Chemistry of collagen cross-linking: biochemical changes in collagen during the partial mineralization of turkey leg tendon

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With age, the proximal sections of turkey leg tendons become calcified, and this phenomenon has led to their use as a model for collagen mineralization. Mineralizing turkey leg tendon was used in this study to characterize further the composition and cross-linking of collagen in calcified tissues. The cross-link profiles of mineralizing collagen are significantly different from those of other collagenous matrices with characteristically low amounts of hydroxylysyl-pyridinoline and the presence of lysyl-pyridinoline and pyrrolic cross-links. However, the presence of the immature cross-link precursors previously reported in calcifying tissues was not supported in the present study, and was found to be due to the decalcification procedure using EDTA. Analysis of tendons from young birds demonstrated differences in the cross-link profile which indicated a higher level of hydroxylation of specific triple-helical lysines involved in cross-linking of the proximal tendon. This may be related to later calcification, suggesting that this part of the tendon is predestined to be calcified. The minimal changes in lysyl hydroxylation in both regions of the tendon with age were in contrast with the large changes in the cross-link profile, indicating differential hydroxylation of the helical and telopeptide lysine residues. Changes with age in the collagen matrix, its turnover and thermal properties in both the proximal and distal sections of the tendon clearly demonstrate that a new and modified matrix is formed throughout the tendon, and that a different type of matrix is formed at each site.

INTRODUCTION

The cross-linking of collagen in mineralized tissues is important for both the structural and mechanical properties of such tissues. Type I collagen molecules are arranged within fibres in the quarter-stagger end-overlap manner. The precise alignment and gap regions [1] resulting from this arrangement allow the nucleation of calcium apatite crystals and intermolecular and interfibrillar cross-linking to occur [2]. In the absence of these cross-links, such as on inhibition with lathyritic agents, bone is greatly reduced in strength [3–5]. In addition, the mineral density of bone decreases with increasing concentrations of β-aminopropiononitrile, suggesting that cross-linking also modifies the mineralization of bone [6].

The cross-link profiles of mineralizing tissues are significantly different from those of other type I collagen matrices. A low level of lysyl hydroxylation, a relatively low hydroxylysylpyridinoline (HL-Pyr) content and the presence of lysylpyridinoline (L-Pyr) are characteristic of mineralized collagen. These low pyridinoline levels have been suggested to be due to inhibition of keto-imine maturation by mineralization [7,8]. However, this proposal is not supported by the *in vitro* incubation of mineralized bones from young animals, whereby the keto-imine is readily converted into the pyridinoline and pyrrole, and we have suggested that the apparent mature cross-link deficit in bone due to the low levels of pyridinoline is accounted for by the pyrrolic cross-link [9], the formation of which would be favoured in collagen with low lysyl hydroxylation [10,11].

Yamauchi and co-workers have suggested that calcifying collagen is also unusual in that it lacks cross-links at the N-terminus [12] and contains a free C-terminal aldehyde [13,14]. However, others [15–17] have located pyridinium cross-links at both C- and N-terminal telopeptides in bone and dentine. The dissociation of the cross-links to produce free aldehyde was proposed to be due to osmotic or mechanical forces generated by the growing apatite crystal. However, it seems surprising, in view of the mechanical requirements of bone, that the mineralization process should have evolved to weaken the collagenous framework. Furthermore, studies of a mineralizing bone cell culture system demonstrated a progressive loss of precursor with increasing levels of immature cross-links, until, with the histological appearance of a heavily mineralized matrix, cross-link precursors were no longer apparent [18].

To clarify the role and nature of collagen cross-links in calcifying tissues, a comprehensive study of the mineralizing turkey leg tendon (MTLT) was undertaken. The MTLT has been used extensively as a model for calcification for many years; however, the majority of studies have centred upon the nature of the inorganic phase of the tendon. Mineralization occurs from approximately 13 weeks of age from a point mid-way along the tendon and proceeds proximally into the muscle [19]. Fibroblast proliferation occurs at about 12 weeks, that is before mineralization is observed, consistent with elevated collagen synthesis and turnover. Likens et al. [20] suggested that, based on the lower hydroxylysine/lysine ratio in mineralized tendon, only 20 % of the collagen was replaced, and concluded that a particular form of collagen is not required for calcification. The decrease in lysine hydroxylation was supported by Yamauchi and Katz in studies on adult tendon [14]. These studies demonstrated that, although the collagens of both the non-mineralized and mineralized sections of the adult tendon are type I collagen, they are different by virtue of their post-translational modifications, the mineralized tendon being similar to bone collagen.

This MTLT model evidently raises additional questions of whether new and/or different matrix is formed during tendon mineralization and if, prior to mineralization, the collagen of the

Abbreviations used: MTLT, mineralizing turkey leg tendon; HL-Pyr, hydroxylysylpyridinoline; L-Pyr, lysylpyridinoline; MMP, matrix metalloproteinase; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; HLKNL, hydroxylysinoketonorleucine; LKNL, lysinoketonorleucine; DSC, differential scanning calorimetry.

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proximal tendon is already different from that of the non-calcifying, distal, section. The partial mineralization of avian tendon was therefore used in the present study to investigate: (i) the relationship between lysine hydroxylation, cross-linking and mineralization in calcifying tissues, (ii) whether the tendon is predestined to be mineralized, and (iii) whether a new matrix is formed during mineralization.

