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# Amino Acid Sequence of a Basic *Agkistrodon halys blomhoffii* Phospholipase A2. Possible Role of $NH_2$-Terminal Lysines in Action on Phospholipids of *Escherichia coli*†

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ABSTRACT: A basic (p*I* = 10.2) phospholipase A2 of the venom of the snake *Agkistrodon halys blomhoffii* is one of a few phospholipases A2 capable of hydrolyzing the phospholipids of *Escherichia coli* killed by a bactericidal protein purified from human or rabbit neutrophil granules. We have shown that modification of as many as 4 mol of lysine per mole of the phospholipase A2, either by carbamylation or by reductive methylation [Forst, S., Weiss, J., & Elsbach, P. (1982) *J. Biol. Chem. 257*, 14055–14057], had no effect on catalytic activity toward extracted *E. coli* phospholipids or the phospholipids of autoclaved *E. coli*. In contrast, modification of 1 mol of lysine per mole of enzyme substantially reduced activity toward the phospholipids of *E. coli* killed by the neutrophil protein. To explore further the role of lysines in the function of this phospholipase A2, we determined the amino acid sequence of the enzyme and the incorporation of [$^{14}$C]cyanate into individual lysines when, on average, 1 lysine per molecule of enzyme had been carbamylated. After incorporation of approximately 1 mol of [$^{14}$C]cyanate per mole of protein, the phospholipase A2 was reduced, alkylated, and exhaustively carbamylated with unlabeled cyanate. The amino acid sequence was determined of the $NH_2$-terminal 33 amino acids of the holoprotein and of peptides isolated after digestion with trypsin and *Staphylococcus aureus* V-8 protease. The protein contains 122 amino acid residues, 17 of which are lysines. The $NH_2$-terminal region is unique among more than 30 phospholipases A2 previously sequenced because of its high content of basic residues (His-1, Arg-6, and Lys-7, -10, -11, and -15). The four $NH_2$-terminal lysines accounted for approximately 50% (about equally distributed) of the total incorporated [$^{14}$C]cyanate. On the basis of these findings and evidence that the $NH_2$-terminal region of phospholipases A2 is a functionally important $\alpha$-helix, we raise the possibility that the cluster of basic residues in the outwardly directed face of this helix is involved in the action of the basic *A. h. blomhoffii* phospholipase A2 on *E. coli* killed by the bactericidal protein of neutrophils.

Phospholipases A2 represent a class of lipolytic enzymes with many common structural and functional features. Their highly conserved nature has been revealed by amino acid sequence analysis of >30 secretory phospholipases A2 from multiple animal sources (Dufton et al., 1983) and by X-ray crystallographic studies (Dijkstra et al., 1983; Brunie et al., 1985). The invariant regions are known to contain the sites concerned with catalysis, $Ca^{2+}$ binding, and substrate recognition (Verheij et al., 1981). Although phospholipases A2 are very similar in many respects, in their action on particular substrates they exhibit major functional differences (Verheij et al., 1980; Hanahan et al., 1980; Zwaal et al., 1975; Dufton & Hider, 1983; Weiss et al., 1979) that presumably are determined by variable structural properties. How such structural differences are related to the ability of a given phospholipase A2 to act in a specific biological event has not been carefully examined.

Our work concerns the determinants of phospholipase A2 action on the envelope phospholipids of *Escherichia coli* (Weiss

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et al., 1979; Elsbach et al., 1979, 1985; Forst et al., 1982). Of 14 purified phospholipases A2 examined, only a few are able to degrade the phospholipids of *E. coli* killed by a potent bactericidal/permeability-increasing protein (BPI)[1] purified from human and rabbit neutrophils (Weiss et al., 1979; Forst et al., 1982; unpublished observations). Thus the ability of phospholipases A2 to act on BPI-killed *E. coli* is a variable property that is shared by only a few phospholipases A2. A highly basic (p*I* = 10.2) phospholipase A2 from the venom of the snake *Agkistrodon halys blomhoffii* is the most active of the BPI-responsive phospholipases A2 tested so far.

Chemical modification of this enzyme by carbamylation or reductive methylation of, on the average, 1 lysine per molecule reduced BPI-dependent hydrolysis by approximately 70%, whereas modification of as many as 4 lysines per molecule had no effect on the hydrolysis of artificial substrates, including extracted *E. coli* phospholipids (Forst et al., 1982).

