetic acid. The protein concentration was determined by spectrophotometry using a molar extinction coefficient[1] of 7.4×10^3 (instead of 18.01×10^3 for native toxin).

Acetylated toxin II was then treated with 0.5 M hydroxylamine, pH 7.4, to reverse acetylation of tyrosine residues. After 1 h, the excess hydroxylamine was eliminated by filtration on the same Sephadex column. The molar extinction coefficient recovered its original value.

In the case of toxin I, acetylation was performed in the same conditions as for toxin II but with unlabeled acetic anhydride.

Citraconylation. Citraconylation was performed according to Dixon and Pehram (1968) on 3.3 mg of protein dissolved in 0.1 M Tris-HCl, pH 8.0 (0.25 mM), at 4 °C. Citraconic anhydride was added as aliquots of, respectively, 10, 10, 5, and 5 μl every 10 min. The pH was maintained constant in a Radiometer pH-stat. After 2 h, the solution was filtered through a column of Sephadex G-15 fine (3 × 20 cm) equilibrated in 0.1 M ammonium acetate, pH 8.5.

Decitraconylation was performed by leaving the citraconylated protein at pH 3.6 and 6 °C for 48 h. The protein was then filtered through a Sephadex G-15 column (3 × 20 cm) equilibrated in 1 M acetic acid.

Guanidination. Toxin II (3.4 mg) was dissolved in a solution of 1.4 M *O*-methylisourea, pH 9. Protein concentration was 0.24 mM. The solution was incubated at 0 °C to avoid a possible denaturation at alkaline pH. Aliquots were taken at different times for toxicity tests. After 380 h, the reaction was stopped by filtration through a Bio-Gel P_2 column (3.2 × 18 cm) equilibrated in 30% acetic acid. The extent of guanidination was estimated after acid hydrolysis by the decrease of lysine content and the appearance of homoarginine. The ninhydrin color constant of arginine was employed in calculating the amount of homoarginine.

Chemical Modifications of Lysine and Histidine Residues by Iodoacetic Acid. Iodoacetic acid was neutralized in 0.1 M Tris-HCl before addition to the toxin dissolved in the same buffer. The resulting protein concentration was 0.3×10^{-3} M. The final iodoacetic acid concentration was 0.48 M, 0.48×10^{-1} M, or 0.48×10^{-2} M. The reaction was carried out at pH 7.7 or 8.6 and was followed kinetically; 0.7-ml aliquots were taken at different times and filtered through a Bio-Gel P_2 column (3.2 × 18 cm) equilibrated in 0.1 M ammonium bicarbonate, pH 8.0. The extent of carboxymethylation was measured after acid hydrolysis by the decrease of lysine and

[1] This value has been determined according to amino acid yield.

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                1                   5                  10                  15                  20                  25
Toxin  I    NH2-Lys-Arg-Asp-Gly-Tyr-Ile-Val-Tyr-Pro-Asn-Asn-Cys-Val-Tyr-His-Cys-Val-Pro-Pro-   -   -Cys-Asp-Gly-Leu-

Toxin  II   NH2-Val-Lys-Asp-Gly-Tyr-Ile-Val-Asp-Asp-Val-Asn-Cys-Thr-Tyr-Phe-Cys-Gly-Arg-Asn-Ala-Tyr-Cys-Asn-Glu-Glu-

Toxin  III  NH2-Val-Arg-Asp-Gly-Tyr-Ile-Val-Asp-Ser-Lys-Asn-Cys-Val-Tyr-His-Cys-Val-Pro-Pro-   -   -Cys-Asp-Gly-Leu-


                        30                  35                  40                  45                  50
Cys-Lys-Lys-Asn-Gly-Gly-Ser-Ser-Gly-Ser-Ser-Cys-Phe-Leu-Val-   -Pro-Ser-Gly-Leu-Ala-Cys-Trp-Cys-   -Lys-

Cys-Thr-Lys-Leu-Lys-Gly-Glu-Ser-Gly-Tyr-   -Cys-Gln-Trp-Ala-Ser-Pro-Tyr-Gly-Asn-Ala-Cys-Tyr-Cys-Tyr-Lys-

Cys-Lys-Lys-Asn-Gly-Ala-Ser-Ser-Gly-Ser-Ser-Cys-...


                    55                  60                  65
Asp-Leu-Pro-Asp-Asn-Val-Pro-Ile-Lys-Asp-Thr-Ser-Arg-Lys-Cys-Thr-COOH

   -Leu-Pro-Asp-His-Val-Arg-Thr-Lys-Gly-Pro-Gly-Arg-   -Cys-His-NH2
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FIGURE 1: Complete primary structures of toxins I and II and N-terminal amino acid sequence of toxin III.

histidine residues since the carboxymethylated derivatives overlap other amino acid residues in the analysis.

