Phosphodiesterase Activity of Alkaline Phosphatase in ATP-initiated Ca\(^{2+}\) and Phosphate Deposition in Isolated Chicken Matrix Vesicles

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Inorganic pyrophosphate is a potent inhibitor of bone mineralization by preventing the seeding of calcium-phosphate complexes. Plasma cell membrane glycoprotein-1 and tissue nonspecific alkaline phosphatase were reported to be antagonistic regulators of mineralization toward inorganic pyrophosphate formation (by plasma cell membrane glycoprotein-1) and degradation (by tissue nonspecific alkaline phosphatase) under physiological conditions. In addition, they possess broad overlapping enzymatic functions. Therefore, we examined the roles of tissue nonspecific alkaline phosphatase within matrix vesicles isolated from femurs of 17-day-old chick embryos, under conditions where these both antagonistic and overlapping functions could be evidenced. Addition of 25 \(\mu\text{M}\) ATP significantly increased duration of mineralization process mediated by matrix vesicles, while supplementation of mineralization medium with levamisole, an alkaline phosphatase inhibitor, reduces the ATP-induced retardation of mineral formation. Phosphodiesterase activity of tissue nonspecific alkaline phosphatase for bis-\(p\)-nitrophenyl phosphate was confirmed, the rate of this phosphodiesterase activity is in the same range as that of phosphomonoesterase activity for \(p\)-nitrophenyl phosphate under physiological pH. In addition, tissue nonspecific alkaline phosphatase at pH 7.4 can hydrolyze ADPR. On the basis of these observations, it can be concluded that tissue nonspecific alkaline phosphatase, acting as a phosphomonoesterase, could hydrolyze free phosphate esters such as pyrophosphate and ATP, while as phosphodiesterase could contribute, together with plasma cell membrane glycoprotein-1, in the production of pyrophosphate from ATP.

Matrix vesicles (MVs), released by budding from specialized areas of plasma membranes of osteoblasts, chondrocytes, or odonblasts, are involved in the initial step of mineralization in all calcifying tissues, by promoting the seeding of basic calcium phosphate crystals of hydroxyapatite in its interior (1–3). MVs are markedly enriched in tissue nonspecific alkaline phosphatase (TNAP) (2), which plays an essential physiological role by providing inorganic phosphate (P\(_i\)) from various phosphorylated substrates during mineralization, by hydrolyzing inorganic pyrophosphate (PP\(_i\)), a potent inhibitor of mineralization (4–6) and by modulating bridging of MVs to matrix collagen (4, 7). The alkaline phosphatases (EC 3.1.3.1) are usually described as exo-phosphomonoesterases (exo-PME), which catalyze the hydrolysis of the phosphoryl group of phosphomonoesters at alkaline pH, by forming P\(_i\) and alcohol (8). It has been reported that ATP is hydrolyzed by human alkaline phosphatase by stepwise production of P\(_i\) and alcohol (8). TNAP is a metalloenzyme with three divalent cations (two Zn\(^{2+}\) and one Mg\(^{2+}\)) and a serine residue in the active site (10). It has been classified into a superfamily of phospho-/sulfo-coordinating enzymes catalyzing the hydrolysis of phosphate monoesters, diesters, triesters, and sulfate esters (11–15).

Mammalian plasma cell membrane glycoprotein-1 (PC-1, phosphodiesterase 1: EC 3.1.4.1/nucleotide pyrophosphatase: EC 3.6.1.9) is the type II transmembrane protein with a small intracellular and a large extracellular domain comprising the catalytic site (16, 17). The PC-1 is a metalloenzyme with two divalent cations (Mn\(^{2+}\), Zn\(^{2+}\), Ca\(^{2+}\), or Mg\(^{2+}\)) and a threonine residue in the active site (12, 15, 18–20). By comparing the structural model of the catalytic core of PC-1 with that of TNAP, it has been shown that the ion binding and active site residues in PC-1 and TNAP are superimposed (15). Moreover, the threonine residue at catalytic site of PC-1 coincides with the serine residue at catalytic site of TNAP (15), suggesting a similar catalytic mechanism for both types of enzymes.

Because at physiological pH, TNAP hydrolyzes P\(_i\), and PC-1 produces P\(_i\), from nucleotide triphosphates, the two enzymes have been recognized as antagonistic regulators of the mineralization process by modulating the extracellular P\(_i\) concentration (Fig. 1) (6, 21, 22). Alkaline phosphatases and PC-1, which belong to the same phospho-/sulfo-coordinating metalloenzyme superfamily, exhibit a broad substrate specificity. PC-1 is able to catalyze the reactions involving both phosphate diesters and phosphate monoesters (15, 23). In the case of alkaline phosphatase from Escherichia coli, the enzyme exhibits a phosphodiesterase (PDE) activity and a low sulfatase activity in addition to its well established PME activity (14). Furthermore, bovine intestinal alkaline phosphatase has been suggested to hydrolyze P\(_{i}\)-[1-(2-nitrophenyl)]-ethyl ester of ATP, an analogue of nucleotide without free phosphate monoester (24). Although previous reports demonstrated that mammalian alkaline phosphatases can act as type I phosphodiesterases (9, 25–29), there is no direct evidence about the role of PDE activity of mammalian alkaline phosphatase under physiological conditions.

