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Removal from the Membrane Affects the Interaction of Rat Osseous Plate Ecto-Nucleosidetriphosphate Diphosphohydrolase-1 with Substrates and Ions

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Abstract We have characterized the kinetic properties of ectonucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1) from rat osseous plate membranes. A novel finding of the present study is that the solubilized enzyme shows high- and low-affinity sites for the substrate in contrast with a single substrate site for the membrane-bound enzyme. In addition, contrary to the Michaelian chraracteristics of the membrane-bound enzyme, the site–site interactions after solubilization with 0.5% digitonin plus 0.1% lysolecithin resulted in a less active ectonucleoside triphosphate diphosphohydrolase, showing activity of about 398.3 nmol Pi min^{-1} mg^{-1}. The solubilized enzyme has M_r of 66–72 kDa, and its catalytic efficiency was significantly increased by magnesium and calcium ions; but the ATP/ADP activity ratio was always <2.0. Partial purification and kinetic characterization of the rat osseous plate E-NTPDase1 in a solubilized form may lead to a better understanding of a possible function of the enzyme as a modulator of nucleotidase activity or purinergic signaling in matrix vesicle membranes. The simple procedure to obtain the enzyme in a solubilized form may also be attractive for comparative studies of particular features of the active sites from this and other ATPases.

Keywords Osseous plate · Endochondral ossification · Digitonin · Lysolecithin · Suramin · Ectonucleoside triphosphate diphosphohydrolase 1

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Introduction

Bone tissue formation is a complex biological process mediated by the concerted action of physicochemical and biochemical activities leading to the deposition of a mineral phase into a specific organic matrix (Anderson 1995). The pioneering work of Robison (1923), who first associated alkaline phosphatase activity with calcification, led to intensive investigations of the multiple activities of this enzyme (Anderson et al. 2005; Leone et al. 1997; Millán 2006). However, it is now well established that alkaline phosphatase is not the sole enzyme in matrix vesicle membranes that may be relevant for calcification (Anderson et al. 2005; Leone et al. 1997; Millán 2006).

Matrix membrane-invested vesicles are structures where the first crystals of calcium hydroxyapatite mineral are generated during biomineralization of growth plate cartilage (Anderson et al. 2005). Mineralization of growing bone resembles that of growth plate cartilage in its initiation associated to submicroscopic, extracellular matrix vesicles. Although bone matrix vesicles are smaller and more rapidly calcified, they do resemble those of cartilage by being extracellular and not connected to cells (Anderson et al. 2005). The deposition of calcium phosphate also involves ATP-dependent transport of the cation into matrix vesicles (Ali and Evans 1973), thus suggesting the involvement of an ATPase in the mineralization process (Hsu and Anderson 1996; Pizauro et al. 1998). In spite of some circumstantial evidence, it remains unknown whether it is alkaline phosphatase or a specific ATPase that triggers the calcification of cartilage and bone since both enzymes can hydrolyze ATP (Anderson et al. 2005; Demenis and Leone 2000). The lack of substrate specificity and the multifunctional properties attributed to alkaline phosphatase added to the difficulty of obtaining alkaline

phosphatase–depleted matrix vesicle membranes have, until recently, been the main reasons for the elusive kinetic and molecular characterization of this specific ATPase (Pizauro et al. 1998).

As part of efforts to understand the relationship between calcification and osseous alkaline phosphatase, we have developed a method to prepare large quantities of this enzyme from rat bone, using the bone-forming system described by Reddi and Huggins (1972), which avoids the use of collagenase and organic solvents (Curti et al. 1986). Previous studies had revealed that the alkaline phosphatase present in rat osseous plate membranes is a glycosylphosphatidylinositol-anchored metalloprotein, consisting of two apparently identical subunits of M_r 65 kDa, that requires zinc and magnesium ions for maximal activity and shows multifunctional activities (for review, see Leone et al. 1997). Following treatment of osseous plate membranes with phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis*, a membrane-bound ATPase showing properties somewhat different from those reported for classical ATPases was identified and kinetically characterized as ectonucleoside triphosphate diphosphohydrolase-1 (NTPDase1), becoming the first report of the presence of an E-NTPDase1 in rat osseous plate membranes (Demenis et al. 2003). Up to the present, the physiological function of this NTPDase1 in rat osseous plate has not been established.

The NTPDase family is comprised of cell surface membrane-bound enzymes that hydrolyze extracellular nucleoside triphosphates (NTPs) and in some cases nucleoside diphosphates (NDPs), thereby modulating purinergic signaling. However, due to the small amounts of most NTPDases in plasma membranes, allied to their high sensitivity to detergents generally used to solubilize membrane-bound proteins, successful purification from rabbit skeletal muscle transverse tubules (Treuheit et al. 1992), human umbilical vessel (Yagi et al. 1992), chicken oviduct (Strobel et al. 1996), chicken liver (Knowles et al. 2002), chicken gizzard (Stout and Kirley 1994) and human placenta (Christoforidis et al. 1995) has only been recently accomplished. Reports on extensive purification of NTPDase from pig pancreas (Sévigny et al. 1995), bovine lung and aorta (Sévigny et al. 1997a, 1997b) and chicken stomach and liver (Knowles et al. 2002; Lewis-Carl and Kirley 1997) have also appeared. The crystal structure of recombinant rat NTPDase2 has also recently been reported (Zebisch and Strater 2008).

Considering that E-NTPDase1 may act as a switch, turning NTP and NDP hydrolysis on and off at points of intercellular communication or alternatively between cells and the extracellular matrix, the characterization of the molecular and kinetic properties of this enzyme may facilitate future investigations of its physiological function as a possible modulator of nucleotidase activity or purinergic signaling in matrix vesicle membranes aimed at understanding the calcification process.

Materials and Methods

Materials

Solutions were prepared using MilliQ ultrapure apyrogenic water (Millipore, Bedford, MA). Bovine serum albumin, theophylline, Tris, oligomycin, levamisole, ouabain, trichloroacetic acid (TCA), sodium azide, P^1,P^5-(adenosine) pentaphosphate (Ap_5A), *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), bafilomycin A_1, thapsigargin, ethacrynic acid, ATP, AMP, sodium β-glycerophosphate, glucose-1-phosphate, glucose-6-phosphate, suramin, digitonin, *p*-nitrophenyl phosphate (PNPP), lysolecithin, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and $C_{12}E_8$ were obtained from Sigma (St. Louis, MO). Dimethylsulfoxide (DMSO), sodium metavanadate and magnesium and calcium chlorides were from Merck (Darmstadt, Germany). Purified phosphatidylinositol-specific phospholipase C (PIPLC) from *B. thuringiensis* was purchased from Oxford GlycoSciences (Oxford, UK). Omeprazole was a gift from Libbs Farmaceutica (São Paulo, Brazil). Analytical-grade reagents were used without further purification.

