# Phosphodiesterase activity is a novel property of alkaline phosphatase from osseous plate

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Phosphodiesterase activity is a novel property of the still-enigmatic alkaline phosphatase from osseous plate. Bis-(*p*-nitrophenyl) phosphate was hydrolysed at both pH 7.5 and 9.4 with an apparent dissociation constant ($K_{0.5}$) of 1.9 mM and 3.9 mM respectively. The hydrolysis of *p*-nitrophenyl-5′-thymidine phosphate followed hyberbolic kinetics with a $K_{0.5}$ of 500 $\mu$M. For *p*-nitrophenyl phenylphosphonate, site–site interactions [Hill coefficient ($h$) = 1.3] were observed in the range between 0.2 and 100 $\mu$M, and $K_{0.5}$ was 32.8 mM. The hydrolysis of cyclic AMP by the enzyme followed more complex kinetics, showing site–site interactions ($h$ = 1.7) and $K_{0.5}$ = 300 $\mu$M for high-affinity sites. The low-affinity sites, representing 85% of total activity, also showed site–site interactions ($h$ = 3.8) and a $K_{0.5}$ of about 22 mM. ATP and cyclic AMP were competitive inhibitors of bis-(*p*-nitrophenyl) phosphatase activity of the enzyme and $K_i$ values (25 mM and 0.6 mM for cyclic AMP and ATP respectively) very close to those of the $K_{0.5}$ (22 mM and 0.7 mM for cyclic AMP and ATP respectively), determined by direct assay, indicated that a single catalytic site was responsible for the hydrolysis of both substrates. Non-denaturing PAGE of detergent-solubilized enzyme showed coincident bands on the gel for phosphomonohydrolase and phosphodiesterase activities. Additional evidence for a single catalytic site was the similar p$K_a$ values (8.5 and 9.7) found for the two ionizing groups participating in the hydrolysis of bis-(*p*-nitrophenyl) phosphate and *p*-nitrophenyl phosphate. The alkaline apparent pH optima, the requirement for bivalent metal ions and the inhibition by methylxanthines, amrinone and amiloride demonstrated that rat osseous-plate alkaline phosphatase was a type I phosphodiesterase. Considering that there is still confusion as to which is the physiological substrate for the enzyme, the present results describing a novel property for this enzyme could be of relevance in understanding the mineralization process.

## INTRODUCTION

Although several lines of evidence suggest diverse substrates for alkaline phosphatase during biological calcification of tissues, the physiological substrate of this ubiquitous enzyme still remains elusive [1–4]. Alkaline phosphatase from cartilage and bone have been considered a non-specific phosphomonohydrolase which hydrolyses a variety of substrates including ATP and pyrophosphate [5–12]. Although phosphodiesters have not been considered to be substrates for alkaline phosphatases [13], it has been reported that phosphodiesterases of different origins have properties similar to those of alkaline phosphatases [14,15]. These include an alkaline apparent pH optimum, requirement for bivalent metal ions and inhibition by phosphate ions, methylxanthines and structural analogues. $Mg^{2+}$ and $Mn^{2+}$ are effective activators of phosphodiesterases. Furthermore, as reported for alkaline phosphatases, $Zn^{2+}$ exerts a strong inhibitory effect on the phosphodiesterases [14,16–18]. This led to the suggestion that a single molecule might be responsible for these two activities [15–17].

Although it is claimed that a possible function as a phosphodiesterase for cartilage and bone alkaline phosphatases is unlikely *in vivo* [19], bone-metabolism studies show that increasing levels of cyclic AMP stimulate not only collagen synthesis but also alkaline phosphatase activity [20–24]. Furthermore, calmodulin, a known activator of cyclic nucleotide phosphodiesterases [25], was recently shown to activate chick alkaline phosphatase [26]. Although physiologically relevant, there is no evidence as yet that this result is related to biomineralization.

Rat osseous-plate alkaline phosphatase is a membrane-bound metalloenzyme made up of two apparently identical subunits of $M_r$ 65000 which require $Zn^{2+}$ and $Mg^{2+}$ for maximal activity [10,27]. This enzyme is multifunctional, showing non-specific phosphomonohydrolase, pyrophosphatase, ATPase and phosphotransferase activities stemming from a single protein molecule [10–12,27].

Here we characterize the phosphodiesterase activity of rat osseous-plate alkaline phosphatase as well as the effects of some inhibitors on its catalytic activity. Kinetic evidence is presented showing that both mono- and di-esters of phosphate are hydrolysed at the same site on the enzyme molecule. Additional kinetic evidence also showed that the enzyme is a type I phosphosphodiesterase.

## MATERIALS AND METHODS

### Materials

All solutions were made up with glass-distilled deionized water. *Naja Naja* (Indian cobra) venom, Bis-(*p*-nitrophenyl) phosphate (BIS-PNPP), *p*-nitrophenyl phenylphosphonate (PNPP-PP), 2-amino-2-methylpropan-1-ol (AMPOL), theophylline, Hepes, β-naphthyl phenylphosphonate, cyclic AMP, levamisole and *p*-nitrophenyl phosphate (PNPP) were from Sigma. *p*-Nitrophenylthymidine 5′-phosphate (PNP-T-5′-P) was from Merck.

