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CHEMICAL MODIFICATION OF THE HISTIDINE RESIDUE IN BASIC PHOSPHOLIPASE A_2 FROM THE VENOM OF *NAJA NIGRICOLLIS* *

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Summary

Phospholipase A_2 from *Naja nigricollis* venom was separated into three fractions by chromatography on a column of CM-Sephadex C-25. The p*I* values of fractions CMS-5, CMS-6 and CMS-9 were determined to be 7.6, 8.3 and 10.6, respectively. Fraction CMS-9 was further purified on a DEAE-Sephacel column and the homogeneity was verified. The specific activity of CMS-9 was found to be 1300 units per mg and lethal toxicity 0.3 mg per kg mouse.

The most basic and toxic fraction, CMS-9, was subjected to chemical modification with *p*-bromophenacyl bromide. The enzyme lost both the enzyme activity and lethal toxicity, however, the antigenicity remained unchanged. Although both 8-anilinonaphthalenesulfonate and Ca^{2+} showed pronounced protection on the inactivation process, the mechanism of 8-anilinonaphthalenesulfonate protection is different from that of Ca^{2+}. Amino acid analysis showed that only one (His-47) out of three histidine residues was modified.

Although both native and His-modified CMS-9 were perturbed by the presence of Ca^{2+}, the modified enzyme lost the characteristic tryptophan blue shift suggesting that the modified enzyme is unable to exert a charge effect upon Ca^{2+} binding in the vicinity of the tryptophan group. Scatchard plots revealed only one type of binding sites for 8-anilinonaphthalenesulfonate in the presence of Ca^{2+}. On the other hand, the modified enzyme lost the ability to bind 8-anilinonaphthalene. It is suggested tentatively that the hydrophobic pocket in which 8-anilinonaphthalenesulfonate is bound may be the site of the enzyme that interacts with phospholipid.

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Introduction

The relationship between the toxicity and the catalytic activity of phospholipase A_2 (EC 3.1.1.4) is, as yet, poorly understood. Whilst there is evidence that the catalytic activity is essential for toxicity [1], the relatively low toxicity of other venom phospholipase A_2 which has high catalytic activity suggests that the relationship is not a straightforward direct one.

Many presynaptic neurotoxins possess phospholipase A_2 activity, besides their potent neurotoxicity [2]. Together with the recent finding that β-bungarotoxin has an absolute requirement of Ca^{2+} for its phospholipase A_2 activity [3] and the involvement of Ca^{2+} in the neurotransmitter release, it is interesting to clarify the relationship between the critical His residue and the dependence of phospholipase A_2 activity on Ca^{2+}.

In this study the most basic and highly toxic phospholipase A_2 was purified from *Naja nigricollis* venom and subjected to chemical modification with *p*-bromophenacyl bromide. Evidence for the metal ion-protein interaction was provided from the spectral properties of the Ca^{2+}-enzyme complex and the pronounced protective effects of Ca^{2+} and 8-anilinonaphthalenesulfonate. The possible role of His-47 of the enzyme is also discussed.

Materials and Methods

N. nigricollis venom (Lot No. NGE45-IZ) was obtained from Miami Serpentarium Lab., Miami. *p*-Bromophenacyl bromide, sodium deoxycholate and iodoacetic acid were purchased from Sigma Chemical Co. Sephadex G-25 and G-100, CM-Sephadex C-25 and DEAE-Sephacel were obtained from Pharmacia Fine Chemicals. Ammonium acetate, ammonium bicarbonate, calcium chloride and Tris were obtained from E. Merck, Darmstadt, 8-anilinonaphthalene sulfonate (ammonium salt) from Pierce Chemical Co. Ampholine carrier ampholytes from LKB-Produkter AB, Sweden, Dithioerythritol and trypsin-TPCK were the products of Worthington Biochemical Corp. All other reagents were of analytical grade.

Isolation and purification of phospholipase A_2 from the venom of N. nigricollis. 0.5 gm of crude *N. nigricollis* venom was dissolved in 5 ml of 0.05 M ammonium acetate buffer, pH 4.5, and applied on a column (2 × 27 cm) of CM-Sephadex C-25 equilibrated with the same buffer. The column was eluted with 0.05 M ammonium acetate buffer (pH 4.5) for 6 h, then 0.1 M ammonium acetate buffer (pH 6.0) for 5 h, thereafter, by two-stage gradients; a linear gradient of 800 ml from 0.1 (pH 6.0) to 0.5 M ammonium acetate (pH 6.8) and a non-linear gradient from 300 ml 0.5 (pH 6.8) to 600 ml 1.0 M ammonium acetate (pH 6.8). The flow rate was 50 ml per h and the effluent was monitored at 280 nm with an ISCO model UA-5 absorbance monitor. Three peaks showed phospholipase A_2 activity, and were lyophilized separately, the fractions comprising each peak having been pooled.

Fraction CMS-5 was further purified on a DEAE-Sephacel column (2 × 27 cm) equilibrated with 0.1 M ammonium bicarbonate buffer, pH 8.5. The column was eluted by a linear gradient of 400 ml from 0.1 (pH 8.5) to 0.2 M ammonium bicarbonate (pH 7.8).

Rechromatography of CMS-6 was conducted on a column (2 × 27 cm) of CM-Sephadex C-25 as described in the preceding paragraph. Further purification of CMS-9 was conducted on DEAE-Sephacel column (1.5 × 25 cm) equilibrated with 0.01 M ammonium bicarbonate buffer, pH 8.9. The column was eluted by a non-linear gradient from 400 ml 0.01 (pH 8.9) to 800 ml 0.05 M ammonium bicarbonate (pH 7.8).

Isoelectric focusing. Isoelectric focusing was performed in 110 ml LKB electrofocusing column according to the instruction manual of LKB 8100 Ampholine using a stabilizing medium of sucrose and a combination of 1% each of pH 6—8 and 8—9.5 Ampholine (for CMS-5) or 2% of pH 8—9.5 Ampholine (for CMS-6). For CMS-9, sorbitol was used as a stabilizing medium and the Ampholine used was pH 9—11. Electric focusing was carried out at 4°C and 800 V. After 102 h, the column was drained by downward displacement with deionized water. The effluent was monitored at 280 nm and the pH measured.