Chondrocytes in the growth plate export continually nucleotides (ATP/UTP) that may regulate cell maturation, energy metabolism and...
matrix mineralization (30–33). The nucleotide concentration of the extracellular fluid aspirated from the hypertrophic zone of rat calciﬁng epiphysis cartilage was reported to reach 1–2 mM (34). Nucleotides (ATP/UTP) are also released both by non-stimulated (35, 36) or stimulated osteoblasts (37, 38) in response to mechanical stimulus. The measured amount of secreted ATP in solution by murine osteoblast-like cell in response to shear was in the order of 100 nM, however this does not accurately show the local ATP concentration at the cell surface immediately on its release (38). In addition, the osteoblasts have been shown to express a membrane-bound ecto-nucleoside diphosphokinase (ecto-NDPK), which allows the ADP→ATP conversion in extracellular calcification medium (35). Because of the physiological role of extracellular ATP in bone formation and remodeling, and its persisting presence in the calcification environment, ATP is expected to be one of the main sources of both P1 and PPi, which play opposite roles in bone formation and remodeling.

The purpose of the present work was to examine the roles of TNAP during the ATP-initiated deposition of calcium phosphate complexes by MVs isolated from femurs of 17-day-old chick embryos. We found that chicken TNAP possesses not only a PME activity that hydrolyzes PPi, but also a PDE activity that may delay the mineralization process by producing PPi from ATP.

**MATERIALS AND METHODS**

*Extraction of MVs—Collagenase-released MV were isolated from bone and epiphyseal cartilage slices of 17-day-old chicken embryos according to Wu et al. (39), with slight modiﬁcations. Slices of bone tissues were digested at 37 °C for 20 min in a synthetic cartilage lymph (SCL) containing 1 mM Ca++ and 0.1% trypsin (from bovine pancreas, ICN Biomedicals Inc.). SCL contained 1.42 mM Pi, 104.5 mM Na+, 133.5 mM Cl−, 63.5 mM sucrose, 16.5 mM TES, 15.5 mM K+, 5.55 mM d-glucose, 1.83 mM HCO3−, 0.57 mM Mg2++, and 0.57 mM SO42−. Then, the trypsin solution was discarded. Slices of bone tissues were rinsed with SCL 5–6 times to completely remove trypsin. Afterwards, the slices were digested at 37 °C for 3–3.5 h in an SCL solution containing 1 mM Ca++ and collagenase (type I, ICN Biomedicals Inc., 200 units/g of tissue with a volume of 4 ml/g of tissue) at 37 °C for 3–3.5 h. The partially digested tissue was vortexed, and the suspension was centrifuged at 13,000 × g for 20 min. The pellet was discarded, and the suspension was centrifuged again at 80,000 × g for 1 h. The MV pellet was suspended as a stock suspension of 1.0 mg of vesicle protein/ml in SCL at 4 °C for further use. Protein concentration in the vesicles was determined by the method of Bradford (40).

*Electron Microscopy of MVs—A drop of the suspension of MVs diluted to 25 μg of MV protein/ml was transferred to carbon-coated grids. Before the complete drying of MV sample, the grid was recovered by an aliquot of 2% uranyl acetate solution according to the negative staining method (41, 42) and dried. The grids were observed by means of an electron microscope Philips CM140 at 80 kV accelerating voltage.*

**Mineralization Assay**—The light scattering method (43) was employed for real time measurement of mineral formation by MV. MVs were suspended in SCL to a ﬁnal concentration of 10 μg of MV protein/ml, different ions (Ca++, P3+, P2+), substrates (ATP, ADP, AMP) or inhibitors (levamisole) were added into the SCL medium. Their respective concentrations are indicated in the ﬁgure legends. The samples were then incubated at 37 °C, and the absorbances read at 340 nm at 15-min intervals.

**Treatment of MVs by Phosphatidylinositol Phospholipase C (PI-PLC)—**

MVs (0.3–0.5 mg of MV protein/ml) were incubated in SCL containing 1–2 units/ml PI-PLC overnight at 37 °C under gentle vortexing. The supernatant (sMV) and the pellet (pMV) were separated by centrifugation at 100,000 × g for 30 min. The pMV was suspended in SCL in the same volume as before centrifugation.