Results and Discussion

The complete primary structures of toxins I and II and N-terminal amino acid sequence of toxin III have already been established (Rochat, H., et al., 1970a,b, 1972, 1974a) and are shown in Figure 1.

Partial Cleavage of Disulfide Bridges (Toxin II). Toxin II contains four disulfide bridges and no cysteine residue. After 1 h of reduction, there was no loss of toxicity and no formation of methylcysteine by treatment with methyl iodide. After 18 h of reaction time the loss of toxicity was 38%[2] and the amount of methylcysteine formed was 0.8 residue per mol. The loss of toxicity seems to be related to the cleavage of one disulfide bridge. If we assume that a methyl group will minimize steric or ionic effects in comparison with carboxymethyl- or carboxamidomethyl groups, methyl iodide is the best choice of alkylating agent. Experimentation does not show whether the cleavage of a disulfide bridge is selective or statistical. In the latter case, the observed loss of activity seems to show that the cleavage of any of the disulfide bridges will probably nullify the toxicity of the entire molecule.

Formylation of Tryptophan (Toxin II). Toxin II contains only one tryptophan residue in position 38. As is shown in Figure 2, the kinetics of toxicity loss do not follow the chemical modification of the tryptophan residue. On the other hand, leaving the modified protein in solution at neutral pH for a few days at 4 °C resulted in the spontaneous recovery of 50% of the toxicity. This was not due to reversion of *N*-formyltryptophan into tryptophan since (1) this reaction only proceeds at high pH, and (2) it was verified spectroscopically that, in any case, this reaction did not take place here. The loss of toxicity might be related to side reactions which have not been identified. Amino acid analyses of the native and the formylated neurotoxins are identical. In addition, neurotoxin II retained full activity after dissolution either in anhydrous formic acid or in 1 N HCl, and a contact period of 100 min. By themselves, the kinetics do not allow a conclusion regarding the importance

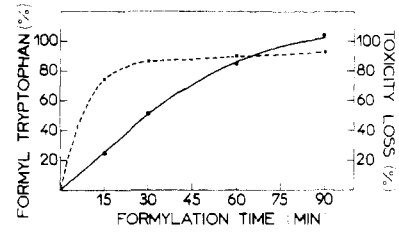

FIGURE 2: Kinetics of the formylation and loss of toxicity of toxin II. *N*-Formyltryptophan, — ● —; loss of toxicity, - - ■ - -.

of the tryptophan residue for toxic activity; the inactivation due to side reactions might be faster than the inactivation due to tryptophan modification. But, since 50% of the activity is regained, two hypotheses can be formulated: (1) side reactions led to irreversible denaturation of 50% of the molecules and to reversible denaturation of the other molecules which have recovered spontaneously and which are 100% active although formylated; (2) side reactions are completely reversed; then all molecules—which are still formylated—are 50% as active as native toxin II. In any of these two assumptions—as far as the formylation leads to a significant change in this molecule—the tryptophan residue cannot be said to be essential for activity. Moreover, the second hypothesis concerning a residual activity of 50% for a molecule specifically modified on one amino acid residue is less generally admitted. Thus, we may conclude that Trp 38 is not essential for toxicity of neurotoxin II (in toxin I, the only tryptophan residue is in position 45).

Chemical Modification of Carboxyl Groups (Toxin II). Toxins II contains seven carboxylic groups. The C-terminal amino acid is a histidinamide residue (Rochat, H., et al., 1972). The method of Hoare and Koshland produces the modification of five carboxylates with a complete loss of toxicity. On the immunochemical level, this molecule is still precipitable by an antiserum against native toxin II, even without prior treatment by hydroxylamine.

We know that, if the reaction is carried out in the presence of urea, the seven carboxylates of the molecule are modified (Chicheportiche, 1970). Therefore, it can be concluded that two carboxylates are buried in toxin II.

Acetylation (Toxins II and I). Acetylation of toxin II produces the incorporation of 13–14 acetyl groups/mol of toxin.

[2] It was verified that toxin II incubated for 18 h at room temperature in the presence of methyl iodide (without prior reduction) retains full toxicity.

TABLE I: Amino Acid Compositions of Native and Modified Toxins after Acid Hydrolysis.