Determination of phospholipase A_2 activity. Phospholipase A_2 activity was determined by using the titrimetric method described by de Haas et al. [4] with a slight modification. Titration was carried out with 0.001 mM NaOH on an ABU 1b Radiometer Auto-burette with Titrigraph SBR 2c at pH 8.0 and 25°C. An aqueous emulsion of egg yolk was used as substrate for which one egg yolk was homogenized with 300 ml water and supplemented with 2.7 mM deoxycholate and 20 mM $CaCl_2$. For each determination 10-ml of the substrate solution were used and a suitable amount of enzyme (0.2 to 2.0 μg) in 10 μl was added. There was a linear relationship between the activity and the amount of enzyme, and the curves were linear up to 3 min. 1 unit of enzyme activity was defined as the release of 1 microequivalent of fatty acid per min.

Chemical modification with p-bromophenacyl bromide. Modification with *p*-bromophenacyl bromide was performed at a molar ratio of protein : reagent of 1 : 10 in 0.025 M Tris-HCl buffer, pH 8.0. Enzyme (0.34 mg/ml) in 0.5 ml Tris buffer was incubated with 0.25 mM *p*-bromophenacyl bromide at 30°C. Aliquots were taken from the reaction mixture at suitable time intervals for the assay of enzyme activity. For preparative runs, 125 μl of 40 mM *p*-bromophenacyl bromide in acetone were added to 5 ml of phospholipase A_2 solution (1.25 mg/ml). The reaction was allowed to proceed for 40 min, then acidified with glacial acetic acid to pH 4.0 to stop the reaction. Excess reagents were removed by passing the mixture through a column of Sephadex G-25 and the protein fraction was lyophilized.

Identification of the p-bromophenacylated His residue. In order to determine the position of the modified His residue in the sequence, the modified derivative was reduced and S-carboxymethylated by using the procedure described by Crestfield et al. [5], followed by tryptic digestion. His-containing peptides from tryptic digests were separated by a combination of high-voltage paper electrophoresis at pH 5.4 and descending paper chromatography, as previously described [6]. Peptides on the map were developed with 0.2% ninhydrin in acetone and His-containing peptides were detected with Pauly reagent. The His-containing peptides were cut out from the duplicate run of the unstained peptide map and eluted with 0.1 M ammonium bicarbonate for amino acid analysis.

Ultraviolet difference spectroscopy. Difference spectra were recorded on a

Cary model 17 double-beam spectrophotometer using 1-cm path length cells.

Fluorescence measurement. All titration and fluorescence spectra were recorded with a Hitachi model 204 fluorescence spectrophotometer equipped with high-stability xenon lamp. In the experiments carried out in the presence of 8-anilinonaphthalenesulfonate, exciting wavelength was at 365 nm. The excitation and the emission slit-widths were 5 nm.

Amino acid analysis. About 30 nmol of protein samples were hydrolyzed with constant boiling (5.7 M HCl at 110°C for 24 h in evacuated sealed tubes). Amino acids were determined on a JOEL JLC-6AH fully automatic amino acid analyzer according to the procedure of Spackman et al. [7]. Tryptophan content was determined by a spectrophotometric method with *N*-bromosuccinimide [8].

Measurements of lethal toxicity. Lethality was measured by intraperitoneal injection of a serial 2-fold dilution of phospholipase A_2 preparations into mice (16—18 g) with the amount of 0.2 ml per mouse as previously described [9]. Six mice of both sexes were used for each dilution, and the LD_{50} was calculated according to the 50% end-point method of Reed and Muench [10].

Results

Isolation and purification of phospholipase A_2 from the venom of N. nigricollis

Phospholipase A_2 from *N. nigricollis* venom was separated into three fractions by chromatography on a column of CM-Sephadex C-25 (Fig. 1). Fraction CMS-5 was further purified on a DEAE-Sephacel column and fraction CMS-6 was rechromatographied on a CM-Sephadex C-25 column to eliminate the small amount of contaminated CMS-5. Fraction CMS-9 was further purified

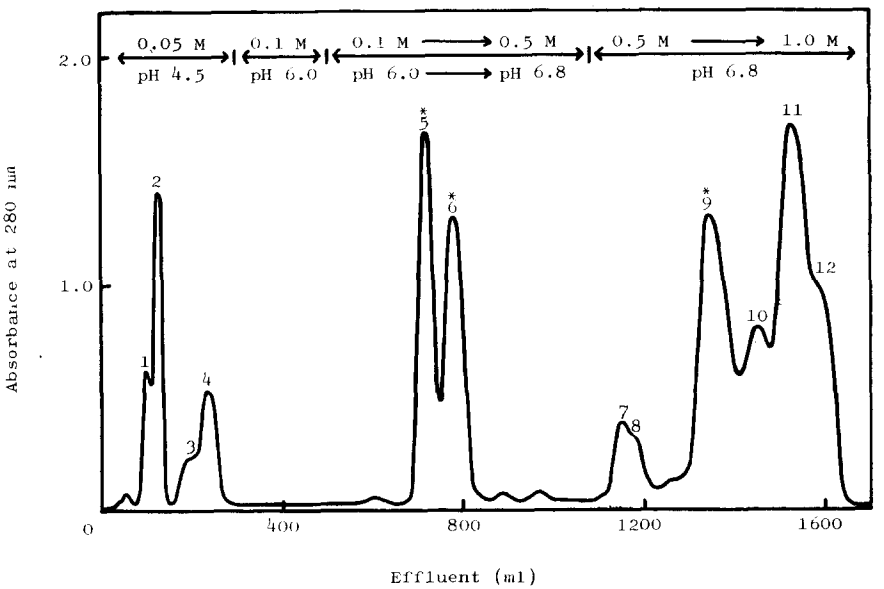

Fig. 1. CM-Sephadex C-25 column chromatography of *N. nigricollis* venom. 0.5 gm of crude *N. nigricollis* venom was dissolved in 5 ml of 0.05 M ammonium acetate buffer, pH 4.5, and applied on a column (2 X 27 cm) of CM-Sephadex C-25 equilibrated with the same buffer. The asterisks indicate phospholipase A_2 activity.

