Phospholipase C from *Bacillus cereus*

EVIDENCE FOR ESSENTIAL LYSINE RESIDUES

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1. Phospholipase C was inactivated by exposure to the three amino-group reagents, ethyl acetimidate, 2,4,6-trinitrobenzenesulphonic acid and pyridoxal 5'-phosphate plus reduction. 2. Inactivation by pyridoxal 5'-phosphate showed the characteristics of Schiff's base formation with the enzyme. The pyridoxal 5'-phosphate-treated enzyme after reduction had an absorbance maximum at 325 nm and 6-*N*-pyridoxyl-lysine was the only fluorescent component after acid hydrolysis. 3. For complete inactivation, 2 mol of pyridoxal 5'-phosphate or 7 mol of 2,4,6-trinitrophenyl were incorporated/mol of enzyme. 4. The two apparently essential lysine residues were much more reactive to pyridoxal 5'-phosphate than the other 19 lysine residues in the enzyme. 5. Binding of phospholipase C to a substrate-based affinity gel caused marked protection against inactivation by pyridoxal 5'-phosphate. For complete inactivation of the gel-bound enzyme, 5 mol of pyridoxal 5'-phosphate were incorporated/mol of enzyme and there was no evidence for two especially reactive lysine residues. 6. On application of pyridoxal 5'-phosphate-treated enzyme (remaining activity 30% of original) to a column of the affinity gel, some material bound and some did not. The latter contained very little enzyme activity and was heavily incorporated with reagent (9.06 mol/mol of enzyme). The former had a specific activity of 34% of that of the control and contained 1.29 mol of reagent/mol of enzyme. 7. Thus phospholipase C appears to contain two lysine residues that are essential for enzyme activity, but probably not for substrate binding.

Phospholipase C (phosphatidylcholine choline phosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* catalyses the hydrolysis of certain phospholipids at the phosphate moiety. The enzyme has been purified to homogeneity (Zwaal *et al.*, 1971; Otnaess *et al.*, 1972; Little *et al.*, 1975) and certain aspects of its structure characterized (Little & Otnaess, 1975). We are now in the process of examining the active centre of the enzyme using amino acid-selective reagents. The present results refer to studies on the possible involvement of amino groups, in particular lysine ϵ -amino groups in the enzyme activity.

Materials and Methods

Pyridoxal 5'-phosphate and 2,4,6-trinitrobenzenesulphonic acid were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and ethyl acetimidate from Eastman Kodak, Rochester, NY, U.S.A. 6-*N*-Pyridoxyl-lysine was prepared from poly-L-lysine hydrobromide (Sigma) as described by Schnackerz & Noltman (1971). Phospholipase C from *Bacillus cereus* was prepared as described (Little *et al.*, 1975) except that in the heat-treatment step, the enzyme preparation was maintained at 69–

70°C for 10 min. The final preparations of enzyme used appeared homogeneous in the polyacrylamide-disc-gel-electrophoresis systems of Davis (1964) and Laemmli (1970). Enzyme prepared by the present method has also been shown to be homogeneous according to immunological and chemical criteria (A.-B. Otnaess, C. Little, K. Sletten, R. Wallin, S. Johnsen & R. Flengsrud, unpublished work). The enzyme was stored in veronal-buffered saline (pH 7.4) (Hjort, 1957) containing 1 mM-Zn²⁺ and dialysed twice at 4°C against 1.5 litres of 0.05 M-sodium phosphate buffer (pH 7.5) before use. Enzyme assays were carried out by the method of Zwaal *et al.* (1971) as described (Little *et al.*, 1975). The agarose-linked egg-yolk-lipoprotein affinity gel was prepared by the method of Takahashi *et al.* (1974) with the modifications of Little *et al.* (1975).

Inactivation by pyridoxal 5'-phosphate

Unless otherwise stated, enzyme and pyridoxal 5'-phosphate in the concentrations given in the Results section were incubated together for 65 min at 22°C in the dark in 0.05 M-phosphate buffer (pH 7.5). A freshly prepared aqueous solution (0.25 ml) of NaBH₄ (10 mg/ml) was then added and the system

incubated for 5 min. If the extent of pyridoxal 5'-phosphate incorporation was to be measured, the reaction system was then dialysed twice at 4°C against 1.5 litres of the above phosphate buffer. For further cycles of treatment with pyridoxal 5'-phosphate, the above procedure was repeated on the dialysed sample.

