

Collagen Cross-Links

David R. Eyre (✉) · Jiann-Jiu Wu

Department of Orthopaedics and Sports Medicine, Orthopaedic Research Labs,
University of Washington, P.O. Box 356500, Seattle, WA 98195-6500, USA
deyre@u.washington.edu

1	Introduction	208
2	Fibril-Forming Collagens (Types I, II, III, V/XI)	208
3	Fibril-Associated Type IX Collagen of Cartilage	214
4	Basement Membrane Type IV Collagen	217
5	Lysyl Hydroxylases Regulate Tissue-Dependent Patterns of Cross-Linking	218
6	Bone and Mineralized Tissue Collagens	220
7	Collagen Cross-Links as Biomarkers	221
8	Other Mechanisms of Collagen Cross-Linking	223
8.1	Cystine Disulfides	223
8.2	Gamma-Glutamyl Lysine Cross-Links	223
8.3	Tyrosine-Derived Cross-Links	224
8.4	Types VIII and X Collagens	224
9	Outlook	225
	References	225

Abstract Collagen is the main source of extracellular support for multicellular animals. The mechanical strength of collagen fibrils depends on a highly regulated mechanism of intermolecular cross-linking. The basis of this cross-linking from the most primitive to the most advanced multicellular animals and across a diversity of vertebrate tissue types, is the formation of covalent bonds from aldehydes produced from lysyl and hydroxylysyl side-chains by lysyl oxidase. In the last decade it has become clear that such bonds form not only between collagen molecules of the same type in homopolymeric fibrils but also between different types of collagen molecule that have evolved to interact and form heteromeric structures. Furthermore, cross-linking amino acids and peptides containing them from collagen degradation, have received attention as bone resorption biomarkers in clinical studies and drug trials in the osteoporosis field. This review summarizes recent research directions with examples of advances in understanding complex interactions in cartilage collagen and the role of lysyl hydroxylase isoforms in regulating the pathway of cross-linking chemistry.

Keywords Extracellular matrix · Lysyl oxidase · Lysyl hydroxylases · Pyridinolines · Bone

1

Introduction

Collagen fibrils provide the mechanical support that enabled large multicellular animals to evolve on earth [1, 2]. The tensile strength of collagen depends on the formation of covalent intermolecular cross-links between the individual protein subunits [3]. All the fibril-forming collagen types in higher vertebrates (types I, II, III, V and XI) are cross-linked through a mechanism based on the reactions of aldehydes generated enzymically from lysine (or hydroxylysine) side-chains by lysyl oxidase [4, 5]. This pathway operates in the collagen fibrils of sponges (Porifera), the most primitive extant multicellular animals, through to mammals [6, 7]. Certain other collagen types (e.g., collagen type IX of cartilage) are also cross-linked by the lysyl oxidase mechanism. In the last decade, perhaps the most important conceptual advance in this field is that different collagen types have evolved to cross-link heterotypically in the assembly of multi-component fibrils. Templates of one class of collagen (type V/XI microfibrils) provide the scaffold on which the more recently evolved fibrillar collagen molecules (types I and II) co-assemble to form large fibrils. In cartilage, a third type of collagen, type IX (of the FACIT sub-family), becomes cross-linked to the surface of this copolymer and all three molecular types are cross-linked internally and to each other through the lysyl oxidase mechanism. In considering the evolution of the different molecular classes of collagen, it is clear that gain of function mutations in the protein subunits have created new cross-linking opportunities for heterotypic intermolecular bonding. This presumably extends the range of collagen functional properties cells can express.

In addition to physiologically regulated cross-linking on synthesis by lysyl oxidase, collagens are also susceptible over time to further cross-linking through the undesirable reactions of reducing sugars [8–13], particularly glucose (non-enzymic glycosylation or glycation) and lipid oxidation products [14, 15]. There is an extensive and growing literature on the chemistry of such age-related changes, which in general have pathological effects, but this chemistry will not be reviewed here.

2

Fibril-Forming Collagens (Types I, II, III, V/XI)

Most of the research defining the cross-linking pathways from precursor lysine and hydroxylysine aldehydes in bulk collagen fibrils of major connective tissues was done over 20 years ago (reviewed in [4, 16–19]). The basic pathways are shown in Figs. 1 and 2. In the last decade the prominence of pyrrole cross-links as maturation products in bone collagen and their molecular location have been established [20].

The two basic pathways, lysine aldehyde vs hydroxylysine aldehyde-initiated, appear in general to occur in loose vs stiff connective tissues respectively. In

