HHS Public Access

Author manuscript

Matrix Biol. Author manuscript; available in PMC 2017 May 01.

Published in final edited form as:

Matrix Biol. 2016 ; 52-54: 43–59. doi:10.1016/j.matbio.2016.01.007.

Intrinsically Disordered Proteins and Biomineralization

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Abstract

In vertebrates and invertebrates biomineralization is controlled by the cell and the proteins they produce. A large number of these proteins are intrinsically disordered, gaining some secondary structure when they interact with their binding partners. These partners include the component ions of the mineral being deposited, the crystals themselves, the template on which the initial crystals form, and other intrinsically disordered proteins and peptides. This review speculates why intrinsically disordered proteins are so important for biomineralization, providing illustrations from the SIBLING (small integrin binding N-glycosylated) proteins and their peptides. It is concluded that the flexible structure, and the ability of the intrinsically disordered proteins to bind to a multitude of surfaces is crucial, but details on the precise-interactions, energetics and kinetics of binding remain to be determined.

Keywords

Biomineralization; Intrinsically Disordered Proteins; Phosphophoryn; Hydroxyapatite

1. Introduction

1.1 Composition of Mineralized Tissues

Biomineralization occurs in a variety of organisms, from unicellular bacteria containing gold deposits [1] to diatoms [2] having silicon based mineral deposits, shells with calcium carbonate phases (aragonite, calcite and occasionally vaterite as well as amorphous calcium carbonate) [3], to hierarchical structures in vertebrate bones and teeth containing the calcium phosphate, hydroxyapatite (HA)[4]. Common features of these mineralized structures are (i) formation on an organic matrix, produced by the cells of that organism. The organic matrix within each organism or within their tissues, extra- or intra-cellular, plays a role in the mineralization process. This is well-known due to the observation that the removal or

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Conflict of Interest

The authors report no conflict of interest.

modification of the matrix, or any given component within the matrix, alters its properties and thus the manner in which the mineral is formed (Table 1). (ii) An additional feature of the matrix proteins regulating mineralization is that a large percentage of them, greater than that within the general protein data base [5], are intrinsically disordered proteins (IDPs). In this review, we suggest the reasons IDPs[1] are essential for the biomineralization process and provide examples to illustrate its importance.

1.2 Overview of the Process of Mineralization

Mineral deposition is a physical-chemical process that, in living organisms, is regulated by cells and by the intra- and extra-cellular matrices they produce. In purely chemical terms, a mineral will not precipitate unless its solubility is exceeded, either by having an increase in the concentration of its component ions, a change in temperature or pressure, or the addition of a surface that lowers the activation energy for the initial mineral formation (Figure 1). Precursor phases such as pre-nucleation clusters [6] and amorphous calcium phosphate can form in solution, reducing the activation energy barrier. Within the organism, the cells and vascular system provide the necessary increases in the concentration of component ions for initial crystal formation (critical nucleus). Intra- or extra-cellular matrices may also provide surfaces that facilitate this initial mineral deposition. Inhibitors of mineralization protect the matrix or extracellular matrix from being mineralized, when mineral is neither needed nor wanted, and are modified or removed by enzymes produced by the cell. This entire process is highly complex, even in the simplest organism, yet common themes exist; the elevation of local concentrations of precipitating ions and the regulation of the process by the extracellular matrix.

1.3 Protein Regulation of Mineralization

According to classic nucleation theory, deposition of crystals requires the formation of nuclei which support the growth and proliferation of mineral crystals. Nucleation can take place, *de novo*, when solution ionic concentrations are increased and ions collide with sufficient energy to form a critical nucleus. This critical nucleus has the same structure, only a few unit cells in size, as does the final crystal. Once a critical nucleus is formed, less energy is required for the addition of ions to the nucleus, thus allowing for growth or branching which facilitates the spread of the crystals. Pre-nucleation clusters or foreign surfaces can resemble the critical nucleus or the aggregates of these pre-nucleation clusters might themselves transform into crystals which will then grow, facilitating mineral deposition. It is believed that in the process of biomineralization, the matrix has a structure resembling that of the crystal phase being formed, and epitaxial nucleation takes place upon that surface. We know that if a protein can bind to the mineral acting as an epitaxial nucleator, it also could coat the mineral surface and regulate the size or shape of that mineral. This phenomenon has been seen recently in HA binding studies using peptides [16] and proteins [13]. It is also likely that the functions of proteins that control mineralization in multicellular organisms are redundant, both because the pattern of mineralization may differ

[1]ABBREVIATION; BSP-bone sialoprotein; CAP – carbonate hydroxyapatite; CD- circular dichroism; DD- dentinal dysplasia; DGI-dentinogenesis imperfecta; DMP1- dentin matrix protein 1; DPP- phosphophoryn; DSP – dentin sialophosphoprotein; DSPP – dentin sialophosphoprotein gene; HA – hydroxyapatite; IDP – intrinsically disordered protein; KO- knockout; MEPE-matrix extracellular phosphoglycoprotein; OI- osteogenesis imperfecta; OPN- osteopontin; SIBLING – small ligand binding N-glycoslated.

in different tissues throughout the organism, and because mineralization is so important for life itself. In more complex organisms, a template, such as collagen for bone, demonstrated by atomic-level molecular modeling [17] or the self-assembled matrix of amelogenin nano-spheres during enamel formation [18] may interact with the other proteins that regulate the mineralization process. It is here, that the IDPs become important.

2. Intrinsically Disordered Proteins (IDPs)

IDPs are proteins whose structures are highly variable [19, 20], and do not assume typical secondary structures (α-helix, β-sheet, etc.). Some of these proteins have a total random coil-structure; others, have intrinsically disordered regions. IDPs are often hydrophilic, having multiple amino acid repeats, often with either very negatively or positively charged domains. IDPs gain more folded-features when post-translationally modified and/or when bound to their numerous partners (Figure 2). Such partners may also have diverse structures and functions. IDPs account for ~30% of the human proteome [21]. The study of IDPs has increased extensively in recent years because these proteins are associated with major diseases such as schizophrenia and Alzheimer's disease, among others [22]. In vertebrate bio-mineralization, with the exception of collagen which does, in fact, have disordered teleopeptides [23], small leucine rich proteoglycans and some enzymes, the majority of proteins associated with HA formation and growth, recently reviewed [24,5], are IDPs. These IDPs may stabilize the ions or ion clusters, the critical nucleus, provide epitaxial sites for initial mineral deposition, and/or stabilize the crystal. The flexible nature of these structures enables them to perform these and many other functions. The observation that unstructured proteins were involved in mineralization was first reported in 1977 in a circular dichroism (CD) study of phosphophoryn (DPP), in the presence of Ca^{2+}, which, with the addition of this ion, changed from a random linear chain to a β-pleated sheet [25]. Phosphophoryn, also known as DPP or DMP2, is the product of the cleavage of DSPP into DPP and dentin sialoprotein (DSP). DSPP is the most abundant protein in dentin [26] and is also expressed in enamel and in bone. Absence of or mutations in DSPP, result in dentin abnormalities, discussed below.

