Identification of the apparently essential lysine residues in phospholipase C (*Bacillus cereus*)

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Phospholipase C (*Bacillus cereus*) contains two apparently essential and very reactive lysine residues that may be labelled selectively by pyridoxal 5'-phosphate [Aurebekk & Little (1977) *Biochem. J.* **161**, 159–165]. One of these lysine residues was found in the 25-amino acid N-terminal fragment liberated by CNBr digestion of the pyridoxal-labelled enzyme and identified as lysine-6. Two of the labelled peptides isolated from the chymotryptic digest of pyridoxal-labelled enzyme contained proline, suggesting that the other labelled lysine residue is situated in the same region of the primary structure as the single proline residue of the enzyme.

Evidence has been presented indicating that phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) contains lysine, arginine and histidine residues and carboxy groups that appear to be essential in some way for catalytic activity (Aurebekk & Little, 1977*a,b*; Little & Aurebekk, 1977; Little, 1977). In addition, tightly bound Zn^{2+} seems essential for catalytic activity (Little & Otnæss, 1975). No information is available about the location of the essential amino acid side chains in the primary structure of the enzyme. In the present work, an attempt has been made to identify the two apparently essential and very reactive lysine residues.

Materials and methods

Pyridoxal 5'-phosphate, CNBr and bovine pancreatic chymotrypsin (3 × crystallized) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Carrier-fixed trypsin (trypsin-30, ENZYGEL) was obtained from Boehringer Mannheim G.m.b.H., Mannheim, Germany. Analytical t.l.c. plates of silica gel (0.25 mm layer thickness) or cellulose (0.1 mm layer thickness) were obtained from E. Merck, Darmstadt, Germany, as were the t.l.c. plates of silica gel (2 mm layer thickness) used for preparative purposes. Dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride and polyamide sheets were from BDH, Poole, Dorset, U.K.

Phospholipase C was isolated from cultures of *Bacillus cereus* as described previously (Little *et al.*, 1975; Myrnes & Little, 1980). Enzyme samples used appeared homogeneous in the polyacrylamide-disc-gel-electrophoresis systems of Davis (1964) and

Laemmli (1970). Enzyme assays were carried out by continuous titration (Zwaal *et al.*, 1971; Little *et al.*, 1975).

Preparation of pyridoxal-labelled phospholipase C

Phospholipase C (200 mg in 100 ml of 0.05 M-sodium phosphate buffer, pH 7.5) was incubated with 1.5 mM-pyridoxal 5'-phosphate for 65 min at 22°C in the dark with continuous stirring. Afterwards, NaBH_4 from a freshly prepared aqueous solution was added to a final concentration of 0.2 mg/ml and the system incubated for a further 5 min. The modified enzyme was then loaded on a column (2.4 cm × 5 cm) of agarose-linked egg-yolk lipoprotein (Takahashi *et al.*, 1974; Little *et al.*, 1975) at room temperature. The column was then thoroughly washed with 0.05 M-phosphate buffer (pH 7.5) until the eluate had no A_{325} . Enzyme was then eluted from the column with a freshly prepared solution of urea (8 M) in the above phosphate buffer. Enzyme eluted from the column was dialysed twice for 2 h against 2 litres of 0.05 M-phosphate buffer (pH 7.5). Modified phospholipase C (97 mg) had a specific activity 30% of that of untreated enzyme and an apparent pyridoxal 5'-phosphate content of 1.3 mol/mol of enzyme.

Cleavage conditions

For cleavage by chymotrypsin, phospholipase C (0.3 mg/ml) in 0.1 M- NH_4HCO_3 was incubated for 2 h at 37°C with chymotrypsin (1% of the weight of phospholipase C). Digestion was stopped by the addition of acetic acid (final concn. 5%, v/v). Precipitated material was removed by centrifuga-

tion (12000 g for 10 min) and the supernatant frozen and freeze-dried.

For cleavage by CNBr, phospholipase C (1 mg/ml) in 70% (w/v) formic acid was treated with a 200-fold molar excess of CNBr in a tightly stoppered tube for 24 h at room temperature. The reaction mixture was then frozen and freeze-dried.

For cleavage by trypsin, 0.5 mg of the small CNBr fragment of phospholipase C in 2 ml of 0.1 M-phosphate buffer (pH 7.5) was incubated with 0.25 vol. of swollen carrier-fixed trypsin for 4 h at 24°C. The immobilized proteinase was then removed by filtration.

