Molecular Basis for Elastic Energy Storage in Mineralized Tendon

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Animals store elastic energy in leg and foot tendons during locomotion. In the turkey, much of the locomotive force generated by the gastrocnemius muscle is stored as elastic energy during tendon deformation. Little energy storage occurs within the muscle. During growth of some avians, including the turkey, leg tendons mineralize in the portions distal to the attached muscle and show increased tensile strength and modulus as a result. The purpose of this study is to test the hypothesis that the degree of elastic energy storage and recovery in mineralizing turkey tendon is directly related to the tendon mineral content. To test this hypothesis, the stress–strain behavior of tendons was separated into elastic and viscous components. Both the elastic spring constant and the elastic energy stored, calculated up to a strain of 20%, were found to be proportional to tendon mineral content. It is concluded that mineralization is an efficient means for increasing the amount of elastic energy storage that is required for increased load-bearing ability needed for locomotion of adult birds. Examination of molecular models of the hole region, where mineralization is initiated within the collagen fibril, leads to the hypothesis that elastic energy is stored in the tendon by direct stretching of the flexible regions. Flexible regions within the collagen molecule fall within the positively stained bands of the collagen D period. It is proposed that mineralization increases the stored elastic energy by preventing flexible regions within the positively stained bands from stretching. These observations suggest that mineralization begins in the hole region due to the large number of charged amino acid residues found in the d and e bands.

Introduction

Elastic energy storage in tendons in the legs, feet, and wings of many animals is an important mechanism that saves substantial quantities of muscular energy during locomotion.1, 2 Elastic recoil, primarily by the tendons, converts most of the stored energy back to kinetic energy as the foot of the animal leaves the ground.2 In the pig, the digital flexor tendons involve direct stretching of strain energy, and at maturity they have twice the tensile strength and elastic modulus but only about half of the strain energy dissipation of the corresponding extensor tendons.3 Shadwick3 pointed out that the density and type of cross-links within tendon collagen fibers change with age as well as with fibril morphology. The increased tensile strength and decreased strain at failure of the flexor tendon appear to be a consequence of increased cross-linking within the collagen fibril.3

In the turkey, direct measurement of force and fiber length in the lateral gastrocnemius muscle reveals that the active muscle produces high force but little work while the tendon produces much of the work because of elastic deformation and recovery.2 Unlike the pig flexor tendon, the turkey tendon mineralizes during aging. It has been proposed that mineralization is an efficient means for preserving elastic energy storage while providing for the increased load-bearing ability required for locomotion of adult birds.3 Recently, elastic energy storage within tendons has been hypothesized to involve direct stretching of the flexible regions within the collagen molecule.6 Evidence suggests that the ends of the molecule and its cross-links are probably not as flexible as regions of the triple helix devoid of proline and hydroxyproline.5

The mechanical properties of tendon arise as a direct result of its constituent components and their arrangement in the tissue. Tendon consists primarily of cells, collagen fibers, proteoglycans, and water. Type I collagen is the most abundant protein in tendon and is a fibril-forming protein that self-assembles into cross-striated fibrils having a 67 nm repeat. The molecule is a triple helix composed of approximately 1000 amino acids and extending about 300 nm in length.7 Collagen has been reported to be a rodlike molecule with little flexibility and high mechanical strength;6 however, some measurements indicate that the type I collagen molecule has numerous bends and is not completely rigid.9, 10 Analysis of the amino acid sequence and physical properties in solution indicates that some regions of the molecule are more flexible than others.6, 10 In theory, the attachment of

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eral to and around these flexible regions would prevent them from stretching when the tendon is strained.

Results of previous studies conducted by the authors indicate that the mechanical properties of the turkey gastrocnemius tendon change during mineralization. The elastic modulus increases between weeks 12 and 17 post-hatching of the animals, and this is the time period during which mineralization begins. While it has been proposed that mineralization increases the ability of turkey tendon to store elastic energy, no report has been made concerning a correlation between changes in viscoelastic properties of the turkey tendon and its mineral content.

The purpose of this study was to test the hypothesis that the degree of elastic energy storage in mineralizing turkey tendon is directly related to its mineral content and to propose a model of how mineralization of collagen increases elastic energy storage. Results reported below indicate that both the elastic spring constant and the elastic energy storage are directly proportional to the mineral content of this avian tendon.