	Toxin II				Toxin I	
	Unmodified[a]	Guanidinated	Alkylated[b]	Citraconylated and Alkylated[b]	Unmodified[a]	Alkylated[b]
Asp	8.1 (8)	8.1	8.1	7.9	8.9 (9)	9.2
Thr	2.8 (3)	2.8	2.7	2.8	2.0 (2)	2.0
Ser	1.8 (2)	1.8	1.7	1.8	5.2 (6)	5.5
Glu	4.1 (4)	4.2	4.1	4.1	0.0 (0)	0.0
Pro	3.1 (3)	2.8	3.1	2.9	6.3 (6)	6.1
Gly	7.0 (7)	6.9	6.9	7.0	6.0 (6)	6.0
Ala	3.0 (3)	3.0	3.0	3.2	1.0 (1)	1.1
Half-Cys	5.8 (8)	5.6	5.6	6.8	5.5 (8)	5.6
Val	3.9 (4)	3.9	3.4	3.8	4.5 (5)	4.8
Met	0.0 (0)	0.0	0.0	0.0	0.0 (0)	0.0
Ile	0.85 (1)	0.82	0.75	0.85	1.6 (2)	1.8
Leu	2.0 (2)	2.0	2.0	2.1	4.0 (4)	4.2
Tyr	6.6 (7)	6.8	6.6	6.6	2.9 (3)	2.9
Phe	1.0 (1)	1.0	1.0	1.0	1.0 (1)	1.0
Lys	4.9 (5)	1.7	1.7	4.8	5.9 (6)	0.58
His	2.0 (2)	2.0	0.6	0.0	1.0 (1)	0.0
Arg	2.9 (3)	3.1	2.9	3.0	2.0 (2)	2.0
Har		3.3				

[a] Number of theoretical residues in brackets. [b] Only results concerning the last point of kinetics ($\simeq$20 h) are reported here in order to show that no other amino acid residues, than those concerned, are modified. For the same reason, in the case of toxin I, only results of experiments performed with the highest iodoacetic acid concentration (0.48 M) are reported.

After treatment with hydroxylamine, only 6.5 acetyl groups remain associated with the protein. It can be concluded that, before treatment with NH_2OH, the five lysine residues, the seven tyrosine residues, and the N-terminal residue are acetylated. After treatment with NH_2OH, only the amino functions are still acetylated.[3] Toxicity tests show that the acetylated toxin is fully inactive in any case.

Acetylation of toxin II nullifies the precipitation of the protein by a monospecific antiserum against the native toxin almost entirely. After treatment with NH_2OH, i.e., when only the amino groups are still acetylated, the protein recovers the ability to be precipitated by the antiserum but it shows spurring in immunodiffusion when compared to the native toxin II (Figure 3). This modified neurotoxin is still inactive. Studies performed in this laboratory on the neutralizing capacity of the antiserum obtained with this acetylated toxin (treated by NH_2OH) showed that this molecule is a good anatoxin (Delori et al., 1974).

In the case of toxin I, complete acetylation abolishes the toxic activity. After immunodiffusion in agarose gel, formation of an arc of precipitation between acetylated toxin I and the antibodies against native toxin I still occurs, but immunological identity is only partial when compared to the native toxin. Hydroxylamine-treated acetylated toxin gave identical results. However, it has been shown that hydroxylamine-treated acetylated toxin I is a better anatoxin than hydroxylamine-treated acetylated toxin II (Delori et al., 1974).

Citraconylation (Toxins II and III). Citraconylation of toxins II and III leads to the complete loss of toxicity. Decitraconylation restores full activity.

Guanidination (Toxin II). After 380 h of reaction time, a 40% loss of toxicity was observed. Of a total number of five lysine residues, 3.30 were transformed into homoarginine. No side reaction could be detected by amino acid analysis (Table I); the α-amino group was not guanidinated.

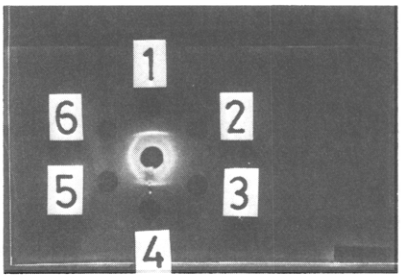

FIGURE 3: Immunodiffusion in agarose gel; central wells, antisera to native toxin II; lateral wells 1 and 4, native toxin II (0.8 μg); 2 and 3, acetylated toxin II (0.8 and 1.6 μg); 5 and 6, acetylated hydroxylamine-treated toxin II (1.6 and 0.8 μg).

There are two possible explanations for these results: (1) 100% of the molecules are partially guanidinated but keep a residual activity of 60%; (2) if we suppose that the chemical modification of one particular lysine residue induces a loss of toxicity of 100%, it can be concluded from the results (3.30 modified lysine residues, 60% loss of toxicity) that it is possible to modify the most accessible residues of the molecule by guanidination without loss of toxicity.

