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HISTIDINE AND LYSINE RESIDUES AND THE ACTIVITY OF PHOSPHOLIPASE A_2 FROM THE VENOM OF *BITIS GABONICA* *

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Summary

Chemical modification of phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4) from the venom of gaboon adder (*Bitis gabonica*) showed that histidine and lysine residues are essential for enzyme activity. Treatment with *p*-bromophenacyl bromide or pyridoxal 5′-phosphate resulted in the specific covalent modification of one histidine or a total of one lysine residue per molecule of enzyme, respectively, with a concomitant loss of enzyme activity. Competitive protection against modification and inactivation was afforded by the presence of Ca^{2+} and/or micellar concentrations of substrate analogue, lysophosphatidylcholine. Neither modification caused any significant conformational change, as judged from circular dichroic properties.

Amino acid analyses and the alignment of peptides from cyanogen bromide and proteolytic cleavage of modified enzyme preparations delineated His-45 as the only residue modified by *p*-bromophenacyl bromide. However, pyridoxal 5′-phosphate was shown to have reacted not with a single lysine but with four different ones (residues 11, 33, 58 and 111) in such a manner that an overall stoichiometry of one modified lysine residue/molecule enzyme resulted. Apparently, the essential function of lysine could be fullfilled by any one out of these four residues.

Introduction

Studies to relate structure and function of the lipolytic enzyme, phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4), have shown certain amino

* This publication is dedicated to Professor D.J.J. Potgieter, teacher of biochemistry for 21 years at the University of Pretoria.

Abbreviations: Buffers used as Good et al. [36]: MOPS, 3-(*N*-morpholino)propanesulphonic acid; MES, 2-(*N*-morpholino)ethanesulphonic acid; CHES, 2-(cyclohexylamino)ethanesulphonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; LL, lysophosphatidylcholine; PLP, pyridoxal 5′-phosphate.

acid residues as functionally significant. Wells [1] demonstrated that two tryptophan residues and an abnormally ionising lysine are important for activity of the phospholipase A_2 from the venom of *Crotalus Adamanteus*. In the well-studied phospholipase A_2 from porcine pancreas, Volwerk et al. [2] pinpointed histidine-53 as an active site residue, while the amino-terminal region of the enzyme requires a very precise architecture for its interaction with lipid-water interfaces [3,4]. In the enzyme from the venom of *Bitis gabonica* (gaboon adder), tryptophan-28 was shown to be an active site residue, possibly involved in substrate binding [5].

A catalytic function has been suggested for the histidine residue in the pancreatic enzyme and for the lysine residue in the enzyme from *C. adamanteus*. It is thought that these two phospholipases A_2 follow a general base-catalysed mechanism of action, with the histidine or lysine serving as the general base [6]. Gaboon adder phospholipase A_2 was therefore further investigated by means of chemical modification in order to explore the role that histidine or lysine residues may play in the activity of the enzyme.

Materials and Methods

Chemicals. 1,4-Dithiothreitol, pyridoxal 5'-phosphate, and $NaBH_4$ were bought from E. Merck A.G. (Germany). CNBr was obtained from Fluka A.G. (Switzerland). Lysophosphatidylcholine from egg phosphatidylcholine, grade II, and *p*-bromophenacyl bromide were supplied by Applied Science Laboratories (U.S.A.) and Koch-Light Laboratories (England), respectively. NaB^3H_4 (specific activity 8.62 Ci/g) was a product of New England Nuclear Corp. (U.S.A.). Aquagel from Chemlab (Pty) Ltd., Pinegowrie, Transvaal, Republic of South Africa, was used as scintillation fluid. Trypsin was purchased from Seravac Laboratories, Cape Town, Republic of South Africa, as a twice crystallized, diphenylcarbamyl chloride-treated, salt-free preparation while α-chymotrypsin (three times crystallized) was obtained from Worthington. DEAE-cellulose DE-52 (Whatman) and Sephadex (Pharmacia) were prepared for column chromatography as recommended by the manufacturers.

Enzyme source and enzyme assay. Phospholipase A_2 was prepared from the venom of *B. gabonica* as described by Botes and Viljoen [7]. The enzyme was assayed for activity by the method reported previously [5]. Since Tris buffer was found to compete for pyridoxal 5'-phosphate in the pyridoxylation reaction, assays were carried out in 0.05 M MOPS, pH 8.0.

Chemical modifications. Phospholipase A_2 was modified with *p*-bromophenacyl bromide as described by Volwerk et al. [2] and Halpert et al. [8]. Enzyme (0.08%) was dissolved in 0.1 M HEPES, pH 7.0, containing 0.1 M NaCl. *p*-Bromophenacyl bromide (4 mg dissolved in 1 ml acetone) was added to a 5-fold molar excess, the reaction mixture being stirred magnetically. At suitable intervals aliquots of the reaction mixture were withdrawn for enzyme activity assays. At the completion of the reaction, excess reagent was removed on a column of Sephadex G-25 (50 × 0.9 cm) in 0.1 M NH_4HCO_3.

The number of *p*-bromophenacyl residues incorporated per mol of enzyme was calculated by the method of Halpert et al. [8], which compares the molar

difference extinction coefficient of modified vs. native protein with that of the reagent.

