ORIGINAL RESEARCH

Association of Calcium and Phosphate Ions with Collagen in the Mineralization of Vertebrate Tissues

William J. Landis · Robin Jacquet

Abstract Among the vertebrate species, collagen is the most abundant protein and is associated with mineralization of their skeleton and dentition in all tissues except enamel. In such tissues, bones, calcifying tendon, dentin, and cementum are comprised principally of type I collagen, which has been proposed as a template for apatite mineral formation. Recent considerations of the interaction between type I collagen and calcium and phosphate ions as the major constituents of apatite have suggested that collagen polypeptide stereochemistry underlies binding of these ions at sites within collagen hole and overlap regions and leads to nucleation of crystals. The concept is fundamental to understanding both normal and abnormal mineralization, and it is reviewed in this article. Given this background, avenues for additional research studies in vertebrate mineralization will also be described. The latter include, for instance, how mineralization events subsequent to nucleation, that is, crystal growth and development, occur and whether they, too, are directed by collagen stereochemical parameters; whether mineralization can be expected in all spaces between collagen molecules; whether the side chains of charged amino acid residues actually point toward and into the hole and overlap collagen spaces to provide putative binding sites for calcium and phosphate ions; and what phenomena may be responsible for mineralization beyond hole and overlap zones and into extracellular tissue regions between collagen structural units.

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These questions will be discussed to provide a broader understanding of collagen contributions to potential mechanisms of vertebrate mineralization.

