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AN ESSENTIAL TRYPTOPHAN IN THE ACTIVE SITE OF PHOSPHOLIPASE A_2 FROM THE VENOM OF *BITIS GABONICA*

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Summary

The role of tryptophan in phospholipase A_2 (EC 3.1.1.4) from the venom of the gaboon viper, *Bitis gabonica*, has been investigated. Modification of the enzyme with *N*-bromosuccinimide and 2-nitrophenylsulfenylchloride showed that the two tryptophan residues in the enzyme, viz. Trp-28 and Trp-59, differ in reactivity towards the reagents. Only Trp-28 reacted with *N*-bromosuccinimide while a preferential reaction occurred between Trp-59 and 2-nitrophenylsulfenylchloride. In each case it was found that loss of enzyme activity was specifically correlated with modification of Trp-28. CD spectra indicated that neither the local nor the gross conformation of the enzyme was altered by modification of Trp-28 and it was therefore concluded that Trp-28 is crucial for enzyme activity. The active enzyme was protected against *N*-bromosuccinimide inactivation by micellar concentrations of substrate or substrate analogue, suggesting that Trp-28 is involved in substrate binding.

Introduction

Phospholipases A_2 (EC 3.1.1.4) from various sources, have been studied fairly extensively as regards their specificity [1], primary structure [2—7], kinetic parameters [9,10] and mechanism of action [11—20]. However, relatively little is known about the specific amino-acid residues contributing to the active sites of the enzymes. Several workers found that organophosphorous compounds have no inhibitor effect on phospholipase A_2 [21—23] but there is a report implicating a reactive serine in the enzyme from *Crotalus atrox* which could be inhibited by diisopropylfluorophosphate [24]. Photooxidation results led Salach et al. [22] to suggest histidine as an essential functional residue in the phospholipase A_2 of *Naja naja* venom, while Wells [25] showed that an

abnormally ionising lysine and two tryptophan residues of the dimeric enzyme from *Crotalus adamanteus* are important for activity.

Chemical modifications carried out on the phospholipase A_2 from porcine pancreas demonstrated that the α-amino group is essential for an activity of the enzyme [26] and in a recent publication Volwerk et al. [27] described studies on the functional significance of histidine-53 in this enzyme.

We report here on chemical modification and spectroscopic investigations of a phospholipase A_2 of known primary structure from the venom of the gaboon adder, *Bitis gabonica* [4] whereby the role played by its tryptophan residues was elucidated.

Materials and Methods

Chemicals. 1,4-Dithiothreitol, trifluoroacetic acid and *N*-bromosuccinimide were products from E. Merck A.G. (Germany) while 1,2-dipalmitoyl-L-α-lecithin, 2-nitrophenylsulfenylchloride, CNBr and phenylisothiocyanate were obtained from Fluka A.G. (Switzerland). Dicaproyl-L-α-lecithin and lysolecithin from egg lecithin, grade II, were supplied by Applied Science Laboratories (U.S.A.) and Sigma Chemical Co. (U.S.A.), respectively. α-Chymotrypsin (three times crystallized) was bought from Worthington. Sephadex G-50, purchased from Pharmacia (Sweden) was prepared for column chromatography as recommended by the manufacturers.

Enzyme source. Phospholipase A_2, purified from *B. gabonica* venom [28] was used as enzyme source.

Enzyme assay. Phospholipase A_2 activity measurements were based on its ability to clear phospholipid-containing egg-yolk suspensions as described by Marinetti [29]. Reaction rates were monitored at 740 nm on a Varian Techtron spectrophotometer Model 635. The reaction was carried out in reaction mixtures of 2.5 ml total volume.

Modification with N-bromosuccinimide. Modification of phospholipase A_2 with *N*-bromosuccinimide was carried out according to the procedure described by Spande et al. [30]. Depending on the scale of preparation 10—100-μl aliquots of 0.01 M *N*-bromosuccinimide were added serially to an 0.04—0.1% enzyme solution at the desired pH. Where necessary, the reaction mixtures contained the additions indicated in Table I. After each addition of *N*-bromosuccinimide an aliquot of the reaction mixture containing 2 μg of enzyme, was withdrawn for the determination of enzyme activity. The number of tryptophan residues oxidised per molecule of enzyme was calculated from the equation [30]:

$$\text{Number of tryptophan residues oxidised} = \frac{\Delta A_{278\ \text{nm}} \times 1.31}{5500 \times \text{enzyme molarity}}$$

Protein concentration was determined from absorbance values and $\epsilon_{278} = 2.52 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [31].