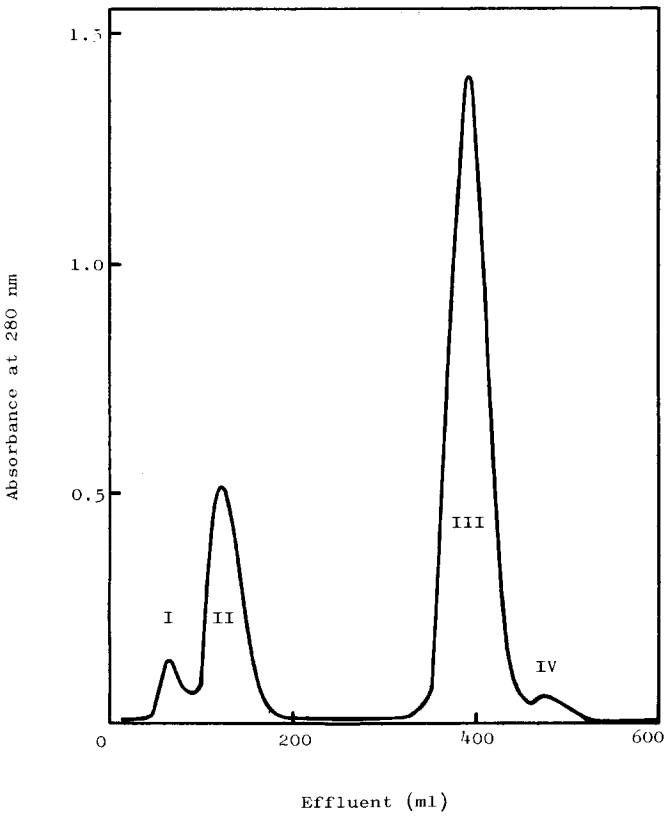

Fig. 2. Further purification of CMS-9 on a DEAE-Sephacel column. 200 mg of the lyophilized fraction CMS-9 were dissolved in 5 ml of 0.01 M ammonium bicarbonate buffer, pH 8.9, and applied on a DEAE-Sephacel column (2 X 27 cm) equilibrated with the same buffer. Only peak III showed high phospholipase A_2 activity and the fractions comprising this peak pooled and lyophilized.

on a DEAE-Sephacel column as shown in Fig. 2, in which only peak III showed high phospholipase A_2 activity. The summary of isolation and purification of three phospholipase A_2 preparations from *N. nigricollis* venom is listed in Table I. As shown in Fig. 3, all the purified phospholipase A_2 was revealed as a single band on polyacrylamide gel at pH 4.5. By isoelectric focusing the p*I* values of CMS-5, CMS-6 and CMS-9 were determined to be 7.6, 8.3 and 10.6, respectively.

Chemical modification of CMS-9 by p-bromophenacyl bromide and the protective effects of Ca^{2+} and 8-anilinonaphthalenesulfonate

As seen from Fig. 4, the inactivation of CMS-9 by *p*-bromophenacyl bromide behaved like a pseudo-first order reaction and the presence of Ca^{2+} showed pronounced protection on the inactivation process. Whilst the hydrophobic probe, 8-anilinonaphthalenesulfonate, did not show much protection in the absence of Ca^{2+}, it enhanced the protective effect of Ca^{2+} more than 1.8-fold. Titration of the inactivation reaction of CMS-9 by *p*-bromophenacyl bromide with various concentrations of Ca^{2+} in the absence or presence of 8-anilino-

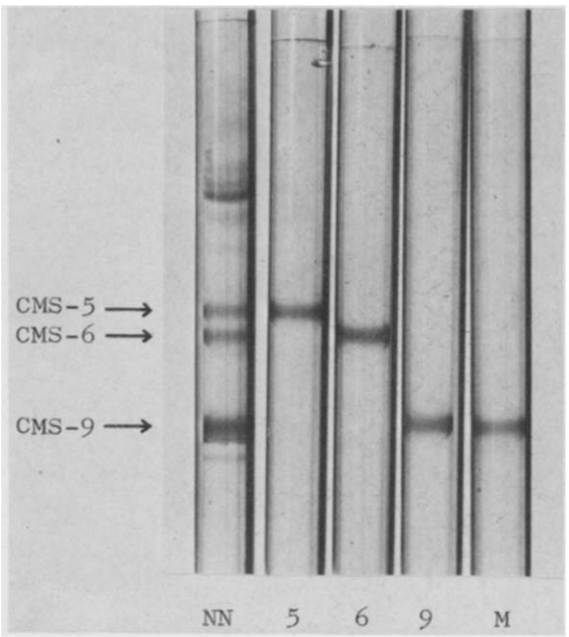

Fig. 3. Disc electrophoresis of the purified phospholipase A_2 from *N. nigricollis* venom. Disc electrophoresis on 7% polyacrylamide gel was carried out as described by Gabriel [11]. Electrophoresis was performed at 4°C by applying a current of 4 mA per gel for 75 min. NN, crude *N. nigricollis* venom; 5, CMS-5; 6, CMS-6; 9, CMS-9; M, His-modified CMS-9.

TABLE I

SUMMARY OF ISOLATION AND PURIFICATION OF PHOSPHOLIPASE A_2 FROM THE VENOM OF *N. NIGRICOLLIS*

The concentrations of the protein were determined spectrophotometrically using $E^{\%}_{1\text{-cm}} = 18.2$ at $\lambda = 280$ nm. Figures in parentheses indicate the *n*-fold increase in specific activity.