To explore further the role of lysines in BPI-mediated hydrolysis by the basic *A. h. blomhoffii* phospholipase A2, we have localized the 17 lysines in the amino acid sequence of this molecule and have identified the lysines that are modified when, on average, 1 mol of [$^{14}$C]cyanate was incorporated/mol of protein.

Our findings reveal the presence of a cluster of four lysines in the $NH_2$-terminal region of this phospholipase A2 that is unique among all phospholipases A2 sequenced so far.[2] These four lysines may play a role in the action of the basic phospholipase A2 on BPI-killed *E. coli*.

## Experimental Procedures

### *Materials*

Lyophilized *A. h. blomhoffii* snake venom was obtained from Miami Serpentarium, Miami, FL (lot no. AH 811 STLZ); L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK)–trypsin (lot no. 310882) was from Worthington Biochemicals; Staphylococcal V-8 protease (lot no. 14) was from Miles; DL-dithiothreitol and iodoacetic acid were from Sigma; [$^{14}$C]potassium cyanate (54 mCi/mmol) was from New England Nuclear; sodium cyanate was from Aldrich Co.; HPLC-grade ammonium acetate was from Fisher; sequanol-grade trifluoroacetic acid was from Pierce Chemicals; DEAE-cellulose 52 and CM-cellulose 52 were from Whatman; ampholines, pH 9–11, (20%) were from LKB; acrylamide, bis(acrylamide), sucrose, and TEMED were from Bio-Rad; Bio-Solv 3 was from Beckman; 2,5-bis(5-*tert*-butyl-2-benzoxazolyl)thiophene (BBOT) was from Packard. All other reagents were of the purest grade commercially available.

### *Methods*

*Purification of the Basic A. h. blomhoffi Phospholipase A2.* The basic phospholipase A2 was purified by the procedure of Martin et al. (1975). However, the basic phospholipase A2 was not retained by DEAE-cellulose. Subsequent chromatography on CM-cellulose equilibrated in 50 mM sodium acetate (pH 6.0), followed by stepwise elution (200 and 400 mM sodium acetate), yielded pure basic phospholipase A2 as judged by SDS–PAGE, basic pH gradient isoelectric focusing, and $NH_2$-terminal sequence analysis.

*Basic pH Gradient Isoelectric Focusing in a Polyacrylamide (6%) Gel.* The gel (Guo & Bishop, 1982) contained 2% ampholines, pH 9–11. The samples were placed directly on the precooled (10 °C) gel surface, and the gel was run at 600–1000 V for 3 h under nitrogen gas and in the presence of sodium hydroxide pellets. The pH gradient was found to be linear from pH 7.5 to pH 10.5. The isoelectric point of the basic phospholipase A2 was 10.2.

*Carbamylation of Approximately 1 mol of Lysine/mol of the Basic A. h. blomhoffi Phospholipase A2 Using [$^{14}$C]-Cyanate.* Carbamylation with [$^{14}$C]cyanate (0.46 nCi/nmol) was carried out as previously described (Forst et al., 1982). The reaction was terminated after 40 min, at which time 0.93 mol of [$^{14}$C]cyanate/mol of protein had been incorporated. The partially modified phospholipase A2 was dialyzed against distilled water, lyophilized, and subsequently exhaustively carbamylated with unlabeled cyanate as described below.

*Exhaustive Carbamylation of the Basic A. h. blomhoffii Phospholipase A2 with Unlabeled Cyanate.* Either native or partially [$^{14}$C]cyanate modified phospholipase A2 was reduced and alkylated (Halpert & Eaker, 1975), extensively dialyzed against 1% acetic acid, lyophilized, and resuspended in 0.1 M sodium borate (pH 8.7) to a final concentration of 2 mg/mL. Recrystallized sodium cyanate was added to a final concentration of 0.3 M, and the reaction mixture was incubated for 14 h at 37 °C. Exhaustively carbamylated phospholipase A2 was dialyzed extensively against 20 mM ammonium acetate (pH 5.8) and lyophilized.

*Proteolytic Cleavage of Carbamylated Phospholipase A2 with Trypsin.* Typically, 2 mg (143 nmol) of the carbamylated phospholipase A2 was dissolved in 500 μL of 0.1 M ammonium bicarbonate and preincubated for 10 min at 37 °C. Digestion was carried out in two ways: (1) TPCK–trypsin was added to a final molar ratio (trypsin:phospholipase A2) of 1:100, and incubation was carried out for 4 h at 37 °C, at which time a second dose of trypsin was added (1:200), followed by an additional 4 h of incubation; (2) Incubation was carried out for 20 min at 37 °C at a molar ratio of 1:100. Digestions were terminated by quick freezing and lyophilization.

