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Characterization of the phosphatidylinositol-specific phospholipase C-released form of rat osseous plate alkaline phosphatase and its possible significance on endochondral ossification

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Abstract

Alkaline phosphatase activity was released up to 100% from the membrane by incubating the rat osseous plate membrane-bound enzyme with phosphatidylinositol-specific phospholipase C. The molecular weight of the released enzyme was 145,000 on Sephacryl S-300 gel filtration and 66,000 on PAGE-SDS, suggesting a dimeric structure. Solubilization of the membrane-bound enzyme with phospholipase C did not destroy its ability to hydrolyse PNPP, ATP and pyrophosphate. The hydrolysis of ATP and PNPP by phosphatidylinositol-specific phospholipase C-released enzyme exhibited 'Michaelian' kinetics with $K_{0.5}$ = 70 and 979 μM, respectively. For pyrophosphate, $K_{0.5}$was 128 μM and site-site interactions were observed (n = 1.4). Magnesium ions were stimulatory ($K_{0.5}$ = 1.5 mM) and zinc ions were a powerful noncompetitive inhibitor (K_i = 6.2 μM) of phosphatidylinositol-specific phospholipase C-released enzyme.

Phosphatidylinositol-specific phospholipase C-released alkaline phosphatase was relatively stable at 40°C. However, with increasing temperature from 40–60°C, the enzyme was inactivated rapidly following first order kinetics and thermal inactivation constants varied from 5.08×10^{-4} min^{-1} to 0.684 min^{-1}.

Treatment of phosphatydilinositol-specific phospholipase C-released alkaline phosphatase with Chellex 100 depleted to 5% its original PNPPase activity. Magnesium ($K_{0.5}$ = 29.5 μM), manganese ($K_{0.5}$ = 5 μM) and cobalt ions ($K_{0.5}$ = 10.1 μM) restored the activity of Chelex-treated enzyme, demonstrating its metalloenzyme nature. The stimulation of Chelex-treated enzyme by calcium ions ($K_{0.5}$ = 653 μM) was less effective (only 26%) and occurred with site-site interactions (n = 0.7). Zinc ions had no stimulatory effects.

The possibility that the soluble form of the enzyme, detected during endochondral ossification, would arise by the hydrolysis of the PI-anchored form of osseous plate alkaline phosphatase is discussed. (Mol Cell Biochem **152:** 121–129, 1995)

Key words: alkaline phosphatase, p-nitrophenyl phosphate, hydrophobic chromatography, phosphatidylinositol-specific phospholipase C, osseous plate, phospholipase C

Introduction

The intense focus given on the dynamics of protein-membrane interactions demonstrates that many prokaryotic and eukaryotic membrane-bound proteins contain covalently attached lipids [1, 2]. Further, it has been established that these lipids are glycosyl-phosphatidylinositol moieties which anchor the protein to the membrane surface [3, 4]. While the physiological role of this protein-membrane association is not well understood, it is believed that it might have functions

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other than to bind the protein to the membrane surface [5].

Among the several proteins which are known to be anchored by a lipid moiety [4, 6], alkaline phosphatase, a widespread nonspecific phosphohydrolase in mammalian tissues, was the first enzyme reported to show such association [7–9].

Matrix-vesicles are small membrane-bound structures which are particularly rich in alkaline phosphatase [10–13], an enzyme which has been associated with biomineralization since the pioneering work of Robison [14]. Recent findings have established that this enzyme from matrix-vesicles is attached to the membrane through a phosphatidylinositol anchor [15]. Although often described as a membrane enzyme, the existence of a soluble form has also been suggested [16, 17]. Several roles have been attributed to this ubiquitous enzyme, however its physiological function in calcification remains unclear as yet [18].

An important point of controversy is whether the soluble form of alkaline phosphatase from matrix-vesicles also participates in biomineralization [16, 17]. Earlier studies from our laboratory show that a soluble and a membrane-bound form of alkaline phosphatase are simultaneously present during endochondral calcification [19]. The soluble form of the enzyme was purified and characterized by us [20]. However, it remains doubtful whether this soluble enzyme is either induced during endochondral calcification or is released from the membrane by the action of a phospholipase [2, 5, 21–28].

We report here the biochemical characterization of a rat osseous plate alkaline phosphatase, selectively released from the membrane by a phosphatidylinositol-specific phospholipase C. It was shown that PIPLC-released enzyme has several properties in common with that of soluble form. However thermal inactivation, MW, specific activity and hydrophobicity of PIPLC-released enzyme were quite different from that of soluble enzyme. The results obtained are discussed focussing the probable origin of the soluble form also found by us, during endochondral ossification.

Materials and methods

All solutions were made up with glass distilled deionized water. Phospholipase C (PLC) from *B. cereus* and *B. perfringens*, 2-amino-2-methyl-1-propanol (AMPOL), Tris, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (Hepes) and p-nitrophenyl phosphate (PNPP) were from Sigma Chem. Co. Purified phosphatidylinositol-specific phospholipase C (PIPLC) from *B. thuringiensis* was kindly provided by Dr. Martin G. Low (Columbia University, USA) and hyaluronidase was a gift from Laboratórios Apsen do Brasil. Analytical grade reagents were used without further purification.

