Human PHOSPHO1 exhibits high specific phosphoethanolamine and phosphocholine phosphatase activities

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Human PHOSPHO1 is a phosphatase enzyme for which expression is upregulated in mineralizing cells. This enzyme has been implicated in the generation of P_i for matrix mineralization, a process central to skeletal development. PHOSPHO1 is a member of the haloacid dehalogenase (HAD) superfamily of Mg^{2+}-dependent hydrolases. However, substrates for PHOSPHO1 are, as yet, unidentified and little is known about its activity. We show here that PHOSPHO1 exhibits high specific activities toward phosphoethanolamine (PEA) and phosphocholine (PCho). Optimal enzymic activity was observed at approx. pH 6.7. The enzyme shows a high specific Mg^{2+}-dependence, with apparent K_m values of 3.0 μM for PEA and 11.4 μM for PCho. These results provide a novel mechanism for the generation of P_i in mineralizing cells from PEA and PCho.

Key words: bone, haloacid dehalogenase (HAD) superfamily, mineralization, PHOSPHO1, phosphocholine (PCho), phosphoethanolamine (PEA).

INTRODUCTION

Matrix vesicle (MV)-mediated mineralization is a process central to the formation of bone, cartilage and teeth. Inside the MV, calcium phosphate accumulates until sufficient amounts are present for precipitation to occur. This is then converted to an intermediate, octa-calcium phosphate, crystals of which are transformed into the less soluble hydroxyapatite [1]. The MV membranes then breakdown and release preformed hydroxyapatite into the extracellular fluid. Calcium accumulation is controlled by Ca^{2+}-binding molecules, such as annexin I and phosphatidylserine [2,3]. P_i accumulation is associated with the action of alkaline and acid phosphatases [4,5]. The most abundant of these being tissue non-specific alkaline phosphatase (TNAP), an isoenzyme of alkaline phosphatase expressed in bone, liver and kidney [6]. In addition to its structural role, P_i has also been shown to regulate multiple genes during osteoblast differentiation, including the immediate response gene, *Nrf2* [7].

Deficiency of P_i in skeletal tissue (termed hypophosphatasia) is highly variable in its clinical expression, ranging from death *in utero* with an unmineralized skeleton to premature loss of teeth [8]. Hypophosphatasia is usually attributed to a reduction in TNAP activity. In newborn TNAP knockout mice, bone development and mineralization appear to be normal, although hypomineralization and other abnormalities of the skeleton and dentition have subsequently been observed [9–11]; failure occurs in the propagation of the mineral from the MV to the surrounding extracellular matrix [12,13]. Support for this concept comes from earlier work [14], which shows that the catalytic activity of TNAP decreases in direct proportion to the extent that MVs induce mineral formation. TNAP is known to hydrolyse inorganic PP_i [15], which is a potent inhibitor of hydroxyapatite crystal formation [16]. Abnormalities found in TNAP knockout mice are, however, not present in TNAP/PC-1 double-knockout mice [9]. PC-1 (now known as NPP1) encodes the enzyme, phosphodiesterase I in mineralizing cells and generates PP_i from nucleotide triphosphates [17]. Studies have also shown that TNAP can be removed from some preparations of MVs without reducing their potential to mineralize [18], whilst specific inhibitory studies on TNAP provide additional evidence that other phosphatases are present within mineralizing chondrocytes [19]. These observations suggest that the primary role of TNAP in skeletal development is to hydrolyse PP_i, preventing its inhibition of mineral crystal growth. It therefore appears that TNAP is not essential, at least for the initial events leading to MV-induced mineralization and implies that other phosphatases are involved.

Recently a novel phosphatase, PHOSPHO1, was identified which is expressed at levels approximately 100-fold higher in mineralizing chondrocytes than in non-skeletal tissues [20]. Immunolocalization studies have since shown that PHOSPHO1 is specifically localized to mineralizing regions of skeletal tissue [21]. The amino acid sequence of PHOSPHO1 contains three peptide motifs that are conserved within the haloacid dehalogenase (HAD) superfamily of Mg^{2+}-dependent hydrolases. Human PHOSPHO1 shares approximately 30 % homology at the amino acid level with the LePS2 family of phosphatases [22,23]. Molecular modelling of human PHOSPHO1, based upon the crystal structure of phosphoserine phosphatase from *Methanococcus jannaschii*, shows that all the characteristic features of the catalytic site, with regard to the HAD superfamily, are preserved [24]. Despite these structural data, little is known about the phosphatase activity of PHOSPHO1. We report here a biochemical characterization of the enzyme and establish its substrate specificity and conditions for optimal activity. The data allow us to propose a new pathway for the generation of P_i in mineralizing cells that is coupled to the degradation of phospholipids.

EXPERIMENTAL

Materials

SaOS-2 osteosarcoma cells were purchased from the European Collection of Cell Cultures (ECACC; CAMR Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wilts, U.K.).

Abbreviations used: BAP, brain alkaline phosphatase; CDP-Cho, cytidine 5′-diphosphocholine; CDP-EA, cytidine 5′-diphosphoethanolamine; HAD, haloacid dehalogenase; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; MV, matrix vesicle; Ni-NTA, nickel-nitrilotriacetate; PEA, phosphoethanolamine; PCho, phosphocholine; PNPase, purine nucleoside phosphorylase; TBS, Tris-buffered saline; TNAP, tissue non-specific alkaline phosphatase.