MATERIALS AND METHODS

Materials

Leg tendons from commercially reared turkeys killed at 11, 14 and 22 weeks of age (before, during and after mineralization of the proximal tendon section respectively) were used in this study.

Potassium boro[^{3}H]hydride (37 MBq/mmol) was obtained from Amersham International; ninhydrin and buffers for amino acid analysis from Pharmacia LKB Biochrom Ltd.; amino acid standards and enzyme inhibitors from Sigma; CF1 cellulose from Whatman; 40 % acrylamide/bis solution from Bio-Rad. Purified matrix metalloproteinase-2 (MMP-2) was obtained from Biogenesis (Poole, Dorset, U.K.). All other chemicals were from BDH. Aluminium pans for differential scanning calorimetry (DSC) were obtained from Perkin–Elmer (Beaconsfield, Bucks., U.K).

Tendon preparation

The distal and proximal sections from MTLTs of approximately the same size were dissected from the legs of the turkeys on the day of slaughter and stored at −20 °C until use. The central part of the tendon was avoided at all ages in order to exclude the transition zone between mineralizing and non-mineralizing sections, and the tendon sheath was removed prior to analysis.

The same tendons were used for the cross-link analysis, hydroxylysine quantification, collagen quantification and DSC. Different tendons were used for collagen type determination, pyrrole quantification and MMP analysis.

Collagen biochemistry

Collagen type

The tendons were analysed for collagen type by CNBr peptide analysis on SDS/PAGE followed by quantitative optical scanning of the gel. Comparison of α1(I)CB8 and α1(III)CB5 bands was used to quantify the type I/type III ratio as detailed previously [21]. All samples were treated sequentially overnight with 0.5 M EDTA, pH 7.5, 4 M guanidine hydrochloride/0.05 M Tris, pH 7.5, and hyaluronidase (1:100 wet weight of tissue), in order to remove proteoglycan and decalcify the tissue prior to CNBr digestion.

Intermolecular cross-links

(i) Quantification by amino acid analysis. The samples (tendons from nine 22-week-old, six 14-week-old and four 11-week-old birds) were frozen in liquid nitrogen and powdered using a Micro-dismembrator (Braun Biotech, Aylesbury, U.K.) prior to reduction with potassium borohydride. The reduced material was then washed, freeze-dried and hydrolysed in 6 M HCl at 110 °C for 24 h and the acid removed by freeze-drying.

Hydrolysates were initially separated on a CF1 cellulose column to remove the non-cross-linking amino acids. The reducible intermediate and mature cross-links were then analysed by separation employing ion-exchange chromatography on an automatic amino acid analyser (Alpha Plus; Pharmacia). The buffer gradients used to separate both the reduced immature and the mature cross-links have been described previously in detail [22].

The reduced cross-links were identified as dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL). The mature cross-links were the pyridinolines HL-Pyr and L-Pyr. The location of the cross-links on the analyser had previously been confirmed with samples of the authentic compounds prepared in this laboratory. The quantification was based on the ninhydrin colour reaction and known leucine equivalents.

(ii) Quantification of pyrrolic cross-links. Tendon samples (tendons from three each of 22- and 14-week-old birds and two 11-week-old birds) were prepared as described earlier for removal of proteoglycans and for decalcification. The pyrrole content was assayed, after trypsin solubilization of the heat-denatured samples, by reaction with 4-dimethylaminobenzaldehyde, and the absorbance of the coloured product was measured at 572 nm. The yield was calculated from the molar absorption coefficient, based on that of an *N*-methylpyrrole [10]. Pyridinoline has been shown not to react with the Ehrlich reagent used to assay the pyrrole.

Lysine hydroxylation

The extent of hydroxylation of the lysine residues was determined by standard amino acid compositional analysis on an Alpha Plus amino acid analyser of the acid hydrolysates of tendon.

Quantification of collagen

Collagen was quantified by determination of hydroxyproline levels from acid hydrolysates [23] using a continuous-flow autoanalyser (Chemlab). The resulting data were then used to calculate the cross-link, hydroxylysine and DSC parameters per mol of collagen.

Collagen degradation

Quantitative zymography was performed using protocols adapted from established methods [24–26]. In brief, three tendons from each age group were powdered, freeze-dried and weighed, and extraction buffer (0.1 % Brij 35, 20 mM triethanolamine) was added. An aliquot of each homogenate was taken for determination of hydroxyproline. Solubilized proteins were extracted by agitation at 4 °C for 18 h, and insoluble material was removed by centrifugation. Aliquots for analysis were mixed with non-reducing 2 × SDS sample buffer, heated to 60 °C and loaded on to 10 % acrylamide gels co-polymerized with 0.5 mg/ml gelatin. Sample volumes were loaded such that each well contained material extracted from homogenate with equal collagen content, and subsequent analyses were correlated to the total collagen. After electrophoresis, the gels were washed in 2.5 % Triton X-100 and incubated for 18 h in proteolysis buffer (50 mM Tris/HCl, pH 7.4, 50 mM $CaCl_2$, 0.5 M NaCl and 0.5 mM aminophenylmercuric acetate).

Zones of proteolytic activity were visualized by background staining of non-degraded gelatin with Coomassie Blue. Gels were scanned using an Agfa Studioscan II, and the images analysed (NIH Image 1.5) in order to quantify the proteolytically clarified zones, as previously described [27]. Samples were directly compared with known standards on each gel, and enzyme levels were expressed as a percentage of the activity of these standards.