Pyridoxylation of phospholipase A_2 was carried out at room temperature while the reaction vessel was protected from light by aluminium foil. Solutions of phospholipase A_2, containing 0.05—0.2% enzyme in 0.05 M MOPS, pH 8.0, were incubated with stirring with variable amounts of pyridoxal 5′-phosphate for a period of 15 min. Where indicated the resulting Schiff's base was reduced by adding a 10-fold molar excess over pyridoxal 5′-phosphate of $NaBH_4$ solution (in distilled water) to the reaction mixture. A drop of octanol in the reaction mixture prevented excessive foaming. Suitable aliquots were removed during the course of the reaction for enzyme activity assays.

After complete reduction, excess $NaBH_4$ was destroyed by acidification with 10 M acetic acid to a pH of about 4.0 and the reaction mixture incubated for an additional 10 min. The modified enzyme was subsequently desalted on a column of Sephadex G-25 in 0.1 M acetic acid.

^3H-labelled pyridoxylated enzyme was prepared in analogous manner with 3.29 mCi NaB^3H_4 being added with the 10-fold molar excess carrier.

The amount of pyridoxal 5′-phosphate incorporated irreversibly per molecule of enzyme was determined spectrophotometrically from a molar extinction coefficient of 10 000 $M^{-1} \cdot cm^{-1}$ for *N*-phosphopyridoxyllysine at 325 nm [9], by amino acid analysis and by radioactivity measurements.

The incorporation of the phosphopyridoxyl group into the enzyme in the form of the unstable Schiff's base, before borohydride reduction, was followed by absorbance changes at 441 nm, which represents the absorbance difference maximum between enzyme-pyridoxal 5′-phosphate and pyridoxal 5′-phosphate solutions. Absorption maxima above 410 nm in protein-pyridoxal 5′-phosphate complexes are attributed to a structure in which the phenolic hydroxyl of the phosphopyridoxyl moiety is hydrogen bonded to the imine nitrogen of the Schiff's base [10].

Protein determination. Phospholipase A_2 concentrations were determined from absorbance values and $\epsilon_{279} = 2.52 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [11]. Modification of phospholipase A_2 by pyridoxal 5′-phosphate followed by reduction with $NaBH_4$ does not significantly alter the absorbance at 279 nm. However, reaction with *p*-bromophenacyl bromide increased the molar extinction coefficient of the enzyme at 279 nm to $3.7 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ and this value was used for the spectrophotometric calculation of the concentration of modified enzyme. Peptide content of radioactive samples was evaluated by using the Folin-Ciocalteu phenol reagent as described by Lowry et al. [12].

Reduction and S-carboxymethylation of modified enzyme and amino acid analyses. Reduction with dithiothreitol, S-carboxymethylation with iodoacetic acid and amino acid analyses were performed as reported in a previous paper [7]. In pyridoxylated derivatives of phospholipase A_2, tryptophan was determined spectrophotometrically by the method of Edelhoch [13].

Fragmentation by CNBr. Cyanogen bromide cleavage of modified enzyme which had been reduced and S-carboxymethylated, was effected in 70% formic acid as described by Steers et al. [14].

Enzymic digestion. 3 μmol of pyridoxylated, reduced and S-carboxymethylated enzyme was digested with 1% chymotrypsin (w/w) or 1% trypsin (w/w) at 37° C for 2 h in 0.1 M NH_4HCO_3.

Peptide separation. The peptide mixtures obtained by cyanogen bromide splitting of modified enzyme were separated on Sephadex G-50 as described [15]. Peptides obtained by chymotryptic and tryptic digestion of pyridoxylated phospholipase A_2 were separated on Sephadex G-50 (150 × 1.9 cm) in 0.1 M NH_4HCO_3 and DEAE-cellulose (15 × 0.9 cm). DEAE-cellulose columns were developed by a linear salt gradient of 0.02—0.6 M NH_4HCO_3 over 2 l. Pyridoxyl peptides were located in the effluent by measuring the absorbance at 325 nm. Chymotryptic peptides which required further purification after ion-exchange chromatography, were separated by paper electrophoresis at pH 1.9 and pH 4.5, using the paper buffer systems and detection methods described previously [15]. Pyridoxylated peptides were made visible on the paper as fluorescent spots under ultraviolet light.

Radioactivity measurements. Radioactivity of tritiated pyridoxyl protein or peptide samples was determined in a Packard Tri-Carb liquid scintillation spectrometer. Efficiency was evaluated by using *n*-[1,2-^{3}H]hexadecane (specific activity 2.00 μCi/g) as internal standard.

Spectroscopy. Spectra were recorded in small volume, 4 mm wide, 1 cm pathlength fused quartz cuvettes in a Varian Techtron Model 635 double beam spectrophotometer, coupled to a Hitachi Model QPD73 recorder. CD spectra were obtained with a Jasco Model J-20 recording spectropolarimeter using cells of 1 cm pathlength in the near ultraviolet region and cells of 0.02 cm light path below 250 nm. Mean residue ellipticity was calculated using a mean amino acid residue weight of 113, based on the known amino acid composition of the enzyme [7]. Helix and β-structure contents were estimated as reported recently [5].