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Reverse transcriptions were carried out using the Gibco SuperScript™ First-Strand Synthesis System for RT-PCR. Custom DNA oligonucleotides were purchased from MWG-Biotech UK Ltd. (Milton Keynes, U.K.). Plasmid isolation and purification was carried out using the Promega Wizard DNA purification kit. The chemically competent *Escherichia coli* TOP10 cells, the pBAD TOPO TA expression kit and the V5 antibody were purchased from Invitrogen (Paisley, U.K.). Restriction enzymes were purchased from New England BioLabs (Hitchin, Herts., U.K.). Thermal cycling was performed using a Hybaid PCR Express Thermal Cycler. Nickel-nitrilotriacetate (Ni-NTA)-agarose was purchased from Qiagen (Crawley, West Sussex, U.K.). Complete® protease inhibitor cocktail was purchased from Roche. SDS/PAGE was performed on precast 10 % acrylamide mini-gels using a Bis-Tris buffered system (NuPAGE, Invitrogen). Gels were stained directly for protein using Coomassie Brilliant Blue R (Sigma). Tryptic digests and matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI–TOF-MS) were carried out by the Functional Genomics Unit at the Moredun Research Institute. BioDesign dialysis tubing (8,000 Molecular mass cut-off) was purchased from VWR International. Human PHOSPHO1 concentrations were determined in 96-well plates using the Bio-Rad protein assay kit with gammaglobulin as standard. Optical spectroscopy of 96-well plates was performed using a Dynatech MR7000 plate-reader unless otherwise stated. Ammonium molybdate, L-arabinose, ATP, β-glycerol phosphate, fructose 6-phosphate, glycone phosphate, Hepes, imidazole, $MgCl_2$ (99 % pure), Malachite Green, NaCl, *p*-nitrophenylphosphate, PCho (phosphocholine), PEA (phosphoethanolamine), phospho-L-serine, phospho-L-tyrosine, pyridoxal-5-phosphate, pyrophosphate sodium salt, ribose-5-phosphate and Trizma base were purchased from Sigma Chemical Co. $CaCl_2$, $CoCl_2$, $NiCl_2$ and $MnCl_2$ (all AnalaR grade) were purchased from BDH–Merck Ltd. (Lutterworth, Leics., U.K.). $ZnCl_2$ (99.99 % pure) was purchased from Acros Organics (Den Bosch, The Netherlands).

Production of recombinant human PHOSPHO1

RNA was isolated from SaOS-2 osteoblast-like cells by phenol/chloroform extraction and reverse transcribed. cDNA corresponding to Met^{19}–Cys^{267} of human PHOSPHO1 was amplified with the specific primers, hs_phos1-f1 primer (5′-ATGGCCGCGCAGGGC-3′) and hs_phos1-r1 primer (5′-GCACGACTTCAGCACCTGTTGC-3′). This strategy was adopted in view of the ambiguity concerning the initiation codon of PHOSPHO1 and, therefore, we expressed a protein containing only the region that would be common to all predicted forms. The cDNA fragment was subcloned into the pBAD TOPO TA vector. The construct was designed to express PHOSPHO1 fused to a V5 epitope and 6 His-tag at the C-terminus. A clone (pBAD-PHOSPHO1) containing the PHOSPHO1 fragment in the correct orientation was identified by restriction digestion of plasmid minipreps. The *E. coli* cells were grown in Luria–Bertani broth (10 litres, 37 °C) and recombinant protein expression was induced by treatment with 0.1 % (w/v) L-arabinose for 4 h. Bacteria were harvested by centrifugation and were resuspended in a lysis buffer containing 50 mM potassium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole and 1.6 mg/ml of Complete® protease inhibitor cocktail. Cells were lysed using a French press (16 000 p.s.i., 16 °C). A clarified lysate was prepared by centrifugation at 20 000 ***g*** for 1 h. A 5 ml Ni-NTA–agarose column was equilibrated with 50 ml of lysis buffer. Following equilibration, a 20 ml aliquot of the clarified lysate was applied to the column. The column was then washed with 50 ml of Tris-buffered saline (TBS) pH 8.0 containing 20 mM imidazole, and eluted in 5 ml fractions each by addition of a single column volume of TBS, pH 8.0, containing 250 mM imidazole. The fraction containing the pure recombinant protein was then dialysed three times in TBS, pH 7.2 (5 l, 4 °C, 24 h) and stored at 4 °C prior to use.

Western blotting

Following dialysis, 1 μg of purified protein was subjected to SDS/PAGE as described above and transferred to nitrocellulose filters using the transfer buffer supplied by the manufacturer. After transfer, filters were blocked for 1 h in PBS containing 4 % dried milk powder and 20 % horse serum. The filters were then incubated in blocking solution containing 1:3000 dilution of mouse monoclonal horseradish peroxidase-labelled anti-V5 antibody and washed three times in PBS. They were then developed by incubation in a solution containing 25 mM Tris/HCl, pH 7.2, 75 mM NaCl, 0.25 mg/ml diaminobenzidine, 0.1 mg/ml $CoCl_2$, 0.15 mg/ml urea hydrogen peroxide.

Phosphatase assays

The standard discontinuous colorimetric assay used was based on that of Baykov et al. [25]. The reactions were measured in 96-well plates containing 200 μl of 25 % (w/v) glycerol, 20 mM TBS, pH 7.2, 25 μg/ml BSA, 2.5 mM substrate, 2 mM of the corresponding divalent metal chloride salt and 600 ng of purified recombinant PHOSPHO1. For investigation of the effect of pH on PHOSPHO1 activity, 20 mM Mes was used to obtain pH values between 5.0 and 6.7, and 20 mM 3-(cyclohexylamino)propane-1-sulphonic acid (Caps) for pH 9.0, in place of TBS. The ionic strength of each buffer was adjusted to that of 20 mM TBS by addition of NaCl. Standard solutions containing known concentrations of KH_2PO_4 were included in each plate. Reactions were allowed to proceed for 15 min at 37 °C then stopped by the addition of 50 μl of 3.75 M sulphuric acid containing 3 % ammonium molybdate, 0.2 % Tween 20 and 0.12 % Malachite Green. The absorbance of each well at 630 nm was measured and the specific activity was calculated in units of activity per mg of enzyme, where 1 unit of activity represents the hydrolysis of 1 nmol of phosphate per min.