Chemical Modification of Lysine and Histidine Residues by Iodoacetic Acid (Toxins II and I). N-Alkylation by iodoacetic acid affects both amino groups and histidine residues. In the case of toxin II (5 Lys, 2 His), increasing the pH of the reaction from 7.7 (Figure 4A) to 8.6 (Figure 4B) results in a relative enhancement of alkylation of lysine residues compared to that of histidine residues. At pH 8.6, the progressive disappearance of 0.5 valine residue was observed, probably caused by alkylation of the α-amino group of the N-terminal residue (Table I). At this point it was not possible to correlate the loss of toxicity of the molecule with the specific chemical modification of amino groups or histidine residues. To discriminate between the respective role of these residues, we first protected

[3] Amino acid analysis shows that no other amino acid is irreversibly modified.

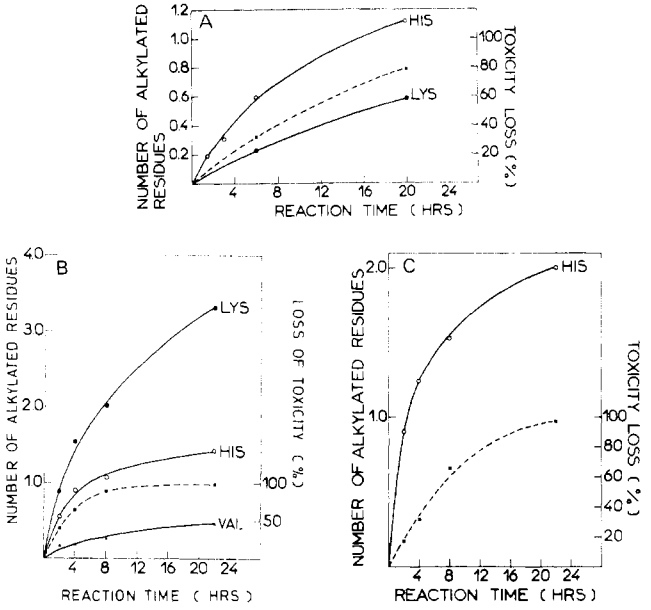

FIGURE 4: Kinetics of alkylation and loss of toxicity of toxin II (0.3×10^{-3} M) by reaction with iodoacetate (0.48 M). (A) Reaction performed at pH 7.7; (B) reaction performed at pH 8.6; (C) reaction performed on citraconylated toxin II at pH 8.6 (- - ■ - -) Loss of toxicity; (— O —) number of alkylated histidine residues; (— ● —) number of alkylated lysine residues; (— ▲ —) number of alkylated valine residues.

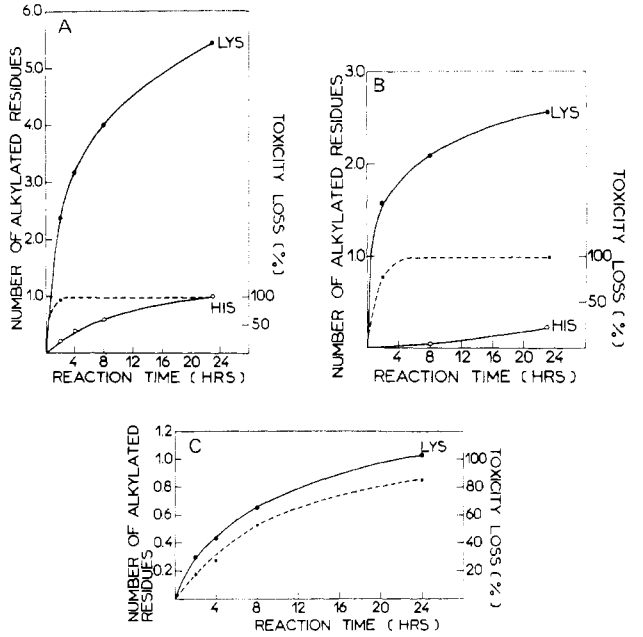

FIGURE 5: Kinetics of alkylation and loss of toxicity of toxin I (0.3×10^{-3} M) by reaction with iodoacetate at pH 8.6. Iodoacetate concentrations: (A) 0.48 M; (B) 0.48×10^{-1} M; (C) 0.48×10^{-2} M. (- - ■ - -) Loss of toxicity; (— O —) number of alkylated histidine residues; (—● —) number of alkylated lysine residues.