In 1994, Evans and Chan using P^{31} and H^{1} –NMR, showed the folding of DPP was dependent on pH and reported the presence of hinge regions that allowed the charged flexible regions to interact [27]. Their model predicted the most compact (greatest folding) at low pH. Fisher in 2001 also used NMR to show that osteopontin (OPN) and bone sialoprotein (BSP) also had flexible (random coil or disordered) structures [28]. OPN, BSP, DSPP and matrix extracellular phosphor glycoprotein (MEPE), dentin matrix protein-1 (DMP1) are all part of the SIBLING protein gene family, where SIBLING indicates small integrin-binding ligand N-linked glycoproteins. They are all found on human chromosome 4, mouse chromosome 5, and have many sequence similarities [29]. They all include repeat sequences with groups capable of phosphorylation and the presence of multiple aspartate or glutamate repeats. Our group used vibrational spectroscopy to show the transition from random coils in OPN and DMP1 to slightly more structured forms in the presence of HA, calcium ions and collagen [30.31]. One of the features of the flexible structures of IDPs is that post-translational modifications (phosphorylation, glycation, acetylation, sulfation, cleavage) can be facilitated by virtue of an open chain [32]. In fact, post-translational

modifications were shown to have a preference for disordered regions in a large number of proteins with known NMR-structures [33]. The post-translational modified structures will also fold differently when they bind to their partners. The importance of post-translational modification (cleavage and phosphorylation) for mineralization was suggested in these studies, since the non-phosphorylated proteins did not interact with HA or Ca^{2+} [34]. Thus IDPs, by having variable post-translational modifications, can regulate their interactions with mineral phases and modulate the mineralization process A second key aspect of the flexible structure is that different parts of the IDP may fold on interaction with different partners. This could allow the IDP to fold one region to stabilize the amorphous cluster and leave another region unfolded to interact with growth factors, the nascent crystal, or other noncollagenous proteins.

IDPs are also involved in the formation of mineral in the invertebrate species. Calcium carbonate formation in shells, for example, is regulated by IDPs. One shell protein, AP7, first shown to form supramolecular-assemblies and nucleate single crystals of aragonite [36] is also involved in the regulation of calcite growth [37]. The C-RING-like sequence is an important site for AP7 self-association and mineral nucleation; self-assembling, to form protein phases that congregate amorphous calcium carbonate nanoparticles, into hybrid, protein–mineral supramolecular networks, and create nano-porosities on the surfaces of the calcite that forms. Chang et al. [37] speculated that the repeated deposition of AP7 on the calcite surface allows for the creation of these nano-porosities. The ability of IDPs to interact with and regulate more than one mineral phase is another important theme that will occur throughout this discussion. Thus, it is likely that where multiple crystalline phases may form, a pre-cursor or well crystallized phase interaction with different proteins can stabilize and direct the nature of the phase that is formed and the way in which it grows.

Another IDP associated with calcium carbonate formation, SM50, the most abundant protein in sea urchin spicules, stabilizes amorphous calcium carbonate [38]. Sea urchins spicules are believed to form by first depositing a hydrated amorphous calcium carbonate onto the forming spicule surface, which dehydrates and crystallizes to form calcite [38]. SM50 has different domains that are important for calcium binding; directing proper growth of the mineral and directing the development of mineralized spicules [39]. Studies of SM50's conformational changes during these processes were reported by the Evan's group [40] who noted an unusual 20-residue proline-asparagine-containing repeat sequences, which were investigated by CD dichroism and other methods, showing the repeat motif adopts an extended “twist” structure, consisting of turn- and coil-like regions. They suggested that this domain acts either as a mineral-binding domain, a protein-protein docking domain, or as an internal “molecular spacer” for the SM50 protein during spicule formation. Proof for these roles is yet to be determined. Variation in conformation and function, however, is a common theme among the IDPs involved in mineralization.

A third example of an IDP involved in calcium carbonate deposition is Star-maker, a protein made in otoliths (ear organs) of fish. This protein, similar to many of the IDPs involved in calcium phosphate formation, is highly phosphorylated via post-translational modifications; and like the examples discussed below, folds to become more compact on phosphorylation [41,42]. Calcium binding also causes a condensation of the molecule, leading to the

appearance of a secondary structure (loss of random coil) and the consequent decrease in hydrodynamic radius.

2.1 HA formation in Enamel: Role of IDPs

Enamel formation, a complex process in which large mineral (HA) crystals are formed as the organic matrix is removed, is similarly dependent on IDPs. Janet Moradian-Oldak and her collaborators John Evans and Wendy Shaw investigated the secondary structure of amelogenin and ameloblastin, two major enamel proteins. Both were shown to be IDPs [43,44], their binding partners were other IDPs (enamelin and tuftelin) and HA. Enamel formation does not occur on a collagen matrix; rather it forms under the direction of the previously mentioned IDPs. The enamel forming cells (ameloblasts), produce several IDPs: amelogenin [45], ameloblastin [46], and enamelin [47,48]. These IDPs are eventually cleaved into smaller fragments by enzymes, e.g., enamelysin (matrix metalloproteinase 20, Mmp-20) and kallikrein 4 [49]. The ways in which these proteins guide the formation of HA in enamel is unknown. There are, however, some important clues from recent studies. In the teeth of mice that produce a truncated form of ameloblastin, enamel fails to appear [50,51]. These mice also have reduced expression of amelogenin with no apparent change in the levels of several other ameloblast-produced proteins [52,53] indicating that there must be some interaction between amelogenin and ameloblastin during the mineralization process. Studying mouse ameloblasts at different stages of development, Moradian Oldak [54] reported amelogenin and ameloblastin co-localized near the secretory face of ameloblasts at the earliest stages of their formation; with maturation, ameloblastin was lost from the enamel surface. Additionally, they found using circular dichroism, that amelogenin and ameloblastin could form stable complexes. C-terminal polypeptides of ameloblastin were cleaved into smaller peptides and lost from the extracellular matrix [55] maturation is complete. This implies that the interactions of these IDPs may facilitate specific cleavage. The observation that the amelogenin protein in some patients with amelogenesis imperfecta formed aggregates supports the concept that having an IDP-like flexible structure is germane to the mineralization process [56]. Further, Wendy Shaw's group used NMR spectroscopy to examine the WT mouse and mutant amelogenins to confirm the pre-mature self-assembly of the mutant proteins [57]. Other studies show how the amelogenin structure is modified during its interaction with HA, phospholipids, other enamel proteins [58] and even collagen (remember that amelogenin does not interact with collagen in situ) [59] suggesting that during the complex interactions leading to HA formation other conformational changes are occurring.