**Materials and Methods**

The mechanical behavior of mineralizing turkey tendons at different stages of tissue development reported by Landis et al. was reanalyzed in this study. Stress–strain curves obtained from constant strain-rate experiments conducted at a strain rate of 100%/min at 22 °C, reported by Landis et al., were converted into elastic and viscous stress–strain curves after correction using the elastic fractions reported by Silver et al. and measured using incremental stress–strain tests on turkey tendons. The elastic fraction is defined as the fraction of stress that remains at equilibrium, when the sample is held at constant length. The slope of the elastic stress–strain curve for collagen fibers has been observed to be strain-rate independent while the slope of the viscous stress–strain curve is strain-rate dependent (manuscript in preparation). Elastic stress–strain curves are obtained from total stress–strain curves by multiplying the total stress–strain curves by the elastic fraction. Viscous stress–strain curves are obtained from the total stress–strain curves by subtracting the elastic stress–strain curves from the total stress–strain curves. The total stress–strain data reported by Landis et al. (see Figure 1 for an example) were multiplied by elastic fractions of 0.8 at a strain of 0.05 and by elastic fractions of 0.74 at all higher strains as reported by Silver et al. to generate an elastic stress–strain curve (Figure 1). Viscous stress–strain curves were obtained by subtracting elastic stress–strain curves from the total stress–strain curves (see Figure 1). Elastic and viscous stress–strain curves were approximated by straight lines using a curve fitting program within Cricket Graph. The slope of the elastic stress–strain curve was converted into a collagen elastic spring constant after correction for the volume fraction of polymer, the ratio of the macroscopic to molecular strain, and the ratio of the amount of molecular slippage of collagen molecules in turkey tendon to that of rat tail tendon. The slope of the elastic stress–strain curve was corrected for the collagen content by dividing by the volume fraction of collagen (0.5). The slope was corrected for the ratio of macroscopic to molecular strain by multiplying it by 10 and then corrected for molecular orientation and cross-linking differences by multiplying by the change in strain for the linear region of the elastic stress–strain curve of turkey tendon divided by the change in strain for the linear region of the elastic stress–strain curve of rat tail tendon as described previously. The value of 10 represents the ratio of macroscopic strain to molecular strain based on X-ray diffraction data for rat tail tendon while the change in strain of the linear region for turkey tendon divided by the change in strain of the linear region for rat tail tendon corrects for differences in the molecular tilt angle and slippage of the collagen molecules in each of these tissues.

Lines representing viscous stress–strain curves were converted into fibril lengths as previously described by converting the extensional viscosity into a shear viscosity and then into an axial ratio using hydrodynamic theory. Equations of the lines representing linear fits to the initial portions of viscous stress–strain curves were divided by (a) the strain rate (0.01/min), (b) 3.0, representing the value of the relationship between shear modulus and tensile modulus for incompressible materials, (c) solvent viscosity, and (d) polymer volume fraction, to yield a value of the axial ratio, Z. Fibril lengths were calculated by multiplying the axial ratio by the fibril diameter, 250 nm, as described previously.

Collagen molecular and fibrillar flexibility profiles were prepared to determine whether there was a relationship between the location of charged amino acid residues that may bind calcium and phosphate ions and the location of flexible sites that could undergo conformational changes. Conformational changes that occur in flexible regions of the collagen molecule may explain why mineralization first occurs in the hole region within the collagen fibril. The flexibility indices of different amino acid sequences were obtained from conformational plots of pairs of amino acid residues constructed using the coordinates of a standard dipeptide as previously described. The flexibility indices were assigned to be proportional to the area of allowed
conformations obtained from the conformational map for sets of dipeptides. Flexibility indices were assigned for different dipeptides as described in Table 1. Assignment of indices for other sets of amino acids was based on their structural similarity to pairs listed in Table 1. The sequence of flexibility indices of a three-chain collagen molecule was obtained by assuming that the collagen molecule consisted of three identical α-chains and that the chains were staggered by one residue axially with respect to the other two α-chains. The flexibility profile for the molecule was then obtained by summing the flexibility indices across three staggered chains. The flexibility profile of the collagen fibril was determined by summing the flexibility indices across a quarter-staggered array of collagen molecules. The resulting flexibility profile was then compared to the positive staining pattern of collagen fibrils previously reported.12

Elastic energy stored during mechanical testing was calculated as the area under the elastic stress–strain curves for strains up to 20%. Elastic energy was approximated by fitting a straight line to the elastic stress–strain curve and finding the area (0.5 base times height) up to a strain of 20%. The elastic energy was calculated up to 20%, since one specimen failed at a strain between 15% and 20%.