Modification with 2-nitrophenylsulfenylchloride. Phospholipase A_2 was sulfenylated with 2-nitrophenylsulfenylchloride as described by Scoffone et al. [32]. Enzyme was dissolved in 30% acetic acid to give an 0.5% protein solution to which the modifying reagent was added in the desired amounts. The reaction

TABLE I

OXIDATION OF PHOSPHOLIPASE A_2 WITH *N*-BROMOSUCCINIMIDE UNDER DIFFERENT REACTION CONDITIONS

Reaction mixtures containing 0.1% enzyme were titrated with 0.01 M *N*-bromosuccinimide. The pH indicated was obtained by using the following buffers: pH 3.5: 0.11 M phosphate/citrate; pH 5.5: 0.1 M ammonium acetate; pH 6.5: 0.1 M ammonium acetate; pH 7.8: 0.1 M Tris · HCl. 1,2-Dipalmitoylglycerophosphocholine (DPL) was added as a solution in 10 μl of ethanol. LL, lysolecithin

Mol of *N*-bromo-succinimide per mol of enzyme	Mol of tryptophan modified/mol of enzyme at								
	pH 3.5	pH 5.5	pH 5.5 + 1 mM Ca^{2+}	pH 5.5 + 30 μM DPL	pH 7.8	pH 7.8 + 1 mM Ca^{2+}	pH 7.8 + 97 μM LL	pH 7.8 + 1 mM Ca^{2+} + 97 μM LL	pH 7.8 + 1 mM Ca^{2+} + 39 μM LL
1	0.42	0.24	0.26	0.18	0.48	0.50	0.31	0.30	0.45
2	0.92	0.44	0.50	0.42	0.75	0.71	0.64	0.42	0.61
3	1.22 [a]	0.82	0.78	0.72	0.90	0.83	0.73	0.51	0.65
4	1.50 [a]	0.96	1.00	0.82	0.99	0.94	0.73	0.52	0.70
Residual activity (%)	2 [b]	2.5	3	n.d.	4.2	5	30	44	25

[a] Apparent tryptophan modification (see text).

[b] Obtained at a 2.2-fold molar excess of *N*-bromosuccinimide. Residual activities at all other pH values are those obtained after addition of a 4-fold molar excess.

n.d. not determined.

was allowed to proceed for 1 h after which excess reagent was removed on a column of Sephadex G-25 (50 × 1.9 cm) in 0.05 M ammonium acetate. The extent of sulfenylation was estimated spectrophotometrically at 365 nm from $\epsilon_{365} = 4000\ M^{-1} \cdot cm^{-1}$ [32]. The concentration of modified enzyme was determined by using $\epsilon_{278} = 2.52 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ and correcting for the absorbance contribution due to derivatization of tryptophan [33].

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 described in a previous paper [28]. Tryptophan was determined spectrophotometrically by the method of Edelhoch [34].

Cleavage by CNBr. The procedure of Gross and Witkop [35] as modified by Steers et al. [36] was employed. 6 μmol of enzyme modified by *N*-bromosuccinimide and subsequently reduced and S-carboxymethylated, was dissolved in 4 ml 70% formic acid and reacted with a 100-fold excess of CNBr for 48 h. Reagents were removed under reduced pressure and the residue taken up in dilute ammonia. The resultant peptide mixture was separated by gel chromatography on Sephadex G-50 (450 × 1.9 cm), elution being effected by 0.1 M NH_4HCO_3.

Digestion with chymotrypsin. 3 μmol of reduced, S-carboxymethylated and sulfenylated enzyme was digested with chymotrypsin at 37° C for 2 h in 0.1 M NH_4HCO_3 at a chymotrypsin to protein ratio of 1.5 : 100 w/w). The digest was chromatographed on Sephadex G-50 (150 × 1.9 cm). Electrophoresis and the location of peptides on paper was carried out as described previously [4].

Sequence determination. Sequence analysis of peptide fragments was performed by a manual manipulation of the Edman degradation technique [37] as modified by Peterson et al. [38]. Phenylthiohydantin derivatives of amino acids were identified by thin-layer chromatography and gas chromatography [4].

