The role of alkaline phosphatase in mineralization

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Purpose of review

Alkaline phosphatase is an important component in hard tissue formation, highly expressed in mineralized tissue cells. It is appropriate to review the current status of this important enzyme.

Recent findings

The mechanism with which this enzyme carries out its function is not completely understood, but it appears to act both to increase the local concentration of inorganic phosphate, a mineralization promoter, and to decrease the concentration of extracellular pyrophosphate, an inhibitor of mineral formation. The enzyme is localized to the outside of the plasma membrane of cells, and of the membrane of matrix vesicles. It is attached to the membrane by a glycophosphatidylinositol anchor, and is found in membrane microdomains known as rafts. Alkaline phosphatase has also been implicated in cardiovascular calcification which appears to proceed by an osteogenic mechanism. Significant interest in alkaline phosphatase expression has also come from tissue engineering experiments, where enzyme expression is a good predictor of neotissue mineralization.

Summary

The high level of activity in this field is sure to provide new and important information into the fundamental mechanisms of hard tissue formation, provide therapeutic opportunities for treatment of bone diseases, and enhance our ability to create useful bone biomaterials.

Keywords

alkaline phosphatase, calcification, matrix vesicle, mineralization, pyrophosphate

Curr Opin Orthop 18:444–448. © 2007 Lippincott Williams & Wilkins.

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Current Opinion in Orthopaedics 2007, 18:444–448

Abbreviations

ALP	alkaline phosphatase
ePPi	extracellular inorganic pyrophosphate
GPI	phosphatidyl inositol-glycophospholipid
NPP1	nucleotide pyrophosphatase phosphodiesterase-1
PPi	inorganic pyrophosphate
SIBLING	small integrin-binding ligand, N-linked glycoprotein
TNAP	tissue nonspecific alkaline phosphatase

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Introduction

The metalloenzyme known as alkaline phosphatase (ALP) [phosphate-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1] exists as several tissue-specific isozymes encoded by separate genes. The enzyme, which is expressed in many species (plants, bacteria and animals), catalyzes the hydrolysis of phosphomonoesters, R-O-PO_3, with little regard to the identity of the 'R' group. The catalytic mechanism involves the formation of a serine phosphate at the active site which reacts with water at alkaline pH to release inorganic phosphate from the enzyme [1]. In the presence of high concentrations of an organic alcohol, a transphosphorylation reaction results when the organic alcohol releases the enzyme-bound inorganic phosphate and becomes phosphorylated [2]. In vertebrates, the enzyme is an ectoenzyme, which is attached to the outer face of the plasma membrane through a phosphatidyl inositol-glycophospholipid (GPI) anchor covalently attached to the C-terminus of the enzyme [3]. Humans have four ALP genes corresponding to intestinal, placental, placental-like and liver/bone/kidney (tissue nonspecific; TNAP) gene products [4]. ALP has many different functions in the many organisms and tissues where it occurs, but the focus of this review is to appreciate the role of ALP in mineralization.

More than 80 years ago, the high level of ALP expression in bone was noted, and the first hypothesis put forward to explain why ALP was important for hard tissue formation [5]. In this formulation, ALP was postulated to increase the local concentration of inorganic phosphate, a concept known as the 'booster hypothesis'. The role of ALP in hard tissue formation has been vigorously debated since then, with two divergent outcomes. As more has been learned about the biology of hard tissues and mineral metabolism, the role of ALP as a marker for osteogenic activity has been consistently solidified. In contrast, the detailed mechanism of how ALP functions in hard tissues is often considered controversial. Its crucial role is now undisputed since it became apparent that the genetic diseases known as hypophosphatasia (OMIM: 171760) result from mutations in the gene coding for TNAP, and lead to a phenotype characterized by undermineralization of bone [6].

In the past 2 years, more than 280 publications have appeared which contain information about ALP and hard tissues. Of these, more than 200 represent the use of ALP as a marker of hard tissue cell differentiation or measurements of enzyme blood levels. Tissue engineering is an

emerging area of interest in ALP, where, as in osteogenesis, success is measured by robust ALP expression, as it inevitably leads to mineralization of the neotissue (e.g. see [7–12]). In contrast, only about 30 papers are aimed at unraveling the mechanism through which ALP functions. Thus, while measurement of increased ALP expression enzymatically, histochemically or at the mRNA level is taken as a reliable indication of the osteoblastic, chondrocytic or odontoblastic phenotype, the function of this elevation in ALP expression is considered less clear. The purpose of this review is to examine the current status of the role of ALP in mineralization.

