CHEMICAL MODIFICATION OF HISTIDINE AND LYSINE RESIDUES OF CROTOXIN

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1. Introduction

An essential histidine residue involved in phospholipase A2 (EC 3.1.1.4) activity has been demonstrated in porcine pancreatic phospholipase [1] and in several snake-venom phospholipases which may or may not be neurotoxins (e.g., notexin [2], β-bungarotoxin [3]) and the phospholipase from the South African gaboon adder *Bitis gabonica* [4]. Recently, a half-site reactivity for chemical modification of a histidine residue of phospholipase A2 from cobra venom *Naja naja naja* was reported [5].

Crotoxin is the neurotoxic principle of the Brazilian rattlesnake, *Crotalus durrissus terrificus* and also carries phospholipase A2 activity [6]. The study of the role of histidine and other amino acid residues in these activities is of interest because it could shed light on the mode of action of this toxin. It has been demonstrated that crotoxin represents a complex of an acidic subunit (crotoxin A) and a basic subunit (crotoxin B) with isoelectric points about 3.8 and 8.4, respectively, and molecular weights around 9000 and 12 000 [7,8]. The neurotoxicity of crotoxin depends upon the synergistic interaction between the two subunits, while the phospholipase A2 activity is associated with the B subunit alone. Both crotoxin B and crotoxin complex showed similar indirect hemolytic activity [7–9] which is generally regarded as equivalent to phospholipase A2 activity. However, the relation between phospholipase activity and neurotoxicity remains as yet obscure [3,9].

The present study describes the effects of *p*-bromophenacyl bromide, ethoxyformic anhydride and acetic anhydride on the crotoxin complex or its subunits. The presence of a histidine residue reactive towards *p*-bromophenacyl bromide and essential for both phospholipase activity and neurotoxicity was demonstrated in crotoxin B, but the histidine in component A was unreactive, and so were all three histidines in the crotoxin complex. Calcium ion was found not to protect the protein against *p*-bromophenacyl bromide modification, while partial protection was achieved by calcium in conjugation with a low concentration of the substrate, α-dicapyryl lecithin.

Acylation by the acid anhydrides affected only the amino groups, and caused progressive inactivation of B, which was interpreted as indicating that blocking of more than two amino groups caused harmful conformational disturbance. In crotoxin A only one amino group reacted, and that without noticeable consequences.

2. Materials and methods

Crotoxin, crotoxin A, and crotoxin B were prepared as in [7], usually by following the primary separation in 6 M urea on DEAE-cellulose by CM-cellulose chromatography also in urea (6 M, 0.01 M phosphate buffer, pH 6.5), with elution by a linear gradient of 0–0.5 M NaCl [9].

Modification with *p*-bromophenacyl bromide was done at 24°C in 2 ml 0.1 M sodium cacodylate–HCl buffer, pH 6.0 or pH 7.2, containing 0.1 M NaCl. Small aliquots of a 14 mM solution of *p*-bromophenacyl bromide in acetone were added to the protein solution up to a 5-fold molar ratio of reagent to protein. At suitable time intervals the samples were acidified with 50 μl glacial acetic acid to stop the reaction. Excess reagent was removed by gel

filtration through a Sephadex G-25 column (1 X 30 cm) in 0.01 M ammonium formate buffer, pH 4.0, or in 0.01 M sodium phosphate buffer, pH 6.5.

Cyanogen bromide fragmentation of the modified enzyme, without reduction and carboxymethylation, was done in 70% formic acid as in [10]. The polypeptide fragments were separated by gel filtration on a Sephadex G-50 column (1.6 X 60 cm) equilibrated with 10% formic acid. Peptides were hydrolyzed in redistilled 6 N HCl at 110°C for 24 h and amino acids analyzed with a Beckman 120C amino acid analyzer.

Modification with acid anhydrides (acetic anhydride, at times ^{14}C-labeled) was done as in [11]; severall small aliquots of acid anhydride solution in dioxane were added to proteins in half-saturated sodium acetate. Modified proteins were desalted by filtration on Sephadex G-25 columns (1 X 30 cm) in 0.01 M sodium acetate, pH 4.5. Protein concentration was determined spectrophotometrically using pre-determined extinction coefficients. Protein concentration was also estimated as in [12].

Phospholipase activity was assayed as in [9] using egg-yolk substrate, 5 mM sodium deoxycholate and 20 mM calcium chloride at 37°C. The first 5 min titration with 0.02 N NaOH was recorded by a pH stat. Lethality was determined over a 24 h period after intraperitoneal injection of 20 g, male Swiss-Webster white mice obtained from Simonsen Laboratory, Gilroy, CA.

