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Erythrocyte ghost cell–alkaline phosphatase: construction and characterization of a vesicular system for use in biomineralization studies

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Abstract

Alkaline phosphatase is required for the mineralization of bone and cartilage. This enzyme is localized in the matrix vesicle, which plays a role key in calcifying cartilage. In this paper we standardize a method to construction a resealed ghost cell–alkaline phosphatase system to mimic matrix vesicles and examine the kinetic behavior of the incorporated enzyme. Polidocanol-solubilized alkaline phosphatase, free of detergent, was incorporated into resealed ghost cells. This process was time-dependent and practically 50% of the enzyme was incorporated into the vesicles in 40 h of incubation, at 25 °C. Alkaline phosphatase–ghost cell systems were relatively homogeneous with diameters of about 300 nm and were more stable when stored at −20 °C.

Alkaline phosphatase was completely released from the resealed ghost cell-system using only phospholipase C. These experiments confirm that the interaction between alkaline phosphatase and the lipid bilayer of resealed ghost cell is exclusively via glycosylphosphatidylinositol (GPI) anchor of the enzyme.

An important point shown is that an enzyme bound to resealed ghost cell does not lose the ability to hydrolyze ATP, pyrophosphate and *p*-nitrophenyl phosphate (PNPP), but the presence of a ghost membrane, as a support of the enzyme, affects its kinetic properties. Moreover, calcium ions stimulate and phosphate ions inhibit the PNPPase activity of alkaline phosphatase present in resealed ghost cells.
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Keywords: Alkaline phosphatase; Osseous plate; Glycosylphosphatidylinositol (GPI) anchor; Ghost cell; Endochondral ossification

1. Introduction

Biomineralization or biological calcification is a tightly regulated process in which different types of tissues, cells, organelles and biomolecules participate in the coordination and regulation of metabolic events involved in accumulating great amounts of calcium phosphate [1–9].

Several studies on mineralizing tissues revealed that calcification initial events occur in matrix vesicles strategically inserted in the mineralizing matrix. It has been proposed that these matrix vesicles, present in mineralizing tissues such as cartilage, bone and dentin, are organelles carrying all important cellular potentialities that trigger the initial crystal formation of calcium phosphate. Studies at molecular level have shown that these vesicles contain alkaline phosphatase, adenosine-5′-triphosphatase (ATPase) and other enzymes that could be the key point through which matrix vesicles adjust the initial stage of mineral deposition [1,2,6,7,10–14]. The crucial role of alkaline phosphatase in the calcification process has been stressed by the inborn disease called hypophosphatasia [15].

Some authors have shown that alkaline phosphatase from cartilage and bone is a phosphatidylinositol-anchored membrane ectoprotein [8,16] in contact with extracellular cartilage fluid, in which natural putative substrates are present at nanomolar or micromolar concentrations. The phosphatidylinositol structure is a phosphatidylinositol-glycolipid anchor, which is covalently attached to the carboxyl terminus (C-terminus) of the protein through an amide linkage. This anchor structure of alkaline phosphatase results in lateral mobility in the membrane and allows the release of the protein from the membrane through the action of phospholipases [8,16,17]. The functional significance of this structural feature in mineral formation remains obscure.

The research on the roles of matrix vesicles in the calcification process has shown that these vesicular systems

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are essential for the calcification process and must be maintained in strict control. It has been shown that vesicle membrane perturbation caused by 10% butanol nulls the property of calcium phosphate salt deposition mediated by ATP [12]. In addition, the treatment of membrane vesicles with Triton X-100 or deoxycholate (when concentrations are below the critical micellar concentration) has stimulated the calcification process twofold. Apparently, this stimulation is not related to the activation of ATPase, alkaline phosphatase or pyrophosphatase. In contrast, cationic detergent eliminates calcium phosphate deposition associated to the inhibition of ATPase activity, suggesting, therefore, that perturbation of the membrane vesicles associated to the presence of ATPase has an important role in the calcium phosphate deposition in cartilaginous matrix [12]. Studies using dipalmitoylphosphatidylcholine-liposomes as vesicle compartments to sequester ions (these systems mimic only matrix vesicles free of several enzymes) revealed it to be a good strategy for calcium phosphate deposition into the tissue substrates [18,19]. Thus, any conclusion concerning the importance of lipid complexes in calcification, mediated by matrix vesicles, needs more detailed study, specially in a system where the pH and the electrolyte levels are similar to those found inside the vesicle or in the native extra cellular fluids.

The ability of synthetic or natural vesicles to mimic the organizational structure and function of biomembrane makes this structure an advantageous and convenient experimental model. Besides, interest in drug and biological macromolecule delivery mediated via carriers has increased considerably in the past few years. Several systems to control these processes have been proposed, including liposomes, erythrocytes ghost cells, biodegradable nanoparticles and microespheres [20–25].

This study reports the construction and characterization of a vesicular system, as a membrane model, with the possibility to mimic matrix vesicles, free of other important proteins, to better study the role of the alkaline phosphatase in the biomineralization process.