Results

Alkylation with p-bromophenacyl bromide

Phospholipase A_2 could within 2—2.5 h be completely inactivated by treatment with *p*-bromophenacyl bromide. From the extinction values and absorbance readings of the reagent and the protein at 279 nm, it could be calculated that one *p*-bromophenacyl moiety was incorporated per molecule of enzyme. Amino acid analysis confirmed that the only residue modified was one of the two histidines of gaboon adder phospholipase A_2 (Table I). Methionine, which is another possible site for alkylation by the reagent [16], was unaffected.

Gaboon adder phospholipase A_2 contains two histidine residues in position 45 and 107, respectively (cf. ref. 15 and Fig. 1). The modified histidine was located after treating the alkylated enzyme with CNBr and isolating the two histidine-containing peptides CN-3 and CN-4 [15]. Table I gives the amino acid analyses and the positions assigned to the two peptides in the sequence of phospholipase A_2. By amino acid composition peptides CN-3 and CN-4 correspond to the segments Gly-13 → Met-52 and Gly-53 → Cys-118, respectively (cf. ref. 15 and Fig. 1), except that peptide CN-3 from the modified enzyme lacked a histidine residue. His-45 was therefore identified as the site of modification by *p*-bromophenacyl bromide.

Under the experimental conditions used, relatively low concentrations (50 μM) of micellar lysophosphatidylcholine [17], a substrate analogue of phos-

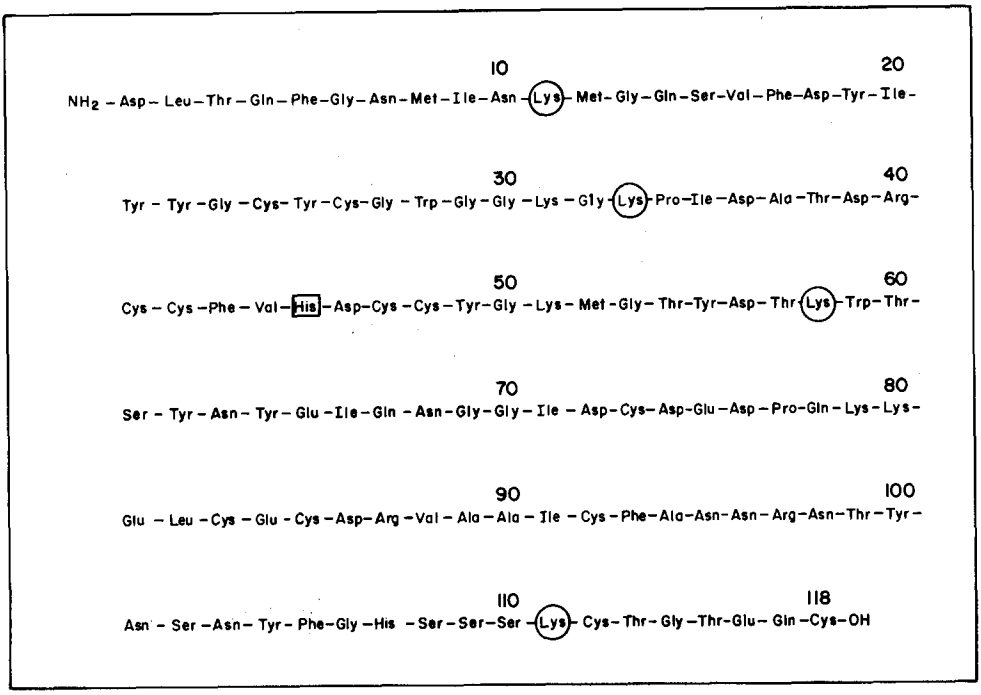


Fig. 1. Primary structure of gaboon adder phospholipase A_2 [15]. Histidine and lysine residues that were modified are indicated.

pholipase A_2 [2,18], failed to protect the reactive histidine against modification by *p*-bromophenacyl bromide. The half-time of the reaction was observed to be similar to that of the reaction in the absence of lysophosphatidylcholine ($t_{1/2}$ = 8 min). However, protection was afforded by increasing the concentration of lysophosphatidylcholine to 150 μM ($t_{1/2}$ = 11 min). Ca^{2+} was also found to protect against *p*-bromophenacyl bromide inactivation ($t_{1/2}$ = 14 min) while the best protection could be obtained with both Ca^{2+} and lysophosphatidylcholine present ($t_{1/2}$ = 17 min).

Pyridoxylation

Pyridoxal 5'-phosphate can be used to identify reactive lysine residues in enzymes which do not require this compound as cofactor [19]. Fig. 2 depicts the time course of inactivation of gaboon adder phospholipase A_2 at various pyridoxal 5'-phosphate concentrations. Enzyme activity declined within 8—10 min to a constant value which depended on the amount of inhibitor used. The curves show that it was not possible in the absence of borohydride reduction to abolish activity completely and even at the highest pyridoxal phosphate excess (200 : 1, molar basis), the enzyme still possessed 40% activity.

Results similar to those reported here, were found by Chen and Engel [20] for the reaction of glutamatic dehydrogenase with pyridoxal 5'-phosphate. They reasoned that the formation of a non-covalent enzyme-modifier complex

(which is still active because of rapid dissociation of the complex) is in equilibrium with a covalent step in which inactive enzyme is produced by the generation of a Schiff's base (see below).

