Inactivation of Phospholipase C from *Bacillus cereus* by a Carboxyl Group Modifying Reagent

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Phospholipase C from *Bacillus cereus* was inactivated by incubation with either of the carboxyl reagents, a water-soluble carbodiimide plus a nucleophile or Woodward's reagent K. With the former reagent, the incorporation into the enzyme of the first mol of nucleophile caused a 4–5-fold increase in the K_m for dihexanoyllecithin with no significant effect on the V_m . The second mol of nucleophile incorporated caused no further change in K_m but destroyed most of the catalytic activity. Modification of the enzyme by carbodiimide plus nucleophile did not alter the relative activity of the enzyme towards micelles and monomolecularly dispersed solutions of diheptanoyllecithin. Furthermore, inactivation by this reagent did not significantly decrease the ability of the enzyme to bind to a substrate-based affinity gel. It was concluded that phospholipase C contains a single carboxyl group that is essential for catalytic activity. The enzyme also contains a total of 4–5 reactive/exposed carboxyl groups.

Recent studies of phospholipase C from *B. cereus* (phosphatidylcholine choline phosphohydrolase, EC.3.1.4.3) have indicated the presence in the enzyme of zinc atoms and lysine residues that are essential for catalytic activity.^{1,2} As part of an examination of the enzyme's active site using amino acid-selective reagents, the influence of carboxyl-group modifying chemicals on the enzyme was investigated. Several enzymes have been shown to contain essential carboxyl groups and in some cases, e.g., lysozyme, carboxyl groups appear to play a direct role in the catalytic reaction. With phospholipase C an active centre carboxyl could also be involved in substrate binding by electrostatic attraction of phosphatidylcholine. Water-soluble carbodiimides in the presence

of suitable nucleophiles have been widely used as a means of selective modification of carboxyl side chains³ and the results of a study of the effects of such reagents on phospholipase C are now presented.

MATERIALS AND METHODS

Materials. Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3'-sulfonate) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Sigma, St. Louis, Mo., USA and *N*-(2,4-dinitrophenyl)ethylenediamine.HCl (DNP-ethylenediamine) from Calbiochem AG, Lucerne, Switzerland. The concentration of this nucleophile and its extent of incorporation into the enzyme were investigated using an extinction coefficient for the 2,4-dinitrophenyl group (DNP) $E_{360}^{1\%}$ of 15 000.

Phospholipase C was isolated from the culture supernatant of *B. cereus*.^{2,5} Dihexanoyl- and diheptanoyllecithins [O-(1,2-diacyl-*sn*-glycero-3-phosphoryl)choline, 3-*sn*-phosphatidylcholine] were synthesized by the method of Cubero Robles and Van den Berg.^{6,7} Sepharose-bound egg yolk lipoprotein was prepared using the method of Takahashi *et al.*^{5,8}

Enzyme assays. Enzyme activity was assayed at 22–23 °C by measuring the rate of acid production either from pure lecithin substrates⁷ or from crude egg yolk extract.^{5,9} The assays were carried out by continuous titration using Radiometer pH stat equipment. Determinations of V_m and K_m were made using dihexanoyllecithin as substrate at pH 7.5. The kinetic constants were calculated from Lineweaver-Burk plots constructed from 8–10 rate measurements over a substrate concentration range 0.2–2 mM for each enzyme sample. Substrate was dissolved in 0.15 M NaCl. When the effect of enzyme modification on the micelle effect was studied, the rates of hydrolysis of diheptanoyllecithin in 0.15 M NaCl were measured. For each enzyme sample two different

Fig. 1. Inactivation of phospholipase C by carboxyl reagents. ● enzyme incubated with EDC plus DNP-ethylenediamine. ○ control containing all but the diimide. ▲, 12 μ M enzyme in 10 mM sodium phosphate buffer incubated with 2.6 mM Woodward's reagent K. The arrows indicate further additions of reagent to increase the final concentration by 2.6 mM. △, control containing no reagent. Enzyme activity was measured against egg yolk substrate and further experimental details are given in the Materials and Methods section.

substrate concentrations were used, 1.5 and 3 mM. The critical micelle concentration for this lecithin is approximately 2 mM.¹⁰

Inactivation conditions. Inactivations were carried out at 22 °C with the pH of the reaction mixture being maintained using a pH stat. For inactivation by the diimide, a pH of 4.75 was used and unless otherwise stated, the conditions used were: phospholipase C (40 μ M), EDC (61 mM) and DNP-ethylenediamine (18 mM). The reaction medium was 0.15 M NaCl.