Fraction	Protein (mg)	Specific activity (units/mg)	Total activity (units × 10^3)	Yield (%)	LD_{50} (mg/kg mouse)
Crude venom	500	510 (1.0)	225	100	
CM-Sephadex C-25					
CMS-5	18.5	1690 (3.3)	31.3	12.3	
CMS-6	29.0	3012 (5.9)	87.3	34.2	
CMS-9	52.5	1108 (2.2)	58.2	22.8	
DEAE-Sephacel					
CMS-5	13.7	1900 (3.7)	26.0	11.5	0.67
CM-Sephadex C-25 (rechromatography)					
CMS-6	22.6	3100 (6.1)	70.6	31.4	0.64
DEAE-Sephacel					
CMS-9	32.2	1380 (2.7)	44.4	20.0	0.30

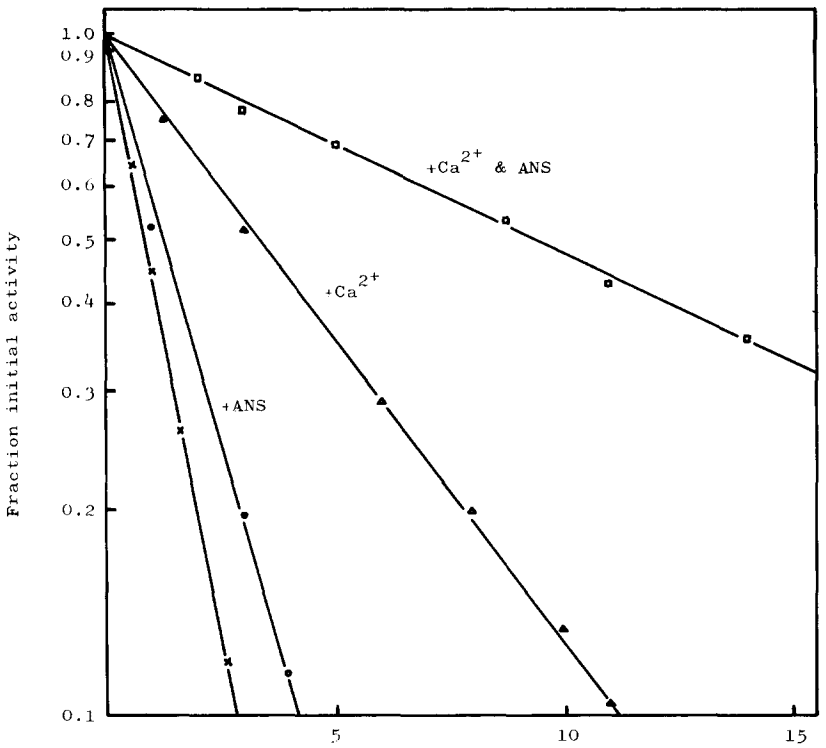

Fig. 4. Effects of Ca^{2+} and 8-anilinonaphthalenesulfonate (ANS) on the rate of inactivation of CMS-9 by *p*-bromophenacyl bromide. 0.5 ml of enzyme solution (0.337 mg/ml) was inactivated by the addition of 25 μl of 5 mM *p*-bromophenacyl bromide (X———X); in the presence of 2 mM 8-anilinonaphthalenesulfonate (○———○), 4 mM $CaCl_2$ (△———△) or 4 mM $CaCl_2$ plus 2 mM 8-anilinonaphthalenesulfonate (□———□). The reaction was conducted in 0.025 M Tris-HCl buffer (pH 8.0) at 25°C. The fraction initial activity is expressed by the ratio of the residual activity to the initial activity.

naphthalenesulfonate revealed a hyperbolic dependence of protection on Ca^{2+} concentration.

The modified enzyme not only lost the enzyme activity but also the lethal toxicity. The LD_{50} of the modified enzyme increased from 0.3 mg to greater

TABLE II

COMPARISON OF PHYSICAL AND BIOLOGICAL PROPERTIES OF NATIVE AND His-MODIFIED CMS-9

K_d of Ca^{2+} was determined at pH 8.0 by following the maximum or minimum of the Ca^{2+}-induced difference spectra. K_d of 8-anilinonaphthalenesulfonate was derived from the degree of saturation of the emission intensity in the presence of Ca^{2+} at pH 8.0

Physical and biological properties	Native	His-modified
Phospholipase A_2 activity (units/mg)	1 380	0.64
Ca^{2+} binding (K_d)	0.20 mM	0.29 mM
8-Anilinonaphthalenesulfonate binding (K_d)	8.7 μM	—
Molecular weight by gel filtration on Sephadex G-100		
with 0.1 M $CaCl_2$	12 800	12 800
without $CaCl_2$	15 100	15 100
by SDS-gel electrophoresis	13 800	13 800
LD_{50} (mg/kg mouse)	0.3	>10

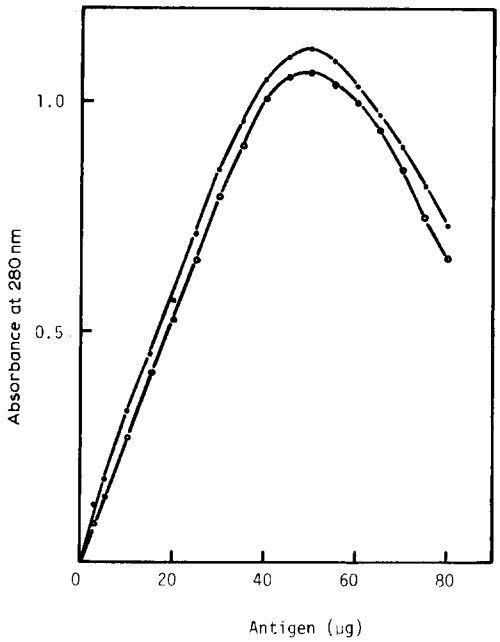

Fig. 5. Quantitative precipitin reactions of native and His-modified CMS-9 with antiserum against CMS-9. The quantitative precipitin reactions were carried out as described by Kabat and Mayer [12]. Increasing amounts (2.5—80 μg) of native (○———○) or His-modified (●———●) CMS-9 in 0.02 M Tris-0.15 M NaCl buffer (pH 7.5) were added to a constant amount of antiserum in a total volume of 0.5 ml. The tubes were incubated for 30 min at 37°C and then left overnight at 4°C. The precipitates were washed three times with cold 0.15 M NaCl, after which they were dissolved in 2 ml of 0.1 M NaOH, and the absorbance values at 280 nm were measured.

than 10 mg per kg mouse (Table II). However, the antigenicity of the His-modified phospholipase A_2 as measured by quantitative precipitin reactions (Fig. 5) did not change at all, suggesting that the antigenic sites might differ from the catalytic site of the enzyme.

