Collagen as a scaffold for biomimetic mineralization of vertebrate tissues

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Collagen is a well known protein component that has the capacity to mineralize in a variety of vertebrate tissues. In its mineralized form, collagen potentially can be utilized as a biomimetic material for a variety of applications, including, for example, the augmentation and repair of damaged, congenitally defective, diseased or otherwise impaired calcified tissues such as bone and cartilage. In order to effect an optimal response in this regard, the manner in which collagen becomes mineralized is critically important to understand. This paper provides details concerning collagen–mineral interaction and its implications with respect to designing biomimetic mineralizing collagen that will be functionally competent in its biological, chemical, and biomechanical properties.

Introduction

Collagen is one of the most common proteins found in nature and an abundant constituent of many of the tissues in the body of humans and other vertebrates. As such, it provides a number of important functions, principal among them being its capacity to mineralize normally in tissues such as bone, cartilage, tendon, dentin and cementum. Mineralized collagen offers the framework for mechanical support imparted by the skeleton; a reservoir for cations, anions and small molecules; and a means for strain energy storage.10,11,26,39,41–43

Mineralized collagen also has a potential to be utilized as a biomimetic material to augment or repair calcified tissues that may be impaired, diseased, or defective. In this context of fabricating such a functional construct, the manner by which collagen becomes mineralized must be carefully understood.

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The precise molecular events leading to collagen–mineral interaction are not, however, completely defined.

The structure of the major type I collagen in vertebrates and its assembly into higher order arrays appear to dictate the location and organization of mineral associated with it. Type I collagen is a triple helix consisting of three left-handed polyproline peptide chains intertwined in a right-handed fashion. Two of the three chains have an identical amino acid sequence. These two α1(I) chains are distinct, then, from the slightly different amino acid composition of the α2(I) chain. The triple helix of type I collagen is approximately 300 nm long and 1.5 nm wide and consists of about 1000 amino acids per chain.

In vivo, type I collagen self-assembles in two-dimensions by forming molecules staggered by approximately 22% of their length with respect to their nearest neighbor, the so-called quarter-staggered model of Hodge and Petruska. These authors, and later Katz and Li, defined two regions in the model, the hole or gap (47 nm in length) and overlap (20 nm in length) zone, that comprised the periodic staggered distance, D. Based on direct conventional electron microscopy and more recently on high voltage electron microscopic tomography, collagen was found to be packed in three-dimensions through strict and contiguous alignment of its composite hole and overlap zones.

This exact registration of the hole and overlap sites among molecules provides channels or gaps in their assemblages where the earliest detectable crystals of apatite nucleate. On subsequent independent growth and development of crystals within many hole zone channels and overlap sites, mineral is elaborated as numbers of thin, irregularly shaped platellets. These small particles develop preferentially in length along their crystallographic c-axes. In addition, they become oriented with their c-axes generally parallel to one another as well as to the long axis of the collagen fibrils accommodating them. Growth and development of the width of the platelets occurs along the contiguous collagen hole zone channels. A further result of the development of platelets is that they are coplanar and form a series of parallel crystal sheets.

The manner by which type I collagen assembles in two- and three-dimensions in vivo is not entirely clear. Neither is the mechanism(s) that determines the nucleation sites of apatite platelets within hole and overlap zones of collagen assemblages, parallel orientation and alignment of platelet c-axes along collagen long axes, and platelet coplanar growth and development. These features of collagen–mineral interaction presumably depend in part on at least two factors. They are the cross-linking character of the molecules as well as specific stereochemical properties of the particular amino acid residues and the nature of their bonding comprising the collagen hole and overlap zones and the hole zone channels.