When the extent of DNP incorporation into the enzyme was to be measured, samples of inactivated enzyme were dialysed against 0.05 M sodium phosphate buffer, pH 6 (4 dialyses against 1 l portions of buffer) at 4 °C. Any precipitate was removed by centrifugation. Protein was determined by the method of Lowry *et al.* using bovine serum albumin as standard.¹¹

RESULTS

Inactivation by carboxyl reagents. Incubation of phospholipase C with EDC in the presence of DNP-ethylenediamine resulted in rapid inactivation which was effectively complete after about 45 min (Fig. 1). Similar results occurred when the ethyl ester of glycine was used as

nucleophile (results not shown). The inactivation was not reversed when the enzyme was dialysed for 5 h at 4 °C and pH 7 against 0.5 M hydroxylamine. When the enzyme was incubated with Woodward's reagent K, another carboxyl reagent,^{12,13} loss of activity occurred, but further inactivation ceased after a few minutes. More extensive inactivation occurred upon the addition of more reagent (Fig. 1). These effects are consistent with the known instability of this reagent in aqueous solution.¹⁴

Stoichiometry of the inactivation. The remaining enzyme activity was measured as a function of the extent of enzyme modification by EDC plus DNP-ethylenediamine. In view of the warning given by Carraway and Koshland,³ no attempt was made to quench the reaction with acetate buffer. Instead, the reaction was stopped by dialysis. As a consequence, it was difficult to obtain enzyme samples that were less than about 40 % inactivated. The data are shown in Fig. 2 and suggest that the incorporation of a single mol of DNP/mol of enzyme would cause total inactivation. It should, however, be noted that the straight line constructed in the figure passes through relatively few experimental points and that for extents of inactivation above 70 %, the data deviate strongly from the

Fig. 2. Stoichiometry of the inactivation by EDC plus DNP-ethylenediamine. Portions of the reaction mixture were removed at different times and the remaining enzyme activity towards egg yolk substrate and the extent of DNP incorporation measured. For further details see Materials and Methods section.

straight line. Essentially similar results were obtained whether the activity was assayed by measuring the rate of acid production in crude egg yolk extract or by using the turbidimetric assay method.^{15,1}

Kinetics of the modified enzyme. Changes in enzyme activity following chemical modification may arise from changes in the maximum velocity and/or changes in K_m . In view of the rather dubious linearity between the extent of inactivation and the amount of reagent incorporation into the enzyme, the kinetic parameters of the modified enzyme were examined and compared with those of the native enzyme. For this purpose, the water-soluble artificial substrate dihexanoyllecithin was chosen and used at concentrations well below its critical micelle concentration of about 14 mM.¹⁰ In keeping with previous results, this compound served as a good substrate.⁷ Lineweaver-Burk plots constructed from the rate data obtained for the native and for the modified enzyme suggest that the incorporation of one mol of DNP/mol of enzyme does not influence the maximum velocity but causes a marked increase in K_m (Fig. 3). The incorporation of 1.4 mol of DNP/mol of enzyme leads to little or no further change in K_m but is as-

Fig. 3. Lineweaver-Burk plots for hydrolysis of dihexanoyllecithin by native and modified enzyme. The reaction rates have been corrected for differences in protein concentration between the samples.

Table 1. Effect of the extent of modification on the kinetic constants of phospholipase C. Dihexanoyllecithin was used as substrate.

DNP incorporation (mol DNP/mol enzyme)	Relative V_m (%)	K_m (mM)
0	100	0.4
0.77	87	0.8
1.0	100	1.7
1.4	62	2.0
2.14	5	1.7
3.14	<1	—

Table 2. Influence of enzyme modification on the micelle effect. Diheptanoyllecithin was used as substrate.

DNP incorporation (mol DNP/mol enzyme)	Rate with micelles/ rate with monomers
0	6.4
1.4	6.8
2.14	6.0

sociated with a decrease in the maximum velocity. Further kinetic data are presented in Table 1, from which it would appear that the first mol of DNP incorporated into the enzyme is associated with a 4–5-fold increase in K_m , whereas the second mol of DNP to be incorporated leads to a near total loss of catalytic activity.

Phospholipase C from *B. cereus* shows a markedly higher activity towards lecithin micelles than it does towards the same lecithin in a monomolecularly dispersed form.^{7,16} The effect of modifying the enzyme by EDC plus DNP-ethylenediamine on this micelle effect was examined. The results (Table 2) clearly demonstrate that the stimulation in enzyme activity brought about by using micellar as opposed to monomolecularly dispersed substrates is not influenced by the incorporation into the enzyme of up to 2.14 mol of DNP/mol of enzyme.

Effect of modification on the binding of phospholipase C to an affinity gel. The substantial increase in K_m resulting from DNP incorporation into the enzyme could arise from, amongst other possibilities, an impairment of substrate binding. Such an effect might then lead to a

decrease in the tightness of enzyme binding to a substrate-based affinity gel. This affinity gel comprising egg yolk lipoprotein covalently bound to agarose is the basis of the enzyme purification procedure.⁵ The gel binds the enzyme very tightly and fairly specifically and it is possible that the enzyme-gel interaction is of an enzyme-substrate nature, although other interactions cannot be ruled out. When a sample of modified enzyme containing 0.7 mol of DNP/mol of enzyme was applied to a column of this gel, the whole sample bound and required high concentrations of urea for its removal from the gel. Applying a sample of extensively modified and totally inactive enzyme containing 4.45 mol of DNP/mol of enzyme resulted in 97–98 % of the applied sample binding tightly to the gel and again requiring elution of the column with concentrated solutions of urea to remove the bound enzyme (results not shown). It is clear therefore that even quite extensive modification of the enzyme does not seriously diminish the binding to the gel.

