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THE METAL ION DEPENDENCE OF PHOSPHOLIPASE C FROM *BACILLUS CEREUS*

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Summary

1. The zinc content and metal ion dependence of phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* have been examined.

2. The native enzyme contained about 2 atoms of tightly bound zinc/molecule.

3. Incubation of the enzyme with EDTA or with *o*-phenanthroline caused inactivation. The inactivation was accompanied by the removal of one zinc atom from the enzyme and could be fully reversed by the addition of Zn^{2+} or Co^{2+} to the enzyme and partly reversed by Mn^{2+} or Mg^{2+}.

4. Prolonged exposure to *o*-phenanthroline removed the second zinc atom also and produced an enzyme species which was reactivated by Zn^{2+} only. Full reactivation was accompanied by the binding of about two zinc atoms to the enzyme.

5. The results are consistent with the view that phospholipase C is a zinc metalloenzyme.

Introduction

The activity of several of the phospholipases is enhanced by the presence of divalent metal ions. In some cases, these metal ions represent genuine enzyme cofactors [1,2] and in other cases a substrate effect may be involved [3]. With phospholipase C from *Bacillus cereus*, a third possibility exists, namely that the enzyme is a metalloprotein. Thus, the enzyme is active when assayed in the presence of high levels of EDTA [4,5], but inactivated by preincubation with this chelating agent or with *o*-phenanthroline [4,6]. Full reactivation occurs upon adding zinc atoms to the enzyme [4,6] and there is some evidence for the binding of zinc to the protein [4]. However, no definitive conclusions can be reached since many of the crucial experiments were carried out using impure preparations of enzyme.

The present report examines the inactivation of the enzyme by the chelating agents EDTA and *o*-phenanthroline, together with subsequent reactivation by various divalent metal ions. In addition, zinc analyses are reported on samples of highly purified enzyme after varying degrees of exposure to the chelating agents and also following enzyme reactivation by Zn^{2+}.

The data suggest that phospholipase C from *B. cereus* is a zinc metalloenzyme.

Materials and Methods

Enzyme purification

Phospholipase C produced by a mutant of *B. cereus* strain ATCC (AB-1) [7] was purified as described [6] with certain modifications. The bacteria were harvested at an absorbance at 600 nm of 0.7—0.75. Lyophilization was carried out prior to dialysis. After $(NH_4)_2SO_4$ precipitation, the dissolved sediment was dialysed 3 h against 1 l 0.01 M sodium phosphate buffer (pH 8.0) and then loaded onto a DEAE-Sephadex A-50 column (4 × 20 cm) previously equilibrated against the above buffer. The enzyme was eluted from the column with the same buffer. Sephadex G-75 replaced Sephadex G-50 in the gel filtration step.

Final enzyme preparations were analyzed by sodium dodecylsulphate disc electrophoresis following treatment of the protein with mercaptoethanol [8] and also by the discontinuous Tris · HCl/Tris/glycine-polyacrylamide gel electrophoresis system described by Davis [9]. After staining, the gels were examined using a Gilford 2400-S spectrophotometer with gel scanning attachment. On the basis of these criteria, enzyme samples used in the zinc assays were of >95% purity. In certain other experiments, enzyme preparations of somewhat lower purity (>70%) were occasionally used. However, all crucial results were confirmed with high purity enzyme. Enzyme preparations (0.01 mg/ml) used had no detectable hemolytic activity when incubated for 2 h at 37°C with 2.5% suspension of human red blood cells in veronal-buffered saline. Furthermore, when sphingomyelin replaced lecithin in the phosphate release assay (see below), the enzyme caused no release of acid-soluble phosphate. The molecular weight of the enzyme was taken as 23 000 [6].

Enzyme assay

Unless otherwise stated, phospholipase C activity was assayed by the turbidimetric method of Ottolenghi [10] with the following modification: Colbeck EY broth (Difco) was diluted (1 : 1) with veronal-buffered saline (pH 7.4) [11]. To remove any contaminatory metal ions, the diluted substrate was passed through a column of Bio-Rad Chelex-100 resin and subsequently adjusted to 5 μM in EDTA before use. Assays were performed at 27°C in a Beckman DB-GT spectrophotometer with recorder using the 0—50% T scale. Assay of activity using the thromboplastinase method [12] was carried out as described [6].