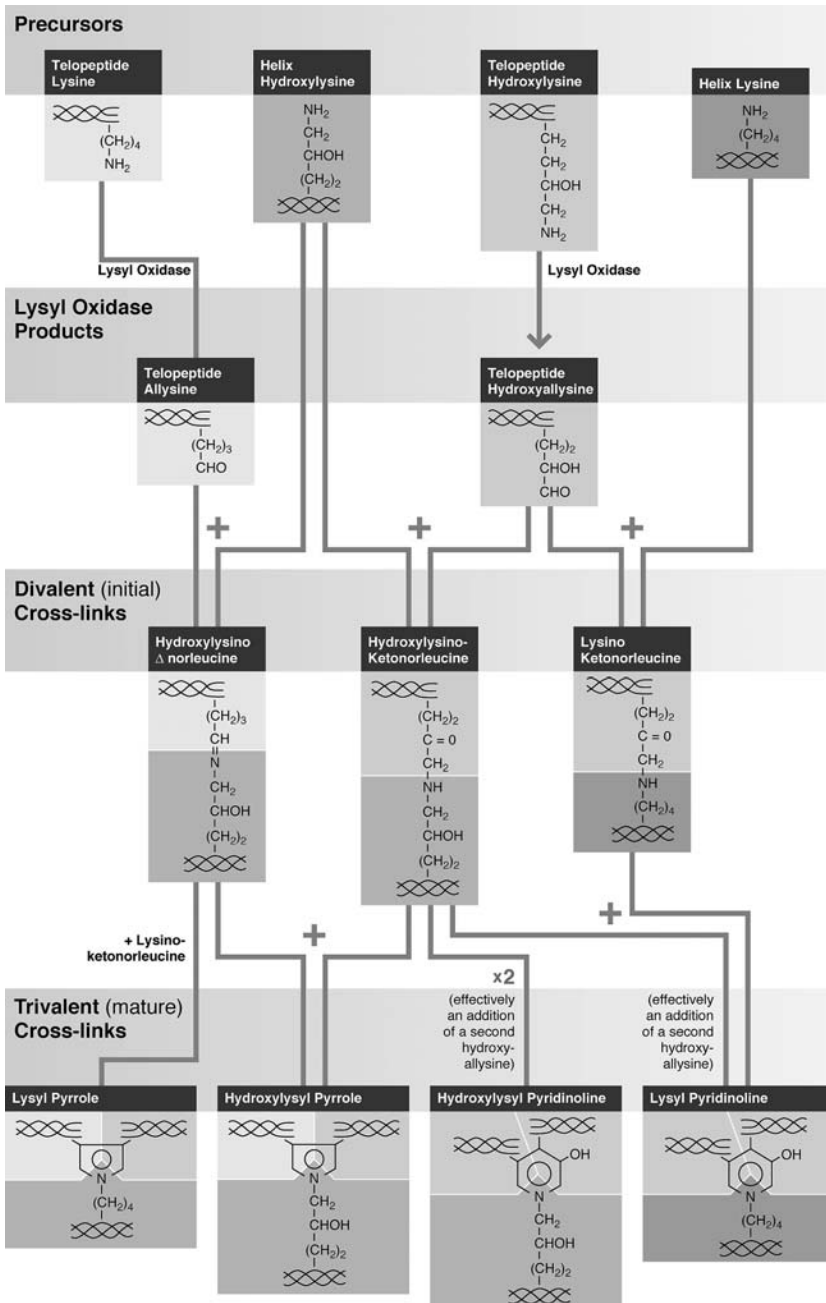


Fig. 1 The hydroxyallysine cross-linking pathway. Hydroxylysine residues are the source of aldehydes formed by lysyl oxidase for intermolecular cross-linking reactions. Mature cross-links are trivalent pyridinolines. Bone collagen is unusual in that pyrrole cross-links are also prominent because both hydroxyallysine and allylsine participate as precursors

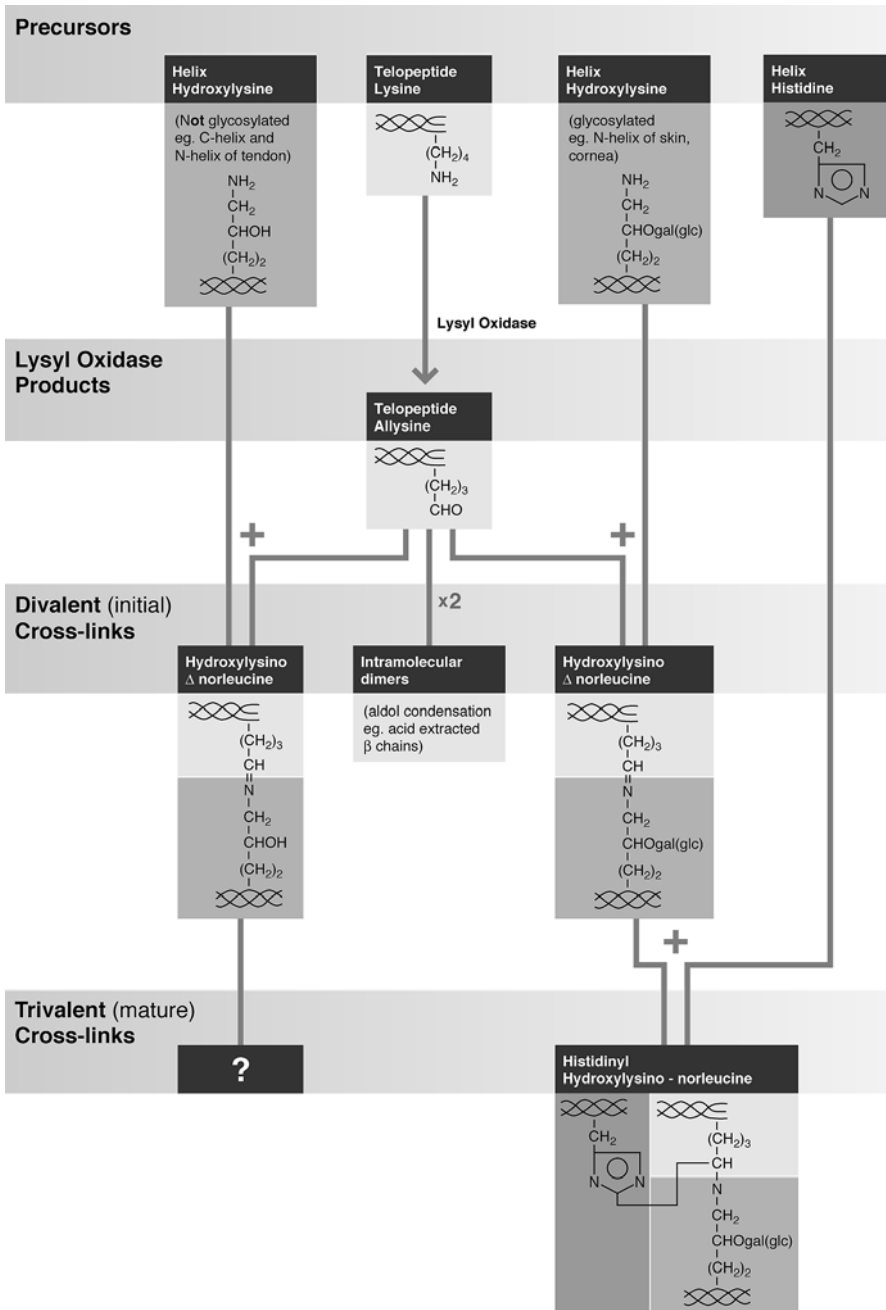


Fig. 2 The allsine cross-linking pathway. Lysine residues are the source of aldehydes formed by lysyl oxidase for intermolecular cross-linking reactions. Histidine can participate in mature cross-link formation, notably in skin collagen

detail, the tissue specificities and the pathways are more diversified, with elements of both pathways and both precursor aldehydes combined in some specialized tissues, for example in bone type I collagen [20]. Historically, the lysine aldehyde pathway (Fig. 2) was easier to study since the initially-formed aldimines are cleaved at low pH allowing the collagen monomers to be solubilized from rat skin and tail tendon in 0.5 M acetic acid [19]. On the hydroxylysine aldehyde pathway (Fig. 1), neither the initial cross-links nor their maturation products were labile so these collagens were insoluble making molecular analysis more difficult. The natural fluorescence of the pyridinoline cross-links (Fig. 1) allowed peptides to be isolated and their molecular sites of origin to be worked out [21–23]. In the last decade the greatest attention in the literature to pyridinoline residues, and to collagen cross-links in general, is from publications reporting assays of these residues and peptides containing them in blood and urine as biomarkers of bone resorption and connective tissue degradation. Because pyridinolines cannot be metabolized, their levels in blood and urine provide a measure of the amount of collagen and hence tissue from which they were proteolytically derived.