Polyacrylamide gel electrophoresis with or without 8 M urea was done as in [13]. Gel filtration on a Sephadex G-75 column was done as in [14].

All chemicals were commercially available reagent grade products used without further purification.

3. Results

3.1. *Modification by p-bromophenacyl bromide*

Both the crotoxin complex and crotoxin A retained their biological activity or potential activity, respectively, upon *p*-bromophenacyl bromide treatment at pH 6.0 or pH 7.2 for 2 h. This was not surprising, since ultraviolet absorption spectra and amino acid analyses indicated that no chemical modification had occurred (table 1).

In contrast, inactivation of crotoxin B by *p*-bromophenacyl bromide followed pseudo first-order kinetics with half-time of inactivation of about 12 min. The extent of modification, estimated by the increase in specific A_{280} after removing excess reagents, was further confirmed by amino acid analysis. A histidine residue was the only amino acid found changed in modified crotoxin B (table 1). A molar extinction coefficient change of about 11 000 was found for 1 histidine alkylation/mol crotoxin B. The specific modification of a histidine residue was further supported by the one-to-one correlation of extent of modification and residual lethality (fig.1).

In the presence of 5 mM calcium and 10 mM α-dicapyryl lecithin, a slowly split substrate, crotoxin B was partially protected against *p*-bromophenacyl bromide modification, the half-time of inactivation increasing from 12–25 min under these conditions. Calcium alone did not have any effect on the modification reaction even at 25 mM. The modification product formed in the presence of the substrate showed the same characteristics of specific histidine modification as that formed in its absence. The alkylated crotoxin B, though enzymatically inactive, retained its ability to form the typical complex with crotoxin A,

Table 1
Reactivity of histidines in crotoxin and components towards *p*-bromophenacyl bromide

	Histidine residues (mol/mol)		Molar extinction coefficient[a]		Lethality[b] (LD_{50} mg/kg)	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
Crotoxin complex	2.5	2.5	30 360	30 360	0.075	0.075
Crotoxin B component	1.5	0.6	21 480	31 600	0.075	1.5
Crotoxin A component	1.0	1.0	7 100	7 100	0.075	0.075

[a] Determined at pH 7.0 in 0.01 M phosphate buffer
[b] Lethality of components was determined in the complex state with other component untreated

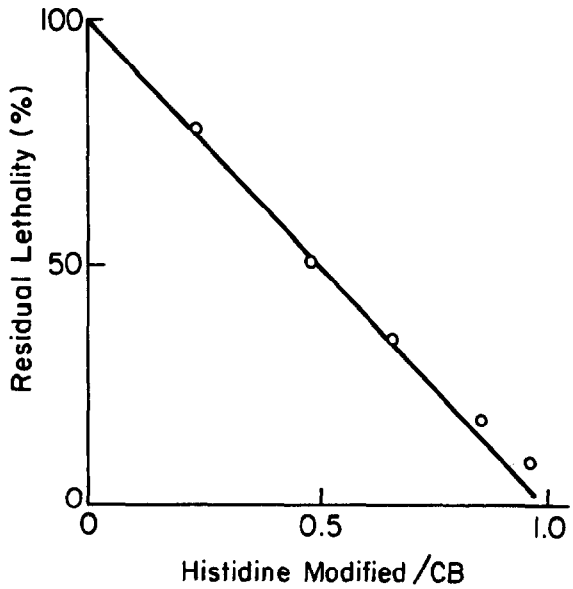

Fig.1. Loss of lethality of crotoxin B (CB) as a function of histidine alkylation by *p*-bromophenacyl bromide. The abcissa is a plot of the mol histone modified/mol CB.

showing the same electrophoretic mobility as native complex in a 7% polyacrylamide gel at acidic pH [13].

Cyanogen bromide treatment of crotoxin B gives rise to an octapeptide, containing the N-terminal histidine, from the N-terminal end of the polypeptide chain [15]. Upon cyanogen bromide treatment of alkylated crotoxin B, an intact N-terminal octapeptide was obtained, as well as a big polypeptide chain with alkylated histidine (table 2) and the C-terminal fragment. It is thus concluded that the active site histidine is the one located internally on the polypeptide chain of the protein, but not the N-terminal histidine.