Results

Elastic and viscous stress–strain curves were obtained from total stress–strain curves11 after multiplication by the elastic fractions reported previously5 (see Figure 1). Elastic and viscous stress–strain curves are plotted in Figures 2 and 3, respectively. In general, the elastic stress–strain curves for tendons with low mineral content (0.0) are on the right-hand side of Figure 2 while those with higher mineral content (above 0.2) are shifted to the left of Figure 2 and have an increased slope. The mineral content appears not to be directly related to the age of an animal since one of the 14 week old birds had a mineral content of 0.295 while the other 14 week old bird had a mineral content of 0.00.

At low mineral content, the elastic stress–strain curve has a very long low modulus region. As the tendon mineral content increases, the low modulus region is then replaced with an almost linear relationship between elastic stress and strain. The slope of the elastic stress–strain curve was obtained in the linear region from Figure 2 and is tabulated in Table 2. The slope increases from about 62 MPa at low mineral weight fraction to 260 MPa at a mineral weight fraction of about 0.25. This change translates into an increase in the elastic spring constant of type I collagen from about 3 GPa to between about 7 and 8 GPa after correction for the macroscopic strain as described in the Methods section. A plot of elastic spring constant vs mineral weight fraction was approximated by a straight line and had a correlation coeffi-

Table 1. Typical Flexibility Indices for Dipeptides Calculated from the Area under Conformational Plots as Described by Silver

<table>
<thead>
<tr>
<th>flexibility index</th>
<th>dipeptide</th>
<th>flexibility index</th>
<th>dipeptide</th>
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</thead>
<tbody>
<tr>
<td>Pro-Pro</td>
<td>8</td>
<td>Pro-Hyp</td>
<td>8</td>
</tr>
<tr>
<td>Ala-Ala</td>
<td>45</td>
<td>Ala-Asp</td>
<td>40</td>
</tr>
<tr>
<td>Ala-Gly</td>
<td>57</td>
<td>Ala-Hyp</td>
<td>29</td>
</tr>
<tr>
<td>Ala-Pro</td>
<td>28</td>
<td>Arg-Arg</td>
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<tr>
<td>Arg-Hyp</td>
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<td>Hyp-Arg</td>
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<td>Glu-Asp</td>
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<td>Glu-Glu</td>
<td>13</td>
</tr>
<tr>
<td>Pro-Glu</td>
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<td>Glu-Ser</td>
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<tr>
<td>Gly-Ser</td>
<td>49</td>
<td>Lys-Pro</td>
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</tr>
<tr>
<td>Pro-Lys</td>
<td>1</td>
<td>Glu-Asp</td>
<td>12</td>
</tr>
</tbody>
</table>

![Figure 2](image-url) Elastic stress–strain curves of mineralizing turkey tendons with different mineral weight fractions. Elastic stress–strain curves were obtained from the total stress–strain curves11 after multiplication by the elastic fraction.5 The elastic stress–strain curves are given as a function of the tendon mineral weight fraction (shown in parentheses) and the animal age.

![Figure 3](image-url) Viscous stress–strain curves of mineralizing turkey tendons with different mineral weight fractions. Viscous stress–strain curves were obtained as the difference between the total and elastic stress–strain curves shown in Figures 1 and 2. Viscous stress–strain curves are presented as a function of the mineral weight fraction (shown in parentheses) and the animal age.

Table 2. Slopes and Intercepts of Straight Lines Used to Approximate the Elastic and Viscous Stress–Strain Curves for Turkey Tendons of Different Ages and Mineral Weight Fractions: All Data Calculated Based on Landis et al.11 Using Elastic Fractions from Silver et al.5

<table>
<thead>
<tr>
<th>sample</th>
<th>mineral weight fraction</th>
<th>elastic slope (MPa)</th>
<th>viscous slope (MPa)</th>
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<tr>
<td>12</td>
<td>0.000</td>
<td>62.1 (−12.39)</td>
<td>3.52 (−0.285)</td>
</tr>
<tr>
<td>13</td>
<td>0.215</td>
<td>116.5 (−5.05)</td>
<td>23.9 (−0.793)</td>
</tr>
<tr>
<td>14</td>
<td>0.000</td>
<td>51.6 (−7.16)</td>
<td>4.71 (−0.359)</td>
</tr>
<tr>
<td>14</td>
<td>0.295</td>
<td>133.9 (−2.37)</td>
<td>33.4 (0.0819)</td>
</tr>
<tr>
<td>15</td>
<td>0.245</td>
<td>259.9 (−0.991)</td>
<td>79.7 (1.223)</td>
</tr>
</tbody>
</table>

a CC* = correlation coefficient.
The data shown in Table 3 (not shown) represent the best least-squares fit to strain and the ratio of the strain in the linear region to that of rat tail tendon. The equation shown represents the best least-squares fit to the data and \( R^2 \) is the correlation coefficient. Note there are two points at a mineral weight fraction of 0 making \( N = 5 \).