Spectroscopy. Absorbance measurements were done in 1-cm quartz cuvettes on a Cary Model 15 spectrophotometer. C.D. spectra were obtained with a Jasco Model J-20 recording spectropolarimeter, using 1-cm pathlength cells in the near ultraviolet region and 0.1- or 0.02-cm pathlength cells below 250 nm. A mean amino-acid residue weight of 113, based on the known amino-acid composition [28] was used in the calculation of mean residue ellipticity. The content of helix and β-structure was calculated according to the following equations [42]:

$$\text{percent helix} = \frac{-[\theta]_{208\ nm} - 4000}{33\,000 - 4000} \times 100$$

$$\text{percent } \beta\text{-structure} = \frac{[\theta]_{204\ nm} + 17\,500}{10\,000 + 17\,500} \times 100$$

The spectral studies were carried out in 0.01 M Tris/0.05 M NaCl, pH 7.8.

Results

Modification with N-bromosuccinimide

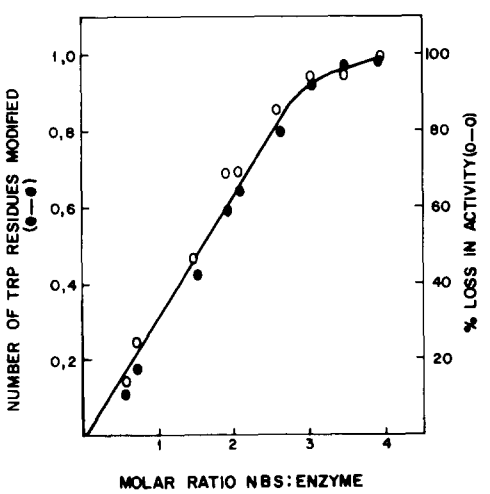
Table I summarizes the results obtained after oxidation of phospholipase A_2

with *N*-bromosuccinimide under various reaction conditions.

Treatment of the enzyme with *N*-bromosuccinimide at pH 3.5 and reagent/enzyme molar ratios above 2 destroyed one tryptophan residue, with a concomitant complete loss in enzyme activity. A control subjected to the same conditions in the absence of *N*-bromosuccinimide retained full activity. The values of 1.22 and 1.50 modified tryptophan residues obtained at 3 : 1 and 4 : 1 molar ratios, respectively, represent only apparent values, since amino-acid analysis indicated that tyrosine modification set in when *N*-bromosuccinimide to enzyme ratios were increased above 2 at pH 3.5.

At higher pH-values, a higher (4-fold) molar excess of *N*-bromosuccinimide was required to modify one tryptophan/enzyme molecule, but in all cases the enzyme was completely inactivated at the stage when one residue of tryptophan had been oxidised. Fig. 1 is representative of results obtained at higher pH-values and illustrates that the extent of modification of tryptophan at pH 5.5 was exactly paralleled by the loss of enzyme activity. At this pH, the presence of Ca^{2+} in the reaction mixture had no effect on the extent of modification and only slight protection was afforded by a micellar concentration of the substrate 1,2-dipalmitoylglycerophosphocholine (cf. Table I).

However, at pH 7.8, the optimum for enzyme activity [10], a definite protective effect against modification by *N*-bromosuccinimide was exerted by micellar concentrations [39] of lysolecithin, a substrate analogue of phospholipase A_2 [20,27]. Only 73% modification was achieved in the presence of $9.7 \cdot 10^{-5}$ M lysolecithin and a residual enzyme activity of about 30% was detected (cf. Table I). The protection was significantly enhanced by the simultaneous presence of Ca^{2+} (44 vs. 30%). With Ca^{2+} present, better protection was



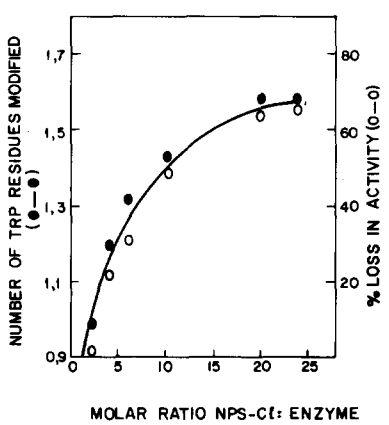


Fig. 1. The extent of tryptophan modification and loss of enzyme activity. Phospholipase A_2 (0.1% in 0.1 M ammonium acetate, pH 5.5) was titrated with 0.01 M *N*-bromosuccinimide.

Fig. 2. Reaction of phospholipase A_2 with 2-nitrophenylsulfenylchloride. An 0.5% solution of enzyme in 30% acetic acid was reacted with the indicated amounts of 2-nitrophenylsulfenylchloride dissolved in glacial acetic acid.

obtained by increasing the micellar concentration of lysolecithin from $3.9 \cdot 10^{-5}$ to $9.7 \cdot 10^{-5}$ M.