2. Materials and methods

All solutions were made using Millipore MilliQ ultra pure apyrogenic water. Bovine serum albumin, Tris hidroxymetil amino methane (Tris), 2-amino-2-methyl-propan-1-ol (AMPOL), trichloroacetic acid (TCA), *N*-(2-hydroxyethyl) piperazine-*N*′-ethanesulfonic acid (HEPES), adenosine 5′-triphosphate disodium salt (ATP), sodium dodecylsulfate (SDS), *p*-nitrophenyl phosphate (PNPP), bromelain and polyoxyethylene 9-lauryl ether (polidocanol) were from Sigma Chemical Co. (St Louis, MO, USA); pyrophosphate (PPi), ethylenediaminetetraacetic acid (EDTA) and magnesium chloride were from Merck (São Paulo SP, Brazil). Calbiosorb resin was from Calbiochem (San Diego, CA, USA) and purified phosphatidylinositol-specific phospholipase C (PIPLC) from *Bacillus thuringiensis* was obtained from Oxford University (UK) by customer service of OGS-Oxford Glycosciences PLC. Analytical grade reagents were used without further purification.

2.1. Preparation of rat osseous plate alkaline phosphatase

Membrane-bound alkaline phosphatase was prepared from rat osseous plate according to Curti et al. [26]. Briefly, 10 to 20 mg of rat bone demineralized diaphyseal bone matrix was introduced through a small incision in the dorsal subcutaneous tissue of ether anesthetized young male Wistar rats (50–60 g). Fourteen days after implantation (the period required for the development of maximal alkaline phosphatase activity), the osseous plate formed was 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 buffer/g osseous plate) in a high speed shearing homogenizer, for 2 min. The homogenate (crude extract enzyme) was centrifuged at $15{,}000 \times g$ for 20 min and the supernatant was dialysed overnight, at 4 °C, against 5 mM Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 0.15 M NaCl. The dialysed homogenate was centrifuged for 1 h at $160{,}000 \times g$. The pellet, corresponding to the membrane-bound enzyme, was resuspended in 5 mM Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$. Aliquots of 1 ml (containing about 0.3 mg/ml of protein showing 500 U/mg) were frozen in liquid nitrogen and stored at −20 °C for a month without appreciable loss of activity.

2.2. Solubilization and purification of rat osseous plate alkaline phosphatase with polyoxyethylene 9-lauryl ether

Samples containing 0.2 mg/ml of membrane bound alkaline phosphatase were solubilized with 1% polidocanol (final concentration) for 2 h with constant stirring, at 25 °C. After centrifugation ($160{,}000 \times g$) at 4 °C for 1 h, the solubilized enzyme was concentrated and purified as described by Ciancaglini et al. [27]. The solubilized enzyme was purified on a Sephacryl S-300 column (130×1.7 cm), equilibrated and eluted in 5 mM Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, 150 mM NaCl and 0.01% polidocanol. The fractions showing PNPPase activity were pooled, concentrated on an YM-5 Amicon filter, dialysed overnight at 4 °C and rechromatographed in the same conditions as described above. Finally, the purified polidocanol-solubilized alkaline phosphatase was dialyzed overnight, at 4 °C, against 5 mM Tris·HCl buffer, pH 7.5, containing 0.01% polidocanol. Note that the solubilized enzyme corresponds to 6% of the total membrane protein, showing a specific activity about of 6000 U/mg (using PNPP as substrate) and was electrophoretically pure [27].

To remove the excess of detergent, 1 ml of the polidocanol-solubilized enzyme (~ 0.03 mg/ml) was added to 200 mg of Calbiosorb resin as described by Camolezi et al. [28,29]. Briefly, the suspension was mixed for 2 h at 25 °C and stored at 4 °C. The supernatant, obtained after centri-

fugation (3000 × *g*) at 4 °C for 1 min, is the source of detergent-free, solubilized enzyme and was used for the incorporation to resealed ghost cells.

2.3. Preparation of enzymatically released alkaline phosphatase

Aliquots containing 2 mg/ml membrane-bound alkaline phosphatase were incubated in 50 mM Tris·HCl buffer, pH 7.25, with phosphatidylinositol-specific phospholipase C (0.1 U), or with bromelain (25 U) for 1 h, under constant rotary shaking, at 37 °C. The incubation mixture was centrifuged at 160,000 × *g* for 1 h, at 4 °C. The pellet was resuspended in 5 mM Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, to the original volume, and centrifuged as before. Both supernatants were source of enzymatic release of alkaline phosphatase and purified as described by Pizauro et al. [16] and Camolezi et al. [28,29]. The solution of alkaline phosphatase released present in the supernatant of phosphatidylinositol-specific phospholipase C treatment 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 (for more details, see Pizauro et al. [16]).

The purification of alkaline phosphatase released by the bromelain treatment was rapidly done using a procedure previously described for purification of polidocanol-solubilized alkaline phosphatase using chromatography on Sephacryl S-300 column (130 × 1.7 cm), equilibrated and eluted in Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 150 mM NaCl. The fractions showing PNPPase activity were pooled, concentrated on a YM-5 Amicon filter, dialysed overnight at 4 °C and rechromatographed in the same conditions as described above. The purified polidocanol-solubilized alkaline phosphatase was dialyzed overnight, at 4 °C, against 5 mM Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ (for more details, see Camolezi et al. [28,29]).

