The Histidine Residues of Phospholipase C from *Bacillus cereus*

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The inactivation of phospholipase C from *Bacillus cereus* at pH 6 by diethyl pyrocarbonate paralleled the *N*-ethoxyformylation of a single histidine residue in the enzyme. The inactivation arose from a decrease in the maximum velocity of the enzymic reaction with no effect on the K_m value. The inactivation did not apparently alter the ability of the enzyme to bind to a substrate-based affinity gel. The native enzyme contained only one reactive histidine residue. Removal of the two zinc atoms from the enzyme increased the number of reactive histidine residues to five, whereas in the totally denatured enzyme nearly eight such residues were available for reaction with diethyl pyrocarbonate. The enzyme thus appears to contain one histidine residue that is essential for catalytic activity and four that may be involved in co-ordinating the zinc atoms in the structure.

In previous papers (Little & Otnæss, 1975; Aurebekk & Little, 1977a; Little & Aurebekk, 1977; Aurebekk & Little, 1977b) we demonstrated in phospholipase C from *Bacillus cereus* that the presence of Zn^{2+} , lysine residues, a carboxyl group and an arginine residue are essential for catalytic activity. By using a wide range of amino acid-selective reagents, we found no evidence for the involvement of cysteine, cystine, methionine, tyrosine, tryptophan or serine residues in the enzyme activity (B. Aurebekk & C. Little, unpublished work). Chemical modification of histidine residues was, however, associated with enzyme inactivation, and a report of a study into the histidine groups of phospholipase C is now presented.

Materials and Methods

Diethyl pyrocarbonate and pyridoxal 5'-phosphate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Phospholipase C was purified from the culture supernatant of *B. cereus* as described previously (Little *et al.*, 1975). Dihexanoylphosphatidylcholine (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine) was synthesized by the method of Cubero Robles & Van den Berg (1969) as described by Little (1977).

Routine enzyme assays were performed at 23°C as described by Zwaal *et al.* (1971). For enzyme-kinetic studies, dihexanoylphosphatidylcholine dissolved in 0.15 M-NaCl was used as substrate. The rate of acid production was measured by continuous titration in Radiometer pH-stat equipment with a 0.25 ml burette. The reaction vessel contained 5 ml of substrate and was thermostatically controlled at 25°C. The end point was pH 7.5 and 0.02 M-NaOH was used as titrant.

Agarose-immobilized egg-yolk-lipoprotein affinity gel was prepared by the method of Takahashi *et al.* (1974). Zinc was determined by atomic-absorption spectroscopy as described previously (Little & Otnæss, 1975). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and also by the A_{280} ($\epsilon = 51\,000$ litre·mol⁻¹·cm⁻¹) in sodium phosphate buffer (pH 6.0) (C. Little, unpublished work). The binding of pyridoxal 5'-phosphate to the enzyme was carried out as described by Aurebekk & Little (1977a), with the extent of incorporation being calculated on the basis of the molar absorption coefficient at 325 nm for the reduced aldimine ($\epsilon = 10\,700$ litre·mol⁻¹·cm⁻¹) (Fischer *et al.*, 1963).

The reaction of the enzyme with diethyl pyrocarbonate was carried out in 0.05 M-sodium phosphate buffer, pH 6.0, at room temperature (22–23°C). Solutions of the reagent in ethanol were prepared immediately before use. The extent of histidine modification was estimated from the increment in A_{240} of the enzyme solution ($\epsilon = 3200$ litre·mol⁻¹·cm⁻¹) (Ovadi *et al.*, 1967). Absorption measurements were made in a Gilford 240 spectrophotometer with a recorder. The density of the reagent was taken as 1.12 g/ml at 25°C (Melchior & Fahrney, 1970). The purity of the reagent was estimated as 80% by measuring the increase in A_{240} when diethyl pyrocarbonate (approx. 0.15 mm) was treated with 5 mm-histidine in sodium phosphate buffer, pH 6.

Results

Inactivation by diethyl pyrocarbonate

Diethyl pyrocarbonate is a reagent commonly used for the selective modification of histidine

Fig. 1. Inactivation of phospholipase C by diethyl pyrocarbonate

Enzyme (31 μ M) was incubated with 2.8 mM-diethyl pyrocarbonate, and samples of the reaction mixture were tested for enzyme activity at different times (●). The control (□) contained all except the reagent.