Abbreviations used: BIS-PNPP, Bis-(*p*-nitrophenyl) phosphate; PNPP, *p*-nitrophenyl phosphate; PNPP-PP, *p*-nitrophenyl phenylphosphonate; PNP-T-5′-P, *p*-nitrophenyl thymidine-5′-phosphate; AMPOL, 2-amino-2-methylpropan-1-ol; $K_{0.5}$, apparent dissociation constant; $h$ (site–site interaction), Hill coefficient; LBK, liver/bone/kidney.

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Amiloride and amrinone were a gift from Merck Sharp and Dohme and The Sidney Ross Co. respectively.

### Preparation of purified rat osseous-plate alkaline phosphatase

Purified rat osseous-plate alkaline phosphatase was prepared as described elsewhere by Curti et al. [28]. Briefly, 10–20 mg of demineralized matrix were introduced through a small incision on to the dorsal subcutaneous tissue of ether-anaesthetized young male Wistar rats (50–60 g body wt.). At 14 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 Superohm homogenizer for 5 min. The homogenate was centrifuged at 15000 *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 cm × 83 cm) equilibrated and eluted in the same buffer. The purified enzyme was layered into a continuous sucrose density gradient (10–50%, w/v, in Tris/HCl buffer, pH 7.5, containing 2 mM $MgCl_2$) and the centrifugation was carried out for 5 h at 38000 *g* using a Sorvall SV-288 vertical rotor at 4 °C. Active fractions were pooled and dialysed overnight at 4 °C against 5 mM Tris/HCl, containing 2 mM $MgCl_2$, with several changes of the buffer. Finally, 1.0 ml aliquots were frozen in liquid $N_2$ and stored at −20 °C for 1 month without appreciable loss of activity.

### Enzymic-activity measurements

Phosphodiesterase activity was assayed continuously at 37 °C by monitoring the liberation of *p*-nitrophenolate ion ($E_{410}^{1cm,pH9.4}$ 17600 $M^{-1} \cdot cm^{-1}$) in a Varian DMS-80 spectrophotometer equipped with thermostated cell holders, using BIS-PNPP as substrate. Standard assay conditions were 50 mM Hepes, pH 7.5 (or 50 mM AMPOL, pH 9.4), containing 2 mM $MgCl_2$ in a final volume of 1.0 ml. The hydrolysis of PNP-PP and PNP-T-5′-P were assayed as described above. For PNPP, the activity was measured as described by Ciancaglini et al. [27].

For cyclic AMP the activity was assayed discontinuously at 37 °C by measuring the amount of $P_i$ liberated as described by Heinonen and Lahti [29]. The reaction was initiated by the addition of alkaline phosphatase and, at appropriate time intervals, the reaction was stopped by placing the stoppered tubes on a boiling-water bath for 15 min. Aliquots of 5′-nucleotidase (50 μg of *Naja Naja* venom in 100 μl of 50 mM Hepes buffer, pH 7.5) were added to each tube. After 15 min incubation at 37 °C, the reaction was stopped by the addition of 0.5 ml of 30% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation at 4000 *g* just before phosphate determination. Standard assay conditions were the same as described above for BIS-PNPP.

The initial velocities were constant for at least 90 min, provided that less than 5% of substrate was hydrolysed. Controls without added enzyme were included in each experiment to allow for the non-enzymic hydrolysis of substrate. One enzyme unit was defined as the amount of enzyme hydrolysing 1.0 nmol of substrate/minute at 37 °C.

### pH-sensitivity of catalysis

Attempts to identify catalytic groups were made by using the method of Tipton and Dixon [30] to analyse data obtained for the effect of pH on enzyme activity. BIS-PNPP and PNPP were used as substrates, and the activities were assayed over the pH range 7.0–10.5. Hepes was used as a buffer over the range pH 7.0–9.0 and AMPOL from pH 9.0 to 10.5. The assays were carried out in 20 mM buffer containing 2 mM $MgCl_2$. The pH of the reaction mixture was measured both before and after the assay, and it did not vary more than 0.05 unit.

### Determination of protein concentration

Protein concentrations were determined as described by Hartree [31], using BSA as standard.

### PAGE

Gel electrophoresis of detergent-solubilized enzyme was carried out in a 7% gel as described by Davis [32]. Phosphomonohydrolase activity on the gel was detected in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$, 0.12% α-naphthyl phosphate and 0.12% Fast Blue RR at 37 °C. Phosphodiesterase activity on the gel was assayed as above by using 0.12% β-naphthyl phenylphosphonate as substrate. The solubilization of the membrane enzyme was carried out as described by Ciancaglini et al. [11]. A sample containing 0.2 mg of the membrane enzyme (final concn)/ml was solubilized with 1% polidocanol (final concn.) for 2 h at room temperature. After centrifugation at 40000 *g* for 2 h, the solubilized enzyme was concentrated by using CF25 Centriflo-Amicon cones and used without further purification.

### Estimation of kinetic parameters

Kinetic parameters $K_m$, $V_{max.}$, $K_{0.5}$, $K_i$ and $n$ obtained from substrate hydrolysis were fitted on an IBM/PC microcomputer by using SIGRAF [33]. Data are reported as the mean of duplicate determinations which differed by less than 5%.

