The Mechanism of Mineralization and the Role of Alkaline Phosphatase in Health and Disease

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Abstract

Biomineralization is the process by which hydroxyapatite is deposited in the extracellular matrix. Physiological mineralization occurs in hard tissues, whereas pathological calcification occurs in soft tissues. The first step of mineralization is the formation of hydroxyapatite crystals within matrix vesicles that bud from the surface membrane of hypertrophic chondrocytes, osteoblasts, and odontoblasts. This is followed by propagation of hydroxyapatite into the extracellular matrix and its deposition between collagen fibrils. Extracellular inorganic pyrophosphate, provided by NPP1 and ANKH, inhibits hydroxyapatite formation. Tissuenonspecific alkaline phosphatase (TNAP) hydrolyzes pyrophosphate and provides inorganic phosphate to promote mineralization. Inorganic pyrophosphate, pyridoxal phosphate, and phosphoethanolamine are thought to be the physiologic substrates of TNAP. These accumulate in the event of TNAP deficiency, e.g., in cases of hypophosphatasia. The gene encoding TNAP is mapped to chromosome 1, consists of 12 exons, and possesses regulatory motifs in the 5'-untranslated region. Inhibition of TNAP enzymatic activity suppresses TNAP mRNA expression and mineralization in vitro. Hypophosphatasia is an inherited systemic bone disease characterized by hypomineralization of hard tissues. The phenotype of hypophosphatasia is varied. To date, more than 200 mutations in the TNAP gene have been reported. Knockout mice mimic the phenotypes of severe hypophosphatasia. Among the mutations in the TNAP gene, c.1559delT is frequent in the Japanese population. This frameshift mutation results in the expression of an abnormally long protein that is degraded in cells. DNA-based prenatal diagnosis using chorionic villus sampling has been developed, but requires thorough genetic counseling. Although hypophosphatasia is untreatable at present, the recent success of enzyme replacement therapy offers promise. The problems presented by impaired mineralization in age-related chronic diseases, such as pathologic calcification and decreasing physiological mineralization are growing in importance. Strategies for preventing pathologic calcification using TNAP and NPP1 are in development. A nutrigenomic approach, based on the relationship between TNAP gene polymorphism and bone mineral density, is also discussed.

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Introduction

Biomineralization is the process by which minerals are deposited within or outside the cells of a variety of organisms¹. In vertebrate tissues, the deposited minerals are composed of hydroxyapatite, a calcium phosphate, and are found in the extracellular matrix. Physiological mineralization occurs in hard tissues, i.e. bone, growth-plate cartilage, and in dentin, in which several tissue-specific cells are responsible for mineralization². In growth-plate cartilage. mineralization occurs in the hypertrophic zone, where late proliferative and early hypertrophic chondrocytes are responsible for hydroxyapatite formation³⁴. In bone, osteoblasts lining the osteoid are responsible for the formation of hydroxyapatite, as are odontoblasts in teeth. In contrast to physiological mineralization, pathological calcification soft tissues. Articular occurs in cartilage, cardiovascular tissues, and the kidney are prone to pathological mineralization⁵. Aging is associated with an increase in defective mineralization. The decrease in bone mass that leads to osteoporosis is due to a defect in physiological mineralization. Increasing cardiovascular calcification increases morbidity and mortality, and articular calcification results in joint stiffness⁵. Interestingly, it is likely that similar mechanisms underlie physiological and pathological mineralization. Recent research has shown that matrix vesicles play an important role in mineralization^{3,4}.

Matrix Vesicles in the Mineralization

Matrix vesicles are extracellular, membraneinvested vesicles, 50-200 nm in diameter, that are formed by polarized budding from the surface membrane of chondrocytes, osteoblasts and odontoblasts². The signals that release matrix vesicles are not well understood, although concentrations of intracellular calcium and extracellular phosphate may be important.

The lipid composition differs between the membrane of the matrix vesicle and the membranes

of the cells from which they originate. Matrix vesicles are rich in several phospholipids, especially phosphatidylserine, a lipid with a high affinity for Ca^{2+ 6}. Matrix vesicles are also rich in annexins A2 (II), A5 (V), and A6 (VI), and in Ca ATPase, calbindin D_{9K}, carbonic anhydrase, collagen X, alkaline phosphatase (ALP), NPP1 (nucleotide pyrophosphatase phosphodiesterase 1), type III sodium-phosphate (Na/Pi) cotransporter and PHOSPHO1²³⁵⁷.