The hole and overlap zones of type I collagen are conspicuous on electron microscopy and can be identified by negative staining the protein in different tissues. Following positive staining of collagen, the D period appears to consist of 12 bands of various lengths, designated a1-4, b1-2, c1-3, d, and e1-2. The primary amino acid sequence for type I collagen is known and available models relate it to the molecular location of both hole and overlap zones. Maitland and Arsenault utilized such data together with their observations of mineral occurring in tendon collagen. They identified hydrophobic and hydrophilic sites as potentially mediating mineralization in this tissue. Sequence information, viscoelastic measurements and electron microscopy resulted in the suggestion that type I collagen contains regions that are more flexible than others. Silver et al. proposed that the flexible portions of collagen are not comprised of proline or hydroxyproline residues. These regions corresponded to the α1, β2, and c3 bands first defined by Hodge and Petruska.

Most recently, further studies have examined the relationship between the fine structure of the periodic banding pattern of type I collagen, the onset and progression of mineralization, and elastic strain energy storage during tensile deformation of the protein. Such work has led to biomechanical considerations of elastic energy storage in collagen and the molecular basis for elastic and viscous deformation as well as for energy storage of collagen. A model incorporating the measured data from deformation of unmineralized and mineralized type I collagen provides insight into the molecular basis of collagen–mineral interaction. Here, tensile deformation of collagen is proposed to involve stretching of the flexible regions in its hole and overlap zones. Elastic energy storage is suggested to result from interactions between charged pairs of amino acid residues constituting those regions. Thus, under load, collagen is stretched and the widths of the D period of the 12 positively stained bands comprising the collagen D period are opened to provide possible binding sites for calcium and phosphate ions involved in mineralization. Both calcium and phosphate ions have been mapped to a type I collagen flexible region located within the D period of the fibril and very close to proteoglycan binding sites on collagen.

Refinements are now being made in the current model of collagen–mineral interaction in association with the continuously flexible regions comprising the hole zone channels of the protein. Additional computer modeling has been initiated to gain more complete understanding of secondary and tertiary collagen structure and its implications in mineral nucleation, growth and development. The results presented below summarize the status of these studies, which are ultimately significant for elaborating the normal physico-chemical mechanism of collagen mineralization as well as for developing a functional biomimetic mineralized collagen.

Materials and methods

Two model systems have been developed and studied in order to investigate collagen–mineral interactions more completely. The first system involved the preparation of self-assembled type I collagen fibers in vitro and their mineralization in saturated solutions of calcium and phosphate as described previously. Following mineral formation under different conditions, the ability of the fibers to store energy was examined. The second system elaborated was a molecular model, designed and built to calculate the theoretical amount of energy stored for collagen with associated calcium and phosphate ions. The purpose of utilizing these models was to gain insight and understanding as to whether the association of
calcium and phosphate ions with collagen altered the ability of the protein to store elastic energy. The ability of collagenous matrices to store elastic energy is critical for locomotion and homeostasis of vertebrates.\textsuperscript{44}

**Preparation of type I collagen**

Type I collagen from rat-tail tendon in 0.01 M HCl at 4 °C was centrifuged, salt precipitated, redissolved in 0.01 M HCl, filtered through filters of 0.65 and 0.45 μm pore sizes, dialyzed against phosphate buffer (PBS), and redissolved in 0.01 M HCl as previously described by Pins et al.\textsuperscript{37} The final concentration of collagen in solution was 10 mg ml\textsuperscript{-1}.

Collagen fibers were formed by coextruding the collagen solution and fiber formation buffer (FFB) through 18 gauge polyethylene tubing into a container of FFB at 37 °C.\textsuperscript{6} After 24 h, FFB was replaced with fiber incubation buffer (FIB) and the solution was allowed to incubate for an additional 24 h at 37 °C. The solution buffer was replaced with distilled deionized water for 1 h at 37 °C. Collagen fibers were then removed from the container and dried across the edges of a drying rack for 24 h at 20 °C.\textsuperscript{6,37} This method produces collagen fibers with relatively few crosslinks but having a subfibrillar structure and orientation along their fiber axes that may resemble a collagenous tissue in its early stages of development.\textsuperscript{37}