Alkaline Phosphatase–Rich Rat Osseous Plate Membranes

Alkaline phosphatase–rich rat osseous plate membranes were prepared according to Curti et al. (1986).

Removal of Alkaline Phosphatase from Osseous Plate Membranes

Osseous plate membranes (2 mg/ml) in 50 mM Tris-HCl buffer (pH 7.25) containing 200 mM sucrose were incubated with 0.1 U PIPLC from *B. thuringiensis* for 1 h at 37°C, under constant rotary shaking according to Pizauro et al. (1995). After centrifugation at 150,000 × *g* for 1 h at 4°C, the pellet was resuspended in 5 mM Tris-HCl buffer (pH 7.5) containing 2 mM $MgCl_2$ and 200 mM sucrose and chromatographed on a Sepharose 4B column equilibrated and eluted with the same buffer. Active fractions (4 ml) against ATP and ADP hydrolysis were pooled and dialyzed against 5 mM Tris-HCl buffer (pH 7.5) containing 200 mM sucrose. Finally, 1.0-ml aliquots were rapidly frozen in liquid nitrogen and stored at −20°C. No appreciable loss of activity was seen after 2-month storage. This protocol yielded NTPDase-rich membranes containing <6% alkaline phosphatase

activity (which is completely inhibited by 5 mM theophylline).

Solubilization of E-NTPDase1

An aliquot (1 ml) of alkaline phosphatase–depleted membranes (2 mg/ml) was mixed with 1 ml of 5 mM Tris-HCl buffer (pH 7.5) containing 2 mM $MgCl_2$, 1% digitonin and 0.2% lysolecithin. After standing for 45 min at room temperature (~27°C) with occasional gentle stirring, the resulting mixture was centrifuged at 150,000 × *g* for 1 h at 4°C. To remove excess digitonin and lysolecithin, the resulting supernatant was chromatographed on a Sephacryl S-300 (41 × 0.8 cm) column, equilibrated and eluted with 5 mM Tris-HCl buffer (pH 7.5) containing 5 mM theophylline, 5 μM $MgCl_2$, 0.05% (w/v) digitonin and 0.01% (w/v) lysolecithin. Fractions of 2 ml were collected (flow rate of 20 cm^3/h) and assayed for absorbance at 280 nm and ATP hydrolysis at 37°C.

Enzymatic Activity Measurements

Nucleotide phosphatase activity was estimated discontinuously at 37°C by measuring the amount of inorganic phosphate liberated according to Pizauro et al. (1998). Standard conditions were 50 mM HEPES buffer (pH 7.5) containing 5 mM theophylline and magnesium or calcium ions (concentrations are given in legends to tables and figures) in a final volume of 1.0 ml. The reaction was initiated by addition of the enzyme, stopped with 0.5 ml of 30% TCA at appropriate times and centrifuged at 4,000 × *g* just before phosphate determination. Inorganic phosphate present in the supernatant was determined by the procedure of Heinonen and Lahti (1981).

Protein Estimation

Protein concentrations were estimated according to Read and Northcote (1981) using bovine serum albumin as the standard.

Effectiveness of Inhibitors on E-NTPDase1 Activity

Initial rates were measured in 50 mM HEPES buffer (pH 7.5) containing 1 mM ATP, 5 μM $MgCl_2$ (or 3 mM ADP, 0.1 mM $MgCl_2$) and the inhibitor in a final volume of 1.0 ml. Except for thapsigargin and bafilomycin A_1, both dissolved in DMSO, as well as suramin, dissolved in distilled water, all inhibitors were prepared in 50 mM HEPES buffer (pH 7.5).

SDS-PAGE and Western Blot Analysis

SDS-PAGE was carried out on 10% slab gels according to Laemmli (1970) using silver nitrate for protein staining. Electroblotting was carried out according to Towbin et al. (1979). Polyclonal antibody against Sigma potato apyrase developed in rabbits (Vasconcelos et al. 1996) and monoclonal rabbit antimouse CD-39 (Boeck et al. 2002) were a gift from Dr. J. J. F. Sarkis from the Universidade Federal do Rio Grande do Sul (Brazil).

Estimation of Kinetic Parameters

Maximum velocity (*V*), apparent dissociation constant ($K_{0.5}$) and Hill coefficient (n) were fitted using the SigrafW software (Leone et al. 2005; SigrafW can be freely downloaded from the site http://portal.ffclrp.usp.br/sites/fdaleone/downloads). All kinetic parameters are calculated values and represent means ± SD of three different preparations of solubilized enzyme. Representative curves obtained from a typical preparation are presented in every figure.

Results

Solubilization of E-NTPDase1 from Rat Osseous Plate Membranes

Immediately after detergent addition to the alkaline phosphatase–depleted membranes, a decrease of about 37% of nucleotidase activity was observed. Unexpectedly, during centrifugation at 150,000 × *g*, an additional 50% loss of E-NTPDase1 activity was verified. After centrifugation, up to 65% NTPDase1 activity was found in the supernatant, in contrast to 13% obtained when only 0.5% digitonin was used. Treatments with 0.2% Triton X-100, 0.5% polyoxyethylene-5 decyl ether, 1.4% Lubrol WX, 1% Lubrol PX, 1% polidocanol, 2.5% CHAPS, 1% $C_{12}E_8$ and 30 mM octylglucoside, either alone or in combination with 0.2% lysolecithin, resulted in inactivation of the enzyme (not shown). Removal of excess digitonin and lysolecithin on the Sephacryl S-300 (Fig. 1) resulted in the elution of solubilized E-NTPDase1 (peak II) showing an ATPase activity of about 398.3 nmol Pi min^{-1} mg^{-1}, which was used throughout this study. Due to the lack of nucleotidase activity, peak I was discarded. Table 1 summarizes the recovery of E-NTPDase1 activity after solubilization with digitonin plus lysolecithin.