**SDS-PAGE and Visualization of Alkaline Phosphatase Activity by BCIP-NBT Method—**

Electrophoresis was performed in 7.5% or 12% (w/v) SDS-polyacrylamide gel, according to Laemmli (44). Under mild denaturing conditions, which can preserve the activity of alkaline phosphatase, the samples were prepared in Tris buffer containing 2% SDS, but without addition of β-mercaptoethanol and without heating before migration. Proteins were stained with Coomassie Brilliant Blue R-250. After SDS-PAGE under mild denaturing conditions, gels were incubated in a buffer containing 0.1 mM Tris-HCl, pH 9.6, 100 mM NaCl, 5 mM MgCl2, 0.25 mM nitroblue tetrazolium (NBT), and 0.24 mM bromochloro-indolyl phosphate (BCIP) until the bands of blue color were clearly visible. The BCIP-NBT revealed speciﬁcally active alkaline phosphatase.

**Extraction of Alkaline Phosphatase from an SDS-PAGE Gel—**

SDS-PAGE in 7.5% (w/v) polyacrylamide gel under mild denaturing conditions, the small bands containing active alkaline phosphatase, as visualized by BCIP-NBT, were cut to extract the enzyme. Briefly, the sliced gel was crushed and vigorously vortexed in 100 mM Tris-HCl, pH 7.4, 10 mM Mg2++, and 0.5 μM Zn2+ to resolubilize TNAP. The supernatant was separated from the gel by centrifugation, and then washed with the same buffer by filtering 5–6 times the TNAP solution through Centricon-30 (Amicon) to remove the detergent and to restore TNAP activity.

**PME Activity Assay—**

PME activity of alkaline phosphatase was measured spectrophotometrically using p-nitrophenyl phosphate (pNPP) as substrate (45). A 5-μl aliquot was added to 1 ml of the reactive solution containing 25 mM piperazine, 25 mM glycylglycine and 10 mM pNPP at pH 10.4 or at pH 7.4. The change in absorbance of released p-nitrophenolate chromophore at 37 °C was monitored at 420 nm (ε = 18.5 cm−1·mol−1·cm−1 at pH 10.4, ε = 9.2 cm−1·mol−1·cm−1 at pH 7.4). One unit of the alkaline phosphatase PME activity was deﬁned as the amount of enzyme hydrolyzing 1 μmol of pNPP per min under described conditions.

**PDE Activity Assay—**

PDE activity was measured spectrophotometrically using bis-p-nitrophenyl phosphate (bis-pNPP) as substrate. A 5-μl aliquot was added to 1 ml of the reactive solution containing 25 mM piperazine, 25 mM glycylglycine, and 2 mM bis-pNPP at pH ranging from 7.0 to 10.5. The changes in absorbance because of the released p-nitrophosphonate chromophore at 37 °C were monitored at 420 nm. The activities determined at different pH were calculated using the absorbance coefﬁcients (ε) of p-nitrophosphonate at different pH. One unit of the PDE activity was deﬁned as the amount of enzyme hydrolyzing 1 μmol of bis-pNPP per min under described conditions.

**Determination of Hydrolysis of Sodium ADPR—**

The kinetics of the ADPR (Sigma) hydrolysis by MVs were determined in 200 μl of the reactive buffer containing 100 mM Tris-HCl, pH 7.4, 0.5 μM Zn2++, 10 mM Mg2++ at different ADPR concentration ranging from 0.2 mM to 2...
The pH effect on the hydrolysis of ADPR by MV was performed in 200 μL of piperazine/glycylglycine buffer (25 mM/25 mM) containing 0.5 mM Zn²⁺, 10 mM Mg²⁺, and 1 mM ADPR at different pH ranging from 7.0 to 10.5. After addition of enzyme, solutions were incubated at 37 °C for 6–10 h. The reaction was stopped by addition of an 800-μL reactive solution containing 0.5% ammonium molybdate, 2% H₂SO₄, and 0.6 M ascorbic acid. After 1–2 h incubation at 37 °C, the Pi levels were determined by measuring absorbance of assay solution at 740 nm according to Murphy and Riley (46). One unit of the ADPR hydrolysis activity was defined as the amount of enzyme hydrolyzing 1 μmol of ADPR per hour in the presence of 1 mM ADPR at pH 7.4 under described conditions.

**Immunoblotting Assay for the Detection of Caveolin 1**—Proteins of MVs were separated by SDS-PAGE in a 12% (w/v) polyacrylamide gel and then electrotransferred onto nitrocellulose membrane (Hybond™-ECL™, Amersham Biosciences). The result of transfer was visualized by Ponceau S (0.2% Ponceau S, 3% trichloroacetic acid). The nitrocellulose membrane was blocked with 3% gelatin solution in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.5, 500 mM NaCl) for 1 h at room temperature. The mouse monoclonal IgG against chicken caveolin type 1 (BD Biosciences) was diluted according to the manufacturer’s instructions in 1% gelatin solution in TBS-Tween 20 (TTBS, 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20) and incubated at 4 °C overnight. The nitrocellulose membrane was washed in TTBS and incubated for 1 h with goat anti-mouse IgG conjugated with alkaline phosphatase (Immun-Blot Assay Kit, Bio-Rad) in the same buffer as for primary antibody. The membrane was washed, and bands were visualized by addition of color-developing solution according to the manufacturer’s instructions.