Fig. 2. Stoichiometry of inactivation of phospholipase C by diethyl pyrocarbonate

Enzyme (31 μ M) was incubated with 2.8 mM- (●) or 0.8 mM- (■) diethyl pyrocarbonate. Alternatively, enzyme (0.53 mM) was incubated with 0.95 mM (□) or 0.58 mM (○) reagent. Samples were withdrawn from the reaction mixtures at various times up to 35 min after the addition of reagent and tested for enzyme activity. The extent of *N*-ethoxyformylation of histidine was followed spectrophotometrically (see the Materials and Methods section). In the case of the very concentrated enzyme solutions, the sample was diluted with the reaction buffer before analysis.

residues in proteins. Incubation of phospholipase C with this reagent resulted in enzyme inactivation (Fig. 1). Under the reaction conditions used, the inactivation process followed closely pseudo-first-order kinetics until at least 75% inactivation. Use of the same concentration of enzyme but lesser amounts of reagent resulted in lower rates of inactivation with deviations from pseudo-first-order kinetics. The

Table 1. Effect of diethyl pyrocarbonate on the essential lysine residues

Enzyme (20 μ M) was incubated with diethyl pyrocarbonate until the remaining activity was 4% of the original. The sample was then dialysed for 3 h against 0.05 M-sodium phosphate buffer, pH6, and then for 3 h against 0.05 M-sodium phosphate buffer, pH7.5. After incubation with 10 mM-pyridoxal 5'-phosphate, followed by NaBH₄ then dialysis (see Aurebekk & Little, 1977a), the extent of pyridoxal 5'-phosphate incorporation into the enzyme was measured. The control enzyme mixture was treated in the same way, except that the diethyl pyrocarbonate step was omitted.

Material	Pyridoxal 5'-phosphate content (mol/mol of enzyme)
Control enzyme	2.0
Diethyl pyrocarbonate-treated enzyme	2.01

latter effect is consistent with the known instability of diethyl pyrocarbonate in aqueous solution (Melchior & Fahrney, 1970).

The extent of *N*-ethoxyformylation of histidine may be measured directly by spectrophotometry. When the enzyme activity was measured as a function of the extent of histidine modification, a good linear relationship was found (Fig. 2). Extrapolation of the data suggested that full inactivation of the enzyme was associated with the *N*-ethoxyformylation of a single histidine residue. This stoichiometry was observed with molar excesses of reagent over enzyme as high as 90 and as low as 1.1. It would seem from these results that phospholipase C contains one histidine residue that is essential for catalytic activity. Diethyl pyrocarbonate, however, may also react slowly with amino groups and, since the enzyme contains essential lysine residues, the possibility must be considered that the inactivation involves the modification of unusually reactive lysine groups. One approach to this problem is to use hydroxylamine. Unlike *N*-ethoxyformylhistidine, the reaction product between diethyl pyrocarbonate and amino groups is very stable towards hydroxylamine (Bleszynski & Leznicki, 1967). Unfortunately hydroxylamine inactivated the native enzyme. The two essential lysine residues of phospholipase C show a much greater reactivity towards pyridoxal 5'-phosphate than do the other lysine residues in the enzyme (Aurebekk & Little, 1977a). Consequently the reaction between pyridoxal 5'-phosphate and the diethyl pyrocarbonate-treated enzyme was examined. If enzyme inactivation by the latter reagent involved blocking of essential lysine residues, this would be expected to lessen the extent of reaction of the enzyme with pyridoxal 5'-phosphate. The results in Table 1 clearly demonstrate that both native enzyme and

diethyl pyrocarbonate-treated enzyme are able to bind freely 2 mol of pyridoxal 5'-phosphate/mol of enzyme, thus suggesting that in both enzyme samples the essential lysine residues were intact. The possible role of tyrosine in the inactivation was ruled out by the observation that treatment with diethyl pyrocarbonate failed to alter the absorption of the enzyme at 278 nm. Ethoxyformylation of tyrosine causes decreases in protein absorption at 278 nm (Mühlrad *et al.*, 1967).

As a check for possible side reactions between the reagent and lysine or arginine at pH 6, 0.15 mM-diethyl pyrocarbonate was incubated with 5 mM-histidine in phosphate buffer (pH 6) in the presence or absence of either 5 mM-lysine or 5 mM-arginine. The yield of *N*-ethoxyformylhistidine was unaffected by the presence of arginine and decreased by less than 5% by the presence of lysine, thus confirming that the imidazole ring of histidine is by far the preferred target for this reagent at pH 6.