Collagen Organization, Structure, and Mineral Formation

The mechanisms of mineralization of the vertebrate skeleton and dentition are understood and generally accepted in some detail, although still to a limited extent. Typically, mineralization occurs in compartments of extracellular tissue spaces in intimate association with organic matrix constituents there. Type I collagen, a helical polypeptide consisting of three individual chains (two identical $\alpha_1[I]$ chains and one $\alpha_2[I]$ chain), is known to be the predominant component of the extracellular organic matrices of bone, calcifying tendon, dentin, and cementum; and in these tissues the protein has been thought classically to function in the capacity of a framework for mineral deposition [1–4]. Underlying such function, type I collagen organization is critical. Extracellular self-assembly of collagen molecules ∼300 nm in length creates so-called hole and overlap zones of ∼40 and 27 nm, respectively, in two-dimensional (2D) molecular or microfibrillar arrays [3, 5]. A resulting characteristic periodicity of ∼67 nm is observed by electron microscopy for the three-dimensional (3D), cross-linked, higher-ordered protein fibril or fiber [1–4, 6, 7]. The appearance of periodicity is evidence that arrays of collagen in 2D maintain precise registration of their hole and overlap regions in 3D so that channels or gaps exist in collagen fibril and fiber assemblages [8–10] (Fig. 1).
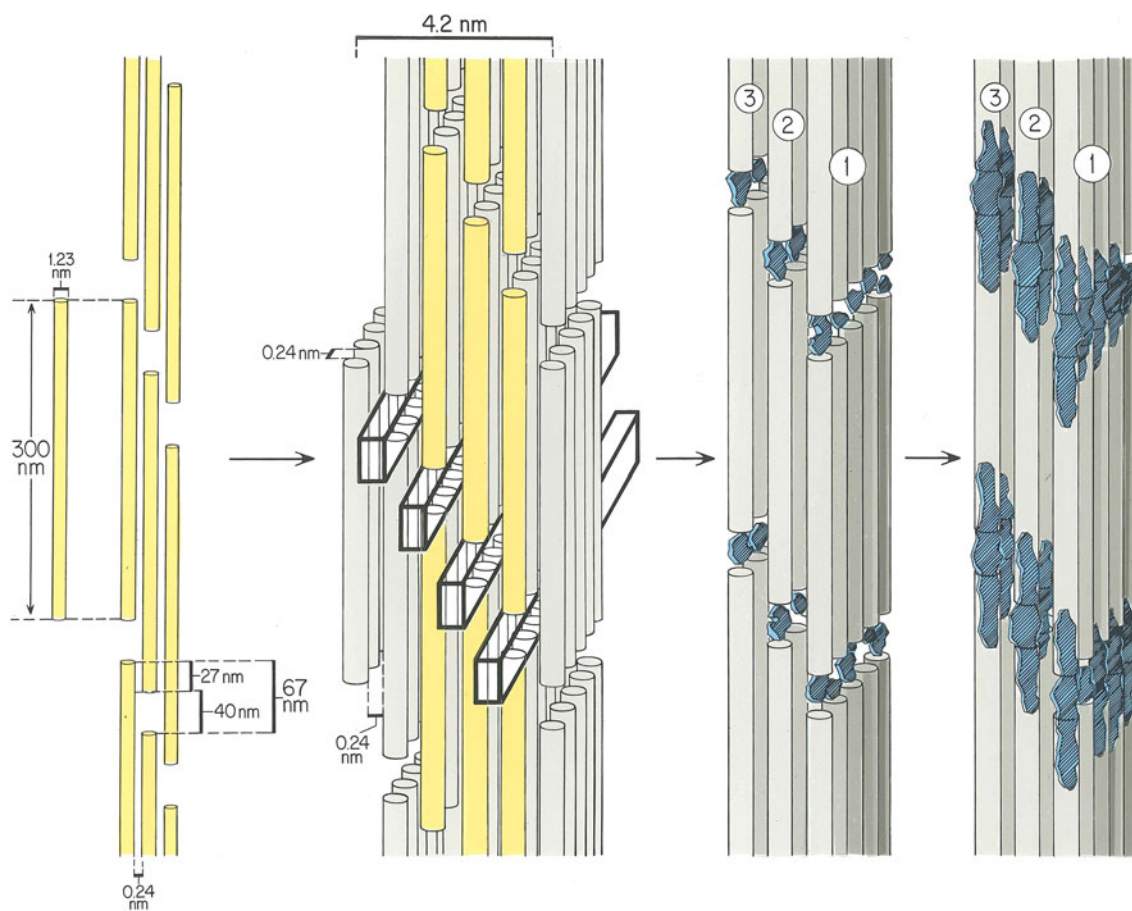
Fig. 1 A depiction of intrafibrillar collagen mineralization showing the 2D and 3D organization, assembly, and initial mineralization of collagen molecules. The schematic follows the Hodge and Petruska [3] and Landis et al. [10] models that suggest the formation of channels or gaps within collagen assemblages. Such channels are considered to be the principal sites in which nucleation of mineral crystals occurs within collagen. The pore spaces (0.24 nm) [51] between adjacent collagen molecules constitute narrow regions whose capacity to accommodate mineral is unknown. In accord with Fig. 9 from Landis et al. [10], a single collagen molecule, 1.23 nm in diameter and 300 nm in length [51], is shown at the *left* of the diagram, then assembled into a 2D aggregate, cross-linked into a quarter-staggered array with characteristic hole (40 nm) and overlap (27 nm) zones [3], and shown in *yellow*. Packing of consecutive 2D arrays into 3D assemblages occurs with strict registration of all hole and overlap zones so that channels are created throughout the model [10]. Once nucleated principally in the collagen hole zones, the crystals, shown in *blue*, grow preferentially in their *c*-crystal axis direction. The (100) planes describing the largest developing face of the crystals lie generally parallel to each other and to the collagen molecules associated with the crystals. Other features of the assembly and mineralization of collagen have been described in detail previously [10]

While the organizational platform briefly described above provides the structural basis for mineral association with type I collagen, additional previous work has reported that mineral deposition occurs both inside (intrafibrillar deposition) and outside (interfibrillar deposition) type I collagen molecules through apparently distinct events [4, 11]. Earlier work has also shown that the mineral is composed of thin, platelet-shaped, carbonated apatite crystals [12, 13] that are aligned generally parallel with one another within type I collagen [8, 10] and whose *c*-crystal axes are oriented parallel to the long axis of collagen with which they associate [4, 8–10]. More recent work has suggested that type I collagen polypeptide stereochemistry and other factors are fundamental in the initial events of mineral deposition within the intrafibrillar spaces of the molecule [14, 15]. In this regard, the side chains of polypeptide charged amino acids in the vicinity of the type I collagen hole and overlap zones [3] are thought to contribute binding sites for calcium and phosphate ions found in the circulating supersaturated fluid of the tissues [14]. Resulting bound calcium and phosphate are located in close association such that they form initial nuclei of mineral crystals in the hole and overlap regions of the intrafibrillar collagen [14]. Type I collagen, then, appears to act as a template for crystal nucleation; and it does so without the need for other non-collagenous molecules in the tissues [14, 15]. On the other hand, the latter conclusion may be appropriate for intra-fibrillar collagen mineralization only and may not

necessarily apply to interfibrillar mineral formation between type I collagen fibrils, fibers, and additional structural hierarchical forms of collagen [14]. If so, the means for mineral deposition surrounding collagen are uncertain and require greater clarification, possibly involving roles and actions in part of noncollagenous proteins and other molecules, as has been suggested [11, 14].