the amino groups by citraconylation. Then alkylation by iodoacetic acid was carried out under the latter experimental conditions (pH 8.6, 30 °C) and affected the histidine residues selectively (Table I). After decitraconylation of the amino groups at pH 3.6, the toxicity of the molecule was tested and amino acid analysis was performed. From the results (Figure 4C), it is concluded that one histidine residue can be alkylated without loss of toxicity (the loss is only 20% for one alkylated histidine residue). When the extent of alkylation increases, however, the toxic activity of the molecule is nullified, either by modification of this first residue into dicarboxymethylhistidine, or by alkylation of the second histidine residue of the molecule.[4]

Moreover, if the results of alkylation of the native and citraconylated molecule are compared (Figures 4B and 4C), it appears that the loss of activity is slower when the molecule is citraconylated before alkylation even though alkylation of histidine residues is more rapid. Since it is improbable that the specific reactivity of the two histidine residues could be reversed by citraconylation of the protein, we may conclude that one or several amino groups are essential for the biological function of this neurotoxin.

The fact that alkylation of the histidine residues is faster after citraconylation suggests that citraconylation induces a change in the conformation of the molecule which increases the reactivity of the histidine residues.

Figure 5A shows that alkylation of the lysine residues in toxin I (6 Lys,[5] 1 His) is relatively more pronounced than alkylation of the lysine residues in toxin II (Figure 4B) performed under the same experimental conditions. Obviously the loss of toxicity of the molecule is due mainly to the modification of lysine residues and not to that of the histidine residues. This was confirmed by the results of other experiments using decreasing iodoacetic acid concentrations from 0.48 to 0.48×10^{-1} M (Figure 5B) and 0.48×10^{-2} M (Figure 5C). With the latter concentration, the only histidine residue in the molecule is not alkylated; only lysine residues are modified and the loss of toxicity follows closely on the alkylation of one lysine residue. Table I shows that for both toxins no other amino acid than those concerned is modified by alkylation with iodoacetic acid.

Conclusions

On the basis of the results of chemical modifications performed in these studies on scorpion neurotoxins, the following conclusions can be derived.

(1) The integrity of the disulfide bridges is necessary for toxic activity of toxin II.

(2) Amidification of carboxylates in toxin II destroys the toxic activity, but this effect cannot be ascribed to the specific modification of one special carboxylate; introduction of five glycine ethyl ester groups on the seven possible groups may induce a change in the conformation of the molecule large enough to explain the toxicity loss.

(3) Aromatic residues are not believed to participate in the active site of neurotoxin II. On the one hand, toxin II partially loses its toxicity by formylation, but this loss of toxicity is not correlated with the chemical modification of the only tryptophan residue of the molecule and is probably related to side reactions. On the other hand, the most accessible residues of tyrosine in toxins I and II could be iodinated without loss of toxicity; 1.34 iodine atom could be incorporated as iodotyrosines (0.58 MIT, 0.38 DIT) without inactivation of toxin II (5% only) (Rochat, C., et al., 1972). In the case of toxin I, the relative reactivities to iodine of the three tyrosine residues were in the ratios 3:1, 2:1, and 1:1 for residues 8, 14, and 5, respectively, and it was demonstrated that the tyrosine in position 8 was not involved in the toxicity of the molecule.

(4) Acetylation and citraconylation lead to the loss of toxicity. However, acetic and citraconic anhydride are not suitable for kinetic studies because of their rapid hydrolysis in water.

[4] Amino acid analysis did not permit the determination of the histidine derivatives as they overlap other amino acids.

[5] Lys is the N-terminal residue for toxin I.

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                        10        20        30        40        50        60        70
AaH IT      KKNGYAVDSS-KKAPECII---XSYCEXDC

AaH I       KRDGYIVYPN-NCVYHCVPP-----CDGLCKKN-GGSSGSSCFLV-PSGLACWC-KDLPDNVPIKDTSRKCT
AaH I'      KRDGYIVYPN-NCVYHCIPP-----CDGLCKKN-GGSSGSSCFLV-PSGLACWC-KDLPDNVPIKDTSRKCT
AaH III     VRDGYIVNSK-NCVYHCVPP-----CDGLCKKN-GASSGSSC ...

AaH II      VKDGYIVDDV-NCTYFCGR---NAYCNEECTKL-KGESG-YCQWASPYGNACYCYK-LPDHVRTKGPGR-CH
Bot III'    LKDGYIVDDR-NCTYFCGT---NAYCNEECVKL-KGE ...
Bot III     VKDGYIVDDR-NCTYFCGR---NAYCNEEC ...
Lqq V       LKDGYIVDDK-NCTFFCGR---NAYCNNEC ...
Am  II      LKDGYIIEDI-NCVFFCGR---NAYCDXXC ...