**Identification of Mineral Complexes by Infrared Spectroscopy**—From the mineralization assay, the formed minerals were centrifuged and washed two times with water, then dried under N₂. Dry material (0.5 mg) was incorporated into KBr (150 mg) to form pellets. The infrared spectra were measured by means of Nicolet FTIR spectrometer model 510 M. The optical resolution was 4 cm⁻¹ but spectral points were encoded every 2 cm⁻¹. 128 scans were recorded. The infrared spectrum of hydroxyapatite standard (Sigma) was prepared and measured to identify the mineral complexes.

**RESULTS**

**Characterization of MVs**—The MVs isolated from femurs of 17-day-old chick embryos were found to be membranous structures of diameters ranging from 100 to 200 nm, as revealed by electron microscopy (Fig. 2A). They were similar to those extracted from growth plate cartilage of 6–8-week-old chicken (47). The specific PME activity of TNAP in MVs at pH 10.4 was 300 units/mg membrane protein. SDS-PAGE

**FIGURE 2. Characterization of MVs isolated from femurs of 17-day-old chicken embryos.** A, morphology of MVs observed under electron microscopy as described under “Materials and Methods.” The size range of spherical to oval MVs was 100–200 nm in diameter. B, SDS-PAGE of MVs and Western blot of MVs for the detection of caveolin. 1. Lane 1, protein standards; lane 2, 15 μg of MV protein profiles stained with Ponceau S; lane 3, 15 μg of MV proteins after Western blotting assay; lane 4, 15 μg of plasma membrane proteins after Western blotting assay. C, mineralization induced by MVs: symbols: (●), mineralization in SCL containing 2 mM Ca²⁺, 1.42 mM Pi, and 10 μg of protein/ml MVs; (○), control of mineralization in SCL containing 2 mM Ca²⁺ and 1.42 mM Pi, but without MV. The mineral formation was assessed by light scattering at 340 nm. D, infrared spectra of mineral deposits produced by MVs. Trace i, infrared spectrum of mineral deposits formed under the same conditions as for turbidimetry measurements; trace ii, infrared spectrum of hydroxyapatite standard; trace iii, infrared spectrum of mineral deposits formed in SCL containing 2 mM Ca²⁺, 3.42 mM Pi, 0.33 mM ATP, and 10 μg of protein/ml MVs, incubation at 37°C.
conditions for the turbidity measurements were analyzed by infrared initialization of mineralization. The mineral formed under the same changes in turbidity were almost negligible over a 12-h incubation time (Fig. 2).

The presence of MV proteins was indicated by the 1652-cm−1 band of the mineral formed in the presence of 0.33 mM ATP showed that there was no hydroxyapatite-like mineral but only other calcium-phosphate complexes as characterized by the broad bands in the 1082 cm−1 to 1031 cm−1 region (Fig. 2D, trace iii). However, in the presence of a relatively low concentration of ATP (< 0.2 mM), MVs were still capable to initiate the formation of hydroxyapatite (Fig. 4, traces i-v), showing a dependence of hydroxyapatite formation on the concentration of ATP in the mineralization medium. Addition of ATP concentration up to 1 mM in the mineralization medium did not lead to the accumulation of calcium-PPi complexes as revealed by the absence of the characteristic 930 cm−1 band of the calcium-PPi complex (51) in the infrared spectra of minerals formed by the MVs (Fig. 4, trace vi).