While the regulation and control of interfibrillar collagen mineralization are conjectural in vertebrate extracellular spaces, other aspects of type I collagen-mineral interaction are similarly poorly understood and unresolved. For example, there is generally little insight into the occurrence of the observed parallel alignment of the thin platelets of newly formed crystals and the relatively strict orientation of their c-crystal axes along type I collagen long axes. There is also incomplete evidence documenting how growth and development progress beyond nucleation events in association with type I collagen, and there are few immediate data relevant to whether intrafibrillar mineral deposition may take place in the narrow longitudinal confines between adjacent type I collagen molecules (pore spaces). Each of these four potential features of vertebrate mineralization will be addressed in the paragraphs to follow.

Formation of mineral within and outside type I collagen remains unclear to some degree. The assembly of type I collagen into the 2D quarter-stagger model of hole and overlap zones [3, 5] admits a specificity of amino acids that can be translated to 3D through the work of Landis et al. [9, 10] based on high-voltage electron tomography and Orgel et al. [16, 17] derived from X-ray crystallography. In those studies, neighboring collagen molecules are considered to be organized in microfibrillar arrays (six molecules in the quasi-hexagonal model of microfibrillar assembly and molecular packing structure in 3D [17]) that are staggered by a 2–5 multiple of 234 amino acids. The polypeptides comprising each molecule and defined according to the known analysis of the primary sequence of the human $\alpha_1[I]$ and $\alpha_2[I]$chains for type I collagen (obtained from the National Institutes of Health website, www.ncbi.nlm.nih.gov/protein/NP_000079) provide insight into intrafibrillar relationships among the amino acids [14]. For each type I collagen molecule, the identity and position of the charged amino acids are characterized by several interesting features [14]. Among them [14], the same charged amino acid, for example, is frequently found adjacent in each of the three collagen polypeptide chains; or if the amino acids are not identical, they possess the same positive or negative charge in the three chains. In each type I collagen molecule, the same two or three adjacent amino acids of like charge (glutamic/aspartic acid or lysine/arginine) are located in close proximity to counterpart two or three adjacent amino acids of the same charge (aspartic/glutamic acid or arginine/lysine). In addition, for each type I

collagen molecule, the same two or three amino acids of one charge and the same two or three amino acids of the opposite charge are in close proximity to each other (glutamic acid and lysine or arginine residues or aspartic acid and lysine or arginine residues). Finally, in each type I collagen molecule, one of the three lysine or arginine residues found in adjacent polypeptide chains may be replaced by a hydroxylysine residue of the same positive charge. Thus, there is frequent clustering of charged amino acids in every collagen molecule, a result that leads to high charge densities at discrete intrafibrillar sites within the molecules [14, 18]. These sites correspond to 12 different bands or regions sequentially designated c_2–c_3 that stain with heavy metal salts in the type I collagen hole or overlap zones [3, 14, 18, 19] (Fig. 2).

Intrafibrillar and Interfibrillar Collagen-Mineral Interactions

The 3D arrangement and distribution of charged amino acids in type I collagen molecules properly staggered and organized in microfibrillar arrays provide sites in which the resulting stereochemistry has been proposed to bind calcium and phosphate ions from the solution bathing the molecules (Fig. 2) [14]. The configurations would conceptually bring these ions into close association and lead to their nucleation in the type I collagen hole and overlap zones [14]. Apatite growth and development would follow initial nucleation events to elaborate platelet-shaped crystals growing preferentially longer in their c-crystal axial direction and wider along the channels created by contiguous hole zones in the highly ordered collagen assemblies. Based on results of electron diffraction [4, 20], crystals have their c-crystallographic axes aligned in the direction of the long axes of the collagen with which they associate and, from high-voltage electron tomography [10], are oriented generally parallel to each other (within ~ 15–$20°$) where several crystals may be nucleated independently and close to one another in the same channel region. Crystals, then, are both aligned and oriented with respect to collagen and each other as their growth and development proceed in an intrafibrillar manner [4, 8–10] (Fig. 1).