Mineralization occurs in two steps². It begins with the formation of hydroxyapatite crystals within matrix vesicles, followed by propagation of hydroxyapatite through the membrane into the extracellular matrix. Calcium-binding phospholipids including phosphatidylserine, calcium-binding proteins including calbindin D_{9k}, and bone sialoprotein promote the accumulation of calcium within matrix vesicles²³. Annexins in the matrix vesicles form a calcium channel and incorporate calcium into the matrix vesicles78. Phosphates are provided by the type III Na/Pi cotransporter located on both the cell membrane and the matrix vesicle membrane^{9,10}. The cytosolic phosphatase PHOSPHO1 produces phosphate also by hydrolyzing phosphocholine and phosphoethanolamine, which are membrane derived from phospholipids by phospholipase C¹¹⁻¹³. The origin of calcium ion and phosphate in the matrix vesicles, however, has not been fully elucidated. When the accumulation of calcium and phosphate exceed the solubility point for CaPO₄, CaPO₄ deposition in the form of hydroxyapatite occurs within the matrix vesicles. In the second step of mineralization, hydroxyapatite crystals penetrate the matrix vesicle membrane and are elongated into the extracellular space. This elongation of hydroxyapatite requires appropriate concentrations of calcium and phosphate outside the matrix vesicles3. The extracellular fluid in a growthplate matrix contains sufficient Ca²⁺ and inorganic phosphate (Pi) to support continuous formation of new hydroxyapatite crystals. The hydroxyapatite is propagated in clusters around matrix vesicles and fills the space between collagen fibrils in the skeletal matrices. Matrix vesicles and neighboring collagen





Fig. 1 Schematic illustration of the process of mineralization. Mineralization begins with hydroxyapatite formation in the matrix vesicles budding from hypertrophic chondrocytes and osteoblasts. Hydroxyapatite is formed from Ca²⁺ incorporated by the annexin calcium channel and from inorganic phosphate (Pi) that is provided by type-III Na/Pi cotransporter and by PHOSPHO1, which hydrolyzes phosphocholine (PCho) and phosphoethanolamine (PEA) derived from the membrane. The hydroxyapatite crystals then penetrate the matrix vesicle membrane, elongate due to the effect of tissue-nonspecific alkaline phosphatase (TNAP), and are deposited between the collagen fibrils. Inorganic pyrophosphate (PPi), which inhibits hydroxyapatite formation, is hydrolyzed by TNAP. PPi is generated by NPP1 and is transported outside the cells by ANKH. This balance between the activities of TNAP, NPP1, and ANKH is crucial for the second step of mineralization.

cooperate in this propagation step². The ratio of Pi to inorganic pyrophosphate (PPi) is crucial in the second step of mineralization. PPi, which inhibits the formation of hydroxyapatite¹⁴, is formed by NPP1 from nucleotide triphosphates. PPi is also provided by ANKH (a homolog of the mouse progressive ankylosis (*ank*) gene product), which is localized on the membranes of hypertrophic chondrocytes and osteoblasts¹⁵. ALP hydrolyzes PPi to generate Pi. The balance between the concentrations of PPi and Pi is thought to be critical in mineralization¹⁵ (**Fig. 1**).

Murine progressive ankylosis is a disorder resulting from a naturally occurring mutation of the *ank* gene (*ank/ank*), which causes hypermineralization and progressive ankylosis in vertebrae¹⁶. ANKH is the human homolog of the *ank* gene product, and transports PPi from the cytosol to the extracellular matrix¹⁵. Another hypermineralization mouse, the tiptoe-walking mouse (*ttw/ttw*), shows calcification of articular cartilage and aorta. The causative mutation is present in the *Enpp1* gene that encodes NPP1¹⁷. *Enpp1* knockout mice also show a similar

phenotype¹⁸. In humans, idiopathic infantile arterial calcification (IIAC) is associated with mutations in the ENPP1 gene¹⁹. Heterozygous mutations of the ANKH gene result in craniometaphyseal dysplasia, which is characterized by overgrowth and sclerosis of the craniofacial bones and abnormal modeling of the metaphysis of tubular bones²⁰. An increase in the function resulting from dominant mutations of the ANKH gene is calcium pyrophosphate dihydrate deposition disease (CPPD), which is characterized by chondrocalcinosis of articular cartilage resulting from increased extracellular PPi²¹. In mice with a double-knockout of $Akp2^{-/-}$ (a knockout of the tissuenonspecific ALP gene) and *ank/ank*, or $Akp2^{-/-}$ and $Enpp1^{-/-}$, showed a seemingly normal phenotype, which indicates that ALP, NPP1, and ANK regulate mineralization in concert^{22,23}.