Properties of the inactivated enzyme

To obtain further information on the nature of the inactivation by the histidine reagent, the kinetics of the modified enzyme were examined and compared with those of the control enzyme. As substrate for the enzyme dihexanoylphosphatidylcholine, a short-chain water-soluble phosphatidylcholine was chosen

and used at concentrations well below its critical micelle concentration of 14 mM (de Haas *et al.*, 1971). The results, in the form of a Lineweaver-Burk plot (Fig. 3), indicate that inactivation of the enzyme by diethyl pyrocarbonate arises from a decrease in $V_{\max}$ of the enzyme with no significant change in the K_m value. The sample of reagent-treated enzyme used in the present work had been inactivated by 62% as indicated by enzyme assay against micelles of crude egg-yolk phosphatidylcholine. With dihexanoylphosphatidylcholine as substrate, an identical extent of inactivation was observed. Clearly, therefore, diethyl pyrocarbonate destroys the activity of phospholipase C towards micelles and towards monomolecularly dispersed substrates at the same rate.

Previously we have used the enzyme's capacity to bind a substrate-based affinity gel as a measure of the ability of the enzyme to bind substrate (Aurebekk & Little, 1977a,b; Little & Aurebekk, 1977). When a sample of diethyl pyrocarbonate-inactivated enzyme was applied to a column of such gel, more than 98% of the applied sample bound tightly and was released on elution of the column with 8 M-urea (Fig. 4). It thus appears that blocking the most reactive histidine residue in the enzyme has no effect on this aspect of substrate binding.

Fig. 3. Lineweaver-Burk plots for the native and diethyl pyrocarbonate-treated enzymes

Enzyme (18 μ M) was inactivated to 38% of the original activity by incubation for 20 min with 1.2 mM-diethyl pyrocarbonate. The reaction was stopped by dialysis against 0.05 M-sodium phosphate buffer, pH 6, at 4°C. The rates of hydrolysis by native (●) and inactivated enzyme (□) of solutions of dihexanoylphosphatidylcholine were then measured. The reaction rates have been corrected for differences in protein concentration between the samples.

Fig. 4. Elution profile of diethyl pyrocarbonate-inactivated enzyme from the affinity column

Enzyme (31 μ M) was inactivated to 31% of the original activity with 2.8 mM-diethyl pyrocarbonate. The inactivated enzyme (0.8 ml) was applied to a column (3 cm $\times$ 0.6 cm) of Sepharose-immobilized egg-yolk lipoprotein. The column was washed with 4 ml of 0.05 M-sodium phosphate buffer, pH 6, and then eluted with 8 M-urea dissolved in the same buffer. Fractions (1 ml) were collected and the A_{280} was measured directly.

Fig. 5. Reaction of diethyl pyrocarbonate with phospholipase C under different conditions

Enzyme (18–34 μM) after pretreatment was incubated with 5.6 mM-diethyl pyrocarbonate. A, Native enzyme, no pretreatment; B, enzyme dialysed for 4 h at 22–24°C against 8 M-urea in 0.05 M-sodium phosphate buffer, pH 6; C, enzyme dialysed for 4 h at 22–24°C against 2 mM-EDTA in 0.05 M-sodium phosphate buffer, pH 6; D, enzyme dialysed for 4 h at 22–24°C against a solution containing 2 mM-EDTA and 8 M-urea in 0.05 M-sodium phosphate buffer, pH 6; E, enzyme boiled for approx. 2 s, then sodium dodecyl sulphate (1%, w/v, final concn.) was added to solubilize the resultant turbidity.

Extent of histidine modification

In an attempt to measure the total number of available histidine residues in phospholipase C, the enzyme was incubated over relatively long time periods with diethyl pyrocarbonate (Fig. 5). Only one histidine residue in the native enzyme seemed reactive to the reagent. The addition of more reagent after 30 min failed to increase the extent of reaction. Attempts were then made to denature the enzyme and uncover further histidine residues. Carrying out the reaction in 8 M-urea merely increased the rate of histidine modification without altering the extent of reaction. The presence of 1% (w/v) sodium dodecyl sulphate appeared to denature the enzyme, but rather slowly, and more than one histidine residue was able to react with the reagent (results not shown). High concentrations of ethanol seemed more effective at denaturing the enzyme, so that in the presence of 25% (v/v) ethanol about seven histidine residues were able to react with diethyl pyrocarbonate (Table 2). The exact extent of the reaction was difficult to ascertain because of the formation of turbidity in the solution. The most effective method for exposing the hidden histidine groups was to boil the enzyme very briefly (approx. 2 s) and then solubilize the denatured protein with sodium dodecyl sulphate (1%, w/v, final concn.).