*Proteolytic Cleavage with Staphylococcal V-8 Protease (Drapeau, 1977).* Digestion with staphyloccal V-8 protease of uncarbamylated, reduced, and alkylated phospholipase A2 (3 mg in 0.75 mL) or carbamylated tryptic peptides (64 nmol in 0.1 mL) in 0.1 M ammonium bicarbonate was carried out for 60 or 90 min, respectively, at 37 °C at a molar ratio of 1:100. The digestion was terminated by lyophilization.

*Acid Cleavage of Reduced and S-Carboxymethylated Basic Agkistrodon Phospholipase A2 (Marcus et al., 1982).* The reduced and S-carboxymethylated basic phospholipase A2 was dissolved in 0.015 N HCl to a final concentration of 2 mg/mL. Incubation was carried out in a boiling water bath for 10 min. The sample was quickly frozen and lyophilized.

*Separation of Peptides by HPLC. (I) Camphorsulfonate–Propanol/Trifluoroacetic Acid (TFA).* An Altex Ultrasphere ODS ($C_{18}$) column (5 μm; 4.6 × 250 mm) was used to fractionate peptides. The column was equilibrated with buffer A (0.1% camphorsulfonic acid, pH 3.1). Peptides were eluted with a gradient of 0–50% buffer B (0.1% TFA, pH 2.1, in 50% 1-propanol).

*(II) Ammonium Acetate–Acetonitrile.* Peptides were resuspended in buffer A (20 mM ammonium acetate, pH 5.8), centrifuged, and applied to the ODS column equilibrated in the same buffer. Peptides were eluted with a gradient of buffer

[1] Abbreviations: BPI, bactericidal/permeability-increasing protein; HPLC, high-pressure liquid chromatography; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[2] Two other snake venom phospholipases A2 have recently been found to have lysines in identical positions (Maraganore et al., 1984). In a third enzyme, the basic phospholipase A2 of the *Agkistrodon halys palas* venom, the $NH_2$-terminal sequence is identical with that of the basic *A. h. blomhoffii* except that His-1 is replaced by Asn-1.

Table I: Amino Acid Composition of Peptides Generated by Digestion of Phospholipase A2 with Trypsin and/or Staphylococcal V-8 Protease[a]

| | T1 | T2 | SP1 | SP2 | T4 | T5 | T6a | T6b | T7 | total no. of residues |
|---|---|---|---|---|---|---|---|---|---|---|
| CMC | | 1.2 (2) | 1.6 (4) | 2.6 (3) | 0.32 (2) | 0.2 (1) | | | 1.0 (2) | (14) |
| Asx | | | 3.6 (3) | 5.9 (6) | 1.1 (1) | | 2.2 (2) | | 1.2 (1) | (13) |
| Thr | | 1.0 (1) | 1.2 (1) | 2.0 (2) | | | 1.1 (1) | 0.9 (1) | | (5) |
| Ser | | 2.0 (2) | 0.6 (0) | 1.3 (1) | 1.3 (0) | | | | 3.0 (3) | (6) |
| Glx | 1.2 (1) | 1.1 (1) | 1.0 (1) | 1.1 (1) | 1.9 (2) | | | | 1.1 (1) | (6) |
| Pro | | 1.0 (1) | 1.0 (1) | 2.2 (2) | | | | | 1.1 (1) | (5) |
| Gly | | 4.5 (5) | 1.6 (1) | 3.7 (4) | | | | 0.6 (0) | | (10) |
| Ala | | 0.9 (1) | 1.2 (1) | | | 3.0 (3) | | | 1.0 (1) | (6) |
| Val | | 0.4 (1) | 1.0 (1) | 1.6 (2) | | | | | | (4) |
| Met | | 1.8 (2) | | | | | | | 0.9 (1) | (3) |
| Ile | | 2.0 (2) | | 0.6 (1) | 0.7 (1) | 1.0 (1) | | | 0.9 (1) | (6) |
| Leu | 2.2 (2) | | | | | | 1.0 (1) | | 0.8 (1) | (4) |
| Tyr | | 2.7 (3) | 1.0 (1) | 2.1 (2) | | | 1.0 (1) | 1.0 (1) | 2.2 (2) | (9) |
| Phe | 0.7 (1) | 1.2 (1) | 0.9 (1) | | | 1.1 (1) | | | | (4) |
| His | 1.0 (1) | | 0.9 (1) | | | | | | | (2) |
| Lys[b] | | 1.2 (4) | 1.4 (2) | 5.5 (6) | | | 0.4 (1) | 0.5 (2) | 0.6 (2) | (17) |
| Arg | 1.0 (1) | 0.8 (1) | 0.8 (1) | | 1.0 (1) | 1.0 (1) | | 1.0 (1) | | (6) |
| Trp | | | | nd (2) | | | | | | (2) |
| sequence position | 1–6 | 7–33 | 34–52 | 53–84 | 84–90 | 91–97 | 98–103 | 102–106 | 107–122 | |
| nmol recovered | 144 | 22 | 27 | 53 | 29 | 173 | 110 | 3 | 115 | |
| yield | 51% | 8% | 10% | 25% | 10% | 62% | 40% | 1% | 41% | |

