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Effect of Some Divalent Metal Cations on Phospholipase C from *Bacillus cereus*

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Incubation of phospholipase C from *Bacillus cereus* with certain divalent metal cations caused enzyme inactivation with Cu(II) being particularly effective. The inactivation arose from the reversible exchange of Zn(II) in the enzyme with the metal cations. Both zinc atoms in the enzyme exchanged rapidly with Cu(II) whereas only one exchanged spontaneously with Co(II). With lecithin substrates, CoZn-phospholipase C had a specific activity of 3.6 –11.3 % of that of ZnZn-phospholipase C, whereas the CoCo-enzyme was <1 % active relative to the native enzyme. The CoZn-enzyme had the same K_m value for dihexanoyllecithin as had the native enzyme, but the V_m value was markedly lower. ZnZn-, CoZn- and CoCo-phospholipase C all had very low activities towards sphingomyelin micelles, although for the CoCo-enzyme, the sphingomyelinase activity was 4–7-fold greater than for the native enzyme.

Most phospholipases are dependent for their catalytic activity on divalent metal cations. Although phospholipase C from *Bacillus cereus* is fully active in the absence of free metal cations,[1] the enzyme contains two tightly bound Zn atoms,[1] together with, apparently, other relatively low affinity binding sites for Zn(II).[2] The tightly bound Zn atoms seem to play a major role in stabilizing phospholipase C[3–5] and may also be involved in the catalytic reaction. Thus, the Zn(II)-free apoenzyme and the mono Zn(II) enzyme are inactive.[1] However, substitution of one Zn(II) in the native enzyme with certain other divalent metal cations permits retention of catalytic properties, whereas the substitution of both Zn(II) ions results in total inacrivation.[1] We have found that the exposure of native phospholipase C to certain divalent metal cations causes enzyme inactivation. This process has been further examined and some properties of the inactivated enzyme are reported.

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MATERIALS AND METHODS

Materials. Phospholipase C was isolated from cultures *Bacillus cereus* by the methods of Little *et al.*[6] and Myrnes and Little.[7] 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.[8,9] Chromatographically pure dipalmitoyllecithin and brain sphingomyelin were obtained from Koch Light (Colnbrook, England).

Enzyme assays. Enzyme activity was measured at 23–24 °C by measuring the rate of acid production in various substrate solutions using Radiometer automatic titration equipment with the titration point set at pH 7.55. One unit of enzyme activity catalyzes the liberation of 1 μmol titratable H^+/min. Crude egg yolk substrate was prepared as described previously[6,10] except that Zn(II) was omitted. Sphingomyelin and dipalmitoyllecithin were mixed (1 mg/ml) with 0.15 M NaCl containing 0.25 % (w/v) sodium deoxycholate and sonicated until optically clear. Short chain lecithins were mixed with 0.15 M NaCl and sonicated until clear. Kinetic constants were calculated by the method of Eisenthal and Cornish-Bowden,[11] using 12–13 rate measurements over a substrate concentration range of 0.2–2.0 mM. Metal concentrations were determined by atomic absorption spectrometry.[1] Free divalent metal ions were removed prior to the determination of enzyme-bound metal by passage of the sample through a short column (0.6 × 0.2 cm) of Bio-Rad Chelex-100 chelating resin. Protein was determined by the method of Lowry *et al.*[12] using bovine serum albumin as standard and the molecular weight of the enzyme was taken as 23 000.[13]

RESULTS

1. Inactivation of enzyme by incubation with divalent metal ions. Phospholipase C was incubated at pH 6.0

with different molar ratios of several different divalent metal ions (Table 1). After 24 h incubation, inactivation was noted in the case of Cu(II), Ni(II), Hg(II), Cd(II), Co(II) and Ag(II). More prolonged incubation resulted in inactivation of the enzyme by a 100-fold molar excess of Mn(II). No inactivation was noted with Ca(II) or Mg(II). The most potent inactivator amongst the metal ions used was Cu(II), which when present in equimolar proportions with the enzyme caused approximately 80 % inactivation. Enzyme inactivation could, in all cases, be fully reversed upon prolonged dialysis of the inactivated enzyme against solutions of Zn(II).