Cryo-TEM investigations on the initial formation of enamel crystals have provided high resolution insight into the way an IDP, amelogenin, interacts with pre-nucleation clusters. In calcium phosphate solution, the presence of amelogenin stabilized small particles, aggregates with two or three particles and larger aggregates (pre-nucleation clusters). While needle shaped particles of hydroxyapatite were eventually formed, the amorphous aggregates were stabilized by amelogenin, leading to the statement that amelogenin inhibited (retarded) mineralization [60,61]. It is of interest to note that atomistic modeling of prenucleation clusters for calcium carbonate suggested that they also consisted of chains of

cations and anions [62]; the formation of such chains could also be true of the calcium phosphate prenucleation clusters.

2.2 Pathologic Calcification: Role of IDPs

Calcifications occurring in unwanted places – the aorta, salivary glands and soft tissues, are all associated with IDPs. To illustrate, salivary stones occur when mucins, such as statherin, an un-folded protein [63], are defective [64]. Statherin, a salivary IDP that lubricates enamel, binds bacteria, and regulates the amount of calcium and phosphate to which enamel is exposed, is involved in prevention of dental calculus formation. Statherin, which also controls the nucleation and growth HA in saliva, contains an N-terminus with small acidic motif (DSpSpEE) that is also found in SIBLING proteins [65]. When solubilized, statherin has a random coil conformation. When bound to HA, it takes on a more alpha-helical structure exposing a bacterial binding site [63], leading to bacterial entrapment.

Another example is the role of the IDP, DMP1, in preventing kidney and cardiovascular calcification. The double knockout mouse lacking both DMP1 and Klotho (a kidney transmembrane protein that regulates phosphate levels), develops kidney and cardiovascular calcifications associated with inability to manage high phosphate levels in the absence of DMP1 [66]. A third example is provided by a collagen-disease. The hinge region of type VII collagen, associated with dystrophic epidermolysis bullosa, is also intrinsically disordered [67]. Additionally, the SIBLING proteins, all IDPs, are also associated with vascular [68,69] and other soft tissue calcifications [70]. Since their genes are expressed in cells associated with these deposits they cannot have accumulated simply due to their affinities for HA or collagen, rather, they must have some function in the deposition of these unwanted calcifications.

2.3 Abundance of IDPs in Mineralization

Structure predictions demonstrate that almost all proteins associated with mineralization in the Swiss Protein Database are IDPs [5]. Many of these proteins share the common repeat sequence: aspartate-serine-serine (DSS) or glutamate-serine-serine (ESS), where the serine is often phosphorylated. An example of such proteins we and others have associated with the mineralization process, are the so-called SIBLING proteins: osteopontin [30,71,72,73], dentin matrix protein 1 [33,74,75], matrix extracellular phosphoglycoprotein [76], bone sialoprotein [77,78] and the cleaved products of dentin sialophosphoprotein, dentin phosphophoryn [79] and dentin sialoprotein [80]. It should be noted in this discussion that enamelin, originally considered a SIBLING, lacks cell binding activity in some species and thus is no longer considered a SIBLING protein [28]. Much like sibling children, they share common heritage (genes) and some structural homology, often appearing to play different roles in regulating biomineralization in various states of health and disease of dentin and bone (Table 2). All bind to and regulate cell differentiation via different factors [81]. They all have numerous repeat sequences, most are charged. To illustrate, we developed a PERL-based script to identify all possible triplet amino acids repeat sequences in human SIBLING proteins (Table 3). For the sake of simplification “N” represents glutamic acid (E) or aspartate (D), “P” represents serine (S), Threonine (T) and Tyrosine (Y), with high probabilities of being phosphorylated based on NETPHOS software. We also include

SPARC (osteonectin), a protein involved with mineralization (linked in some ways to osteoporosis) that has some IDP regions.

The SIBLINGs all bind to HA, however characterizing adsorption of proteins to solid surfaces is challenging. The energy controlling the absorption of proteins comes from intermolecular forces, such as Coulombic, van der Waals, Lewis acid-base, and hydrophobic interactions. Temperature, pH, ionic strength, protein concentration, protein polarizability, and surface polarizability can influence these forces. Together, they control the conformational entropy of the protein structure and the motilities of the protein over solid surfaces [97,98,99]. The proteins more frequently studied are large globular proteins, such as enzymes [99,100], which show some conformational changes, decreases in secondary structure or loss of three dimensional structure upon surface adsorption [101,102,103,104,105]. Globular proteins show a loss of native structural stability upon adsorption to solid interfaces due to increased entropy as proteins are absorbed to a solid surface. These conformational rearrangements in the native structure are thought to affect the protein functions [102, 106]. In contrast, IDPs are charged proteins, thus they can control their interfacial absorption onto solid surfaces [106]. Experiments, *in silico*, suggest that intrinsically disordered peptides absorbed to a surface with a complementary pattern form a well-defined structure (α-helix) indicating that a specific surface can stabilize the structure of an IDP peptide. The effect of a complementary surface pattern surface on protein structure *in vitro* was demonstrated by Wendy Shaw's group. They used a peptide from Leucine-Rich Amelogenin Protein (LRAP) to analyze the changes in the secondary structure when this peptide was absorbed to HA and carbonated HA (CAP). The structure of LRAP peptide absorbed to CAP was consistent with an α-helix, whereas when bound to HA the structure corresponded more to a random coil [107]. We studied DPP peptides by FTIR spectroscopy and found the phosphorylated peptides in solution in the presence of HA formed α-helical structures and lost their random coil characteristics [108]. Perhaps, through evolution, the most efficient strategy found by nature for protein-surface interactions, was using IDPs to bind to solid surfaces and in this way control the nucleation, growth and morphology of biominerals while still being able to interact with other partners. IDPs do not have a structure to lose on binding and thus can form more favorable secondary structures on binding.