Inactivation of enzyme bound to affinity gel

Phospholipase C (3–4 mg) was loaded on to a column (5 cm × 1.6 cm) of affinity gel. The column was briefly washed with 0.05 M-phosphate buffer (pH 7.5) and then a solution of pyridoxal 5'-phosphate applied. This was left to percolate into the column until yellow material was observed in the column effluent. Flow was then stopped. After 65 min at 22°C, 1.8 ml of a freshly prepared solution of NaBH₄ was added to the column and allowed to run through the gel slowly over about 5 min. The gel was then washed with about 75 ml of the phosphate buffer. About one-third of the gel was then removed to another column and the enzyme eluted with 5 M-urea (Little *et al.*, 1975). The protein fractions were dialysed against the phosphate buffer at 4°C and examined for activity and pyridoxal 5'-phosphate content. The remaining gel was subjected to another cycle of pyridoxal 5'-phosphate treatment. Half of the remaining gel was removed and eluted with urea as above and the remainder given a further cycle of pyridoxal 5'-phosphate treatment and then eluted with urea. As far as possible, the whole process was carried out in the dark.

Identification of 6-N-pyridoxal-lysine in phospholipase C

Enzyme (about 10 µM) was treated with pyridoxal 5'-phosphate (11 mM) at pH 7.5 and reduced as described above. Part of the sample was removed and the rest subjected to five more cycles of pyridoxal 5'-phosphate treatment. The enzyme samples were dialysed three times against 1.5 litres of 0.1 M-NH₄HCO₃ solution before being freeze-dried, dissolved in water and freeze-dried again. The samples were then hydrolysed in 6 M-HCl at 110°C *in vacuo* for 24 h in sealed vials. The hydrolysates were evaporated to dryness and dissolved in the minimum volume of distilled water. The acid hydrolysates together with 6-N-pyridoxyl-lysine were then analysed by t.l.c. as described by Ernest & Kim (1974) and the developed chromatograms examined for fluorescence using an excitation wavelength of 366 nm. Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Measurements of pyridoxal 5'-phosphate incorporation into the enzyme 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). Measurements of trinitrophenylation were based on a molar absorption coefficient at 367 nm of 10888 litre·mol⁻¹·cm⁻¹ (Goldfarb, 1966; Coffee *et al.*, 1971).

Results

Inactivation by amino-group reagents

Incubation of phospholipase C with the amino-group reagents ethyl acetimidate, 2,4,6-trinitrobenzenesulphonic acid or pyridoxal 5'-phosphate resulted in inactivation (Fig. 1). Under the reaction conditions used, these reagents are generally believed to react specifically with amino groups (Dworschak *et al.*, 1975; Ernest & Kim, 1974; Means & Feeney, 1971) except that 2,4,6-trinitrobenzenesulphonic acid may also modify thiol groups (Okuyama & Satake, 1960). However, phospholipase C apparently contains no free thiol groups and is not inactivated by thiol-specific reagents (C. Little, unpublished observation). Consequently, it appears very likely that the enzyme contains essential amino groups.

Characterization of the reaction with pyridoxal 5'-phosphate

When the enzyme was treated with pyridoxal 5'-phosphate and then reduced, a rapid but incomplete

Fig. 1. Inactivation of phospholipase C by three different amino-group reagents

Enzyme (0.2 mg/ml) was incubated with the three different reagents at 22°C and samples of the reaction mixture were tested for enzyme activity at different times. All reactions were carried out in 0.05 M-phosphate buffer, pH 7.5. The reagents used were: 0.7 mM-2,4,6-trinitrobenzenesulphonic acid (time-scale in h) (○), 0.12 M-ethyl acetimidate (time-scale in min) (□) and 4.3 mM-pyridoxal 5'-phosphate (time-scale in min) (△). After incubation with pyridoxal 5'-phosphate, the system was reduced before enzyme activity was assayed (see the Materials and Methods section).