The kinetics of inactivation were studied using Cu(II) and Ni(II). At relatively high levels of the metal ion [100 mM Ni(II) or 5 mM Cu(II)] the inactivation followed apparently first order kinetics, whereas at much lower metal ion concentrations, the inactivation was pseudo-first order during the initial stage only (Fig. 1). The effect of the concentration of metal ion used on the value of the pseudo-first order rate constant for the inactivation was examined. It was found that the rate constant seemed to reach a limiting value which was fairly

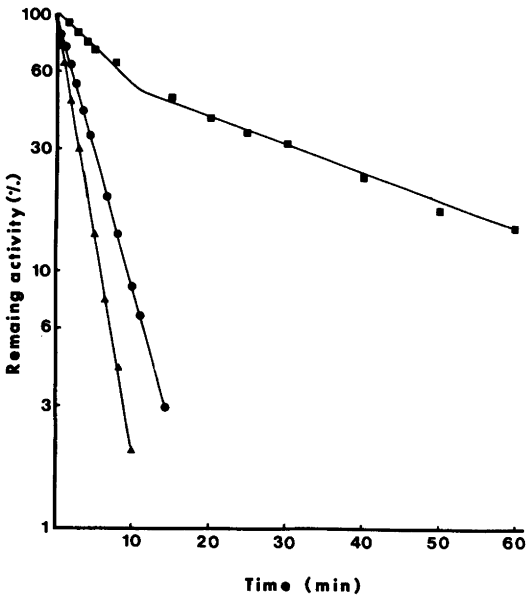

Fig. 1. Inactivation of phospholipase C by Cu(II) and Ni(II). Phospholipase C (50 μM) was incubated at 23 °C in 0.1 M sodium acetate buffer (pH 6.0) together with 2.5 mM Ni(II) (■), 100 mM Ni(II) (●) or 5 mM Cu(II) (▲). Enzyme activity was measured against egg yolk substrate.

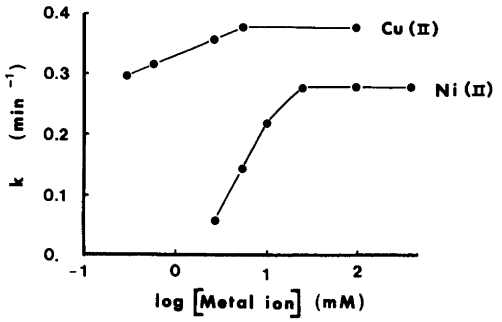

Fig. 2. Effect of metal ion concentration on the rate of inactivation. The apparent first order rate constant for enzyme inactivation was measured from inactivation data obtained at different concentrations of Cu(II) and Ni(II). Other conditions were identical to those in Fig. 1.

similar for the two metal ions used (Fig. 2). The concentration needed to reach this limiting value was considerably higher in the case of Ni(II) than with Cu(II).

2. Metal exchange studies. The mechanism of inactivation by the divalent metal ions might involve exchange with one or both of the tightly bound metal ions in the enzyme or, alternatively, interaction with the low affinity Zn(II)-bonding.

To examine this problem further, enzyme was inactivated to different extents by Cu(II) and Co(II) and the content of tightly bound metal in the enzyme was measured. 0.15 M NaCl/30 mM sodium 5,5-diethylbarbiturate buffer (pH 7.3) was used instead of 0.1 M sodium acetate buffer (pH 6.0) because it was noted that, especially with Cu(II), enzyme precipitation occurred during inactivation in the latter but not in the former buffer. With Cu(II), during the inactivation, a one for one substitution of Zn(II) by Cu(II) occurred (Fig. 3). The relation between metal exchange and the loss of activity was non-linear. Total inactivation corresponded to the substitution of both zinc ions for copper ions. In the case of treatment of the enzyme with Co(II), again a one for one substitution of Zn(II) by Co(II) occurred (Fig. 4). However a linear relationship was found between the extents of inactivation and of metal substitution. Only one Zn(II) exchanged spontaneously with Co(II). Even after prolonged dialysis against solutions of Co(II) (one week dialysis, with daily changes against solutions of 1 mM Co(II) in the barbiturate buffer), the enzyme retained about 10 %