## RESULTS

Figure 1 shows the effect of increasing concentrations of BIS-PNPP on phosphodiesterase activity of membrane-bound rat osseous-plate enzyme at pH 7.5 and 9.4. Despite the simple curve

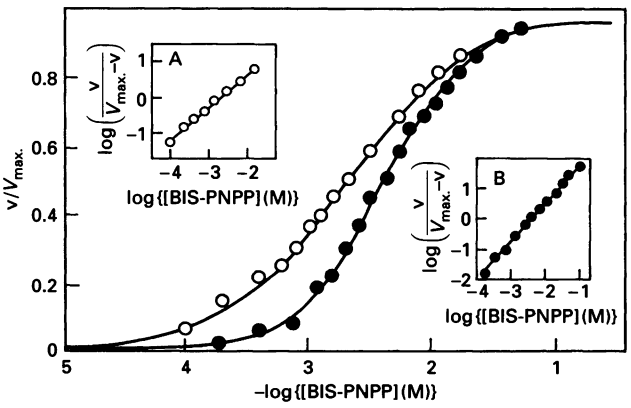


**Figure 1 Effect of BIS-PNPP concentration on phosphodiesterase activity of rat osseous-plate alkaline phosphatase at pH 7.5 (○) or 9.4 (●)**

The protein concentration used was 16 μg. Enzymic activity was assayed in 50 mM Hepes buffer pH 7.5 (or 50 mM AMPOL buffer, pH 9.4) containing 2 mM $MgCl_2$. Inset A, Hill plot for the data obtained at pH 7.5. Inset B, Hill plot for the data obtained at pH 9.4.

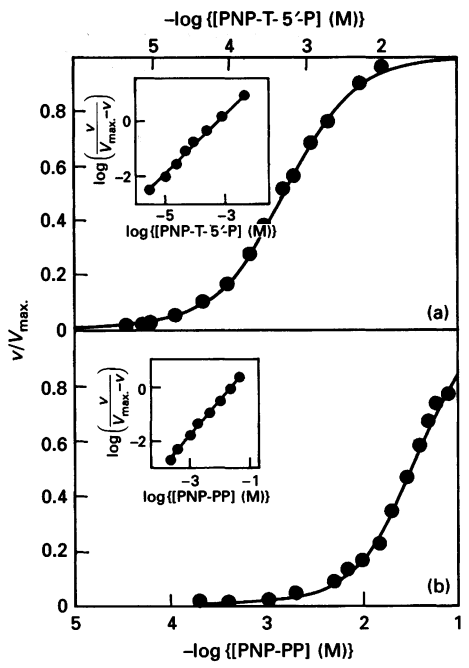


**Figure 2 Effect of chromogenic phosphodiesters concentration on phosphodiesterase activity of rat osseous-plate alkaline phosphatase**

The amount of protein used was 17 $\mu$g and 31 $\mu$g for (**a**) PNP-T-5′-P and (**b**) PNP-PP respectively. Enzymic activity was assayed in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$. Insets: Hill plots for the above results: (**a**) PNP-T-5′-P; (**b**) PNP-PP.

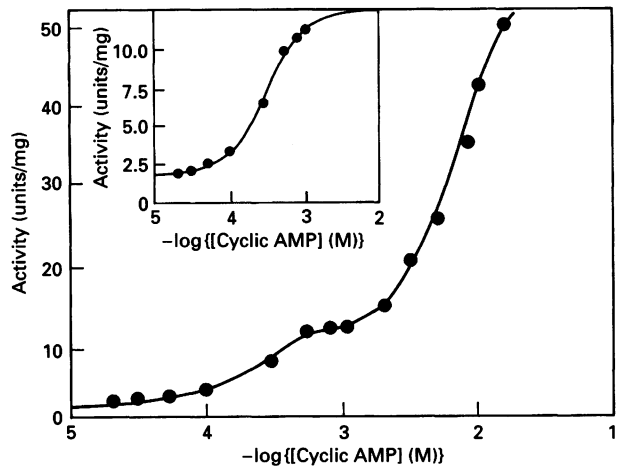


**Figure 3 Effect of cyclic AMP concentration on phosphodiesterase activity of rat osseous-plate alkaline phosphatase**

The amount of protein used was 24 $\mu$g, and the enzymic activity was assayed in 50 mM Hepes buffer, pH 7.5, containing 2 mM $MgCl_2$. The inset shows a high-affinity-hydrolysing-sites curve for cyclic AMP.

**Table 1 Kinetic parameters for the hydrolysis of several phosphodiesters by rat osseous-plate alkaline phosphatase**

Standard assay conditions for the hydrolysis of substrates were 50 mM Hepes buffer, pH 7.5 (or 50 mM AMPOL buffer, pH 9.4), containing 2 mM $MgCl_2$, as described in the Materials and methods section. Results are means of duplicate determinations and differed by less than 5%.

| Substrate | pH | $K_{0.5}$ or $K_m$ (mM) | $V_{max.}$ (units/mg) | $n$ |
|---|---|---|---|---|
| BIS-PNPP | 7.5 | 1.9 | 48 | 1.0 |
| BIS-PNPP | 9.4 | 3.9 | 170 | 1.3 |
| PNP-T-5′-P | 9.4 | 0.5 | 7110 | 1.0 |
| PNP-PP | 9.4 | 32 | 1616 | 1.3 |
| Cyclic AMP | 7.5 | 0.3* | 13 | 1.7 |
| | | 22† | 84 | 3.8 |

* High-affinity sites.
† Low-affinity sites.