The continuous spectrophotometric assay was performed using the EnzChek® Phosphatase Assay Kit (Molecular Probes, Eugene, OR, U.S.A.), which is based upon the purine nucleoside phosphorylase (PNPase)-coupled assay reported by Webb [26]. The reactions were measured in 96-well plates containing 25 % (w/v) glycerol, 20 mM Mes, pH 6.7, 500 mM NaCl, 2 mM $MgCl_2$, 0.2 unit PNPase, 200 μM MESG (2-amino-6-mercapto-7-methylpurine ribonucleoside) and 144 ng of purified recombinant PHOSPHO1 at 37 °C. PNPase and MESG concentrations were optimized to ensure that the phosphatase activity was rate-limiting. PHOSPHO1 substrate concentrations were varied accordingly. Absorbances were measured continuously at 355 nm using a VICTOR HTS plate-reader.

RESULTS

Purification of recombinant human PHOSPHO1

Recombinant His-tagged PHOSPHO1 protein in fractions eluted from a Ni-NTA–agarose column was assayed by SDS/PAGE. Typically, fraction 2 yielded a single band of the expected mass (32 kDa) consistent with > 99 % purity (Figure 1A). The final yield of protein was approx. 35 mg per 10 litres of culture. Western blotting of the purified protein yielded a band of expected

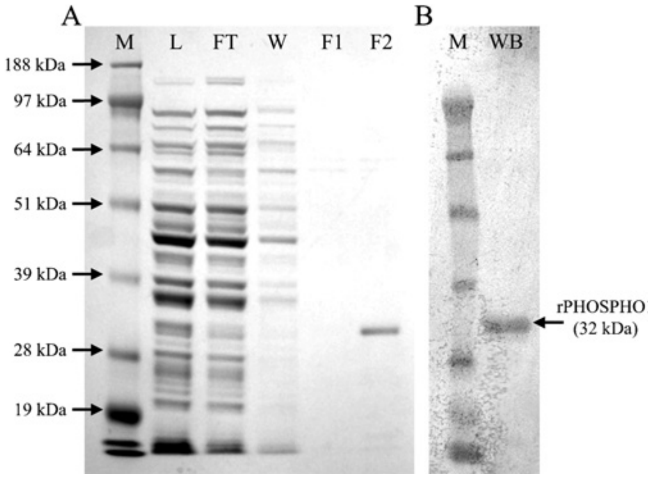


Figure 1 SDS/PAGE and Western analysis of purified recombinant human PHOSPHO1

(**A**) The cell lysate (L) and the flow-through (FT), wash (W) and eluted fractions 1 and 2 (F1 and F2) from each stage of Ni-NTA purification were subjected to SDS/PAGE under reducing conditions and visualized by Coomassie Blue staining. Molecular mass standards are also shown (M). (**B**) Western blot (WB) of the purified protein (1.2 μg) with anti-V5 antibody, which recognizes the V5 epitope tag fused to the recombinant protein near its C-terminus.

Table 1 Substrate specificity of recombinant human PHOSPHO1

Recombinant human PHOSPHO1 (3 μg/ml) was incubated with each substrate and assayed for phosphatase activity by the discontinuous assay at 37 °C. The 200 μl reaction mixture contained 25 % (w/v) glycerol, 20 mM TBS, pH 7.2, 25 μg/ml BSA, 2.5 mM substrate and 2 mM $MgCl_2$. The results are the means $\pm$ S.E.M. of triplicate assays.

Compound	Specific activity (units/mg)
Phosphoethanolamine	4600 ± 582
Phosphocholine	2980 ± 335
Ribose 5-phosphate	74.8 ± 6.2
p-Nitrophenyl phosphate	64.5 ± 36.6
β-Glycerol phosphate	39.6 ± 6.2
Pyridoxal-5-phosphate	17.6 ± 12.4
Pyrophosphate	< 0.1
Phospho-L-serine	< 0.1
Glycone phosphate	< 0.1
Fructose 6-phosphate	< 0.1
Phospho-L-tyrosine	< 0.1
ATP	< 0.1

size and showed the presence of the V5-epitope tag (Figure 1B). The purified protein was also confirmed as recombinant PHOSPHO1 by MALDI–TOF MS of tryptic fragments (results not shown).

Catalytic properties of recombinant human PHOSPHO1

Twelve phosphate compounds were investigated as potential substrates for human PHOSPHO1. The resultant specific activities are shown in Table 1. PHOSPHO1 was found to have the highest specific activities toward PEA and PCho, with PEA being hydrolysed approx. 1.5 times faster than PCho. Six of the potential substrates tested (PP_i, phospho-L-serine, glycone phosphate, fructose 6-phosphate, phospho-L-tyrosine and ATP) yielded no detectable phosphatase activity.

The concentration of $MgCl_2$ was varied between 2 μM and 200 mM and activity was found to be maximum at 2 mM $MgCl_2$.