As noted above, mineral deposition in vertebrate tissues occurs both within and outside type I collagen assemblages. The arrangement of apatite crystals in each case is different and may be distinguished by selected area electron diffraction that specifically samples mineral associated with either intrafibrillar or interfibrillar collagen. With regard to the former, the diffraction patterns consist of a series of arced reflections (crystallographic indices of 002, 004, and so on) indicative of crystals that are highly oriented with respect to collagen and that correspond to the aligned and oriented crystals observed and documented by
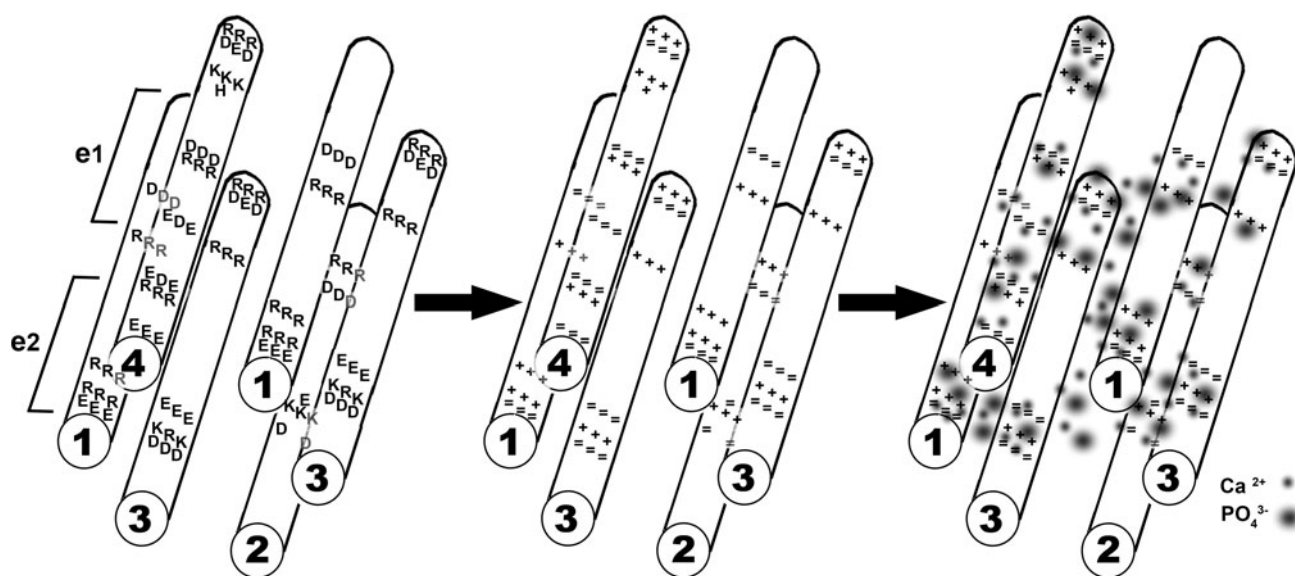
Fig. 2 Representation of the arrangement of the e1 and e2 bands of high charge density in the neighborhood of type I collagen molecules constituting a portion of the hole zone. The model follows Fig. 6 of Silver and Landis [14], which depicts the e1 band alone. Here, the six collagen molecular segments bordering the hole zone are shown in pseudohexagonal packing [16, 17]. The location and distribution of the charged amino acids comprising the e1 and e2 bands of the individual molecular segments are obtained from the National Institutes of Health website (www.ncbi.nlm.nih.gov/protein/NP_00 0079) as noted above and given in Fig. 2 of Silver and Landis [14]. Collagen molecular segments are labeled *1–4* to designate the relative stagger of 234 amino acid residues compared to segment 1. Thus, segments 2, 3, and 4 are respectively staggered 234, 2 × 234, and 3 × 234 residues from the beginning of the triple helix defining segment 1. Shown in the *left* and *center* groupings of segments, the amino acids are identical or have the same charge in almost all instances at the adjacent sites in $\alpha_1[I]$ and $\alpha_2[I]$ chains comprising collagen molecules. In the *right* grouping of segments, the charged amino acids are presented as attracting and binding individual calcium and phosphate ions to respective negative and positive side chains of the corresponding residues. Sufficient numbers of ions lead to nucleation in the vicinity of the hole zones, as described in more detail previously [14]