inactivation resulted (Fig. 1). Although the extent of maximum inactivation varied with the amount of reagent used, even very large molar excesses of pyridoxal 5'-phosphate failed to cause complete inactivation (Table 1). More extensive inactivation could be obtained if the same sample of enzyme was exposed to more than one cycle of incubation with pyridoxal 5'-phosphate followed by reduction, the low-molecular-weight compounds being removed from the system by dialysis between each cycle of inactivation (e.g., see Fig. 3a). A further characteristic of the inactivation was the necessity of reducing the system after incubation with pyridoxal 5'-phosphate. Failure to do this led to reactivation of the enzyme during assay. These observations are consistent with the reversible reaction of pyridoxal 5'-phosphate with enzyme amino groups to form aldimines, these being converted into stable secondary amines on reduction by NaBH₄. To characterize further the inhibition product, the absorption spectrum of a sample of pyridoxal 5'-phosphate-treated enzyme was examined after reduction and dialysis. Apart from the normal protein absorption peak at about 280nm, the spectrum of this partially active enzyme showed another absorption peak at about 325 nm. No absorption was found at wavelengths higher than about 390nm. This second absorption band, which was not seen with the native enzyme, is characteristic of 6-*N*-pyridoxyl-lysine (Fischer *et al.*, 1963). Additional evidence for the identity of the residue modified by pyridoxal 5'-phosphate is possible since the secondary amine formed on reduction of the aldimine is stable

to acid hydrolysis and may be identified chromatographically. T.l.c. was carried out on authentic 6-*N*-pyridoxyl-lysine and on the acid hydrolysates of two different samples of enzyme, one that had been exposed to a single cycle of inactivation and reduction and another that had been exposed to six such cycles (see the Materials and Methods section). When the developed chromatogram was examined for fluorescence, it was seen that the hydrolysates and the standard 6-*N*-pyridoxyl-lysine exactly co-chromatographed (*R_F* = 0.3).

Stoichiometry of inactivation by pyridoxal 5'-phosphate and 2,4,6-trinitrobenzenesulphonic acid

The extent of reagent incorporation into the enzyme was measured as a function of the amount of

Table 1. *Effect of pyridoxal 5'-phosphate concentration on the extent of inactivation*
Phospholipase C (0.192mg/ml) was incubated with different concentrations of pyridoxal 5'-phosphate. After reduction, the remaining activity was measured.

Molar excess of pyridoxal 5'-phosphate to enzyme	Activity (% of original)
0	100
36	74
170	68
360	58
1350	39

Fig. 2. *Stoichiometry of inactivation by pyridoxal 5'-phosphate and 2,4,6-trinitrobenzenesulphonic acid*
(a) Free enzyme: phospholipase C (0.25 mg/ml) was incubated at 22°C with 0.7 mM-2,4,6-trinitrobenzenesulphonic acid (○) or 0.4–8 mM-pyridoxal 5'-phosphate (Δ). Samples were taken from the reaction mixtures after various periods of time (and then reduced in the case of pyridoxal 5'-phosphate treatment) and dialysed twice against 1.5 litres of the reaction buffer (0.05M-phosphate, pH 7.5). The remaining enzyme activity and extent of reagent incorporation were then measured (see the Materials and Methods section). For high degrees of inactivation by pyridoxal 5'-phosphate, several cycles of inactivation were necessary. (b) Affinity gel-bound enzyme: affinity gel-bound phospholipase C was incubated with solutions of pyridoxal 5'-phosphate (8–40mM) at 22°C and pH7.5. Other experimental details are given in the Materials and Methods section.

Fig. 3. Effect of affinity gel-binding on the sensitivity of phospholipase C to modification and inactivation by pyridoxal 5'-phosphate

Gel-bound enzyme was incubated with pyridoxal 5'-phosphate (20 mM). Free enzyme (0.25 mg/ml) was incubated with 8 mM-pyridoxal 5'-phosphate. Three cycles of inactivation were carried out, and portions of the reaction mixture removed and examined for enzyme activity and extent of pyridoxal 5'-phosphate incorporation after each cycle. Further details are given in the Materials and Methods section. □, Free enzyme; ●, bound enzyme. (a) Enzyme inactivation; (b) extent of pyridoxal 5'-phosphate incorporation.