Variations in cross-linking chemistry appear to be more tissue-specific than collagen type-specific. This is understandable if a single cell type synthesizing multiple collagens passes them through the same processing enzymes and endoplasmic reticular pathway. The basic pathway of cross-linking is regulated primarily by the hydroxylation pattern of telopeptide and triple-helix domain lysine residues. The flanking sequences around the cross-linking lysine residues, however, can affect the ensuing chemistry. An example is the participation of a histidine residue in the formation of the mature trivalent cross-links found in skin type I collagen (Fig. 2). A histidine in the $\alpha 2(I)$ chain (of a third collagen molecule) reacts with a vicinal aldimine cross-link formed between a lysine aldehyde and a hydroxylysine residue in two 4D-staggered collagen I molecules [24, 25]. The pitch of collagen molecules packed in skin collagen fibrils is thought to facilitate this addition reaction to histidine [26], but whether it is fortuitous or provides a functional advantage to dermal collagen is unknown. Older studies had observed that the helical domain hydroxylysine at this location in the $\alpha 1(I)$ chain was glycosylated in skin, but not in tendon [27]. Notably, the histidine-containing mature cross-link HHL (histidinyl hydroxylysino-norleucine), is not found in tendon collagen, which raises the possibility that glycosylation might drive HHL formation. It has been observed that pyridinoline residues (hydroxylysyl pyridinoline) can still be formed when glycosylated hydroxylysine occurs in the helix, for example at the C-telopeptide to N-helix site in bone collagen [20]. But at the N-telopeptide to C-helix cross-linking site, the helical hydroxylysine is not glycosylated. Again the biological significance of these differences is unknown.

Edman N-terminal sequence analysis of cross-linked peptides isolated from protease digests of tissue collagens has revealed intermolecular cross-linking between types I and III collagens (e.g., from aorta [28, 29]) and types I and II collagens (e.g., from intervertebral disc [30]). Unequivocally proving covalent

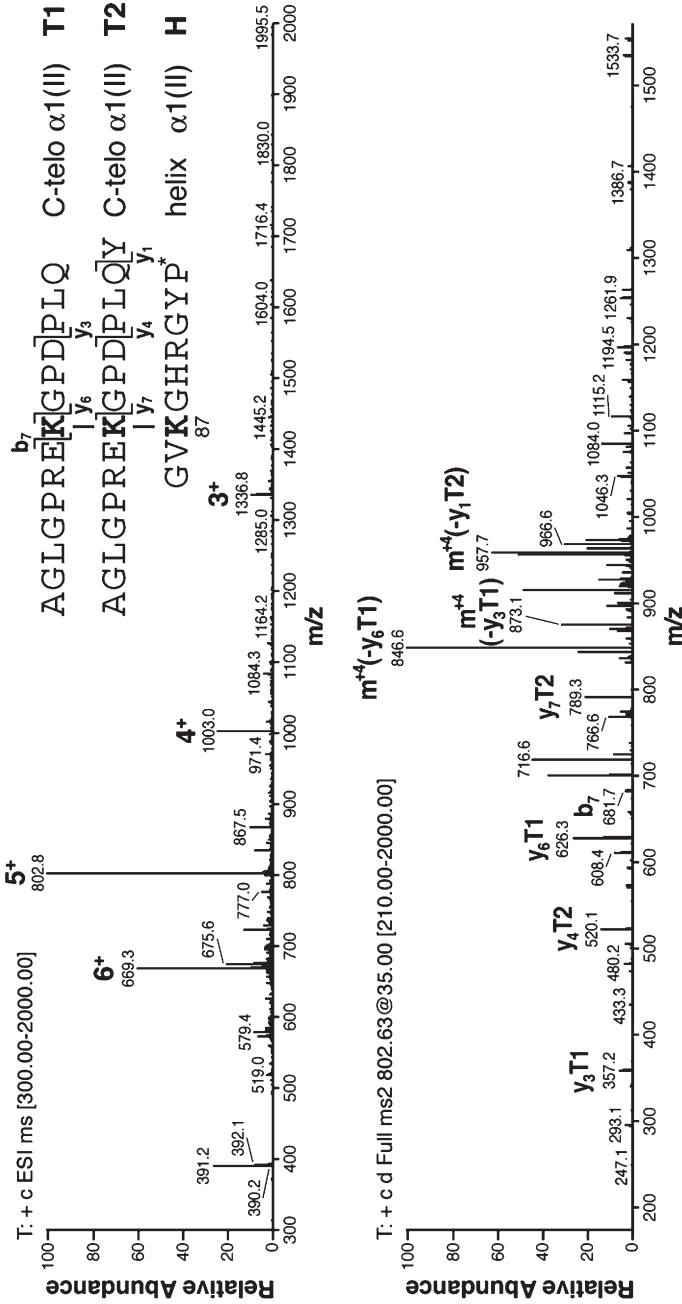


Fig. 3 Electrospray tandem mass spectrometry of a pyridinoline cross-linked peptide isolated from type II collagen (unpublished)

structures rather than peptide mixtures is difficult using chromatography and N-terminal sequence analysis alone. Mass spectrometry promises to be a valuable tool in such studies. Preliminary data from purified cross-linked trimeric peptides indicate characteristic MS/MS fragmentation patterns. Figure 3 shows an example of MS/MS data from tandem mass spectrometry of a homotypic fragment from human cartilage type II collagen.

Types V and XI collagens are quantitatively minor molecular species that are found copolymerized with collagens I and II respectively, and are believed to form a filamentous scaffold or template on which the bulk fibrillar collagens co-polymerize. The gene products represented in these molecules can in fact assemble in a variety of heterotrimeric chain combinations, not just type V ($[\alpha 1(V)]_2\alpha 2(V)$) and type XI ($[\alpha 1(XI)] [\alpha 2(XI)] [\alpha 3(XI)]$) molecules, but also novel tissue-specific chain associations [31–33]. Collagen type V/XI is best considered as a distinct sub-family of the fibril-forming collagen molecules. The same basic pattern of cross-linking, employing lysyl oxidase, and the telopeptide-to-helix location of intermolecular bonds occurs in these molecules. Homologous cross-linking sites (lysines) to those in types I, II and III collagens are evident in the protein sequences (Fig. 4), consistent with their evolutionary origin from a single founder gene [2].