2.4. Enzymatic activity measurements

p-Nitrophenylphosphatase (PNPPase) activity was assayed discontinuously at 37 °C in a Spectronic (Genesys 2) spectrophotometer by following the liberation of *p*-nitrophenolate ion (ε 1 M, 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. The reaction was initiated by the addition of the enzyme and stopped with 1.0 ml of 1.0 M NaOH at appropriate time intervals.

For ATPase, the activity was also assayed discontinuously by measuring the amount of inorganic phosphate liberated, according to the procedure previously described [16], 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. The reaction mixtures were centrifuged at 4000 × *g* prior to phosphate determination. Standard assay conditions were 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 2 mM ATP. For pyrophosphatase activity assays (PPiase), standard conditions were 50 mM Tris·HCl buffer, pH 8.0, containing 2 mM $MgCl_2$ and 2 mM sodium pyrophosphate, and processes were as described for ATPase activity.

All determinations were carried out in triplicate 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 nonenzymatic hydrolysis of substrate.

2.5. Protein quantification

Protein concentrations were estimated according to Hartree [30] in the presence of 2% (w/v) SDS. Bovine serum albumin was used as standard.

2.6. Resealed ghost cells preparation

Inside-out vesicles of ghost cells were prepared as described by Steck et al. [31], modifying the original buffers which were sodium phosphate based to Tris·HCl. This substitution was made because phosphate is an alkaline phosphatase inhibitor and it would be impossible to monitor the enzyme activity in its presence. Washer buffer: 23 mM Tris·HCl buffer, pH 7.4, containing 145 mM NaCl and 1 mM EDTA; hemolysis buffer: 41.2 mM Tris·HCl buffer, pH 8.0, containing 5 mM NaCl and 1 mM EDTA; and finally, resealing buffer: 82.4 mM Tris·HCl buffer, pH 8.0, containing 100 mM NaCl, 0.01 mM $CaCl_2$ and 4 mM $MgSO_4$. In all solutions, NaCl was used for the correction of the ionic strength reproducing the same as described by Steck buffers [31]. The resulting resealed cells (0.6 mg/ml of total proteins) were separated in 1-ml samples and stored at − 70 °C.

The resealed ghost cells prepared in Tris·HCl buffer have shown disc-shaped images in the optical microscope, suggestive of the presence of closed vesicles with an average diameter of 200–300 nm.

2.7. Incorporation of alkaline phosphatase to resealed ghost cell

The polidocanol-solubilized detergent-free alkaline phosphatase, obtained as described in Section 2.2, was incorpo-

rated into resealed ghost cells by mixing equal volumes of both solutions at room-temperature. Samples of 100 μl were removed at predetermined intervals and centrifuged at 150,000 × *g* for 40 min. PNPPase activities before centrifugation, in the supernatant and resuspended pellet were assayed and used to calculate the percent incorporation.

2.8. Characterization of resealed ghost cell–alkaline phosphatase system

The resealed ghost cell–alkaline phosphatase system (0.5 mg of protein) was characterized by chromatography filtration on a Sephacryl S-300 column (130 × 1.7 cm), equilibrated and eluted in 5 mM Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 150 mM NaCl, at a flow rate of 13 ml/h. Fractions of 3 ml were collected and aliquots were assayed for absorbance at 280 nm and PNPPase activity. Elution profiles of resealed ghost cells (0.5 mg) and polidocanol-solubilized alkaline phosphatase (0.05 mg) were also obtained. When polidocanol-solubilized alkaline phosphatase was used, the column was equilibrated and eluted in the same buffer containing 0.01% polidocanol.

2.9. Electron microscopy

The resealed ghost cells with incorporated alkaline phosphatase were examined by electron microscopy. The pellet obtained after centrifugation at 150,000 × *g* was fixed using glutaraldehyde for 1 h followed by 1% (w/v) osmium solution. After 1 h, the sample was washed in 0.1 M sodium phosphate buffer, pH 7.3, centrifuged and dehydrated with acetone/water at increasing acetone concentration. The last dehydration was done with 100% acetone during 10 min for three times. Finally, the pellet was infiltrated in an araldite resin (1:1) at 37 °C for 48 h and included in pure araldite for 72 h at 60 °C. A thin plate (0.5 μm) was cut using a microtome and contrasted with 4% (w/v) uranile acetate, pH 12, for 10 min followed by 0.3% (w/v) plumb citrate, pH 12, for another 10 min. After this, the sample was examined using a Philips electron microscope Model 208.

2.10. Histochemistry of resealed ghost cell–alkaline phosphatase system

The alkaline phosphatase activity was histolocalized in the membrane of resealed ghost cells using a method described by Mayahara et al. [32]. A 1-ml sample containing resealed ghost cells (0.6 mg/ml) and enzyme (0.03 mg/ml) in 5 mM Tris·HCl buffer pH 7.5, in the presence of 2 mM $MgCl_2$, was incubated at room temperature for 40 h and centrifuged at 100,000 × *g* for 1 h. The pellet obtained corresponds to a resealed ghost cell–alkaline phosphatase system (of about 0.5 mg of total protein). It was assayed with PNPP in the presence of lead ammonium citrate/acetate complex for 2 h and then fixed with 3% (v/v) glutaraldehyde; the pellet was then processed by electron micrography.