**Mineralization of self-assembled collagen fibers**

Fibers were mineralized in a dual chamber bath, one side of which contained a CaCl\textsubscript{2} solution and the other a K\textsubscript{2}PO\textsubscript{4} solution as described previously.\textsuperscript{5} The mineralization process is briefly described as follows. Dialysis tubing was filled with 0.05 M Trizma solution (pH 7.4) and the fiber/frame system was placed inside the tubing. The filled tubing was mounted in a window that was placed into the dual chamber bath and 4 liters of 0.1 M CaCl\textsubscript{2} solution were added to one side of the bath and 4 liters of 0.1 M K\textsubscript{2}PO\textsubscript{4} solution were added to the other side. Both solutions were buffered to pH 7.4 with 0.05 M Trizma and maintained at 20 °C. A stir plate was placed under each chamber and both sides of the bath were simultaneously mixed. The collagen fibers were allowed to mineralize in the dual chamber for 4 or 7 d at 20 °C.\textsuperscript{6,37} Earlier work has documented the nucleation of hydroxyapatite in association with collagenous matrices at pH of 7.4 and 20 °C.\textsuperscript{5}

Following mineralization of the collagen, the fiber/frame system was carefully removed from the dialysis tubing and placed in PBS at pH 7.4 and 20 °C. Collagen fibers were then cut from the plastic frame, removed from PBS, and allowed to dry by draping them between two wooden stands.

**Mechanical testing of unmineralized and mineralized self-assembled collagen fibers**

The mineralized and unmineralized collagen fibers obtained following their dual chamber bathing and drying were tested on an MTS Tytron 250 tensionometer. As detailed previously,\textsuperscript{37} both mineralized and unmineralized fibers were attached to vellum paper frames (20 mm gauge length) using an epoxy adhesive prior to mechanical testing. The dry diameter of each fiber was measured with a light microscope equipped with a calibrated eyepiece. Diameters were measured at three places along the individual fiber lengths and the average of these values was recorded as a final dry diameter. Fibers mounted on the vellum frames were then soaked for 30 min in PBS at 20 °C and wet fiber diameters were measured in the same manner to yield a final wet diameter.

Prior to mechanical testing, the vellum paper frame containing single fibers was placed in a set of small grips to be connected to the MTS machine; the grips were lined with sandpaper to prevent the frame from slipping. One of the small grips was then fitted directly into the MTS machine while the other was held in place by the pneumatic grips of the machine. Screws in the jaws of the grips were tightened with a screwdriver to hold the specimen securely. Samples were immersed in a PBS bath at 20 °C to keep the collagen fibers hydrated. Before testing, slits were cut along the frame sides to permit fibers to be pulled or stretched in the MTS machine without any frame interference. Fibers were strained to a final strain at a rate of 10% per min, they were allowed to relax to equilibrium, and they were then subjected to a subsequent strain increment equal to the first. The process was repeated until fiber failure.

**Building molecular sections**

Molecular models of the type I collagen molecule and a type I collagen microfibril were constructed using a sequence of steps summarized in Table 1. Calculations were performed for both unmineralized and mineralized type I collagen microfibrils to model the effects of mineralization on energy storage during stretching. All molecular modeling was performed on a Silicon Graphics Octane2 workstation using SYBYL (v6.7, v6.8) software developed by TRIPPOS Associates, Inc. (St. Louis, MO). This software has functions to build and refine protein structure.\textsuperscript{45}

A triple helical template of the type I collagen molecule was initially modeled. The template consisted of nine repeats of the amino acid sequence Glycine–Proline–Hydroxyproline (Gly–Pro–Hyp) in each chain. The triple helix was built according to dimensions of a previously calculated poly (Gly–Pro–Pro) model,\textsuperscript{32} which uses energy minimizations performed