Preparation of rat osseous plate alkaline phosphatase

Matrix-induced membrane-bound alkaline phosphatase was prepared from rat osseous plates according to the procedure described by Curti *et al.* [19]. Briefly, 10–20 mg of demineralized matrix were introduced through a small incision in the dorsal subcutaneous tissue of ether anesthetized young male Wistar rats (50–60 g). Fourteen days after the implantation (the period of maximal alkaline phosphatase activity) the plaques formed were removed, rinsed in ice-cold 0.9% (w/v) NaCl and homogenized with 10 mM Tris.HCl buffer, pH 7.5, containing 0.15 M NaCl (3 ml of buffer/g of plaque) in a high speed shearing homogenizer, for 5 min. The homogenate was centrifuged at 15,000 × g for 20 min and the supernatant was dialysed overnight against 5 mM Tris.HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 0.15 M NaCl. The dialysed homogenate was applied to a Sepharose 4B column (3 × 83 cm) equilibrated and eluted in the same buffer. Active fractions of the membrane-bound form of the enzyme were pooled, dialysed overnight against 5 mM Tris.HCl buffer, pH 7.5 and rechromatographed in the same conditions. The purified enzyme was layered into a continuous sucrose density gradient (10–50%, w/v, in 5 mM Tris.HCl buffer, pH 7.5 containing 2 mM $MgCl_2$) and the centrifugation was carried out for 5 h at 38,000 × g using a Sorval SV-288 vertical rotor, at 4°C. Active fractions were pooled and dialysed overnight against 5 mM Tris.HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, at 4°C, with several changes of the buffer. Finally, 1.0 ml aliquots were frozen in liquid nitrogen and stored at –20°C for a period of one month without appreciable loss of activity.

Preparation of enzymatically-released alkaline phosphatase

Aliquots containing 2 mg/ml rat osseous plate membrane-bound alkaline phosphatase were incubated in 50 mM Tris.HCl buffer, pH 7.25 with either phospholipases C or hyaluronidase for 1 h, under constant rotary shaking, at 37°C. The incubation mixture was them centrifuged at 160,000 × g for 1 h, at 4°C. After careful removal of supernatant, the pellet was resuspended with 5 mM Tris.HCl buffer, pH 7.5 to the original volume and centrifuged in the above conditions. The pellet and the supernatant were both assayed for p-nitrophenylphosphatase, adenosine-5'-triphosphatase and pyrophosphatase activities. As control, alkaline phosphatase was incubated in the same conditions with phospholipases and hyaluronidase previously denatured by heating on stopered tubes for 1 h.

Phenyl-Sepharose CL-4B hydrophobic chromatography

The supernatant of enzymatically-released rat osseous plate alkaline phosphatase (PIPLC-released enzyme) was adjusted to 1 μM $ZnCl_2$, 2 mM $MgCl_2$ and 2.7 M NaCl with gentle stirring, at 4°C. The pH was then adjusted to 7.5 and an aliquot of 3.6 ml was applied to a Phenyl-Sepharose CL-4B column (1 × 10 cm) previously equilibrated with 5 mM Tris.HCl buffer, pH 7.5 containing 1 μM $ZnCl_2$, 2 mM $MgCl_2$ and 2.7 M NaCl. Stepwise elution was carried out with decreasing NaCl concentration in the buffer. Fractions of 1.5 ml were collected at a flow rate of 18 ml/h. The active fractions were pooled, concentrated by ultrafiltration on an Amicon cell equipped with a YM-5 membrane and then dialyzed overnight against 5 mM Tris.HCl buffer, pH 7.5 containing 2 mM $MgCl_2$, at 4°C. Samples of 1.0 ml were frozen and stored at –20°C for a period no longer than a month without appreciable loss of activity.

Enzymatic activity measurements

p-Nitrophenylphosphatase (PNPPase) activity was assayed discontinuously, at 37°C, in a Hitachi U-2000 spectrophotometer by following the liberation of p-nitrophenolate ion (ε 1M, pH 9.4 = 17,600 M^{-1} cm^{-1}) at 410 nm. Standard conditions were 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 1 mM PNPP in a final volume of 1.0 ml.

For adenosine-5'-triphosphate, the activity was also assayed discontinuously by measuring the amount of inorganic phosphate liberated, according to the procedure described by Heinonen and Lahti [29], adjusting the assay medium to a final volume of 1.0 ml. The reaction was initiated by the addition of the enzyme, stopped with 0.5 ml of cold 30% TCA at appropriate time intervals and centrifuged at 4,000 × g just before phosphate determination. Standard assay conditions were 50 mM AMPOL buffer, pH 9.4 (or 50 mM Hepes buffer, pH 7.5) containing 2 mM $MgCl_2$ and 2 mM ATP. For pyrophosphate, standard conditions were 50 mM Tris.HCl buffer, pH 8.0, containing 2 mM $MgCl_2$ and 2 mM sodium pyrophosphate.