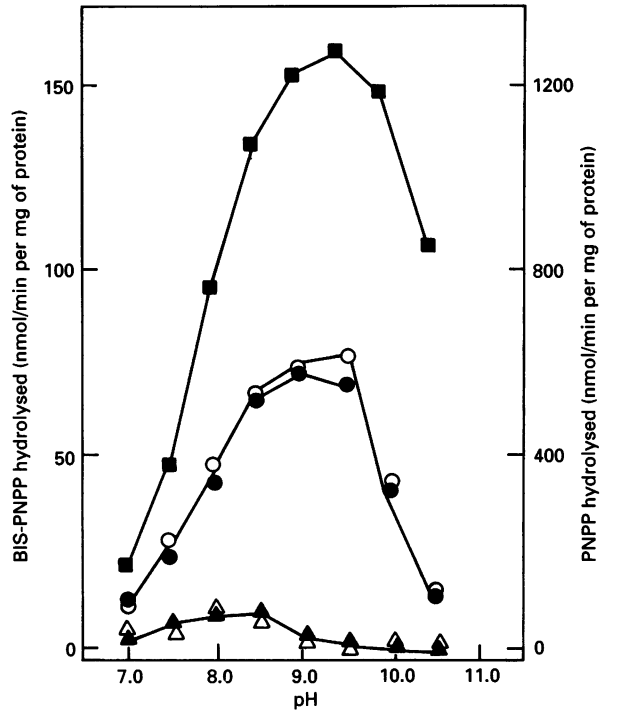


**Figure 4 Effect of substrate concentration on the apparent pH optima of PNPP and BIS-PNPP hydrolysis by rat osseous-plate alkaline phosphatase**

The concentrations of substrate used were 0.2 mM (▲, △), 2 mM (●, ○) and 20 mM (■). Hepes was used as buffer over the pH range of 7–9 and AMPOL from 9 to 10.5. The assays were carried out in 50 mM buffer, containing 2 mM $MgCl_2$, and using 16 $\mu$g of protein. Closed and open symbols represent values obtained for BIS-PNPPase and PNPPase activities respectively.

obtained, the kinetics of hydrolysis of this substrate at each pH were quite different. While at pH 7.5 the substrate was hydrolysed with hyperbolic kinetics, site–site interactions ($n = 1.3$) were observed at pH 9.4. The $K_{0.5}$ value for the hydrolysis of BIS-PNPP was 2-fold greater at pH 9.4 ($K_{0.5} = 3.9$ mM) when compared with that obtained at pH 7.5 ($K_{0.5} = 1.9$ mM).

Chromogenic substrates other than BIS-PNPP were also hydrolysed by the enzyme at pH 9.4 (Figure 2). The hydrolysis of PNP-T-5′-P proceeded through a single class of substrate-hydrolysing sites following hyperbolic kinetics with a $K_m$ of 0.5 mM (Figure 2a). For PNPP-PP, site–site interactions ($n = 1.3$) were observed in the range of concentrations between 0.2 and 100 mM and $K_{0.5}$ was 32.8 mM (Figure 2b). Interestingly,

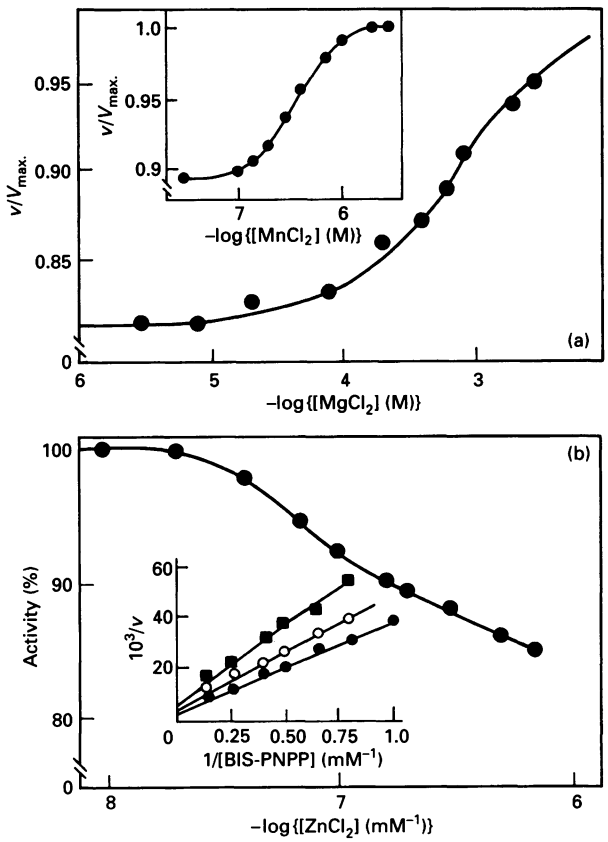


**Figure 5 Effect of bivalent metal ions on bis-(*p*-nitrophenyl) phosphatase activity of rat osseous-plate alkaline phosphatase**

The assays were carried out in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$, and using 16 $\mu$g of protein. (**a**) Stimulatory effect of $Mg^{2+}$. Inset: stimulatory effect of $Mn^{2+}$. (**b**) Inhibitory effect of $Zn^{2+}$. Inset: reciprocal plot of $1/v$ versus BIS-PNPP concentration using 0.2 mM $ZnCl_2$ (■), 0.1 mM $ZnCl_2$ (○) or no $ZnCl_2$ added (●).