Zymography is quantitative within certain limits [24]. In order to establish that analyses were performed within the linear response range of the technique, a dilution series was analysed

using purified human MMP-2. Such analyses demonstrated a linear response from 0.1 to 6 μg of total MMP-2 loaded [28]. All of the samples analysed fell within this quantitative range of proteolytic gel clearance.

Thermal analysis

Tendons from eleven 22-week-old and three 11-week-old birds were analysed in a Perkin-Elmer DSC-2C fitted with intracoolers and computer-controlled at a heating rate of 10 °C/min from 5 to 95 °C, using an empty pan as a reference. Temperature and energy scales were calibrated with water and indium as standards. Mineralized samples were decalcified using acetic acid, since mineralized tendon could not be analysed because denaturation does not occur within the temperature range used. The contents of the pans were hydrolysed after denaturation and assayed for collagen by hydroxyproline analysis, as described above.

Effects of mineralization and demineralization on cross-links

Decalcification procedures

Two commonly used demineralization procedures were carried out on the reduced and non-reduced tendon to determine their effects on the intermolecular cross-links. Calcified and non-calcified sections from the MTLTs of three 22-week-old turkeys were pooled, and powdered in a Micro-dismembrator. Samples from both tendon sections were dialysed in either 0.5 M EDTA, pH 7.4, or 0.5 M acetic acid, both containing enzyme inhibitors, for 3 weeks at 4 °C with two volume changes, followed by dialysis against distilled water for 3 days [13]. Aliquots were also left untreated as controls. Samples were then reduced with potassium boro[^{3}H]hydride and dialysed against distilled water for 3 days prior to freeze-drying, and then hydrolysed with either acid or alkali. A complementary series of experiments was undertaken with the immature cross-links stabilized by reduction with potassium boro[^{3}H]hydride prior to decalcification treatment.

Chromatography of radioactive cross-links and precursors

To identify and isolate the cross-links and their precursors, hydrolysates were separated by preparative ion-exchange. A Technicon analyser with Duolite 225 cation-exchange resin and pyridine/formate buffers were employed as previously described in detail [29]. Initial standardization of the column was by analysis of pure amino acids and cross-link standards. Sample fractions were analysed by scintillation counting. Confirmation of cross-link identity was achieved by re-analysis of the relevant peaks on the Alpha Plus amino acid analyser as described above.

Smith degradation of the reduced precursors

To confirm the identity of the cross-link precursors, Smith degradation of the relevant pooled fractions was undertaken [30]. Samples were rotary-evaporated and redissolved in citrate buffer, pH 3.0, and sodium periodate was added to a final concentration of 0.01 M. The reaction proceeded at 4 °C for 5 min in the dark. The pH was adjusted to 7.5 and potassium borohydride was added to reduce the reaction products. After 30 min at room temperature, the solution was acidified to pH 2.2 and rechromatographed on a Technicon analyser to determine the presence of [^{3}H]proline and/or [^{3}H]hydroxynorvaline. The identities of the resultant proline and hydroxynorvaline peaks were validated by TLC and amino acid analysis. Fractions containing DHLNL were also treated as positive controls for the Smith degradation process.

DSC analysis

The non-mineralizing sections of tendons from eleven 22-week-old turkeys were treated with acetic acid or EDTA as above, and aliquots were taken for DSC and cross-link analysis as described earlier. In addition, aliquots from six tendons were reduced prior to EDTA treatment and analysed by DSC and for cross-links.

Statistical analysis

In the majority of cases, the variances of the data sets were found to be homogeneous and the residuals normally distributed. Therefore, for comparison of the data between age groups, one-way ANOVAs were used; paired t tests were used to determine the significance of differences between decalcification treatments and between tendon sites.

RESULTS

Collagen biochemistry

Collagen type

The CNBr peptide pattern was identical for both sections of the tendons at all ages (results not shown). The predominant collagen was type I, the proportion of type III collagen being less than 5 % and therefore not accurately quantifiable by the techniques used [21].

The following data are summarized in Table 1.

Lysine hydroxylation

The levels of lysine hydroxylation of the distal (non-calcifying) and the proximal (calcifying) MTLT increased ($P < 0.01$) and decreased ($P < 0.01$) respectively with increasing age.

Intermolecular cross-links

The changes in total lysine hydroxylation were relatively small when compared with those observed in the cross-link levels. The ratio of DHLNL to HLNL doubled with age in the distal tendon ($P < 0.01$), while there was a 5-fold decrease in that in the proximal tendon ($P < 0.001$).

The changes in the cross-link profile of the non-calcifying tendon were as expected, i.e. a decrease in the immature and an increase in the mature cross-links with age. However, the changes observed in the proximal tendon were more complex. DHLNL was consistently higher in the proximal than in the distal region at all ages ($P < 0.005$), whereas HLNL was lower until, at 22 weeks, the levels were eight times higher in the proximal than in the distal section ($P < 0.001$). HL-Pyr was present in both sections of the tendon at all ages, while L-Pyr was only detectable in the 22-week-old calcified tendon.

Pyrrole cross-links

The pyrrole cross-link profile closely mirrored that of the total pyridinoline cross-links. The pyrrole levels increased, although not significantly, with age in the distal tendon ($P < 0.06$), while levels decreased and then increased in the proximal tendon.