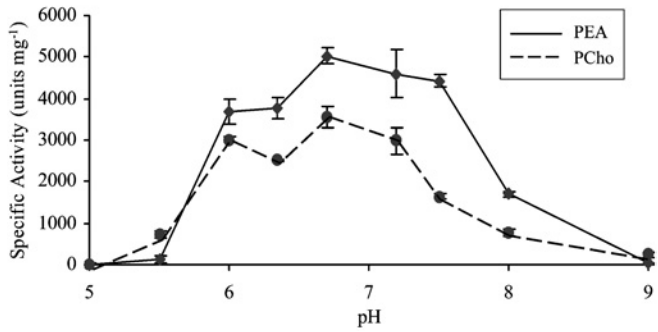


Figure 2 The pH optimum for activity of recombinant PHOSPHO1

Enzymic activity was measured in the presence of 3 μg/ml enzyme and 2 mM Mg^{2+} by the discontinuous assay (as described in the Experimental procedures) for phosphoethanolamine (solid line) and phosphocholine (broken line).

In the presence of 2 mM Mg^{2+} at 37 °C, the recombinant enzyme exhibited a pH optimum around 6.7 for both PEA and PCho (Figure 2). High catalytic activity (> 70 % of maximum) was observed between pH 6.0 and 7.2. This high level of activity extends up to at least pH 7.5 for PEA but begins to decline significantly for PCho at pH values higher than pH 7.2. The kinetic constants of recombinant PHOSPHO1 were determined for PEA and PCho using the continuous coupled assay in the presence of 2 mM Mg^{2+} at 37 °C. The enzyme exhibited Michaelis–Menten kinetics for both substrates (Hill coefficients = 1.00). A plot of reaction rate versus substrate concentration for PEA and PCho and also Lineweaver–Burke plots, allowing the calculation of K_m and V_{max} values, are shown in Figure 3. PHOSPHO1 displayed an apparent K_m of 3.0 μM and a k_{cat} of 2.27 s^{-1} for PEA, and a K_m of 11.4 μM and a k_{cat} of 1.98 s^{-1} for PCho.

Requirement for metals

To investigate the requirement of metal ions for the recombinant enzyme, the purified enzyme solution was extensively dialysed against metal-free buffer to remove any weakly bound metal ions. The effect of different metal ions on the hydrolysis of PEA and PCho was assessed by addition of 2 mM concentrations of various metal salts. As controls, reactions were also carried out in buffers without added metal ions. The results are shown in Figure 4. The phosphatase activity was approximately 80-fold higher for both substrates in the presence of Mg^{2+} compared with the metal-free control. Co^{2+}, Mn^{2+} and Ni^{2+} also stimulated activity but to a lesser extent than Mg^{2+}, whereas the presence of Ca^{2+} and Zn^{2+} had no significant effect on activity compared with the control ($Mg^{2+} > Co^{2+} > Mn^{2+} > Ni^{2+} > Ca^{2+} = Zn^{2+} =$ no metal). Interestingly, PHOSPHO1 has a higher activity toward PCho than to PEA in the presence of Co^{2+} and Mn^{2+}. This is most probably due to an allosteric effect caused by a difference in the metal-binding properties of each enzyme–substrate complex.

DISCUSSION

The results presented here show that PHOSPHO1 has activity which is typical of most enzymes within the HAD superfamily, with a strong Mg^{2+}-dependence and a pH optimum within the acid-to-neutral pH range [27–29]. A high level of activity extends to pH values at least as high as pH 7.5 for PEA but begins to decline significantly for PCho at pH values higher than pH 7.2. The pH of the extracellular fluid of growth plate cartilage is close to pH 7.6 [30] and so the activity of PHOSPHO1 may be restricted