The kinetics of the reaction between enzyme and pyridoxal 5'-phosphate alone was studied under conditions where the reagent was added in excess. Linear first-order plots were obtained (Fig. 3) which indicate that the modification does not involve groups with different reactivities. Rate constants calculated from the slopes of the first-order plots in Fig. 3 showed a hyperbolic dependence on the concentration of the modifier suggesting that a specific binding site for pyridoxal 5'-phosphate is progressively saturated. The following mechanism for the inactivation of enzyme by pyridoxal 5'-phosphate (PLP) was put forward by Chen and Engel [20]:

TABLE I

AMINO ACID COMPOSITION AND ALIGNMENT OF PEPTIDES DERIVED FROM CNBr CLEAVAGE AND PROTEOLYTIC HYDROLYSIS OF MODIFIED PHOSPHOLIPASE A_2

Values in parentheses are those expected from the known sequences.

Amino acid	Bromophenacylated Enzyme		Pyridoxylated enzyme			
	CN-3	CN-4	T-1	T-2	T-3	T-4
S-CM-Cys	5.89 (6)	5.64 (6)	2.10 (2)	3.52 (4)	0.94 (1)	1.94 (2)
Asp	3.89 (4)	11.63 (12)	4.05 (4)	2.82 (3)	5.71 (6)	2.86 (3)
Thr	0.82 (1)	5.52 (6)	0.92 (1)	0.80 (1)	2.83 (3)	2.71 (3)
Ser	0.79 (1)	4.82 (5)	0.96 (1)		0.97 (1)	3.63 (4)
Glu	0.95 (1)	7.69 (8)	2.17 (2)		4.17 (4)	2.09 (2)
Pro	1.12 (1)	0.92 (1)		0.83 (1)	0.70 (1)	
Gly	6.82 (7)	5.08 (5)	5.83 (6)	2.01 (2)	2.88 (3)	2.01 (2)
Ala	0.81 (1)	2.83 (3)		0.95 (1)		
Val	1.85 (2)	1.09 (1)	1.03 (1)	0.79 (1)	0.79 (1)	
Met	1* (1)		1.81 (2)		0.70 (1)	
Ile	1.81 (2)	2.89 (3)	1.93 (2)	0.69 (1)	1.74 (2)	
Leu		0.93 (1)	0.97 (1)			
Tyr	4.72 (5)	4.89 (5)	3.69 (4)	0.74 (1)	2.65 (3)	1.88 (2)
Phe	1.96 (2)	1.99 (2)	1.88 (2)	0.86 (1)		0.88 (1)
Lys	3.91 (4)	4.02 (4)	1.10 † (2)	1.03 † (2)	1.88 † (2)	0 †† (1)
His	0.05 (1)	0.87 (1)		0.89 (1)		0.73 (1)
Arg	1.10 (1)	1.99 (2)		0.89 (1)		
Trp	(1)	(1)	(1) **		(1) **	
Sequence alignment	Gly-13 → Met-52	Gly-53 → Cys-118	Asp-1 → Lys-31	Gly-32 → Lys-51	Met-52 → Lys-80	Asn-98 → Cys-118
ϵ_{325}	—	—	—	—	—	—
Specific activity (cmp/μmol)	—	—	$4.96 \cdot 10^5$	$3.18 \cdot 10^5$	$5.01 \cdot 10^5$	$4.15 \cdot 10^5$

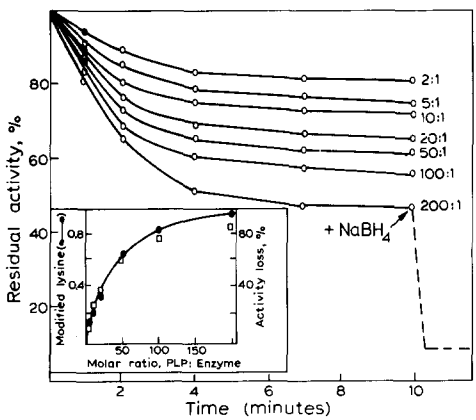
* Methionine was determined as homoserine and its lactone.
** Determined by the method of Edelhoch [13].
*** Ehrlich positive peptide.
† These values do not include lysine derivatized as phosphopyridoxyllysine.
†† These peptides contained only phosphopyridoxyllysine.

$$E + PLP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \ldots PLP \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E - PLP \xrightarrow{NaBH_4} E' - PLP$$

Non-covalent complex; Covalent complex (Schiff's base); Reduced complex

where $k_2/k_1 = K$, the dissociation constant for the non-covalent enzyme-modifier complex. A Michaelis-Menten type of treatment of the slow first-order process of covalent bond formation preceded by rapid reversible binding of pyridoxal 5'-phosphate allows calculation of the dissociation constant, K, and k_3, the rate constant for the interconversion of the non-covalent complex to the covalent Schiff's base. This treatment gave values of $1.5 \cdot 10^{-4}$ M for K and 0.44 min^{-1} for k_3. Second-order rate constants obtained by dividing the observed first-order rate constants by the modifier concentration decreased with increasing inhibitor concentration, which serves as further evidence for the validity of the kinetic mechanism outlined above [21].