Collagen degradation

The zymograms are illustrated in Figure 1. MMP-2 was the major enzyme detectable by this method, with MMP-9 only being present in very small quantities. Total MMP-2 levels markedly increased in both the calcifying and the non-calcifying

Table 1 Summary of the post-translational modifications of MTLT collagen with age and mineralization

Values are expressed as means ± S.E.M., with units of mol/mol of collagen. For the pyrrole data: 11 weeks old, $n = 2$; 14 and 22 weeks old, $n = 3$. For the remainder of the data: 11 and 14 weeks old, $n = 6$; 22 weeks old, $n = 9$.

	Modification (mol/mol of collagen)					
	Non-calcifying (distal) tendon			Calcifying (proximal) tendon		
	11 weeks	14 weeks	22 weeks	11 weeks	14 weeks	22 weeks
DHLNL	0.724 ± 0.050	0.407 ± 0.015	0.327 ± 0.026	0.886 ± 0.044	0.483 ± 0.047	0.483 ± 0.023
HLNL	0.279 ± 0.024	0.193 ± 0.019	0.046 ± 0.013	0.134 ± 0.009	0.137 ± 0.014	0.398 ± 0.040
Total immature cross-links	1.003 ± 0.068	0.600 ± 0.021	0.373 ± 0.028	1.020 ± 0.049	0.622 ± 0.049	0.881 ± 0.062
DHLNL/HLNL ratio	2.623 ± 0.154	2.230 ± 0.336	4.874 ± 0.643	6.657 ± 0.404	3.682 ± 0.481	1.262 ± .0795
HL-Pyr	0.276 ± 0.023	0.387 ± 0.057	0.488 ± 0.055	0.427 ± 0.020	0.306 ± 0.028	0.335 ± 0.030
L-Pyr	0	0	0	0	0	0.186 ± 0.012
Total pyridinoline cross-links	0.276 ± 0.023	0.387 ± 0.057	0.488 ± 0.055	0.427 ± 0.020	0.306 ± 0.028	0.521 ± 0.038
Pyrrole	0.282 ± 0.021	0.378 ± 0.025	0.394 ± 0.038	0.308 ± 0.025	0.255 ± 0.015	0.373 ± 0.057
Hydroxylysine	34.74 ± 1.62	31.83 ± 2.71	41.43 ± 1.71	38.13 ± 0.87	30.16 ± 1.22	30.98 ± 1.57

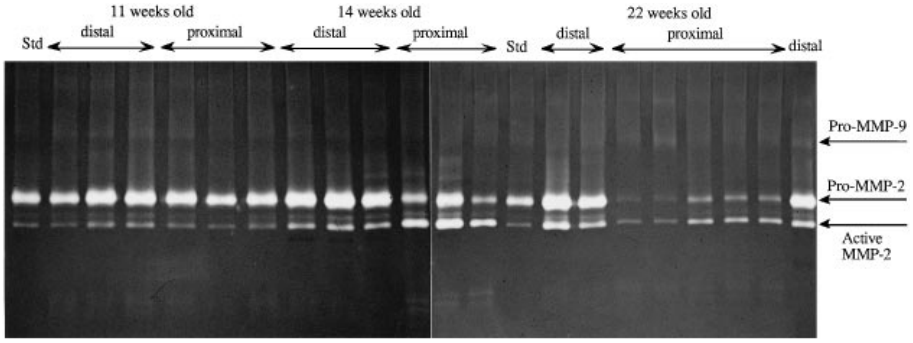


Figure 1 Gelatin zymography of MTLT during aging and calcification

Enzyme extracts from powdered samples of distal and proximal tendons from 11-, 14- and 22-week-old birds were analysed on SDS/PAGE (co-polymerized with 0.5 mg/ml gelatin). Sample volumes were loaded relative to each homogenate collagen content, so that direct comparisons between lanes could be made. After electrophoresis, gels were washed in Triton X-100 and incubated for 18 h in proteolysis buffer.

Table 2 Summary of the levels of MMP-2 activity in MTLT collagen: effects of age and mineralization

Gelatin zymography gels were scanned and the images analysed to quantify the proteolytically clarified zones. The enzyme levels are expressed as a percentage of the activity of the standards. Active MMP-2 is also expressed as a percentage of total MMP-2 present. Values are means ± S.E.M.; $n = 3$ for all ages.

	Activity (% of control)					
	Non-calcifying (distal) tendon			Calcifying (proximal) tendon		
	11 weeks	14 weeks	22 weeks	11 weeks	14 weeks	22 weeks
Pro-MMP-2	139 ± 7	176 ± 6	205 ± 6	127 ± 9	102 ± 34	16 ± 4
Active MMP-2	23 ± 6	45 ± 2	84 ± 2	16 ± 4	107 ± 21	24 ± 5
Total MMP-2	163 ± 23	221 ± 9	289 ± 27	142 ± 1.5	209 ± 55	40 ± 9
Percentage active MMP-2	14 ± 2	20 ± 1	29 ± 2	11 ± 2	52 ± 5	61 ± 2

tendons between weeks 11 and 14. This trend continued in the non-calcifying tendons up to 22 weeks, but in the calcifying group it was reversed, to give levels at 22 weeks below those seen at 11 weeks.