Identification of the modified His residue

The results of amino acid analysis of the modified enzyme showed that only one out of three His residues was modified (Table III). All other amino acids remained essentially unchanged.

In order to determine the position of the modified His residue in the sequence of CMS-9, native and modified enzymes were digested with trypsin after reduction and S-carboxymethylation. The digests were separated into 17 spots by two-dimensional paper electrophoresis and descending paper chromatography of which five spots (T-3, T-4, T-9, T-10 and T-11) gave positive color reactions to Pauly reagent. Among them only spots T-3 and T-4 shifted to T-3′ and T-4′ on the map of His-modified CMS-9. Therefore, these four spots were cut out and eluted for amino acid analysis. From the amino acid composition (Table III), T-3 and T-4 were identified to be peptides with amino acid sequences from Gly-34 to Lys-56 and Cys-43 to Lys-56, respectively (Fig. 6). As seen from the results of amino acid analysis, spots T-3′ and T-4′ on the map of the modified CMS-9 corresponded to the spots T-3 and T-4 of native CMS-9,

TABLE III

AMINO ACID COMPOSITION OF NATIVE, MODIFIED CMS-9 AND TRYPTIC PEPTIDES, T-3, T-4, T-3′ AND T-4′

All values for CMS-9 are expressed as molar ratios based on glycine = 12.0. All values for tryptic peptides are expressed as molar ratios based on alanine = 1.0.

Amino acid	CMS-9	Modified CMS-9	Tryptic peptides			
			T-3	T-4	T-3′	T-4′
Aspartic acid	16.1	15.9	8.4	2.8	8.2	2.7
Threonine	3.5	3.6	0.9	—	0.9	—
Serine	3.7	3.8	—	—	—	—
Glutamic acid	5.2	5.3	2.5	1.6	2.4	1.8
Proline	4.2	3.9	0.9	—	0.9	—
Glycine	12.0	12.0	2.2	1.0	2.1	1.0
Alanine	8.6	8.6	1.0	1.0	1.0	1.0
Half-cystine	14.2	14.1	1.2	1.7	1.5	1.7
Valine	5.2	5.3	1.4	0.7	1.5	0.8
Methionine	2.2	2.0	—	—	—	—
Isoleucine	2.8	3.0	—	—	—	—
Leucine	5.7	5.9	2.5	—	2.2	—
Tyrosine	8.5	8.7	1.3	0.6	1.2	0.6
Phenylalanine	3.6	3.9	—	—	—	—
Lysine	10.4	10.4	2.0	1.7	1.9	1.8
Histidine	2.7	1.9	0.8	0.6	0.0	0.0
Arginine	5.4	5.4	1.0	—	0.8	—
Tryptophan	2.9	2.9	—	—	—	—
Total residue	118	117	—	—	—	—

respectively. The only difference was in both spots T-3′ and T-4′ missing the His residue at position 47 in the sequence of CMS-9.

Ca^{2+}-induced difference spectra of native and His-modified CMS-9

The perturbations caused by Ca^{2+} at pH 8.0 are illustrated in Fig. 7A. A blue

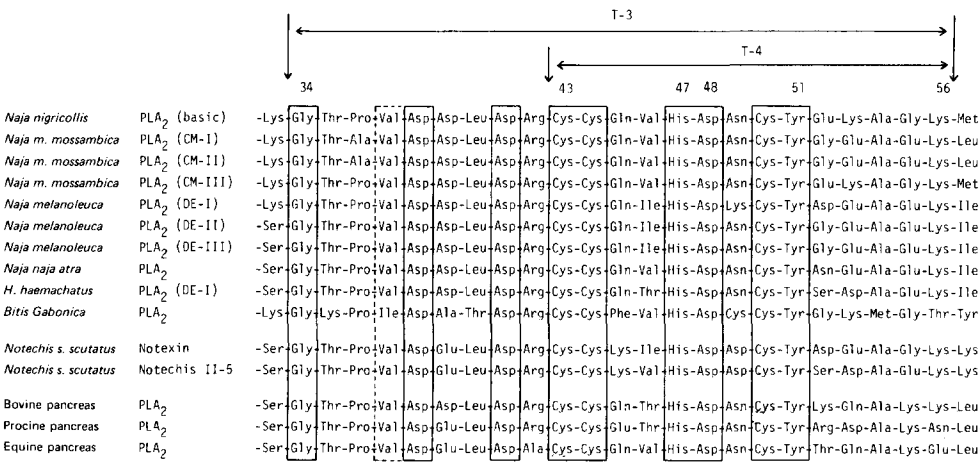

Fig. 6. Partial sequences around the active site His residue of phospholipase A_2 (PLA_2) from snake venoms and mammalian pancreas. The positions of invariant amino acid residues are given in region enclosed in solid lines and similarities of side chain function of the residues in the region enclosed in dashed lines.