Bop II      GRDAYIADDX-NCAYXCAL---XXYCN ...
Bop I       GRGVYIADIA-NCAY ...
Bot I       GRDAYIAQPE-NCVYECAE---NSYCNDWC ...
Bot I'      VRDAYIAQNY-NCVYTCFK---NEYCNDLCXXN-G ...
Bot I''     GRDAYIAQPE-NCVYECAK ...
Bot II      GRDAYIAQPE-NCVYECAK---NWYCND ...
Lqq III     VRDAYIAKNY-NCVYECFR---DSYCNDLC ...

Css III     -KEGYLVSKSTGCKYECLKLGDNDYCLRECKQQYGKSSGGYCYAF-----ACWC-EALPDHTQVW-VPNKCT
Css I       -KEGYLVSKSTGCKYECLKLGDNDYCL ...
CsE I       -KDGYLVEK-TGCKKTCYKLGENDFCNRECKWKHIGGSYGYCYGF-----GCYC-EGLPDSTQTWPLPNKCT
CsE v1      -KEGYLVKKSDGCKYDCFWLGKNEHNTCECKAKNQGGSYGYCYAF-----ACWC-EGLPESTPTYPLPNKCSS
CsE v2      -KEGYLVNKSTGCKYGCLKLGENEGNKCECKAKNQGGSYGYCYAF-----ACWC-EGLPESTPTYPLPNKCSS
CsE v3      -KEGYLVKKSDGCKYGCLKLGENEGNTCECKAKNQGGSYGYCYAF-----ACWC-EGLPESTPTYPLPNKCSS
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FIGURE 6: Amino acid sequences of scorpion toxins. AaH I, I', II, and III: mammal toxins I, I', II, and III of *Androctonus australis* Hector (Rochat, H., et al., 1970a,b, 1972, 1974a). AaH IT: insect toxin from the same venom (Zlotkin et al., 1973). Am II: mammal toxin II of *Androctonus mauretanicus* (Rochat, H., et al., 1974a). Bop I and II: mammal toxins I and II of *Buthus occitanus paris* (Rochat, H., et al., 1974a). Bot I, I', I'', II, III, and III': mammal toxins I, I', I'', II, III, and III' of *Buthus occitanus tunetanus* (Rochat, H., et al., 1974a). CsE I: mammal toxin I of *Centruroides sculpturatus* Ewing (Babin et al., 1975). CsE v1: variant 1 of *Centruroides sculpturatus* Ewing (Babin et al., 1974). CsE v2: variant 2 of *Centruroides sculpturatus* Ewing (Babin et al., 1974). CsE v3: variant 3 of *Centruroides sculpturatus* Ewing (Babin et al., 1974). Css I and III: mammal toxins I and III of *Centruroides suffusus suffusus* (Rochat, H., et al., 1974a). Lqq III and V: mammal toxins III and V of *Leiurus quinquestriatus quinquestriatus* (Rochat, H., et al., 1974a). X: not determined amino acid residue; (−) deletion. The last 17 amino acid residues of Css III were placed in order to obtain a maximum homology with CsE I.

Too many amino acid residues are modified and the role of amino groups cannot be clearly determined. On the immunochemical level, acetylation is a valuable technique for obtaining anatoxins. Moreover, tyrosine residues in toxin II are probably involved in some of the antigenic sites since treatment of the acetylated toxin II with NH_2OH restores its precipitability by the monospecific antiserum against native toxin almost completely. On the other hand, in the case of toxin I tyrosine residues are probably not involved in antigenic sites since both acetylated and acetylated NH_2OH-treated toxin I gave the same pattern by immunodiffusion in agarose gel with specific anti-native toxin I antiserum. This was confirmed recently (Rochat, H., et al., 1974b); it is possible to purify the radioiodinated toxin I labeled on tyrosine residues by precipitation with the specific anti-native toxin I antiserum.

Modifications introduced by citraconylation are perfectly reversible: decitraconylation restores full toxicity. Citraconic anhydride was used to discriminate between the respective roles of histidine and lysine residues in toxin II.

(5) Transformation of the lysine residues in toxin II into homoarginine by guanidination does not destroy the activity as far as the most accessible residues of the molecule are concerned. In this case positive charges of the lysine residues are preserved.

(6) The role of the amino groups has been determined by the use of iodoacetic acid which is a relatively small radical[6] and which introduces negative charges on lysine residues. Iodoacetic acid has been already used for alkylation of lysine and histidine residues in ribonuclease (Heinrikson, 1966). Alkylation of amino groups in both toxins leads to the loss of toxicity. In the case of toxin I, one lysine residue is superreactive, when compared to toxin II, and essential for the toxicity. The most interesting hypothesis is that this residue may be one of the three lysine residues in homologous position in toxins I and II (Figure 1). If this is not the case, it is also possible that the N-terminal lysine residue may be either α- or ϵ-alkylated or both. Some preliminary experiments suggest that the N-terminal residue of the toxins may be buried (for instance, we know that leucine aminopeptidase is inactive upon native toxin II).