Electrophoretic Analysis of Solubilized E-NTPDase

SDS-PAGE and Western blot analyses are shown in Fig. 2. A single diffuse protein band of M_r 66–72 kDa appearing on the SDS-PAGE (lane A) was coincident with Western blotting using polyclonal antibody against potato E-NTPDase (lane B) and with rabbit anti-mouse CD39 antibody

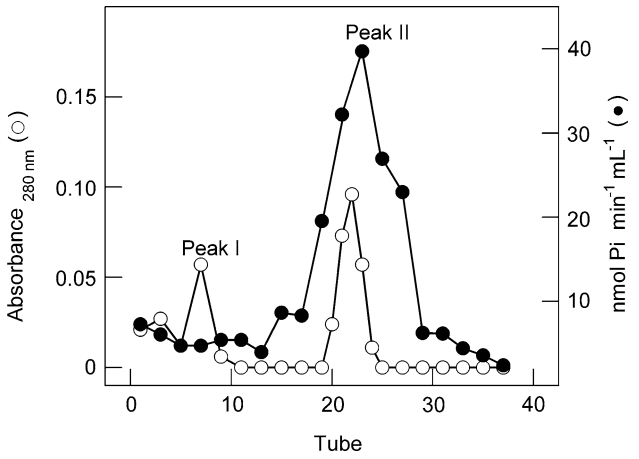

Fig. 1 Sephacryl S-300 chromatography of detergent-solubilized E-NTPDase1 from rat osseous plate membranes. The column (41 × 0.8 cm) was equilibrated and eluted by 5 mM Tris-HCl buffer (pH 7.5) containing 2 mM $MgCl_2$, 5 mM theophylline, 0.05% (w/v) digitonin and 0.01% (w/v) lysolecithin. Fractions of 2 ml were collected at a flow rate of 20 cm^3 h^{-1} and assayed for absorbance at 280 nm and phosphohydrolase activity. Only peak II, corresponding to the solubilized E-NTPDase1, was used. A representative profile of a typical chromatograph is presented

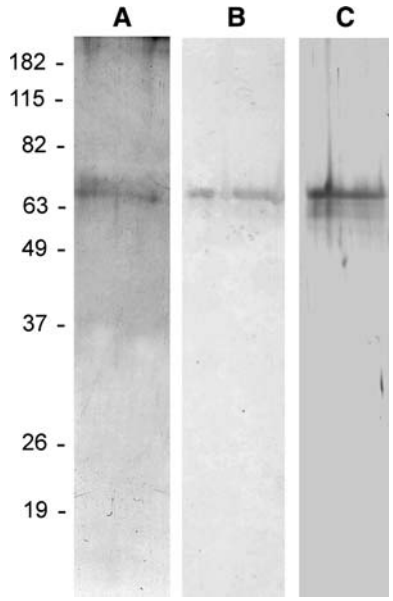

Fig. 2 Electrophoretic analysis of detergent-solubilized E-NTPDase1 from rat osseous plate membranes. *Lane A* Silver staining. *Lane B* Western blot using rabbit polyclonal antibody against potato E-NTPDase. *Lane C* Western blot using rabbit anti-mouse CD39 antibody. Electrophoresis was carried out in 10% acrylamide gels using 6.8 and 30 μg protein for SDS-PAGE and Western blot, respectively. Analyses were repeated using aliquots from three different preparations of solubilized enzyme; representative results from a typical preparation are presented

(lane C). The strongly immunoprecipitated band with rabbit anti-mouse CD39 antibody was diffuse; thus, it was not possible to conclude whether the solubilized enzyme had been purified to homogeneity. However, taken together, the results strongly suggest that the solubilized enzyme is indeed an E-NTPDase1 and is sufficiently pure to permit kinetic studies.

Dependence of Hydrolysis Rate on Substrate Concentration

The dependence of the hydrolysis rate of NTPDase1 on ATP and ADP concentration at pH 7.5, in the presence of magnesium or calcium ions, is shown in Fig. 3. Independent of the substrate and the metal ion, two families of hydrolyzing sites were observed. In the presence of 5 μM $MgCl_2$, ATP was hydrolyzed at maximal rates of about 308.3 ± 12.3 nmol Pi min^{-1} mg^{-1} with $K_{0.5} = 2.9 \pm 0.1$ μM at high-affinity sites and $V = 105.1 \pm 4.2$ nmol Pi min^{-1} mg^{-1} with $K_{0.5} = 41.0 \pm 2.0$ μM at low-affinity sites. Cooperative effects for the homotropic interaction of ATP with the enzyme at both high- and low-affinity sites were observed. ATP nonstimulated activity of about 81.6 ± 3.2 nmol Pi min^{-1} mg^{-1} was estimated at ATP concentrations as low as 100 nM (Fig. 3a). In the presence of 0.1 mM $MgCl_2$, ADP hydrolysis was approximately half of that observed for ATP, 166.1 ± 4.9 nmol Pi min^{-1} mg^{-1} with $K_{0.5} = 95.0 \pm 5.7$ μM at high-affinity sites and 78.8 ± 3.1 nmol Pi min^{-1} mg^{-1} with $K_{0.5} = 800.0 \pm 9.6$ μM at low-affinity sites. Cooperative effects for the homotropic interaction of ADP with the enzyme at both high- and low-affinity sites were also observed. ADP nonstimulated activity of about 99.6 ± 4.9 nmol Pi min^{-1} mg^{-1} was determined at ADP concentrations as low as 10 μM (Fig. 3a). In the presence of calcium ions, quite similar results were obtained (Fig. 3b). When ATP was used as substrate, no significant variations were observed for $K_{0.5}$ values, although V was approximately

Table 1 Solubilization of E-NTPDase1 from rat osseous plate membranes

Step	U ml^{-1}	Total U	Total protein (mg)	U mg^{-1}	Yield (%)
PIPLC-treated membranes	1,163.6	1,058.9	0.85	1,245.7	100
Solubilized NTPDase-1	763.1	228.9	0.90	254.3	21.6
Sephacryl S-300 chromatography	7.23	65.0	0.163	398.3	6.1

Initial rates were measured in 50 mM HEPES buffer (pH 7.5) containing 5 mM theophylline, 1 mM ATP and 5 μM $MgCl_2$. The table shows representative data from a typical preparation. U is given as nmol Pi min^{-1} mg^{-1}