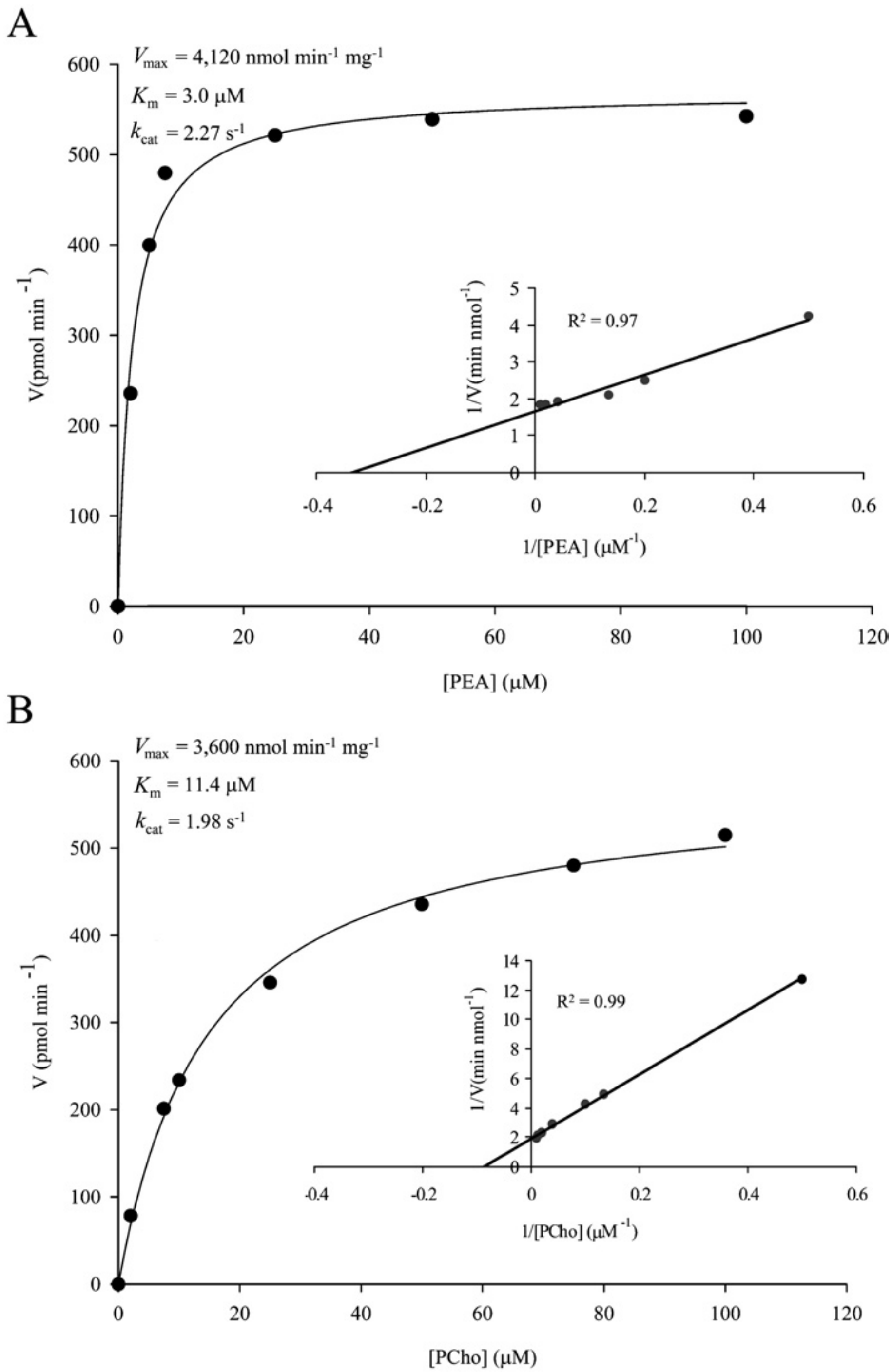


Figure 3 Kinetic analysis of the hydrolysis reactions catalysed by recombinant PHOSPHO1

Kinetic activity toward (**A**) PEA and (**B**) PCho, measured using a method based upon the purine nucleoside phosphorylase-coupled assay [26] as described in the Experimental procedures. Shown are the reaction velocities (V) as a function of substrate concentration. *Insets*, Lineweaver–Burke plots from which K_m and V_{max} values were calculated.

mainly to PEA at this region. Overall, PHOSPHO1 has a high specific activity toward PEA and PCho compared with the other phosphomonoesters investigated. The results are highly significant for the mineralization process in cells. Both PCho and PEA are present in mineralizing cells and are the two most abundant phosphomonoesters in cartilage [31]. The very low K_m values for both PEA and PCho (μM range) suggest that they would be half-saturated at levels of 3 and 11.4 μM, respectively. This indicates that under the reported conditions both substrates would be rapidly hydrolysed. These compounds are therefore likely to be natural substrates of PHOSPHO1.

The hydrolysis of PEA and PCho is known to occur *in vivo*, although the enzyme responsible has not been identified previously. It has been hypothesized that PEA is a natural substrate for TNAP [8,32] due to an increase in its urinary excretion in patients diagnosed with hypophosphatasia [33]. However, this appears unlikely following examination of kinetic data for the TNAP-catalysed hydrolysis reaction at physiological pH, with

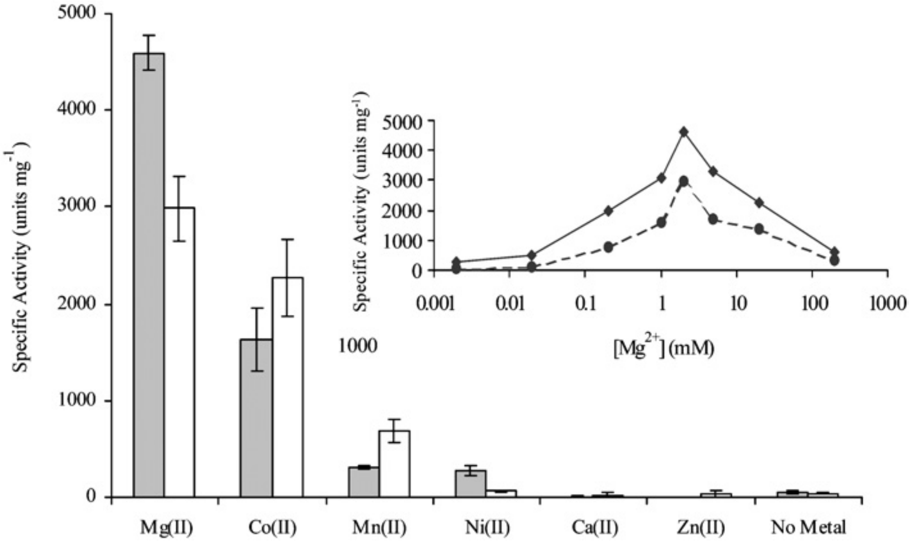
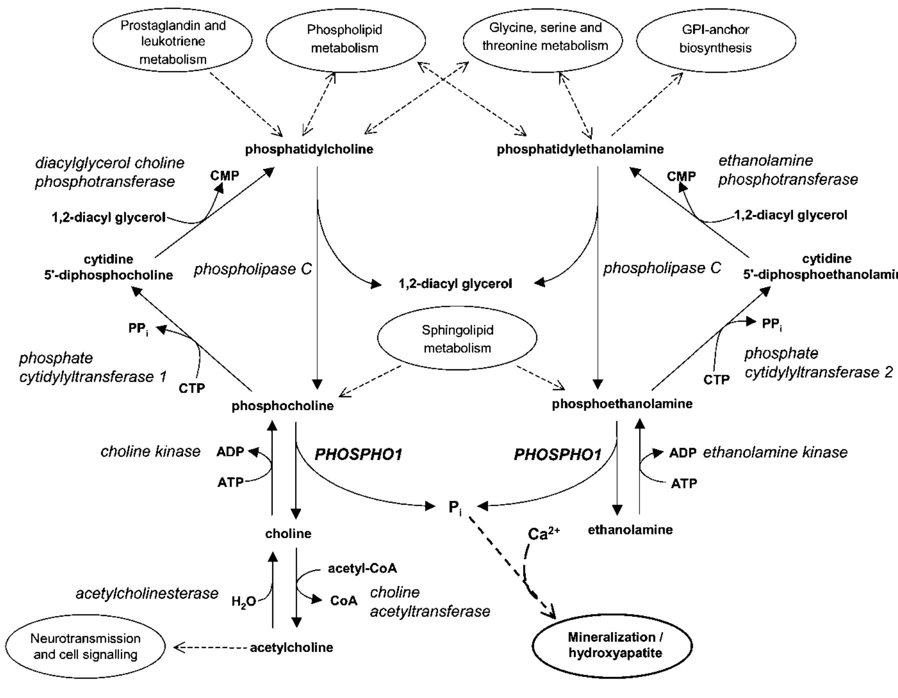