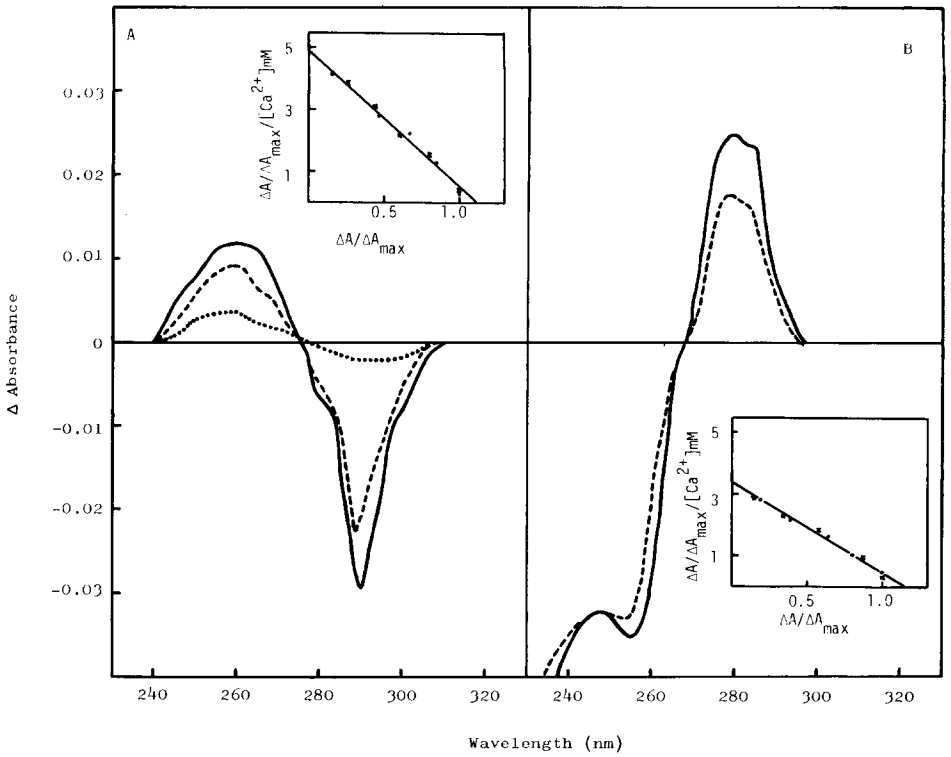

Fig. 7. Spectral changes of native and His-modified CMS-9 induced by Ca^{2+} in 0.025 M Tris-0.1 M NaCl buffer (pH 8.0) at 30°C and the Scatchard plots (inserts). The sample cuvette contained 0.45 mg/ml of native (A) or His-modified CMS-9 (B) in the presence of Ca^{2+} (20 mM, ———; 1 mM, - - - - -; 0.5 mM, · · · · · ·). The cell in the reference position contained 0.45 mg/ml of the respective enzyme and spectra recorded with a 0—0.05 absorbance scale. Scatchard plots of data obtained by Ca^{2+}-induced difference absorbances were monitored at 260 nm (native CMS-9) or 280 nm (His-modified CMS-9). X and · refer to two separate experiments and the solid lines were determined by a linear regression method.

shift of the spectra was noted between 310 and 275 nm while a red shift was observed at shorter wavelength with a peak at 260 nm. Both perturbations could be titrated as a function of Ca^{2+} concentration. The difference spectra of His-modified CMS-9 differ greatly from that of native enzyme in lacking the trough between 310 and 275 nm and the peak at 260 nm, while a new positive perturbation at about 280 nm was observed instead (Fig. 7B). This peak is most likely due to the newly added chromophore, the *p*-bromophenacyl group, which absorbed strongly between 240 and 300 nm with a maximum at 265 nm. This peak was also manifested as a function of Ca^{2+} concentration. The dissociation constants of Ca^{2+} were measured from the Scatchard plot which was obtained by the titration data derived from spectral changes induced by Ca^{2+}. As seen from the inserts in Fig. 7, the dissociation constant of native CMS-9 for Ca^{2+} was 0.20 mM and that of His-modified CMS-9 for Ca^{2+} was 0.29 mM, indicating that the binding ability did not change much after the *p*-bromophenacyl group was incorporated into the enzyme.

Fluorescence study

The interaction between CMS-9 and 8-anilinonaphthalenesulfonate is

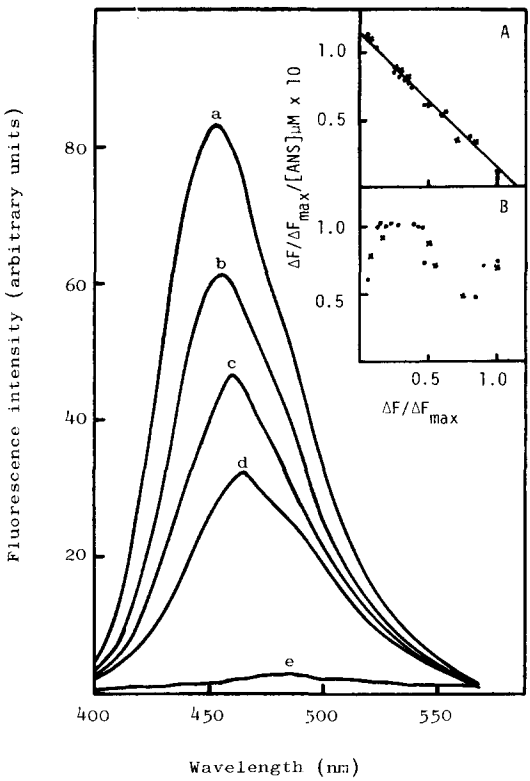

Fig. 8. Effect of Ca^{2+} on the interaction of CMS-9 with 8-anilinonaphthalenesulfonate and the Scatchard plot (inserts). Protein concentration of 0.135 mg/ml and 14 μM 8-anilinonaphthalenesulfonate in 0.025 M Tris-0.15 M NaCl, pH 8.0 (at 25°C), were used in the experiments. Native CMS-9 in the presence of various concentration of Ca^{2+}; a, 0 mM; b, 5 mM; c, 10 mM; d, 20 mM. His-modified CMS-9 in the presence (20 mM) or absence of Ca^{2+} and the fluorescence of 8-anilinonaphthalenesulfonate without protein (e). Scatchard plot with data obtained by fluorescence emission enhancement at 460 nm of various concentration of 8-anilinonaphthalenesulfonate with 2 μM CMS-9 in the presence of 10 mM Ca^{2+} (A) or absence of Ca^{2+} (B). · and X refer to two separate experiments and the straight line was aligned (A) by a linear regression method.