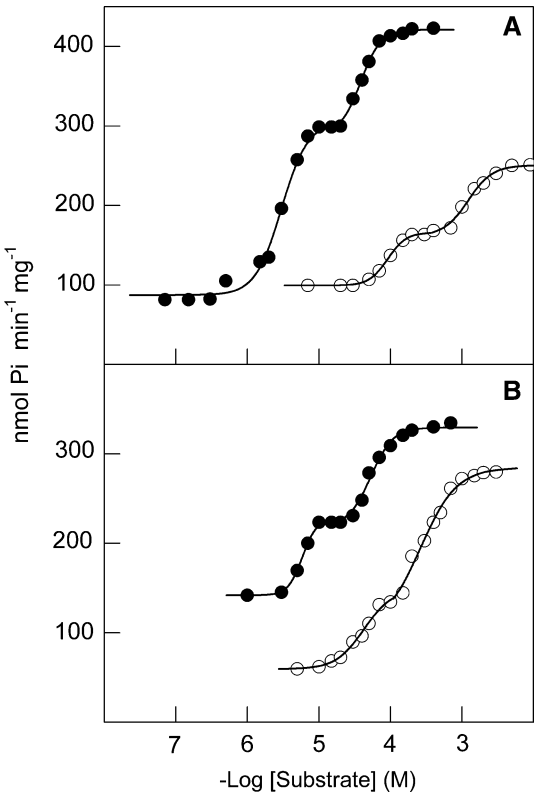

Fig. 3 Effect of substrate concentration on detergent-solubilized E-NTPDase1 activity in the presence of magnesium or calcium ions. **a** Hydrolysis of ATP (●) and ADP (○) in the presence of 5 μM or 0.1 mM $MgCl_2$, respectively. **b** Hydrolysis of ATP (●) and ADP (○) in the presence of 10 μM or 1 mM $CaCl_2$, respectively. Activities were assayed using 1.8 μg protein in 50 mM HEPES buffer (pH 7.5) containing 5 mM theophylline. All determinations were carried out in duplicate using aliquots from three different preparations of the solubilized enzyme. Initial rates remained constant for up to 60 min, provided that <5% of substrate was hydrolyzed. Controls without added enzyme were included in each experiment to account for nonenzymatic hydrolysis of substrate in each experiment. Representative curves from a typical preparation are shown

78% of that observed in the presence of magnesium ions. An ATP nonstimulated activity of about 127.7 ± 6.1 nmol Pi min^{-1} mg^{-1} was estimated for ATP concentrations as low as 1 μM. For ADP, significant changes in $K_{0.5}$ at both high- and low-affinity sites occurred, and V was nearly 15% higher than that observed in the presence of magnesium ions. ADP nonstimulated activity of about 61.9 nmol Pi min^{-1} mg^{-1} was also estimated at ADP concentrations as low as 10 μM (Fig. 3b). Table 2 summarizes the values for the kinetic parameters calculated for ATP and ADP hydrolysis by purified E-NTPDase1 at pH 7.5 in the presence of magnesium or calcium ions. In the presence of magnesium ions, catalytic efficiency ($V/K_{0.5}$) of the enzyme for ATP was significantly higher than that obtained for ADP. In contrast, in the presence of calcium ions, catalytic efficiency for ADP was higher than that observed with ATP. Interestingly, the $K_{0.5}$ value estimated for ADP hydrolysis at high-affinity sites was up to 33 times higher than that determined for ATP hydrolysis; for low-affinity sites it was almost 20-fold greater. Glucose-6-phosphate, glucose-1-phosphate, AMP, PNPP and β-glycerophosphate were not hydrolyzed to a significant extent by solubilized E-NTPDase1 (not shown).

Effect of Magnesium and Calcium Ions on Substrate Hydrolysis

The effect of magnesium and calcium ions on ATP and ADP hydrolysis at pH 7.5 by purified E-NTPDase1 from rat osseous plate membranes is shown in Fig. 4. At saturating concentrations of ATP (1 mM) or ADP (3 mM), cooperative kinetics was observed for the hydrolysis of both substrates with increasing concentrations of Mg^{2+} (or Ca^{2+}). The calculated kinetic parameters were $V = 392.6 \pm 19.6$ nmol Pi min^{-1} mg^{-1} with $K_{0.5} = 1.4 \pm 0.1$ μM and $V = 255.4 \pm 12.7$ nmol Pi min^{-1} mg^{-1} with $K_{0.5} = 9.8 \pm 0.4$ μM for ATP and ADP, respectively (Fig. 4a). No variation of V was observed as Mg^{2+} concentrations increased to 1 mM for both ATP and ADP. Importantly, Mg^{2+} concentrations as low as 5×10^{-7} M trigger stimulation of nucleotidase activity to about 316.8 ± 11.1 nmol Pi min^{-1} mg^{-1} for ATP and 90.5 ± 3.6 nmol Pi min^{-1} mg^{-1} for ADP. For calcium

Table 2 Kinetic parameters calculated for ATP and ADP hydrolysis at pH 7.5 by detergent-solubilized E-NTPDase1 of rat osseous plate membranes in the presence of magnesium and calcium ions

S	Solubilized enzyme									Membrane-bound enzyme[a]			
	Metal ion	High-affinity sites				Low-affinity sites							
		V	n	$K_{0.5}$ (μM)	$V/K_{0.5}$	V	n	$K_{0.5}$ (mM)	$V/K_{0.5}$	V	n	K_M (μM)	V/K_M
ATP	5 μM Mg^{2+}	308.3 ± 12.3	2.3	2.9 ± 0.1	106.3	105.1 ± 4.2	4.5	41.0 ± 2.0	2.6	1,278.7 ± 38.4	1.0	83.3 ± 2.5	15.3
	10 μM Ca^{2+}	228.4 ± 11.4	3.1	5.2 ± 0.2	43.9	96.2 ± 5.8	3.7	54.0 ± 2.7	1.8	1,346.5 ± 53.8	1.0	85.3 ± 3.4	15.8
ADP	0.1 mM Mg^{2+}	166.1 ± 4.9	2.5	95.0 ± 5.7	1.7	78.8 ± 3.1	2.8	800.0 ± 9.6	0.1	473.9 ± 18.9	1.0	150.6 ± 6.0	3.1
	1 mM Ca^{2+}	134.5 ± 5.4	2.7	38.0 ± 1.5	3.5	147.0 ± 5.9	2.2	323.3 ± 6.0	0.5	783.9 ± 27.4	1.0	196.2 ± 6.9	3.9

[a] Data from Demenis et al. (2003)

Initial rates were measured in 50 mM HEPES buffer (pH 7.5) containing 5 mM theophylline in the presence of Mg^{2+} (5 μM or 0.1 mM for ATP and ADP, respectively) or Ca^{2+} (10 mM or 1 mM for ATP and ADP, respectively) and variable concentrations of ATP and ADP ranging from 10^{-7} to 10^{-2} M. Data shown are means ± SD of three different preparations of the solubilized enzyme. V is given as nmol Pi min^{-1} mg^{-1}