[a] Data are expressed as residues/mol of peptide without correction for hydrolytic destruction. The numbers in parentheses indicate the amino acid composition as determined by amino acid sequence analysis. [b] Carbamylated lysine is converted back to native lysine at a variable rate during acid hydrolysis of carbamylated peptdies.

B (20 mM ammonium acetate, pH 5.8, in 70% acetonitrile) (Figure 2).

*(III) Trifluoroacetic Acid–Acetonitrile.* This procedure was the same as (II) except that trifluoroacetic acid (0.1%; pH 2.1) was substituted for ammonium acetate.

*Measurement of Incorporated Cyanate into Holoprotein, Peptides, and Individual Lysine Residues.* To quantitate cyanate incorporation, carbamylation was carried out with [$^{14}$C]cyanate of known specific radioactivity. The radioactivity and mass of the holoprotein and of purified peptides were determined by liquid scintillation counting and amino acid analysis (Forst et al., 1982), respectively, for calculation of the molar amounts of cyanate incorporated per mole of protein or peptide. To determine cyanate incorporation into individual lysine residues of peptides containing more than one lysine, phenylthiohydantoin- (PTH-) carbamyllysines were isolated by automated Edman degradation and HPLC. Radioactivity and the integrated surface area of the chromatographic peak of each PTH-carbamyllysine were measured for calculation of the relative specific radioactivity of each lysine, providing the fractional distribution of cyanate incorporated into individual lysines within a given peptide. From this fractional distribution and the known moles of cyanate incorporated per mole of the parent peptide, the molar amounts of cyanate incorporated into individual lysine residues were computed.

*Amino Acid Composition and Sequence Analysis (Goni & Frangione, 1983).* Amino acid analysis of protein and peptides hydrolyzed with 6N HCl/0.1% phenol for 20 h at 110 °C was performed on a Durrum D-500 analyzer. Automated Edman degradation was carried out by using a Beckman 890C sequencer and an 0.1 M Quadrol program. Polybrene was used when needed. Phenylthiohydantoin derivatives were identified by HPLC with a μBondapak $C_{18}$ column and automated amino acid analysis with a Durrum D-500 analyzer after hydrolysis in 0.2 mL of 6 M HCl containing 5 μL of 5% $SnCl_2$ under reduced pressure for 4 h at 150 °C.

*Measurement of Radioactivity.* Incorporation of [$^{14}$C]-cyanate into phospholipase A2 was measured as conversion of radiolabel into 10% trichloroacetic acid precipitable material (Forst et al., 1982). The radioactivity associated with individual peptides and lysines was determined by mixing the sample with toluene/BBOT/Bio-Solv 3 and counting in a Beckman liquid scintillation counter.

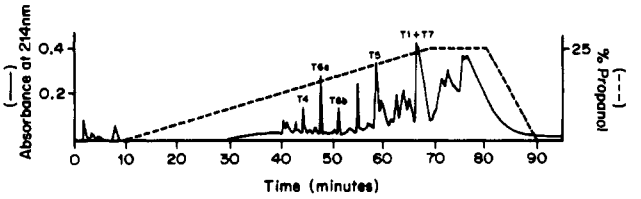


FIGURE 1: HPLC separation of peptides generated by trypsin digestion of exhaustively carbamylated phospholipase A2 (procedure I). Peptides were eluted with solvent system I (see Experimental Procedures) at a flow rate of 1 mL/min.