All determinations were carried out in duplicate and the initial velocities were constant for at least 90 min provided that less than 5% of substrate was hydrolyzed. Controls without added enzyme were included in each experiment to allow for the non-enzymic hydrolysis of substrate. One enzyme unit (U) was defined as the amount of enzyme releasing 1.0 μmol of product per minute, at 37°C.

Polyacrylamide gel electrophoresis

Electrophoresis of purified PIPLC-released enzyme was carried out in 6% gels according to Davis [30], using silver nitrate for protein staining [31]. Phosphohydrolytic activity on the gel was detected in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$, 0.12% Fast Blue RR salt and 0.12% α-naphthyl phosphate, at 37°C. When necessary, protein samples were concentrated by using CF-25 Centriflo Amicon ultrafiltration membrane cones.

Determination of protein

Protein concentrations were determined according to Hartree [32] and Read and Northcote [33] for the membrane-bound enzyme and purified PIPLC-released enzyme, respectively. Bovine serum albumin was used as standard in both cases.

Molecular weight determination

The molecular weight of purified PIPLC-released enzyme was estimated by filtration on a Sephacryl S-300 column (1.5 × 98 cm) equilibrated and eluted with 5 mM Tris.HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 150 mM NaCl. Myosin, bovine serum albumin dimer, phosphorylase b, β-galactosidase and carbonic anhydrase were used as molecular markers.

Dissociative SDS-polyacrylamide electrophoresis

Dissociative electrophoresis of purified PIPLC-released enzyme was performed according to Weber and Osborn [34]. Egg albumin, bovine serum albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase and trypsinogen were used as molecular markers.

Thermal inactivation

Samples of PIPLC-released enzyme (5 μg/ml) in 5 mM Tris.HCl buffer, pH 7.5 containing 2 mM $MgCl_2$ were incubated in a water bath at different temperatures for varying periods. Immediately after the treatment 150 μl samples were chilled in an ice-water bath and the remaining PNPPase activity was assayed as described above.

Treatment of PIPLC-released enzyme with Chelex 100

Metal ions were removed by incubating PIPLC-released enzyme with Chelex-100, sodium form (200–400 mesh), according to the procedure described by Ciancaglini *et al.* [35].

Estimation of kinetic parameters

V, v, $K_{0.5}$, K_i and n obtained from substrate hydrolysis were fitted on an IBM/PC microcomputer as described elsewhere [36]. Data are reported as the mean of duplicate determinations which differed by less than 5%.

Results

The effect of PLC and hyaluronidase on PNPPase activity of rat osseous plate membrane-bound alkaline phosphatase is shown in Table 1. Treatment of the enzyme with PIPLC from *B. thuringiensis* was the only to release substantial levels of PNPPase activity into the supernatant. Furthermore, the effect of PIPLC from *B. thuringiensis* was concentration-dependent with a maximum at 0.1 U PIPLC/ml incubation mixture (Fig. 1).

Table 2 shows comparative data on the phosphohydrolytic activity of PIPLC-released enzyme toward PNPP, ATP and pyrophosphate as substrates. It was evident that PIPLC-released enzyme had no limited specificity for any of the above substrates. Furthermore, the released enzyme also showed phosphodiesterase and phosphotransferase activities (data not shown). While 96% of the original activity of membrane-bound alkaline phosphatase was released into the supernatant, a significant amount of ATPase activity, both at pH 7.5 and 9.4, remained bound to the membrane. The same occurred with pyrophosphatase activity of the membrane-bound enzyme, but to a minor extent.

The purification of PIPLC-released enzyme on Phenyl Sepharose CL-4B, resulted in the separation of two fractions with phosphohydrolytic activity (Fig. 2). Peak I, which represented more than 95% of total activity (4 μg of total protein), showed a specific activity of 290 U/mg and was used in the further studies.

In non-denaturing conditions, the purified PIPLC-released enzyme migrated on a 7% polyacrylamide gel as a single diffuse band coincident with phosphohydrolytic activity, indicating that the enzyme was homogeneous (not shown).

Gel filtration on Sephacryl S-300 indicated an apparent molecular weight of 145,000 (not shown). However, in denaturing conditions the purified PIPLC-released enzyme exhibited an apparent molecular weight of 66,000 (not shown). Together, these results suggested that the enzyme was a dimer of apparently identical subunits.