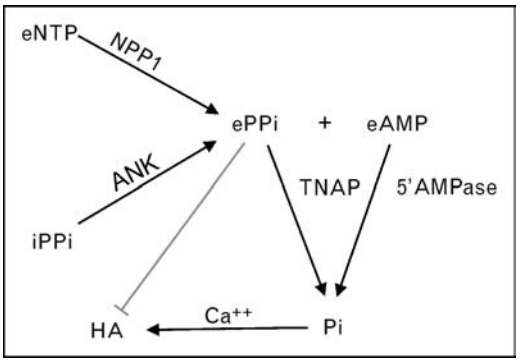
Alkaline phosphatase expression

A key to understanding the role of ALP in mineralization is provided by studies of the phased expression of genes during osteoblastic differentiation and growth plate cartilage calcification. In both tissues, bone and calcifying cartilage, ALP is expressed early in development, and is soon observed on the cell surface and in matrix vesicles. Later in the developmental program, while other genes (e.g. osteocalcin) are upregulated, ALP expression declines. Clearly, ALP must function in the initial phases of the process. The mechanisms though which ALP expression is regulated are complex; a web of interlaced signaling pathways, the details of which are just emerging. The principal regulatory pathways controlling osteoblastic differentiation, chondrogenesis and ALP expression are the BMP/RUNX2 (CBAf1, AML3)/Osterix system, and the WNT signaling cascade, which also interact with each other [13,14]. A very recent study of the BMP/RUNX2 system has implicated a number of patterning gene products, including HOX10A, which has been shown both to stimulate osteogenesis in mesenchymal stem cells and to directly regulate expression of TNAP (and other genes) through chromosomal remodeling [15••]. Additional control of ALP expression is exerted through the actions of 1,25-$(OH)_2$-vitamin D, retinoic acid and PTH, which can modulate ALP expression through unique pathways, and by interacting with the principal regulatory systems [16–23]. A thorough examination of the regulation of ALP expression is beyond the scope of this review, but it is clear from these studies that ALP is vitally involved in the early stages of hard tissue formation.

The role of alkaline phosphatase in initiating calcification

ALP is among the first functional genes expressed in the process of calcification. It is therefore likely that at least one of its roles in the mineralization process occurs at an early step. A clue to the role of ALP in calcification came from studies of hypophosphatasia patients, whose disease results from mis-sense mutations in the gene coding for TNAP leading to decreased or absent ALP activity. Blood and urine levels of phosphoethanolamine, pyridoxal-5′-phosphate and inorganic pyrophosphate (PPi) are elevated in these patients, suggesting that these compounds are substrates for TNAP [6]. In recent years, interest has focused on the mineralization inhibitor, PPi, as the key regulator of calcification (more fully reviewed by C.J. Williams elsewhere in this issue) [24]. Studies of transgenic mice have led to an emerging consensus which emphasizes the central role of the enzyme's TNAP and nucleotide pyrophosphatase phosphodiesterase (NPP1/PC-1), in conjunction with the cell-associated ankylosis protein in regulating the onset of calcification [25–29]. The currently held doctrine posits that ankylosis protein and NPP1 function to suppress mineralization by increasing the extracellular concentration of the calcification inhibitor, pyrophosphate (ePPi), while TNAP functions to promote mineralization by decreasing the concentration of ePPi and increasing the concentration of the mineralization promoter, inorganic phosphate [30]. In this scheme, ePPi is formed from extracellular nucleoside triphosphates by NPP1 and exported from cells through the action of ankylosis protein; it is hydrolyzed to inorganic phosphate by TNAP (see Fig. 1).

Figure 1 Generation of extracellular inorganic pyrophosphate (ePPi) by nucleotide pyrophosphatase phosphodiesterase (NPP1) from extracellular nucleoside triphosphates (eNTP) or by ankylosis (ANK) protein from intracellular PPi (iPPi) blocks mineral (HA) formation



The action of TNAP decreases ePPi and increases inorganic phosphate.