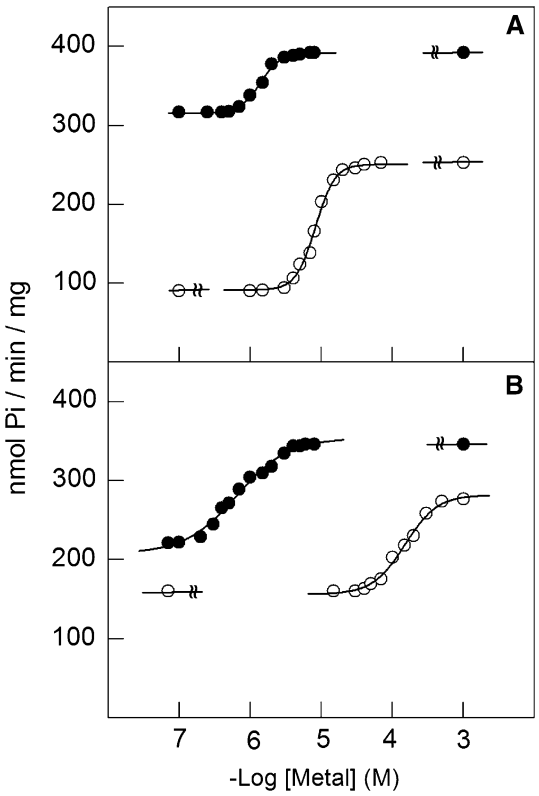

Fig. 4 Modulation by magnesium or calcium ions of detergent-solubilized E-NTPDase1 activity. **a** Magnesium ions. **b** Calcium ions. Activities were assayed using 1.8 µg protein in 50 mM HEPES buffer (pH 7.5) containing 1 mM ATP (●) or 3 mM ADP (○). Experiments were performed using duplicate aliquots from three different preparations of solubilized enzyme; representative curves from a typical preparation are shown

ions, similar results were obtained (Fig. 4b), the calculated kinetic parameters being $V = 344.2 \pm 20.6$ nmol Pi min^{-1} mg^{-1} with $K_{0.5} = 0.7 \pm 0.04$ µM and $V = 281.1 \pm 14.1$ nmol Pi min^{-1} mg^{-1} with $K_{0.5} = 100.0 \pm 4.0$ µM for ATP and ADP, respectively. As observed for Mg^{2+}, nucleotidase activity was triggered by Ca^{2+} concentrations as low as 10^{-7} M up to 222.1 ± 9.9 nmol Pi min^{-1} mg^{-1} (for ATP) and 157.0 ± 7.8 nmol Pi min^{-1} mg^{-1} (for ADP). Independent of the substrate, addition of 5 mM EDTA resulted in nucleotidase activities lower than 5 nmol Pi min^{-1} mg^{-1} for both metal ions. Table 3 summarizes the effect of Mg^{2+} and Ca^{2+} on ATP and ADP hydrolysis. It should be mentioned that no significant additive effect on hydrolysis rate of ATP and ADP was observed in the simultaneous presence of magnesium and calcium ions (not shown).

Effectiveness of Inhibitors on the Hydrolysis of ATP and ADP

The relative effectiveness of several reagents on ATP and ADP hydrolysis by solubilized E-NTPDase1 is shown in Table 4. At saturating concentrations of ATP (1 mM) or ADP (3 mM) and 0.1 mM Mg^{2+}, suramin and sodium azide inhibited E-NTPDase1 activity, while oligomycin, ouabain, bafilomycin, thapsigargin, omeprazole, vanadate, ethacrynic acid and theophylline showed no significant effects. Together with those of SDS-PAGE analysis (see Fig. 2), these results support the conclusion that the solubilized enzyme is free of contaminating ATPases. The lack of inhibition by theophylline and levamisole unequivocally rules out any contamination by alkaline phosphatase. Interesting results emerged when solubilized E-NTPDase1 was assayed in the presence of suramin, a polysulfonated naphthylamine (Fig. 5). When the substrate was added at levels just sufficient to saturate the high-affinity site (10^{-6} M to 5×10^{-5} M), suramin inhibited 75% and 50% ATP and ADP hydrolysis, respectively (Fig. 5a). However, when the enzyme was completely saturated by the substrate, suramin inhibited only 25% and 43% of ATP and ADP hydrolysis, respectively (Fig. 5b). Apparently, suramin is a noncompetitive inhibitor of the enzyme (Fig. 5a, inset). Sodium azide (20 mM) inhibited up to 57% and 68% ATP and ADP hydrolysis, respectively (Fig. 5b, inset).

Table 3 Modulation by magnesium and calcium ions of ATP and ADP hydrolysis by detergent-solubilized E-NTPDase1 of rat osseous plate membranes

Metal ion	Substrate	Detergent-solubilized enzyme				Membrane-bound enzyme[a]			
		V	n	$K_{0.5}$ (µM)	$V/K_{0.5}$	V	n	K_M (µM)	V/K_M
Mg^{2+}	ATP	392.6 ± 19.6	3.2	1.4 ± 0.1	280.4	1,367.2 ± 41.0	1.0	595.3 ± 17.8	2.6
	ADP	255.4 ± 12.7	2.1	9.8 ± 0.4	26.1	458.2 ± 20.6	1.5	420.6 ± 18.9	1.1
Ca^{2+}	ATP	344.2 ± 20.6	2.0	0.7 ± 0.04	491.7	1,084.7 ± 32.5	1.1	377.8 ± 11.3	2.8
	ADP	281.1 ± 14.1	2.0	100.0 ± 4.0	2.8	635.1 ± 28.6	1.2	144.3 ± 6.5	4.4

[a] Data from Demenis et al. (2003)

Initial rates were measured in 50 mM HEPES buffer (pH 7.5) containing 5 mM theophylline, 1 mM ATP (or 3 mM ADP) and variable concentrations of magnesium (10^{-7} to 10^{-2} M) or calcium (10^{-6} to 10^{-3} M) ions. Data are means ± SD of three different preparations of the solubilized enzyme. V is given as nmol Pi min^{-1} mg^{-1}

Table 4 Relative effectiveness of several compounds on ATP and ADP hydrolysis by detergent-solubilized E-NTPDase1 of rat osseous plate membranes