transmission electron microscopy [4, 8–10]. With regard to the latter, the diffraction patterns are a series of intact and complete rings, rather than arcs, that demonstrate that the crystals are randomly oriented outside type I collagen. While the manner of intrafibrillar collagen-apatite crystal growth and interaction may be understood in the context of calcium and phosphate binding to a template of charged amino acids of the type I collagen polypeptides as previously described [14], the means of interfibrillar collagen-crystal interplay is more uncertain. The two distinct crystallographic diffraction patterns, highly oriented and random, imply different and independent mechanisms of crystal nucleation at intrafibrillar and interfibrillar collagen sites [11, 21, 22].

It is unclear what factors may mediate crystal formation and growth in interfibrillar spaces, that is, along and on type I collagen surfaces and within regions between collagen fibrils. Certainly, the surfaces are marked by amino acid residues of the collagen polypeptides that comprise these regions of fibrils, fibers, and related structural units of collagen; and such residues may bind calcium and phosphate ions and lead to crystal nucleation. The sites of hole zone channel entry or access to the interior of these collagen assemblages also exist at the surfaces, and various molecules that themselves have binding sites for calcium and phosphate ions (see below) may be located specifically at these channel entrance sites or in their vicinity. Over collagen surfaces, however, identification of specific amino acid residues is unreported and localization both in vitro and in vivo of molecular species potentially important for mineral ion binding remains unresolved. Studies of the type I collagen surface, then, are necessary for more complete understanding of potential structural, stereochemical, or other features that support or influence interfibrillar collagen mineral formation.

Nucleation Events

Mineral initiation and subsequent growth and development associated with intrafibrillar or interfibrillar collagen regions may be considered from two different perspectives. First and widely understood from classical crystallization, apatite formation may be defined by heterogeneous nucleation within a solution supersaturated in calcium (Ca^{2+}) and phosphate ions (effectively the two species of $H_2PO_4^-$ and HPO_4^{2-} in equimolar amounts at pH 7.4, ionic strength 1.5, and 25 °C) [23, 24]. In such solutions, the

presence of molecules or particles (or their surfaces) other than apatite results in lowering the Gibbs free energy barrier to the formation of stable crystal nuclei [25]. Besides collagen itself, examples of molecules thought to contribute to this effect are several proteins, notably members of the SIBLING (small integrin-binding ligand, N-linked glycoprotein) family [26, 27]. Bone sialoprotein [26–34], osteopontin [26–28, 34–38], and dentin phosphophoryn [34, 39] among the SIBLINGs have been intensively studied in experiments in vitro to examine solution changes in reaction thermodynamics and kinetics of apatite nucleation and crystallization. Once formed, these initial nuclei grow by accumulation of additional ions, the process driven spontaneously as free energy decreases with increasing size of individual nuclei.

On the other hand, a second existing and now growing literature perspective has proposed that homogeneous, rather than heterogeneous, nucleation is the basis for crystal formation in the diversity of mineralizing biological systems [40]. In this regard, recent work has demonstrated prenucleation clusters of stable accumulations of calcium and phosphate ions within solutions such as simulated body fluid [41]. In the presence of a nucleating surface, these initial ion clusters resulting from homogeneous reactions were transformed to an amorphous calcium phosphate phase as a precursor to more crystalline apatite in the system [41, 42]. The context of prenucleation clusters in mediating mineral formation is potentially very significant. Their possible relation with polymeric analogues such as polyaspartic acid examined in work concerning polymer-induced liquid precursors [43] or potential chaperone molecules [44] may provide a novel approach and insight into mechanisms for both mineral deposition and inhibition in vertebrate tissues.