2.11. Effects of phosphate and calcium ions in the PNPPase activity of resealed ghost cell–alkaline phosphatase system

The effect of concentration of sodium phosphate and calcium ions in PNPPase activity was assayed discontinuously at 37 °C in a Spectronic (Genesys 2) spectrophotometer by following the liberation of *p*-nitrophenolate ion as described in Section 2.4. Standard conditions were 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$, 1 mM PNPP and calcium chloride or sodium phosphate in a final volume of 1.0 ml.

Kinetics parameters obtained from the effect of ions in the hydrolysis were fitted using a microcomputer according to the Hill equation using the procedure described by Atkins (for details see Ref. [27]). *V*, $K_{0.5}$, K_d and *n*, which appear in this paper as computed values, stand for maximal velocity, half-maximum effect, apparent dissociation constant and Hill coefficient, respectively. The values plotted are the means of triplicate determinations which differed by less than 5%.

3. Results

3.1. Obtaining resealed ghost cell–alkaline phosphatase system

Fig. 1 shows the incorporation curve of alkaline phosphatase, solubilized with polidocanol and detergent-free,

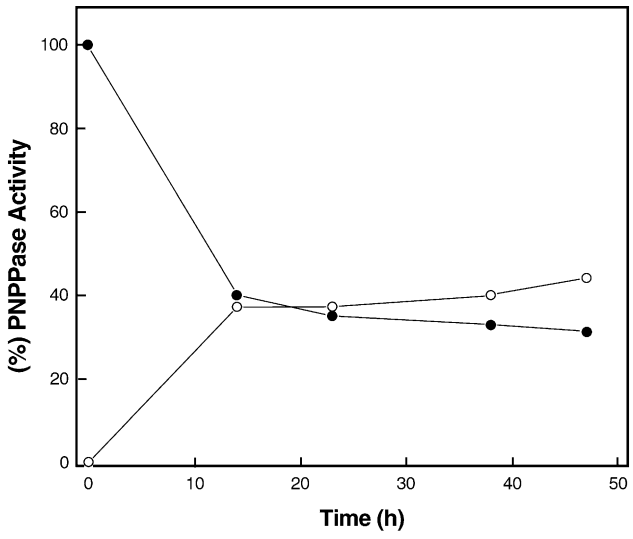

Fig. 1. Incorporation of detergent solubilized alkaline phosphatase into resealed ghost cells. Resealed ghost cells (0.60 mg/ml) and enzyme (0.03 mg/ml) in 5 mM Tris·HCl buffer pH 7.5, containing 2 mM $MgCl_2$ were incubated at room temperature. Samples were assayed before and after centrifugation for PNPPase activity and values are the mean of triplicate determinations which differed by less than 5%: (○) pellet (enzyme incorporated into resealed ghost cells) and (●) supernatant (enzyme-free).

into resealed ghost cells. As it can be observed, almost 50% of the alkaline phosphatase was incorporated after about 48 h of incubation at 4 °C.

The resealed ghost cell–alkaline phosphatase system has shown to be relatively stable when stored either at 4 or −20 °C. In fact, only around 20% of the PNPPase initial activity was lost after 12 days.

It should be stressed that only the alkaline phosphatase solubilized with polidocanol and detergent free presents the ability to incorporate to resealed ghost cells, even after an incubation period of 72 h. The Phosphatidylinositol-specific phospholipase C treatment selectively removes the diacylglycerol molecule, which is covalently attached to the alkaline phosphatase C-terminus, while bromelain treatment, presumably, causes an unspecific cleavage of the polypeptide chain from the alkaline phosphatase, removing the whole glycosylphosphatidylinositol (GPI) anchor. That is, these treatments result into an enzyme form that loses its capacity to incorporate to ghost cell membranes.

3.2. Characterization of resealed ghost cell–alkaline phosphatase system

With the objective of characterizing the alkaline phosphatase–resealed ghost cell system, we have performed a set of experiments in Sepharose 6B gel filtration.

The elution profile of the resealed ghost cells monitored by absorbance at 280 nm (Fig. 2A) resulted in a chromatogram with two protein peaks. The first peak (around fraction 8), which was apparently excluded from the column (coincides with the Blue Dextran elution peak V_o), can be related to the resealed ghost cells. The second peak, eluted around