Analyses of cross-linked peptides isolated from type XI collagen of cartilage showed only divalent cross-links (hydroxylysino-5-*keto*-norleucine), not the mature form of pyridinoline (hydroxylysyl pyridinoline) that predominates in type II collagen, the bulk fibril-forming subunit of the copolymer [34]. In addition, analysis of cross-linked peptides showed that most of the intermolecular bonds had formed between collagen XI molecules (N-telopeptide to C-helix). Any inter-type cross-linking was between type II C-telopeptides and the type XI N-helical site. This is best interpreted if collagen XI had self-polymerized to form its own filamentous network, at least initially, consistent with its suspected role as a template for the growth of thick banded fibrils. Similar properties have been

Type	Chain	N-telo	N-helix	C-helix	C-telo
I	$\alpha 1(I)$	YDEKSTGGI	GMKGHR	GIKGHR	PPQEKAHDG
	$\alpha 2(I)$	YDGKGVGLG	GFKGIR	GLKGHN	no lysine
II	$\alpha 1(II)$	FDEKAGGAQ	GVKGHR	GLKGHR	GPREKGPDP
III	$\alpha 1(III)$	YDVKSGVAV	GMKGHR	GIKGHR	IGGEKAGGF
V/XI	$\alpha 1(V)$	AGSKGPMVS	GEKGHR	GEKGHP	no lysine
	$\alpha 2(V)$	LDEKSGLGS	GLKGHR	GQKGHR	no lysine
	$\alpha 3(V)$	GSFKGPPVS	GEKGQR	GEKGHI	no lysine
	$\alpha 1(XI)$	DGSKGPTIS	GDKGHR	GEKGHP	PILSSKKTRR ¹
	$\alpha 2(XI)$	GGDKGPVVA	GEKGHR	GEKGHP	PIQMPKKTRR ¹

1. Lysines appear not to be involved in cross-linking

Fig. 4 Amino acid sequences at the four primary cross-linking sites in fibrillar collagens. Related sequence motifs can be found in all the fibrillar collagen gene products

found for type V collagen isolated from bone [35]. The primary cross-links were divalent (hydroxylysino-5-*keto*-norleucine) and they linked type V molecules between N-telopeptides and the C-helix site in a head-to-tail manner. Cross-links from the C-telopeptide of type I collagen to the N-helix site of type V were also found. The data on mature bone fit a special role for a hybrid type V/XI collagen molecule, [$\alpha 1(V)\alpha 1(XI)\alpha 2(V)$] [32], as a component of the filamentous template of the type I collagen-based fibrillar network of bone matrix.

3

Fibril-Associated Type IX Collagen of Cartilage

In addition to the fibril-forming collagen molecules (and basement membrane type IV collagen), 30 or more other molecular forms of collagen function in the extracellular matrix or at cell surfaces. Of these, only type IX collagen has been established to use the lysyl oxidase-mediated pathway of cross-linking. Within the diverse FACIT sub-family of collagen molecules (fibril-associated collagen with interrupted triple-helix [36]) therefore, collagen IX is the only member known to cross-link by the lysyl oxidase mechanism. Collagen IX cross-linking is extensive, highly evolved and presumably central to the protein's function as an adapter molecule on the surface of nascent type II collagen fibrils. All three collagen IX chains, which in birds and mammals are each the product of a distinct gene, appear to have diverged from a common ancestor after earlier whole or partial genome duplications. Each chain has two or three sites through which lysine-mediated cross-links can form and each displays unique specificity in its evolved cross-linking interactions. Through a series of studies over the last decade, we have defined the cross-linking sites and nature of the cross-linking of collagen type IX [37–41] (Fig. 5). The experimental approach has focused on isolating and structurally identifying peptides containing cross-linking residues (using fluorescence to track pyridinolines, and NaB^3H_4 to stabilize and tritium-label divalent cross-links). Conventional Edman-sequencing and, more recently, liquid chromatography/electrospray mass spectrometry (LCMS [41]) have been the methods of choice. LCMS is clearly a powerful tool for the future for defining complex cross-linking mechanisms in collagens and other extracellular matrix proteins.

Collagen IX has evolved to cross-link to collagen type II. The positioning of cross-linking sites along the molecule predicts their spatial inter-relationship. Figure 6 shows a molecular interaction model that can accommodate all six known cross-linking sites, and their interaction partner residues in type II collagen and other type IX collagen molecules. This packing arrangement requires an antiparallel relationship between the central type IX COL2 triple-helical domain and type II collagen molecules on a fibril surface and the type IX COL1 domain to be folded back on the COL2 domain as shown. All the known bonds [22, 37, 38, 41] can then be accommodated with the correct axial spatial alignments for cross-link formation.

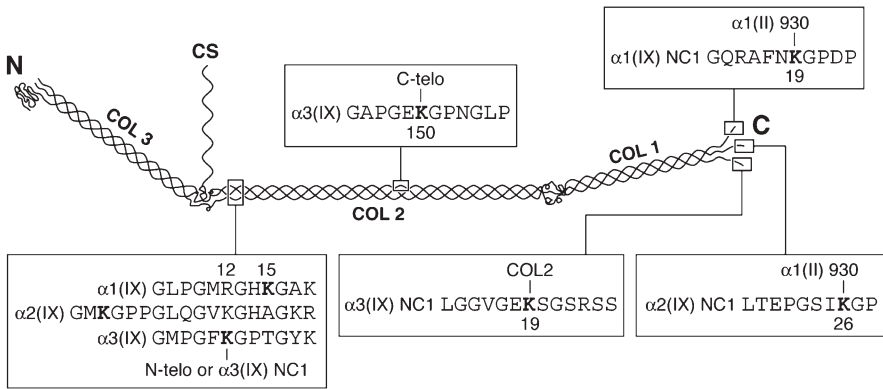


Fig. 5 Cross-linking sites in type IX collagen. Seven sites (hydroxylysine or lysine residues) in total have been identified, two each in $\alpha 1(\text{IX})$ and $\alpha 2(\text{IX})$ and three in $\alpha 3(\text{IX})$

Clearly it will be important to define the molecular interactions that regulate this complex cross-linking cascade, which also includes the participation of collagen type XI, acting as a template filament within the composite fibril. Are monomers of collagen IX transported extracellularly before they interact with nascent (thin) collagen II/XI co-assemblies, or is a discrete element of the collagen II/IX/XI heteromer pre-fabricated inside the cell in a secretory compartment, which can polymerize extracellularly with the addition of type II collagen monomers? It is known that collagens IX and XI are most concentrated (with their highest ratios to collagen II) in developing cartilage and in the immediate pericellular zone of chondrocytes [42–44]. As cartilage matures, the ratios of collagen II to collagens XI and IX rise to about 96:3:1 from 80:10:10 in fetal cartilage [39].