SIBLING Proteins and Collagen Mineralization

Whether the more widely accepted heterogeneous or recently proposed homogeneous mechanism underlies the nucleation of calcium and phosphate ions in the relevant biological systems, there is little disagreement that type I collagen (or other types of collagen) is involved in the reaction schemes. As already described above, whereas the association between calcium and phosphate ions and collagen has been reported in considerable detail for intrafibrillar heterogeneous nucleation events [8–10, 14, 19, 22], less is known with respect to the collagen surface and between microfibrils, fibrils, and fibers. Both ions and different molecular species such as certain of the protein members of the SIBLING family noted previously have been suggested as mediating collagen surface and interfibrillar heterogeneous nucleation. Most of these molecules,

however, appear to be too large (molecular weight >40 kDa) to enter the collagen structure, in either the predicted intrafibrillar channels or the pore regions between adjacent collagen molecules [45, 46]. Only single ions and molecules with molecular weight <6 kDa such as osteocalcin are thought to be small enough to diffuse and make their way into such regions [45]. Fetuin, a noncollagenous, circulating plasma protein [47, 48], and most of the SIBLING proteins in particular are excessive in size and, as a result, apparently restricted to the spaces at the surface and outside collagen [46].

Numerous studies based principally on solution analysis have demonstrated that the SIBLING family members are acidic proteins with structurally flexible peptide chains and limited long-range order. Some of them, notably bone sialoprotein, osteopontin, dentin phosphophoryn, and dentin matrix protein 1, contain regions that recognize collagen as well as domains characterized by charged amino acids and sites of glycosylation, sulfation, and phosphorylation capable of mineral ion interaction [26, 28–30, 32–35, 38, 39]. In regard to these features, their molecular weights in excess of 6 kDa, and certain evidence from electron microscopy localizing dentin phosphophoryn [49] and bone sialoprotein [31] to collagen, they are considered to bind to collagen surfaces where their charged or phosphorylated conformations may possibly extend into interfibrillar spaces, attract calcium and phosphate ions, and lead to nucleation (Fig. 3). Such interactions may contribute in large part to the commonly observed deposition of mineral between collagen fibrils and fibers in extracellular tissue spaces. On the other hand, the same molecules have been documented as inhibitory to mineral formation [34, 35], and the extent of inhibition may be such that mineral deposition is highly controlled and constrained to collagen alone and not to other tissue constituents and regions [45, 46].