N-Terminal amino acids were identified by using dansyl chloride as described by Hartley (1970) and Gray (1972).

For amino acid analysis, samples were hydrolysed in 6 M-HCl in evacuated sealed tubes for 24 h at 110°C. Analyses were carried out with a Jeol JLC-6AH automatic amino acid analyser. Fluorescence determinations were made in a Perkin-Elmer MPF-3 fluorescence spectrophotometer with t.l.c.-plate-scanning accessory at room temperature, with an excitation wavelength of 325 nm and an emission wavelength of 392 nm. Measurements of the amount of pyridoxal 5'-phosphate incorporated into the enzyme or peptides therefrom were based on the molar absorption coefficient at 325 nm for the reduced aldimine formed between pyridoxal 5'-phosphate and the ϵ -amino group of lysine (ϵ 10700 litre·mol⁻¹·cm⁻¹) (Fischer *et al.*, 1963). Fluorescence from t.l.c. plates was also examined with a portable u.v. lamp, with inspection by eye.

Fig. 1. Gel filtration of chymotryptic digest
Freeze-dried chymotryptic digest was dissolved in 4 ml of 0.02 M-NH₄HCO₃ and applied to a column (2.8 cm × 78.5 cm) of Bio-Gel P-6. The column was eluted with 0.02 M-NH₄HCO₃ and fractions (5 ml) were collected and analysed for pyridoxal fluorescence (----) and A₂₈₀ (—). The horizontal bar denotes the fractions that were pooled, and the material therein was subsequently purified further.

The homogeneity of the isolated chymotryptic fragments was studied by two-dimensional t.l.c. on silica plates, with butan-1-ol/acetic acid/water (4:1:1, by vol.) in the first dimension and chloroform/methanol/25% (v/v) NH₃ (2:1:1, by vol.) in the second dimension. The homogeneity was also checked by one-dimensional t.l.c. on cellulose plates with chloroform/methanol/25% NH₃ (2:1:1, by vol.) or butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.).

Results and discussion

Isolation of pyridoxal-labelled chymotryptic fragments

After treatment with pyridoxal 5'-phosphate, phospholipase C was submitted to chymotrypsin digestion. After acidification and centrifugation, the digest was submitted to gel filtration on Bio-Gel P-6 (Fig. 1), and fractions giving pyridoxal fluorescence

Fig. 2. Analytical t.l.c. of chymotryptic digest before (a) and after (b) gel filtration

T.l.c. was carried out on silica plates, with chloroform/methanol/25% NH₃ (2:2:1, by vol.) as solvent system. The spots indicated were ninhydrin-positive, with the shaded spots also showing pyridoxal fluorescence. The origin is marked X.

were pooled and concentrated by freeze-drying. The results of t.l.c. of the digest before and after gel filtration are shown in Fig. 2. Components indicated as (1), (2) and (3) were strongly fluorescent and well separated from the non-fluorescing components. Consequently the fluorescent fraction from gel filtration was submitted to preparative t.l.c. on silica plates of 2 mm layer thickness and then developed in the same system as in Fig. 2. The fluorescent components could, by virtue of their very low mobility, be simply scraped off the plate as one fraction and so separated from the other non-fluorescent material. Fluorescent material was then eluted from the silica with 0.5% (w/v) NH_3 . The material was then freeze-dried, dissolved in 0.1 M NH_4HCO_3 and loaded on a column of DEAE-Sephadex A-25. The column was eluted with 260 ml of a linear gradient of 0.1–0.6 M NH_4HCO_3 . The elution profile showed two overlapping peaks of fluorescence and another fairly well separated one. Two separate pools of fractions were collected, as indicated in Fig. 3, and freeze-dried. Fraction 2 (Fig. 3) was then rechromatographed on DEAE-Sephadex and eluted with a linear gradient of 0.3–0.6 M NH_4HCO_3 . Two clearly resolved peaks of fluorescent material were obtained (Fig. 4), and fractions in these two peaks (indicated as 2A and 2B in Fig. 4) were pooled separately and freeze-dried. The homogeneity of the peptides from peak 1 (Fig. 3) and peaks 2A and 2B (Fig. 4) was examined by t.l.c. on both silica and cellulose plates (see the Materials and methods section).