Alkaline Phosphatase

Alkaline phosphatases [ALPs; orthophosphoricmonoester phosphohydrolase (alkaline optimum), EC. 3.1.3.1] are membrane-bound ectoenzymes that hydrolyze monophosphate esters at a high pH (pH 8–10)²⁴. Human ALP is classified into 4 types—tissuenonspecific, intestinal, placenta, and germ cell—of which the tissue-nonspecific type is ubiquitously expressed in many tissues, including liver, bone, and kidney, and is therefore referred to as the liver/ bone/kidney (LBK) type²⁵. ALP is linked to the membrane via glycosylphosphatidylinositol (GPI) anchors by means of posttranslational modification. Tissue-nonspecific alkaline phosphatase (TNAP) is expressed on the cell membrane of hypertrophic chondrocytes, osteoblasts, and odontoblasts, and is also concentrated on the membranes of the matrix vesicles budding from these cells^{27,28}.

Although the three-dimensional structure of TNAP has not been elucidated, computer simulations of the three-dimensional structures of human placental alkaline phosphatase (PLAP)²⁹ and E. coli alkaline phosphatase³⁰ revealed structural aspects of alkaline phosphatase. Human TNAP and PLAP show 57% identity and 74% homology³¹. Human PLAP is a homodimer with 2 disulfide bonds and has 2 zinc-binding sites, a magnesium-binding site, and a calcium-binding site in the interface between monomers. The active site consists of 2 Znbinding sites, a Mg-binding site, and serine 102, to which phosphate binds. Mammalian ALP has a specific domain called the crown domain, which is a flexible loop³¹. These structural features suggest the possibility of catalytic activity in mutant ALPs³².

The physiological substrates of TNAP are thought to be PPi, pyridoxal 5'-phosphate (PLP), and, perhaps, phosphoethanolamine^{25,33,34}. TNAP hydrolyzes PPi during the process of mineralization. This decreases PPi, which is an inhibitor of hydroxyapatite formation, and provides Pi for the formation of hydroxyapatite. PLP, an activated form of vitamin B₆, is needed as a cofactor in neuronal cells to form neurotransmitters, dopamine, serotonin, histamine, yaminobutyric acid (GABA), and taurine²⁵. PLP cannot be transported into cells. Instead, TNAP dephosphorylates PLP to pyridoxal, which is able to enter cells, after which it is transformed back to PLP in neuronal cells, where PLP is involved in the formation of neurotransmitters. It has not been

confirmed that phosphoethanolamine is a substrate of TNAP. Like PPi and PLP, phosphoethanolamine is elevated in the serum and urine of TNAP knockout mice and TNAP-deficient patients, but the pathological significance of phosphoethanolamine elevation is not known²⁵.

The gene encoding human TNAP (the ALPL gene) is mapped to chromosome 1p36.1-34³⁴ and consists of 11 coding exons (exon 2 to exon 12)³⁵ and 2 leader exons (exon 1B and 1L)³⁶, whereas 3 tissue-specific ALP genes are clustered on chromosome 2q34-37^{37,38}. The first leader exon highly expressed in bone (exon 1B) has a TATA box in the 5'-untranslated region. Promoter activity of exon 1L, the second leader exon expressed in liver, is weaker in human neutrophilic granulocytes⁴⁰; this is also the case for the murine second leader exon in most tissues⁴¹. In addition to the TATA box, 2 Sp1 binding sites and a retinoic acid response element have been identified in the regulatory region of exon 1B42. Sp343, progestin44, granulocyte colony-stimulating factor (G-CSF)⁴⁵, cyclic AMP plus retinoic acid⁴⁶ are regulatory factors of human TNAP expression and are known to increase TNAP expression in human cells. Retinoic acid, an activated form of vitamin A and a regulator of growth, regulates TNAP expression through the interaction of retinoic acid receptor complex and the retinoic acid response element on the promoter region of the TNAP gene⁴². 1,25-Dihydroxyvitamin D 3, an activated form of vitamin D₃ and a regulator of mineralization, also promotes TNAP expression, not by increasing transcriptional activity, but by posttranscriptional modulation via an increase in TNAP mRNA stability47.48. In addition, phosphates of TNAP. activate the expression β-Glycerophosphate activates both TNAP expression and mineralization49,50. Inhibition of TNAP activity suppresses mineralization and TNAP mRNA level in vitro, which suggests that phosphate derived from β glycerophosphate by TNAP regulates TNAP expression^{49,51}.