3.2. *Modification by ethoxyformic anhydride*

Ethoxyformic anhydride is a very reactive reagent with partial specificity. Under appropriate conditions it acylates mainly the amino groups and histidine residues of proteins [16,17].

Less than 10% of the neurotoxicity and phospholipase activity were retained when the crotoxin complex was treated with over 1000-fold excess of ethoxyformic anhydride. However, the modified protein did not show any change in ultraviolet absorption spectrum, indicating that tyrosine was not

Table 2
Amino-acid compositions of cyanogen bromide fragments of crotoxin B[a]

	CB-CN-I		CB-CN-II and CB-CN-III	
	Native	Alkylated	Native	Alkylated
Asp	1	1	8	8
Thr			6	6
Ser			6	6
Glu	1	1	7	7
Pro			4	4
Gly			10	10
Ala			6	6
½ Cys			14	14
Val			2	2
Met[b]	1	1	1	1
Ile			4	4
Leu	2	2	4	4
Tyr			9	9
Phe	1	1	5	5
His	1	1	1	0
Lys	1	1	8	8
Arg			8	8
Tryp[c]			3	3
Total	8	8	106	105

[a] Molar ratios compared to 1 and 7 glutamic acid residues, respectively
[b] Methionine found as homoserine lactone
[c] Based on ultraviolet absorption

affected [18]. The spectrum further showed that no histidine modification had occurred, since there was no increase in A_{242} [19]. The extent of ethoxyformylation of amino groups was estimated by the decrease of ninhydrin color reaction [12], and further checked by electrophoresis of the acylated protein in polyacrylamide gels in the presence of 8 M urea. The modified protein gave several discrete bands due to charge differences, thus reflecting different extents of modification. The extents of modification estimated by both methods agree with each other. Up to an average of four amino groups blocked, there was no detectable histidine modification, while phospholipase activity and neurotoxicity were decreased to about 30% of those of the native toxin (table 3).

Crotoxin B showed a similar reactivity toward ethoxyformic anhydride. However, the density of modification of amino groups that crotoxin B could stand without loss of activity was much less than that of the crotoxin complex. Crotoxin B retained only

Table 3
Extent of acylation by ethoxyformic anhydride

Molar ratio anhydride/protein	Amino groups acylated (and residual lethality)[a]		
	Crotoxin complex	Crotoxin A	Crotoxin B
50	2.0 (100)	0.9 (100)	1.8 (80)
100	3.1 (60)	0.9 (100)	2.9 (30)
200	4.3 (30)	0.9 (100)	4.1 (17)
1000	>6.5[b] (<10)	1.0 (100)	5.5 (<10)

[a] Residual lethality expressed, as % control, in parenthesis; the residual lethality of components A and B was determined in the complexed state with the other component untreated

[b] Products were turbid due to low solubility. Estimation of extent of acylation was done after solubilization with urea to final conc. 8 M

30% of its activity compared to 60% for the crotoxin complex when an average of three amino groups had been ethoxyformylated. In crotoxin A only one amino group reacted, and the acylated protein retained its activity of potentiating crotoxin B to show neurotoxicity (table 3). All soluble acylated components could still form complexes although these showed slightly different electrophoretic mobilities compared to that of native complex due to their charge differences.

3.3. *Acetylation*

The role of amino groups in crotoxin was further investigated by acetic anhydride modification. The results of acetylation are essentially the same as those observed upon ethoxyformylation. Only amino groups were found modified. Besides ninhydrin color reaction and polyacrylamide gel electrophoresis in the presence of urea, incorporation of radioactivity from ^{14}C-labeled acetic anhydride was used to estimate the extent of modification with increasing amounts of reagent. These data, and particularly the electrophoretic patterns (fig.2) indicate a population of molecules of different modification densities. Furthermore, the distribution of modification density among the population indicated that acetylation of crotoxin is a non-cooperative reaction. No predominant species with a certain number of acetyl groups was formed with increasing amounts of acetic anhydride. Attempts to isolate products of homogeneous modification density by ion-exchange chromatography were unsuccessful. However, a partial separation of modified crotoxin B into low modification density (less than 4 acetyl groups, av. acetyl groups 2 h/mol) and high modification density (av. > 3 acetyl groups/mol) was achieved by using DEAE-cellulose chromatography in 10 mM phosphate buffer, pH 6.5, in the presence of 8 M urea. The increase of residual toxicity after the removal of the high modification density population suggested that the inactivation process was due to a discrete change in the conformation of the molecule induced by the loss of positive charges. The semi-log plot of residual activity against average