remaining enzymic activity for pyridoxal 5'-phosphate and 2,4,6-trinitrobenzene sulphonic acid (Fig. 2a). Essentially linear relationships were obtained in which extrapolation to complete inactivation indicated the incorporation into the enzyme of about 2 mol of pyridoxal 5'-phosphate or 7 mol of trinitrophenyl/mol of enzyme. It appeared very difficult to completely inactivate the enzyme and about 5% of activity remained even after four cycles of treatment with pyridoxal 5'-phosphate. However, this probably just reflects the reversible/equilibrium nature of the initial binding of pyridoxal 5'-phosphate to lysine residues. Because of the very long time-periods involved, no attempt was made to cause full inactivation by 2,4,6-trinitrobenzenesulphonic acid.

Inactivation of the affinity gel-bound enzyme by pyridoxal 5'-phosphate

Affinity gels formed by covalently binding egg-yolk lipoprotein to CNBr-activated agarose have been used in the purification of phospholipase C from two different sources (Takahashi *et al.*, 1974; Little *et al.*, 1975). Such gels bind the enzyme very tightly and with a high degree of specificity (Little *et al.*, 1975). The sensitivity of the affinity gel-bound enzyme to pyridoxal 5'-phosphate was examined. It was found that binding to the gel afforded considerable protection to the enzyme. Thus one cycle of treatment of the free enzyme with 8 mM-pyridoxal 5'-phosphate caused 80% inactivation, whereas one treatment of the gel-bound enzyme with 20 mM-pyridoxal 5'-phosphate caused a mere 24% inactivation (Fig. 3a). This marked protective effect could arise from simple steric factors which might hinder contact between pyridoxal 5'-phosphate and the gel-bound enzyme.

Fig. 4. Relative reactivities to pyridoxal 5'-phosphate of the essential and non-essential lysine residues

Enzyme (0.2 mg/ml) was exposed to cycles of inactivation by 31 mM- (Δ) or 8 mM- (○) pyridoxal 5'-phosphate. Portions of the reaction mixtures were examined for the extent of reagent incorporation between cycles (see the Materials and Methods section).

Another possibility is that rather than reacting with the enzyme, pyridoxal 5'-phosphate reacted with the egg-yolk proteins that are attached to the gel. That both of these possibilities are unlikely is illustrated in Fig. 3(b). Clearly, even with gel-bound enzyme, pyridoxal 5'-phosphate incorporation occurred relatively easily. Another noteworthy aspect of Fig. 3(b)

is the shape of the incorporation curves. With free enzyme, the first cycle of treatment led to the incorporation of 1.7 mol of pyridoxal 5'-phosphate/mol of enzyme, whereas the next two cycles together resulted in the further incorporation of a mere 0.3 mol of pyridoxal 5'-phosphate/mol of enzyme. With the gel-bound enzyme, a fairly constant incorporation of about 1 mol of pyridoxal 5'-phosphate/mol of enzyme occurred per cycle over three cycles.

The stoichiometry of inactivation of the affinity gel-bound enzyme is shown in Fig. 2(b). Extrapolation of the data to complete inactivation shows the incorporation of almost 5 mol of pyridoxal 5'-phosphate/mol of enzyme.

Relative reactivities to pyridoxal 5'-phosphate of the essential and non-essential lysine residues

The results in Fig. 3(b) suggest that the first 1–2 mol of pyridoxal 5'-phosphate might be incorporated into the free enzyme more readily than subsequent ones. This possibility is examined in more detail in Fig. 4 where it is apparent that the first 2 mol of pyridoxal 5'-phosphate are in fact incorporated into the enzyme much more readily than subsequent ones. Since enzyme inactivation is associated with the incorporation of the first 2 mol of pyridoxal 5'-phosphate, it is clear that the essential lysine residues are much more reactive to pyridoxal 5'-phosphate than the other non-essential ones. Another feature of Fig. 4 is the sudden increase in the amount of incorporation between the cycles 5 and 6 of the treatment. This occurred both with 8 mM-pyridoxal 5'-phosphate and with 31 mM-pyridoxal 5'-phosphate and may suggest that more extensive reaction with pyridoxal 5'-phosphate leads to enzyme denaturation.