The proportion of active MMP-2 relative to total MMP-2 increased with age for both tendon sites, with a dramatic increase between 11 and 14 weeks in the mineralizing tendon. On the other hand, the actual levels of activated MMP-2 increased sharply between 11 and 14 weeks in calcifying tendon, subsequently returning to 11-week levels at 22 weeks. Again, non-calcifying tendon demonstrated a gradual increase with age in MMP-2 levels. These data are summarized in Table 2.

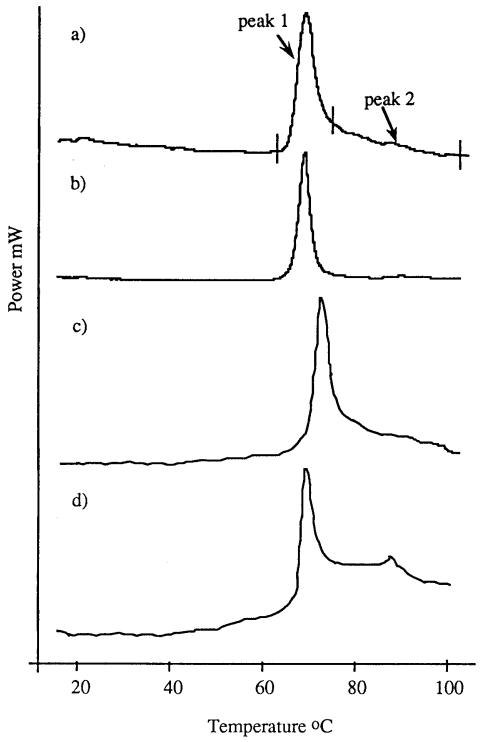


Figure 2 DSC thermograms of distal and proximal sections of tendons from 11- and 22-week-old turkeys

A heating rate of 10 °C/min from 5 to 95 °C was used. Thermograms (**a**) and (**b**) are 11-week distal and proximal tendon respectively; (**c**) and (**d**) are 22-week distal and proximal tendon respectively. The proximal tendon from 22-week-old birds was decalcified with acetic acid prior to analysis.

Thermal characteristics

The changes in the thermal properties with age and calcification were varied and complex. The data demonstrated differences between the proximal and distal sections prior to calcification, in addition to changes associated with aging and actual mineralization. Figure 2 illustrates typical DSC thermograms obtained from both proximal and distal sections of 11- and 22-week MTLTs. The thermograms of adult MTLTs were similar to those that we have previously reported for bone [5], with two distinct regions to the endotherm. Data gathered from these thermograms are summarized in Table 3.

The temperature at which the main denaturation peak occurred (peak 1; T_{max}) increased with age in the distal tendon ($P < 0.001$), while the size (enthalpy) and shape (peak width and height) of the endotherm remained the same. In contrast, the size ($P < 0.001$) and shape ($P < 0.001$), but not the T_{max}, altered with age (and mineralization) in the proximal tendon.

Peak 2 (the later, broad portion of the endotherm) became more prominent in the tendons from 22-week-old birds; this was more pronounced in the proximal tendon sites, resulting in an 11-fold decrease in the ratio of peak 1 to peak 2 with age and mineralization ($P < 0.001$).

Interestingly, the total enthalpy of the proximal tendon once mineralization had occurred was greatly decreased from a consistent ~ 60 to ~ 40 J/g of collagen. This specific drop in enthalpy for type I collagen has only previously been noted for the calcifying collagen of bone [5].

Table 3 Summary of the changes in the thermal characteristics of MTLT collagen with age and mineralization

Samples were analysed by DSC, and the thermal parameters were measured from the resulting thermograms. Values are expressed as means ± S.E.M. For tendons from 11-week-old turkeys, $n = 3$; from 22-week-old birds, $n = 11$. Comparison of age (same site): $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. Comparison of site (same age): $\dagger\dagger\dagger P < 0.001$; $\dagger\dagger P < 0.01$; $\dagger P < 0.05$.

	Non-calcifying (distal) tendon		Calcifying (proximal) tendon	
	11 weeks	22 weeks	11 weeks	22 weeks
Enthalpy of denaturation ΔH (J/g of collagen)				
Peak 1	38.3 ± 4.2	33.9 ± 1.6	53.8 ± 0.1	13.8 ± 0.5$^{***}\dagger\dagger\dagger$
Peak 2	24.2 ± 3.6	25.5 ± 1.5	10.4 ± 1.8	28.6 ± 2.3***
Total	62.5 ± 0.7	59.7 ± 1.7	68.4 ± 5.0	41.8 ± 0.1$\dagger\dagger\dagger$
Peak 1/peak 2 ratio	1.74 ± 0.50	1.39 ± 0.12	5.58 ± 1.18	0.49 ± 0.07$^{***}\dagger\dagger$
Peak 1				
T_{max} (°C)	68.0 ± 0.5	71.7 ± 0.3***	67.5 ± 0.1	68.4 ± 0.8$\dagger\dagger$
Height (J/g of collagen)	10.11 ± 1.97	6.92 ± 0.81	17.17 ± 1.11	2.87 ± 0.20$^{***}\dagger\dagger\dagger$
Width (ΔT °C)	3.7 ± 0.3	4.7 ± 0.3	2.8 ± 0.3	4.1 ± 0.1***
Height/width ratio	2.86 ± 0.79	1.66 ± 0.31	6.37 ± 1.05	0.74 ± 0.07$^{***}\dagger$