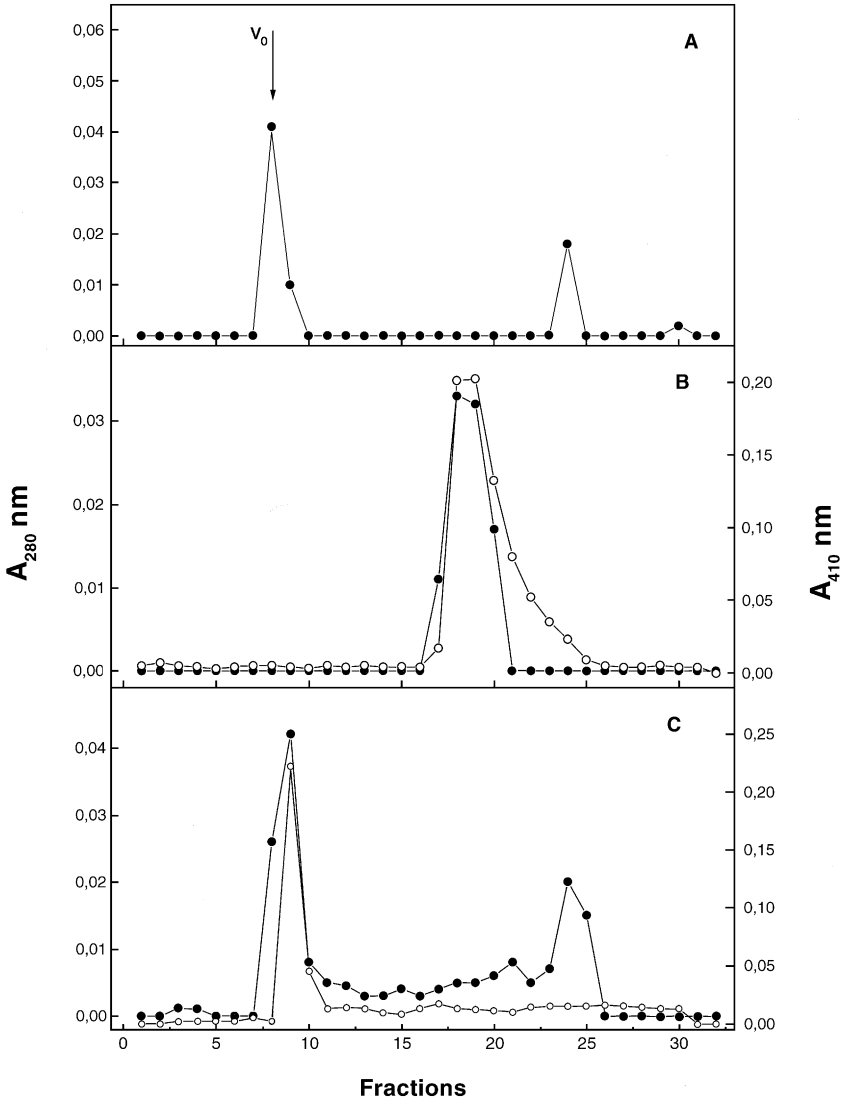

Fig. 2. Elution profiles of: (A) resealed ghost cells; (B) polidocanol-solubilized alkaline phosphatase* and (C) alkaline phosphatase-resealed ghost cells system; in Sephacryl S-300 column (130 × 1.7 cm) equilibrated and eluted with 5 mM Tris·HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 150 mM NaCl, at a flow rate of 13 ml/h. Fractions of 3 ml were collected and aliquots were assayed for: (●) protein concentration (A_{280nm}) and (○) PNPPase activity (A_{410nm}). *For this sample, 0.01% (w/v) of polidocanol was added in the elution buffer (details see Materials and methods).

fraction 24, can be probably related to proteins that were released from the ghost cell during the filtration process. It is important to comment that none of fractions presents PNPPase, ATPase or PPiase activities in the experimental condition assayed.

In Fig. 2B the elution profile of solubilized alkaline phosphatase is shown monitoring absorbance at 280 nm for protein and 410 nm for PNPPase activity. As it can be observed, the enzyme is eluted in a single peak close to fraction 20, showing relatively symmetrical peaks for protein and enzymatic activity as well.

Fig. 2C shows the elution profile of the resealed ghost cell–alkaline phosphatase system. Note that the enzyme was excluded from the column together with the resealed ghost cells, suggesting that the alkaline phosphatase is associated to the membrane of the ghost cells. Observe that the second peak, eluted around fraction 24, does not show enzymatic activity, probably due to the proteins that are released from the ghost cells membrane, which was previously observed in the absence of the enzyme (Fig. 2A).

The electron microscopy of the ghost cells which anchored the alkaline phosphatase shows that the vesicles formed are closed and can form even multilamellar type associations, where a vesicle closes around another with diameters varying from 200 to 400 nm (see Fig. 3). Also observe that the enzyme insertion does not break the membrane of the resealed cells.

When resealed ghost cell–alkaline phosphatase system was submitted to histochemical analysis, it was possible to observe that the enzyme is located on the surface of the resealed cell (Fig. 4). It is important to note that the treatment of the resealed ghost cell–alkaline phosphatase system with phosphatidylinositol-specific phospholipase C solubil-