The TNAP knockout mouse was developed as a model of TNAP deficiency⁵²⁵³. The mouse has at least 4 isozymes, of which the *Akp2* gene corresponds to the human *ALPL* gene²⁵. Inactivation of the mouse TNAP gene ($Akp2^{-/-}$) results in

seizures, due to defective metabolism of PLP⁵², and skeletal hypomineralization⁵³, which indicates that this knockout mouse is a model of severe form of human TNAP deficiency, i.e., hypophosphatasia⁵⁴; heterozygotes $(Akp2^{+/-})$ appear to be normal⁵². Hydroxyapatite crystal proliferation and growth is inhibited in the matrix surrounding the matrix vesicles of growth plate and bone in $Akp2^{-/-}$ mice, even though mineral crystals are initiated within matrix vesicles⁵⁵. Osteoblasts from Akp2^{-/-} mice differentiate normally, but cannot initiate mineralization in vitro56. Mineralization is delayed in heterozygous osteoblasts⁵⁶.

Hypophosphatasia

Hypophosphatasia (OMIM # 241500, 241510, 146300) is a rare inherited systemic bone disease caused by mutations of the ALPL gene^{57,58}. It is characterized by hypomineralization of hard tissues and is classified into at least 5 forms-perinatal, infantile, childhood, adult, and odontohypophosphatasia—according to disease severity and age of onset⁵⁸. The perinatal form is the most severe, and often results in the death of affected fetuses. The infantile form occurs within 6 month of birth; respiratory failure is the main cause of death. The childhood form presents within 2 years of birth and is characterized by milder symptoms, deformity of the extremities, and premature loss of deciduous teeth. The adult form of the disease presents during middle age, with mild symptoms. Odontohypophosphatasia is characterized by dental manifestations, but not skeletal defects 58. Hypophosphatasia is inherited as an autosomal recessive trait, but, in some milder cases, autosomal dominant inheritance was reported^{59,60}. To date, more than 200 mutations in the ALPL gene have been reported (http://www.sesep.uvsq.fr/Database.html)^{61,62}. The mutations are distributed widely in the ALPL gene, although some specific mutations are frequent in certain populations: c1559delT and p.F327L are frequent in Japanese63.64, and p.E191K and p.D378V are frequent in Caucasian^{65,66}. Relationships between phenotypes and genotypes are not fully understood^{67,68}; siblings with different phenotypes have been reported^{69,70}. The enzymatic activity and characteristics of mutant proteins have been investigated. Secreted-type mutant proteins had different kinetic properties for PPi and PLP than those of the routinely used artificial substrate, *p*-nitrophenylphosphate⁷¹. When mutant cDNAs were introduced to U₂OS cells, the cells expressing more than 35% of the enzymatic activity mineralize their extracellular matrix via the addition of β -glycerophosphate⁷².

Although hypophosphatasia has been diagnosed on the basis of ultrasonographic findings in fetuses with the severe form of disease73, serum ALP activity is the usual means of obtaining a diagnosis. Measurement of the substrates of TNAP also has diagnostic value. Although urinary PEA is easily measurable and widely used, it not is pathognomonic⁵⁸. Plasma PLP levels are sensitive⁷⁴, but contamination due to oral vitamin B₆ supplementation must be avoided⁵⁸. Elevation of plasma PLP levels causes convulsions in some, but not all, patients74. Plasma PPi levels also have diagnostic value, although it is difficult to determine whether an increase in plasma PPi directly reflects accumulation of PPi in bone75. DNA-based diagnosis is the gold standard for diagnosis⁶², although sometimes only 1 mutation in an obligatory homozygote is found⁶⁸. Prenatal diagnosis was attempted by using chorionic villus sampling of ALP activity; however, this method was not reliable because of the difficulty in obtaining pure samples⁷⁶. DNA-based diagnosis using genomic DNA from the chorionic villi is the most reliable method, assuming the absence of maternal DNA contamination77.78. Because siblings with different phenotypes have been reported, genetic counseling after prenatal diagnosis must be carefully conducted⁷⁰.