The effect of ATP concentration on the duration of induction phase provided more insight into its characteristic and origin of the retardation of mineral formation. The mineralization process was initiated in SCL containing 2 mM Ca2+ and 3.42 mM Pi with various ATP concentrations ranging from 0 to 75 μM (Fig. 5A). To match the physiological conditions in the extracellular medium, the ATP concentration range was within the affinity value of mammalian-cartilage ATPase for ATP (20 μM) (53). In addition, human osteoblast ecto-NDPK convert efficiently 10 μM ADP into 4 μM ATP in the presence of 20 μM GTP (ADP+GTP ⇌ ATP+GDP) (36). Supplementation of SCL with 2 mM Pi reduced the induction phase of mineralization to about 0.5–1 h (Fig. 3), allowing us to obtain a better estimation of the retardation effect of ATP during the mineralization. Addition of 25 μM ATP was sufficient to induce a significant 3-hour retardation in the delay of mineral formation; and the duration of induction phase was prolonged from 0.5 to 18 h when the ATP concentration increased from 0 to 75 μM (Fig. 5A). A similar delay of induction phase of mineralization was also observed in the presence of PPi, but at a concentration range about 7–8 times lower than that of ATP (Fig. 5B), consistent with the potent inhibitory effect of PPi on the mineralization (4, 5). Neither P2, AMP, nor ADP could promote an 18-h delay of mineral formation under the same conditions (results not shown). These findings suggested that either ATP or PPi, may be
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FIGURE 5. Retardation of mineralization induced by ATP and by PP. MVs (10 µg/ml) were incubated in SCL buffer containing 2 mM Ca\(^{2+}\), 3.42 mM Pi, and ATP or PP, at various concentrations. Mineral formation was assessed by light scattering at 340 nm. Only induction phase and rapid mineral formation are shown. A, ATP (●), 25 µM (○), 50 µM (△); B, PP (●), 0.5 µM (○), 1 µM (△), 2 µM (▲), 4 µM (▲▲).

FIGURE 6. Effects of levamisole on the inhibition of mineralization by ATP. Mineralization conditions: 10 µg/ml MVs in SCL containing 2 mM Ca\(^{2+}\), 3.42 mM Pi, 50 µM ATP, and levamisole at different concentrations: 0 (■), 0.5 mM (△), 1 mM (○), 2 mM (▲), 4 mM (▲▲).

FIGURE 7. Chemical structure of the ADPR, ATP, and the possible cleavage positions in their hydrolysis. ADPR can be hydrolyzed either by a phosphodiesterase enzyme corresponding to the cleavages at positions i and ii or by phosphoanhydrase enzyme corresponding to the cleavage at position iii. In the case of the hydrolysis of ATP, both phosphodiesterase activity (i) and phosphoanhydrase activity (ii) may lead to the formation of pyrophosphate, the inhibitor of mineralization.

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responsible for the ATP-induced lag time in mineralization (Fig. 5). In the presence of levamisole, an inhibitor of alkaline phosphatase, the delay of retardation of mineral formation caused by the addition of 40 µM ATP was reduced with the increase of levamisole concentration from 0 to 4 mM (Fig. 6), suggesting that TNAP may be involved in the ATP-induced retardation of mineralization.

Experimental Evidence Indicates That TNAP Could Produce PP.—TNAP has a well established PME/phosphoanhydrase activity, which could hydrolyze ATP sequentially by releasing Pi (9), and cleave PP (56), but also a less known PDE activity (14, 28, 29). Given the structural similarity in the active centers of TNAP and PC-1 (15), one cannot exclude that TNAP could have a hydrolytic activity contributing to the formation of PP, from ATP. Therefore, a nucleotide analogue, ADPR, was used as a substrate to examine whether TNAP could have a PDE or a phosphoanhydrase activity, because there is no free terminal phosphate available for exo-PME activity of TNAP in ADPR as in ATP (Fig. 7).

To separate TNAP from MVs, MVs were incubated with PI-PLC. After centrifugation, TNAP without GPI anchor was detected in the supernatant (sMV), while MVs devoid of their GPI-anchored membrane proteins formed the pellet (pMV). As PC-1 is a transmembrane protein (16, 17), it should remain in the pMV. To control the efficiency of the separation of TNAP from MVs, SDS-PAGE under mild denaturing conditions was performed, and active TNAP was stained by BCIP-NBT (Fig. 8). Active TNAP with GPI anchor formed a band with an apparent molecular mass of 116 kDa, as indicated in Fig. 7 (lanes 1 and 3). Released TNAP without GPI anchor, obtained by PI-PLC treatment, migrated more slowly as indicated by a band of apparent molecular mass of ∼130 kDa (Fig. 8, lane 2), in accordance with the observed behavior of the mammalian alkaline phosphatase lacking its GPI anchor (57). 80–95% TNAP were usually released from MVs after the PI-PLC treatment as determined by the PME activity at pH 10.4 (TABLE ONE), consistent with a previous report (52). The PME activities at pH 10.4 (PME\(_{10.4}\)) and at pH 7.4 (PME\(_{7.4}\)) were determined by using pNPP as substrate, while the PDE activity at pH 7.4 (PDE\(_{7.4}\)) was followed by determining the hydrolysis of bis-pNPP (PDE\(_{-2.4}\)). The hydrolysis of ADPR at pH 7.4 (ADPR\(_{-2.4}\)), reflecting a PDE or a phosphoanhydrase activity (Fig. 7), was determined by measuring the Pi level. TABLE ONE represents the PME, PDE, and ADPR hydrolytic activities of MVs, and fractions sMV and pMV.