2.4 Self-Assembly of IDPs

IDPs frequently undergo self-assembly. The extended conformation of IDPs facilitates the intermolecular interactions between them and promotes formation of supramolecular architecture [109]. This is illustrated in some of the mineralized tissues discussed above. Silaffins, for example, are protein constituents of biosilica, playing an active role in silica formation. These small proteins with numerous phosphoserine repeats self-assemble via electrostatic interactions; a prerequisite for biosilica formation [110,111]. SM50, from the mineralized larval of sea urchin spicules, contains C-type lectin and IDPs repeat domains. The C-terminal region of the IDP domain containing an asparagine and proline (Asn- and Pro)-rich region, with glycine (Gly)- and glutamine (Gln)-rich repeats mid-sequence is implicated in self- assembly. Based on analytical ultracentrifugation, the glycine-rich region oligomerizes to form large protein assemblies. The proline-rich region also shows a self-

assembly due to the hydrophobic interactions between pyrolidine rings and hydrogen bonding interactions between the Asn residues [39,112].

In enamel, amelogenin conformational changes upon self-assembly differ from those observed with interactions with calcium phosphate mineral [113]. *In vitro* studies with amelogenin reported a disorder-to-order transition during the self-assembly process. The amelogenin molecules at pH 7.2 spontaneously self-assemble into oligomers and nanospheres. These subsequently bind together to form micro-ribbons [58]. Beniash et al suggest that this amelogenin self-assembly is attributable to formation of intramolecular hydrogen bonds between extended unordered regions of the protein, leading to the formation of tightly bound intrafibrillar β-strands [88]. As a final example, two peptides from dentin matrix protein 1 cleavage can self-assemble *in vitro* to form micro-fibrils. This was suggested to be crucial for HA mineral nucleation [59]. Self-assembly is thus another way that the IDP proteins can regulate the process of biomineralization.

3. Potential Mechanisms of IDP Action: Biomineralization

There must be precise reasons why so many IDP proteins are associated with mineralization. It is our suggestion that among these reasons are: i) the energetics of initial mineral deposition in the presence of IDP is more favorable. Namely, the driving force (free energy) needed to bind IDP to either collagen, precursor mineral phase or HA, whichever initiates crystal deposition, is lower, due to the change in entropy Δ S, when an unstructured protein is bound and folded (transiently), is smaller than when a structured protein is bound ($\Delta G = \Delta H - T\Delta S$). This concept may be debated, however, due to the relative order of the IDP vs. the dis-order of water upon IDP-partner binding in aqueous media. ii) An open structure gives an IDP the ability to bind to more than one partner and present different surfaces facilitating the regulation of mineralization. iii) An open structure facilitates post translational modifications, which can alter IDP net charge by phosphorylation and glycosylation, etc., providing for a greater structural variability, opening the door to new ligands, enabling the IDP to act as either nucleators, inhibitors, or both. It is likely that different combinations of each of the above exist in situ.

IDPs in general have multiple functions. Biomineralization-related IDPs, with their flexible chains, have several possible functions, which, depending on the above-proposed mechanisms, may include [114]:

(a) Stabilization of calcium phosphate nano-clusters or amorphous calcium phosphate, ACP. For example, unstructured chains of the milk proteins casein [64] and the SIBLING protein osteopontin [34] are known to stabilize ACP. A recent neutron spectroscopy, small-angle X-ray and neutron scattering of osteopontin-calcium phosphate interactions, confirmed that the 1-149 fragment of osteopontin maintained its flexible linear chain structure in solution, stabilizing ACP particles [115]. Additionally, stabilization of calcium-carbonate nanoparticles and creation of nano-porosities within them is due to the IDP mollusk protein, AP7 [37], discussed above, implying this could be a common IDP –mineral regulation mechanism. As reviewed elsewhere [116] the initial

mineral that forms, be it an amorphous liquid, or pre-nucleation chains or clusters, may exhibit polyamorphism. One or more amorphous phases that are formed may be stabilized by IDPs, in some cases facilitating the transformation to a crystalline phase and in others providing a means for remaining in an amorphous state. Some of these amorphous structures have short range order [117] and may be considered proto-crystalline [116]. For amorphous calcium carbonate, this short range order is only noted in the presence of additives, such as poly-aspartic acid. Since the amorphous structures aggregate to form prenucleation clusters, this suggests that the prenucleation clusters also have short-range order, and this is likely to be associated with the presence of IDPs, which in turn could determine whether there is an amorphous to crystalline transformation and what phases are formed.

(b) Enabling formation of supramolecular structures, as discussed above and as seen for i) enamel and ameloblastin [53], ii) complexes of collagen, IDP and mineral, or iii) peptide-amphiphiles being developed for bone regeneration [118]. Ameloblastin self-associates into long (10-100 nm) ribbon-like structures averaging 18 and 0.34 nm, in width and thickness [44]. How this regulates enamel mineralization is under investigation but it important to remember that mineral formation does not occur when ameloblastin is absent [51].

(c) Allowing for variations in post-translational processes, as exists for the various SIBLING proteins of tooth and bone. This is illustrated by the SIBLING proteins, which undergo extensive and variable post translational modification in different tissues, facilitating their interactions with their binding partners (cell surface integrins, HA, Ca^{2+} and collagen). When phosphorylated, they are all highly acidic. The extent of phosphorylation of these proteins and their peptides affects their ability to bind to HA and to regulate mineralization [119]. Modeling of the most frequently appearing repeat peptide in DPP [108] by molecular dynamics simulations, showed the phosphorylated peptide bound with greater affinity and a more organized structure to the surface of hydroxyapatite (Figure 3A,B). The change to a more-ordered structure after phosphorylation and interactions with either calcium ions or hydroxyapatite was verified by circular dichroism, small angle scattering and vibrational spectroscopy (Figure 4). Based on modeling, the phosphorylated peptide folded differently when associated with a model of type I collagen than did the non-phosphorylated peptide (Figure 5). These changes in conformation associated with post-translational modifications, could facilitate nucleation and growth of mineral crystals upon and within the collagen matrix.