The kinetic characteristics of several effectors of PIPLC-released enzyme are summarized in Table 3. The remarkable 10-fold difference on K_i values for theophylline and levamisole is noteworthy. Except for pyrophosphate, in which site-site interactions (n = 1.4) were observed, for PNPP and ATP, the enzyme followed Michaelis-Menten kinetics. The hy-

Table 1. Effect of treatment with phospholipases and hyaluronidase on PNPPase activity of rat osseous plate membrane-bound alkaline phosphatase

Condition	% PNPPase activity	
	Pellet	Supernatant
Non-treated enzyme (Control)	98.8 ± 4.9	1.3 ± 0.1
PLC from *B. cereus* (0.1 U)	99.2 ± 3.9	3.9 ± 0.2
PLC from *C. perfringens* (0.1 U)	99.7 ± 4.5	2.8 ± 0.1
PLC from *B. thuringiensis* (0.1 U)	6.5 ± 0.2	106.5 ± 7.4
Hyaluronidase (0.1 U)	97.8 ± 3.4	3.9 ± 0.1

Aliquots containing 2 mg/ml rat osseous plate alkaline phosphatase in 50 mM Tris.HCl buffer, pH 7.25 were incubated with the indicated units of phospholipases (PLC) or hyaluronidase for 60 min, at 37°C. For PLC from *B. thuringiensis*, 50 mM Tris.HCl buffer, pH 7.25 was used. Values are the mean (± SD) of four independent experiments.

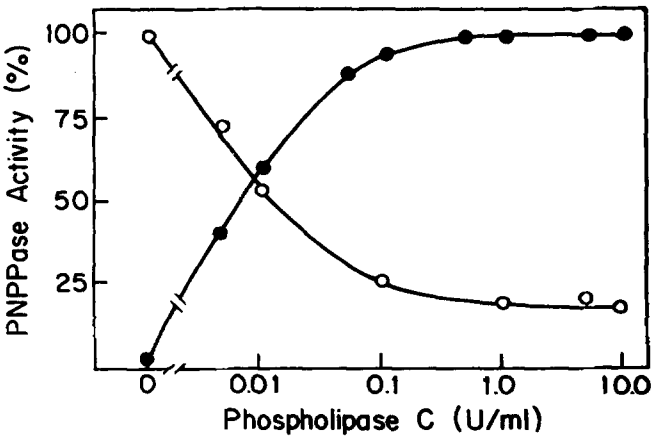


Fig. 1. Effect of concentration of PIPLC from *B. thuringiensis* on the release of osseous plate alkaline phosphatase from the membrane. Two milligrams of membrane enzyme were incubated with the indicated concentrations of phosphatydilinositol-specific phospholipase C in 1.0 ml of 50 mM Tris.HCl buffer, pH 7.25 for 60 min, at 37°C. Alkaline phosphatase activity was assayed using PNPP as substrate as described in Materials and methods. (●) per cent of incubated alkaline phosphatase activity released into the supernatant. (○) per cent of alkaline phosphatase activity remaining in the membrane pellet.

Table 2. Effect of treatment with phospholipase C from *B. thuringiensis* on rat osseous plate alkaline phosphatase activity

Substrate	pH	nmoles/min/ml			
		Non-treated enzyme		PIPLC-treated enzyme	
		Pellet	Supernatant	Pellet	Supernatant
PNPP	9.4	744.3 ± 11.2	11.4 ± 0.2	48.6 ± 0.7	754.5 ± 11.2
ATP	7.5	286.0 ± 5.7	17.4 ± 0.3	260.5 ± 5.2	28.2 ± 0.6
ATP	9.4	389.0 ± 11.7	16.3 ± 0.5	272.2 ± 8.2	171.3 ± 5.1
Pyrophosphate	8.0	76.5 ± 3.1	6.7 ± 3.3	14.0 ± 3.8	91.1 ± 3.6

The assays were carried out in 50 mM AMPOL buffer (pH 9.4) or 50 mM Hepes buffer (pH 7.5) containing 2 mM $MgCl_2$ and the substrate, at 37°C, as described in Materials and methods. For pyrophosphate, standard conditions were 50 mM Tris.HCl buffer, pH 8.0, containing 2 mM $MgCl_2$. Values are the mean (±SD) of five independent experiments.

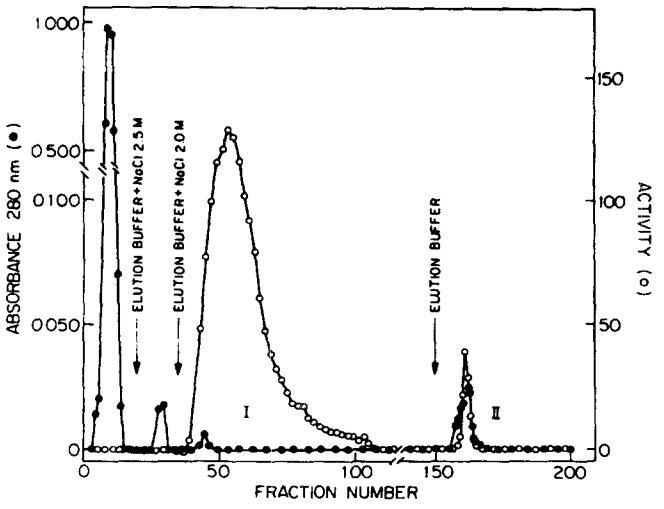


Fig. 2. Phenyl Sepharose CL4B chromatography of PIPLC-released alkaline phosphatase. A sample of 3.6 ml (5,563 U) was applied to a Phenyl Sepharose CL-4B column (1 × 10 cm) equilibrated with elution buffer containing 2.7 M NaCl. Fractions of 1.5 ml were collected and elution was performed at 4°C at a flow rate of 18 ml/h. Active fractions were pooled and concentrated on YM-5 Amicon membrane. Activity is given in nmoles of p-nitrophenolate ion released per minute per ml enzyme. The elution buffer was 5 mM Tris.HCl (pH 7.5) containing 1 μM $ZnCl_2$ and 2 mM $MgCl_2$.