**Table 2 Kinetic characteristics of several reversible inhibitors of bis-(*p*-nitrophenyl) phosphatase activity of rat osseous-plate alkaline phosphatase at pH 9.4**

The activity was assayed in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$, 20 mM BIS-PNPP and the indicated concentration of inhibitor in a final volume of 1 ml, as described in the Materials and methods section. Results are means of duplicate determinations and differed by less than 5%.

| Inhibitor | Type | $K_i$ (mM) |
|---|---|---|
| Sodium metavanadate | Competitive | 0.02 |
| Sodium arsenate | Competitive | 0.06 |
| $ZnCl_2$ | Non-competitive | 0.21 |
| Theophylline | Uncompetitive | 0.37 |
| Levamisole | Uncompetitive | 0.50 |
| ATP | Competitive | 0.60 |
| Amrinone | Competitive | 0.90 |
| Sodium phosphate | Competitive | 2.25 |
| Amiloride | Non-competitive | 4.55 |
| Cyclic AMP | Competitive | 25.0 |

**Table 3 Hydrolysis of BIS-PNPP in the presence of several phosphate monoesters by purified alkaline phosphatase from osseous plate at pH 9.4**

The activity was assayed in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$, 20 mM BIS-PNPP and the indicated concentration of phosphate monoester in a final volume of 1 ml, as described in the Materials and methods section. Data are reported as the mean of duplicate determinations which differed by less than 5%.

| Substrate | Concn. (mM) | Activity (%) |
|---|---|---|
| BIS-PNPP | 20.0 | 100 |
| Glucose 1-phosphate | 10.0 | 59 |
| Glucose 6-phosphate | 40.0 | 62 |
| ATP | 2.0 | 36 |
| ADP | 2.0 | 36 |
| AMP | 0.5 | 31 |
| $\beta$-Glycerophosphate | 2.0 | 66 |

only 80% of maximal activity of the enzyme was achieved for concentrations of PNP-PP up to 100 mM.

The hydrolysis of cyclic AMP by the enzyme at pH 7.5 is shown in Figure 3. Two species of substrate-hydrolysing sites were observed for cyclic AMP concentrations varying from 10 $\mu$M to 100 mM. The high-affinity sites (inset to Figure 3) showed site–site interactions ($n = 1.7$), and $K_{0.5}$ of about 300 $\mu$M. The low-affinity sites, which represented 85% of total activity, showed site–site interactions ($n = 3.8$) with a $K_{0.5}$ of 22 mM. Table 1 summarizes the kinetic parameters obtained for the hydrolysis of several phosphodiesters at pH 7.5 and 9.4.

The apparent optimum pH for the hydrolysis of PNPP and BIS-PNPP by the purified enzyme was affected in a similar way by the concentration of substrate (Figure 4). As substrate concentration decreased, the apparent optimum pH became more acidic. Furthermore, the analysis of $\log V_{max.}$ as a function of pH revealed that two ionizing groups with $pK_a$ of 8.5 and 9.7 were involved in the hydrolysis of both PNPP and BIS-PNPP.

Site–site interactions ($n = 0.8$) were observed for the 20% stimulation by $Mg^{2+}$ with a $K_{0.5} = 870$ $\mu$M (Figure 5a). For $Mn^{2+}$, site–site interactions ($n = 2.4$) were also observed (inset to Figure 5a) and $K_{0.5}$ of about 0.35 mM was similar to that obtained for $Mg^{2+}$. On the other hand, concentrations of $Zn^{2+}$ higher than 50 nM were inhibitory (Fig. 5b). This inhibition was non-competitive and plots of $1/V_{max.}$ versus $[Zn^{2+}]$ were linear, yielding a $K_i$ value of 0.21 mM.

The kinetic effects of several inhibitors on the hydrolysis of BIS-PNPP by the enzyme are given in Table 2. Sodium phosphate was a competitive inhibitor of the enzyme ($K_i$ 2.25 mM), as were sodium arsenate ($K_i$ 0.06 mM) and sodium metavanadate ($K_i$ 0.02 mM). Cyclic AMP was a competitive inhibitor of the hydrolysis of BIS-PNPP, and the $K_i$ value of about 25 mM is in excellent agreement with the $K_{0.5}$ value of about 22 mM determined by direct assay (Table 1 and Figure 3). ATP was also a true competitor of BIS-PNPP for the active site of the enzyme, and the $K_i$ value, 0.6 mM, was in good agreement with $K_{0.5}$ value of about 0.7 mM, determined by direct assay. Amiloride was a non-competitive inhibitor, yielding a $K_i$ of 4.55 mM. Theophylline was an uncompetitive inhibitor ($K_i$ 0.37 mM), and amrinone was a competitive inhibitor ($K_i$ 0.9 mM). All secondary plots of $1/V_{max.}$ (or $1/K_m$) versus inhibitor concentration were linear.

Interestingly, besides cyclic AMP and ATP, several other physiological phosphate monoesters were also inhibitors of phosphodiesterase activity of the enzyme (Table 3) indicating that mono- and di-esters of phosphate were hydrolysed at the same site.