Among the mutations in the *ALPL* gene, a deletion of thymine at nucleotide 1559 is important in Japanese population^{63,79}. To date, this mutation has only been found in Japanese, among whom it is the most frequent mutation in the *ALPL* gene^{63,64}. The deletion causes a frameshift from leucine 503, eliminates the stop codon at 508, and adds 80 amino acids to the C-terminus⁸⁰. Analysis of the expression of c.1559delT mutation revealed that the mutant protein forms a disulfide-bonded aggregate, undergoes polyubiquitination, and is then degraded in the proteasome⁸¹. The protein lacks a glycosylphosphatidylinositol anchor, and a small amount of the mutant proteins is released into circulation^{80.81}. Sera from probands and heterozygous parents show a high-molecular weight band in electrophoresis⁸⁰.

Attempt to treat hypophosphatasia have been disappointing. The results of a trial of enzyme replacement therapy (ERT) using the serum of a patient with Paget disease were unsatisfactory⁸². Transplantation using bone fragments and cultured osteoblasts was partially successful⁸³. More recently, mesenchymal stem cell transplantation was reported to be successful⁸⁴. In a $Akp2^{-/-}$ mouse model, ERT using a deca-aspartate-tagged enzyme that had high affinity to hydroxyapatite was successful⁸⁵. A clinical trial using this modified enzyme is in progress. However, because this therapy requires daily or thrice-weekly injections of the enzyme, gene therapy by means of a single injection may prove to be a superior treatment.

Impaired Mineralization in Age-related Chronic Diseases

Age-related chronic diseases are major health issue in industrialized countries. Impaired mineralization is a cause of a number of such diseases, including osteoporosis, and calcification in articular cartilage, aorta, and large arteries, as well as valvular calcification in the heart³. The mechanism underlying pathological calcification is similar to that of physiological mineralization: the presence of smooth muscle cells in the large arterial wall leads to budding of matrix vesicles. However, the factors that trigger this budding are not known⁴⁵. Once hydroxyapatite is formed in the matrix vesicles, it propagates into the extracellular matrix and forms calcified crystals. Uremia causes hyperphosphatemia and pathological calcification in aorta⁸⁶. Increased TNAP activity has been reported in aortas from uremic rats⁸⁷. Enpp1^{-/-} mice display accelerated aortic calcification88, and induction of TNAP into human vascular smooth muscle cells

calcifies the extracellular matrix via addition of β glycerophosphate⁸⁹. Therefore, suppression of TNAP and activation of NPP1 is a strategy that might prevent pathologic calcification⁹⁰.

A decrease in physiological mineralization leads to diminished bone mineral density and bone mineral content, followed by osteoporosis⁹¹. There are several factors that influence bone mineral density and content⁹², including TNAP. In addition, there are a number of single nucleotide polymorphisms (SNPs) in the human ALPL gene. One exonic SNP resulting in an amino acid substitution is the 787T>C (p.Y263H) polymorphism. In elderly women, the CCtype (His residue at amino acid 263) was associated with significantly higher bone mineral density, as compared to TC-type and TT-type93. Expression of the CC-type protein results in lower Km for pnitrophenylphosphate, artificial substrate an routinely used in the ALP assay. These findings suggest that the CC-type (His at 263) protein has higher affinity for the substrate^{93,94}. It is not known, however, how this enzymatic property enhances bone mineral density in elderly women. The interaction between diet and genes is also important. It is therefore essential to examine the relationship between diet, especially calcium and phosphate intake, and SNPs. This nutrigenomic approach should lead to clues in developing individualized nutrition plans that prevent osteoporosis.

Conclusions

Mineralization is a fundamental activity of living things. Elucidation of the mechanisms underlying mineralization is crucial not only for understanding normal physiological development, but also for treating impaired mineralization. The recent increase in age-related impaired mineralization should lead to a new focus on preventing impaired mineralization and pathological calcification.

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