Figure 4. Plot of elastic spring constant vs mineral weight fraction for turkey tendon. The elastic spring constant was calculated from the slope of the elastic stress– strain curve (Figure 2) up to a strain of 20%. The equation shown represents the best least-squares fit to the data and \( R^2 \) is the correlation coefficient.

Figure 5. Plot of elastic energy stored vs mineral weight fraction for turkey tendon. Elastic energy stored was calculated as the area under the elastic stress–strain curve (Figure 2) up to a strain of 20%. The equation shown represents the best least-squares fit to the data and \( R^2 \) is the correlation coefficient.

The purpose of these experiments was to elucidate the physiological role of mineralization of turkey leg tendon. Previously, mineralization in this avian tissue was found to be associated with an increase in the slope of the tendon stress–strain curve (modulus) as well as an increase in ultimate tensile strength. Turkey tendons are known to be composed of a complex assembly of somewhat flexible, highly aligned collagen fibrils with different diameters. Smaller collagen fibrils are observed to branch from larger fibrils or to aggregate into fibrils of greater size. Mineral is deposited in the turkey tendon between and within the collagen fibrils. The fibril diameters range from about 25 to 500 nm at about 15 weeks post-hatching with about an equal distribution of small and large fibrils. The tendon unit found in the gastrocnemius of the turkey cannot be divided without mechanical disruption so it is not possible to determine whether the mechanical properties of tendon unit fragments are similar to the properties of the whole tendon. Results reported in this paper suggest that the elastic spring constant and the elastic energy storage for type I collagen increase with increased mineral weight fraction.

The collagen molecule has been previously modeled as being composed of alternating rigid and flexible regions. The rigid regions are found at the free amino and free carboxylic acid ends of the molecule where regions containing the sequence Gly-Pro-Hyp are present. They are also found to alternate with flexible sequences to give the molecule “accordion-like” properties. The flexible regions are associated with all of the positively staining regions of the collagen fibril while rigid regions are found between the positively staining bands. Of specific note is the hole region of the collagen fibril which contains the d and e bands. This region appears to be continuously flexible, a result suggesting that possible binding of ions and noncollagenous molecules may change the conformation of the collagen molecule within collagen fibrils at this site. Application of external forces during locomotion of adult birds would result in deformation of the flexible regions, conceptually exposing charged sites that could bind calcium phosphate in the hole region.

Studies of the early phases of mineral deposition in bone and other calcifying vertebrate tissues have revealed that calcium phosphate crystals are not randomly distributed along the collagen molecule within fibrils. Glimcher and McEwen et al. showed that calcium phosphate crystals are first deposited within the hole zone region of collagen imparting a 70 nm axial periodicity to the fibrils. The hole zones of neighboring collagen fibrils are in lateral register with respect to each other over long distances. Results presented here suggest that the hole zone may be the site of...
initial calcium phosphate deposition in mineralizing tissues as a consequence of the flexibility of this region. The increased $D$ period of collagen fibrils in mineralized tissues above that seen in tendon (70 vs 67 nm) indicates that binding of calcium and phosphate to this region of the molecule (Figure 7) may lead to extension of the flexible regions. Since the modeling studies (see Figure 6) suggest that the region of molecular flexibility could be as large as $0.4D$, then mineralization may be associated with molecular deformation and extension of the $D$ period. Examination of the amino acid sequence in the d and e bands indicates that these regions contain both free glutamic and aspartic acid residues and free lysine and arginine residues as well as charged pairs. Such charged pairs have been implicated in promoting collagen self-assembly and may play a role in the binding of noncollagenous molecules to the hole region.

Several phosphorylated and acidic noncollagenous proteins have been implicated in the mineralization of vertebrate tissues (see refs 22 and 23 for reviews). These include osteopontin, bone sialoprotein, osteocalcin, osteonectin, proteoglycans, and phosphophoryn. In bone, phospho-proteins contain both serine and threonine phosphate and sialic acid. It has been proposed that phosphate groups on phosphoproteins complexed to collagen facilitate nucleation of calcium phosphate crystals. Phosphophoryn, which has calcium binding activity, is the most abundant non-collagenous protein in dentin. Studies in vitro of phosphophoryn binding to turkey tendons indicate that association...