What is the function of collagen IX? Why are covalent bonds needed to anchor it to the surface of collagen II fibrils and to link adjacent collagen IX molecules? A mechanical role in strengthening the fibrillar matrix is reasonable to suspect. The interaction scheme shown in Fig. 6 suggests how interfibrillar cross-links might also form and so add to network stability, but the existence of such bonding will be hard to rule in or out. It does appear that the presence of collagen IX is not crucial for skeletal growth, since mice engineered with homozygous null genes for COL9A1, in which type IX collagen is functionally knocked out [45], appear normal at birth [45, 46]. They do, however, develop osteoarthritis with progressive destruction of joint cartilages [46], as do mice and humans expressing mutated forms of collagen IX genes [47, 48]. Collagen IX therefore may be required for mechanically durable mature cartilages. Perhaps the three-dimensional meshwork of developing fibrils is locked as a template outside the cell through the covalent interactions of collagen IX. Without a covalently-stabilized template, the mature collagen network may be more susceptible to proteolysis, detrimental effects of mechanical loads and less able to endure repetitive injury and repair cycles.

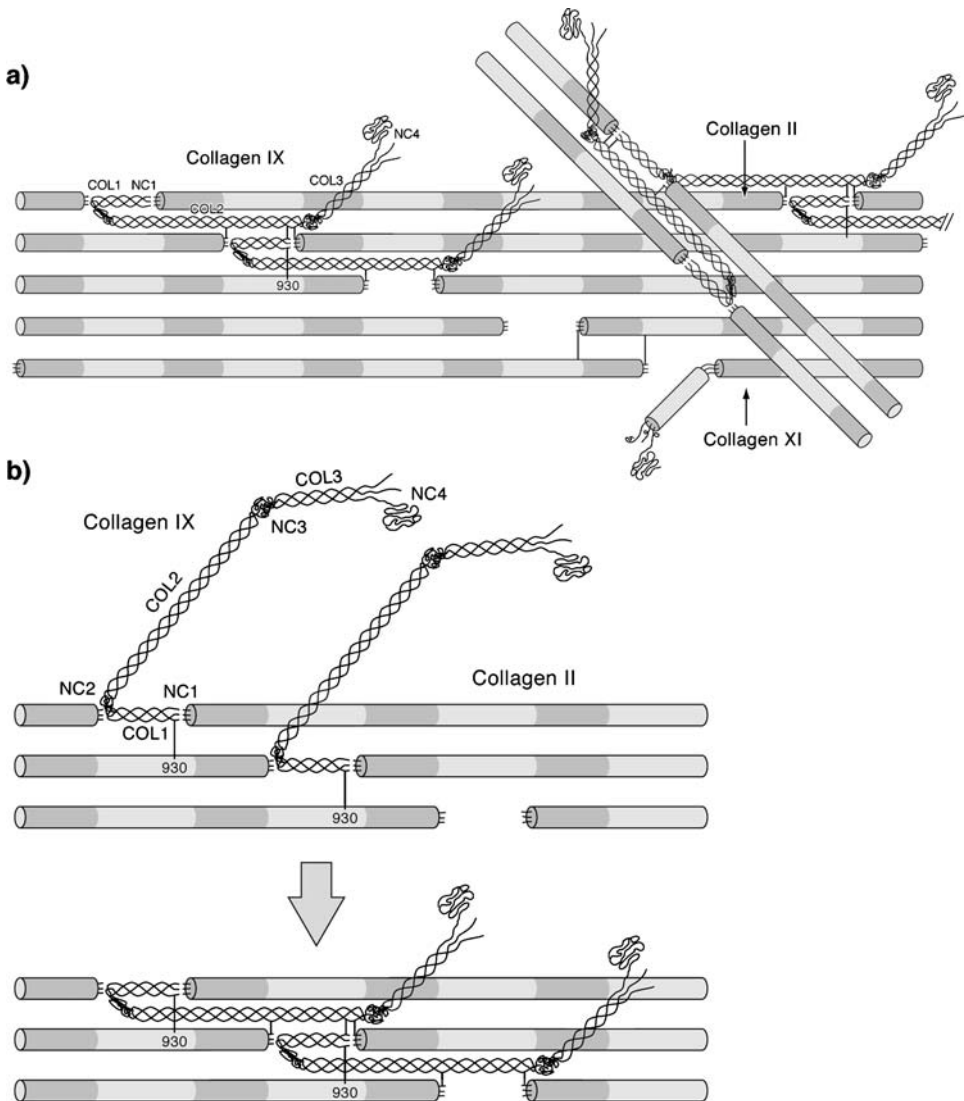


Fig. 6 A proposed model of interaction of type IX collagen with type II collagen that can accommodate all the cross-linking sites shown in Fig. 5. Folding back of the type IX COL1 domain on COL2 and an antiparallel alignment of the molecular on the surface of the type II collagen polymer can accommodate all the cross-linking interactions in the type IX collagen molecule. Potential inter-fibrillar bonds are also illustrated. The type II collagen fibril surface is shown acting as a template to bind and position adjacent type IX collagen molecules to cross-link to each other

The complex structure of the cartilage heterofibril network presents a challenge for understanding how the various enzyme-controlled steps in fibril growth are orchestrated. Lysyl oxidase must continue to create aldehydes on multiple sites on the interacting structural molecules without interfering spatially with the addition of new subunits or the propeptidases that remove the globular propeptides from II and XI procollagen molecules. The procession of protein interactions driving these and other activities on the fibril surface will be important to understand.

Inspection of the gene sequences for the other FACIT family members reveals no obvious homologies to suggest candidate cross-linking lysines in domains comparable to those in type IX collagen. One possible exception is COL21A1, which has a candidate lysine in its C-terminal NC1 domain, which also is short as in the three type IX collagen chains [49]. Little is yet known about the tissue distribution and properties of collagen XXI, for example whether it binds to collagen fibrils and so could act in a similar manner to collagen IX. It does not appear to be present in cartilage.

4 Basement Membrane Type IV Collagen

Basement membrane collagens are ancient (>500 million years [50, 51]), having evolved in primitive metazoa as early or earlier than the fibril-forming collagens. Hydra, a simple organism formed from two cell layers that secrete and sandwich the mesoglea, an extracellular layer, has been shown to express genes for a basement membrane collagen that is homologous in sequence to vertebrate type IV collagen and for a fibril-forming collagen [52, 53].