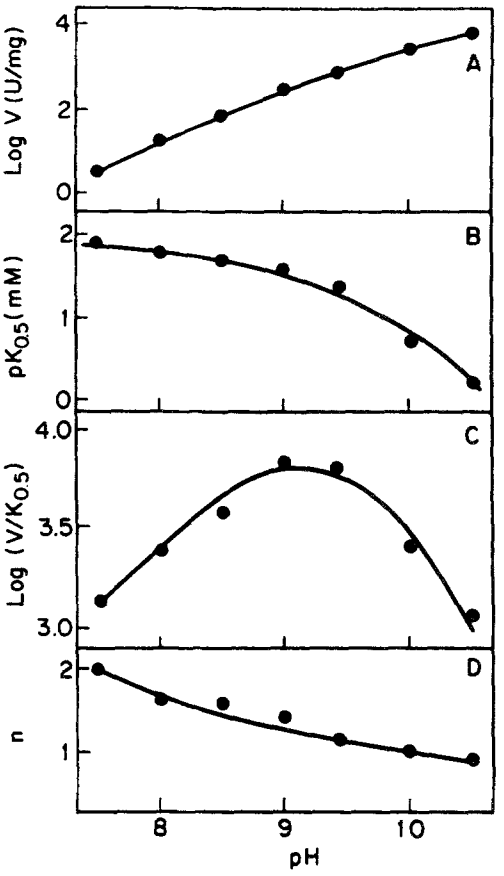
Table 3. Kinetic characteristics of several effectors of purified PIPLC-released alkaline phosphatase from osseous plate

Effector	Type of inhibition	K_i (mM)	V (U/mg)	$K_{0.5}$	n
Vanadate	competitive	0.003	–	–	–
Zinc	non-competitive	0.006	–	–	–
Levamisole	uncompetitive	0.022	–	–	–
Arsenate	competitive	0.023	–	–	–
Theophylline	uncompetitive	0.23	–	–	–
Phosphate	competitive	1.5	–	–	–
Phenylalanine	uncompetitive	6.6	–	–	–
PNPP	–	–	264.3	70	1.0
ATP	–	–	28.4	129	1.4
Pyrophosphate	–	–	42.0	979	1.0

The reaction was carried out in 50 mM AMPOL buffer, pH 9.4 containing 2 mM $MgCl_2$ and PNPP. For ATP, the assays were carried out in 50 mM AMPOL buffer, pH 9.4 containing 2 mM $MgCl_2$. For pyrophosphate, standard conditions were 50 mM Tris.HCl buffer, pH 8.0, containing 2 mM $MgCl_2$. PNPP, ATP and pyrophosphate concentrations varied from 5 μM to 10 mM and 25 ng of protein were used per assay. V is given in umoles of substrate hydrolyzed/min/mg protein. Values are the average of three independent experiments.

drolysis of these three substrates indicated that the release of the enzyme from the membrane with PIPLC did not destroy its multi-functional characteristics. In addition, no inhibition by excess of any of the substrates was observed in the assay conditions used (not shown).

The apparent optimum pH for the hydrolysis of PNPP by purified PIPLC-released enzyme varied markedly with substrate concentration: as substrate concentration decreased,



Flg. 3. pH sensitivity of the catalysis of purified PIPLC-released alkaline phosphatase from osseous plate. Assays were buffered with 50 mM Hepes buffer for pH 7.5-8.0 and with 50 mM AMPOL buffer for pH 8.0–10.0, both containing 2 mM $MgCl_2$. There was no significant differences between the two buffers at pH 8.0. Each saturation curve (not shown) necessary to determine V, $K_{0.5}$ and n comprised at least 15 experimental points and PNPP concentration ranged from 40 μM to 40 mM, using 25 ng protein. A: plot of log V versus pH. B: plot of $pK_{0.5}$ versus pH. C: plot of log ($V/K_{0.5}$) versus pH. D: plot of n versus pH.

optimum pH became more acidic (not shown).

The pH dependence of the hydrolysis of PNPP as substrate by purified PIPLC-released enzyme is shown in Fig. 3. This substrate was recognized better by the enzyme at pH 7.5 rather than at pH 10.5 (see in Fig. 3B that the p $K_{0.5}$ is lower by a factor of 2 at pH 10.5). The apparent pK_a values for catalytic groups, determined from data of Fig. 3C, were 8.5 and 9.7. While site-site interactions were observed for the hydrolysis of PNPP at less alkaline pH, Michaelis-Menten kinetics was observed above pH 9.2 (Fig. 3D).