Figure 4 Metal requirement for activity of recombinant PHOSPHO1

Enzyme activity toward phosphoethanolamine (filled bars) and phosphocholine (open bars) was measured in the presence of the indicated metal ions (final concentration 2 mM). The activity of the apoenzyme was measured in the absence of added metals (No Metal). Inset, concentration dependence of apoenzyme activation by Mg^{2+} for PEA (solid line) and PCho (broken line); enzyme activity was measured by the discontinuous assay.



Scheme 1 Human PEA and PCho metabolism

Proposed metabolic pathways for the generation of PEA and PCho. The basis of the diagram is information from the Kyoto Encyclopedia of Genes and Genomes (KEGG) [41].

reported high K_m values at millimolar concentrations [34–36]. It is therefore possible that the genetic defect assumed to be caused by a loss of TNAP in hypophosphatasia is actually due to a loss or defect of PHOSPHO1. Brain alkaline phosphatase (BAP), an isoenzyme of TNAP, is reported to have PCho phosphatase activity [37]. However, the specific activity of BAP toward PCho in the reported study was measured under alkaline conditions (pH 8.5) and is likely to be much lower at physiological pH. PCho hydrolysis has been studied in hamster heart and is not catalysed by alkaline phosphatase, but by a separate unidentified enzyme [38]. It has also been shown that the hydrolysis of PEA and PCho is due to the action of an acid phosphatase in a variety of tissues, including bone and teeth [39], a study which agrees well with our present finding that PHOSPHO1 displays high activity toward both substrates between pH 6.0 and 7.2.

PEA and PCho are metabolites in the cytidine 5′-diphosphoethanolamine (CDP-EA) and cytidine 5′-diphosphocholine (CDP-Cho) pathways respectively (Scheme 1). These are the main pathways involved in the formation of phosphatidylcholine and phosphatidylethanolamine [40], which are involved in the metabolism of complex glycerolipids, glycosylphosphatidylinositol-anchors, prostaglandins, leukotrienes and the amino acids glycine, serine and threonine [41]. These pathways are also implicated in the pathogenesis of Alzheimer's and Huntington's disease [42,43]. Therefore the identification of a phosphatase with specificity toward PEA and PCho is highly significant. Conversely, phosphatidylethanolamine and phosphatidylcholine may be hydrolysed by phospholipase C to form PEA and PCho respectively [44]. The synthesis of phosphatidylcholine from choline by the CDP-Cho pathway in mineralizing cells has previously been investigated. PCho accumulation is much decreased in neo-natal rat calvaria compared with the liver of the same animal [45]. PCho concentration is usually determined by the relatively higher activity of choline kinase compared with that of phosphate cytidylyltransferase 1. However, the low PCho accumulation in mineralizing compared with non-mineralizing cells may be due to the upregulation of PHOSPHO1. PHOSPHO1 is highly expressed at sites of mineralization [21], and as a consequence will reduce the levels of PCho and PEA in chondrocytes and osteoblasts. P_i may be scavenged from PEA and PCho during the mineralization process in order to generate the concentration required for hydroxyapatite crystal formation.

The MV-membrane is a rich source of both phosphatidylethanolamine and phosphatidylcholine and may act as a pool for PEA and PCho in MVs. Wuthier et al. have found that the phosphatidylethanolamine and phosphatidylcholine composition of the MV membrane decreases during mineralization and that 1,2-diacyl glycerol accumulates in MVs, indicative of phospholipase C activity [46]. However, in the absence of any kinetic data relating to phosphatidylethanolamine or phosphatidylcholine degradation in MVs it is impossible to say at this time whether such a mechanism exists as a viable means of generating PEA or PCho. Ca^{2+} and P_i are present at high levels in MVs even before induction of mineral formation, and are derived from cellular activity prior to MV formation [47]. Since the ambient concentration of P_i in the extracellular fluid is close to 2 mM [48], it is doubtful that the amount of P_i released from MV lipids would be sufficient to increase the overall level of extracellular P_i. However, the local effect of this limited release may be sufficient to facilitate mineral formation.

In conclusion, these results show for the first time that human PHOSPHO1 is a phosphoethanolamine and phosphocholine phosphatase. PHOSPHO1 is known to be upregulated in mineralizing cells, these findings therefore provide a novel means of generating P_i in mineralizing cells and may have implications for the diagnosis of hypophosphatasia and treatment of bone mineralization abnormalities such as osteomalacia and pathological soft-tissue ossification, a process clinically significant in atherosclerosis and heart failure.