Material fully modified at pH 5.5 with *N*-bromosuccinimide was used to identify the reactive residue. Amino-acid analyses of acid hydrolysates of such preparations were identical to those of unmodified enzyme (results not shown), providing indirect evidence that tryptophan was the residue modified. A similar conclusion was reached from the magnitude of the absorbance loss upon *N*-bromosuccinimide oxidation. Since it is known that *N*-bromosuccinimide treatment can cause cleavage of peptide bonds [40], and hence a loss of enzyme activity, three cycles of Edman degradation were performed on the modified phospholipase A_2. This established the unique sequence Asp-Leu-Thr, which is identical to that of the NH_2-terminal and of the native enzyme (cf. Fig. 4 and ref. 4) and excluded the possibility of a split in the polypeptide backbone. The reaction of the modified enzyme with CNBr resulted in a peptide mixture from which peptides CN-3 and CN-4, amongst others [4], could be isolated by gel chromatography on Sephadex G-50. Table II reveals the amino-acid analyses and the positions allocated to these two peptides in the sequence of phospholipase A_2.

TABLE II

AMINO-ACID COMPOSITION AND ALIGNMENT OF PEPTIDES DERIVED FROM CNBr CLEAVAGE AND CHYMOTRYPTIC DIGESTION OF MODIFIED PHOSPHOLIPASE A_2

Amino acid	*N*-Bromosuccinimide-modified enzyme		2-Nitrophenylsulfenylchloride-modified enzyme	
	Peptide CN-3	Peptide CN-4	Peptide C-1	Peptide C-2
S-CM-Cys	5.59 (6)	5.72 (6)	1.83 (2)	
Asp	3.85 (4)	11.92 (12)		0.90 (1)
Thr	0.92 (1)	5.81 (6)		1.75 (2)
Ser	0.90 (1)	4.91 (5)		0.96 (1)
Glu	1.15 (1)	8.23 (8)		
Pro	0.74 (1)	0.97 (1)		
Gly	6.96 (7)	5.12 (5)	2.05 (2)	
Ala	1.02 (1)	2.82 (3)		
Val	1.91 (2)	1.11 (1)		
Met	0.84 (1) [a]			
Ile	2.01 (2)	2.79 (3)	1.10 (1)	
Leu		1.12 (1)		
Tyr	4.51 (5)	4.97 (5)	3.12 (3)	0.99 (1)
Phe	1.81 (2)	2.07 (2)		
Lys	2.86 (3)	4.02 (4)		0.84 (1)
His	0.84 (1)	1.13 (1)		
Arg	0.91 (1)	2.00 (2)		
Trp	1 [b]	1 [c]	1 [d]	1 [e]
Sequence alignment	Gly-13 → Met-52	Gly-53 → Cys-118	Ile-20 → Trp-28	Asp-56 → Tyr-62
ϵ_{280}	$0.6 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$	$1.2 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$	—	—

[a] Determined as homoserine and its lactone.
[b] Present as the oxindole derivative of tryptophan.
[c] Determined by the method of Edelhoch [34].
[d] Ehrlich-positive peptide.
[e] Present as the reaction product of 2-nitrophenylsulfenylchloride.

By amino-acid composition peptides CN-3 and CN-4 correspond to the segments Gly-13 → Met-52 and Gly-53 → Cys-118, respectively (Fig. 4). CN-3 and CN-4 each contain five residues of tyrosine and one tryptophan to yield an expected $\epsilon_{278} = 1.22 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [41]. The observed molar extinction coefficients of $0.6 \cdot 10^4$ and $1.2 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ for peptides CN-3 and CN-4, respectively, suggested that the tryptophan residue of peptide CN-3 had been destroyed. This was corroborated by the positive identification of the phenylthiohydantoin derivative of tryptophan after seven steps of Edman degradation on peptide CN-4. Trp-28 was therefore identified as the site of modification by *N*-bromosuccinimide.

Modification of Trp-59 with *N*-bromosuccinimide could not be achieved even at high reagent excess and low pH-values. The maximum decrease in absorbance at pH 3.5 and a 7-fold molar excess of reagent, gave a value of 1.8 modified tryptophan residues. However, amino-acid analysis showed that three residues of tyrosine had also been oxidised in the process which, together with the loss of one tryptophan (residue 28), would account exactly for the apparent number (1.8) of modified tryptophans obtained from absorbance measurements alone.