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Steps used in building the molecular model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>Select structure for the template</td>
</tr>
<tr>
<td>Step 2</td>
<td>Replace every third proline with hydroxyproline</td>
</tr>
<tr>
<td>Step 3</td>
<td>Minimize the structure using the Powell method</td>
</tr>
<tr>
<td>Step 4</td>
<td>Substitute the template with amino acids from a section of the collagen fibril</td>
</tr>
<tr>
<td>Step 5</td>
<td>Incorporate two repeats of Gly–Pro–Hyp at the beginning and end of each chain</td>
</tr>
<tr>
<td>Step 6</td>
<td>Add essential hydrogens and minimize each structure</td>
</tr>
<tr>
<td>Step 7</td>
<td>Set final strain of all collagen molecule sections to 1, 2, and 3% and minimize</td>
</tr>
<tr>
<td>Step 8</td>
<td>Arrange sections into quasi-hexagonal packing pattern to create a microfibril</td>
</tr>
<tr>
<td>Step 9</td>
<td>Minimize microfibril sections</td>
</tr>
<tr>
<td>Step 10</td>
<td>Place calcium and phosphate ions around microfibril sections</td>
</tr>
<tr>
<td>Step 11</td>
<td>Minimize the microfibril sections with ions</td>
</tr>
</tbody>
</table>
on such a triple helix and incorporates parameters based on agreement of experimental and crystallographic data. The type I collagen sequence, obtained from the NIH website (www.ncbi.nlm.gov/BLASA/fasts.html), was next divided into separate sections based on its positively staining banding pattern. The known amino acid sequence of each section was then identified and substituted into the template. The resulting template sections included both the positive staining bands and interband regions of collagen. In order to maintain the triple helical structure, two sequences of Gly–Pro–Hyp per chain were attached to the beginning and end of each section. Previous studies have shown that this triplet maintains triple helix formation of collagen.1,36

Each section of the molecule was then energy-minimized in batch using the Powell method, a conjugate gradient minimization method for large molecules.45 In order to mimic changes in the collagen molecule resulting from hypothetical macroscopic strains of 10 (0.1), 20 (0.2), and 30% (0.3), the final length of each collagen section was set to an additional 1 (0.01), 2 (0.02), and 3% (0.03), respectively, of its original length after energy minimization. These values for molecular strain were based on a study by Mosler et al. that estimated a molecular strain of 1% (0.01) for every macroscopic strain of 10% (0.1) in tendon.31 Steric energies of the resulting triple helical structures were calculated after minimization to their lowest energy states.

All collagen molecular sections were arranged into the quasi-hexagonal packing pattern containing four molecular segments in the collagen hole zone region or five segments in the collagen overlap zone region in a quarter-staggered array of collagen molecules (Fig. 1). Sections of the microfibril were thereby created that were strained 1% (strain of 0.01), 2% (0.02), and 3% (0.03). These modeled sections were minimized in energy again. The orientation of each molecular section was based on known collagen crosslink location, the axial rise per residue in the protein (3.3 residues per turn in an α chain and approximately 30 residues per turn in the triple helix), and collagen molecular packing pattern.35 Molecular sections were next separated from their neighbors by a center-to-center distance of 15 Å, a length derived from measurements of collagen fibers in tendon. All resulting steric energies for every section of the modeled collagen molecule and microfibril were computed by SYBYL. To mimic early stages of collagen mineralization, calcium and phosphate ions were assigned and located to sites of the quasi-hexagonal molecular pattern based on its amino acid charge distribution and stereochemical considerations. The “mineralized” models were minimized to their lowest energy states in the same manner as that for microfibrils, and, after minimizations were completed, all steric energies were recorded. Although it has been noted that there is a decrease in the distance between collagen molecules after mineralization,28 this change was not incorporated in the model upon the addition of ions.