Reagent	% *V*			
	Solubilized enzyme		Membrane-bound enzyme[a]	
	ATP	ADP	ATP	ADP
Oligomycin (1 μg/ml)	100.5 ± 3.0	97.0 ± 4.8	99.3 ± 4.8	101.3 ± 6.4
Ouabain (1 mM)	100.3 ± 4.1	100.8 ± 4.0	109.3 ± 5.2	104.4 ± 3.5
Bafilomycin A_1 (1 μM)	95.0 ± 4.7	98.0 ± 4.9	96.5 ± 4.3	97.6 ± 4.9
Thapsigargin (1 μM)	95.0 ± 3.4	90.4 ± 6.3	97.2 ± 6.1	98.7 ± 7.1
Omeprazole (5 μM)	98.7 ± 3.9	99.0 ± 2.9	99.8 ± 2.1	100.3 ± 3.2
Vanadate (1 mM)	98.0 ± 5.3	94.5 ± 3.8	95.3 ± 4.7	96.1 ± 4.8
Ethacrynic acid (2 mM)	100.0 ± 3.9	93.0 ± 6.5	91.4 ± 2.8	92.6 ± 3.6
Theophylline (5 mM)	99.8 ± 4.9	100.3 ± 6.0	95.3 ± 4.7	94.8 ± 3.3
Levamisole (3.5 mM)	99.3 ± 3.2	97.9 ± 2.1	–	–
Suramin (1 mM)	75.0 ± 4.5	57.0 ± 2.8	70.1 ± 2.8	59.3 ± 3.4
Sodium azide (10 mM)	56.9 ± 4.4	67.5 ± 2.1	50.0 ± 1.3	32.6 ± 2.3
AP_5A (1 mM)	100.2 ± 3.2	99.1 ± 4.1	96.0 ± 2.9	97.2 ± 4.1
EDTA (5 mM)	1.5 ± 0.2	2.1 ± 0.1	3.2 ± 0.1	2.5 ± 0.1

[a] Data from Demenis et al. (2003)

Initial rates were measured at 37°C in 50 mM HEPES buffer (pH 7.5) containing 5 mM theophylline, 1 mM ATP (or 3 mM ADP) and 5.0 μM $MgCl_2$ (or 0.1 mM $MgCl_2$ for ADP) in a final volume of 1.0 ml. Solubilized enzyme-specific activity of 100% corresponded to 413.4 ± 8.2 and 244.9 ± 4.0 nmol Pi min^{-1} mg^{-1} for ATP and ADP hydrolysis, respectively. For the membrane-bound enzyme, 100% specific activity corresponded to 1,252.3 ± 37.5 and 463.9 ± 18.5 nmol Pi min^{-1} mg^{-1} for ATP and ADP hydrolysis, respectively. Data are means ± SD of three different preparations of solubilized enzyme

Identification of ATP and ADP Hydrolyzing Sites in the Enzyme Molecule

Figure 6 shows that ATP and ADP are hydrolyzed at a single common site on the enzyme molecule. This interpretation came from the fact that in any mixture containing different concentrations of ATP and ADP, total velocity fell between the velocities obtained for each substrate assayed separately in the same concentration used in the mixture. If ATP and ADP were hydrolyzed at two different sites, then the total velocity would be the sum of ATP and ADP hydrolysis (theoretical value shown in Fig. 6). This kinetic result rules out the possibility that ATP and ADP are hydrolyzed by two different nucleotidases. Together with those from Table 4 and Figure 2, these data suggest that the solubilized enzyme may be considered pure enough for kinetic studies.

Discussion

We have characterized the kinetic properties of an NTPDase1 from rat osseous plate membranes. A novel finding of the present study is that the solubilized enzyme shows high- and low-affinity sites for the substrate in contrast with a single substrate site for the membrane-bound enzyme. In addition, the site–site interactions resulting after enzyme solubilization with 0.5% digitonin plus 0.1% lysolecithin oppose the "Michaelian" chraracteristics of the membrane-bound enzyme (Demenis et al. 2003).

The widespread occurrence of NTPDases in plasma membranes from various tissues has been widely documented (references in Knowles et al. 1983; Robson et al. 2006). The presence of this enzyme in rat osseous plate membranes has been reported (Demenis et al. 2003), and its precise location is under investigation in our laboratory. E-NTPDase1 is an important ectonucleotidase that sequentially hydrolyzes the β and γ phosphates of tri- and diphosphonucleosides, modulating their extracellular concentration in a variety of physiological systems (Plesner 1995). In spite of many reports about ATP transport to the extracellular compartment by a variety of primary culture cells, including endothelial ones (Chaudry 1982), the existence of extracellular ATP concentrations high enough to initiate vesicle-mediated calcification remains controversial. However, it is now accepted that nucleotides can be found in significant concentrations outside the cells (Burnstock 1997; Dombrowski et al. 1998; Gordon 1986) and might exert effects on other cells in the vicinity of the secretion site, modulating biological processes by binding to specific receptors (Burnstock 1997; Dombrowski et al. 1998).