C-1	C-2	C-3	C-4	CN-2	CN-3	CN-4
	2.02 (2)		1.73 (2)		5.61 (6)	5.80 (6)
1.96 (2)	1.94 (2)	0.98 (1)		0.92 (1)	3.98 (4)	12.13 (12)
	0.85 (1)	0.75 (1)	1.92 (2)		0.94 (1)	5.75 (6)
0.84 (1)			2.83 (3)		0.85 (1)	4.70 (5)
1.08 (1)			2.12 (2)		1.20 (1)	8.16 (8)
	0.73 (1)				0.86 (1)	0.93 (1)
1.93 (2)	3.01 (3)		1.98 (2)		7.08 (7)	5.05 (5)
	0.95 (1)				1.10 (1)	2.93 (3)
0.84 (1)					1.70 (2)	0.90 (1)
1.60 (2)				1 *	1 *	
0.92 (1)	0.89 (1)			0.72 (1)	2.09 (2)	2.90 (3)
						0.82 (1)
					4.68 (5)	4.83 (5)
1.00 (1)	0.86 (1)				1.90 (2)	1.79 (2)
0 ‡‡ (1)	0.93 ‡ (2)	0 ‡‡ (1)	0 ‡‡ (1)	0 ‡‡ (1)	2.84 ‡ (4)	2.01 ‡ (4)
			0.89 (1)		0.80 (1)	0.83 (1)
	0.90 (1)				1.06 (1)	1.94 (2)
		(1) ***			(1) **	(1) **
Gly-6 → Phe-17	Gly-29 → Phe-43	Asp-56 → Trp-59	Gly-106 → Cys-118	Ile-9 → Met-12	Gly-13 → Met-52	Gly-53 → Cys-118
—	—	—	—	2300	2500	4800
—	—	—	—	—	—	—



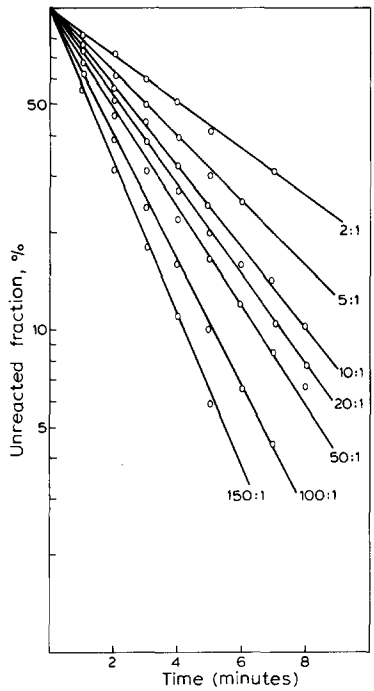


Fig. 2. Time course of inactivation of phospholipase A_2 as a function of pyridoxal 5'-phosphate concentration. Incubations with pyridoxal 5'-phosphate were carried out at the indicated molar excesses on 0.05% enzyme solutions in 0.05 M MOPS, pH 8.0, and 2-μl aliquots were assayed. At the time indicated by the arrow the remainder of the samples was treated with $NaBH_4$ at a 10-fold molar excess over modifier concentration for subsequent amino acid analysis. Inset: The extent of lysine modification and loss of enzyme activity (□————□) as a function of pyridoxal 5'-phosphate concentration. Activities were determined on reduced samples.

Fig. 3. First-order plots of the changes in absorbance at 441 nm with time at different molar excesses of pyridoxal 5'-phosphate over 0.05% phospholipase A_2 in 0.05 M MOPS, pH 8.0.

Whereas treatment with $NaBH_4$ did not show any effect on enzyme alone, addition of this reagent to reaction mixtures containing enzyme-pyridoxal 5'-phosphate complexes abolished activity (cf. Fig. 2), and inactivation thus became irreversible. The relation between the amount of phosphopyridoxyllysine formed and the loss in enzyme activity is illustrated in Fig. 2 (inset). At a 200-fold molar excess of reagent, one molecule of pyridoxal 5'-phosphate is incorporated per molecule of enzyme with a concomitant complete loss of catalytic activity. The observed stoichiometry was confirmed by quantitating the radioactive label in phospholipase A_2 when the reduction step was carried out with tritiated NaB^3H_4 (0.91 radioactive lysine residues/enzyme molecule; specific activity = $4.7 \cdot 10^5$ cpm/μmol enzyme). Amino acid analysis established that only lysine reacted with the modifier since the rest of the amino acid composition agreed with that of native enzyme.

The reactive residue could be protected against pyridoxylation by micellar concentrations of lysophosphatidylcholine in the reaction mixture. Material modified in the presence of 0.6 mM lysophosphatidylcholine (Fig. 4) and subsequently reduced, still possessed catalytic activity to an extent of 47% of the original while 0.5 residue of phosphopyridoxyllysine had formed. The protec-