Effect of pyridoxal 5'-phosphate modification on the binding of phospholipase C to affinity gel

A sample of enzyme that had been treated with a single inactivation cycle by pyridoxal 5'-phosphate was loaded on to a column of affinity gel. A portion of the applied material passed straight through the column without binding, and the rest of the material bound and was later eluted from the column by 5M-urea (Fig. 5). The material that did not bind to the gel had a high absorbance at 325 nm relative to its absorbance at 280 nm. The enzyme eluted from the column in 5M-urea had a much higher absorbance at 280 nm than at 325 nm and contained most of the enzyme activity applied. The material that passed straight through the column was almost totally inactive and was heavily incorporated with pyridoxal 5'-phosphate (about 9 mol/mol of enzyme), whereas the material eluted in 5M-urea contained about 1.3 mol of pyridoxal 5'-phosphate/mol of enzyme and had a specific activity of 30% of that of the native enzyme (Table 2).

Discussion

Wells (1973) demonstrated that certain lysine residues are essential for the activity of phospholipase A₂ from *Crotalus adamanteus* and the present data strongly suggest that the same is the case for phospholipase C from *B. cereus*. This conclusion is based on the following evidence. (1) Incubation of the enzyme with three different amino group-

Fig. 5. Elution profile of pyridoxal 5'-phosphate-modified enzyme from the affinity column

Experimental conditions were as described in Table 2 and in the Materials and Methods section. Fractions (1 ml) were collected and examined directly for A₂₈₀ (—) and A₃₂₅ (----). The arrow indicates elution with 5M-urea.

Table 2. Effect of pyridoxal 5'-phosphate content on binding of enzyme to affinity gel

Phospholipase C (0.3 mg/ml) was incubated with 25 mM-pyridoxal 5'-phosphate then reduced. The sample was dialysed twice against 1.51 litres of 0.05 M-phosphate buffer (pH 7.5). A sample (4 ml) was applied to a column (3 cm × 0.6 cm) of affinity gel. The column was washed with 3 ml of the phosphate buffer and then eluted with 5M-urea (see Fig. 5). Fractions containing material that passed straight through the column were pooled and examined (non-binding fraction). Fractions containing material eluted in 5M-urea were dialysed twice against 1.51 litres of the phosphate buffer before examination (binding fraction).

Material	Pyridoxal 5'-phosphate content (mol/mol of enzyme)	Relative specific activity (%)
Native enzyme	0	100
Applied sample	1.7	30
Non-binding fraction	9.06	3
Binding fraction	1.29	34

selective reagents resulted in the loss of enzyme activity. (2) Inactivation by one of these reagents, pyridoxal 5'-phosphate, showed the typical characteristics of aldimine formation with the enzyme. Thus the inactivation was reversible unless the system was reduced and the inactivation showed a saturation effect at high pyridoxal 5'-phosphate concentrations. (3) The reduced pyridoxal 5'-phosphate-treated enzyme had an absorption maximum characteristic of 6-*N*-pyridoxyl-lysine. In addition, 6-*N*-pyridoxyl-lysine was detected in the acid hydrolysate of such an enzyme sample. This was the sole fluorescent component. (4) The relation between extents of inactivation and of reagent incorporation into the enzyme was essentially linear for pyridoxal 5'-phosphate and trinitrobenzene sulphonic acid. On extrapolation, the data suggested that complete inactivation corresponded to the incorporation of 2 mol of pyridoxal 5'-phosphate or 7 mol of trinitrophenyl/mol of enzyme. (5) Binding the enzyme to a substrate-based affinity gel significantly protected the enzyme against pyridoxal 5'-phosphate inactivation and altered the stoichiometry of the reaction so that on extrapolation, complete inactivation corresponded to the incorporation of about 5 mol of pyridoxal 5'-phosphate/mol of enzyme.