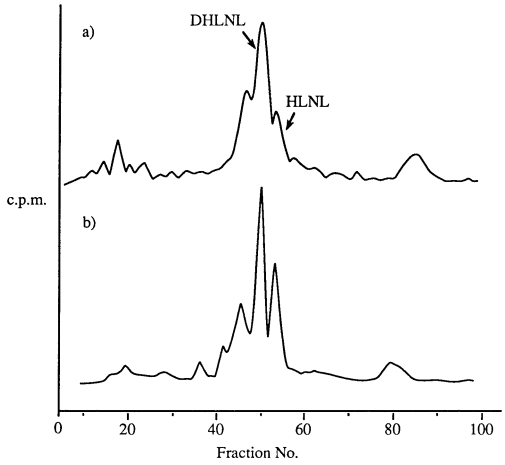


Figure 3 Ion-exchange chromatograms of tendons from 22-week-old turkeys

Samples were reduced with tritiated borohydride and acid- or alkali-hydrolysed prior to analysis by preparative ion-exchange chromatography. (**a**) Distal (non-calcified) tendon; (**b**) proximal (calcified) tendon.

Effects of mineralization and demineralization on cross-links

The mineralized and non-mineralized sections of the adult tendon were both subjected to demineralizing agents prior to, and following, stabilization of the intermediate cross-links by borohydride reduction.

Mineralization

The ion-exchange/pyridine column chromatograms for the untreated mineralized and non-mineralized tendons revealed similar cross-link profiles, with no tritium-labelled peaks being eluted in the positions of the cross-link precursors (Figure 3).

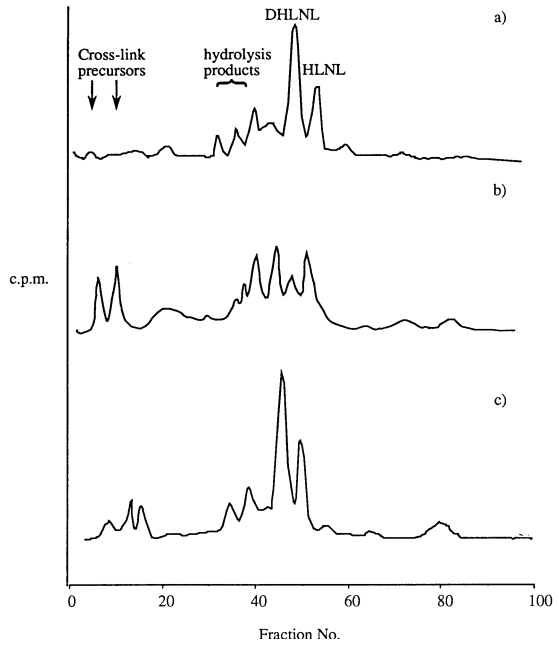


Figure 4 Ion-exchange chromatograms of treated proximal (calcified) tendons from 22-week-old birds

Samples were treated with either (**a**) acetic acid or (**b**) EDTA prior to reduction with tritiated borohydride and acid hydrolysis. The sample in (**c**) was reduced prior to EDTA treatment and acid hydrolysis.

The major reduced cross-links were DHLNL and HLNL. The identities of these peaks were confirmed by amino acid analysis using ninhydrin detection.

Demineralization

Decalcification treatment of both mineralized and non-mineralized tendon with acetic acid before reduction resulted in cross-link profiles similar to those shown in Figure 3, in which DHLNL and HLNL were the major radioactive cross-link peaks (Figure 4a). However, when the tendon was decalcified with 0.5 M EDTA prior to reduction, there was a significant decrease in the levels of both cross-links. More importantly, we noted peaks in the elution positions of the reduced forms of the individual aldehyde cross-link precursors, lysyl-aldehyde and hydroxylysyl-aldehyde (Figure 4b).

These peaks were found to be partially labile to acid hydrolysis, as previously noted for the cross-link precursors, to form chloro-derivatives [29]. Smith degradation of the fractions isolated from alkaline hydrolysates resulted in the formation of tritiated proline and hydroxynorvaline, so confirming their identities as lysyl aldehydes [30]. Prior reduction of the collagen prevented the appearance of the precursors following EDTA treatment in both tendon sites (Figure 4c).

DSC analysis

EDTA treatment had the most significant effect on the thermal characteristics of the tendon (Table 4). When compared with untreated controls, the enthalpy of denaturation of peak 1 was increased ($P < 0.05$), with a concomitant decrease in the enthalpy of the second peak ($P < 0.05$). The enthalpies of peak 1 and peak 2 in samples reduced prior to treatment were comparable with those in untreated tendons (34.7 ± 0.8 and 27.8 ± 1.5 J/g of collagen respectively; $n = 6$). T_{max} was not affected by either treatment.

Table 4 Summary of the effects of decalcification treatment on the thermal characteristics and immature cross-links of collagen from the distal tendons of 22-week-old turkeys

Samples form 11 tendons were treated with either acetic acid or EDTA, or left untreated, prior to DSC and cross-link analysis. Values are expressed as means ± S.E.M. Treated compared with untreated: $^*P < 0.05$.