**Calculation of steric energies.** Each model structure used the gradient method of the Tripos forcefield45 to minimize the steric energy of the respective system.45 The total energy of the system was calculated from the equation

\[
E_{\text{tot}} = E_{\text{bs}} + E_{\text{ab}} + E_{\text{tors}} + E_{\text{oop}} + E_{\text{14vdw}} + E_{\text{vdw}} + E_{\text{14es}} + E_{\text{es}} + E_{\text{fd}}
\]

where \(E_{\text{tot}}\) is the total energy (also called steric energy), \(E_{\text{bs}}\) is the energy attributable to bond stretching, and \(E_{\text{ab}}\) is the energy caused by angle bending. \(E_{\text{tors}}\) is the torsion energy, \(E_{\text{oop}}\) is the out-of-plane bending energy (energy required to move planar atoms out of the plane), \(E_{\text{14vdw}}\) is the energy of all van der Waals interactions from atoms connected by three bonds, and \(E_{\text{vdw}}\) is the van der Waals energy of all non-bonded van der Waals interactions. \(E_{\text{14es}}\) is the 1–4 electrostatic energy from all non-bonded electrostatic interactions of atoms connected by three bonds and \(E_{\text{es}}\) is the electrostatic energy from all non-bonded electrostatic interactions. \(E_{\text{fd}}\) is the fixed distance energy required to create the 1 (0.01), 2 (0.02), or 3% (0.03) strain in the molecule.

![Fig. 1](image-url) **Fig. 1** A representation of collagen molecules in the quarter-staggered array. Top: the banding pattern of the repeat sequences in the overlap and gap regions. The overlap region contains cross-sections of five collagen molecules while the gap region contains only four molecules (see circle on bottom of diagram). Bottom: the overlap and gap regions are identified in a group of self-assembled collagen molecules. The collagen molecules are shifted by a distance \(D\) with respect to their nearest neighbors after they are self-assembled into fibrils in tissues; the overlap region is 0.4 \(D\) and the gap region is 0.6 \(D\) where \(D\) is 65–67 nm. This representation is based on a figure from Freeman and Silver.8
Molecular model for elastic energy storage

As previously stated, molecular models were built in order to predict the amount of elastic energy stored during stretching in unmineralized and mineralized type I collagen fibers. The spring energy stored per unit strain in a type I collagen fiber was calculated in the following manner: The steric energy, in kcal mol$^{-1}$, for each section of the collagen microfibril was calculated using SYBYL. The difference between the values for the strained sections (0.01, 0.02, and 0.03 strain) and unstrained sections was calculated and divided by the difference in section length (in Ångstroms) multiplied by Avogadro’s number ($6.023 \times 10^{23}$ mol$^{-1}$) to obtain an elastic spring constant in kcal Å$^{-1}$ for 0.01, 0.02, and 0.03 strained fibril sections. This model accounts for strain on the molecular and microfibrillar levels.

Individual spring constants were arranged in increasing order and the total displacement of the section pieces was summed until the sum of the strains of each section was equal to the desired total microfibrillar strain (0.01, 0.02, or 0.03). Only the contributions of strains with positive spring constants were considered in spring constant calculations. The spring constants associated with the strains for each section were added together in series using the equation:

$$ R_{mic} = \frac{1}{\sum_{i=1}^{n} \frac{1}{R_i}} $$

where $R_{mic}$ is the spring constant for the entire microfibril at a particular strain (0.01, 0.02, and 0.03), $n$ is the number of times the band appears in the microfibril, and $R_i$ is the spring constant for each section of the microfibril that contributes to the total strain. Calculation was repeated for microfibrillar strains of 2 (0.02) and 3% (0.03) to give the spring constant for a type I collagen microfibril at 0.01, 0.02, and 0.03 strain.