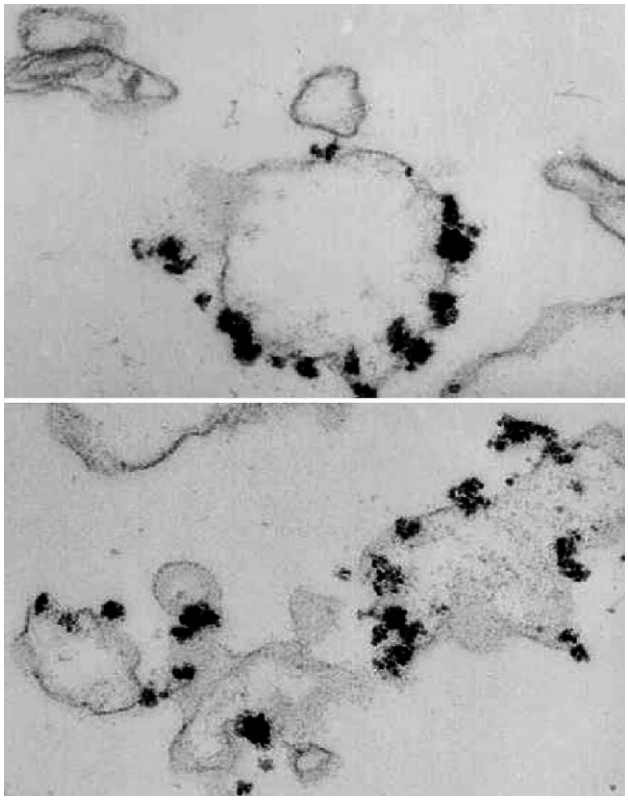

Fig. 4. Histolocalization of rat osseous plate alkaline phosphatase incorporated to resealed ghost cells. Resealed ghost cells (0.6 mg/ml) and enzyme (0.03 mg/ml) in 5 mM Tris·HCl buffer pH 7.5, containing 2 mM $MgCl_2$, were incubated at room temperature for 40 h and centrifuged at 100,000 × *g* for 1 h. Pellets were assayed with PNPP in the presence of lead ammonium citrate/acetate complex for 2 h and next fixed with 3% (v/v) glutaraldehyde. Finally, they were processed by electron micrography (× 100,000).

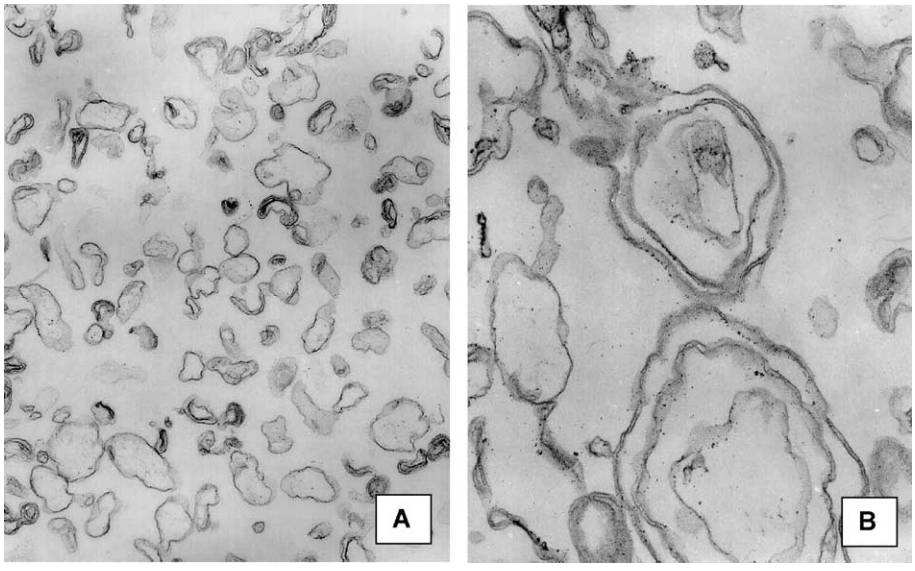

Fig. 3. Electron micrography of rat osseous plate alkaline phosphatase incorporated to resealed ghost cells. Resealed ghost cells (0.6 mg/ml) and enzyme (0.03 mg/ml) in 5 mM Tris·HCl buffer pH 7.5, containing 2 mM $MgCl_2$ were incubated at room temperature for 40 h and centrifuged at 100,000 × *g* for 1 h. Pellets were fixed with 3% (v/v) glutaraldehyde and finally, processed by electron micrography. (A) × 50,000 and (B) × 80,000.

Table 1
Study of ATPase, PNPPase and PPiase activities of alkaline phosphatase incorporated to resealed ghost cells

Activity	(%)
PNPPase	44.2
ATPase	27.6
PPiase	17.9

Values are the means of triplicate determinations which differed by less than 5%. The activity before incorporation was considered 100% and was determined as described in Materials and methods.

izes the enzyme completely. Addition of detergent (1% w/v polidocanol) to the resealed ghost cell–alkaline phosphatase system does not alter its PNPPase activity either before or after the phospholipase C treatment. Furthermore, no significant changes in the enzyme PNPPase activity on release by phospholipase C were observed (not shown). This observation strengthens the hypothesis that the interaction of the alkaline phosphatase with the ghost cells membrane occurs through GPI anchor.

The results on Table 1 show that the enzyme does not lose the ability to hydrolyze any of the studied substrates, when associated to resealed ghost cells. So it can be observed that for PNPPase, the activity remained the same as the initial (considering 50% of incorporation), but for the ATPase and PPiase activities, reductions of about 37% and 60%, respectively, were observed.