Fig. 3. Chromatography on DEAE-Sephadex of the fluorescent peptides obtained after preparative t.l.c. The freeze-dried extract from the preparative t.l.c. step was dissolved in 0.1 M NH_4HCO_3 and applied to a column (1 cm $\times$ 10 cm) of DEAE-Sephadex A-25 that had been equilibrated against 0.1 M NH_4HCO_3 . The column was then washed with 0.1 M NH_4HCO_3 (200 ml) and finally eluted with a linear gradient (----) of 0.1–0.6 M NH_4HCO_3 . Fractions (2.5 ml) were collected and analysed for pyridoxal fluorescence (—). The horizontal bars indicate fractions that were pooled.

In both one- and two-dimensional t.l.c. systems the material from each of the three peaks appeared homogeneous, with a single ninhydrin-positive spot coinciding with the single fluorescent spot. The amino acid compositions and *N*-terminal residues of the three labelled chymotryptic fragments are shown in Table 1. Two of the fragments contained proline, suggesting that one labelled lysine residue occurs in the same area of the primary structure as does the sole proline residue in the enzyme (Otnæss *et al.*, 1977).

Studies on the 25-residue *N*-terminal fragment

After chymotryptic digestion, the reaction mixture was normally acidified to stop the reaction. Considerable precipitation of material occurred on acidification. From non-acidified digest, a fluorescent peptide was isolated which was shown by amino acid analysis to contain methionine (results not shown). Since phospholipase C contains a single methionine residue, methionine-25 (Otnæss *et al.*, 1977), the possibility that apparently essential lysine residue(s) might be located in the *N*-terminal region was investigated. Pyridoxal-labelled phospholipase C was cleaved with CNBr and separated into two fragments by gel filtration. Both fragments possessed typical pyridoxal fluorescence (Fig. 5), suggesting that both contain a labelled lysine residue. The fluorescence of the large fragment was not decreased by a further cycle of CNBr treatment, thus indicating that the fluorescence did not arise from uncleaved enzyme.

The 25-amino acid residue fragment contains

Fig. 4. Further chromatography of the peptides of peak 2 (Fig. 3) on DEAE-Sephadex

After freeze-drying, the peptides of peak 2 (Fig. 3) were dissolved in 0.2 M NH_4HCO_3 and loaded on to a column of DEAE-Sephadex A-25 (1 cm $\times$ 10 cm). The column was first washed with 0.2 M NH_4HCO_3 (50 ml) and then eluted with a concentration gradient (----) of 0.3–0.6 M NH_4HCO_3 . Fractions (2.5 ml) were collected and analysed for pyridoxal fluorescence (—). The horizontal bars indicate fractions that were pooled.

Table 1. Amino acid composition and N-terminal residues of three chymotryptic fragments of pyridoxal-labelled phospholipase C

6-N-Pyridoxyl-lysine was determined spectrophotometrically. Spectrophotometric analysis indicated the absence of tryptophan for all three peptides. Numbers in parentheses indicate the nearest integer.

Amino acid	Content (mol/mol of peptide)		
	Peptide 1	Peptide 2	Peptide 3
Aspartic acid	1.63 (2)	1.66 (2)	3.28 (3)
Threonine	1.90 (2)	0.95 (1)	1.53 (2)
Serine	1.26 (1)	2.52 (3)	2.45 (3)
Glutamic acid	4.10 (4)	3.90 (4)	3.48 (3)
Proline	0 (0)	0.70 (1)	1.08 (1)
Glycine	2.90 (3)	3.12 (3)	3.37 (3)
Alanine	4.31 (4)	1.62 (2)	2.05 (2)
Isoleucine	0.44 (1)	0.54 (1)	0.6 (1)
Leucine	0.50 (1)	0.89 (1)	0.57 (1)
Phenylalanine	0.89 (1)	0 (0)	0.92 (1)
Histidine	0.64 (1)	1.3 (1)	1.18 (1)
Lysine	3.19 (3)	0.98 (1)	1.96 (2)
Arginine	0.37 (1)	0.54 (1)	0.64 (1)
6-N-Pyridoxyl-lysine	1 (1)	1 (1)	1 (1)
No. of residues	25	22	25
N-Terminal residue	Alanine	Glycine	Lysine

Fig. 5. Gel filtration of CNBr-cleaved labelled enzyme. Pyridoxal-labelled enzyme (4 mg) was cleaved with CNBr, freeze-dried, dissolved in 200 μ l of NH_3 (0.5%) and loaded on to a column (0.7 cm $\times$ 60 cm). The column was eluted with 0.5% NH_3 and fractions (0.5 ml) were collected and analysed for pyridoxal fluorescence (----) and A_{280} (—).