For the phosphate release assay, the substrate was prepared by sonicating until clear 1 mg dipalmitoyl-DL-3-lecithin (Koch-Light) in 0.05 M veronal buffer (pH 8.2) containing 0.1% deoxycholate, 0.05 M KCl and 0.23 M mannitol

(8—12 30-s sonications were usually required). The enzyme was incubated for 30 min at 37°C using the sonicated liposomes and then the reaction stopped by the addition of cold 70% $HClO_4$. The solution was filtered through 0.22 μM Millipore filters and the acid-soluble phosphate measured according to Chen et al. [13]. The amount of acid-soluble phosphate released by the native enzyme was taken as 100% activity. Two series of experiments were carried out: one where the protein concentration was such that the fully active enzyme released approx. 55% of the theoretically available phosphate from lecithin and a second where the fully active enzyme released about 22% of the available phosphate. Identical patterns of results were obtained from the two series of assays.

Zinc assay

Zinc was assayed using a Perkin-Elmer atomic absorption spectrometer (model 305A) at 2138 Å. A standard zinc solution was diluted with water for use as reference standards. Zinc levels were measured in the 0.05—0.6 ppm range. The use of glass was avoided wherever possible.

Preparation of samples

Phospholipase C was stored as a solution in veronal/NaCl buffer (pH 7.4) containing 1 mM $ZnCl_2$. Prior to all experiments the enzyme was passed down a column (0.6 × 10 cm) of Bio-Rad Chelex-100-chelating resin previously equilibrated against veronal/NaCl buffer (pH 7.4) and eluted with the same buffer. No activity changes resulted from passage of the enzyme through such a column. The enzyme was then treated with chelating agents as indicated. In the case of samples where the zinc content was to be measured, the enzyme activity was assayed and then, unless otherwise stated, the inactivation reaction was stopped by the addition of 5 mM $CoCl_2$ and the sample immediately applied to a Sephadex G-25 column (1.1 × 17 cm) and eluted with water. Zinc assays were carried out immediately. The addition of Co^{2+} did not affect the measured zinc content of the enzyme, but seemed to give more consistent results, especially with inactivated enzyme. Presumably Co^{2+} prevented the inactivated enzyme from binding contaminating zinc. Unless otherwise stated, veronal-buffered saline (pH 7.4) was used as the reaction medium and elution buffer. Protein was assayed by the method of Lowry et al. [14] using bovine serum albumin as standard.

Results

Previous work [4—6] has suggested that phospholipase C from *B. cereus* may contain intrinsic zinc. To investigate this possibility further, zinc assays were made on highly purified enzyme preparations. The average zinc content of nine different preparations of enzyme was 1.89 g atoms Zn/23 000 g protein, thus suggesting that the native enzyme contains two atoms of zinc/molecule.

The effect of EDTA on the activity and zinc content of the enzyme was next investigated and the results are illustrated in Fig. 1. The loss of activity upon preincubation of the enzyme with EDTA would seem to correlate with the removal of one of the zinc atoms from the enzyme. Similar results were

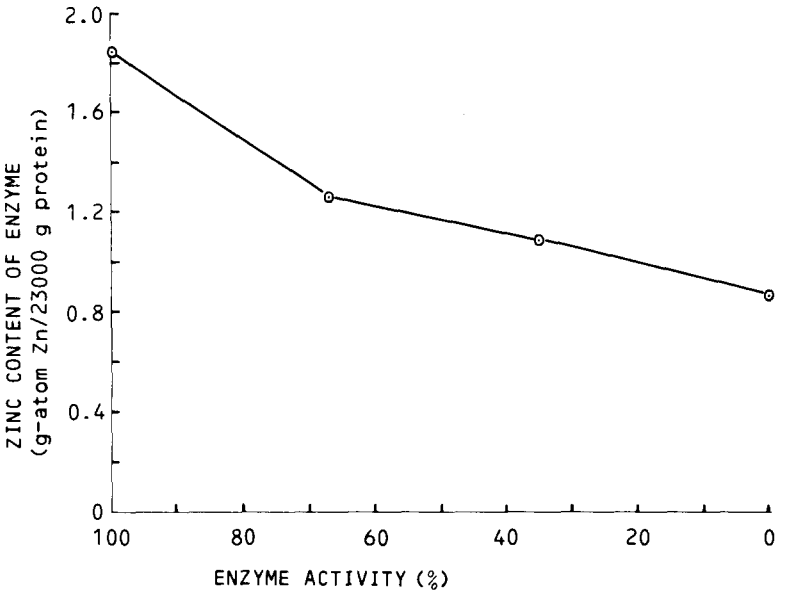

Fig. 1. Effect of preincubation with EDTA on the activity and zinc content of phospholipase C. Phospholipase C (0.1 mg/ml) was incubated at 4°C with 2.5 mM EDTA for periods up to 36 h. Enzyme activity and zinc content were measured at different times as described in Materials and Methods. The activity of an untreated control has been taken as 100%.

obtained using *o*-phenanthroline. Further incubation with EDTA failed to remove the second zinc atom. It would therefore seem that the second zinc atom is either bound more tightly to the enzyme than the first or is not exposed for reaction with EDTA.