The effect of zinc and magnesium ions on PNPPase activity of purified PIPLC-released alkaline phosphatase is shown in Fig. 4. In the presence of 1 μM $ZnCl_2$ the enzyme activity was inhibited 35% (Fig 4A) Competition kinetic measurements indicated that zinc was a non-competitive inhibitor with K_i = 6.2 μM (inset of Fig. 4A). On the other hand, mag-

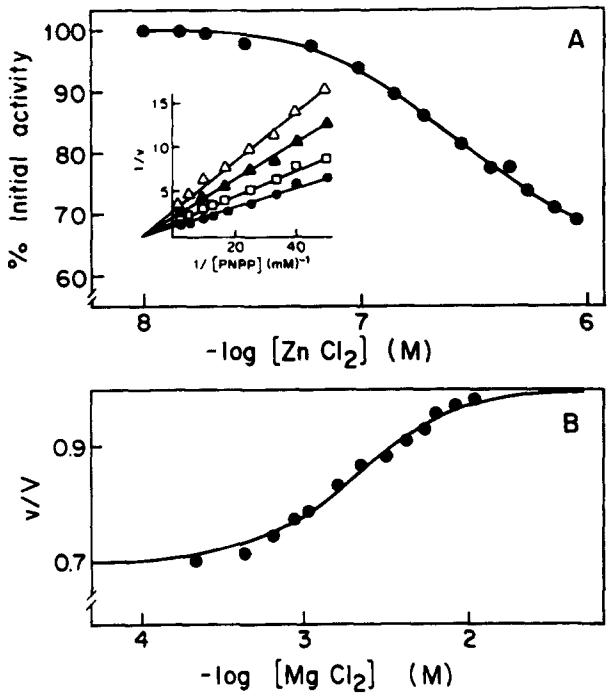


Fig. 4. Effect of divalent metal ions on the hydrolysis of PNPP by PIPLC-released alkaline phosphatase. The activity was measured in 50 mM AMPOL buffer pH 9.4 containing 1 mM PNPP, as described in Materials and methods. Protein concentration used was 25 ng. A: zinc. B: magnesium. Inset of Fig. A-reciprocal plot for the inhibition of PIPLC-AP by zinc ions: (●) zero; (□) 5 μM; (▲) 8.5 μM; (Δ) 10 μM.

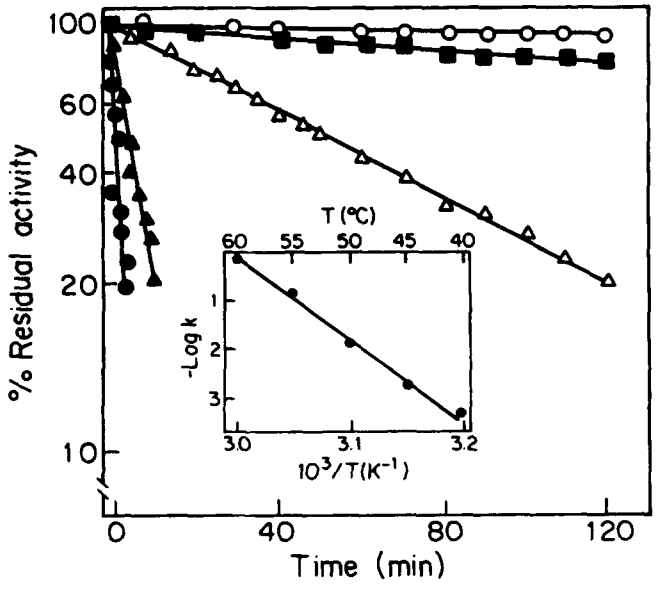


Fig. 5. Thermal inactivation of PIPLC-released alkaline phosphatase. The residual activity was determined by the addition of 50 μl aliquots to 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 1 mM PNPP, in a final volume of 1.0 ml. Values are the mean of duplicate determinations which differed by less than 5% variation. (O) 40°C. (■) 45°C. (Δ) 50°C. (▲) 55°C. (●) 60°C. Inset: Arrhenius' plot for the above data.

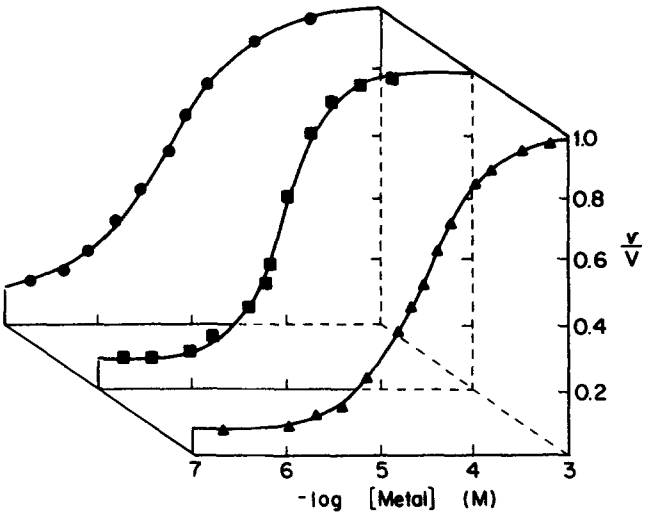


Fig. 6. The metal concentration dependence of the stimulation of the hydrolysis of PNPP by PIPLC-released alkaline phosphatase. The reaction was carried out in 50 mM AMPOL buffer pH 9.4, containing 1 mM PNPP and (▲) $MgCl_2$ or (■) $CoCl_2$ or (●) $MnCl_2$. Protein concentration used was 25 ng.