Chicken TNAP has an optimum activity for pNPP hydrolysis at alkaline pH 10.5 while its activity in MVs at pH 7.4 was about 60 ± 10 times lower (TABLE ONE), consistent with earlier findings (45). The percentage of the PME activity of sMV compared with that of the normal MVs was 92% ± 5% at pH 10.4 and 87% ± 5% at pH 7.4 (TABLE ONE), indicating that more than 87% of PME activity is rather associated with TNAP. The remaining PME activity (−8–13%) in MVs at physiological pH should correspond to the activities of TNAP not completely released by PI-PLC from MVs and to other enzymes in MVs having PME activities (possibly including PC-1).

After PI-PLC treatment, most of PDE\(_{7.4}\) activity (92% ± 5%) was found in sMV, the percentage of PDE\(_{7.4}\) activity was almost identical to the percentage of PME\(_{10.4}\) activity (TABLE ONE). In addition, as PME activity of TNAP can be inhibited by levamisole, the PDE activities of MV, sMV, and pMV using bis-pNPP as substrate were all inhibited by levamisole in the same manner at pH 7.5 (results not shown), suggesting a strong association of PDE activity with TNAP. The effects of pH on the PDE activities were compared with intact MVs, sMV, and pMV. The...
PDE activity for bis-pNPP was maximal at pH 9.0 for all three samples (Fig. 9A), with the same apparent $K_m$ for bis-pNPP of 6 ± 1 mM. This value should be considered as the apparent $K_m$ for TNAP at pH 9.0. PDE activity can also be inhibited by P$_i$, in a competitive manner, with a $K_i$ of 0.20 ± 0.05 mM under our experimental conditions. Taking these findings together, we concluded that TNAP was the principal protein in MV that has a PDE activity for bis-pNPP.

After PI-PLC treatment, pMV kept apparently an almost intact ADPR activity (100% ± 30%) (TABLE ONE), indicating that a transmembrane protein in pMV can hydrolyze ADPR. A number of nucleotide pyrophosphohydrolases have been reported to cleave nucleotide diphosphate sugars (53, 58, 59). Most likely PC-1, the transmembrane protein, is responsible for the ADPR hydrolysis in pMVs. The sMV fraction also exhibited a strong ADPR hydrolysis activity (80% ± 25%), indicating that at least a GPI-anchored membrane protein in sMV is capable to hydrolyze ADPR. When the pH increased from 7.0 to 9.5, only small and similar changes of ADPR hydrolysis activities were observed for the three samples (MV, sMV, and pMV) (Fig. 9B). From pH 9.5, the increase of pH led to a large increase of ADPR hydrolysis of MV and sMV, reflecting the alkaline preference of enzymatic action of TNAP. However, the increase of pH up to 10.5 did not lead to any significant change of ADPR hydrolysis activity of pMV, suggesting that a distinct protein participated in the ADPR hydrolysis in the pMV (PC-1) than the protein in sMV fraction (TNAP). The ADPR hydrolysis activities of MV, sMV, and pMV were all inhibited by levamisole in the same manner (results not shown), suggesting that both TNAP in sMV and PC-1 in pMV can be inhibited by levamisole.

To confirm the ADPR hydrolysis activity of TNAP, we isolated active TNAP from the gel of SDS-PAGE under mild denaturing conditions. Active TNAP from MV (Fig. 8, lane 1) and from sMV (Fig. 8, lane 2) were assayed for ADPR hydrolysis. A $K_m$ of 1.5 ± 0.2 mM and a $V_{max}$ of 6.3 ± 0.5 nmol of ADPR hydrolyzed per min per unit of PME$_{10.4}$ activity of TNAP were found for all three samples (sMV, gel extracted TNAP with and without GPI anchor), indicating that TNAP is the only GPI-anchored membrane protein that hydrolyzes ADPR. Because of the difficulty in the determination of the concentration of TNAP in MVs, the protein concentration of TNAP is expressed by its PME activity measured at pH 10.4 under our experimental conditions. The $K_m$ of PMV for ADPR was identical to the $K_m$ of TNAP for ADPR. Under our experimental conditions, the $V_{max}$ of pMV and sMV (TNAP) for ADPR hydrolysis were in the same order of magnitude, suggesting that TNAP and PC-1 are close enzymes with respect to ADPR hydrolysis at pH 7.4.