In order to estimate the elastic strain energy stored in a collagen fiber, the spring constants for the microfibrils were converted from kcal Å$^{-1}$ to J m$^{-2}$, multiplied by the length of the modeled microfibril (572.72 × 10$^{-9}$ m) and the degree of strain (0.01, 0.02, or 0.03) to yield the amount of energy stored in a microfibril with strains of 1 (0.01), 2 (0.02), and 3% (0.03). These resulting energies were multiplied by the ratio of the gauge length of the collagen fibers used in the mechanical tests, 0.02 m, and the length of the modeled microfibril as above, 572.72 × 10$^{-9}$ m. This number was subsequently multiplied by the ratio of the average wet cross-sectional area of the tested unmineralized collagen fibers, 2.83 × 10$^{-8}$ m$^2$, and the cross-sectional area of the modeled microfibril, 1.02 × 10$^{-17}$ m$^2$. The same technique was used to calculate the energy stored in mineralized collagen fibers; a value for the wet cross-sectional area for fibers mineralized for 7 d, 1.76 × 10$^{-8}$ m$^2$, was applied here. In order to normalize the energy stored by the different models, the calculated energy was divided by the average wet cross-sectional area of the tested fibers. In order to simplify the model, some interactions were considered negligible as explained earlier.

Results and discussion

The effect of mineralization on changes in mechanical properties of collagen fibers has been studied using the self-assembled model of type I collagen fibers described above. Fig. 2$^6$ illustrates that the elastic energy stored in such models of collagen increases after 7 d of mineralization in vitro. Hydroxyapatite is formed in association with the collagen fibers under the conditions used for mineralization in earlier studies.$^4,5$ The mean apatite crystal sizes formed at neutral pH were 53.0, 22.7 and 5.4 nm in length, width and thickness, respectively.$^4$ Comparative results of mechanical tests on the normally mineralizing gastrocnemius tendons from turkey leg$^{21,22}$ and these self-assembled collagen fibers suggest that the latter system qualitatively mimics the behavior of the naturally mineralized turkey tendon.$^9$ Since the gastrocnemius and other leg tendons from the turkey mineralize normally with animal age and maturation,$^{16,21,34}$ the effect of different degrees of mineralization on the properties of tendon as a model for mineralizing collagenous tissues can be examined in turkeys sacrificed at different times during their growth and development.$^{25,41,42}$ In this manner, gastrocnemius mineralization appears to increase the apparent collagen fibril length, a result that limits slippage between fibrils, reduces tendon macroscopic strain, and transfers stress between neighboring collagen fibrils.$^9$ Mineralization in turkey tendon is more efficient than that observed in self-assembled collagen fibers with respect to increasing elastic energy storage putatively by increasing the binding of mineral components to the surface of the fibrils.$^9$ The non-collagenous proteins and other constituents found in mineralizing turkey tendon but not in self-assembled collagen fibers may promote the collagen fibril–mineral interactions in the tissue.$^9$

Modeling mineral deposition associated with collagen fibrils

Mineralization of a self-assembled type I collagen fiber model and of turkey leg tendon discussed above is suggested to lead
to increased load bearing capacity and altered ability to store elastic energy by the fibers.\textsuperscript{25,26,41,42} These published results were compared to predictions of a molecular modeling program used to calculate the changes in stored elastic energy of a collagen fibril subject to axial extension.

The amount of elastic energy stored was calculated using the two computer models (with and without calcium and phosphate ions) and is shown in Fig. 3.\textsuperscript{9} The collagen molecule and fibril were simulated by a series of flexible and rigid domains based on previous modeling results.\textsuperscript{43} The flexible domains coincide to a first approximation with the positive staining bands of the collagen fibril.\textsuperscript{45}

According to the molecular model of collagen,\textsuperscript{9} its most flexible regions in the absence of ions are the a\textsuperscript{2} band, a\textsuperscript{3} band, a\textsuperscript{4} band, the area between the b\textsuperscript{1} and b\textsuperscript{2} bands, the area between the c\textsuperscript{1} and c\textsuperscript{2} bands, and the d band (Fig. 4). When calcium and phosphate ions are added to the model, its most flexible regions are the a\textsuperscript{3} band, a\textsuperscript{4} band, the area between the b\textsuperscript{1} and b\textsuperscript{2} bands, the c\textsuperscript{1} band, c\textsuperscript{2} band, d band, and the area between the d and e\textsuperscript{1} bands of collagen (Fig. 4). The regions that remain the most flexible before and after the addition of ions to the model are the a\textsuperscript{3} band, a\textsuperscript{4} band, the area between the b\textsuperscript{1} and b\textsuperscript{2} bands, and the d band of collagen (Fig. 4).