(d) Regulating protein expression within cells. DMP1, for one, regulates circulating phosphate levels by controlling bone expression of the hormone FGF23 [66]. IDPs assume many different functions because their “unfolded” structure presents with a multitude of surfaces which enables them to interact with a diverse set of enzymes, cell membranes, and other partners [120]. The overall question, however remains, as to why the IDPs are both associated with and required for biomineralization. It might be argued, that IDPs arose by gene

multiplication and are incidental to the mineralization process. (*DSPP*, for example, is a duplication product of *DMP1* [121]). The observation that IDPs are so ubiquitous during mineralization argues against this. IDPs are known to be "promiscuous" [109] with multiple binding partners. This promiscuity most likely is due to the multiple surfaces presented by the IDPs or the low energy requirement associated with their role in mineralization. Thus, it is not surprising that DMP1 can interact with cell membrane receptors (e.g. αvβ3 integrin [122] to regulate signaling [123], FGF23 expression [66], bind to HA crystals so as to regulate HA growth [34], interact with itself [124] and with collagen [125] to modulate dentin and bone mineralization. The ASARM (acidic serine aspartate-rich motif) peptide released from MEPE, DPP and DMP1 has roles in regulation, vascularization, soft-tissue calcification, osteoclastogenesis, mechanotransduction, fat energy metabolism, along with regulation of mineralization [126]. As illustrated in Figure 4, IDPs also interact with HA, as reported by our group, for DPP [79] and OPN [13] and by the Clarkson's group for DSP and DPP interacting with enamel crystals [127].

To date, in addition to the studies mentioned above, atomistic modeling has suggested ways in which IDPs regulate biomineralization. For example, based on modeling of different domains of the bone sialoprotein structure, first reports found ser-136 had to be phosphorylated for HA nucleation to occur [128]. In contrast, another study reported there was no domain that was involved in nucleation [129]. Using a larger portion of the molecule for modeling, it was later suggested that a cationic loop region promoted nucleation by attracting Ca^{2+} ions, with the loop's flexibility allowing for rapid self-assembly with phosphate ions [130], rather than providing a regular template for crystallization. The flexible nature of the osteopontin structure when bound to the HA surface, was also predicted by molecular dynamics [120]; the phosphorylation of peptides modified binding in these studies [131], as in studies of interaction with calcium oxalates [132]. These data led the Hunter group to suggest that adsorption of acidic proteins to Ca^{2+}-rich crystal faces of minerals was governed by electrostatic phenomena and facilitated by the conformational flexibility of the chain [131]. These and other atomistic modeling studies suggest the importance of the flexible conformation of the IDPs for interaction with mineral crystals and with collagen [133]. The best demonstration of the importance of IDPs function in the mineralization process comes from studies of animals and people in which IDP has been manipulated, by nature or by genetic techniques. The effect of mutations in biomineralization-related IDPs on bones and teeth are discussed below.

4. Relationship of IDPs to Bone and Tooth Diseases

In both animal models and human samples, defects in IDPs, have been associated with tooth and bone diseases. Defects in DMP1, for example, are associated with Dentinogenesis Imperfecta type III and autosomal recessive hypophosphatemic rickets [134,135]. Although the observed phenotype may be linked to low phosphate levels and inappropriate FGF23 expression in those with aberrant DMP1, these condition were the first directly linked to a defect in an IDP, and thus deserves mention. Dentinal dysplasias, in general, are relatively rare diseases. When present, they have major effects on the patient's lifestyle, altering the

craniofacial complex and exacerbating tooth decay. They have been associated with mutations in collagen in cases of osteogenesis imperfecta (OI) [136]. Defects in the human DSPP gene are the major cause of dentin disorders identified to date [137,138,139]. Mutations in DSPP are associated with five different types of inherited dentin defects – dentinogenesis imperfecta (DGI) types I, II, III and dentinal dysplasias (DD) types I and II. DGI has an incidence of 1/6000 to 1/8000 and DD an incidence of 1/100,000 [137]. In DGI and DI, the weakened dentin can lead to loss of enamel as it shears off the surface. DGI type I is associated with osteogenesis imperfecta and hence a defect in the underlying collagen matrix [140]. To date, all other types of DGI and DD have been associated with mutations in DSPP. It should be noted, that with the exception of the abnormal collagen in DGI type I, all of the other forms are associated with abnormalities in an IDP. Due to the collagen anomaly we suggest that the IDPs similarly contribute to the phenotype in DGI type I as the IDPs bind to specific sites on the collagen matrix. These sites may be altered in DG I or in OI, thereby altering the structure of the environment in which mineral crystals form. Thus, for most types of OI studied to date, the matrices tend to be hyper-mineralized (due to decreased collagen content) and perhaps normal amounts of HA, albeit with smaller HA crystals because the IDPs that regulate their growth are also altered in structure [141,142,143,144,145,146].

Enamel malformations are also associated with DSPP mutations, although enamel mineralization is not dependent on DSPP [147]. In the Dspp knockout (KO) mice, the teeth are hypo-mineralized [148] and similar to humans with DSPP mutations, the mice develop periodontal disease [149,150]. The dentin phenotype of the Dspp KO mouse mimics that of DGI type III, with widened pulp spaces and predentin and the presence of hypomineralized areas within the circumpulpal dentin [148]. A key study by von Marschall et al [151] suggested that the dentinal dysplasia phenotype is likely associated with the retention of the mutant DSPP in odontoblasts, i.e. a failure to “traffic” the mature DSPP protein out of the cell. Thus if the protein, independent of its mutation, remains trapped in the cell and cannot interact with collagen so as to regulate the mineralization process, the hypo-mineralized phenotype persists. This is likely to be true of other IDPs where mutations could lead to their inability to exit the Golgae and hence form the required complexes needed to initiate and facilitate mineral deposition.

DSPP is also expressed in bone, but patients with *DSPP* mutations have not been reported to have bone abnormalities. This is probably due to the low level of expression *of DSPP* in bone, redundant functions of the protein or differences in the processing of DSPP in bone and dentin [121, 139]. It should be noted that immature *Dspp* KO mice, contrasted with patients with *DSPP* mutations, have hypomineralized bones [89]. It is not known whether this is corrected as the mice age. *In vitro* TEM data suggested that DSPP could induce highly oriented intrafibrillar HA mineralization along the collagen fibril axis of self-assembled collagen gels [125].

Osteogenesis imperfecta (OI) is caused by abnormalities in at least 17 genes, all concerned with type I collagen synthesis, secretion, alignment and mineralization [136, 152,153]. The phenotypic variability of OI in different families and within the same family, with the identical mutations [154], may be attributable to unknown effects on other genes or to

similarly unknown interactions with collagen during development. As noted above, interactions of many of the IDPs can be mapped to distinct sites on the type I collagen matrix [155]. It has been theorized that alterations in these sites can affect the binding of these noncollagenous IDPs [155]. This is supported by the previously reported lower amount of various IDPs in different forms of OI [153,154]. Related to the OI story, the serpins, proteins which regulate protease activities, are IDPs, and serpin F1 mutations are associated with type V OI [153]. Another possible example of defective IDPs leading to bone disease is osteoporosis which has been associated with defects in osteonectin [156], which, as noted above has IDP regions.