Testing the inorganic pyrophosphate hypothesis

Much of the foundation for the PPi hypothesis comes from the analysis of knockout mice. TNAP homozygous mice demonstrate skeletal abnormalities reminiscent of hypophosphatasia, presumably because of elevated ePPi levels. Mice deficient in NPP1 exhibit hypermineralization and ectopic calcification, presumably because of decreased ePPi. When TNAP null mice are crossed with NPP1 null mice, the double mutants exhibit a more normal bone phenotype, which has been interpreted to result from a restored balance in ePPi and inorganic phosphate levels derived from compensatory ablations

of TNAP and NPP1 [25]. More recent evidence shows that the rescue of TNAP deficiency by simultaneous NPP1 deficiency is site-specific, primarily evident in calvaria and spine, but not seen in long bones [31]. The authors of this carefully conducted study document bone formation in wild-type, TNAP deficient, NPP1 deficient and double mutant mice, in calvarium, spine phalanges and long bones at the histological, radiological and ultrastructural levels. Clear differences are seen in the degree of rescue of the hypophosphatasia phenotype by ablation of NPP1 between the various tissues, with strong evidence of rescue in calvaria and spine, and poor bone formation in long bones. Further, the single knockout of NPP1 which displayed hypermineralization in calvarium, was more poorly mineralized than wild-type in long bones. Further, measurement of osteoblastic differentiation *in vitro* reveals similar differences when osteoprogenitors derived from calvarium are compared with those derived from marrow. To explain this site-specific phenotypic variation, the authors construct an ingenious argument to explain their anomalous results. This explanation is based on differential expression of TNAP and NPP1 in calvarium vs. tibia, and suggests that the higher expressing tissues (e.g. calvarium) reach a mineralization promoting balance between inorganic phosphate and ePPi, even in the absence of both TNAP and PPi, while in contrast, the lower enzyme expression seen in long bones results in a lower steady state level of ePPi, which is not inhibitory. This explanation does not account for the inhibition of mineralization observed in TNAP deficiency. If increased ePPi is responsible for the hypophosphatasia phenotype observed in long bones, then the inability of the ablation of NPP1 to decrease ePPi suggests that NPP1 may be less important in regulating ePPi at this site than in calvarium and spine.

Effects of the inorganic phosphate/extracellular inorganic pyrophosphate ratio on gene expression and mineralization

More recent studies have considered the bone matrix small integrin-binding ligand, N-linked glycoprotein (SIBLING) ostoepontin, demonstrating that elevated osteopontin levels also contribute to the hypophosphatasia phenotype, and that increased ePPi, in addition to inhibiting calcification, also upregulates osteopontin expression in osteoblasts [32,33••]. Osteopontin is itself an inhibitor of mineral formation. Increased inorganic phosphate in cementoblasts has been shown to upregulate osteopontin and another SIBLING, dentin matrix protein-1, along with NPP1 and ankylosis protein, while at the same time downregulating TNAP expression [34••]. Thus, alterations in the inorganic phosphate/ePPi ratio have profound effects on biomineralization which go beyond simple physical chemical effects on hydroxyapatite formation. It has been shown, for example, that the quality of mineral produced as the result of TNAP action is critically dependent on the TNAP substrate along with the inorganic phosphate/ePPi ratio [35••]. At low ePPi concentrations, TNAP can hydrolyze PPi, and other potential phosphate-generating species such as AMP, to produce apatite. ATP and high levels of PPi result in the formation of amorphous calcium phosphate and, in the case of PPi, calcium pyrophosphate. While a comprehensive model to explain all the data awaits further investigation, currently available information clearly demonstrates the centrality of TNAP in the formation of mineral in mineralizing tissues probably through its ability to alter the inorganic phosphate/ePPi ratio in the premineralized matrix of mineralizing tissues.