Hofmann \textit{et al.} identified flexible regions of collagen.\textsuperscript{13} They consisted of the regions from the 1st a\textsuperscript{2} band to the 1st e\textsuperscript{2} band, the 2nd a\textsuperscript{4} band to the 2nd a\textsuperscript{2} band, the 3rd b\textsuperscript{2} band to the 3rd a\textsuperscript{4} band, the area from the 4th c\textsuperscript{1} band to and including the 4th c\textsuperscript{2} band, and the 4th c\textsuperscript{3} band to the 5th c\textsuperscript{2} band. All of these regions agree directly with the molecular model presented in this study except for the area from the 1st a\textsuperscript{2} band to the 1st e\textsuperscript{2} band. When situated in a microfibril, the latter region is surrounded by less flexible regions and causes the entire region to be less flexible, a result that also agrees with the molecular model.

The flexible regions identified in this study have amino acid sequences that lack proline or hydroxyproline in the Gly–X–Y triplet.\textsuperscript{43} The absence of these two specific amino acids causes the entire region to be less flexible, a result that also agrees with the molecular model.

Comparisons between elastic energy storage in the collagen model and self-assembled fibers

The plots of elastic energy stored versus strain for the collagen molecular model developed in this study have the same general shape as the graphs for elastic energy storage in self-assembled type I collagen fibers.\textsuperscript{8} The trend in elastic energy stored for strains of 0.01, 0.02 and 0.03 in an unmineralized computer model follows the same trend as the elastic energy stored in the unmineralized fibers for strains up to 0.30 (Fig. 5–6). In both the computer model and tested fibers, there is an area with a
results in a molecular strain of 1% based on data from Mosler et al.\textsuperscript{31} Values from the model and the tested fibers have the same general trend, a low slope region followed by a high slope region. There is an increase in elastic energy stored with increased strain in both the model and the fibers. This plot is based on a figure from Freeman and Silver.\textsuperscript{8}

When plotted (Fig. 5–6), the slope of elastic energy storage appears to increase with strain as collagen is mineralized in both the molecular model and the experimental reconstituted collagen fibers. This result may be caused by the presence of calcium and phosphate ions that limit the movement of collagen flexible regions through bond formation between amino acid side chains, requiring more energy to stretch them (therefore increasing their rigidity). Data from both the collagen fibers and molecular modeling show that calcium and phosphate ions alter the elastic behavior of type I collagen and lead to an increase in elastic energy stored per unit strain (Fig. 5–6). These results are consistent with the suggestion that binding of calcium and phosphate ions to collagen raises the elastic spring constant of the molecule by preventing the stretching of its flexible regions.\textsuperscript{42} The model shows that the phosphate ions form bonds with atoms in the charged areas of the collagen fibril.\textsuperscript{9}

While there is similarity between the computer model predictions and the actual experimental measurements of elastic energy storage, the amount of energy storage predicted by the model is larger than the energy stored in both unmineralized and mineralized collagen fibers (Fig. 5–6). This result may be explained by an absence of water from the model. The presence of water would reduce electrostatic and van der Waals interactions between atoms in the collagen molecule, and this in turn would lower the spring constant for each of its composite regions and the elastic energy stored per unit strain.