## RESULTS

*Amino Acid Sequence Determination of the Basic A. h. blomhoffii Phospholipase A2.* The sequence of the first 33 $NH_2$-terminal amino acids was obtained by automated Edman degradation of reduced and S-carboxymethylated phospholipase A2 (approximately 70 nmol). To determine the rest of the amino acid sequence, peptides generated by proteolysis were purified and sequenced by using three procedures.

*Procedure I.* Peptides generated by trypsin digestion (8 h) of exhaustively carbamylated basic phospholipase A2 (280 nmol) were separated by HPLC as shown in Figure 1. Peptides T4, T5, and T6a were obtained in pure form as judged by amino acid analysis and subsequent sequence analysis. Peptides T1 and T7 were reapplied to the $C_{18}$ column and separated by using a shallower propanol gradient (15–20% in 50 min). The amino acid compositions of these purified peptides are shown in Table I.

*Procedure II.* To reduce secondary cleavages and improve solubility of certain carbamylated peptides, digestion with trypsin was carried out for only 20 min, and the peptides were separated by HPLC using an ammonium acetate/acetonitrile solvent system (Figure 2). This solvent system improved the solubility of larger carbamylated peptides, allowing their purification by HPLC. T1 and T2 were obtained in pure form as judged by amino acid analysis. T7 was purified by using

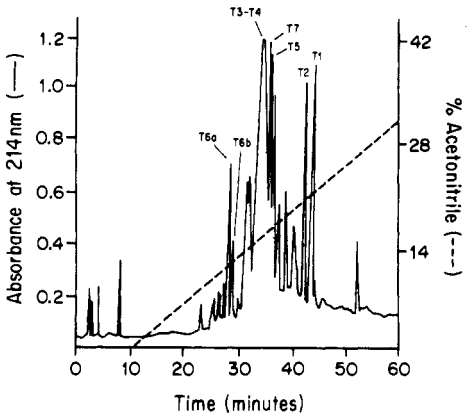


FIGURE 2: HPLC separation of peptides generated by limited trypsin digestion (20 min) of carbamylated phospholipase A2 (procedure II). Peptides were eluted with solvent system II (see Experimental Procedures).

a shallow acetonitrile gradient (10–60% in 90 min). SP1 and SP2 were obtained by digesting T3-T4 with staphylococcal V-8 protease (90 min) under conditions specific for cleavage at glutamic acid residues (Marcus et al., 1982). The resulting peptides were resolved by HPLC using the same shallow acetonitrile gradient. T6a and T6b were purified by HPLC using the trifluoroacetic acid/acetonitrile solvent system. T6b was judged to be pure by amino acid analysis and sequence analysis. Generation and recovery of T6 were found to be variable, probably because of chymotryptic cleavage at Tyr-103 as well as tryptic cleavage at uncarbamylated Lys-101. The amino acid compositions of purified peptides T2, SP1, and T6b are shown in Table I.

*Procedure III.* Peptide SP2 was also generated by staphylococcal V-8 protease digestion of uncarbamylated, reduced, and alkylated phospholipase A2 and purified by HPLC in solvent system III (trifluoroacetic acid/acetonitrile). The composition of uncarbamylated SP2 is shown in Table I. Both the carbamylated peptide SP2 and the uncarbamylated peptide SP2 were subjected to amino acid analysis, yielding identical results.

The complete amino acid sequence of the basic *A. h. blomhoffii* phospholipase A2 is shown in Figure 3. The sequenced peptides (T1, T2, T4, T5, T6a, T6b, T7, SP1, and SP2) were aligned on the basis of homology (approximately 35%) with the >30 phospholipases A2 that have been sequenced to date (Dufton et al., 1983). The carboxyl-terminal sequence of peptide SP2 was confirmed by sequence analysis of a peptide obtained after acid cleavage of the native protein at Asp-79/Pro-80 (Marcus et al., 1982). This peptide was sequenced from Pro-80 to Ile-85, verifying the presence of Lys-82 and Lys-83. Overlap between peptides T6a and T6b permitted proper alignment of these peptides and established the primary structure between Asp-98 and Arg-106, which was confirmed by sequence analysis of peptide T6. The enzyme contains 17 lysine residues out of a total of 122 amino acids. All the lysines are located outside of the invariant positions. The 4 lysines in the $NH_2$-terminal (His-1 to Lys-15) region and the 5 lysines in the carboxyl-terminal region (Lys-101 to Lys-122) contribute to the high net positive charge (5.5 and 4.0, respectively) of these segments of the basic enzyme.