The reactivation of the inactivated enzyme by various divalent metal ions was subsequently examined (Table I). When assays were carried out using the turbidimetric method, the inactivation was fully reversed by Zn^{2+} and partly reversed by the ions of cobalt, manganese or magnesium. No reactivation

TABLE I

REACTIVATION OF PHOSPHOLIPASE C AFTER TREATMENT WITH CHELATING AGENTS

Phospholipase C (0.03 mg/ml) was incubated 24 h at 0 °C with 2.5 mM EDTA or 5 min at 0 °C with 0.1 mM *o*-phenanthroline (turbidimetric and thromboplastinase assays). In the case of the phosphate release assay, the enzyme (0.2 mg/ml) was incubated 30 min at 0 °C with 5 mM *o*-phenanthroline. Enzyme aliquots were removed from the reaction systems, incubated 2 min at 23 °C with 5 mM of the divalent metal chloride and then assayed. In the turbidimetric system, 0.5 mM of the same metal salt was added to the assay system. The activity of the native enzyme in the presence of Zn^{2+} has been taken as 100%.

Addition	Turbidimetric assay	Activity (%)	
		Thromboplastinase assay	Phosphate release assay
No addition	<2	0	17
Zn^{2+}	100	100	100
Co^{2+}	40	100	88
Mn^{2+}	17	35	—
Mg^{2+}	9	25	—

occurred with the divalent ions of cadmium, calcium, nickel, copper, iron or mercury. Identical results were obtained with enzyme that had been freshly inactivated by EDTA and by *o*-phenanthroline.

When enzyme activity was measured with the thromboplastinase method, certain quantitative differences from the results obtained with the turbidimetric method were found. Now, Co^{2+} was as effective as zinc in reactivating the EDTA-treated enzyme (Table I). In order to investigate these differences further, the effect of free metal ions on the activity of the native enzyme in the two assay systems was examined. In the turbidimetric assay, low levels of Zn^{2+} (20 μM) caused a 2.5-fold stimulation of activity, while concentrations of Co^{2+}, Mg^{2+} or Mn^{2+} up to 1 mM had no effect. In the thromboplastinase assay, neither the presence of any of the above four metal ions nor EDTA affected the activity of the native enzyme. Furthermore, when enzyme activity was measured by following the release of acid-soluble phosphate from pure lecithin, Co^{2+} and Zn^{2+} were almost equally effective in reactivating the enzyme (Tabl I). When the stimulatory effect of free Zn^{2+} in the turbidimetric method is taken into consideration, the results obtained with the three assays methods become consistent.

Although Co^{2+} could fully reactivate samples of phospholipase C that had been exposed to *o*-phenanthroline over a period of minutes, prolonged exposure to *o*-phenanthroline resulted in an enzyme form which could be reactivated by Zn^{2+} only (Table II). The effect of time of exposure of the enzyme to *o*-phenanthroline on the degree of reactivation obtained by the subsequent addition to the enzyme of Co^{2+} or Zn^{2+} is illustrated in Fig. 2. *o*-Phenanthroline causes a very rapid inactivation of the enzyme to a form which can be reactivated by Co^{2+} or Zn^{2+}. Longer exposures to *o*-phenanthroline decrease the degree of reactivation obtained with Co^{2+}, although Zn^{2+} reactivation is not initially affected. Still longer exposure appears to cause irreversible inactivation.

One possible reason why *o*-phenanthroline, after inactivating the enzyme, later decreases the degree of reactivation by Co^{2+}, might be that this reagent

TABLE II

REACTIVATION OF PHOSPHOLIPASE C AFTER LONG TERM INCUBATION WITH *o*-PHENANTHROLINE

Phospholipase C (0.03 mg/ml) was incubated 24 h at 0 °C with 0.1 mM *o*-phenanthroline (turbidimetric and thromboplastinase assays). For the phosphate release assay, 0.2 mg/ml enzyme was incubated 18 h at 4 °C with 5 mM *o*-phenanthroline. Enzyme aliquots were then treated with metal ions as described in Table I.