Osteopontin deficiency results in dystrophic calcification in humans [94] and in mice [157]. Studies from our laboratory in 2002 [158] showed that the osteopontin KO animal had greater amounts of mineral in mature areas of the bone than did control mice and mineral crystal size and perfection throughout all bones of the KO mice was significantly increased. The OPN KO animals had a 30% decrease in fracture toughness, and significant reductions in their elastic modulus [94]. In a solution [71] and in ectopic sites, osteopontin is an inhibitor of the formation and growth of HA crystals [157]. FGF23 deficiency leads to renal phosphate wasting, elevated circulating phosphate levels and surprisingly, rickets (hypomineralization). FGF23 KO mice over-express osteopontin [159] which is thought to be the origin of the rickets. Taken together, these data show that osteopontin needs to be flexible in order to i) be phosphorylated and dephosphorylated, ii) to interact with collagen (to facilitate mineralization), (iii) to interact with crystals (regulating their growth ad matrix crack population). Raine's syndrome, a rare condition with an incidence of 1/1,000,000, is due to a mutation in FAM20C the Golgi protein kinase that phosphorylates most secreted phosphoproteins including SIBLING proteins [160,161]. The osteosclerosis characteristic of this skeletal dysplasia provides clinical support for the importance of controlled phosphorylation of IDPs for proper mineralization [162,163].

5. Conclusions

This review has presented evidence that a large number of proteins associated with biomineralization are intrinsically disordered. We also summarized data showing that these proteins are involved in the process of biomineralization, and in some cases, have provided proof that these proteins are required for appropriate mineralization. It is demonstrated that having a flexible structure is important for allowing these proteins to take on a variety of forms in order to interact with their partners. To prove our theory of why IDPs are needed (lowering activation energy), kinetic experiments need to be conducted; but at this point the first parts of our concept on why IDPs are so important, have been validated.

Acknowledgments

Our work as presented in this paper was acquired under NIH grants DE04141 and DE022716. Dr Villarreal-Ramírez acknowledges financial support received from DGAPA-CONACYT and programa UNAM-DGAPA-PAPIIT IA207216. The PERL-based script and SIBLINGs sequences analysis was carried out by Dr. Luis Fernando Lozano-Aguirre Beltran. We appreciate the editorial assistance of Dr. Judah Gerstein.

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HIGHLIGHTS

- A preponderance of the noncollagenous proteins associated with biomineralization are intrinsically disordered (IDPs).
- IDPs regulate the mineralization process through interactions with ion clusters, pre-nucleation clusters, amorphous phases and crystalline phases.
- Examples are provided from shells, enamel, dentin, bones and pathologic calcifications.

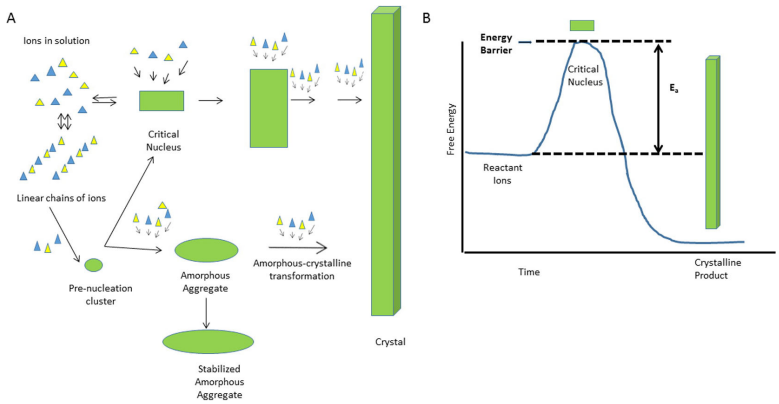

Figure 1.

Physical Chemistry of Mineralization. A) Schematic illustrating how ions in solution can either form linear chains of ions, critical nucleus (the classical concept of nucleation) or unstructured (amorphous) clusters. The critical nucleus continues to accumulate ions and develops into a crystal. The clusters may also associate to form a “critical nucleus”. Proteins can interact with any of these forms to facilitate or inhibit the next step. B) The major expenditure of energy is in the formation of the critical nucleus as illustrated by this plot of activation energy vs. time.

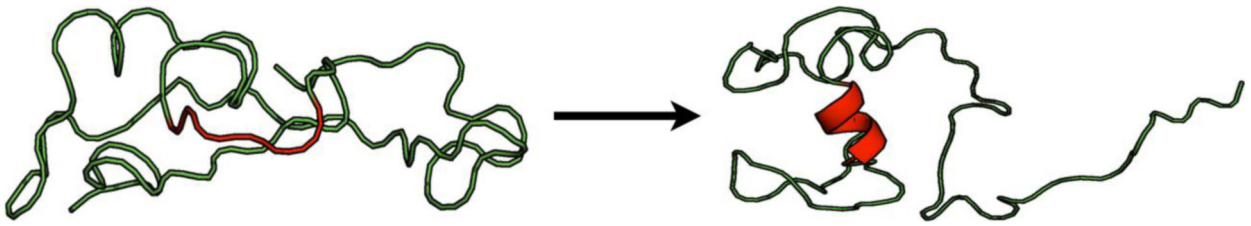

Figure 2.

Intrinsically disordered proteins or regions of proteins (as shown here by the unstructured ribbon) can take on increased secondary structure when interacting with their partners. Here, a domain of the retinal cGMP phosphodiesterase was predicted by molecular dynamics (10 ns) to form an alpha helix (red) on interaction with cGMP. Note that not all of the protein folds on this interaction. The NMR structure for this interaction was reported elsewhere [35].