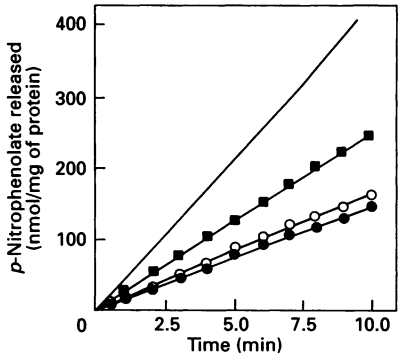


**Figure 6 Time course of BIS-PNPP and PNPP hydrolysis by alkaline phosphatase from osseous plate**

The assays were carried out in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$, using 30 $\mu$g of protein. ●, 20 mM BIS-PNPP; ■, 1 mM PNPP; ○, 20 mM BIS-PNPP plus 1.0 mM PNPP; ——, theoretical value for the hydrolysis of a mixture of 20 mM BIS-PNPP plus 1 mM PNPP, assuming two independent catalytic sites.

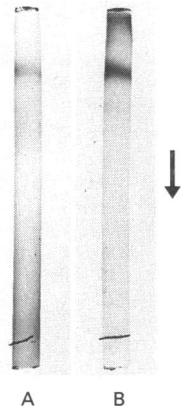


**Figure 7 PAGE of polidocanol-solubilized alkaline phosphatase**

The direction of migration was from cathode to anode and 200 $\mu$g of polidocanol-solubilized enzyme were used without further purification. Lane A, phosphodiesterase activity; lane B, phosphomonohydrolase activity.

Data from Figure 6 show unequivocally that PNPP and BIS-PNPP were hydrolysed by a single common site on the enzyme molecule. This interpretation came from the fact that, in any mixture containing given concentrations of PNPP and BIS-PNPP, total velocity fell between the velocities obtained for each substrate assayed separately at the same concentration used in the mixture.

Finally, as expected from the kinetic data shown, non-denaturating PAGE (Figure 7) of detergent-solubilized enzyme revealed that phosphodiesterase activity of the enzyme (Figure 7a) was coincident with phosphomonohydrolase activity (Figure 7b) on the gel. This result provides additional direct evidence that both activities stem from the same protein molecule.

## DISCUSSION

It has been reported that alkaline phosphatases do not hydrolyse phosphate diesters [13,34,35]. However, in the present study we demonstrate that rat osseous-plate alkaline phosphatase could hydrolyse BIS-PNPP at both pH 9.4 (170 units/mg) and pH 7.5 (48 units/mg). At pH 9.4, phosphodiesterase activity represented one-third of that obtained for *p*-nitrophenyl phosphatase activity [27,28].

Interestingly, this enzyme also hydrolysed cAMP at pH 7.5 (Table 1 and Figure 3). This evidence and the fact that the enzyme also hydrolysed PNP-T-5′-P and PNP-PP (Figure 2), two chromogenic substrates widely used for the characterization of several phosphodiesterases [15,17,36–38], demonstrate that this enzyme can function as a phosphodiesterase.

The effects of $Mg^{2+}$ and $Mn^{2+}$ on bis-(*p*-nitrophenyl) phosphatase activity (Figure 5) were similar to those reported for *p*-nitrophenyl phosphatase activity [39], suggesting, again, a possible role as a phosphodiesterase for osseous-plate enzyme. Although some controversy has been reported for the role of bivalent ions on phosphodiesterase activity [16,17], the inhibition of phosphodiesterase activity by $Zn^{2+}$ was apparently due to the displacement of $Mg^{2+}$ by $Zn^{2+}$, as we reported elsewhere for non-specific phosphohydrolase activity of the enzyme [27,39].

The fact that *p*-nitrophenyl phosphatase [10] and phosphodiesterase activities (Table 2) were competitively inhibited by phosphate ions and analogues, suggests a similar mechanism for the hydrolysis of PNPP, BIS-PNPP and cyclic AMP. It has been suggested that vanadate ions form a trigonal bipyramidal transition state at the active site of alkaline phosphatase, which bears some resemblance to the metastable intermediate occurring during the hydrolysis of phosphate esters [40]. A similar suggestion has been used to explain the hydrolysis of cyclic AMP by phosphodiesterases [41]. The uncompetitive inhibition of phosphodiesterase activity of osseous-plate enzyme by levamisole suggests the formation of a ternary complex during the catalytic cycle of the enzyme. Similar results were reported for *p*-nitrophenyl phosphate activity of cartilage and bone alkaline phosphatases [7,42,43].

Theophylline, a non-specific competitive inhibitor of cyclic nucleotide phosphodiesterases [14], has been often used for kinetic characterization of alkaline phosphatases [13]. However, for cartilage and bone enzymes, *p*-nitrophenyl phosphatase activity has been reported to be uncompetitively inhibited by this drug [19,42,43]. In addition to the fact that the inhibition of osseous-plate enzyme was independent of the nature of the substrate used, the uncompetitive inhibition of phosphodiesterase activity by either theophylline ($K_i$ 0.37 mM) or levamisole ($K_i$ 0.50 mM) reinforces the view that osseous-plate enzyme is also a phosphodiesterase. Moreover, the inhibitory effects observed for amiloride and amrinone were similar to those reported for several phosphodiesterases [44,45].