Addition	Turbidimetric assay	Activity (%)	
		Thromboplastinase assay	Phosphate release assay
No addition	<2	0	14
Zn^{2+}	100	100	100
Co^{2+}	<2	0	14
Mn^{2+}	<2	0	—
Mg^{2+}	<2	0	—

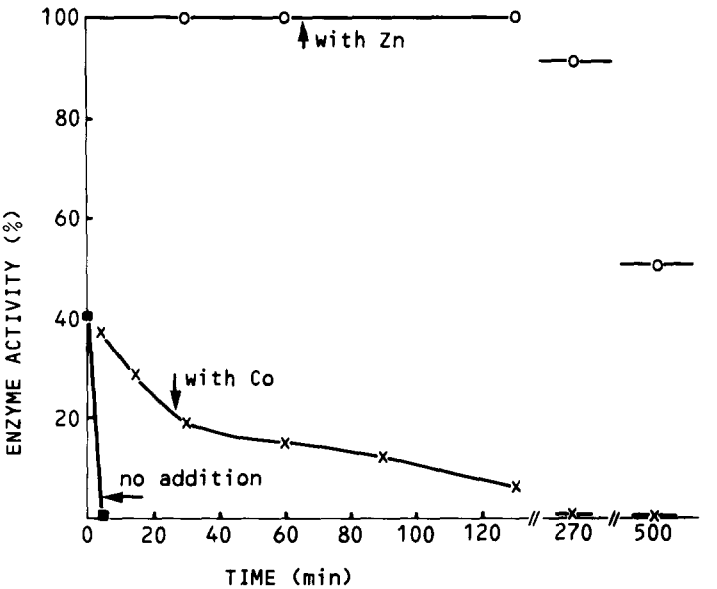

Fig. 2. Effect of time of incubation with *o*-phenanthroline on the reactivation of phospholipase C by Zn^{2+} or Co^{2+}. Phospholipase C (0.3 mg/ml) was incubated with 0.1 mM *o*-phenanthroline. After various times, aliquots were removed and assayed directly (■) or following incubation for 2 min at 23°C with either 2 mM $CoCl_2$ (X) or 2 mM $ZnSO_4$ (○) and the addition of 0.5 mM of the respective salts to the assay system. The activity of the native enzyme in the presence of free Zn^{2+} has been taken as 100%.

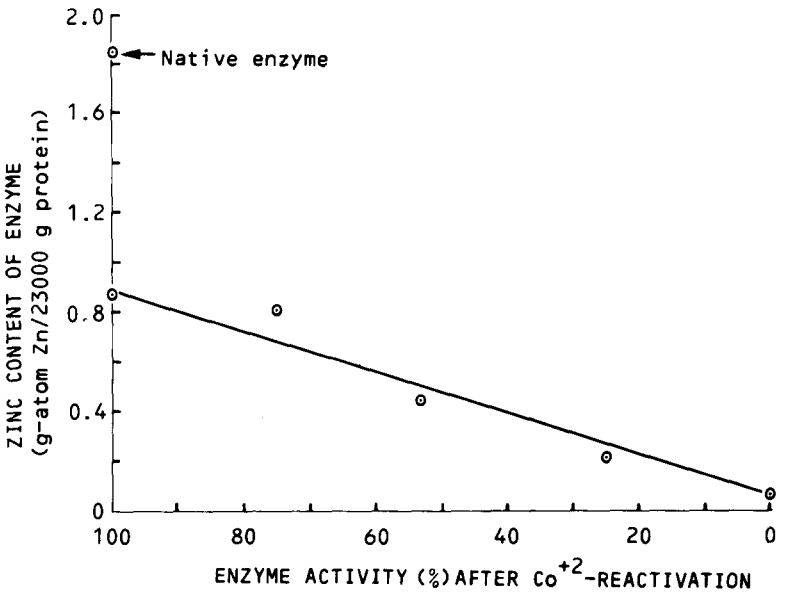

Fig. 3. Effect of incubaton with *o*-phenanthroline on the zinc content and reactivation by Co^{2+} of phospholipase C. Phospholipase C (0.1 mg/ml) was incubated at 4°C with 1 mM *o*-phenanthroline for periods from 5 min to 36 h. Aliquots were withdrawn at various times and assayed after reactivation by Co^{2+} (as in Table I) and the zinc content was measured. All samples were fully inactive before reactivation with Co^{2+}. The activity of the native enzyme in the presence of Co^{2+} has been taken as 100%. Addition of Zn^{2+} fully reactivated all samples except the one with the lowest zinc content (0.1 Zn/molecule).