The open molecular networks that collagen type IV molecules form [54, 55] provide the framework for an assortment of proteins and proteoglycans that characterize the basement laminae that underlie most endothelial and epithelial cell layers. Early work indicated that vertebrate type IV collagen molecules are cross-linked covalently by disulfide bonds and bonds derived through the aldehyde-initiated lysyl oxidase mechanism [56]. Lysyl oxidase-mediated cross-links and disulfides were found in the 7S domain, a cross-linked tetramer of N-terminal globular domains, and also in preparations of the dimeric C-terminal NC1 domains. Recent work has concluded that the apparent non-reducible cross-link (suggesting a lysyl oxidase product) in the NC-1 dimer, was in fact a stable disulfide bond that required a high concentration of mercaptoethanol to break [57]. On the other hand, a high-resolution X-ray crystallographic study has indicated a strong spatial association between a methionine and a lysine side-chain in this domain [58], suggesting a novel covalent intermolecular bond although the chemical nature of the link was not determined. Whether lysyl oxidase-mediated cross-links ever occur in type IV collagen still seems to be an open question. Both divalent cross-links (hydroxylysino-5-*keto*-norleucine; [56]) and evidence for pyrroles [59] have earlier been noted,

but the low yield of divalent cross-links implied that the main cross-links remained unidentified.

An alternative explanation is that the low levels of known collagen cross-links had come from other collagen contaminants rather than from type IV collagen itself, and that in fact type IV collagen networks are not cross-linked by the lysyl oxidase mechanism. To resolve this question, more definitive data on cross-linked purified peptides are needed. On current evidence it seems that cystine disulfides and strong hydrophobic interactions may explain the cross-linking properties of type IV collagen [55]. The collagen type IV gene product of *Hydra vulgaris* lacks a 7S domain in its sequence by comparison with the six vertebrate collagen IV genes, and the stable non-reducible cross-links recently noted in its NC1 domain [52] could be an unusually stable disulfide, as recently concluded for this domain from vertebrate type IV collagen [57]. The highly conserved cysteine distribution patterns in the type IV collagens of Hydra and vertebrates support this possibility [52].

5

Lysyl Hydroxylases Regulate Tissue-Dependent Patterns of Cross-Linking

From the structures of the lysyl oxidase-mediated cross-links and tissue-specific differences, it has long been suspected that one or more telopeptide lysyl hydroxylases must exist to regulate the different cross-linking pathways. Only recently has direct evidence for such a gene product been found. Bank and colleagues discovered that the defect in Brück Syndrome, a heritable disorder of bone resembling osteogenesis imperfecta, eliminated pyridinolines and other hydroxylysine aldehyde-based cross-links from bone collagen [60], implying a defect in the putative telopeptide hydroxylase. In the first family studied, it turned out that the chromosomal locus linked to disease expression was not the hydroxylase, and the effect was probably through an associated gene product. This was revealed later in a study of two other families expressing the Brück Syndrome phenotype and the same abnormal pattern of bone collagen cross-linking, in which disease-causing mutations were identified in PLOD2 (procollagen-lysine 2-oxoglutarate 5-dioxygenase, also known as lysyl hydroxylase 2 or LH2) [61], one of the three human genes that encode lysyl hydroxylase isoforms [62–65]. PLOD2 can be expressed in two alternative splicing variants, LH2a and LH2b, where LH2b contains the product of an extra exon [66]. Studies on skin fibroblasts from patients with systemic sclerosis, which features skin progressively fibrotic in which the collagen had higher than normal levels of hydroxylysine-aldehyde cross-links, showed overexpression of LH2b, which is concluded to be the telopeptide hydroxylase [61].

The molecular basis of another genetic disease, Ehlers-Danlos Syndrome type VI (EDS-VIA) caused by mutations in PLOD1 (LH1) [67–69], have helped in understanding how the chemical quality of collagen cross-linking is con-

trolled *in vivo*. In EDS-VIA, active PLOD1 expression is eliminated and in bone collagen, HP (hydroxylysyl pyridinoline), is replaced by LP (lysyl pyridinoline), which means that the specific triple-helical cross-linking lysines that donate the ring-nitrogen side-chain of pyridinolines are underhydroxylated [70, 71]. In addition, skin type I collagen of EDS-VI patients essentially lacks any hydroxylysine residues, whereas bone type I collagen has about 50% of normal hydroxylysine. EDS-VIA cartilage type II collagen has 90% of the hydroxylysine content of normal cartilage but its HP/LP ratio is abnormally low [71]. Together these findings reveal tissue-specific and molecular site-specific differences in the relative contributions of LH1, LH2 and LH3 to triple-helical domain lysine hydroxylation. Also, the product of PLOD1 (LH1) is a helical lysyl hydroxylase that favors lysine residues as substrates at the two triple-helical sites of cross-linking in fibrillar collagens. In considering how these hydroxylases may regulate cross-linking, another property that may be important is the apparently additional activities of LH3 as both a galactosyl transferase and glucosyl transferase for collagen [72–74]. Since glycosylated hydroxylysines at cross-linking sites can participate in cross-linking, this may turn out to be another regulatory step. Notably in EDS VIA in skin collagen lacking any hydroxylysine, the cross-link later identified as HHL (Fig. 2) was missing (Eyre, unpublished). This suggests that lysine cannot substitute for glycosylated hydroxylysine (which is the helix donor residue) and form the lysine homologue (see Fig. 2).

In bone collagen the lysyl hydroxylase-mediated control mechanisms for cross-linking must be especially fine-tuned. Each triple-helical domain lysine and telopeptide lysine that goes on to form cross-links is partially hydroxylated in a site-specific manner. This produces the characteristic ratio of HP/LP and of pyridinolines to pyrroles that typify bone collagen. Presumably, expression levels of lysyl hydroxylase 1, 2 and 3 by osteoblasts and the local sequence context of the individual chains in which the cross-linking lysines occur, dictate the pattern. Analysis of the eventual cross-link composition of peptides from each locus can be used to estimate the original degrees of lysine hydroxylation [20]. Figure 7

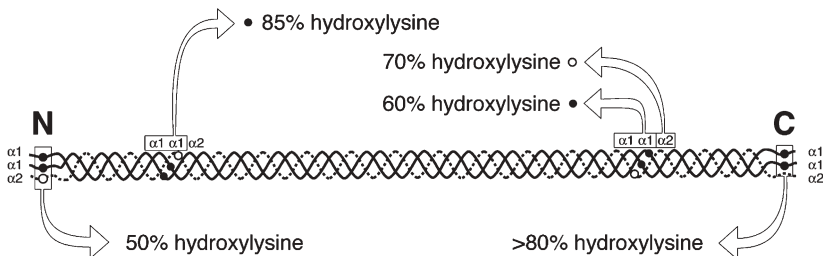


Fig. 7 A pattern of partial hydroxylation of cross-linking lysine residues in bone collagen regulates this tissue's distinctive cross-linking chemistry. The cross-link properties and yields of cross-linked peptides from the four primary loci are the source for the degrees of hydroxylation indicated [20]

summarizes the information from such analyses of normal human bone collagen. How this affects or relates to the lateral organization of molecules in bone collagen fibrils and the process of mineral crystallite deposition is still not clear, though suspected to be fundamental to bone properties [75]. In a study of LH1, LH2 and LH3 in the rat, most tissues expressed mRNAs for all three enzymes, implying a lack of tissue specificity in lysyl hydroxylase function [76]. However, collagen site-specific hydroxylation is probably regulated at more than one level of gene and protein expression.