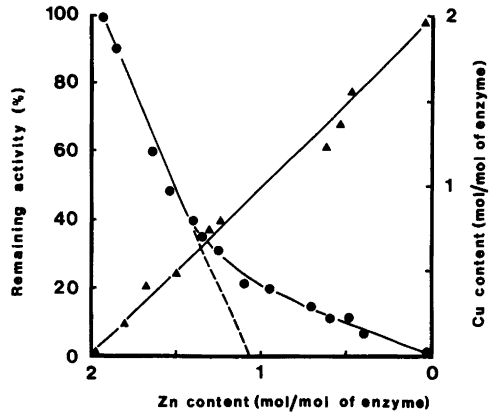

Fig. 3. Copper-zinc exchange in phospholipase C. Enzyme (65 μM) in barbiturate/saline buffer (pH 7.3) was either incubated with or dialyzed against solutions Cu(II) in the same buffer. The exchange was stopped by passage of the enzyme through a column of chelating resin. Enzyme activity (●) was measured and protein-bound copper (▲) and zinc determined.

of its original catalytic activity together with one of its zinc ions.

The metal exchange process was reversible upon dialysis of the sample against Zn(II)-containing buffers. Full reactivation corresponded to the

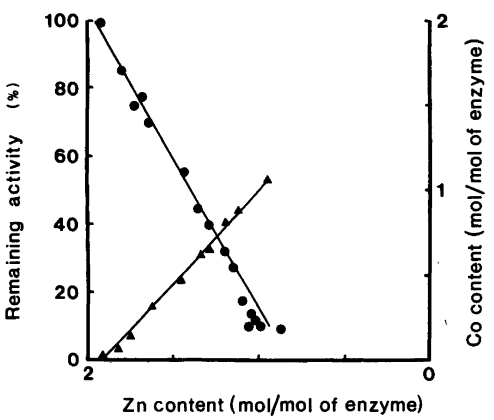

Fig. 4. Cobalt-zinc exchange in phospholipase C. Enzyme (65 μM) in barbiturate/saline buffer (pH 7.3) was dialyzed against solutions of Co(II) in the same buffer. The exchange was stopped by passage of the enzyme through a column of chelating resin. Enzyme activity (●) was measured and protein-bound cobalt (▲) and zinc determined.

restoration of an enzyme species containing two zinc atoms. However, prolonged exposure of phospholipase to high concentrations (1 – 5 mM) of Cu(II) caused irreversible inactivation. The enzyme precipitated under these circumstances and although the precipitate was soluble in 4 M guanidinium chloride, removal of the denaturant resulted in precipitation. It seems likely that high concentrations of Cu(II) lead to covalent modification, presumably oxidation, of the enzyme.

3. Properties of Co-substituted phospholipase C. The catalytic activities of native (ZnZn-enzyme), mono-Co(II)-substituted (CoZn-enzyme) and bi-(Co(II)-substituted (CoCo-enzyme) phospholipase C were compared using different substrates. The results in Table 2 show that relative to ZnZn-phospholipase C, the CoZn-enzyme has an activity of 3.5 – 11.3 % depending on the exact nature of the lecithin substrate used. The CoCo-enzyme had 0.2 – 0.8 % of the activity of the native enzyme. Monomolecularly dispersed (soluble) dihexanoyllecithin was the preferred substrate for both the native and CoZn-enzyme forms whereas for the CoCo-enzyme, crude egg lecithin-deoxycholate micelles gave the highest activity. Sphingomyelin-deoxycholate micelles proved to be an exceedingly poor substrate for all three enzyme forms. To obtain detectable activities, enzyme concentrations of around 10 – 20 μg/ml had to be used. The native enzyme showed a sphingomyelinase activity some 1700 – 5000-fold lower than its lecithinase activity. This very small sphingomyelinase activity was lowered slightly on substitution of one zinc atom by Co(II) and increased upon substitution of both zinc atoms with Co(II) (Table 1). The same pattern of results was obtained with five different batches of enzyme and was independent of whether or not the enzyme had been crystallized and of the purification method used. The exact degree of enhancement of the sphingomyelinase activity upon substitution of both zinc atoms with Co(II) varied somewhat but was typically 4 – 7-fold relative to the activity of the native enzyme. Different amounts of Zn(II) were also removed from the enzyme by dialysis against solutions of 1,10-phenanthroline. Co(II) was added to these samples and the enzyme activity towards crude egg yolk substrate and towards sphingomyelin was measured. Under these circumstances, samples containing one mol Zn(II)/mol of enzyme had enhanced sphingomyelinase activity (Fig. 5). Enzyme samples which had been exposed to more prolonged dialysis against 1,10-phenan-