Owing to the lability of the membrane-bound enzyme to SDS, the membrane-bound enzyme was solubilized with 1% polidocanol. The electrophoretic results (see Figure 7) are direct evidence that both activities stem from the same protein molecule. It must be recalled that 200 $\mu$g of solubilized enzyme were used without further purification.

In addition to this direct result, the question of whether the activity of osseous-plate enzyme towards both phosphate mono- and di-esters was an intrinsic property of this enzyme, and not due to a contaminant membrane-bound phosphodiesterase that co-purifies, has been answered by kinetic evidence. First, phosphomonohydrolase and phosphodiesterase activities were competitively inhibited by phosphate ions and analogues (Table 2), suggesting a similar mechanism for the hydrolysis of both substrates.

The loss of phosphodiesterase activity towards several phosphate monoesters (Table 3) also reinforces the interpretation that phosphomonohydrolase and phosphodiesterase activities are related to a unique protein molecule. Furthermore, the

ionizing groups showing apparent $pK_a$ values of 8.5 and 9.7, which are involved in the catalytic cycle of the enzyme independent of a mono- or a phosphate di-ester being used, were additional evidence that both activities stem from the same protein molecule.

ATP and cyclic AMP, two naturally occurring substrates, were competitive inhibitors of phosphodiesterase activity, showing $K_i$ values (25 mM and 0.6 mM for cyclic AMP and ATP respectively) very close to the $K_{0.5}$ values (23 mM and 0.7 mM for cyclic AMP and ATP respectively) determined by direct assay. These results suggest that both substrates were hydrolysed by the same molecule. Skeletal alkaline phosphatase has been also reported to be competitively inhibited by cyclic AMP with $K_i$ 23 mM [19].

Data from Figure 6 provided more evidence that PNPP and BIS-PNPP were unequivocally hydrolysed by a common catalytic site on the enzyme molecule. According to Dixon and Webb [46], Cornish-Bowden [47], Segel [48] and Keleti et al. [49], these results are a consequence of the action of a unique enzyme on two substrates simultaneously. If we consider, for instance, that the enzyme had two catalytic sites (one for each substrate) or, if the enzyme was contaminated with phosphodiesterase that co-purified, then the total velocity would be necessarily the sum of *p*-nitrophenyl phosphatase and phosphodiesterase activities (theoretical value given in Figure 6), which was not the case.

Finally, it must be emphasized that phosphodiesterases are not present in the membranes of matrix vesicles among other phosphohydrolases which hydrolyse naturally occurring substrates [2].

Taken together, the similar electrophoretic pattern for phosphohydrolytic and phosphodiesterase activities and the kinetic results reported herein support the view that rat osseous-plate alkaline phosphatase has the kinetic properties of type I phosphodiesterases.

Alkaline phosphatase is a multigene-controlled enzyme [50]. Although three isoforms of this enzyme are widespread in humans and higher-primate tissues, the liver/bone/kidney (LBK) isoenzyme is found at a much higher level in bone-forming osteoblast cells [51]. The fact that osteoblasts and osteosarcomas express very high levels of LBK alkaline phosphatase [52,53] is consistent with the possibility that the LBK isoform could be involved in bone mineralization [1]. Considering this evidence and the structural and functional properties of osseous plate enzyme reported elsewhere [10–12,28,33,39,43], it seems likely that it is a product of the LBK gene. However, in spite of such circumstantial evidence, only after cloning and expressing the enzyme gene can we confirm this view.