revealed in Fig. 8. The native enzyme not only enhanced the emission intensity of 8-anilinonaphthalenesulfonate dramatically but also shifted the maximum from 480 to 450 nm. However, the emission intensity of the 8-anilinonaphthalenesulfonate-enzyme complex decreased in parallel with the increasing concentration of Ca^{2+} until the saturation level was reached. The emission intensity at the saturated concentration of Ca^{2+} was still much higher than that in the absence of enzyme, while the His-modified CMS-9 did not enhance the emission intensity of 8-anilinonaphthalenesulfonate at all whether in the presence or absence of Ca^{2+}. The insert A in Fig. 8 shows a Scatchard plot derived from the degree of saturation of the emission intensity in the presence of Ca^{2+}, and from which the dissociation constant of 8-anilinonaphthalenesulfonate for the Ca^{2+}-enzyme complex was calculated to be 8.7 μM. This indicates that there is only one kind of 8-anilinonaphthalenesulfonate binding site in the Ca^{2+}-enzyme complex.

Discussion

Wahlström [13] had isolated two forms of phospholipase A_2 (AI and AII) from the venom of *N. nigricollis* by gel filtration on Sephadex G-75 and chromatography on DEAE-cellulose. AII was an acidic protein with p*I* 5.5 and AI was slightly basic with p*I* 7.8. Although the homogeneity of AII had been verified by polyacrylamide gel electrophoresis and other electrophoresis techniques, the author had noted, from the non-integral values of serine (5.5 residues) and valine (6.5 residues), that the AII preparation may possibly be an approximately equal mixture of two enzymes. Recently, Wu et al. [14] have also purified both acidic and basic phospholipase A_2 from the same venom. By isoelectric focusing with Ampholine (pH 3.5—10), they found that the acidic phospholipase A_2 consists of two isozymes, the major one with p*I* 7.9 and the minor one 7.1. Phospholipase A_2 isozymes with slight differences in amino acid sequence have been isolated from the venoms of *Naja mossambica mossambica* (CM-I, CM-II and CM-III) and *Naja melanoleuca* (DE-I, DE-II and DE-III) (15—17).

In the present study, phospholipase A_2 from the venom of *N. nigricollis* was separated into three fractions by chromatography on a column of CM-Sephadex C-25 with very mild pH and salt gradients. After further purification, all three phospholipase A_2 fractions, CMS-5, CMS-6 and CMS-9, revealed as a single band on polyacrylamide gel, which corresponding to three distinct bands of the crude venom. By using Ampholine carrier ampholytes of narrow pH range, the p*I* values were determined to be 7.6, 8.3 and 10.6 for CMS-5, CMS-6 and CMS-9, respectively. As seen from Table IV, the amino acid compositions of CMS-5 and CMS-6 are almost the same, except that CMS-6 contains one more lysine and valine and two less prolines than that of CMS-5. The amino acid compositions of the so-called two acidic phospholipase A_2 preparations, AII of Wahlström [13] and acidic phospholipase A_2 of Wu et al. [14], are also listed in the table for comparison.

The most basic and highly toxic phospholipase A_2 (CMS-9) was subjected to chemical modification with *p*-bromophenacyl bromide at pH 8.0. Although the His-modified CMS-9 lost both its catalytic activity and lethal toxicity, its antigenicity remained unchanged. This suggests that not only His-47 is not involved in the antigenic site, but also that the conformation of the modified CMS-9 changes little and *p*-bromophenacyl bromide specifically attacks the catalytically essentially His-47. In the case of porcine phospholipase A_2, Meijer et al. [18] have observed that anti-pig phospholipase A_2 Fab fragments exhibit a protective effect against active center modification. The present inference, however, does not exclude this observation, since the antigenic site and the active site may partially overlap or steric hindrance may prevent modification of His-47.

Amino acid analysis showed that only one out of three His residues was modified and the modified residue was indentified as His-47. Halpert et al. [19] found that one out of three His residues of myotoxic phospholipase A_2 notexin, was modified by *p*-bromophenacyl bromide with concomitant loss of enzyme activity and toxicity, and the modified residue was determined to be His-47 in the sequence. The same result was also observed in the case of pre-

TABLE IV

COMPARISON OF THE AMINO ACID COMPOSITION OF THE SO-CALLED ACIDIC PHOSPHOLIPASE A_2 FROM *N. NIGRICOLLIS* VENOM

Amino acid	Wahlström [13]	Wu et al. [14]	CMS-5	CMS-6
Aspartic acid	19	18	17	17
Threonine	4	5	4	4
Serine	5 or 6	4	3	3
Glutamic acid	10	9	10	10
Proline	6	6	5	3
Glycine	12	12	12	12
Alanine	9	10	10	10
Half-cystine	14	14	14	14
Valine	6 or 7	6	6	7
Methionine	1	1	1	1
Isoleucine	2	2	2	2
Leucine	9	9	8	8
Tyrosine	10	9	9	9
Phenylalanine	3	3	3	3
Lysine	7	7	5	6
Histidine	3	3	3	3
Arginine	4	7	5	3
Tryptophan	5	5	5	5
Total residues	130	130	122	122

synaptic neurotoxin with phospholipase A_2 activity, β-bungarotoxin which lost both enzyme activity and toxicity after the critical His residue in the homologous position had been modified by *p*-bromophenacyl bromide [20].

The site-specific nature of *p*-bromophenacyl bromide for phospholipase A_2 was revealed not only by the inactivation process in which only one out of three His residues was modified but also by the pronounced protective effect of the essential co-factor Ca^{2+} which slowed down the inactivation rate markedly. This protective effect of Ca^{2+} has been noted for porcine pancreatic phospholipase A_2 [21], phospholipase A_2 from the venom of *N. naja naja* [22], and the presynaptic neurotoxin, β-bungarotoxin [20]. The inactivation of phospholipase A_2 is extremely rapid, while the free histidine does not react with the reagent at all [21]. Roberts et al. [22] had observed that all organic inhibitors of phospholipase A_2 from the venom of *N. naja naja* were hydrophobic phenyl or naphthyl compounds. These observations implicated the hydrophobic nature of the active center of phospholipase A_2.