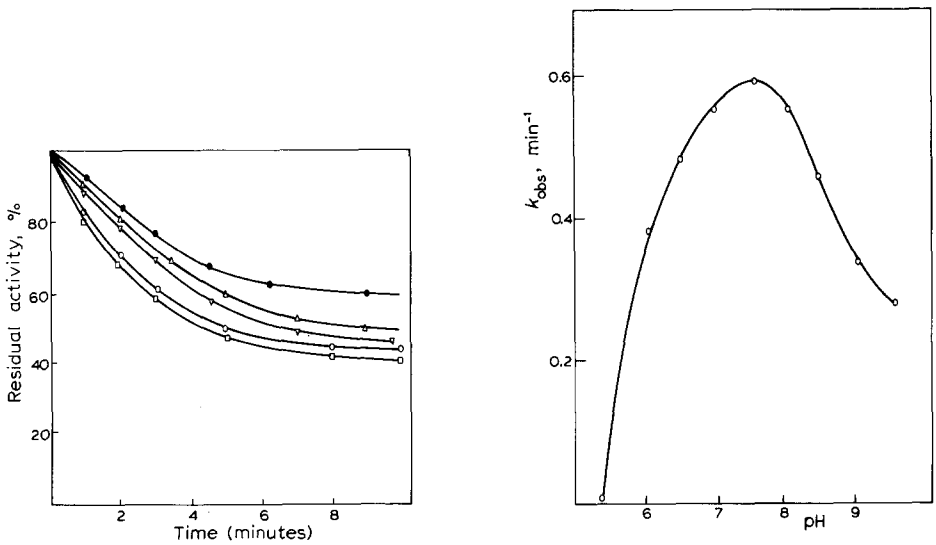


Fig. 4. Protective effect of Ca^{2+} and lysophosphatidylcholine on the inactivation of phospholipase A_2 by pyridoxal 5′-phosphate. Enzyme (0.05%) in 0.05 M MOPS, pH 8.0 was reacted with a 200-fold molar excess of inhibitor. Activities were determined on unreduced samples. ○———○, no additions; □———□, + 1 mM Ca^{2+}; △———△, + 0.6 mM lysophosphatidylcholine; ▽———▽, + 1 mM Ca^{2+} + 0.6 mM lysophosphatidylcholine; ●———●, + 1 mM lysophosphatidylcholine.

Fig. 5. Effect of pH on the rate of inactivation of phospholipase A_2 by pyridoxal 5′-phosphate. 0.05% enzyme was inactivated with a 100-fold molar excess of modifier. Rate constants were calculated from semilogarithm plots similar to those of Fig. 3. The following buffers were used at the various pH values: pH 5.0—pH 7.0, 0.05 M MES; pH 7.5—pH 8.5, 0.05 M MOPS; pH 9.0—pH 9.5, 0.05 M CHES.

tion was more pronounced at a higher concentration (1 mM) of lysophosphatidylcholine, giving a product with 65% residual activity and only 0.3 residue of modified lysine. No protective effect could be seen when Ca^{2+} was added to reaction mixtures (cf. Fig. 4).

In Fig. 5 the results are presented for the incubation of phospholipase A_2 with pyridoxal 5′-phosphate at different pH values. The inactivation rate constant is shown to be pH dependent, reaching a maximum value at about pH 7.5. The first-order rate constants were calculated from the slopes of semilogarithm plots similar to those described in Fig. 3. Groups having pK values of about 6.0 and 8.6 are apparently rate-determining in the reaction between enzyme and modifier.

Inactive pyridoxylated enzyme which had been reduced and S-carboxymethylated, was subjected to proteolytic cleavage in order to identify the reactive lysine residue(s). Gel filtration of a chymotryptic digest followed by DEAE-cellulose chromatography, and where necessary paper electrophoresis, gave four fluorescent peptides having the amino acid composition summarized in Table I. By composition, peptides C-1 to C-4 corresponded to the segments Gly-6 → Phe-17, Gly-29 → Phe-43, Asp-56 → Trp-59 and Gly-106 → Cys-118, respectively. The phosphopyridoxyl moiety was therefore unexpectedly located at four positions in the phospholipase A_2 chain, viz. at Lys-11, Lys-58, Lys-111 and as far as peptide C-2 was concerned at either Lys-31 or Lys-33. The latter ambiguity was resolved by tryptic hydrolysis of tritiated phosphopyridoxyl-phospholipase A_2. The radioactive peptides that were isolated showed that a

tryptic split did not occur at phosphopyridoxyl lysines. Peptide T-1 extended from Asp-1 → Lys-31 while peptide T-2 corresponded to the segment Gly-32 → Lys-51. In addition tryptic peptides also containing the radioactive label were found to extend from Met-52 → Lys-80 (T-3) and Asn-98 → Cys-118 (T-4), respectively. These results unequivocally identified Lys-11, Lys-33, Lys-58 and Lys-111 as the sites of pyridoxylation. Apparently each of these sites incorporated label to a comparable extent since the specific radioactivities reported in Table I for the four peptides ($3.2 \cdot 10^5$—$5.0 \cdot 10^5$ cpm/μmol peptide) are not intolerably different. This finding was further corroborated by isolating cyanogen bromide fragments CN-2, CN-3 and CN-4 corresponding to the segments shown in Table I. The sum of the observed molar extinction coefficients at 325 nm of 2300, 2500 and 4800 $M^{-1} \cdot cm^{-1}$ for peptides CN-2, CN-3 and CN-4, respectively, almost equalled the total molar extinction of 10 000 $M^{-1} \cdot cm^{-1}$ at 325 nm for the modified protein. Peptide CN-2 contains Lys-11, peptide CN-3, Lys-33 and peptide CN-4, Lys-58 and Lys-111.