6 Bone and Mineralized Tissue Collagens

A surge of interest in the last decade in collagen cross-links as clinical biomarkers of bone turnover has drawn attention to the cross-linking properties of bone collagen. It has long been noted that the cross-linking of collagen in bone, and other tissues that mineralize (dentin, calcified tendons) is distinctive, and probably functionally related to the intimate relationship between mineral crystallites and the packing arrangement of collagen molecules in fibrils [77, 78]. The mechanism, using both lysine aldehydes and hydroxylysine aldehydes, is distinctive and produces roughly equal amounts of pyridinolines and pyrroles as the mature cross-linking residues (see Fig. 1). Each type of cross-link is distributed site-specifically. Pyrroles, for example, are concentrated at the N-telopeptide-to- α 2(I) chain C-helix [20]. Lysyl pyridinoline is also more abundant in general at the N-telopeptide-to-C-helix locus.

The structural quality of the trabecular architecture of human cancellous bone was found to be linked to the ratio of pyrrole to pyridinoline cross-links [79]. Also, a change in the cross-linking pattern along turkey tendons just before they mineralize suggests a causative relationship between cross-linking quality and mineralization [80]. In a fracture repair model, the ratio of HP/LP and hydroxylysine content of the callus collagen was strongly related to the degree of fibril mineralization [81]. Methods for detecting cross-linking residues spectroscopically in sections of bone tissue using FTIR show promise in exploring this further [82–84]. Pyrrole cross-links, which are not stable to acid hydrolysis, are difficult to quantify and characterize. A method that uses biotinylated Ehrlich's reagent to derivatize the residues prior to isolation has been reported [85]. Lysyl pyridinoline, a recognized biomarker of bone collagen, has now been synthesized as a standard and reagent source for developing biomarker immunoassays [86].

A further form of pyrrole cross-link, a trivalent pyrrolenone, has been tentatively identified in an acid hydrolysate of dentin collagen (Fig. 8) [87], in addition to the pyridinolines, pyrroles and ketoamines already described.

In another study using an antibody that recognizes the C-telopeptide of the α 1(I) chain, both cross-linked and uncross-linked forms of this domain were isolated from trypsin-digested human bone collagen [88]. Of the trivalent

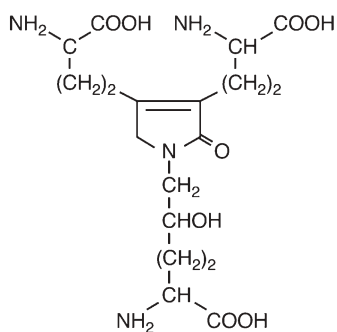


Fig. 8 A novel pyrroleninone cross-link isolated from bovine dentin in addition to pyridinolines, pyrroles and divalent keto-amines

cross-linked structures, a significant fraction could not be explained by pyrroles or pyridinolines, implying an unidentified cross-linking residue.

Higher levels of pyridinoline cross-links and hydroxylysine were found in bone from patients with osteogenesis imperfecta (clinical types I, II and III) compared with normal bone [89]. Although this suggests no gross disturbance in molecular packing of collagen molecules in fibrils, the potential that collagen/mineral inter-relationships are subtly disturbed deserves more attention as a source of the bone fragility.

7

Collagen Cross-Links as Biomarkers

Results from searching the literature on collagen cross-linking are heavily weighted over the last decade with reports from clinical studies that measured pyridinolines or cross-linked telopeptides in body fluids as molecular markers of bone turnover (reviewed in [90–94]). These cross-linking amino acids, and peptides containing them, are found in blood as products of collagen proteolysis which survive into urine. Pyridinolines are quantitatively excreted in the form of the free amino acids and small peptides, there being no degradation pathway in the liver. Initial reports showed that the total pool of pyridinolines (HP plus LP) in urine, quantified after acid hydrolysis by HPLC, can provide a more specific index of systemic bone resorption than hydroxyproline, a long-used marker [95–99]. Even more specific immunoassays were then introduced that targeted short telopeptide fragments attached to the cross-linking residues using specific antibodies [100–102]. It was discovered that peptide degradation products of the two cross-linked domains of bone collagen (N-telopeptide-to-helix or NTx, and C-telopeptide-to-helix or CTx) surviving into blood and urine, fell into discrete chromatographic pools of low molecular weight (<2 kDa) [100–103]. Antibodies could be tailored that recognized the core peptide components as neoepitopes. Immunoassays were developed that are specific to type I