8-Anilinonaphthalenesulfonate, which had been used as a hydrophobic probe to study the conformational change of proteins, also showed a special affinity to phospholipase A_2. As seen from Fig. 8, CMS-9 not only markedly enhanced the emission intensity of 8-anilinonaphthalenesulfonate but also shifted the maximum from 480 to 450 nm. The conformational change induced by Ca^{2+} could also be manifested by the interaction between 8-anilinonaphthalenesulfonate and the enzyme; the emission-enhancing ability of the enzyme decreased as the concentration of Ca^{2+} increased until the saturated level have been reached. However, there are different results obtained with phospholipase A_2 from different sources, i.e., phospholipase A_2 from porcine pancreas

enhanced the emission intensity of 8-anilinonaphthalenesulfonate more dramatically when Ca^{2+} was present [21], phospholipase A_2 from the venom of *Crotalus adamanteus* did not increase the emission intensity at all [23] and the enzyme from *Crotalus atrox* venom increased the emission intensity but the number of binding sites was reduced as Ca^{2+} bound to the enzyme [24].

In the case of basic *N. nigricollis* phospholipase A_2, the specific binding of 8-anilinonaphthalenesulfonate occurred only after the enzyme complexed with Ca^{2+}. Together with the finding that the His-modified CMS-9 lost the ability to enhance the emission intensity of 8-anilinonaphthalensulfonate and the specific binding of 8-anilinonaphthalenesulfonate only to the Ca^{2+}-enzyme complex, it is suggested that 8-anilinonaphthalenesulfonate may bind to the hydrophobic pocket of the active site. As seen from Fig. 4, although 8-anilinonaphthalenesulfonate showed little protection on the inactivation, marked protective effect was observed once the enzyme complexed with Ca^{2+}. This observation further strengthens the interpretation that 8-anilinonaphthalenesulfonate binds to the active site and due to the steric hindrance, the bulky group of *p*-bromophenacyl bromide is prevented from having access to His-47.

It has been recognized by de Haas et al. [25] and Wells [26] that phospholipase A_2 from porcine pancreas and *C. adamanteus* venom have an absolute requirement for Ca^{2+}. During the modification of CMS-9, Ca^{2+} also showed moderate protection and the protective effect of Ca^{2+} was further enhanced by the addition of 8-anilinonaphthalenesulfonate. This indicates that there is still enough space in the active site of the Ca^{2+}-enzyme complex for *p*-bromophenacyl bromide to react with His-47, and it could be completely occupied by 8-anilinonaphthalenesulfonate. Therefore, it is possible that the protection of Ca^{2+} was non-competitive with respect to the reaction between His-47 and *p*-bromophenacyl bromide.

At pH 8.0, the Ca^{2+}-induced difference spectra of CMS-9 are negative and the minima are 290 and 283 nm, indicating perturbation of tryptophan residues [27,28]. The result of Andrews and Forster [29] on model indole compounds may be applied to distinguish between solvent-induced and charge-induced spectral perturbation. If the ratio of the molar absorption change at 283 nm to that at 292 nm is greater than 0.5, the perturbation is most likely solvent-induced, and if the ratio is less than 0.4, the perturbation is most likely charge-induced. Application of this rule suggests that the Ca^{2+}-induced spectral change is primarily charge-induced. At pH 9.0, the negative signs of the Ca^{2+}-induced difference spectra have also been observed for phospholipase A_2 from *N. naja naja* [28] and *C. adamanteus* venoms [29]. The tryptophan perturbation of these two phospholipase A_2 preparations showed both a major negative maximum at 292 nm and a minor one at shorter wavelength, 283 nm for *N. naja naja* phospholipase A_2 and 286 nm for *C. adamanteus* phospholipase A_2. In both cases, the ratios of the magnitudes of the minima at shorter wavelength to those at 292 nm are less than 0.4. This evidence suggests that Ca^{2+} may facilitate a titratable group in the active site to deprotonate (by lowering its pK_a) and exert a charge effect to relieve the tryptophan perturbation [28,30].

The His-modified CMS-9 not only loses the enzymic activity and the ability to bind 8-anilinonaphthalensulfonate but its Ca^{2+}-induced difference spectra

also differ greatly from that of the native enzyme. In spite of these differences, both native and His-modified CMS-9 could be perturbed as a function of Ca^{2+} concentration. The Scatchard plots reveal that there is only one kind of binding site for each protein.

It is interesting to note that Ca^{2+} exerts pronounced protection on the inactivation process and the modified enzyme still possesses the ability to bind Ca^{2+}. Roberts et al. [22] had reported that phospholipase A_2 from *N. naja naja* venom was also protected from inactivation by *p*-bromophenacyl bromide with Ca^{2+} while the modified enzyme still bound Ca^{2+}. A similar phenomenon was also observed by Halpert et al. [19] but the affinity of the modified notexin for Ca^{2+} was reduced 180-fold. It is a requirement that Ca^{2+} and the *p*-bromophenacyl group do not bind to the same residue, although they may be close and it could be inferred that His-47 is not directly involved in Ca^{2+} binding. Van Dam-Mieras et al. [31] and Slotboom et al. [32] observed a second site for Ca^{2+}, in addition to the site modified by *p*-bromophenacyl bromide, and its affinity for Ca^{2+} would not be altered by the incorporation of the bulky group.

Bearing in mind that the binding of Ca^{2+} can not only cause a titratable group to deprotonate, which exerts a charge-releasing effect to relieve the tryptophan blue shift, but also induce a conformational change which enable the enzyme to be more accessible to the substrate. This Ca^{2+}-induced conformational change not only makes the enzyme more active to perform its catalytic function but also renders it less reactive toward *p*-bromophenacyl bromide. The argument above could be supported not only by the observation that native and His-modified CMS-9 were perturbed by Ca^{2+} but also by the specific binding of 8-anilinonaphthalenesulfonate upon complexing with Ca^{2+}

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