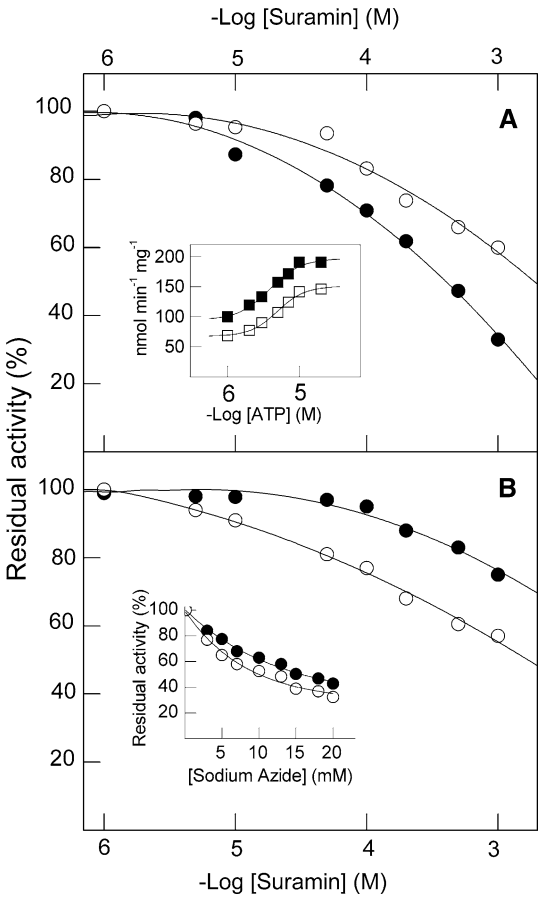

Fig. 5 Suramin inhibition of detergent-solubilized E-NTPDase1 activity in the presence of magnesium ions. **a** Inhibition by suramin of solubilized NTPDase1 partially saturated by ATP (●) or ADP (○). Activities were assayed using 1.8 μg protein in 50 mM HEPES buffer (pH 7.5) containing 5 mM theophylline, 0.01 mM ATP and 5 μM $MgCl_2$ (or 0.1 mM ADP and 0.1 mM $MgCl_2$). *Inset*: ATP hydrolysis in the presence of (■) 0.2 mM suramin and (□) 0.5 mM suramin. **b** Inhibition by suramin of detergent-solubilized NTPDase1 saturated by ATP (●) or ADP (○). Activities were assayed using 1.8 μg protein in 50 mM HEPES buffer (pH 7.5) containing 5 mM theophylline, 1 mM ATP and 5 μM $MgCl_2$ (or 3 mM ADP and 0.1 mM $MgCl_2$). *Inset*: Inhibition by sodium azide of detergent-solubilized E-NTPDase1 activity of ATP (●) or ADP (○) hydrolysis. Activities were assayed using 1.8 μg protein in 50 mM HEPES buffer (pH 7.5) containing 5 mM theophylline, 1 mM ATP and 5 μM $MgCl_2$ (or 3 mM ADP and 0.1 mM $MgCl_2$). All determinations were carried out in duplicate using aliquots from three different preparations of solubilized enzyme. Initial rates were constant for up to 60 min, provided that <5% of substrate was hydrolyzed. Controls without added enzyme were included in each experiment to account for the nonenzymatic hydrolysis of substrate in each experiment. Representative curves from a typical preparation are shown

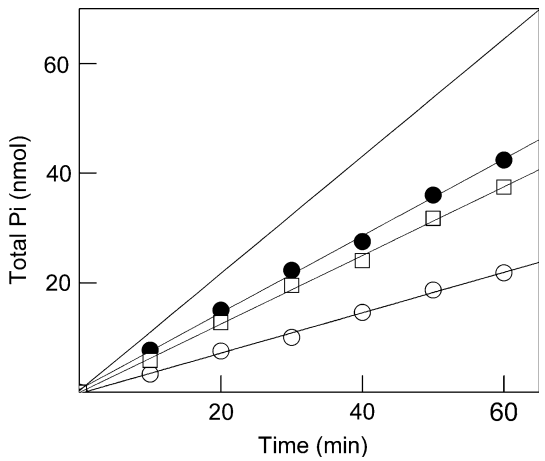

Fig. 6 Time course of ATP and ADP hydrolysis by detergent-solubilized E-NTPDase1. Assays were carried out in 50 mM HEPES buffer (pH 7.5) containing 5 mM theophylline and 0.01 mM $MgCl_2$ and using 1.8 μg protein. (●) 1 mM ATP, (○) 3 mM ADP, (□) 1 mM ATP plus 3 mM ADP, (—) theoretical value for the hydrolysis of a mixture of 1 mM ATP plus 3 mM ADP, assuming two independent catalytic sites

As reported for the membrane-bound enzyme (Demenis et al. 2003), the solubilized enzyme also shows maximum activity at pH 7.5 for hydrolysis of both ATP and ADP. However, removal from the membrane resulted in a form with lower activity, exhibiting high- and low-affinity substrate-hydrolyzing sites. Furthermore, the solubilization process significantly increased the catalytic efficiency of the enzyme in the presence of magnesium or calcium ions, the ATP/ADP hydrolysis ratio remaining below 2.0. Apparently, these differences may be attributed to conformational alterations of the solubilized enzyme, which is free from constraints exerted by the lipid environment at transmembrane domains. There is evidence that the activity of NTPDases shows a striking dependence on their state of oligomerization (Smith and Kirley 1998; Stout and Kirley 1994; Wang et al. 1998). Compounds that promote oligomerization as well as cross-linking reagents have been shown to increase enzyme activity (Caldwell et al. 1999; Stout and Kirley 1996), while detergents and other amphiphilic molecules that prevent oligomerization decrease it (Caldwell et al. 1997, 2001). Yet, different effects of detergents have been reported for NTPDases from several sources, suggesting that oligomerization may be required for full activity (Beeler et al. 1983; Caldwell et al. 1999, 2001; Knowles et al. 2002; Stout and Kirley 1996). Similar to other NTPDases, detergents commonly used for solubilization of membrane proteins rapidly inactivated the rat osseous plate enzyme. The recovery of about 63% of E-NTPDase1 activity immediately after addition of 0.5% digitonin plus 0.1% lysolecithin contrasts with the barely 13% obtained using 0.5% digitonin alone, apparently suggesting a protective effect of the phospholipid. Whether the oligomerization state or a detergent-resistant quaternary structure is responsible for the above recovery of nucleotidase activity remains to be established.

The apparent $K_{0.5}$ values of the solubilized enzyme for ATP and ADP hydrolysis were significantly lower than

those reported for other NTPDase1 (Mans et al. 1998; Valenzuela et al. 1996). ATPase/ADPase ratios lower than 2.0 also contrast with those reported for the enzyme from other sources (Knowles et al. 1983). Inhibition of NTP (or NDP) hydrolysis by an excess of free nucleotides at constant concentration of magnesium (or calcium) ions, a frequently observed feature of enzymes from various sources (Caldwell et al. 1999; Hidalgo et al. 1983), was not observed for the rat osseous plate–solubilized E-NTPDase1.

To date, it is assumed that the same catalytic residues are involved in the hydrolysis of either NTP or NDP by NTPDases. According to Knowles and Nagy (1999), since the enzyme hydrolyzes NTP and NDP, it seems likely that the active site shows some flexibility in order to assume conformations that can bind either NTP or NDP, allowing interactions with the catalytic residues and cleavage of the anhydride bond. This was recently clarified by Zebisch and Strater (2008) through X-ray structure studies of recombinant rat NTPDase2, showing that the hydrolysis of both ATP and ADP occurs at a single site. Independent of the presence of the metal ion, NTPDase1 of rat osseous plate shows $K_{0.5}$ values for ATP lower than those estimated for ADP, apparently reflecting the enzyme preference for ATP and suggesting that higher efficiency in its binding to the active site may be related to additional interactions with the γ phosphate. The results from Knowles and Nagy (1999) showing increased affinity of NTPDase for ADP in the presence of azide, which may occupy the ATP γ phosphate–binding site when ADP is in the active site, support this suggestion.