Alkaline phosphatase and matrix vesicles

The role of matrix vesicles in mineralization is being reviewed by H.C. Anderson elsewhere in this issue. It is however impossible to discuss the role of ALP in mineralization without some reference to these extracellular organelles. Some progress has recently been made in understanding how the composition and organization of matrix vesicle lipids bears on the structure of matrix vesicles, and on their function in serving as the site of initial mineral formation. Matrix vesicles are believed to arise by budding from the plasma membrane of hard tissue forming cells (osteoblasts, chondrocytes, odontoblasts and cementoblasts). This conclusion is supported by comparative lipid and protein studies of the vesicle and the plasma membrane. From these investigations, subtle structural differences in the composition of the two membranes suggest that matrix vesicles originate from specific sites on the plasma membrane. It has previously been shown that matrix vesicles contain higher concentrations of acidic phospholipids (phosphatidylserine and phosphatidylinositol) than the chondrocyte plasma membrane [36]. Overall, plasma membrane contains similar lipid components to matrix vesicles, but in different proportions. We recently studied the lipid organization of matrix vesicles and plasma membrane [37••]. In this study, we determined that chick growth plate matrix vesicles were highly enriched in membrane raft microdomains containing high levels of cholesterol, glycophosphatidylinositol GPI-anchored TNAP and phosphatidylserine localized to the external leaflet of the bilayer. To determine how such membrane microdomains arise during chondrocyte maturation we explored the role of plasma membrane cholesterol-dependent lipid assemblies, in regulating the activities of lipid translocators involved in the externalization of phosphatidylserine. We first isolated and determined the composition of detergent-resistant membranes from chondrocyte plasma membrane. Detergent-resistant membranes isolated from chondrocyte plasma membrane are enriched in ganglioside I and cholesterol as well as GPI-anchored TNAP. Furthermore, these membrane domains are enriched in phosphatidylserine (localized

to the external leaflet of the bilayer) and have significantly higher TNAP activity than noncholesterol-enriched domains. Thus, it is likely that the formation of matrix vesicles requires the formation of plasma membrane assemblies enriched in phosphatidylserine (on the outer leaflet), and TNAP (because of its GPI lipid anchor). It should also be noted that membranes enriched in phosphatidylserine attract annexins and permit them to form calcium channels, facilitating Ca^{2+} entry into matrix vesicles for crystal formation (see review by J. Bandorowicz-Pikula in this issue and [38]).

The role of alkaline phosphatase in cardiovascular calcification

In recent years it has become recognized that pathologic calcification of the cardiovascular system follows an osteogenic mechanism [39,40,41••,42]. While the details of this process mimic that of hard tissue mineralization, there are also some differences in how the process is initiated and regulated. For example, hypercholesterolemia has been shown to accelerate calcification of aorta and vascular smooth muscle cells by a mechanism possibly involving oxidative stress [43]. Hyperlipidemia promotes MSX-2 driven aortic calcification [41••]. In other models of diabetic vascular calcification, evidence has accumulated that hyperglycemia may trigger the osteogenic response [44]. In all these models, expression of ALP is a key part of the process, and presumably acts similarly to its action in hard tissues, i.e. by decreasing ePPi and increasing inorganic phosphate levels. In this regard, it has been shown that polyphenols, which are reputed to be cardioprotective, inhibit ALP activity in vascular smooth muscle cells, perhaps inhibiting artery calcification [45••]. As in hard tissues, the expression of ALP activity is necessary, albeit not sufficient for calcification.

Summary and conclusions

ALP was one of the first key players in the process of osteogenesis to be recognized. Subsequent work over an extended period has solidified the importance of the enzyme in normal and pathological calcification. As a result of this centrality, and the ease of biochemical and histological assay, ALP has become the marker of choice when assessing the phenotype or developmental maturity of mineralized tissue cells. As more detailed information on the regulation of gene expression and cell differentiation by growth factors and the web of signal transduction mechanisms involved in the process becomes available, the number of reports demonstrating modulation of ALP expression will continue to expand. Moreover, the emerging investigations of osteogenic stem cells and tissue engineering have further enhanced interest in ALP. Recent investigations have shed new light on the mechanism of ALP action in promoting mineralization. While Robison's booster hypothesis lives on in modified form, the mechanism has expanded to focus on the balance between mineralization promoters, such as inorganic phosphate, and mineralization inhibitors such as ePPi. While ALP remains a central player, other gene products, such as NPP1, ankylosis protein and the SIBLINGS, have come increasingly to the fore. In addition, investigation of the key association of ALP with cell membranes and matrix vesicles, and the complex interactions of lipids, proteins and ions which ultimately results in the nucleation and propagation of mineral crystals promises to reveal new insights into how cells utilize the unique properties of ALP to form mineral. The high level of activity in this field is sure to provide new and important information into the fundamental mechanisms of hard tissue formation, provide therapeutic opportunities for treatment of bone diseases, and enhance our ability to create useful bone biomaterials.

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