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REFERENCES

1 Sauer, G. R. and Wuthier, R. E. (1988) Fourier transform infrared characterization of mineral phases formed during induction of mineralization by collagenase-released matrix vesicles *in vitro*. J. Biol. Chem. **263**, 13718–13724

2 Wu, L. N. Y., Ishikawa, Y., Sauer, G. R., Genge, B. R., Mwale, F., Mishima, H. and Wuthier, R. E. (1995) Morphological and biochemical characterization of mineralizing primary cultures of avian growth plate chondrocytes: evidence for cellular processing of Ca^{2+} and P_i prior to matrix mineralization. J. Cell. Biochem. **57**, 218–237

3 Anderson, H. C. (2003) Matrix vesicles and calcification. Curr. Rheumatol. Rep. **5**, 222–226

4 Roach, H. I. (1999) Association of matrix acid and alkaline phosphatases with mineralization of cartilage and endochondral bone. Histochem. J. **31**, 53–61

5 Nakano, Y., Kawamoto, T., Oda, K. and Takano, Y. (2003) Alkaline and acid phosphatases in bone cells serve as phosphohydrolases at physiological pH *in vivo*: a histochemical implication. Connect. Tissue Res. **44**, 219–222

6 Anderson, H. C. (1995) Molecular biology of matrix vesicles. Clin. Orthopaed. **314**, 266–280

7 Beck, Jr, G. R., Moran, E. and Knecht, N. (2003) Inorganic phosphate regulates multiple genes during osteoblast differentiation, including *Nrf2*. Exp. Cell Res. **288**, 288–300

8 Whyte, M. P. (1994) Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. Endocr. Rev. **15**, 439–461

9 Hessle, L., Johnson, K. A., Anderson, H. C., Narisawa, S., Sali, A., Goding, J. W., Terkeltaub, R. and Millán, J. L. (2002) Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. Proc. Natl. Acad. Sci. U.S.A. **99**, 9445–9449

10 Narisawa, S., Frohlander, N. and Millán, J. L. (1997) Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. Dev. Dyn. **208**, 432–446

11 Waymire, K. G., Mahuren, J. D., Jaje, J. M., Guilarte, T. R., Coburn, S. P. and MacGregor, G. R. (1995) Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. Nat. Genet. **11**, 45–51

12 Anderson, H. C., Hsu, H. H., Morris, D. C., Fedde, K. N. and Whyte, M. P. (1997) Matrix vesicles in osteomalacic hypophosphatasia bone containing apatite-like mineral crystals. Am. J. Pathol. **151**, 1555–1561

13 Anderson, H. C., Sipe, J. B., Hessle, L., Dhamyamraju, R., Atti, E., Camacho, N. P. and Millán, J. L. (2004) Impaired calcification around matrix vesicles of growth plate and bone in alkaline phosphatase-deficient mice. Am. J. Pathol. **164**, 841–847

14 Genge, B. R., Sauer, G. R., Wu, L. N., McLean, F. M. and Wuthier, R. E. (1988) Correlation between loss of alkaline phosphatase activity and accumulation of calcium during matrix vesicle-mediated mineralization. J. Biol. Chem. **263**, 18513–18519

15 Moss, D. W., Eaton, R. H., Smith, J. K. and Whitby, L. G. (1967) Association of PPi activity with human alkaline-phosphatase preparations. Biochem J. **102**, 53–57

16 Meyer, J. L. (1984) Can biological calcification occur in the presence of pyrophosphate? Arch. Biochem. Biophys. **231**, 1–8

17 Narita, M., Goji, J., Nakamura, H. and Sano, K. (1994) Molecular cloning, expression, and localization of a brain-specific phosphodiesterase I/nucleotide pyrophosphatase (PD-Iα) from rat brain. J. Biol. Chem. **269**, 28235–28242

18 Register, T. C., McLean, F. M., Low, M. G. and Wuthier, R. E. (1986) Roles of alkaline phosphatase and labile internal mineral in matrix vesicle-mediated calcification. Effect of selective release of membrane-bound alkaline phosphatase and treatment with isosmotic pH 6 buffer. J. Biol. Chem. **261**, 9354–9360

19 Hsu, H. H. T. and Anderson, H. C. (1996) Evidence of the presence of a specific ATPase responsible for ATP-initiated calcification by matrix vesicles isolated from cartilage and bone. J. Biol. Chem. **271**, 26383–26388

20 Houston, B., Seawright, E., Jefferies, D., Hoogland, E., Lester, D., Whitehead, C. and Farquharson, C. (1999) Identification and cloning of a novel phosphatase expressed at high levels in differentiating growth plate chondrocytes. Biochim. Biophys. Acta **1448**, 500–506

21 Houston, B., Stewart, A. J. and Farquharson, C. (2004) PHOSPHO1 – a novel phosphatase specifically expressed at sites of mineralization in bone and cartilage. Bone **34**, 629–637

22 Baldwin, J. C., Karthikeyan, A. S. and Raghothama, K. G. (2001) LEPS2, a phosphorus starvation-induced novel acid phosphatase from tomato. Plant Physiol. **125**, 728–737

23 Stenzel, I., Ziethe, K., Schurath, J., Hertel, S. C., Bosse, D. and Kock, M. (2003) Differential expression of the LePS2 phosphatase gene family in response to phosphate availability, pathogen infection and during development. Physiol. Plant. **118**, 138–146

24 Stewart, A. J., Schmid, R., Blindauer, C. A., Paisey, S. J. and Farquharson, C. (2003) Comparative modelling of human PHOSPHO1 reveals a new group of phosphatases within the haloacid dehalogenase superfamily. Protein Eng. **16**, 889–895

25 Baykov, A. A., Evtushenko, O. A. and Avaeva, S. M. (1988) A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. Anal. Biochem. **171**, 266–270

26 Webb, M. R. (1992) A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. Proc. Natl. Acad. Sci. U.S.A. **89**, 4884–4887