Concluding Remarks and Research Challenges

To summarize this brief review concerning aspects of calcium and phosphate mineral deposition in the skeleton and dentition of vertebrates, collagen is widely recognized as a protein critically associated with calcification. Its involvement with mineral ions supersaturated in the extracellular spaces of bone, cartilage, tendon, cementum, and dentin is distinct with respect to mineral formation within collagen structural units and on the surface and outside collagen. The respective intrafibrillar and interfibrillar collagen-mediated mineralization processes, then, occur by different and independent means, the former in which collagen may act as a template for crystal nucleation without the intervention of additional molecules, the latter in which the protein may likewise serve as a template but
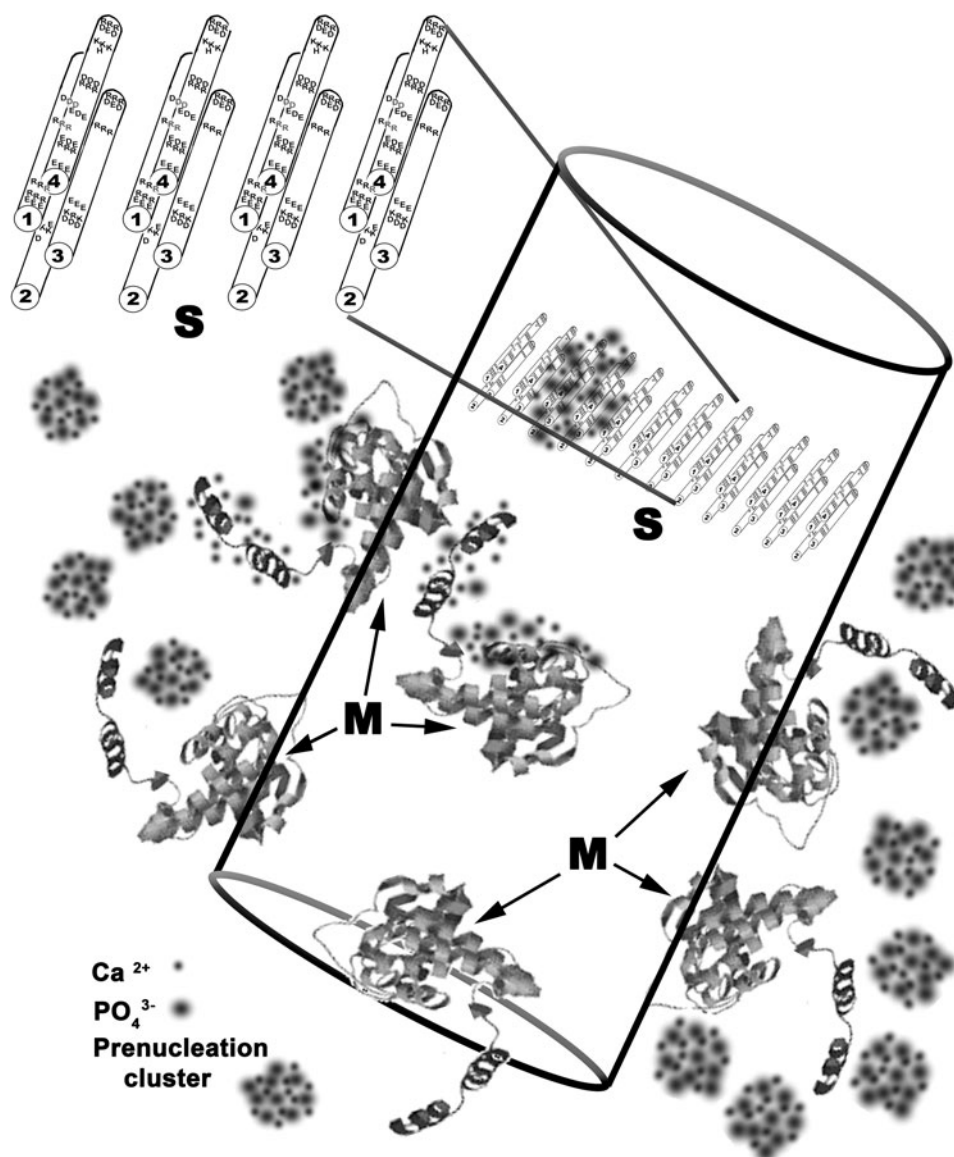
Fig. 3 A conceptual schematic showing a portion of a cylindrically shaped type I collagen fibril undergoing mineralization at both the intrafibrillar and interfibrillar levels of interaction with calcium and phosphate ions. The collagen fibril is composed of numbers of collagen molecules organized in parallel arrays with ordered hole and overlap zones. Four e1 and e2 molecular segments (S, full molecular structures shown in Fig. 2) are presented in one aspect of the fibril, extending across its full diameter. As described above, these e1 and e2 segments, their ten neighboring high charge density segments in hole and overlap regions [3, 5, 14, 18, 19], and the complete molecules are considered to be mineralized through calcium and phosphate ion binding to the template of their composite charged residues [14]. Nucleation, growth, and development of crystals follow for intrafibrillar collagen mineralization and, as shown in Fig. 1, the formation of multiple mineral platelets whose (100) faces are approximately parallel to each other along the collagen long axis.

The schematic also illustrates binding at the surface of collagen by a protein or proteins of a noncollagenous nature, as proposed originally by Nylen et al. [52] and subsequently others [11]. Here, six molecules (M) conceptually consisting of polypeptide loops and extensions are shown attached to the collagen surface at various sites. These molecules, together with collagen residues appearing at the fibril surfaces, are exposed to both calcium and phosphate ions and prenucleation clusters of the ions [41, 42] present in the supersaturated fluid bathing the extracellular matrices of vertebrate calcifying tissues. Binding of ions and clusters is depicted to occur to charged residues of noncollagenous molecules, such as bone sialoprotein or dentin phosphophoryn, and the collagen surface directly to constitute interfibrillar collagen mineralization. By this mechanism, further crystal nucleation, growth, and development lead to mineral formation throughout the extent of extracellular spaces between collagen fibrils and fibers

enhanced by the action of other molecules, themselves bound to collagen and attracting calcium and phosphate ions. In either intrafibrillar or interfibrillar collagen mineralization, nucleation of calcium phosphate crystals results from heterogeneous reactions between collagen and mineral ions. Prenucleation clusters of initial calcium

phosphate are proposed to appear as a consequence of homogeneous reactions between the ions alone, and these clusters may also promote crystal formation in additional heterogeneous events with collagen or collagen-bound molecules. Together, these physicochemical events within, on the surface of, and beyond collagen produce a collagen-mineral composite that completely occupies and calcifies both the collagen and the tissue spaces between collagen.