Modification with 2-nitrophenylsulfenylchloride

Fig. 2 depicts the results obtained upon sulfenylation of phospholipase A_2 with increasing amounts of 2-nitrophenylsulfenylchloride. At a 2-fold molar excess of reagent one mol of tryptophan/mol of enzyme was modified, while the enzyme retained full activity. At higher molar excesses a loss in enzyme activity was observed which corresponded to a progressive modification of a second tryptophan. Material having 50% residual activity was examined by amino-acid analysis. The results showed that only tryptophan was affected by 2-nitrophenylsulfenylchloride since the rest of the amino-acid composition of modified enzyme corresponded to that of native enzyme.

Sulfenylated but fully active enzyme was digested with chymotrypsin and the digest passed through Sephadex G-50. An intensely yellow coloured peptide, C-2, eluting at the total included volume was obtained in pure form while an Ehrlich-positive peptide, C-1, from the preceding peak was further purified by high voltage electrophoresis on paper at pH 1.9. The amino acid compositions of peptides C-1 and C-2 are given in Table II. Peptide C-1 corresponded with segment 20—28 and peptide C-2 with segment 56—62 of the sequence previously reported (cf. Fig. 4). These results clearly indicated Trp-59 as the preferential site of sulfenylation.

Titration of active sulfenylated enzyme at pH 5.5 with *N*-bromosuccinimide rendered the enzyme inactive and modified the remaining tryptophan.

Circular dichroism spectra

The near ultraviolet circular-dichroism spectra of native gaboon adder phospholipase A_2 and the oxidized and sulfenylated forms of the enzyme are given in Fig. 3a. There are three negative Cotton effects centred near 300, 285 and 260 nm in the spectrum of the native enzyme and these CD bands were retained after specific oxidation of Trp-28. Selective modification of the other residue, Trp-59, by 2-nitrophenylsulfenylchloride resulted in a different CD spectrum

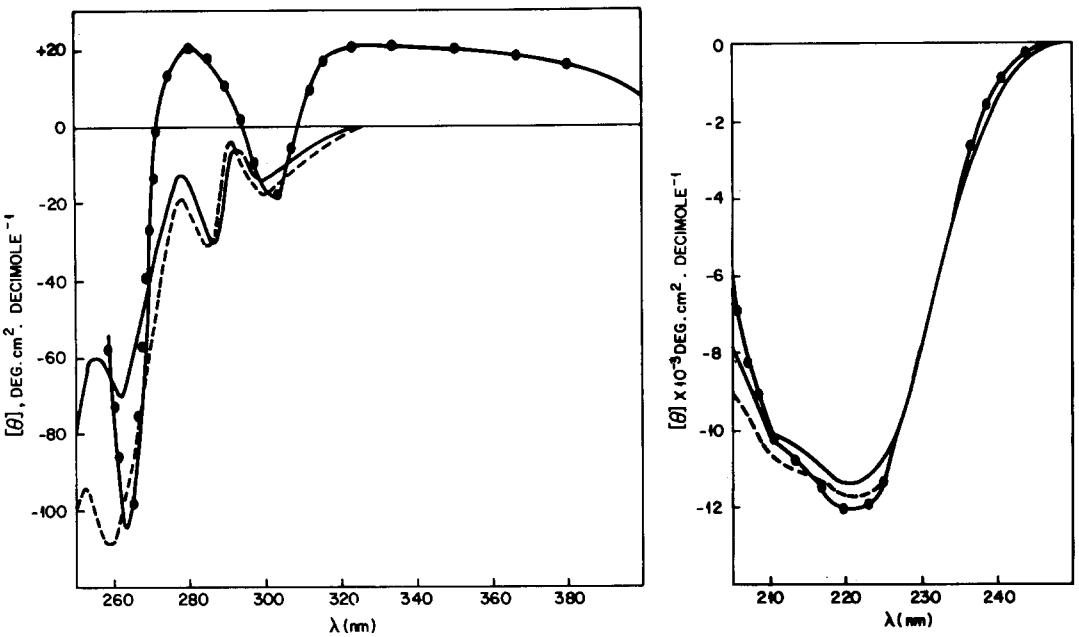


Fig. 3.(a) Circular dichroism spectra of native phospholipase A_2 and enzyme modified with *N*-bromosuccinimide and 2-nitrophenylsulfenylchloride. Enzyme solutions were prepared in 0.01 M Tris/0.05 M NaCl, pH 7.8. ———, phospholipase A_2 (2.2 mg/ml); . . . . . , oxidized phospholipase A_2 (2.4 mg/ml); The enzyme was oxidised with *N*-bromosuccinimide (reagent: enzyme ratio, 4 : 1) at pH 5.5; •———•, sulfenylated phospholipase A_2 (2.3 mg/ml); The preparation was the reaction product obtained at 2-fold molar excess of 2-nitrophenylsulfenylchloride. (b) Deep ultraviolet circular dichroism spectra of phospholipase A_2 and its modified forms. ———, phospholipase A_2 (0.7 mg/ml); . . . . . , phospholipase A_2 modified with *N*-bromosuccinimide (0.6 mg/ml); •———•, phospholipase A_2 modified with 2-nitrophenylsulfenylchloride (0.6 mg/ml).