In Fig. 5, the effect of the concentration of phosphate in the PNPPase activity of the enzyme associated to the ghost cells is shown. As it can be observed, about 50% of the enzyme is inhibited in the presence of 2 mM of sodium phosphate in the reaction mean, presenting a $K_d = 3.8$ mM.

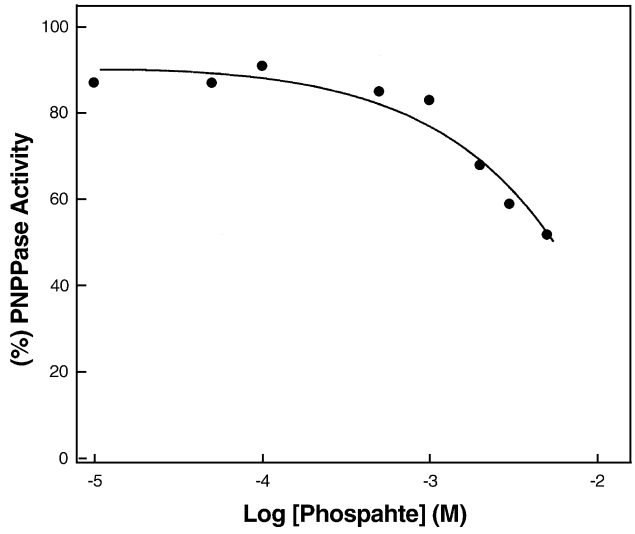

Fig. 5. Effect of sodium phosphate concentration on PNPPase activity of the ghost incorporated alkaline phosphatase at pH 9.4. PNPPase activity was estimated in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$, as described in Materials and methods. The reaction was initiated by adding 100 μl of alkaline phosphatase-resealed ghost cell system. Values are the mean of triplicate determinations which differed by less than 5%.

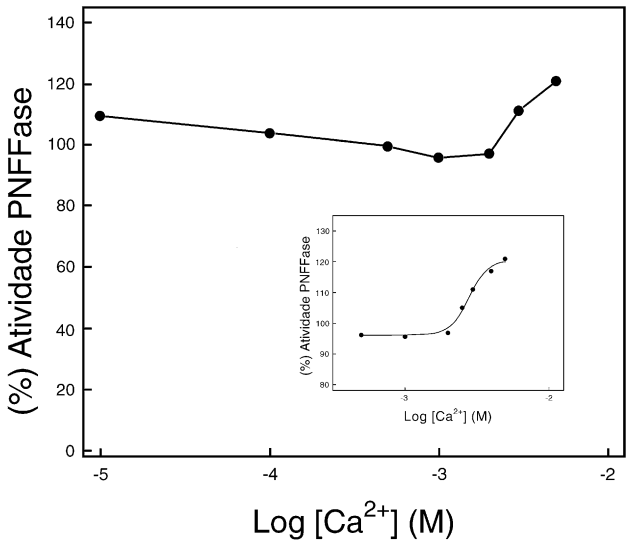

Fig. 6. Effect of calcium ion concentration on the PNPPase activity of ghost incorporated alkaline phosphatase at pH 9.4. PNPPase activity was estimated in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$, as described in Materials and methods. The reaction was initiated by adding 100 μl of alkaline phosphatase-resealed ghost cell system. Values are the mean of triplicate determinations which differed by less than 5%. Insert: sigmoidal adjustment to calcium stimulation effect.

Surprisingly, it was verified that the increase in calcium ions concentration in the PNPPase activity associated to the ghost cells did not inhibit the enzyme (Fig. 6). In contrast, this ion in concentrations up to 5 mM stimulated about 20% the PNPPase activity of the resealed ghost cell–alkaline phosphatase system. The insert of Fig. 6 shows a sigmoidal analysis with a single saturation curve ($K_{0.5} = 2.8$ mM) and cooperative effects ($n = 3$).

4. Discussion

The erythrocyte is a convenient start material to prepare the resealed ghost cell for use in a membrane model because it has no membrane-bound organelles and the plasma membrane is the only membrane present in the blood cell. Moreover, using a Steck et al. [31] method to prepare a resealed ghost cell could result to about 80% of inside-out vesicles, when prepared in the presence of magnesium ions. Sizes of resealed ghost cells of about 7–10 μm or 500 nm can be found and this size depends on the procedure used to obtain the vesicle and the technique in developing the micrograph image [31,33]. Although the average size of resealed vesicles can vary a lot, the modification introduced here allows the obtainment of regular vesicles with average diameters of about 300 nm.

The same proteins are present in the plasma membrane of nearly all vertebrate erythrocytes. The most important proteins present in the membrane are: glycophorin, band 3 protein, band 4.1 protein, spectrin, ankyrin and actin, corresponding to 49% of weight membrane. Lipids are a

second principal component with 44% (25% cholesterol, 60% glycerophospholipids and 5–10% glycolipid) [34].

These erythrocytes vesicular systems are constituted by different membrane proteins from matrix vesicles which are abundant in proteolipids, calcium phospholipids phosphate complexes and anexin. Matrix vesicles also contain enzymes, other than alkaline phosphatase (which it is now recognized as a marker for this organelle), that play a role, such as nucleotide triphosphatase, pyrophosphohydrolase, pyrophosphatase, various phospholipases and matrix-processing enzyme [1–4,7,12–15,35–37].