Table 1. Inactivation of phospholipase C by divalent metal ions. Phospholipase C (43 μM) in 0.1 M sodium acetate (pH 6) was incubated at 22–23 °C in the dark with different molar ratios of bivalent metal ions. The remaining enzyme activity was measured at 24 and 48 h. Values in parentheses refer to the remaining enzyme activity after 48 h when different from that after 24 h incubation. Determinations were made in duplicate. M/E represents the molar ratio of the metal ion to enzyme.

Metal ion	Activity (% of original)				
	M/E = 1	M/E = 2	M/E = 3	M/E = 10	M/E = 100
Ca(II)	100	100	100	100	100
Mg(II)	100	100	100	100	100
Mn(II)	100	100	100	100	100 (79)
Ag(II)	100	100	100	100	1
Co(II)	81	71	46	38	11
Cd(II)	73	54	40	25	5
Hg(II)	86	80	39	16	0
Ni(II)	63	40	15	7	0
Cu(II)	19	8	1	0	0

throline and which contained very little Zn(II) had less elevated sphingomyelinase activities upon exposure to Co(II) (Fig. 5). Kinetic analysis using monomolecularly dispersed dihexanoyllecithin showed that at 23 °C and pH 7.55 in 0.15 M NaCl, the ZnZn-enzyme had a K_m value of 0.4 ± 0.08 mM and a V_m value of 4 300 ± 400 u/mg. The

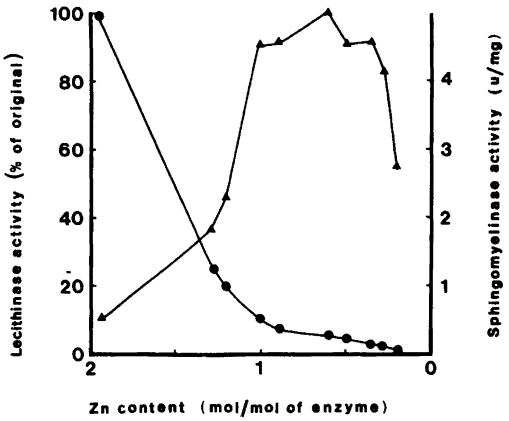

Fig. 5. Effect of cobalt-zinc exchange on the sphingomyelinase activity of phospholipase C. Enzyme (60 μM) in barbiturate/saline buffer (pH 7.3) was dialyzed at 10 °C against 2.5 mM 1,10-phenanthroline in the same buffer. After different periods of dialysis, Co(II) to a final concentration of 2.7 mM was added to the enzyme. The lecithinase activity (●) against crude egg yolk substrate and the sphingomyelinase activity (▲) were measured immediately. The zinc content of the enzyme was also determined.

corresponding values for the CoZn-enzyme were 0.4 ± 0.07 mM and 250 ± 30 u/mg, respectively.

DISCUSSION

The inactivation of phospholipase C (*B. cereus*) by divalent metal ions would appear to be due simply to the spontaneous and reversible exchange of zinc in the enzyme with other metal ions. The low affinity Zn(II)-binding sites are probably not involved. Indeed, the fact that the enzyme is fully active either in EDTA or in the complete absence of divalent metal ions other than the structural zinc indicates that these low affinity sites play little or no role in the catalytic process.