Table 2. Number of available histidine residues in phospholipase C

Enzyme (18–34 μM) was treated with 5.6 mM-diethyl pyrocarbonate and the maximum extent of reaction measured. EDTA/enzyme with and without 8 M-urea and boiled enzyme were prepared as described in Fig. 5. Where metal ions were added to the EDTA-treated enzyme, CoCl_2 or ZnSO_4 was added to a final concentration of 1 mM, 5 min before the addition of diethyl pyrocarbonate. When the reaction was carried out in ethanol, the latter was added to the enzyme just before the histidine reagent.

Reaction conditions	Available histidine residues (mol/mol of enzyme)
Native enzyme	1.05
Native enzyme + 8 M-urea	1.05
EDTA/enzyme	4.95
EDTA/enzyme + 8 M-urea	5.05
EDTA/enzyme + Co^{2+}	1.15
EDTA/enzyme + Zn^{2+}	1.1
Enzyme in 25% (v/v) ethanol	7 (approx.)
Boiled enzyme	7.81

In this case about eight histidine residues were able to react with the reagent (Fig. 5 and Table 2).

Phospholipase C from *B. cereus* is a metalloenzyme containing two tightly bound zinc atoms (Little & Otnæss, 1975). As zinc is often bound in proteins by co-ordination with histidine residues, the effect of removal of the zinc from the enzyme on the number of reactive histidine groups was studied. Dialysis of the enzyme against 2 mM-EDTA resulted in extensive removal of zinc from the enzyme, together with an increase by four in the total number of reactive histidine groups (Fig. 5 and Table 2). Exposure of the enzyme to 2 mM-EDTA in the presence of 8 M-urea failed to increase this number any further (Fig. 5 and Table 2), although the rate of histidine modification was considerably increased. EDTA treatment decreased the zinc content of the enzyme from 2.1 mol of Zn/mol of enzyme to less than 0.2 mol of Zn/mol of enzyme. The addition of Co^{2+} or Zn^{2+} ions to the EDTA-treated enzyme resulted once more in an enzyme species with only one reactive histidine residue.

Discussion

The incubation of phospholipase C from *B. cereus* with diethyl pyrocarbonate at pH 6 under mild conditions resulted in a rapid inactivation which paralleled the modification of a single histidine residue in the enzyme. This stoichiometry applied even when near-equimolar ratios of reagent to enzyme were used. Under these conditions, the chances of multiple reaction of diethyl pyrocarbonate with histidine residues and the subsequent possibility of

fission of the imidazole ring (Loosemore & Pratt, 1976) should be minimal. The pseudo-first-order kinetics of the inactivation are also consistent with the modification of a single essential residue, as is the observation that the inactivation arose from a decrease in the $V_{\max}$ value of the enzyme with no significant effect on the K_m value. However, as experiments to re-activate the enzyme with hydroxylamine could not be carried out, the possibility that diethyl pyrocarbonate inactivates the enzyme as a result of a side reaction with an unusually reactive residue other than histidine must still be considered. Side reactions with this reagent could involve amino and phenolate groups and also possibly guanidine and thiol groups (Melchior & Fahrney, 1970). It is extremely unlikely that thiol or tyrosine residues are involved. The enzyme is not inactivated by thiol or tyrosine reagents and contains no free thiol groups (Otnæss *et al.*, 1972; C. Little & B. Aurebekk, unpublished work). In addition, no evidence for tyrosine modification was obtained during the present work. The most likely side reaction is probably that with amino groups, although in the present work, when high molar excesses of free amino acids over reagent were used, diethyl pyrocarbonate appeared to be virtually specific for histidine with little or no side reaction with α - and ϵ -amino or with guanidino groups. However, phospholipase A₂ from the snake *Crotalus adamanteus* is inactivated by diethyl pyrocarbonate at pH 6 as a result of lysine modification, the histidine residues not being modified in the absence of urea (Wells, 1973). It might perhaps be noted that Wells (1973) appears to have used very high molar excesses of reagent. Phospholipase C contains two lysine residues whose modification results in inactivation and which are selectively modified by pyridoxal 5'-phosphate (Aurebekk & Little, 1977a). The diethyl pyrocarbonate-inactivated enzyme was still able to bind freely 2 mol of pyridoxal 5'-phosphate, thereby suggesting that enzyme inactivation by the histidine reagent did not involve a side reaction with the reactive and essential lysine residues. Side reactions with guanidino groups are also unlikely. Although phospholipase C apparently contains essential arginine, arginine modification in the enzyme is associated with increases in the K_m value and also a destruction of the enzyme's ability to bind to substrate-based affinity gel (Aurebekk & Little, 1977b). Neither of these changes occurred after inactivation of the enzyme by diethyl pyrocarbonate. Taken together, the above evidence strongly supports the view that phospholipase C contains a single essential histidine residue and in this respect resembles phospholipase A₂ from pig pancreas (Volwerk *et al.*, 1974).