The essential lysine residues represent no more than 2 out of a total of 21 such residues in the enzyme (A.-B. Otnaess, C. Little, K. Sletten, R. Wallin, S. Johnsen & R. Flengsrud, unpublished work). Pyridoxal 5'-phosphate thus reacts very selectively with the essential lysine residues and apparently much more selectively than does trinitrobenzene sulphonic acid. A similar observation has been made with rat liver glycogen synthetase, where the modification by pyridoxal 5'-phosphate of a single lysine residue per subunit causes total inactivation, but the incorporation of 2.5 mol of reagent/mol of enzyme is required for complete inactivation by trinitrobenzene sulphonic acid (Ernest & Kim, 1974).

Pyridoxal 5'-phosphate has been suggested to be a reagent for the selective labelling of lysine residues in or near phosphate-binding sites in proteins (Colombo & Marcus, 1974), and it is worthwhile to consider whether this might be the case with phospholipase C and that pyridoxal 5'-phosphate inactivates the enzyme by blocking lysine residues at a binding site for the phosphate moiety of the phospholipid substrate. An insight into this problem can be gained by examining the results obtained using the agarose-linked egg-yolk-lipoprotein affinity gel. The binding of enzyme to the gel presumably involves the formation of something resembling an enzyme-substrate complex. Binding the enzyme to such a gel afforded strong protection against inactivation by pyridoxal 5'-phosphate together with an increase in the amount of pyridoxal 5'-phosphate incorporation needed to cause complete inactivation from about

2 mol to about 5 mol of pyridoxal 5'-phosphate/mol of enzyme. The increase may, in fact, be greater than this since any heavily incorporated enzyme that could not bind to the column would be lost during the column washing. There was no evidence in the gel-bound enzyme for two lysine residues of especially high reactivity to pyridoxal 5'-phosphate, thereby suggesting that the essential lysine residues were somewhat sheltered under these conditions. Perhaps of greater significance is the observation that pyridoxal 5'-phosphate-modified partially active enzyme can still bind to the affinity gel. The results in Table 2 demonstrate that although extensive incorporation of pyridoxal 5'-phosphate into phospholipase C prevents binding to the gel, modification of the essential lysine residues does not.

The material that failed to bind to the affinity gel contained about 9 mol of pyridoxal 5'-phosphate/mol of enzyme. In view of the apparent selectivity with which pyridoxal 5'-phosphate blocks the two apparently essential lysine residues, it is perhaps somewhat surprising that a sample of pyridoxal 5'-phosphate-treated enzyme (content 1.7 mol of pyridoxal 5'-phosphate/mol of enzyme) should contain a fraction of molecules so heavily incorporated with reagent. However, even after one cycle of treatment, a small proportion of molecules would be expected to become incorporated with more than 2 mol of pyridoxal 5'-phosphate, although the number containing 9 mol should be exceedingly small. A possible explanation can be seen in Fig. 4 where there is some indication for protein denaturation after the incorporation of 4–5 mol of pyridoxal 5'-phosphate. The sudden increase in the ease of reagent incorporation after this point could explain the few heavily incorporated enzyme molecules. Such denaturation could also account for the failure of these heavily incorporated enzyme molecules to bind to the affinity gel.

The material that bound to the column and was eluted in 5M-urea contained 1.29 mol of pyridoxal 5'-phosphate/mol of enzyme and had a specific activity of 34% of that of the native enzyme. Clearly, this material was a mixture of enzyme molecules containing different integral molar proportions of pyridoxal 5'-phosphate, most probably 0, 1 and 2, and of different activities.

All fractions eluted from the gel by 5M-urea and which contained protein had essentially similar ratios of A_{280}/A_{325} . Thus, for low degrees of incorporation, the pyridoxal 5'-phosphate content does not influence the tightness of enzyme binding to the gel in the presence of 5M-urea.

Provided that the interaction between phospholipase C and the agarose-linked egg-yolk lipoprotein is indeed an enzyme-substrate-like process, then the above results indicate that low amounts of pyridoxal 5'-phosphate incorporation into the enzyme do not

prevent substrate binding. It therefore seems that phospholipase C from *B. cereus* contains two lysine residues that are in some way essential for enzyme activity. Another possibility is that the enzyme contains one essential and one equally reactive but non-essential lysine residue. Although the present data are compatible with both possibilities, the latter is somewhat less likely since both reactive lysine residues are sheltered when the enzyme binds the affinity gel and therefore both are probably in or very near to the active site.

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