(7) It is clear that the only histidine residue in toxin I is not a part of the active site. In the case of toxin II, it is possible to alkylate (monoalkylation) the most accessible residue without loss of toxicity. Furthermore, both toxins have no histidine residue in homologous position (Figure 1).

Snake neurotoxins also form a family of homologous proteins. They are made of single peptide chains of 60–74 amino acid residues cross-linked by four or five disulfide bridges. They have molecular properties analogous to scorpion neurotoxins (Chicheportiche, 1970; Chicheportiche and Lazdunski, 1972). Some amino acid residues of snake neurotoxins have been chemically modified. The main results were summarized by Yang (1974). Some aromatic amino acids residues (Tyr, Trp) should play a role in the stabilization of an adequate geometry of the active site, and an "essential" role should be ascribed to the ϵ-amino group of lysine-53 and to the guanidino group of arginine-33. It is tempting to draw analogies with scorpion neurotoxins, but until now the mode of action of these two families of neurotoxins has appeared quite different: snake neurotoxins induce a flaccid paralysis in mice and act generally on the postsynaptic neuromuscular junction by binding irreversibly to the acetylcholine receptor (Lester, 1970; Eaker et al., 1971); scorpion neurotoxins, on the other hand, produce a spastic paralysis: studies performed with whole scorpion venom have shown that the neurotoxic action is mainly presynaptic (Adam et al., 1966; Koppenhöffer and Schmidt,

[6] Dansyl chloride has been used without success: no superreactive residue has been found with this reagent, thus differing markedly from the case of snake neurotoxins (Chicheportiche et al., 1972).

TABLE II: Activity of Buthinae Mammal Neurotoxins.

Toxin	Sp Act. (LD_{50}/Absorbance Unit at 280 nm)	Wt of the LD_{50}/kg Mouse (μg)
AaH I	1980	17
AaH I′	1980[a]	17[a]
AaH II	2400	9
AaH III	1290	23
Am I	720	29
Am II	1000	19
Bop I	80	b
Bop II	100	176
Bot I	205	91
Bot I′	40	b
Bot I″	500	b
Bot I‴	125	b
Bot II	126	144
Bot III	956	21
Bot III′	22	810
Lqq I	375	63
Lqq II	544	47
Lqq III	259	64
Lqq IV	265	70
Lqq V	717	25

[a] AaH I′ must have the same activity as AaH I since the mixture of AaH I and I′ purified from the venom of animals living in Tunisia shows the same activity than pure AaH I from animals living in Algeria. [b] Not determined.

1968; Parnas et al., 1970; Narahashi et al., 1972); in a more recent work (Romey et al., 1975) it is shown that toxin I of *Androctonus australis* Hector "affects the losing of the Na^+ channel and the opening of the K^+ channel in giant axons of crayfish and lobster nerves".

Comparison of the primary structures of scorpion toxins may also give information about the relationship between structure and activity (Figure 6). Among the 22 neurotoxins isolated from the scorpion venoms, as far as the sequence has been established, it appears that one neurotoxin is very different from the others: the "insect toxin" isolated from the venom of the scorpion *Androctonus australis* Hector (Zlotkin et al., 1973). In this toxin the first cysteine residue is replaced by a lysine residue, and it is possible that this could induce a change in the position of the disulfide bridges and consequently in the conformation of the molecule. Centrurinae toxins are differentiated from Buthinae toxins essentially by the lack of the first N-terminal amino acid residue, the next important deletion appearing only after the 44th amino acid residue. On a toxicity level it is known that toxins I, II, and III of *Androctonus australis* Hector have chronotropic and inotropic effects upon heart cells in culture at concentration as low as 10^{-8} M (Fayet et al., 1974); toxins I and III of *Centruroides suffusus suffusus* do not act at concentrations of 10^{-6} and 10^{-5} M (Couraud, personal communication). If there is any correlation between the particularities of the sequence of Centruroides toxins and the inactivity with respect to heart cells, further experimentation will probably show.

Of particular interest will be the comparisons of toxins III and III′ of *Buthus occitanus* with toxin II of *Androctonus australis* Hector. Their respective specific toxicities are quite different: toxin II of *Androctonus* is twice as potent than toxin III of *Buthus* and 100 times more potent than toxin III′ of *Buthus* (Table II) (Rochat et al., 1974a; Rosso, personal communication). Comparison of the sequences already established and of the amino acid compositions strongly suggests that they all three have a very high degree of homology.