*Measurement of Carbamylation of Individual Lysines after Modification with [$^{14}$C]Cyanate.* To determine which lysine(s) is (are) modified when, on average, 1 lysine per molecule of phospholipase A2 was carbamylated, the native protein was incubated with [$^{14}$C]cyanate for 40 min, at which time 0.93 mol of cyanate had been incorporated per mole of protein, on the basis of determination of acid-precipitable radioactivity and protein content (Forst et al., 1982) of the dialyzed labeled phospholipase A2. After exhaustive carbamylation with unlabeled cyanate, tryptic peptides were generated and purified as described in procedure II to identify and measure the extent of modification of individual lysines. The purity of isolated peptides was established by amino acid composition and sequence analysis. Determination of moles of [$^{14}$C]cyanate incorporation into radiolabeled peptides and individual lysines was carried out as described under Experimental Procedures. Table II shows the moles of [$^{14}$C]cyanate incorporated per mole of peptide and its distribution among the individual lysines of each peptide. The sum of the moles of cyanate incorporated into the individual radiolabeled peptides was 1.06 mol per mole of protein (closely similar to the amount of incorporated cyanate measured in the holoprotein). The $T_1$ peptide carried less than 5% of the total incorporated radioactivity, confirming previously published evidence that the $\alpha$-amino group is relatively nonreactive under the conditions of these experiments (Forst et al., 1982). Essentially all lysines were labeled. However, Lys-7, -10, -11, and -15 contained approximately 50% of the total incorporated [$^{14}$C]cyanate (each labeled about equally). Lys-37, -104, and -105 each contained 7–8% of the total incorporated [$^{14}$C]cyanate, and the remaining 10 lysines together accounted for approximately 25% of the total [$^{14}$C]cyanate incorporated. Thus, multiple lysines are modified at submolar levels, but the four lysines


FIGURE 3: Amino acid sequence of *A. h. blomhoffii* basic phospholipase A2. The procedures used for amino acid sequence analysis are described under Methods and Results. Lysyl residues are indicated in boldface and enclosed in boxes. Residues that are invariant in more than 30 completely sequenced phospholipases A2 are indicated by a bracket on top. The residue in position 74 has been tentatively identified as Val.

Table II: Measurement of [$^{14}$C]Cyanate Incorporation into Individual Lysines of Basic *Agkistrodon* Phospholipase A2[a]

| peptide | mol of [$^{14}$C]cyanate incorporated/ mol of peptide | position of Lys | mol of [$^{14}$C]cyanate incorporated/ mol of Lys |
|---|---|---|---|
| T2 | 0.52 | | |
| | | 7 | 0.12 |
| | | 10 | 0.11 |
| | | 11 | 0.12 |
| | | 15 | 0.17 |
| SP1 | 0.11 | | |
| | | 35 | 0.04 |
| | | 37 | 0.07 |
| SP2 | 0.15 | | |
| | | 53 | 0.02 |
| | | 58 | 0.02 |
| | | 60 | 0.04 |
| | | 69 | 0.03 |
| | | 82 | 0.03 |
| | | 83 | 0 |
| T6a | 0.04 | | |
| | | 101 | 0.04 |
| T6b | 0.16 | | |
| | | 104 | 0.08 |
| | | 105 | 0.08 |
| T7 | 0.08 | | |
| | | 118 | 0.05 |
| | | 121 | 0.03 |
| total | 1.06 | | |

[a] Peptides were isolated from the basis *Agkistrodon* phospholipase A2 after modification of the protein with [$^{14}$C]cyanate (0.93 mol incorporated/mol of protein), exhaustive carbamylation with unlabeled cyanate, and digestion with trypsin (T) and/or staphylococcal V-8 protease (SP). The moles of [$^{14}$C]cyanate incorporated/mol of peptide and the amount of [$^{14}$C]cyanate incorporated into individual lysines (moles of [$^{14}$C]cyanate/mol of lysine) were calculated as described under Experimental Procedures.

in the $NH_2$-terminal region are preferentially modified.

## Discussion

In a previous study, we showed that carbamylation of, on average, 1 lysine residue per protein molecule reduced the activity of the basic *A. h. blomhoffii* phospholipase A2 toward BPI-killed *E. coli* by approximately 70% (Forst et al., 1982). The demonstration, in this study, that no more than 17% of any single lysine was modified during carbamylation under these conditions (Table I) clearly indicates that modification of any one of a number of lysines impairs enzyme activity toward this particular target, suggesting therefore that multiple lysines are involved in this function.