Fig. 6. Gel filtration of the tryptic digest of the small CNBr fragments from labelled phospholipase C

The retarded CNBr fragment (Fig. 4) was freeze-dried and cleaved with immobilized trypsin (see the Materials and methods section). The tryptic digest was loaded on to a column (0.7 cm $\times$ 60 cm) of Bio-Gel P-2 and eluted with 0.02 M- NH_4HCO_3 . Fractions (0.5 ml) were collected and analysed for pyridoxal fluorescence (----) and A_{220} (—).

lysine at positions 5 and 8 (Otnæss *et al.*, 1977). From the known amino acid sequence of this fragment, it is possible to predict the tryptic-cleavage pattern when either lysine-6 or lysine-8 is blocked. If lysine-6 were blocked, tryptic cleavage would yield the pyridoxal-labelled fragment tryptophan-1 to lysine-8, together with a 5- and a 12-amino acid residue fragment. If lysine-8 were blocked, the labelled fragment would be histidine-7 to arginine-20, and a 5- and a 6-amino acid fragment would also be produced. After tryptic digestion of the small

CNBr-cleavage product, the mixture was separated by gel filtration (Fig. 6). Fractions containing pyridoxal fluorescence were pooled and freeze-dried, and then loaded on to a t.l.c. plate of silica (0.25 mm layer thickness) and the chromatogram was developed in chloroform/methanol/25% NH_3 (2:2:1, by vol.). The developed plate showed one zone of fluorescence and a non-fluorescing ninhydrin-positive zone (results not shown). The former

Table 2. Amino acid composition of the fluorescent tryptic peptide of the 25-residue N-terminal fragment of pyridoxal-labelled phospholipase C

6-N-Pyridoxyl-lysine and tryptophan were determined spectrophotometrically. Numbers in parentheses indicate the nearest integer.

Amino acid	Content (mol/mol of peptide)	
	Observed	Predicted
Aspartic acid	1.3 (1)	1
Glutamic acid	2.4 (2)	2
Glycine	5.1 (5)	0
Alanine	1.3 (1)	1
Histidine	1.35 (1)	1
Lysine	1.1 (1)	1
6-N-Pyridoxyl-lysine	1 (1)	1
Tryptophan	0.9 (1)	1
No. of residues	8*	8

* The glycine residues have been ignored.

zone was scraped off the plate, extracted with 0.5% NH_3 and freeze-dried. Amino acid analysis was carried out on this material (Table 2). Apart from the glycine content, the amino acid composition of the tryptic peptide was the same as that which can be predicted if lysine-6 were blocked. The much higher than expected glycine content may arise, in part at least, from tryptophan degradation.

Thus, one of the two apparently essential and very reactive lysine residues of phospholipase C (*B. cereus*) appears to be lysine-6 and the other one appears to be situated close to the single proline residue in the enzyme.

A search was also made to establish whether other apparently essential side chains might occur within 25 residues of the N-terminal of the enzyme. Consequently, phospholipase C was inactivated by approx. 90% by incubation with either cyclohexane-1,2-dione in borate buffer to modify essential arginine residues (Aurebekk & Little, 1977b) or 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide in the presence of *N*-(2,4-dinitrophenyl)ethylenediamine to modify essential carboxy groups (Little & Aurebekk, 1977) under the reaction conditions described in these two references. Modified enzyme was cleaved with CNBr, and the small fragment was analysed for label incorporation after the carboxy-modifying treatment and for decreased arginine content after treatment with the dione. No evidence for either arginine or carboxy-group modification was found in the small fragment (results not

shown), thus suggesting that the apparently essential arginine and carboxy residues are situated outside the 25-residue N-terminal region.

X-ray-crystallographic studies on phospholipase C have been started (Hough *et al.*, 1978), and it is hoped that the findings in the present paper may eventually assist in establishing the location of the active site in the three-dimensional structure of the enzyme.

Note Added in Proof (Received 31 December 1980)

Recent evidence (A.-B. Otnæss, unpublished work) indicates that the enzyme contains more than one proline residue. Thus the major conclusion in the present paper should be amended to: one of the two apparently essential lysine residues in phospholipase C (*B. cereus*) appears to be lysine-6 and the other one appears to be situated close to a proline residue in the enzyme.

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