The above detail leads to several additional considerations necessary for more complete understanding of the collagen-mediated mineralization process. As a template that presumably guides the binding and close association of calcium and phosphate ions, collagen stereochemistry at the atomic level needs to be examined in greater detail to determine whether the polypeptide backbone and its constituent side chains possess the appropriate features that lead to crystal nucleation. In this regard, precise dimensions of helical turns, direction and orientation of side chains, and additional parameters are paramount to understanding, for example, whether charged residues point into or away from collagen hole zones where initial nucleation events are documented to occur [9, 10]. Distances within the hole zone spaces between neighboring collagen molecules (Fig. 2) are critical to define in order to determine the number of calcium and phosphate ions that may be accommodated in these regions with water and the relative arrangement that may again lead to possible nucleation of ion species. If certain of the charged polypeptide side chains are directed away from hole zone regions, their putative binding of calcium and phosphate ions may introduce wider mineral interactions and biomechanical implications than those resulting from nucleation in hole and overlap zones alone. Indeed, in a broader context, the relationships between the physicochemical interactions of crystal formation and mineral deposition in association with collagen or its specific domains and resulting biomechanical properties of the mineralizing collagen composite remain an area for further study. Further, the so-called pore spaces separating adjacent collagen molecules (Figs. 1, 2) are not understood in terms of their capacity for crystals, mineral, and water. Again, the identity of collagen residues and their side chains and measurements of side chain lengths and directions are critical for clarifying whether mineral deposition may occur in these regions of the protein. Finally, the manner is not clear by which crystals grow preferentially in their crystallographic c-axis and along the long axis of collagen with which they associate. It has been suggested that once more the stereochemistry of the collagen molecule is a principal guide to crystal growth and development [10, 14], but this concept has yet to be demonstrated. Possibly, too, collagen stereochemistry may be restrictive or inhibitory to formation of crystals in regard to development of certain of their faces and shapes. Configurations of collagen polypeptide residues

and side chains; calcium and phosphate ion binding; crystal size, shape, orientation, and alignment; and other features of collagen-mineral interaction may be approached by application of additional methods. These may include immunocytochemistry with electron microscopic tomography, atomic force microscopy, database analysis and computer simulation of protein sequence and structure, and molecular dynamics modeling for new insight and potential resolution of the uncertainties noted above [11, 50].

The role of recently reported prenucleation clusters of calcium and phosphate ions [41, 42] also remains conjectural regarding their putative effect on either or both intrafibrillar or interfibrillar mineral deposition. It is possible considering the present evidence [41, 42] to conceptualize interaction events between such ion clusters and collagen surfaces or SIBLING or other possible noncollagenous proteins themselves attached to the surfaces (Fig. 3). Less easily understood are any possible events between the clusters and collagen or the channels and interior spaces of collagen mediating mineralization. For example, the means of transport, translocation, or delivery of prenucleation clusters to collagen surfaces and channel sites and then, perhaps through effects of capillary forces [43] or molecular chaperones [44], into the intrafibrillar regions of the protein have not been well documented and require more thorough investigation of these aspects of the mineralization process. Moreover, the very localization of noncollagenous molecules with respect to collagen is in need of much more detail. In these circumstances, certain of the methods noted above such as immunocytochemistry and atomic force microscopy as well as solution chemistry studies should provide additional clarity to these incompletely understood areas of mineralization. Finally, recent advances in molecular dynamics modeling and supercomputing capability should yield novel information about mineralization events in collagen channels, hole and overlap zones, and possibly the pore spaces separating adjacent collagen molecules as well as outside those regions and into extracellular tissue spaces between collagen structural units. Indeed, this particular approach could add important insight into the suggested roles of noncollagenous proteins as mineral facilitators or inhibitors, perhaps depending on their stereochemical configuration following their putative binding to collagen surfaces.

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