### DISCUSSION

Origin of Retardation of ATP-initiated Mineralization—In the absence of inhibitors, the rate of mineralization initiated by MVs depends on the $P_i$ and calcium concentrations in the incubation medium (60–63). Although PP$_i$ inhibits hydroxyapatite seeding (62), the uptake of $P_i$ and calcium by MVs is not significantly affected by the presence of ATP or PP$_i$ at low concentrations (51, 61, 64, 65). In this report, we used ADPR to detect the enzymes that are able to release PP$_i$ from ATP. The hydrolysis of ADPR may imply either a cleavage in the middle of the phosphoanhydride bond (cleavage at position i or iii) or a cleavage of phosphodiesterase bonds between ribose and phosphate (cleavage at position i or iii), both of which can lead to the formation of $P_i$ in the case of hydrolysis of ATP (Fig. 7). We found that both sMV and pMV fractions were able to hydrolyze ADPR and that their ADPR hydrolysis activities were inhibited by levamisole, suggesting that the amount of $P_i$ generated from ATP by MVs is reduced in the presence of levamisole. The retardation of mineralization in MVs caused by ATP (Fig. 5A) was equivalent to the retardation caused by $P_i$, at a concentration 7–8 times lower (Fig. 5B). Because neither $P_i$, AMP, nor ADP promoted such retardation, the ATP-initiated retardation of mineralization should be mediated by $P_i$, rather than by ATP itself.

Although we did not determine directly the effects of TNAP on ATP, the rate of ATP pyrophosphohydrolase activity of MVs could be roughly estimated. 4 $\mu$M $P_i$ led to a 4-h retardation of mineralization. To have the same retardation effect, about 30 $\mu$M ATP was needed (Fig. 5A), indicating that about 4 $\mu$M $P_i$ could be formed from 30 $\mu$M ATP. The rate of the conversion of ATP in $P_i$ is rather approximate since ATP is continuously hydrolyzed by TNAP, ATPase, and other PME enzymes (including PC-1) in addition to the uninterrupted hydrolysis of PP$_i$ by TNAP. That accounts to the difficulty for a direct measurement on the effects of TNAP on ATP.

Under our experimental conditions, the rate of ADPR hydrolysis was about 1.2 ± 0.4 $\mu$mol ADPR hydrolyzed per hour by 10 $\mu$g of MV proteins/ml in the presence of 1 mM ADPR. Given the potent inhibitory effect of $P_i$ for mineralization (Fig. 5B), such ATP pyrophosphohydrolase activity is high enough to maintain the inhibition of mineralization until ATP and $P_i$ are completely removed from mineralization medium. This may explain why when the initial ATP concentration was relatively low (≤ 0.2 mM), we could still observe the formation of hydroxyapatite crystals (Fig. 4, traces i-iv), but when the initial concentra-
tration of ATP was sufficiently high (≥ 0.33 mM), ATP and PP, could not be removed completely during complexation of P, and calcium, and other complexes were produced (Fig. 2D, trace iii; Fig. 4, traces v and vi). This finding points up the role of phosphoanhydride activity of TNAP in the removal of PP, (6, 21, 22).

Addition of levamisole failed to inhibit MV-initiated mineralization in the presence of ATP (Fig. 6), which is consistent with the earlier findings made by other investigators suggesting that ATPases are responsible for MV-initiated calcification in the presence of ATP (52, 65). Consequently, the role of ATPases for the hydrolysis of ATP should be even more important when the PME activity of TNAP is inhibited. However, the origin of the ATP-induced retardation of mineralization is associated with the formation of PP, and cannot be explained solely on the basis of the presence of active ATPases. In fact, various enzymes of MVs may compete for the same substrate, e.g., ATP, and their relative amounts could influence the production of PP. Even minor variations in PP, concentrations may have relatively large effects on the mineral formation (Fig. 5B).

Functional Complexity of TNAP in Biomineralization—The sum of ADPR hydrolysis activity of pMV and sMV was generally much higher or even doubled as compared with the hydrolysis of ADPR by MVs not treated with PI-PLC (TABLE ONE), suggesting that the ADPR hydrolysis activity was partially inhibited in intact MVs under our experimental conditions. As MVs were extracted from femurs of 17-day-old chicken embryos, it is tempting to suggest that some unknown factors within MVs of these femurs may restrict the nucleotide pyrophosphohydrolase activity to a minimal level to maintain fast bone growth. We speculate that with aging, the inhibition of this activity is released. This could explain why PC-1 does not account for all nucleotide pyrophosphohydrolase activity in osteoblasts (66), and the PC-1−− osteoblasts still contain ~50% nucleotide pyrophosphohydrolase activity (67). It may also partially answer the question why the specific activity of PC-1 was 2-fold greater in MV fractions of osteoblasts from TNAP−/− mice relative to TNAP−/− mice (21). In all of these cases, TNAP may act as a nucleotide pyrophosphohydrolase, in addition to its expected PME activity (TABLE ONE).