Spectroscopy

Pyridoxylated enzymes which had been reduced with $NaBH_4$ showed the typical protein absorption spectrum of native phospholipase A_2 together with a new absorption band found at 323 nm (Inset, Fig. 6) which is characteristic of the reduced *N*-ε-phosphopyridoxyllysine group [9].

Fig. 6 describes the near ultraviolet CD spectra of native phospholipase A_2 and the bromophenacylated and pyridoxylated forms of the enzyme. The negative CD bands near 300, 285 and 260 nm in the native enzyme were replaced by positive bands with maxima at 293 and 275 nm for enzyme modified by pyridoxal 5'-phosphate. In addition a broad positive band appeared in the visible region around 330 nm. Modification of His-45 with *p*-bromophenacyl

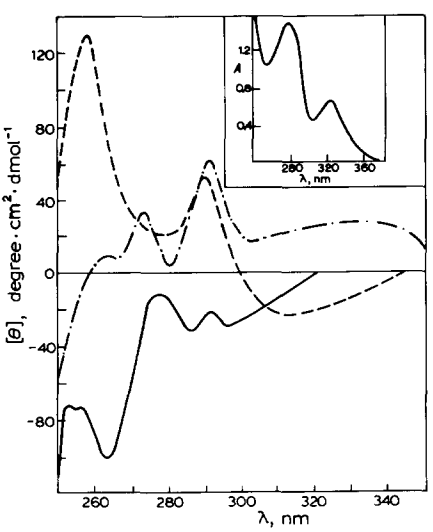


Fig. 6. Near ultraviolet circular dichroism spectra of native phospholipase A_2 and enzyme completely inactivated with *p*-bromophenacyl bromide or pyridoxal 5'-phosphate. Enzyme solutions were prepared in 0.05 M MOPS, pH 8.0. ———, phospholipase A_2 (2.2 mg/ml); - - - - - -, *p*-bromophenacyl bromide-treated phospholipase A_2 (1.4 mg/ml); — · — · —, pyridoxylated phospholipase A_2 (1.1 mg/ml). Inset: The absorption spectrum of pyridoxylated enzyme (1 mg/ml) in 0.05 M MOPS, pH 8.0).

bromide resulted in enzyme showing positive Cotton effects with extrema near 290 and 260 nm, and a new broad negative band at about 315 nm.

Neither modification influenced the deep ultraviolet CD spectrum to any significant extent (results not shown). From the observed ellipticities at 208 and 204 nm relative to those of model polypeptides [22] estimates of 18% helix and 45% β-sheet were obtained for pyridoxylated enzyme. The corresponding values for *p*-bromophenacyl bromide-treated enzyme were 22% helix and 39% β-structure. These figures are for practical purposes the same as the 22% helix and 45% β-structure reported for the native enzyme [5].

Discussion

Studies on the amine-catalysed methanolysis of phosphatidylcholine [23] coupled with investigations on phospholipase A_2 from *C. adamanteus* [1,24,25] led Wells [23] to propose a base-catalyzed mechanism of action for the enzyme according to which a reactive lysine generates the active nucleophile by abstracting a proton from water. Sigman and Mooser [6] suggested that this role is fulfilled in porcine pancreatic phospholipase A_2 by a histidine residue, located as His-53 in the primary sequence of the enzyme [2]. The effect of pH on the activity of gaboon adder phospholipase A_2 showed a group in the enzyme-substrate complex having an apparent pK_a value of 7.5 to be important in catalysis [11], implicating an imidazolium or ammonium group. The chemical modification of this enzyme, which earlier showed Trp-28 to be involved in substrate binding, was therefore extended to cover histidine and lysine residues.

An essential histidine in *B. gabonica* phospholipase A_2 was unambiguously identified by reaction of the enzyme with *p*-bromophenacyl bromide. The modification established that the parallel loss in enzyme activity can be related to reaction with a specific residue identified as His-45 by amino acid analyses and peptide alignment. No reaction took place with the only other histidine of *B. gabonica* phospholipase A_2 viz. His-107 (cf. Fig. 1).

The involvement of His-45 in the active site of the enzyme was further substantiated by the fact that it could be partially protected against reaction with *p*-bromophenacyl bromide by Ca^{2+}. Similar observations have been made by Volwerk et al. [2] and Halpert et al. [8], who, respectively, modified an equivalent histidine with the same inhibitor in pancreatic phospholipase A_2 and notexin, a toxic phospholipase A_2 from *Notechis scutatis scutatis*. These results all suggest that the reactive histidine lies close to the calcium binding site, a suggestion which seems to be borne out by the proposed location of bound Ca^{2+} near His-55 in crystalline pancreatic prephospholipase A_2 [26].

The bromophenacylation rate of His-45 was slowed down by the presence of a substrate analogue such as lysophosphatidylcholine. The fact that protection was more effective at a concentration far above, rather than at the critical micelle concentration (150 vs. 50 μM) suggests a mass action effect *. The protection by Ca^{2+} together with lysophosphatidylcholine was additive.

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* We did not observe an increase in the solubility of the reagent above the critical micelle concentration of egg L-lysophosphatidylcholine, in contrast to its reported behaviour in the presence of micelles of short-chain synthetic D-phosphatidylcholines [2].