The exact role of the essential histidine residue in phospholipase C is not clear. Its modification results in no loss in the enzyme's ability to bind to the affinity

gel and so this histidine residue may not be necessary for substrate binding. Phospholipase C shows a preference for hydrolysing micellar as opposed to monomeric substrates (Little, 1977). Histidine modification decreased the activity to micellar and monomeric substrates by equal proportions, suggesting that the essential histidine residue is not specifically involved in any micelle-interface recognition site that the enzyme might possess.

Phospholipase A₂ from pig pancreas has been shown to possess such an interface recognition site which involves the N-terminal region of the enzyme (van Dam-Mieras *et al.*, 1975). As both this enzyme and phospholipase C contain essential histidine residues and show preferences for micellar substrates (Little, 1977; de Haas *et al.*, 1971), the possibility that the two enzymes interact with their phospholipid substrates by a common mechanism might be considered. Substantial differences between the two enzymes are, however, suggested by the observation that the interface recognition site and the catalytic activity of phospholipase A₂ are destroyed as a result of methionine modification in the enzyme (van Wezel *et al.*, 1976), whereas phospholipase C is totally resistant to iodoacetamide under conditions in which it might have been expected to react with methionine (C. Little, unpublished work).

Native phospholipase C seems to contain only one histidine residue that is able to react with diethyl pyrocarbonate. Other histidine residues may well be involved in co-ordinating the zinc atoms as on removal of zinc from the enzyme, diethyl pyrocarbonate was able to react with five histidine residues, i.e. two histidine residues are exposed per zinc atom removed. Further, the four histidine residues exposed by removal of Zn²⁺ from the enzyme were sheltered once more when Zn²⁺ or Co²⁺ ions were added to the EDTA-treated enzyme. It is unlikely that cysteine is involved in co-ordinating the zinc in the present work; no thiol groups were detected after EDTA treatment to remove Zn²⁺ (results not shown). Carboxyl groups could well contribute towards the co-ordination, since the enzyme seems to contain a large proportion of buried or inert carboxyl groups (Little & Aurebekk, 1977). In attempting to fully denature the enzyme and thereby expose all of the histidine residues for reaction, the very high conformational stability of the enzyme was noted. Native phospholipase C seems almost totally resistant to denaturation by 8M-urea. Even in the zinc-free enzyme, 8M-urea is not able to cause the exposure of any additional histidine groups. This stability is all the more remarkable when it is realized that the enzyme contains no disulphide bridges (C. Little, unpublished work). Denaturation of the enzyme by boiling, and subsequent solubilization with detergent, resulted in about eight histidine residues being made available for reaction with diethyl pyro-

carbonate. This value coincides with the histidine content of the enzyme indicated by amino acid analysis (A.-B. Otnæss, C. Little, K. Sletton, R. Wallin, S. Johnsen & R. Flengsrud, unpublished work).

Phospholipase C from *B. cereus* thus contains a total of eight histidine residues. Of these, one appears to be essential for catalytic activity and this is the sole reactive histidine residue in the native enzyme. Four other histidine residues are possibly involved in co-ordinating the two zinc atoms in the structure, leaving three other histidine residues in the enzyme for which a function has yet to be proposed.

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