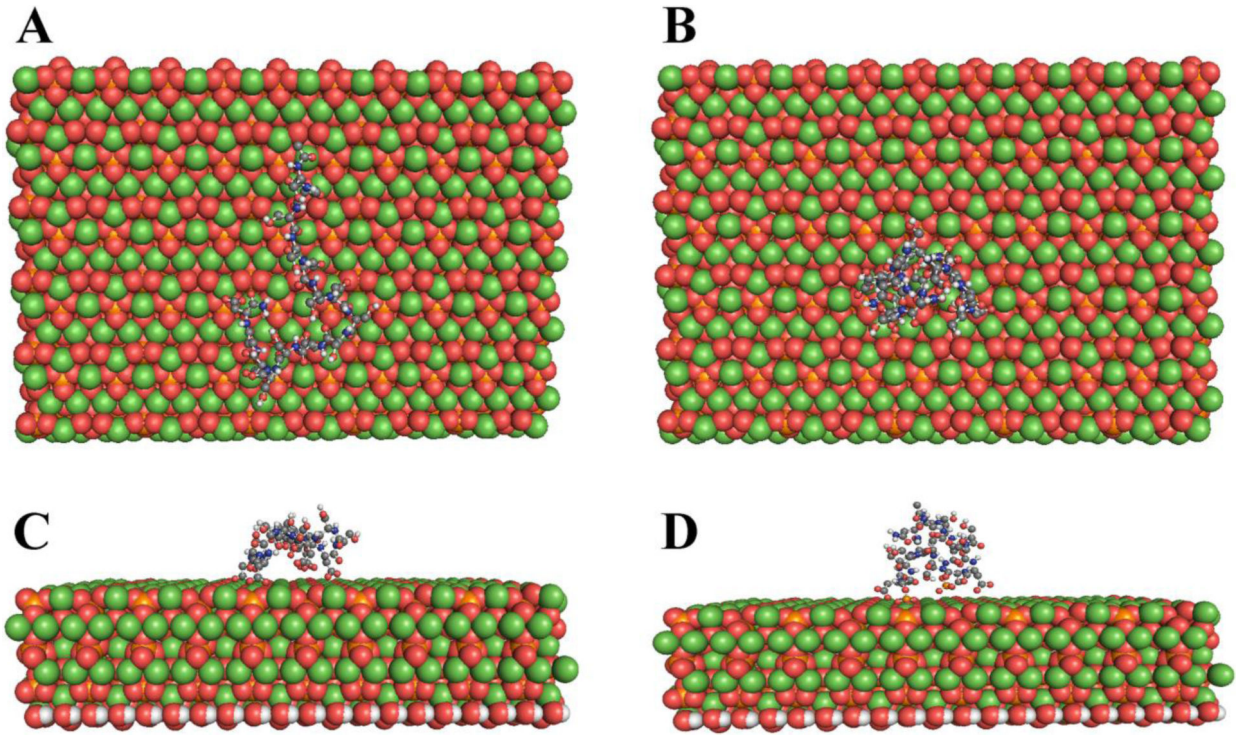

Figure 3.

Molecular Dynamics Simulation: The most commonly repeated peptide of Dentin phosphophoryn (P5 Ace-SSDSSDSSDSSDSSD-NH2 and P5P Ace-SSDpSpSDpSpSDpSpSDpSpSD -NH2) binds in a more extended form to the 100 surface of hydroxyapatite when phosphorylated (A,C) than when unphosphorylated (B,D). A and B show the mineral surface, B and D are viewed perpendicular to the surface. In the mineral model red circles are oxygen (O) atoms, orange circles phosphorus (P) atoms, white circles hydrogen (H) atoms and green circles calcium (Ca) atoms. Water is not shown but was included in the model. For details of the modeling procedures see Villarreal-Ramirez et al., 2014 [108]).

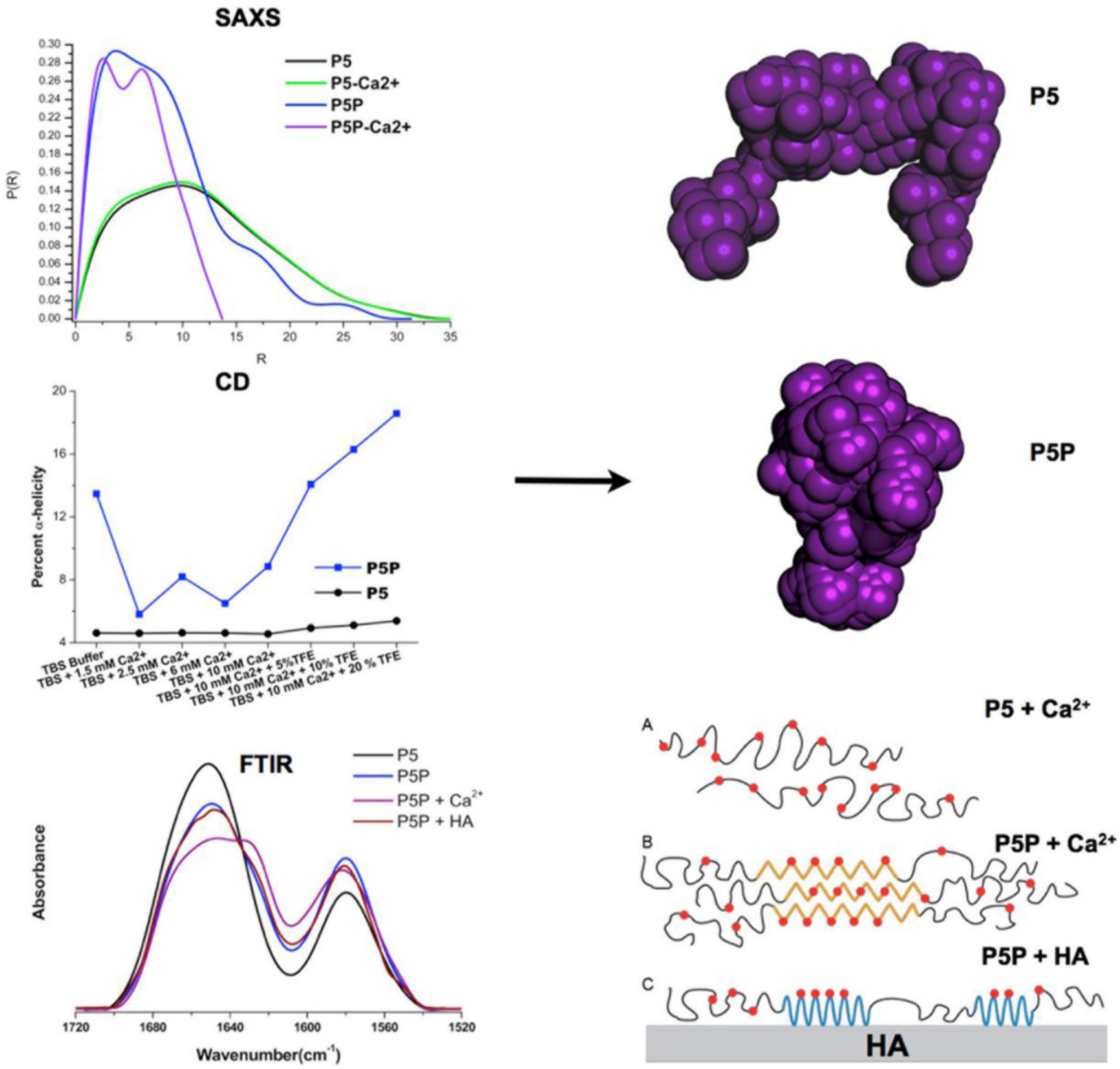

Figure 4.