The phospholipases A_2 investigated to date [15,27—31] all contain a histidine near position 50, and if the sequences are properly aligned [26], this residue appears to be invariant in all these enzymes. The recently published crystal structure [26] of porcine prephospholipase A_2 shows that the side chains of His-55, Asp-56 and Tyr-35 are spatially close to each other. It led the authors to postulate that the aspartate carboxylate could function as nucleophile with the imidazolium and phenolic groups respectively, acting as stabilizer and proton donor to the putative tetrahedral intermediate formed after nucleophilic attack by Asp-56 on the carbonyl carbon of the ester bond in a phospholipid. This would then explain the invariance of the histidine and the loss in activity after selective chemical modification observed for three different phospholipases A_2. It is, of course, possible that the imidazolium group may rather act as generator of the nucleophile in the reaction, as has been suggested [6] earlier for a general base-catalysed mechanism of phospholipase A_2 action.

It is known that pyridoxal 5′-phosphate possesses characteristics which render it suitable as an affinity label for enzymes [32] with specificity for phosphate-containing substrates. The present report confirmed that the interaction of this modifier and gaboon adder phospholipase A_2 is of the active site-directed type, since the inhibition reaction velocities measured as a function of pyridoxal 5′-phosphate concentration showed saturation kinetics and complete loss in enzyme activity was found at the stage where one molecule of pyridoxal 5′-phosphate had been incorporated per enzyme molecule (Fig. 2, inset). The observed stoichiometry and the protection against lysine modification and inactivation by substrate analogues constitute evidence for the specificity of the reaction.

The appearance of extrinsic Cotton effects related to modifier absorption bands in the near ultraviolet CD spectrum of the pyridoxylated enzyme suggested that the covalent attachment of the modifier group occurred in a stereospecific manner and what is more, without affecting (denaturing) the polypeptide backbone conformation, since the deep ultraviolet CD spectrum was unchanged from that of the native enzyme (the same conclusion also applies to the histidine modification with *p*-bromophenacyl bromide described above).

Selectivity of pyridoxylation of enzymes not requiring pyridoxal 5′-phosphate as cofactor is ascribed to the proximity of reactive lysines to phosphate binding sites, the positively charged nature of which would reduce the pK values of the relevant ϵ-amino groups. In turn, this property will favour aldimine formation [19,33]. The bipolar pyridoxal 5′-phosphate, with its negatively charged phosphate separated a similar distance from the positively charged quarternary nitrogen as in phospholipids, may resemble the zwitterionic natural substrate of phospholipase A_2 sufficiently closely to become bound to the enzyme in a manner analogous to substrate. The inactivation rate vs. pH profile in Fig. 5 shows that groups of p$K \approx 6.0$ and ≈ 8.6 control the reaction between modifier and enzyme. These may be related to binding, since the values correspond with the pK values of the phosphate (pK 6.0) and pyridinium nitrogen (pK 8.7) groups of pyridoxal 5′-phosphate [34]. The observed decreases in pyridoxylation rates at acid and alkaline pH would then reflect poorer binding of modifier. If so, the state of ionization of appropriate groups on the enzyme could be expected to complement those on the substrate or inhibitor.

A completely unexpected finding upon localization of the presumed single modified lysine by peptide analyses was that not one, but four different residues, viz. lysines-11, -33, -58 and -111 in the phospholipase sequence (cf. Fig. 1) were modified. Quantitation by either amino acid content, spectrophotometric or radioactivity measurements gave internally consistent values from three different experiments. The specific activity of the modified intact protein ($4.7 \cdot 10^5$ cpm/μmol) fell within the range found for the 4 isolated peptides ($3.2 \cdot 10^5$ —$5.0 \cdot 10^5$ cpm/μmol peptide) implying that the appropriate lysine residues were modified in a mutually exclusive manner.

This would seem to require a close proximity of the relevant residues such that pyridoxylation of one may sterically hinder reaction with any of the other three. A similar explanation was offered when the interaction of RNAase A and pyridoxal 5'-phosphate was studied [19]. In that investigation Raetz and Auld also noted a 1 : 1 stoichiometry while peptide analysis showed that in fact two lysines were modified. Inspection of the stereo plot of the main chain of porcine prephospholipase [26] shows that lysine residues comparable to these occur in a surface region distal to the postulated binding sites of the enzyme for calcium and the hydrophobic fatty acyl chains of phospholipids, but proximal to that for the phosphate moiety of a substrate. A similar spatial arrangement in the adder phospholipase may explain why Ca^{2+} gave no protection against the modification of lysine residues, whereas lysophosphatidylcholine did.

An intriguing alternative is suggested by the explanation offered by Walter and Wold [35] for their analogous finding that the activity loss of ribonuclease A upon acetylation correlated with the sum of the fractional modification of three different lysines located fairly close to each other in the RNAase crystal structure. They namely proposed that a lysine amino group is required for catalysis (e.g. by providing a positive charge whereby the substrate may be fixed in the correct stereospecific orientation), and that this requirement may be satisfied by any one of the lysine residues found close to the substrate binding site. Experiments are at present being undertaken with a view to find supporting evidence for or eliminate some of these alternatives for phospholipase A_2.

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