**Regions with charged residues**

As shown in the schematic diagram of Fig. 4, the number of charged amino acid residues capable of binding either calcium or phosphate ions is highest in bands c\textsubscript{2}, b\textsubscript{2}, b\textsubscript{1}, c\textsubscript{2}, e\textsubscript{1}, and d of the collagen molecule. The potential binding sites for calcium and phosphate in the hole zone model consisting of the bands are presented in Fig. 7. These bands are sites of flexibility in the collagen triple helix and, as indicated from molecular modeling, they are also the regions that show a reduction in free energy associated with stretching. The energy in some of these regions is initially reduced on stretching since bands with neighboring amino acid residues with like charges cause local repulsion. Therefore, stretching and binding of

![Image](Fig_5.png)

**Fig. 5** Elastic energy storage vs. strain curves for unmineralized collagen fibers tested incrementally at 22 °C with a strain rate of 10% per min and the unmineralized computer model. The values on this plot assume that every macroscopic strain of 10% in the collagen fiber results in a molecular strain of 1% based on data from Mosler et al.\textsuperscript{31} Values from the model and the tested fibers have the same general trend, a low slope region followed by a high slope region. There is an increase in elastic energy stored with increased strain in both the model and the fibers. This plot is based on a figure from Freeman and Silver.\textsuperscript{8}

![Image](Fig_6.png)

**Fig. 6** Elastic energy storage vs. strain curves for 4 d mineralized collagen fibers tested incrementally at 22 °C with a strain rate of 10% per min and the mineralized computer model. Values from the model assume that every macroscopic strain of 10% in the collagen fiber results in a molecular strain of 1% based on data from Mosler et al.\textsuperscript{31} Values from the model and the tested fibers have the same general trend, a low slope region followed by a high slope region. The increase in slope is much higher in the model. There is an increase in elastic energy stored with increased strain in both the model and the fibers. This plot is based on a figure from Freeman and Silver.\textsuperscript{8}

![Image](Fig_7.png)

**Fig. 7** Molecular model of the amino acid sequence found in the d band of the hole zone of a collagen fibril. This region contains glutamic acid, arginine and lysine residues that can theoretically bind calcium and phosphate ions. The positively charged side chains of lysine and arginine extend away from the backbone of the triple helix. Molecular modeling suggests that binding of phosphate ions to lysine and arginine positively charged side chains increases collagen flexibility. The diagram illustrates the possible binding sites for calcium and phosphate ions in the hole zone model.
counter ions to these residues lead to a reduction in free energy. A consequence of these observations is that the repulsion of like charges found in collagen flexible regions may play an important role in promoting the binding of calcium and phosphate ions to the protein. There are three similar amino acid chains in collagen, each containing residues such as lysine and arginine. Such charged residues located along one chain would be expected to come into close proximity with identical or other like-charged residues along other chains, enhancing calcium and phosphate binding and limiting repulsive forces.

Conclusions

Self-assembled type I collagen fibrils mineralize in a manner that parallels the normal mineralization of turkey leg tendon in vivo. Mineralization increases the elastic modulus of type I collagen fibrils and modifies the amount of elastic energy that can be stored in the fibers during stretching. Molecular modeling suggests that stretching of collagen fibrils leads to deformation of inherently flexible domains in the collagen molecule. To a first approximation, such regions coincide with positively staining bands of the collagen fibril, an indication that they are sites containing charged amino acid side chains. Molecular stretching increases the steric energy of these flexible domains through bond stretching, angle bending and backbone torsion. Addition of calcium and phosphate ions to the collagen molecular model appears to increase the flexibility of the area specifically between the e and d bands, located within the hole zone region of the fibril. This result suggests that the presence of calcium and phosphate ions in the hole region may modify the conformation of the collagen triple helix. The increase in flexibility of the collagen region between the e and d bands observed with increased strain may promote binding of calcium and phosphate to collagen in its hole zone regions.

The parallel results of molecular modeling and mechanical testing of self-assembled collagen fibers imply that energy storage during stretching is likely to involve steric energy changes to the collagen molecule. Since these changes lead to an increase in the elastic modulus of the collagen fibrils, an extracellular matrix composed of such fibrils will therefore be able to bear higher loads without failing. As a result of the current testing here of mineralizing connective tissues in vivo and the use of computer modeling, the molecular relation between collagen structure and mineral formation is becoming clearer. Such understanding of collagen–mineral interaction is critical for developing the protein as a true biomimetic material with its potential in a variety of basic science and clinical applications.

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