It is conceivable that the integrity of all lysines scattered throughout the molecule is necessary for BPI-dependent hydrolysis. However, judging by the reactivity with cyanate, it appears more likely that not all lysines are equivalent. Ten of the 17 lysines show low reactivity with [$^{14}$C]cyanate and are spread in regions of the molecule with approximately equal numbers of basic and acidic residues. Lys-37, -104, and -105 showed intermediate reactivity (Figure 3 and Table II). Of these lysines two are part of a small cluster of basic residues (Lys-104 and -105; Arg-106). However, it is particularly the four lysines within the $NH_2$-terminal region (Lys-7, -10, -11, and -15) that stand out, not only because of their high reactivity (approximately 50% of total incorporated radioactivity, roughly equally distributed) but also because of the special properties of this region in all phospholipases A2 studied so far (Dufton et al., 1983; Dijkstra et al., 1983; Brunie et al., 1985; Verger et al., 1982; van Dam-Mieras et al., 1975; Randolph & Heinrikson, 1982; van Scharrenburg et al., 1983).

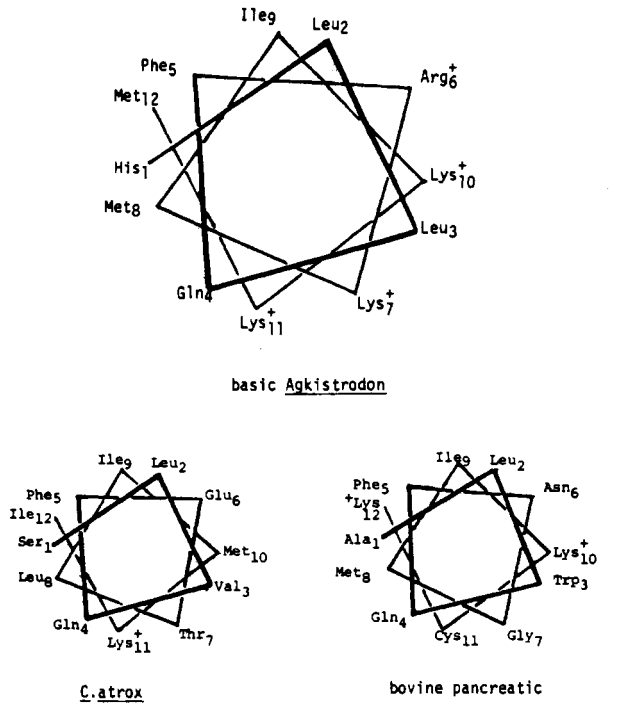


FIGURE 4: Axial projections of the $NH_2$-terminal 12 residues of the basic *Agkistrodon*, *C. atrox* (Brunie et al., 1985), and bovine pancreatic (Dijkstra et al., 1983) phospholipases A2. According to the Chou–Fasman method (Chou & Fasman, 1978), the conformational parameters of residues 1–13 of the basic *Agkistrodon* phospholipase A2 are as follows: $P_\alpha = 1.15$; $P_\beta = 1.08$; $P_t = 0.79$.

X-ray crystallographic analysis of three phospholipases A2 (from pig and cow pancreas and *Crotalus atrox* venom) has shown that the first 13–15 residues are arranged in an $\alpha$-helix (Dijkstra et al., 1983; Brunie et al., 1985). Prediction of the secondary structure of these 3 enzymes plus 29 additional phospholipases A2 indicated an $\alpha$-helical configuration in this region in each case (Dufton et al., 1983). Chou–Fasman analysis (Chou & Fasman, 1978) predicts that the first 13 residues of the basic *A. h. blomhoffii* phospholipase A2 are also arranged in an $\alpha$-helix. An axial projection of this putative structure (Figure 4) demonstrates schematically the asymmetric arrangement of polar and nonpolar amino acids that is common to all phospholipases A2. The model also shows how the basic residues (Arg-6; Lys-7, -10, and -11) are clustered along the polar face of the helix. Moreover, residues 13–16 are predicted to form a reverse turn, suggesting that Lys-15 is close to Lys-7, -10, and -11 and Arg-6. The high reactivity of Lys-7, -10, -11, and -15 with cyanate is consistent with the proposed structure, because clustering of lysine (basic) residues reduces the $pK_a$ of the $\epsilon$-$NH_2$ groups (Quay & Tronson, 1983), thus increasing lysine reactivity with cyanate (Jensen et al., 1973). Such a high concentration of basic residues on the polar face of the $NH_2$-terminal helix (net charge of 5) has been found in only one other venom phospholipase A2 (unpublished data).[2] In 32 of 33 phospholipases A2 previously analyzed, the net charge of this region ranges from −2 to 3 (Dufton et al., 1983). In a phospholipase A2 from pig small intestine (Verger et al., 1982), the charge is 4.