These differences in the biochemical composition and the single similarity as a vesicular lipid–protein compartment (membrane model free of others matrix vesicles proteins) can aid in understanding the role of the alkaline phosphatase anchored during the biomineralization process.

The alkaline phosphatase incorporation into resealed ghost cells was maximum with 48 h of incubation at 4 °C (Fig. 1) and it only occurred when a polidocanol-solubilized enzyme, free of detergent, was used, since only in this case the enzyme presents the phosphatidylinositol anchor.

Similar results were observed in the enzyme incorporation to liposomes, where 60–80% incorporation was obtained with 4-h incubation [28,29]. This fact probably occurs due to the presence of proteins in the erythrocyte membrane, which may cause some difficulty in the approximation of phosphatidinositol alkaline phosphatase anchor, responsible for the incorporation [38]. Another factor that can justify longer incorporation time is that proteins can contribute for a higher "packing" of the phospholipids present in the erythrocyte membrane, thus making the interaction between GPI anchor and the membrane difficult [38,39].

The size exclusion chromatography, done with solubilized alkaline phosphatase, resealed ghost cells and alkaline phosphatase associated to ghost cells samples (Fig. 2), has led us to conclude that the enzyme was incorporated to the membrane, since enzyme activity was detected together with resealed ghost cells. Besides, in Fig. 3, the histochemical study strengthens even more this hypothesis and clearly shows that the enzyme is located on the ghost cell membrane surface. Another factor that corroborates this hypothesis is that once the incorporated system was treated with phosphatidylinositol-specific phospholipase C, all the enzyme was released into the soluble environment. Similar results were obtained by Camolezi et al. [28,29] using liposomes in the place of resealed ghost cells. Furthermore, these authors show that enzyme released from liposomes is dependent on both time and phospholipase C concentration used.

When one considers the physical size of natural matrix vesicles (from 20 to 300 nm in diameter), it is suggested that more than one type of matrix vesicle could exist or the size might be a function of the extraction technique used. Moreover, it is still not known whether multiple sub-classes of matrix-vesicles are produced by a single cell at a specific stage of differentiation or if each cell produces only one class of matrix vesicles [35,40].

Nevertheless, the resulting size of the resealed ghost cell–alkaline phosphatase being in the same natural matrix vesicle's range could act as a vesicular mimetic system.

As for the kinetic characteristics of alkaline phosphatase–ghost cell system, Table 1 shows that the enzyme has not lost its ability to hydrolyze any of the substrates, and surprisingly, while there was not a significant variation of the PNPPase activity, inhibition of ATPase and PPiase activity was observed. This result supports the hypothesis that lipidic membrane, mimicked by ghost cells, could affect the protein structure and/or the substrate approximation to the active site of the protein [18,41]. Changes in the hydrolysis velocity of the substrates were also obtained using an alkaline phosphatase–liposome system [29]. These authors showed that the presence of the membrane phospholipids, as a support for the enzyme, affects its activity in different ways, depending on the substrate used.

Alkaline phosphatase obtained from osseous tissues is a multifunctional enzyme, capable of hydrolyzing in alkaline pH: phosphate monoesters, pyrophosphate, phosphodiesters, and also catalyzing transphosphorylation reactions [27,42–45]. The physiological role of alkaline phosphatase in the calcification process is difficult to understand inasmuch as the enzyme extraction from osseous and/or cartilaginous tissue as well as its solubilization can alter its structure, catalytic activity and properties related to its function.

Sodium phosphate was an inhibitor of enzyme associated to ghost cells (Fig. 5). This behavior was expected since other authors have already described this inhibitory effect (K_i about 1.2 mM) for alkaline phosphatase obtained for different tissues associated to membrane and detergent-solubilized as well [8,46–48].

On the other hand, calcium ions have stimulated PNPPase activity of the enzyme associated to ghost cells (Fig. 6). The presence of specific binding calcium ion sites in alkaline phosphatase is in agreement with the studies by Mornet et al. [49]. Although conflicting results have been described for osseous plate alkaline phosphatase [8,27,44,50], it should also be emphasized that calcium binding sites described herein for osseous alkaline phosphatase are different from those reported for zinc and magnesium ions [27] and are absent in *E. coli* protein structure [49].

Since calcium ion levels in the extracellular fluid [49] vary significantly during the calcification process, we suggested that calcium ions could have a physiological role in the enzyme regulation process.

Another possible explanation for the calcium stimulation effects observed in Fig. 6 can be attributed to: (i) calcium ions captured by phospholipids and/or some proteins present in the membrane; (ii) electrostatic attraction of the substrate in the membrane neighborhood, since calcium ions interact

with phospholipids; and (iii) conformational changes of the enzyme molecule in these experimental conditions [50].

This membrane model constituted by resealed ghost cell–alkaline phosphatase have a compatible size, hydrolyzes different substrates and could also be used for the study of the ions' influence associated with calcium deposition, similar to matrix vesicles. This mimetic systems will be able to elucidate some very important points, inasmuch for the mineralization understanding as for the alkaline phosphatase role during this process.

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