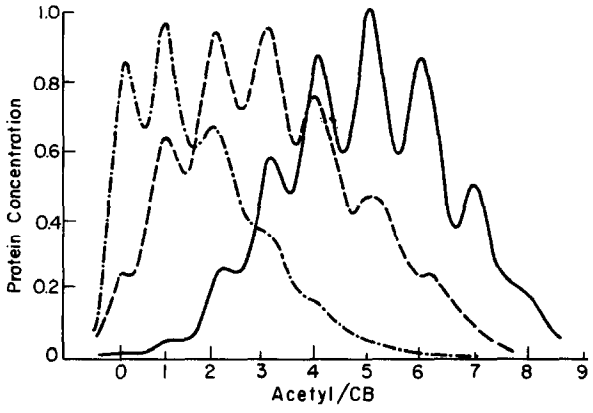

Fig.2. Distribution of modified crotoxin B with different acetylation densities revealed by polyacrylamide electrophoresis, at pH 4.3, in the presence of 8 M urea. Protein concentration, expressed in arbitary units, was estimated by scanning gels stained with amido black 10B. Average modification extent: (.–.–.–) 1.2 mol, (– – –) 3.0 mol, (——) 4.9 mol acetyl group/mol crotoxin B (CB).

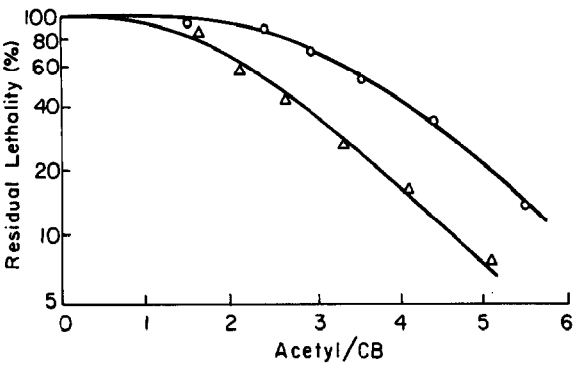

Fig.3. Loss of lethality as a function of average acetylation density of crotoxin B. (△——△) Crotoxin B; (○——○) crotoxin complex. The abcissa is plotted as mol acetyl group/mol crotoxin B (CB).

modification density showed that the biological activity of crotoxin B was retained with up to two amino groups acetylated while the crotoxin complex remained active with three out of their total of ten and fourteen amino groups, respectively, being blocked (fig.3). Like ethoxyformic anhydride, acetic anhydride affected only one of the three amino groups of crotoxin A, and the modified crotoxin A was still fully active (data not shown). The complexes formed from acetylated crotoxin components are indistinguishable from acetylated crotoxin complexes. The acetylated complex was eluted at the same elution volume of a Sephadex G-75 column as the native complex, indicating that no drastic change in apparent volume of the complex had occurred after acetylation. Calcium showed no effect on the extent of acetylation.

4. Discussion

The present study represents a minor contribution in support of a thesis that was first suggested by the classical findings concerning the specific reactivity of an active-site histidine in pancreatic ribonuclease towards bromoacetate. Briefly stated, this thesis is that there exist no specific protein reagents, but only specific protein reactions.

p-Bromophenacyl bromide had been shown specifically to esterify one aspartyl group in pepsin [19,20] long before it was found specifically to alkylate a histidine residue in the active site of phospholipase A2 molecules from widely different sources [1–5,21,22]. We now report that the active site of crotoxin contains a histidine which is not accessible to that reagent until this protein complex is dissociated into its two components. Thus the active site of crotoxin B is masked by crotoxin A.

Ethoxyformic anhydride has also been reported as showing different group affinities in different proteins [16–19,23]. In most proteins it acylates amino and/or imidazole groups. In acting on crotoxin, as well as on component B, which has, as stated above, an alkylatable histidine, ethoxyformic anhydride acylates only amino groups. It resembles in this particular case acetic anhydride, which frequently acylates phenolic besides amino groups (e.g. [24]). (We are disregarding here the reactivity of -SH groups, since these are absent from crotoxin and other proteins under consideration.)

The present finding that the amino groups of crotoxin B react randomly with both reagents, and are only cumulatively 'essential', resembles the situation in several other proteins [17]. The finding that in crotoxin A only one of the three amino groups can be acylated is more unusual and requires further study. It appears of interest that acylation does not affect the biological potential of crotoxin A, and that complex formation between the two components is not prevented by acetylation of a total of up to five amino groups.

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