Extent of reagent incorporation into the enzyme. The above results show that the incorporation of the first 2 mol of DNP into phospholipase C result in activity changes. In order to see how much DNP could be incorporated, the enzyme was incubated over long time periods with EDC in the presence of DNP-ethylenediamine. The results are shown in Fig. 4 and demonstrate that 4–5 mol of DNP/mol of enzyme appears to be the maximum extent of incorporation in the absence of denaturants. The extent of reaction was not limited by the availability of reagent since the addition of extra EDC caused little increase in the total incorporation.

DISCUSSION

Incubation of phospholipase C under mild conditions with two chemically dissimilar reagents known to modify carboxyl groups caused rapid inactivation. The effect of one of these, EDC was examined in some detail. Under the reaction conditions used, the protein side chains which are most sensitive to modification by diimides are carboxyl, sulfhydryl and tyrosine groups.³ The present enzyme appears to contain no free sulfhydryl group¹⁷ and

Fig. 4. Extent of DNP incorporation into phospholipase C. ● Enzyme (20 μ M) was incubated with 60 mM EDC and 20 mM DNP-ethylenediamine. After different time periods, portions of the reaction mixture were removed and analysed for DNP content. ○, after 2 h incubation as above, extra EDC to give a concentration increase of 60 mM was added to the reaction mixture.

is not inactivated by sulfhydryl reagents.¹⁸ Furthermore, it is unlikely that the inactivation involved tyrosine residues since hydroxylamine was unable to reactivate and also, tyrosine reagents do not cause inactivation.¹⁸ The modification of lysine, histidine or aliphatic hydroxyl groups is very unlikely under the reaction conditions used¹⁹ and the involvement of these side chains is made even less likely by the observation (results not shown) that the inactivation proceeded significantly less rapidly at pH 6 than it did at pH 4.75. It would therefore appear that the inactivation of phospholipase C by EDC plus nucleophile may be attributed to the modification of carboxyl groups. Kinetic analysis of the modified enzyme suggested that the inactivation observed arose partly from an effect on K_m and partly from a reduction in V_m . In particular, it seemed that when the enzyme was incubated with EDC plus DNP-ethylenediamine, the incorporation of the first mol of DNP led to a 4–5-fold increase in K_m with no significant effect on V_m .

Incorporation of the second mol of DNP caused little or no further change in K_m , but reduced the V_m to near zero. Thus, phospholipase C from *B. cereus* appears to contain a single carboxyl group that is essential for catalytic activity.

The present enzyme exhibits anomalous rate effects in the region of the critical micelle concentrations for short chain lecithins.⁷ The extent of this micelle effect was not altered by exposure of the enzyme to EDC plus nucleophile. Thus, the activities towards micelles and monomers are reduced by equal proportions upon carboxyl group modification. This suggests that the two most reactive carboxyl groups in the enzyme are more involved with the catalytic centre than with any structure in the enzyme responsible for detecting micelles or lipid/water interfaces.

The first carboxyl group blocked by the carbodiimide is clearly not essential for catalytic activity. However, the associated 4–5-fold increase in K_m could arise from significant changes in the enzyme's ability to bind substrate. The failure of even quite extensive incorporation of DNP into the enzyme to decrease markedly the tightness of enzyme binding to a substrate-based affinity gel indicates that inactivation by the present reagent and the ability of the enzyme to bind to the affinity gel are independent processes and that the reactive carboxyls may not be involved in substrate binding. It should, perhaps, be noted that the gel bound substrate (egg yolk lipoprotein) and the monomeric dihexanoyllecithin used in the kinetic determinations are very dissimilar and the possibility that the enzyme binds the gel through some micelle recognition site should not be overlooked. The results do, however, demonstrate that the reactive carboxyl groups are not essential for the binding of the enzyme to the affinity gel.

The carboxyl groups whose modification results in activity changes are but two out of a total of 48 such residues in the enzyme.¹⁷ Clearly, therefore, EDC modifies these two carboxyl groups with an extremely high degree of selectivity. Protein modification by water-soluble carbodiimides has been advocated as a method for determining the total numbers of exposed and buried carboxyl groups in a

protein.⁸ The present data suggest that in phospholipase C from *B. cereus*, 4–5 carboxyl residues are of very much greater reactivity towards EDC plus DNP-ethylenediamine than are the remaining ones. In the absence of protein denaturants, only 10 % of the carboxyl side chains are reactive towards the present reagent, so that a surprisingly high 90 % of the carboxyl groups appear to be hidden within the structure of the protein.

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