The two (high affinity) Zn(II)-binding sites in the enzyme appear to differ somewhat in their degree of affinity for Zn(II). This difference may explain why only one Zn(II) will exchange spontaneously with Co(II), whereas both zinc ions exchange very readily with Cu(II). However, it is also possible that the exchange of one Zn(II) for Cu(II) lowers the affinity of the protein for the remaining zinc ion. The highly non-linear relation between the extent of Cu(II) incorporation into the enzyme and the extent of enzyme inactivation together with the fact that complete inactivation corresponded to the incorporation of 2 atoms of copper in the enzyme (Fig. 3) is consistent with the possibility of very similar equilibrium constants for the reactions:

$$\text{ZnZn-Enzyme} + \text{Cu(II)} \rightleftharpoons \text{CuZn-Enzyme} + \text{Zn(II)}$$

$$\text{CuZn-Enzyme} + \text{Cu(II)} \rightleftharpoons \text{CuCu-Enzyme} + \text{Zn(II)}$$

Table 2. Activity of native and Co(II)-substituted enzyme forms towards different substrates. CoCo-phospholipase C was prepared by adding Co(II) (1 mM) to apophospholipase C.[5] Except in the case of crude egg yolk substrate, all substrates were present at 1 mg/ml. Further details are given in the Materials and Methods section.

Substrate	Specific activity/units mg^{-1}		
	ZnZn-enzyme	CoZn-enzyme	CoCo-enzyme
Crude egg yolk substrate	1260	142	10
Dipalmitoyllecithin	1040	37.5	3.8
Dihexanoyllecithin	3000	180	5.6
Sphingomyelin	0.6	0.45	1.75

Phospholipase C seems to have a very high affinity for Cu(II). The fact that upon mixing equimolar quantities of ZnZn-enzyme and Cu(II) caused 81 % inactivation suggests that phospholipase C has a much higher affinity for Cu(II) than for Zn(II). Indeed, it would be expected that in the absence of free Zn(II), the enzyme would be very susceptible to inactivation or "poisoning" by traces of metal ions, especially Cu(II). Even in the absence of free Zn(II), phospholipase C is structurally very stable[4,5,14] and after chelating resin treatment, solutions of phospholipase C (1 mg/mg in 0.1 M sodium acetate buffer, pH 6.0) are completely stable for several months at room temperature (C. Little, unpublished work). The instability problems reported by certain previous workers with this enzyme[10,15,16] and which were overcome when Otnæss *et al.*[14] suggested adding Zn(II) during purification and storage, very probably arose from such poisoning effects. With the three different lecithin substrates used, CoZn-phospholipase C had a much lower activity than did the native enzyme. The precise degree of inhibition depended on the exact substrate used and this may suggest some subtle alteration in enzyme substrate interactions, especially since with certain other lecithin substrates the two enzyme forms have more similar activities.[1] Kinetic studies with a soluble as opposed to a micellar substrate indicate that the differences in activity between the two enzyme forms arise primarily from differences in the maximum velocities with no detectable differences in K_m values.

The substitution of Zn(II) by Co(II) in the enzyme may alter the relative substrate specificity. Thus, the CoZn-enzyme formed by dialysis of native enzyme against Co(II) has a much lower lecithinase activity than the ZnZn-enzyme, but a similar small sphingomyelinase activity. The CoCo-enzyme has a significantly higher sphingomyelinase activity than has the native enzyme, but a very much lower lecithinase activity. Removal of Zn(II) by dialysis against 1,10-phenanthroline followed by the addition of an excess of Co(II) indicated that enhanced sphingomyelinase activity could occur when only one zinc atom had been removed. However, since this chelating reagent can remove both zinc atoms from the enzyme, a zinc content of one mol zinc/mol of enzyme could arise from a mixture of enzyme molecules containing two, one and no zinc atoms, so that upon addition of Co(II), the enzyme species are ZnZn-, CoZn- and CoCo-phospholipase C. Near zinc-free species produced by more long term exposure to 1,10-pheanthroline had lower than expected sphingomyelinase activities upon exposure to Co(II). This probably reflects the instability of the apoenzyme in the presence of this chelating agent.[1] Otnæss reported recently that the incubation of phospholipase C with 1,10-phenanthroline followed by the addition of Co(II) produced an enzyme with a sphingomyelinase activity as high as the initial lecithinase activity.[17] It is perhaps of significance that different methods were used to produce the sphingomyelin-deoxycholate micelles used by Otnæss[17] and in the present work. The precise physical form of the substrate seems critical in determining its rate of hydrolysis by the present enzyme. Thus, the native enzyme shows very little activity towards pure sphingomyelin, but when hydrolyzing phospholipids in aged human red cells, lecithin and sphingomyelin are degraded at very similar rates.[18]

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