characterized by replacement of the negative band at 285 nm with a positive one near 280 nm and the appearance of a new broad positive band in the visible region at about 330 nm.

Neither modification affected as the deep ultraviolet CD spectrum of phospholipase A_2 to any significant extent (Fig. 3b). The negative minima at 222 and 210 nm are indicative of the presence of helix in the secondary structure of all three forms of the enzyme. From the observed ellipticities at 208 and 204 nm relative to those of model polypeptides [42] it is estimated that the polypeptide backbone of gaboon adder phospholipase A_2 has approx. 22% helix and 45% β-structure.

Prediction of secondary structure from sequence

The prediction of helical and β-structured regions from the sequence of gaboon adder phospholipase A_2, according to the method of Chou and Fasman [43] is outlined in Fig. 4. Two helical regions consisting of residues 11—17 and 77—94 (i.e. 20% of the total residues) and six β-regions (1—5, 19—27, 35—44, 46—50, 52—57 and 112—118, i.e. 36% of the total residues) are predicted to contribute to the secondary structure of the enzyme.

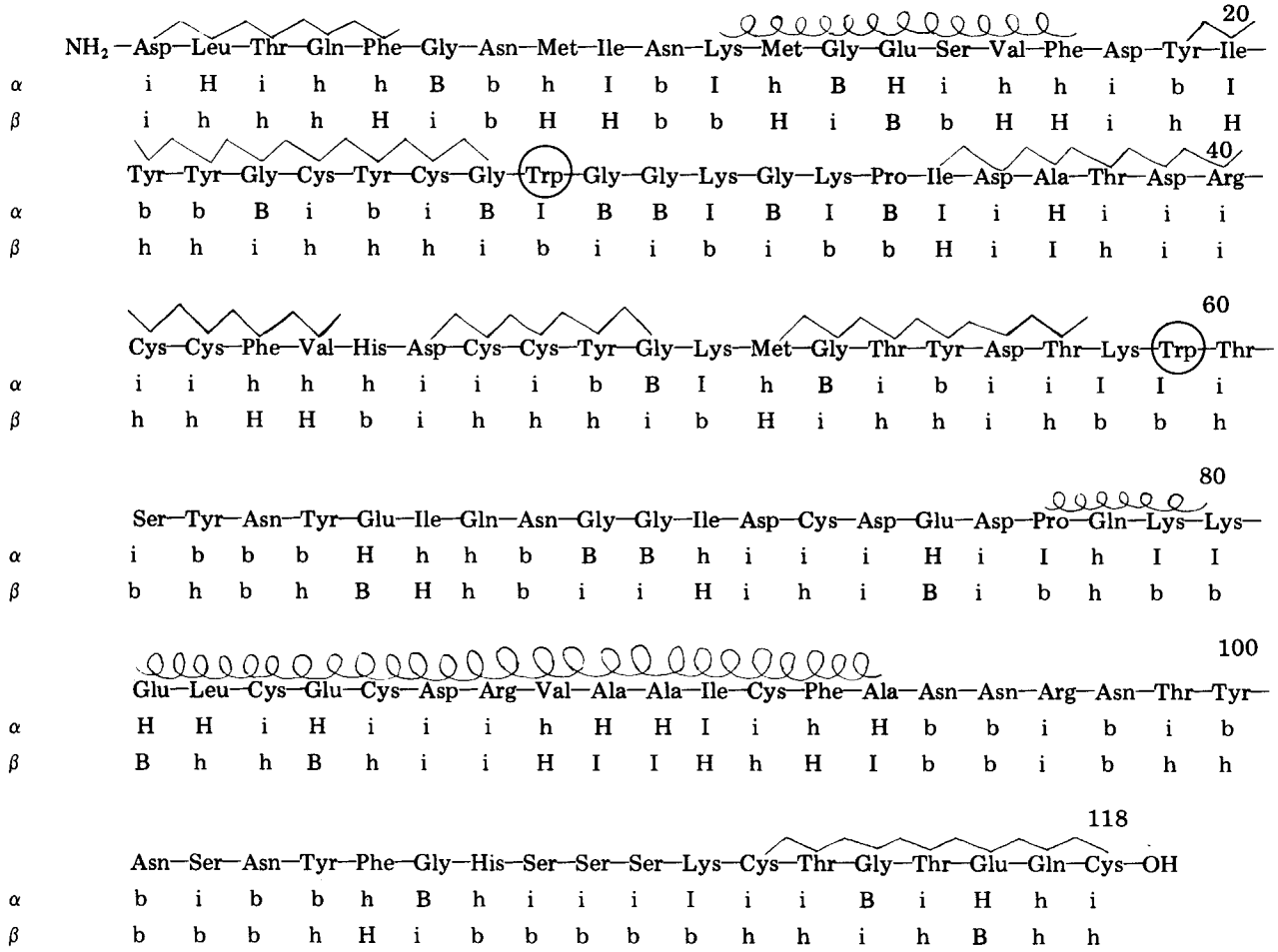


Fig. 4. Prediction of helical and β-structure regions from the sequence of phospholipase A_2 of *B. gabonica* [4]. Assignments under each residue refer to helical (α) and β-structure (β) potential. H, strong former; h, former; I, weak former; i, indifferent; b, weak breaker; B, strong breaker. The predicted α- and β-regions are, respectively, indicated by ~~~~ and ∧∧∧ symbols above the residues. The two tryptophans relevant to the chemical modification experiments (see text) are circled.

Discussion

Previous studies on venom phospholipase A_2 [13,20,25] indicated that aromatic residues are required for enzyme activity. In particular, spectral investigations showed that tryptophan was perturbed by the binding of Ca^{2+} and substrate to the enzyme [13,20]. An examination of the role of tryptophan was therefore undertaken in order to learn more about the active centre of phospholipase A_2 purified from the venom of *B. gabonica*.

Modification of the enzyme by *N*-bromosuccinimide established that the concomitant activity loss can be correlated with the destruction of one specific tryptophan residue per molecule of enzyme. The reactive residue crucial for enzyme activity was identified as Trp-28 by absorbance measurements, amino-acid analyses and peptide alignment. No reaction occurred between *N*-bromosuccinimide and Trp-59, the only other tryptophan residue of *B. gabonica* venom phospholipase A_2 (cf. Fig. 4 and ref. 4), even at low pH or in 8 M urea.