	Untreated	+ EDTA	+ Acetic acid
Enthalpy of denaturation ΔH (J/g of collagen)			
Peak 1	34.0 ± 1.6	38.8 ± 2.2*	32.5 ± 2.3
Peak 2	25.5 ± 1.5	22.4 ± 1.6*	27.6 ± 1.2
Total	59.7 ± 1.7	61.0 ± 1.2	60.7 ± 2.1
Peak 1/peak 2 ratio	1.4 ± 0.1	2.0 ± 0.4	1.2 ± 0.1
Peak 1			
T_{max} (°C)	71.7 ± 0.3	70.6 ± 0.6	71.8 ± 0.8
Height (J/g of collagen)	6.9 ± 2.7	8.5 ± 0.7	6.5 ± 0.4
Width [ΔT (°C)]	4.7 ± 0.3	4.2 ± 0.2	4.1 ± 0.2
Height/width ratio	1.7 ± 0.3	2.0 ± 0.2	1.6 ± 0.1
Cross-links (mol/mol of collagen)			
DHLNL	0.346 ± 0.027	0.273 ± 0.026*	0.365 ± 0.033
HLNL	0.072 ± 0.010	0.063 ± 0.007*	0.070 ± 0.009

The decreases in DHLNL and HLNL with EDTA treatment of these samples were confirmed by amino acid analysis (Table 4).

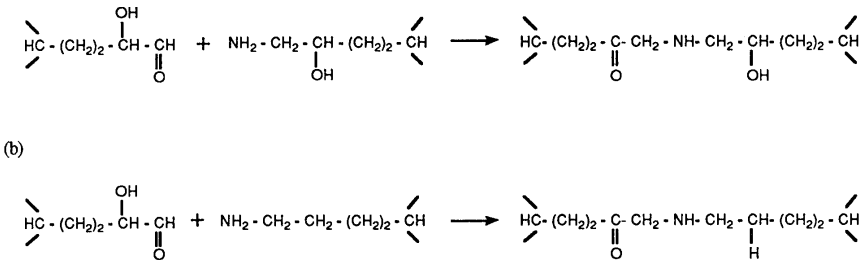
DISCUSSION

The post-translational modifications of the MTLT during aging and mineralization are complex, and it is therefore worthwhile recapitulating at this point on the options available for the formation of different types of intermediate and mature cross-links. In bone and other calcified tissues, the telopeptide hydroxylysyl-aldehyde can react with triple-helical lysine or hydroxylysine to give the lysylketoimine (LKNL; HLNL when reduced) and hydroxylysylketoimine (HLKNL; DHLNL when reduced) respectively (Scheme 1) [30]. These ketoimines, LKNL and HLKNL, then mature into the trivalent cross-links L-Pyr and HL-Pyr respectively by further reaction with another telopeptide, hydroxylysyl-aldehyde (Scheme 2a) [15]. Alternatively the keto-imine could react with a telopeptide lysine-aldehyde to form the trivalent lysyl and hydroxylysyl pyrroles (Scheme 2b) [11]. The structures and mechanism of formation of these pyrroles have not yet been confirmed, but there is considerable circumstantial evidence for their formation. The present study provides further evidence for the pyrrole as an important mature cross-link. The changes in pyrrole levels with age closely mirror the maturational increases in pyridinoline levels in the proximal tendon.

Cross-link changes with age and mineralization

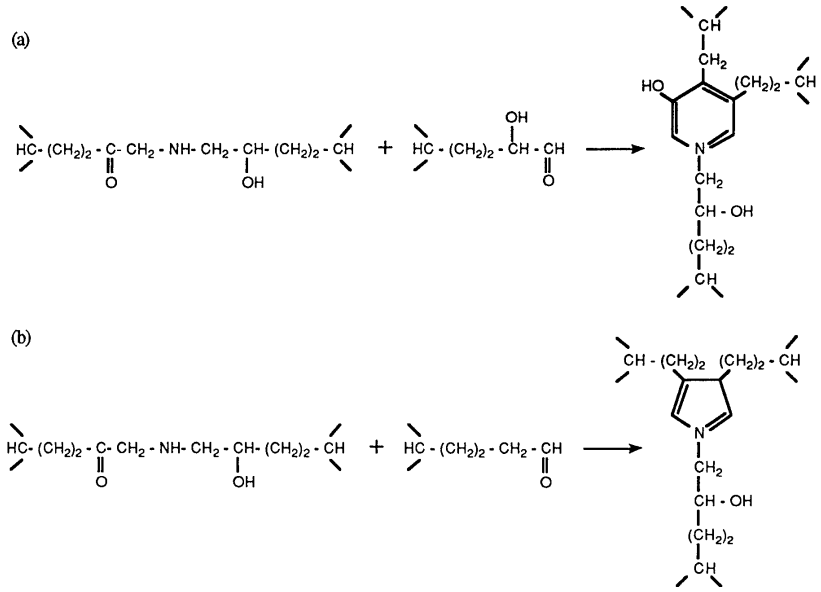
It is apparent, even from the above brief summary, that the post-translational hydroxylation of both the helical and telopeptide lysine residues is of primary importance for subsequent cross-link formation, and the complex nature of the post-translational modifications during both aging and mineralization of the tendon is reflected in the present study.

The minimal changes in overall lysine hydroxylation in both the proximal and distal sections of the MTLT with age were in



Scheme 1 Formation of the immature keto-imine cross-links

(**a**) HLKNL; (**b**) LKNL.