In Situ Validation of Molecular Dynamics Predictions: The peptide detailed in figure 3 was studied by a variety of techniques to confirm the predictions from Molecular Dynamics. Small-angle/wide-angle X-ray scattering (SAXS/WAXS) confirmed the formation of a more structured molecule for P5P in the presence of calcium ions. This was also confirmed by circular dichroism (CD). Fourier transform infrared analysis (FTIR) combined with curve-fitting showed the peptides increased their secondary structure when bound to the HA surface; with phosphorylation (P5P) causing a shift to more helical structures. The ab initio dummy atom models (purple models) on the right were generated with the DAMMIN algorithm with SAXS data. In the lower right corner a cartoon illustrates the conformational changes suggested by these studies.

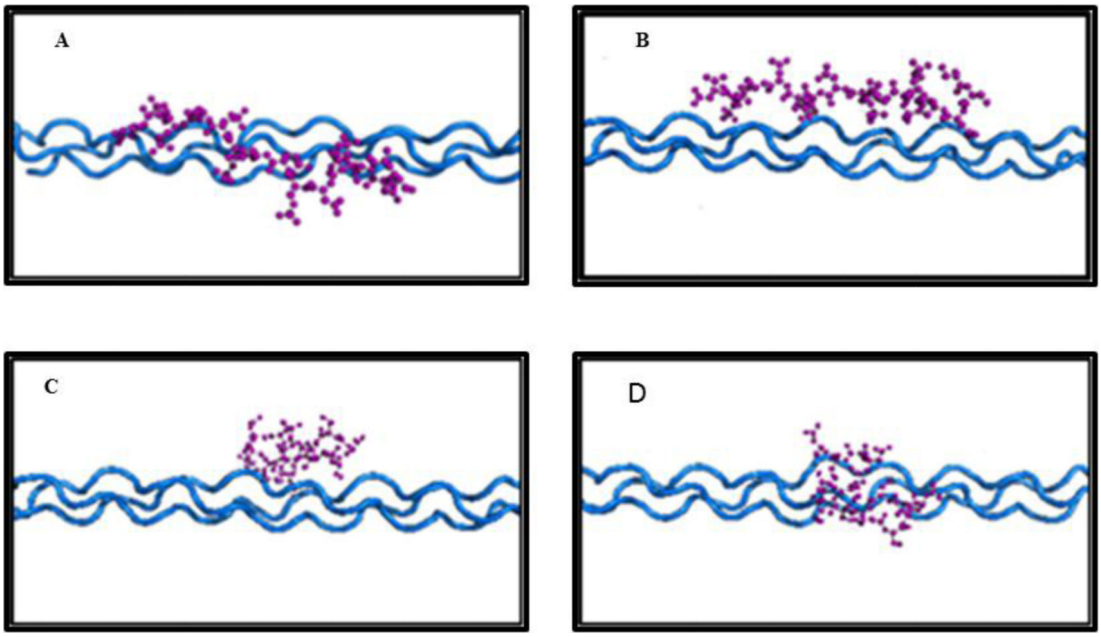

Figure 5.

Molecular Dynamics Simulation of P5 and P5P interactions with a typical type I collagen peptide structure: Collagen binding was predicted using a rigid body docking model. P5P interacts more with fibrillar collagen (A/B) than P5 (C/D). Two different orientations are shown to illustrate the differences in binding. The backbone of the collagen peptide is shown in blue ribbons. The P5 (A-C) and P5P (B-D) peptides are represented as colored balls and sticks in purple. A-B) viewed from the side; C-D) viewed from above.

Table 1

Examples of Alterations in Matrix Composition that Affect the Mineralization Process

Species	Type of mineral	Treatment of Matrix	Change in Mineral
Magnetic Bacteria[7]	Magnetite (Fe_3O_4)	Deletion of operon	Irregular crystals
Avian Egg shell[8]	Calcite	Incubation in Dermatan sulfate	Delays Formation
Abalone shell[9]	Aragonite/Calcite	Perlucin variants	Modulate rate of precipitation
Diatoms[10]	Silicate	Composite of silk and silaffin	Promotes mineralization
Mice[11]	Hydroxyapatite	Knockout or overexpression of ameloblastin	Decreased enamel formation
Mice[12]	Hydroxyapatite	Knockout of phospho-1	Smaller crystals
Vertebrate[13]	Hydroxyapatite	Dephosphorylation of osteopontin	No inhibition of growth
Vertebrate[14]	Hydroxyapatite	Dephosphorylation of all phosphoproteins	Decreases extent of mineralization
Zebra-fish[15]	Hydroxyapatite	Mutation in BMP1 (enzyme)	Defective mineralization

TABLE 2

SIBLING Proteins and their Roles in Vertebrate Mineralization

Protein	Associated Disease	Properties of KO	Effect on HA formation
Bone sialoprotein	?	Impaired bone turnover ; higher bone mass[82] Embryonic delayed mineralization[83]	Promotes (nucleates) mineralization[84]
Dentin matrix protein 1	Dentinogenesis Imperfecta III Autosomal recessive form of hypophosphatemic rickets	Dentin hypomineralization in maturation[85] Reduced dspp levels.[86]	Full protein inhibits[74] Peptides promote[33]
DSPP	Dentin dysplasia	Hypomineralized dentin[87,88] Bone composition varies with age[89]	DPP inhibits HA formation at low concentration and promotes it at high[71]; DSP has little effect[80]
Enamelin	Hypoplastic amelogenesis imperfecta	Impaired enamel mineralization[90]	Inhibited seeded HA growth[91]
MEPE	Oncogenic osteomalacia	Increased bone formation and bone mass[92]	Full protein nucleates; peptides inhibit[93]
Osteopontin	Vascular calcification	Reduced fracture toughness without change in bone mass[941]; Increased mineral content and decreased crystallinity[95]	Full protein inhibits[96] Fragments- variable effects[16]

Table 3

Simplified Triplet Repeat Count

	NNN	NPP	NNP	PNN	PPN	PPP
DSPP	9	167	5	170	3	11
DMP1	14	7	15	11	11	6
BSP	17	8	10	7	2	4
MEPE		5	3		5	1
OPN	7	6	3	9	5	
SPARC	2	1	3	1		

“N” represents glutamic acid (E) or aspartate (D) and “P” represents serine (S), Threonine (T) and Tyrosine (Y) with high probabilities of being phosphorylated (NETPHOS).