The essential nature of Trp-28 was further corroborated by the fact that it could be partially protected against *N*-bromosuccinimide oxidation by micellar concentrations of substrate (1,2-dipalmitoylglycerophosphocholine) or substrate analogue (lysolecithin) with a resultant retention of corresponding amounts of enzyme activity. It is of interest that greater protection was observed upon increasing the micellar concentration of lysolecithin, in contrast to a lowering of its protective effect against *p*-bromophenacylbromide inactivation of porcine pancreas phospholipase A_2 at lysolecithin concentrations above the critical micelle concentration [27]. The latter result was ascribed to an increase in effective reagent concentration due to its greater solubility in lysolecithin micelles, suggesting that *N*-bromosuccinimide does not readily form mixed micelles with lysolecithin. If so, no enhanced inactivation but rather improved protection would be expected at lysolecithin concentrations above the critical micelle concentration, as has been observed in the present study.

The presence of Ca^{2+} alone did not affect the oxidation of Trp-28, but a mixture of Ca^{2+} plus lysolecithin gave better protection than lysolecithin alone, suggesting that a more efficient enzyme-substrate analogue complex is obtained in the presence of Ca^{2+}.

A general base-catalyzed mechanism of action has been proposed for phospholipase A_2, with a specific histidine or lysine residue serving as the general base [44]. Since it is unlikely that tryptophan can act in this manner, a direct catalytic role for Trp-28 appears implausible. The protection observed in the presence of 1,2-dipalmitoylglycerophosphocholine or lysolecithin therefore implies that Trp-28 is involved in substrate binding. *N*-Bromosuccinimide oxidation irreversibly modifies the indole ring [30] and it is conceivable that the hydrophobic interaction of tryptophan with the apolar part of phospholipid substrate would be abolished after *N*-bromosuccinimide treatment, thereby rendering the enzyme inactive. A hydrophobic binding region has, in fact, been postulated to exist on pancreatic phospholipase on the basis of its increased affinity for more non-polar substrate analogues [27]. The possibility that modification of Trp-28 caused a conformational change resulting in loss of enzyme activity appears to be ruled out by the CD results. The near ultraviolet region of the CD spectrum of gaboon adder phospholipase A_2 contains several

bands, some of them (e.g. those above 280 nm) originating in constrained tyrosine and tryptophan side chains. These bands remained unchanged after oxidation of Trp-28 by *N*-bromosuccinimide, though, and the deep ultraviolet CD spectrum of the oxidised enzyme was also the same as that of the native one. From these results it can be concluded that neither the local conformation near Trp-28 nor the gross polypeptide backbone structure was perturbed upon oxidative modification of that residue.