Figure 6. (Top) Diagram illustrating the packing pattern of type I collagen molecules into a subfibrillar unit containing five molecules in cross section. The subunit contains collagen molecules that have a total length of about 4.4D and are staggered laterally by multiples of $D$, which is 67 nm. The $D$-period is the characteristic fingerprint of collagen that arises from the repeat of the hole and overlap regions. The hole region is characterized by the presence of only four molecules in the cross section while the overlap region contains cross sections of five molecules. The hole region is a result of the presence of a 0.6D space between neighboring molecules along the axis of the subunit. When collagen fibrils are viewed in the electron microscope after positive staining, 12 dark staining lines identified as bands $c_2$, $c_1$, $b_2$, $b_1$, $a_4$, $a_3$, $a_2$, $a_1$, $e_2$, $e_1$, and $c_3$ are observed. These bands are diagrammed as flexible springs connected by rigid segments (solid areas) that represent the interband regions. The width of each region is proportional to the number of amino acids. (Bottom) Plot of band flexibility as a function of position along the positive staining pattern for a type I collagen fibril. Collagen fibril flexibility was estimated from the area under the conformational map for different pairs of amino acids in a subfibril containing five quarter-staggered collagen molecules. The plot of flexibility (number of conformations, a unitless variable) vs band number shown indicates that regions $e_2$, $e_1$, d, and c3 are continuously flexible. Other bands such as $b_2$ and $a_1$ are flexible but are separated by relatively rigid interband regions.
occurs predominantly in the collagen e band, an observation suggesting that phosphophoryn regulates mineral deposition within the hole region of the fibrils. Bone sialoprotein has been shown to bind preferentially to the R2 chain in the hole zone of collagen fibrils and may regulate the onset of calcification and cell binding to the protein. Proteoglycans are believed to play an important role in controlling cartilage mineralization. On one hand, these components have been shown to inhibit apatite growth in the presence or absence of collagen fibrils, but on the other hand, proteoglycans appear either to inhibit or promote apatite growth depending on free calcium concentration. Scott has proposed that anionic glycosaminoglycans (keratan, chondroitin, and dermatan sulfates) are stiff chains that result from the repulsion of the numerous negatively charged constituents of these molecules. These glycosaminoglycans are reportedly bound to collagen primarily at the d band and less frequently at the e band, orthogonal to the collagen fibril axis. Bone contains no dermatan sulfate orthogonal to the collagen hole zone (d and e bands), a result therefore suggesting that binding of the stiff proteoglycans to the hole zone prevents mineralization.

The ability of collagen in the form of fibrils to support formation of apatite in vitro was observed over 40 years ago. Katz later reported that a type I collagen matrix reduced the size of crystal nuclei necessary for mineralization. Results of other studies in vitro suggested that collagen matrices induced uptake of calcium and phosphate ions from stable solutions to form a matrix-bound mineral phase. This mineral phase exchanges calcium and phosphate ions with the soluble phase. At low pH, type I collagen in the form of fibrils induces formation of tricalcium phosphate or brushite while at a pH above 6.5 hydroxyapatite is formed. Although the amount of mineral deposited on type I collagen fibrils in vitro is highest at pH less than 6.5, the modulus observed is maximized at a pH between 7.0 and 9.0. At a pH less than 6.5, the mineral is deposited primarily within the fibril. The modulus and ultimate tensile strength of mineralized collagen fibers are each maximized at a pH of about 9.0, a value suggesting that the resistance by collagen to deformation is increased when mineralization occurs within the fibrils. The inhibition of intrafibrillar mineral formation within collagen fibrils at acidic pH suggests that the charged amino acid residues on collagen play a role in collagen mineralization. These residues are fully ionized at a pH below about 9.6 since the pKs of the carboxylic acid residues is about 3.5 while that of e-amino and phenolic groups on collagen is reported to be 10.6. Further evidence suggests that these groups stabilize the triple helix since the helix-to-coil transition temperature for collagen increases from about 32 to about 39 °C when the pH is increased from 2 to 7. These observations support the involvement of charged amino acid residues in the stability of the triple helix and in the formation of apatite.

The presence of charged residues in the collagen d and e bands as well as the inherent and continuous flexibility of the amino acid residues in the hole region (bands c and d) suggest that deposition of calcium and phosphate in these regions would stiffen the collagen triple helix, as diagrammed in Figure 7, and would increase the elastic spring constant (Figure 4). An increase in the elastic spring constant of type I collagen would lead to an increase in elastic energy storage in the turkey gastrocnemius tendon. Thus, mineralization of the flexible regions in the d and e bands comprising the collagen hole zone appears an efficient means for increasing energy storage in developing tendon of the turkey and other avians.

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References and Notes


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