The variability of the polar face of the $NH_2$-terminal helix is in marked contrast to the conservation of hydrophobic residues along the nonpolar face (e.g., residues 2, 5, 8, and 9) that are thought to form a wall in the active site cavity (Randolph & Heinrikson, 1982; van Scharrenburg et al., 1983). Along the polar face, amino acid side chains are exposed to the exterior, permitting interactions with polar groups

at the substrate interface as well as "activator agents" [e.g., BPI, melittin (Shier, 1979)] that facilitate enzyme action. Differences in the properties of the polar face may therefore contribute to differences in activity among phospholipases A2 toward specific targets. Indeed, increasing the net positive charge of the polar face of the $NH_2$-terminal helix of the cow pancreas and *C. atrox* phospholipase A2 increases enzyme activity, particularly toward negatively charged substrates (Randolph & Henrikson, 1982; van Scharrenburg et al., 1983).

Thus, it appears that the composition and sequence of the $NH_2$-terminal region of phospholipases A2 may govern functional differences among members of this class of enzymes. The unusual concentration of basic residues in the functionally important $NH_2$-terminal $\alpha$-helix may also confer special properties that distinguish BPI-responsive from BPI-nonresponsive phospholipases A2. This possibility is supported by unpublished observations on three other highly basic ($pI > 9.6$) venom phospholipases A2 (kindly made available by Drs. J. Maraganore and R. L. Heinrikson of the Department of Biochemistry, University of Chicago).[2] Only one of these, with a cluster of five basic residues identical with the one in the basic *A. h. blomhoffii* phospholipase A2, is active toward BPI-killed *E. coli*, indicating that overall basicity of a given phospholipase A2 is not sufficient per se for BPI-dependent hydrolysis.

How lysines contribute to a conformation of the basic *Agkistrodon* enzyme that is favorable for its action on BPI-killed *E. coli* is unclear. The profound effect on this action of modification of, on average, only a single lysine per molecule, either by carbamylation ($\downarrow$ net charge) or by reductive methylation (no $\Delta$ charge), suggests that subtle conformational changes are sufficient to disturb the BPI-dependent function. The discrete nature of the alterations produced is shown further by the fact that neither modification affects the enzyme's activity toward artificial substrates (Forst et al., 1982).

Our attempt to identify the carbamylated lysines with a functional role in BPI-dependent hydrolysis did not yield conclusive results. Although the four $NH_2$-terminal lysines were, in fact, the most reactive ones in the molecule, the selectivity of modification of individual lysines was not sufficient to exclude the contribution of lysines in other parts of the molecule as codeterminants in BPI-dependent hydrolyxis by this enzyme.

More precise identification of specific residues, including lysines, involved in BPI-dependent hydrolysis may be possible by applying recently developed techniques for semisynthetic modification at discrete sites in the phospholipase A2 molecule (van Scharrenburg et al., 1983) or site-specific substitutions by genetic means. If it can be shown that substitution of residues on the polar face of the $NH_2$-terminal $\alpha$-helix can convert active phospholipases A2 to BPI-unresponsive ones and vice versa, the conclusion would be justified that compositional differences in the $NH_2$-terminal region are critical determinants of the functional behavior of these enzymes.

The phenomenon of BPI-dependent phospholipase A2 action is useful because it serves to bring out subtle functional and biological differences among phospholipases A2 that are not readily apparent otherwise. Our initial observations on BPI-dependent hydrolysis were made on BPI and phospholipase A2 of rabbit neutrophils (Elsbach et al., 1979). The future exploration of the structural properties of the neutrophil phospholipase A2, an enzyme that has BPI-killed *E. coli* as one of its natural targets and that is also a positively charged protein (Elsbach et al., 1979; unpublished observations), should be aided by the studies on the basic *A. h. blomhoffii* phospholipase A2 presented herein.

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