nesium ions stimulated the PNPPase activity of the enzyme by 30% and site-site interactions (n = 1.2) were observed with $K_{0.5}$ = 1.5 mM. Interestingly, cobalt, manganese and calcium ions had no effects on PNPPase activity of purified PIPLC-released enzyme (not shown).

Thermal inactivation studies showed that PIPLC-released enzyme was relatively stable at 40°C (Fig. 5). However, with increasing temperature the enzyme was inactivated rapidly following first order kinetics. From 40–60°C, thermal inactivation constants varied from 5.08×10^{-4} min^{-1} to 0.684 min^{-1}.

Treatment of PIPLC-released enzyme with Chelex-100 depleted to 5% the original PNPPase activity after 150 min incubation with the resin. Atomic absorption spectrometry revealed that neither zinc nor magnesium ions remained bound to the enzyme (not shown). These results suggested that these two metal ions were essential for full activity of purified PIPLC-released alkaline phosphatase.

Figure 6 shows the stimulation of PNPPase activity of Chelex-treated PIPLC-released alkaline phosphatase by divalent metal ions. The monophasic stimulation curves obtained suggested that a unique family of sites is involved on the catalytic cycle of the enzyme. Magnesium, manganese and cobalt ions stimulated significantly (13–20-fold) the activity of Chelex-treated enzyme while calcium ions showed a stimulation of about 26%. In addition, except for manganese ions, site-site interactions were observed for the stimulation of the activity of the enzyme (Table 4).

Table 4. Kinetic parameters for the stimulation of PNPPase activity of Chelex-treated PIPLC-released alkaline phosphatase by divalent metal ions

Parameters	Mg(II)	Co(II)	Mn(II)	Ca(II)
V (U / mg)	276.3	225.9	166.2	33.1
$K_{0.5}$ (μM)	29.5	10.1	5.0	653
n	1.2	1.7	1.0	0.7
Stimulation (fold)	20	17	13	<1

The reaction was carried out in 50 mM AMPOL buffer, pH 9.4 containing 1 mM PNPP. The concentration of metal ion varied from 0.1 μM to 1 mM and 25 ng of Chelex-treated protein were used per assay. Values are the average of three independent experiments.

Discussion

Alkaline phosphatase is a multigene-controlled enzyme [37]. Although three isoforms of this enzyme are widespread in human and higher primate tissues, the liver/bone/kidney isoenzyme is found at much higher levels in bone-forming osteoblast cells [38], a fact which has been related to the mineralization process [14, 18]. These isoforms have been reported to exist also as membrane-bound and soluble forms [16, 19, 20, 39, 40]. However, only the membrane-bound isoform is considered to participate in the calcification process [18, 39, 41].

We have reported previously that a membrane-bound and a soluble form of rat osseous plate alkaline phosphatase appear during the ectopic mineralization process [19, 20]. The membrane-bound form is a multifunctional metalloenzyme hydrolysing not only p-nitrophenylphosphate, pyrophosphate and ATP but cAMP as well [42]. It is constituted of two apparently identical subunits (M_r 65,000) which requires both zinc and magnesium ions for maximal activity [35, 43–45]. While the soluble form revealed structural and some kinetic properties very similar to those of the membrane-bound enzyme, its stimulation by calcium ions was a striking unexpected result [20].

The results shown in Fig. 1 and Table 1 suggested that alkaline phosphatase from rat osseous plate is anchored to the lipid bilayer of the membrane through a phosphatidylinositol glycan, as is reported for other proteins [2, 15, 39]. This conclusion is consistent with substrate specificity of the phospholipases used. While PI-PLC from *B. thuringiensis* specifically hydrolyzes phosphatidylinositol and lysophosphatidylinositol and no other phospholipids [46], phospholipases from *B. cereus* and *C. perfringens* have broad specificities toward phospholipids but they do not hydrolyze phosphatidylinositol or lysophosphatidylinositol [47, 48]. A similar argument has been used by Low and Finean [9] when analysing alkaline phosphatases from several sources.