Scheme 2 Formation of the mature HL-Pyr and putative pyrrole cross-links

(**a**) HL-Pyr; (**b**) hydroxylysylpyrrole.

contrast to the disproportionately large changes in the cross-link hydroxylation profile. In addition, although there was no change in overall lysyl hydroxylation between 14 and 22 weeks of age in the calcifying tendon, there was a dramatic increase in HLNL and the concomitant appearance of its maturation product L-Pyr. These data indicate that hydroxylation of the cross-linking telopeptide and helical lysine residues may be independently and specifically controlled. The existence of two different lysyl hydroxylases has been previously postulated by Royce and Barnes [31], and other studies of cross-linking in calcifying tissues have also supported this proposal [5,31–33].

Despite the demonstration of clear differences in cross-linking between calcifying and non-calcifying tissues, it remained unclear whether a specific matrix was required for mineralization, or if such differences were incidental. In an attempt to clarify this matter, we compared the proximal and distal sections of MTLTs from 11-week-old birds in order to determine whether the proximal tendon was different prior to mineralization and therefore predestined to become mineralized, and/or whether a new matrix was formed during mineralization. The data demonstrated that the young proximal tendon was indeed different, in terms of post-translational modifications, from the distal tendon, again in a manner suggesting specific regulation of lysine hydroxylation. Furthermore, changes with age in the proximal tendon, in addition to the normal cross-link maturation processes, indicated that a new matrix was formed during mineralization.

The formation of a new matrix requires a remodelling of collagen, and this is supported by the changes in the levels of MMP-2. Although, to date, the lack of data on the levels of MMP inhibitors precludes a complete analysis, the data are important in determining the sequence of events in the metabolism of the MTLT. The gradual increases in the total, active and percentage active MMP-2 in the distal tendon with age are consistent with a rapidly growing animal. In contrast, the changes observed in the proximal tendon are more complex. At 14 weeks of age the high levels of all three parameters of MMP-2 activity correspond to the period of fibroblast proliferation and mineralization [20].

Thermal analysis of the MTLT provided further evidence to support the biochemical data in our study, and in addition supported several interesting phenomena of mineralizing tissues previously reported by us. DSC analysis of collagen provides substantial information concerning the nature of collagen within

tissues. Collagen denaturation is greatly influenced by the composition of the molecule, its post-translational modifications, its molecular organization and the environment [34–36]. Changes in the shape of the thermogram have been associated with differences in the post-translational modifications of collagen, e.g. in osteoporotic bone collagen [5].

In the present study, the contrasts in thermogram profiles between sites in young tendon support the differences in the collagen matrix prior to mineralization demonstrated by the cross-link data. The thermal stability of collagen increases with age due to two mechanisms, enzymic cross-link maturation and glycation-induced cross-linking [37,38], and a similar mechanism may be responsible for the increase in T_{max} with age in the distal tendon observed in the present study. The more dramatic differences with age in the thermal characteristics of the calcifying tendon reflect the complex biochemical changes that we have demonstrated during mineralization. The unusually low enthalpy of the calcified tendon has previously been noted for bone collagen [5], and its significance is currently under investigation. The presence of at least two thermally distinct populations of collagen has also been described for bone [5] and mature skin collagen [38], the more thermally stable population being more prominent in calcifying collagen. This phenomenon may be attributed to the heterogeneity of complex collagenous tissues. The changes in the thermally stable cross-link profile associated with aging and calcification would result in a tissue composed of populations of collagen with slightly differing thermal properties, and hence in a broadening of the denaturation peak.

Effect of EDTA-induced demineralization on cross-links

Otsubo et al. [13] have reported high levels of the free lysine and hydroxylysine aldehyde cross-link precursors in both bone and MTLT, and proposed that they were derived from cleavage of the immature cross-links during mineralization. However, in our hands, borohydride reduction of intact mineralized bone did not reveal the presence of free aldehyde precursors. We therefore postulated that the demineralization process caused a reversion of the Amadori product to the more labile form of the immature cross-links, i.e. a retro-Amadori reaction, and hence resulted in the release of the free aldehydes. It is clear from our present results with both mineralized and non-mineralized tendon that EDTA indeed results in a decrease in the immature cross-links and a concomitant increase in their precursors. Demineralization with acetic acid failed to result in any change in the cross-link profile. Similar model compounds, for example glycosyl-lysine, which undergo Amadori rearrangements were also reversed by EDTA, with the release of the original components (L. Knott, R. G. Paul, unpublished work).

DSC analysis of the treated MTLT provided further evidence to support the proposal of cross-link cleavage by EDTA. The increase in the amount of the less thermally stable population of collagen on EDTA treatment, to the detriment of the more stable population, is consistent with the theory of EDTA-induced disruption of the immature cross-links. The fact that stabilization of the cross-links by reduction prior to EDTA treatment prevented this change in the proportions of the two collagen populations further supports this theory.

In conclusion, we have shown that, while there are only minimal changes in the overall hydroxylation of the total lysines in calcifying collagen, there are dramatic changes in the cross-link profile during mineralization, providing further evidence for the existence of at least two lysyl hydroxylase enzymes. The differences in post-translational modifications and MMP activity between the proximal and distal tendon, even prior to mineralization, suggest that the proximal tendon is predestined to mineralize, and that a specific matrix for mineralization may be required. These data demonstrate that the post-translational modifications of collagen play an integral role in matrix mineralization, in contrast with other studies suggesting that the integrity of collagen cross-links is disrupted during calcification.

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