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Physical Studies on the H3/H4 Histone Tetramer†

Tom Moss,* Peter D. Cary, Colyn Crane-Robinson, and E. M. Bradbury

ABSTRACT: High-resolution proton magnetic resonance spectroscopy (270 MHz), circular dichroism, and infrared spectroscopies and ultracentrifugation studies have been carried out on the salt-extracted $(H3/H4)_2$ tetramer from calf thymus. The tetramer contains about 29% α helix and no β structure. It is denatured in 6 M urea but can be renatured simply by dialysis to water. The proton spectrum shows a number of perturbed resonances which are not observed in the spectra of either H3 or H4 alone. The observation of these resonances demonstrates that the tetramer contains some elements of tertiary structure. The overall appearance of the spectrum however is close to that of a partially denatured protein. Sedimentation velocity studies show the tetramer to have a frictional ratio of 1.99 in 50 mM acetate/50 mM bisulfite and thus to be hydrodynamically quite different from a globular protein. Two possible structural models compatible with the data are discussed.

Much recent work has suggested a subunit structure for chromatin in which the four histones H2A, H2B, H3, and H4[1] are complexed with about 200 base pairs of DNA to form a unit of mass about 250 000 daltons. On salt dissociation of chromatin the last histones to be removed are the arginine-rich pair H3 and H4 which are both known to have very highly conserved sequences. Evidence that this pair of histones can form a structural unit has come from cross-linking studies of both salt dissociated histone and of chromatin itself. The conclusion drawn from this work (Kornberg and Thomas, 1974) (Thomas and Kornberg, 1975) was that the unit of structure was a tetrameric $(H3/H4)_2$. The molecular weight of the salt-dissociated H3/H4 complex has been studied by Roark et al. (1974) using sedimentation equilibrium and making a specific correction for primary charge effects. They obtained a molecular weight of 54 000 which is close to the value calculated for an $(H3/H4)_2$ tetramer. At low concentration a significant reduction in the apparent molecular weight was interpreted as a consequence of an equilibrium between tetrameric and dimeric forms. More recently, D'Anna and Isenberg (1974a) demonstrated that, by reassociation of an equimolar ratio of acid-extracted H3 and H4, a complex of apparent molecular weight 54 000 could be obtained. This observation was important since Kornberg and Thomas had suggested that acid extraction of histones irreversibly denatures their native structure. The observations described above indicated the chemical composition and molecular weight of the complex, but no details of secondary or tertiary structure.

D'Anna and Isenberg (1974b) using reconstituted material showed that the complex contained about 25% α helix and apparently no β structure. This helix content is not dissimilar to the average of the separated histones, but the absence of β structure at high ionic strength is characteristic of the complex. The only possible indicators of tertiary structure so far published are the observation of Kornberg and Thomas (1974) that the complex shows a sedimentation coefficient of 3 S, corresponding to a frictional ratio of 1.7, and the observation that the histone tetramer elutes at an apparent molecular weight of ~100 000 from Sephadex G-100 (van der Westhuyzen and Von Holt, 1971). These observations imply that the tetramer is not a fully compact globular structure.

In view of the foregoing, we have undertaken a more detailed investigation of the secondary and tertiary structure of the H3/H4 complex, using circular dichroism (CD), infrared (ir), and high-resolution proton magnetic resonance spectroscopy (^{1}H NMR).[2] The last technique is a particularly sensitive indicator of compact tertiary structure since the close packing of residues in the inner parts of globular regions gives rise to perturbed resonances. The effect is most marked if aromatic

† From the Department of Physics, Biophysics Laboratory, Portsmouth Polytechnic, Portsmouth, Hants., United Kingdom. *Received August 19, 1975.* This research was supported by the Science Research Council of Great Britain.

[1] The new histone nomenclature used here was accepted by the participants at the CIBA Foundation Symposium on Structure and Function of Chromatin, April 3-5, 1974. This new nomenclature, which has been proposed to the appropriate international nomenclature committee, is as follows for each histone where the previous names are given in parentheses: H2A (F2A2, IIb1, ALK), H2B (F2B, IIb2, KSA), H3 (F3, III, ARK), H4 (F2A1, IV, GRK), and H5 (F2C, V, KAS).

[2] Abbreviations used are: ^{1}H NMR, proton magnetic resonance; DSS, sodium 2,2-dimethyl-2-silapentanesulfonate; CD, circular dichroism; DTT, dithiothreitol.