Interestingly, PIPLC-released alkaline phosphatase retained its multifunctional characteristics (Table 3), similarly as did the detergent-solubilized enzyme [43]. PIPLC-released enzyme is a dimer of apparently identical subunits of M_r of 66,000, which is similar to that reported for the soluble form [20] and detergent-solubilized enzyme [43, 44]. In addition, it exhibited kinetic properties (Table 3) very similar to the three other forms of the enzyme already reported by us [20, 43, 45, 49, 50]. The apparent optimum pH more acidic with decreasing substrate concentration corresponded to data reported for soluble [20], detergent-solubilized [50] and membrane-bound enzyme [49]. Substrate inhibition was not observed for PNPPase activity in the pH range from 7.5–10.5. The hydrolysis of PNPP at different pH values (Fig. 3) is consistent with the diprotic model [51], involving two catalytic groups with pK_a of 8.6 and 9.7. Similar values were reported for the catalytic groups of membrane-bound and detergent-solubilized enzyme [44].

It is generally accepted that divalent metal ions are essential not only for the catalytic activity but also for the stability of alkaline phosphatases from several sources [52]. Purified PIPLC-released enzyme also showed metalloenzyme properties since the incubation of the enzyme with Chelex-100 depleted about 95% of its initial activity. On the other hand, removal of metal ions did not promote an irreversible inactivation of PIPLC-released enzyme since magnesium, cobalt and manganese ions restored most of the enzyme activity. These results do not differ significantly from those obtained for membrane-bound [36] and detergent-solubilized enzyme [35]. Although calcium ions were not an efficient stimulator, their effects on the activity of PIPLC-released enzyme was very similar to that reported for the soluble enzyme [20].

Mammalian alkaline phosphatases are distributed in various tissues [52]. While multiple forms of the enzyme from a single organ have been reported, the origin of such different forms is often discussed in terms of post-translational modification [37, 53].

The similarities observed on elution profile, structural and kinetic properties of PIPLC-released enzyme when compared with those of the soluble forms of osseous plate alkaline phosphatase [20], suggested at a first glance that the soluble form was released by the hydrolysis of PI-anchored enzyme, similarly as reported for liver alkaline phosphatase [28]. Nevertheless, both membrane-bound and soluble species may exist as such *in vivo*. If they indeed exist, by which mechanism is the membrane-bound enzyme converted to the soluble form appearing during endochondral ossification process? Recently, Bublitz *et al.* [54] reported that calf intestinal alkaline phosphatase is delivered intralumenally as a GPI species associated with undefined particular materials. Although the delivery mechanisms are still unknown, these authors concluded that no changes on the protein moiety nor on GPI-anchor structure occurred.

By comparing the thermal inactivation data obtained for PIPLC-released enzyme at 55°C ($t_{0.5}$ = 4.3 min) with those of

membrane-bound ($t_{0.5}$ = 4.4 min), detergent-solubilized ($t_{0.5}$ = 10.2 min) and soluble form ($t_{0.5}$ = 8.1 min) of the osseous plate enzyme, interesting results emerged [19, 20, 50]. While $t_{0.5}$ of PIPLC-released enzyme was similar to that of the membrane-bound enzyme, it was about 50% lower than that of the soluble enzyme. On the other hand, if we consider that detergents not only can be bound to GPI-linked proteins [55, 56] but also might protect the enzyme molecule against denaturation [57], $t_{0.5}$ = 10.2 min obtained for detergent-solubilized enzyme could be interpreted on this basis. However, the difference between $t_{0.5}$ obtained for soluble enzyme and that of PIPLC-released form is, in our opinion, large enough to support the view that soluble enzyme present during endochondral ossification could arise through the hydrolysis of PI-anchored form of the enzyme by specific anchor-cleaving activities as was reported for liver alkaline phosphatase [28] The presence of two different forms of alkaline phosphatase, showing different heat sensitivities, in mineralizing tissues, has also been reported by Collin *et al.* [40].

Our interpretation is also supported by Phenyl Sepharose CL-4B chromatography and metal ion stimulation data obtained for soluble and PIPLC-released forms. When subjected to hydrophobic chromatography, soluble enzyme showed at least three species with different hydrophobicities [20] while the PIPLC-released form showed only two. A similar situation has been reported for calf intestinal alkaline phosphatase [54]. Another result which argues against the possibility that the soluble form might arise from the PI-anchored one relates to the stimulation by calcium ions. While these ions had no effect on the PIPLC-released form, a stimulation of about 30% was reported for the soluble form [20]. For PI-linked enzymes self aggregation is a common phenomenon which can enhance the differences in hydrophobic behavior [56]. In addition, exposure of buried specific metal sites through self aggregation is a possibility which cannot be discarded. These possibilities are coherent with M_r (of about 163 kD) obtained for soluble [20] and PIPLC-released forms (of about 145 kD) of rat osseous plate enzyme.

Finally, considering that almost all the mammalian PI-specific phospholipase C is believed to be intracellular [1], our results suggested the soluble form of alkaline phosphatase isolated during endochondral ossification [20], might occur through a pathway which apparently does not involve changes in the protein moiety nor the hydrolysis of the linkage between the phosphate and diacylglycerol moieties. However, our interpretation does not exclude unequivocally the possibility that the soluble form of osseous plate alkaline phosphatase could arise from a different mRNA as was reported for soluble and membrane forms of intestinal alkaline phosphatase [37]. Work in this direction is now in progress in our laboratory.

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