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## REFERENCES

1 Wuthier, R. E. and Register, T. C. (1985) in The Chemistry and Biology of Mineralized Tissues (Butler, W. T., ed.), pp. 113–124, EBSCO Media Inc., Birmingham, AL
2 Anderson, H. C. (1989) Lab. Invest. **60**, 320–330
3 Blumenthal, N. C. (1989) Clin. Orthop. **247**, 279–289
4 Poole, A. R., Matsui, Y., Hinek, A. and Lee, E. R. (1989) Anat. Rec. **224**, 167–179
5 Majeska, R. J. and Wuthier, R. E. (1975) Biochim. Biophys. Acta **391**, 51–60
6 Felix, R. and Fleisch, H. (1976) Calcif. Tissue Res. **22**, 1–7
7 Fortuna, R., Anderson, H. C., Carty, R. and Sajdera, S. W. (1980) Calcif. Tissue Int. **30**, 217–225
8 Cyboron, G. W. and Wuthier, R. E. (1981) J. Biol. Chem. **256**, 7262–7268
9 Hsu, H. H. T., Munoz, P. A., Barr, J., Oppliger, I., Morris, D. C., Vaananen, H. K., Tarkenton, N. and Anderson, H. C. (1985) J. Biol. Chem. **260**, 1826–1831
10 Pizauro, J. M., Curti, C., Ciancaglini, P. and Leone, F. A. (1987) Comp. Biochem. Physiol. **87B**, 921–926
11 Ciancaglini, P. Pizauro, J. M., Rezende, A. A., Rezende, L. A. and Leone, F. A. (1990) Int. J. Biochem. **22**, 385–392
12 Pizauro, J. M., Curti, C., Ciancaglini, P. and Leone, F. A. (1993) Biochim. Biophys. Acta **1202**, 22–28
13 McComb, R. B., Bowers, G. N., Jr. and Posen, S. (1979) Alkaline Phosphatase, pp. 229–287, Plenum Press, New York
14 Drummond, G. I. and Yamamoto, M. (1971) Enzymes 3rd Ed. **4**, 355–371
15 Yamane, K. and Maruo, B. (1978) J. Bacteriol. **134**, 100–107
16 Londesborough, J. (1985) Biochem. J. **225**, 143–147
17 Ito, K., Yamamoto, T. and Minamiura, N. (1987) J. Biochem. (Tokyo) **102**, 359–367
18 Mori, N., Nikai, T. and Sugihara, H. (1987) Int. J. Biochem. **19**, 115–119
19 Farley, J. R., Ivey, J. L. and Baylink, D. J. (1980) J. Biol. Chem. **255**, 4680–4686
20 Hiramatsu, M., Ikeda, E., Kashimata, M., Minami, N., Kodama, H., Sudo, H. and Kumegawa, M. (1983) J. Biochem. (Tokyo) **94**, 1353–1358
21 Peck, W. A. and Klahr, S. (1979) Cyclic Nucleotide Res. **11**, 89–130
22 Nijweid, P. J., Burger, E. H. and Feyen, J. H. M. (1986) Am. J. Physiol. **66**, 855–886
23 Kato, Y., Shimazu, A., Nakashima, K., Suzuki, F., Jikko, A. and Iwamoto, M. (1990) Endocrinology (Baltimore) **127**, 114–118
24 Klem, K. H., Jablonski, G., Saether, O., Jarosz, G., Gautvik, K. M. and Gordeladze, J. O. (1990) Biochim. Biophys. Acta **1054**, 304–310
25 Cheung, W. Y. (1980) Science **207**, 19–27
26 Sivanaesan, L., Kwan, T. K. and Perumal, R. (1991) Biochem. Int. **25**, 561–570
27 Ciancaglini, P., Pizauro, J. M. and Leone, F. A. (1992) Int. J. Biochem. **24**, 611–615
28 Curti, C., Pizauro, J. M., Rossinholi, G., Vugman, I., Mello de Oliveira, J. A. and Leone, F. A. (1986) Cell. Mol. Biol. **32**, 55–62
29 Heinonen, S. K. and Lahti, R. J. (1981) Anal. Biochem. **113**, 313–317
30 Tipton, K. F. and Dixon, H. B. F. (1979) Methods Enzymol. **63**, 183–234
31 Hartree, E. F. (1972) Anal. Biochem. **48**, 422–427
32 Davis, B. J. (1964) Ann. N.Y. Acad. Sci. **121**, 404–427
33 Leone, F. A., Degreve, L. and Baranauskas, J. A. (1992) Biochem. Ed. **20**, 94–96
34 Reid, T. W. and Wilson, I. B. (1971) Enzymes 3rd Ed. **4**, 373–415
35 Arsenis, C., Rudolph, J. and Hackett, M. H. (1975) Biochem. Biophys. Acta **391**, 301–315
36 Hawley, D. M., Hodes, M. Z., Crisp, M., Ellis, G., Karn, R. C. and Hodes, M. E. (1985) Anal. Biochem. **151**, 375–380
37 Kelly, S. J. and Butler, L. G. (1977) Biochemistry **16**, 1102–1104
38 Landt, M., Everard, A. R. and Butler, L. G. (1980) Biochemistry **19**, 138–143
39 Leone, F. A., Pizauro, J. M. and Ciancaglini, P. (1992a) Int. J. Biochem. **24**, 923–928
40 Lopez, V., Steves, T. and Lindquist, R. N. (1976) Arch. Biochem. Biophys. **175**, 31–38
41 Erhardt, P. W. and Chou, Y. (1991) Life Sci. **49**, 553–568
42 Cyboron, G. W., Vegins, M. S. and Wuthier, R. E. (1982) J. Biol. Chem. **257**, 4141–4146
43 Curti, C., Pizauro, J. M., Ciancaglini, P. and Leone, F. A. (1987) Cell. Mol. Biol. **33**, 625–635
44 Carpenedo, F., Debetto, P., Floreani, M., Guarnieri, A. and Luciani, S. (1987) Biochem. Pharmacol. **36**, 778–780
45 Ito, M., Tanaka, T., Saitoh, M., Masuoka, T. N. and Hidaka, H. (1988) Biochem. Pharmacol. **37**, 2041–2044
46 Dixon, M. and Webb, E. C. (1979) in Enzymes, 3rd edn., pp. 72–75, Academic Press, New York
47 Cornish-Bowden, A. (1976) Principles of Enzyme Kinetics, pp. 43–44, Butterworth, London
48 Segel, I. H. (1976) Enzyme Kinetics, pp. 113–118, John Wiley and Sons, New York
49 Keleti, T., Leoncini, R., Pagani, R. and Marinello, E. (1987) Eur. J. Biochem. **170**, 179–183
50 Lowe, M., Strauss, A. W., Alpers, R., Seetharam, S. and Alpers, D. H. (1990) Biochim. Biophys. Acta **1037**, 170–177
51 Goldstein, D. J., Rogers, C. and Harris, H. (1982) Clin. Clin. Acta **125**, 63–75
52 Weiss, M. J., Ray, K., Henthorn, P. S., Lamb, B., Kadesch, T. and Harris, H. (1988) J. Biol. Chem. **263**, 12002–12010

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