TABLE III

ZINC BINDING AND ENZYME REACTIVATION

Phospholipase C (0.1 mg/ml) was incubated 18 h at 4 °C in 1 mM *o*-phenanthroline. The activities of the native and the inactivated enzymes were measured before and after addition of Co^{2+} or Zn^{2+} as described in Table I. Zinc assays were carried out on the inactivated enzyme before and after 5 min incubation at 0 °C with either 5 mM $CoCl_2$ or 5 mM $ZnSO_4$ and on the native enzyme. Excess metal ion was removed by gel filtration as described in Materials and Methods. The activity of the native enzyme under the three different assay conditions was in each case taken as 100%.

Sample	Zinc content gatom Zn/ 23 000 g protein	No metal	Activity (%)	
			With Co^{2+}	With Zn^{2+}
Native enzyme	1.85	100	100	100
Inactivated enzyme	0.25	<2	31	95
Inactivated enzyme after addition of $ZnSO_4$	1.67	—	—	100

can also remove the second zinc atom from the enzyme. That this is indeed the case is seen in Fig. 3. The first effect of *o*-phenanthroline is a rapid enzyme inactivation. This freshly inactivated enzyme, like the enzyme that had been inactivated by EDTA, was fully reactivated by Co^{2+} and contained about one atom of zinc. The diminution by *o*-phenanthroline of the degree of enzyme reactivation by Co^{2+} correlates well with the removal of the final zinc atom from the enzyme.

As well as investigating the removal of zinc from the enzyme by chelating agents, the binding of zinc upon subsequent reactivation was also examined. Table III shows the effect of the addition of Zn^{2+} to enzyme after long term incubation with *o*-phenanthroline. Virtually full reactivation resulted together with the restoration of the zinc content to a value almost identical with that of the native enzyme.

Discussion

As purified by the method of Otnæss et al. [6], phospholipase C contains two tightly bound zinc atoms which are essential for enzyme activity. Incubation of the enzyme with EDTA or *o*-phenanthroline initially causes enzyme inactivation together with the removal of one zinc atom from the enzyme. If allowance is made for the stimulatory effect of Zn^{2+} in the turbidimetric assay, then both Zn^{2+} and Co^{2+} cause full reactivation of this enzyme species with Mn^{2+} and Mg^{2+} causing partial reactivation. Longer incubation with *o*-phenanthroline removes the second zinc atom and prevents reactivation by metals other than zinc. The reactivation by Zn^{2+} restores the zinc content of the enzyme to that of the native enzyme.

Studies on the metal dependence of enzymes made using chelating agents may be complicated by the binding of such agents to the apoprotein. In the case of EDTA such binding may increase the metal ion binding capacity of the enzyme [15]. With *o*-phenanthroline, hydrophobic binding of the reagent of enzymes may cause inactivation [16]. It is unlikely that the present results are

attributable to either of these effects. The purified native enzyme could not be contaminated with EDTA since the purification procedure involves no soluble chelating agents. Enzyme inactivations by both EDTA and *o*-phenanthroline were accompanied by the removal of zinc from the enzyme and reactivation by zinc atoms was accompanied by the binding of the metal to the enzyme.

Thus, the present results suggest that phospholipase C from *B. cereus* has two metal binding sites, one which is zinc specific and which binds the metal very tightly and another which will accept zinc, and probably cobalt, manganese and magnesium also and which binds zinc somewhat less tightly. The presence of other weak binding sites for zinc cannot be ruled out, since, in the turbidimetric assay, free Zn^{2+} caused a 2.5-fold stimulation. However, an effect of Zn^{2+} on the substrate may be responsible since no such stimulation occurred when tissue thromboplastin or pure lecithin were used as substrates. Previous workers [4,5] have reported that the enzyme is active when assayed in the presence of high concentrations of EDTA. In addition, neither the enzyme activity nor the two zinc atoms were removed when the enzyme was passed through a column of chelating resin. It would therefore seem that the zinc atoms are so tightly bound to the enzyme that they may be regarded as essential and integral parts of the protein structure rather than as enzyme cofactors. Thus, the present results would appear to confirm the tentative suggestion by Ottolenghi [4] that phospholipase C from *B. cereus* is a zinc metalloenzyme.

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