The dependence of ATP and ADP hydrolysis by the solubilized enzyme on magnesium (or calcium) ions provides strong kinetic evidence for an E-NTPDase1 activity similar to that reported by others (Mita et al. 1998; Picher et al. 1994; Plesner 1995; Torres et al. 1998; Valenzuela et al. 1996). Furthermore, enzyme activity stimulation by equimolar concentrations of calcium and magnesium ions suggests that each ion can substitute for the other during the catalytic cycle, but the absence of a significant additive effect on activity in the presence of both ions excludes any possible contribution of a (Ca^{2+}–Mg^{2+})-ATPase to E-NTPDase1 activity.

Data from Table 4 unequivocally confirm that the E-NTPDase1 preparation from rat osseous plate is free of F_oF_1-, (Na^+, K^+)-, V-, Ca^{2+}-, H^+- and Na^+- or K^+-ATPase contamination. Furthermore, Ap_5A, an effective inhibitor of adenylate kinase (Feldhau et al. 1975), did not affect the activity of solubilized E-NTPDase1, excluding the possibility that ATPases or AMPases would produce phosphate from ATP and AMP, both resulting from the conversion of ADP by adenylate kinase. Finally, the absence of inhibition by vanadate, levamisole and theophylline rules out the possibility that nonspecific alkaline phosphatase activity may contribute to ATP and ADP hydrolysis by E-NTPDase1.

Figure 6 provides strong evidence that ATP and ADP are hydrolyzed at a common catalytic site on the enzyme molecule. According to Cornish-Bowden (1976), Segel (1976) and Keleti et al. (1987), these results are a consequence of the action of a unique enzyme on two substrates simultaneously. If we eventually assume that the enzyme had two catalytic sites, one for each substrate, or that the enzyme was contaminated by either a phosphohydrolase or a different NTPDase, then the total velocity would be necessarily the sum of ATP and ADP hydrolysis, which was not the case. Data are in close agreement with those reported by Zebisch and Strater (2008) demonstrating that ATP and ADP hydrolysis occurs at the same hydrolytic site.

Sensitivity to azide was earlier used as a criterion to distinguish NTPDase1 from NTPDase2 (Knowles and Nagy 1999; Picher et al. 1994; Plesner 1995; Smith and Kirley 1998). The significant inhibition of ATP and ADP hydrolysis by sodium azide at millimolar concentration (Table 4) is similar to that reported for fish, chicken and rat synaptosomal enzymes (Schetinger et al. 2001) and suggests the existence of an E-NTPDase1 in rat osseous plate membranes. Furthermore, the higher inhibition of ADP hydrolysis agrees with data reported for the chick oviduct enzyme (Knowles and Nagy 1999) but not with those from fish, chicken and rat synaptosomes (Schetinger et al. 2001). According to Knowles and Nagy (1999), the differences in the effects of azide on ADP and ATP hydrolysis could be explained by several factors: (1) the enzyme binds ATP more tightly than ADP (for solubilized osseous plate E-NTPDase1 the $K_{0.5}$ value for ATP is almost 33 times lower than that for ADP); (2) in the presence of ATP, azide binds more weakly to the enzyme than in the presence of ADP (Fig. 5b inset shows that osseous plate E-NTPDase1 yields a higher I_{50} for ATP than for ADP); (3) the inhibition of Mg-ATP hydrolysis by azide is noncompetitive, while that of Mg-ADP is uncompetitive or of a mixed type (for osseous plate E-NTPDase1 no information about the type of inhibition is available). The inhibition of ATP and ADP hydrolysis by suramin in a concentration-dependent manner agrees with data reported for rat hippocampal (Bonan et al. 1999); fish, chicken and rat synaptosome (Schetinger et al. 2001); and *Torpedo* electric organ (Marti et al. 1996) enzymes. There is evidence that ATPases and P_2 receptors have similar ATP binding domains since suramin, a potent antagonist of P_2 receptors, affects many ATP-utilizing enzymes (Hoyle et al. 1990). However, it should be stressed that suramin is a nonspecific inhibitor of NTPDase1 since it does not inhibit rat E-NTPDase1 heterologously expressed in CHO cells (Heine et al. 1999).

Although the presence of E-NTPDase1 activity in chondrocytes was demonstrated by Sévigny et al. (1997a) using polyclonal antibodies against a conserved region of NTPDase, the kinetic properties of the membrane-bound form of E-NTPDase1 in rat osseous plate membranes have only been recently characterized (Demenis et al. 2003). However, its physiological function in the calcification process has not yet been established. Considering that NTPDase modulates the activities of adenylyl cyclase and 5′-nucleotidase by changing the ratio (ATP + ADP)/AMP, a possible role of NTPDase1 could be the control of 5′-nucleotidase activity since this action is inhibited by ATP and ADP and stimulated by AMP (Dornand et al. 1978). Thus, AMP produced by the action of E-NTPDase1 could be hydrolyzed by 5′-nucleotidase to adenosine, which in contrast to AMP can cross the membrane and become phosphorylated in the cytosol, restoring ATP supplies (Burnstock 1997; Vasconcelos et al. 1993). Several lines of evidence supporting this view are (1) levels of NTP in cartilage extracellular fluid are vanishingly small, in contrast to those of NDP and NMP, and may exert only minimal influence on calcification, occurring in the extracellular matrix in vivo (Wuthier and Register 1985); (2) the presence of 5′-nucleotidase in matrix vesicle membranes is well established (Anderson et al. 2005); (3) hydrolysis of ATP could proceed directly to AMP without liberating free ADP as an intermediate (Heine et al. 1999), and alkaline phosphatase could be involved in the subsequent regulation of apatite formation by reducing pyrophosphate levels at sites of extravesicular mineralization (Genge et al. 1988); (4) in the matrix vesicles, adenylyl cyclase could be stimulated by adenosine occurring in the synaptic space (Burnstock 1997), thus increasing cAMP concentration, which in turn may influence bone mineralization in vivo; (5) accumulating evidence suggests that extracellular nucleotides, signaling via P_2 receptors, play a role in modulating bone cell function (Hoebertz et al. 2003).

To conclude, the partial purification and kinetic characterization of rat osseous plate E-NTPDase1 in a solubilized form should encourage future investigation of its physiological function as a modulator of nucleotidase activity or purinergic signaling in matrix vesicle membranes in plasma and/or matrix vesicle membranes. Further, the ease of obtaining the enzyme in a solubilized form may be attractive for future comparative studies of some features of the active sites of this and other ATPases.

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