While Trp-59 could not be attacked by *N*-bromosuccinimide, this residue preferentially reacted with 2-nitrophenylsulfenylchloride. Conversion of Trp-59 to the sulfenylated derivative did not alter enzyme activity, so that Trp-59 does not participate in enzyme action. However, activity was diminished upon the subsequent reaction of Trp-28 with 2-nitrophenylsulfenylchloride. Residual activity in this instance also correlated with the amount of Trp-28 modified, which serves as additional evidence for the importance of the latter residue.

Modification of Trp-59 by 2-nitrophenylsulfenylchloride resulted in a near ultraviolet CD spectrum which differed from the native enzyme spectrum. The appearance of a broad CD band associated with the absorption maximum of the reagent in the visible region suggests that the nitrophenyl moiety has a relatively fixed orientation on the sulfenylated enzyme. Since the indole ring of Trp-59 retains its conjugated character after modification with sulphenyl halides [32], it may be involved in the induction of the extrinsic Cotton effect in the attached nitrophenylsulfenyl group, especially since its appearance is accompanied by changes in the 280—285 nm CD bands. The gross conformation of the enzyme was, however, not altered by modification with 2-nitrophenylsulfenylchloride, as the deep ultraviolet CD features characteristic of the spatial structure of the polypeptide backbone remained the same.

The secondary structure of phospholipase A_2 from the gaboon adder is like those from porcine pancreas [45] and *C. adamanteus* [12], characterized by the presence of helix in the polypeptide backbone. The amount of helix (22%), is however, appreciably less than the values reported for the pancreas (55%) and *C. adamanteus* (70%) enzymes. Part of this difference (at least between the adder and pancreas enzymes of which the complete sequences are known) can be ascribed to extensive variations in primary structures, a 28% identity notwithstanding. It would furthermore appear that the amount of helix present in the pancreas enzyme has been somewhat overestimated, since the observed ellipticity of approx. —14 500 degree cm^2/dmol at 208 nm (cf. Fig. 3b, ref. 45) yields a value of about 36% helix rather than the reported 55%. The CD spectrum of adder phospholipase shows that the enzyme possesses approx. 45% β-structure as well.

Several approaches exist for the prediction of the secondary structure of proteins on the basis of amino acid sequences and one of these, the method of Chou and Fasman [43], was applied to phospholipase A_2 from *B. gabonica* (cf. Fig. 4). Apart from the fact that there is acceptable agreement between the predicted and CD values (20 vs. 22% for helix, 36 vs. 45% for β-structure, respectively), the exercise also revealed that the functionally significant tryptophan (residue 28) lies outside any periodically ordered region at the edge of a β-structured segment. A similar treatment of the sequence of pancreas phospholipase gives 28% helix and 16% β-structure with His-53, which is essential for

catalytic activity [27], also outside regions with secondary structure.

Although the sequences of a number of phospholipases A_2 are available, only His-53 of the enzyme from porcine pancreas has thus far been pinpointed as an active site residue [27]. Wells [25] demonstrated that a lysine and a tryptophan are functionally important residues in the phospholipase A_2 from *C. adamanteus*, but specific assignments were not possible from the incomplete sequence. Trp-28 is therefore the first example of a residue that can be specifically assigned to the active centre of a snake venom phospholipase A_2. An homologous tryptophan residue is not found in Elapid venom phospholipases A_2 [5] or the porcine pancreatic enzyme [2]. However, in a comparison of the partial sequence of the *C. adamanteus* enzyme with those of the phospholipase A_2 derived from bee venom and *B. gabonica* venom, Tsao et al. [46] aligned Trp-30 of *C. adamanteus* phospholipase A_2 with Trp-28 of the enzyme from *B. gabonica*. In view of the quite similar characteristics found for both enzymes [10,20], Trp-30 of *C. adamanteus* may well be the residue corresponding in function to Trp-28 of the phospholipase A_2 from *B. gabonica*.

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