27 Morais, M. C., Zhang, W., Baker, A. S., Zhang, G., Dunaway-Mariano, D. and Allen, K. N. (2000) The crystal structure of *Bacillus cereus* phosphonoacetaldehyde hydrolase: insight into catalysis of phosphorus bond cleavage and catalytic diversification within the HAD enzyme superfamily. Biochemistry **39**, 10385–10396

28 Wu, J. and Woodard, R. W. (2003) *Escherichia coli* YrbI is 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase. J. Biol. Chem. **278**, 18117–18123
29 Klutts, S., Pastuszak, I., Edavana, V. K., Thampi, P., Pan, Y.-T., Abraham, E. C., Carroll, J. D. and Elbein, A. D. (2003) Purification, cloning, expression, and properties of mycobacterial trehalose-phosphate phosphatase. J. Biol. Chem. **278**, 2093–2100
30 Howell, D. S., Pita, J. C., Marquez, J. F. and Madruga, J. E. (1968) Partition of calcium, phosphate and protein in the fluid phase aspirated at calcifying sites in epiphyseal cartilage. J. Clin. Invest. **47**, 1121–1132
31 Kvam, B. J., Pollesello, P., Vittur, F. and Paoletti, S. (1992) ^{31}P NMR studies of resting zone cartilage from growth plate. Magn. Reson. Med. **25**, 355–361
32 Whyte, M. P., Landt, M., Ryan, L. M., Mulivor, R. A., Henthorn, P. S., Fedde, K. N., Mahuren, J. D. and Coburn, S. P. (1995) Alkaline phosphatase: placental and tissue-nonspecific isoenzymes hydrolyse phosphoethanolamine, inorganic pyrophosphate, and pyridoxal 5′-phosphate. Substrate accumulation in carriers of hypophosphatasia corrects during pregnancy. J. Clin. Invest. **95**, 1440–1445
33 Rasmussen, K. (1968) Phosphorylethanolamine and hypophosphatasia. Dan. Med. Bull. **15**, Suppl. 2, 1–112
34 Fedde, K. N., Lane, C. C. and Whyte, M. P. (1988) Alkaline phosphatase is an ectoenzyme that acts on micromolar concentrations of natural substrates at physiologic pH in human osteosarcoma (SAOS-2) cells. Arch. Biochem. Biophys. **264**, 400–409
35 Muller, K., Schulz, J. and Oemus, R. (1989) Phosphoäthanolamin – ein substrat der alkalischen phosphatase aus ratten-calvaria. Biomed. Biochim. Acta **48**, 495–504
36 Tenenbaum, H. C. and Palangio, K. (1987) Phosphoethanolamine- and fructose 1,6-diphosphate-induced calcium uptake in bone formed *in vitro*. Bone Miner. **2**, 201–210
37 Sok, D.-E. (1999) Oxidative inactivation of brain alkaline phosphatase responsible for hydrolysis of phosphocholine. J. Neurochem. **72**, 355–362
38 Hatch, G. M. and Choy, P. C. (1987) Phosphocholine phosphatase and alkaline phosphatase are different enzymes in hamster heart. Lipids **22**, 672–676

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39 McDonald, D. F., Schofield, B. H., Geffert, M. A. and Coleman, R. A. (1980) A comparative study of new substrates for the histochemical demonstration of acid phosphomonoesterase activity in tissues which secrete acid phosphatase. J. Histochem. Cytochem. **28**, 316–322
40 Walkey, C. J., Donohue, L. R., Bronson, R, Agellon, L. B. and Vance, D. E. (1997) Disruption of the murine gene encoding phosphatidylethanolamine N-methyltransferase. Proc. Natl. Acad. Sci. U.S.A. **94**, 12880–12885
41 Kanehisa, M., Goto, S., Kawashima, S. and Nakaya, A. (2002) The KEGG databases at GenomeNet. Nucleic Acids Res. **30**, 42–46
42 Ellison, D. W., Beal, M. F. and Martin, J. B. (1987) Phosphoethanolamine and ethanolamine are decreased in Alzheimer's disease and Huntington's disease. Brain Res. **417**, 389–392
43 Farber, S. A., Slack, B. E. and Blusztajn, J. K. (2000) Acceleration of phosphatidylcholine synthesis and breakdown by inhibitors of mitochondrial function in neuronal cells: a model of the membrane defect of Alzheimer's disease. FASEB J. **14**, 2198–2206
44 van Dijk, M. C., Muriana, F. J., de Widt, J., Hilkmann, H. and van Blitterswijk, W. J. (1997) Involvement of phosphatidylcholine-specific phospholipase C in platelet-derived growth factor-induced activation of the mitogen-activated protein kinase pathway in Rat-1 fibroblasts. J. Biol. Chem. **272**, 11011–11016
45 Stern, P. H. and Vance, D. E. (1987) Phosphatidylcholine metabolism in neonatal mouse calvaria. Biochem. J. **244**, 409–415
46 Wu, L. N. Y., Genge, B. R., Kang, M. W., Arsenault, A. L. and Wuthier, R. E. (2002) Changes in phospholipid extractability and composition accompany mineralization of chicken growth plate cartilage matrix vesicles. J. Biol. Chem. **277**, 5126–5133
47 Wu, L. N., Wuthier, M. G., Genge, B. R. and Wuthier, R. E. (1997) *In situ* levels of intracellular Ca^{2+} and pH in avian growth plate cartilage. Clin. Orthop. **335**, 310–314
48 Wuthier, R. E. (1977) Electrolytes of isolated epiphyseal chondrocytes, matrix vesicles, and extracellular fluid. Calcif. Tissue Res. **23**, 125–133