As discussed previously, TNAP is the principal enzyme in MV that possesses PME activity at pH 7.4. Although TNAP also has a significant ADPR hydrolysis activity that may contribute to the formation of PP, from ATP, its main physiological function is the removal of PP, from mineralization medium due to its PME/phosphoanhydride activity. In humans, a large number of hypophosphatasia cases is linked to the inactivation of TNAP (for TNAP mutation-hypophosphatasia data base, see www.sesep.uvsq.fr/Data base.html). From our findings, PME, PDE, and ADPR hydrolytic activities of TNAP should share the same active site, since these activities are all inhibited by levamisole. In the case of the mutation that inactivates TNAP, the loss of ADPR hydrolysis activity could still be complemented by that of PC-1, but the loss of PME/phosphoanhydride activity cannot be remedied by others, resulting in the increase of extracellular PP, concentration in calcification environment.

Concluding Remarks—Although TNAP is considered as an antagonistic enzyme to PC-1 toward ATP-induced mineral formation, because of its hydrolysis activity toward PP, a reevaluation of multiple roles of TNAP in bone formation and remodeling is required because of our observation of significant ADPR hydrolysis activity of TNAP in isolated MVs. Our findings provide more insights into a new role of alkaline phosphatase as a phosphodiesterase/ATP pyrophosphohydrolase during biomineralization, showing the functional complexity of TNAP and the intricacy of bone mineralization process in which many enzymes are involved. In this respect, this type of activity of mammalian alkaline phosphatase could play an important role not only in biomineralization but also in other normal physiological and in pathological processes.

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REFERENCES
1. Ali, S. Y. (1992) in Bone Biology and Skeletal Disorders (Whitehead, C. C., ed) pp. 19–38, Carfax/Abingdon, London.
2. Anderson, H. C. (1995) Clin. Orthop. Relat. Res. 314, 266–280
3. Boskey, A. L. (1996) Connect. Tissue Res. 35, 357–363
4. Meyer, J. L. (1984) Arch. Biochem. Biophys. 231, 1–8
5. Johnson, K., Vaingankar, S., Chen, Y., Moffa, A., Goldring, M. B., Sano, K., Jin-Hua, P., Sali, A., Goding, J. W., Terkeltau, R., and Millan, J. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9445–9445
6. Mornet, E., Stura, E., Lia-Baldini, A. S., Stigbrand, T., Menen, A., and Le Du, M.-H. (2001) J. Biol. Chem. 276, 31171–31178
7. Holl, W. E., Halford, S. E., Gutfried, H., and Sykes, B. D. (1976) Biochemistry 15, 1547–1561
8. Moss, D. W., Eaton, R. H., Smith, J. K., and Whitby, L. G. (1967) Biochem. J. 102, 37295
9. Moss, D. W., and Walli, A. K. (1969) Biochim. Biophys. Acta 191, 476–477
10. Kim, E. E., and Wyckoff, H. W. (1991) J. Mol. Biol. 218, 449–464
11. Strater, N., Lipscomb, W. N., Klubunde, T., and Krebs, B. (1996) Angew. Chem. Int. Ed. Engl. 35, 2024–2055
12. Wilcox, D. E. (1996) Chem. Rev. 96, 2435–2458
13. Galperin, M. Y., Bairoch, A., and Koonin, E. V. (1998) Protein Sci. 7, 1829–1835
14. O’Brien, P. J., and Herschlag, D. (2001) Biochemistry 40, 5691–5699
15. Gijbers, R., Ceulemans, H., Stalmans, W., and Bollen, M. (2001) J. Biol. Chem. 276, 1361–1368
16. Goding, J. W., Terkeltau, R., Maurice, M., Deterre, P., Sali, A., and Belli, S. I. (1998) Immunol. Rev. 161, 11–26
17. Goding, J. W. (2000) J. Leukocyte Biol. 67, 285–311
18. Belli, S. I., Sali, A., and Goding, J. W. (1994) Biochem. J. 304, 75–80
19. Belli, S. I., Mercut, F. A., Sali, A., and Goding, J. W. (1995) Eur. J. Biochem. 228, 669–676
20. Stefan, C., Stalmans, W., and Bollen, M. (1996) Eur. J. Biochem. 241, 338–342
21. Johnson, K. A., Hesse, L., Vaingankar, S., Wenneberg, C., Mauro, S., Narisawa, S., Goding, J. W., Sano, K., Millan, J. L., and Terkeltau, R. (2000) Am. J. Physiol. Regul. Integr. Comp. Physiol. 279, 1365–1377
22. Harney, D., Hesse, L., Narisawa, S., Johnson, K. A., Terkeltau, R., and Millan, J. L. (2004) Am. J. Pathol. 164, 1199–1209
23. Clair, T., Lee, H. Y., Liotta, L. A., and Stracke, M. L. (1997) J. Biol. Chem. 272, 996–1001
24. Zhang, L., Buchet, R., and Azzar, G. (2004) Biochem. J. 387, 3873–3881
25. Moss, D. W., Eaton, R. H., Smith, J. K., and Whitby, L. G. (1967) Biochem. J. 102, 1911–1912.