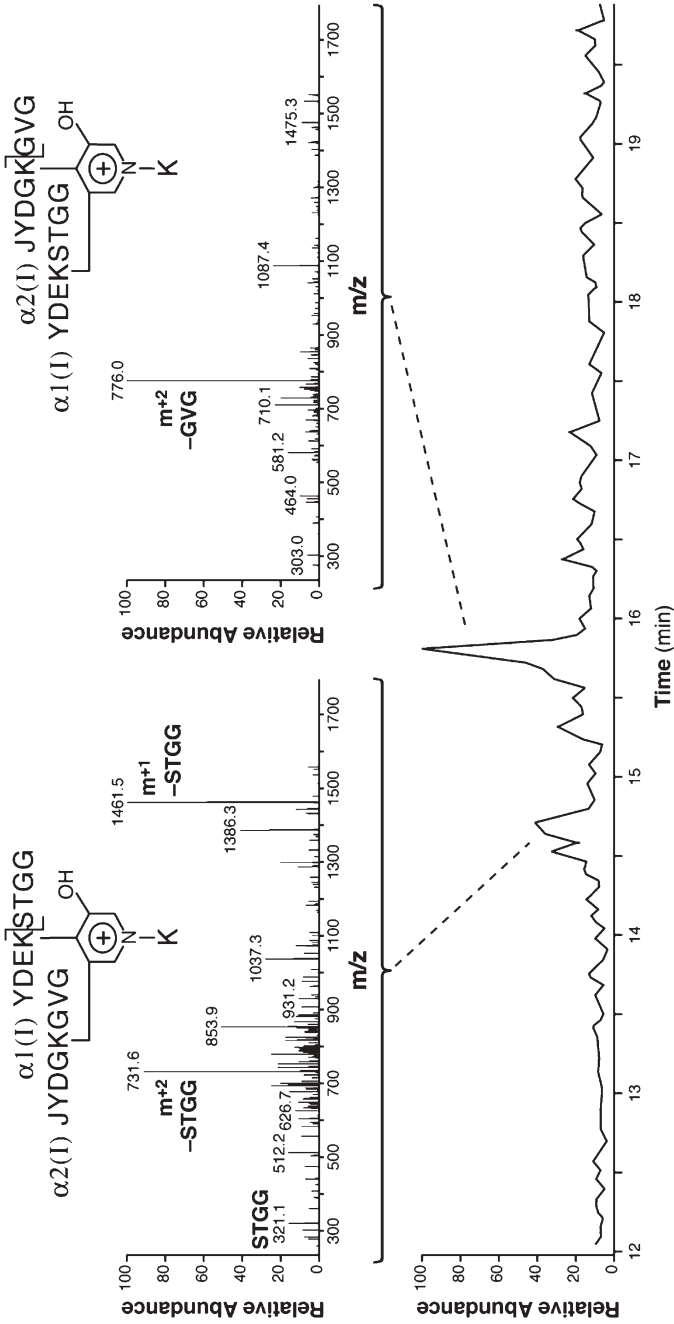


Fig. 9 Stereoisomers of a cross-linked type I collagen peptide (NTx) from human urine resolved by LCMS (liquid chromatography/mass spectrometry). The peptides differ in having the pyridinium ring positions of their telopeptide donor arms reversed. The ring nitrogen donor peptide sequence has lost all attached amino acids (presumably through proteolysis in the kidney before excretion)

collagen (peptide sequence specificity) and to cleavage products peculiar to the cathepsin K-initiated pathway of proteolysis which osteoclasts use to degrade bone collagen [104, 105].

Figure 9 shows an NTx peptide structure recovered from urine. Using in-line microbore reverse-phase HPLC and electrospray mass spectrometry, two stereoisomers of the peptide can be resolved, which differ in MS/MS fragmentation pattern. This can be explained by an interchanged placing of their N-telopeptide side-arms ($\alpha 1(I)$ and $\alpha 2(I)$) on the 3-hydroxy-pyridinium ring of the trivalent cross-linking residue as shown. Mass spectrometry reveals a favored primary fragmentation product on MS/MS that we presume depends on the pyridinium ring positions of the peptide arms. This information can help in defining the origin and preferred path of interactions of the three precursor collagen sequences giving rise to each trivalent structure. Liquid chromatography/mass spectrometry also resolved the HP and LP forms of the cross-links (Fig. 9 shows the spectra for the LP stereoisomers).

Although bone collagen is the principal source of pyridinoline cross-links in urine, other tissue collagens also contribute. For example, specific fragments from cartilage type II collagen have been identified [106] and targeted for immunoassay as a biomarker of cartilage breakdown [107]. The main source in urine of these discrete peptides from type II collagen we believe is from osteoclastic breakdown of mineralized cartilage by osteoclasts. Levels are extremely high in growing children with open growth plates, supporting this conclusion [108]. Patients with osteoarthritis show on average higher levels than control subjects [109] and, in a study of high-performance athletes, runners showed higher levels than swimmers or rowers [110]. Accelerated joint remodeling is the likely explanation for the raised levels in adults.

8 Other Mechanisms of Collagen Cross-Linking

8.1 Cystine Disulfides

For most non-fibrillar collagens of the extracellular matrix (e.g., type IV collagen, see earlier) cystine cross-links may be the only source of covalent intra- and inter-molecular bonds. Type VI collagen forms a characteristic banded filamentous network in which the chains are cross-linked as molecules, dimers and tetramers by disulfides. No lysine-mediated or other cross-links are present [111].

8.2 Gamma-Glutamyl Lysine Cross-Links

In addition to lysyl oxidase-mediated cross-links, there is indirect evidence that transglutaminase-mediated cross-links might also be involved in the process

of polymerization of collagen networks [112–114]. Candidate glutamine substrate sites have been identified for transglutaminase, but no partnering lysine residues for natural cross-link formation have been defined.

Tissue transglutaminases, related to Factor XIII in the blood-clotting cascade, are a family of Ca^{++} -dependent enzymes thought to be active in the covalent cross-linking of certain extracellular matrix proteins. They catalyze the linkage of specific glutamine and lysine side-chains by a transamidation reaction to form epsilon (gamma-glutamyl) lysine cross-links [112–114]. A role in bone matrix is suspected [115]. Methods of identification of transglutaminase-mediated cross-links rely on the use of tritiated putrescine or cadaverine to label candidate glutamyl cross-linking sites. No new technique has been developed in the last decade. Several [^3H]putrescine-binding sites have been identified in collagens III, V, XI and XVI. The potential glutamine sites are present in the aminopropeptide of type III collagen, the non-triple-helical telopeptides of $\alpha 1(\text{V})$ and $\alpha 1(\text{XI})$ chains, and the N-terminal noncollagenous domain (NC11) of $\alpha 1(\text{XVI})$ chain [116–118]. However, none of the other cross-linking partners, the lysine sites, have been identified or suggested.

8.3

Tyrosine-Derived Cross-Links

The cuticles of *C. elegans* and other nematodes consist of short-helix collagen polymers cross-linked by dityrosines and trityrosines [119, 120]. The lysyl oxidase mechanism does not operate. Instead a peroxidase is responsible. The enzyme catalyzing worm cuticle collagen cross-linking is a membrane-bound dual oxidase/peroxidase referred to as Duox [121]. A homologue to this enzyme is expressed in human tissues, but whether it has a similar function in generating extracellular di- and tri-tyrosine cross-links is unknown. Low levels of such cross-links can be found in vertebrate tissues but whether they are formed specifically or as an oxidative byproduct (e.g., in inflammation) is still unclear.

8.4

Types VIII and X Collagens

These homologous short chain molecules form hexagonal lattices. There is evidence that collagen type X, restricted to the hypertrophic and mineralized zones of growth plate cartilages, is cross-linked by the lysyl oxidase mechanism [122, 123], in addition to interchain disulfide bonding. However, site-specific cross-linked peptides need to be isolated to establish a role for lysyl oxidase-mediated cross-links. It is clear, nevertheless, that unusually strong hydrophobic interactions occur between the C-terminal globular domains in the hexagonal networks that these collagens form.

9 Outlook

The functional significance of the differences in cross-linking chemistry between tissue types is not clear. Imposed packing constraints on collagen molecules through the placement of cross-links may be more significant than the chemistry of the links themselves. On the other hand a reversible cross-linking chemistry may offer benefits, for example, in facilitating the mineralization of collagen fibrils in bone by allowing mineral crystallites to interdigitate and push apart the filamentous elements making up a fibril. Pyrrole cross-links are also thought to confer special qualities to bone collagen fibrils, perhaps related to the unique capacity of bone collagen to mineralize. The function of the pyrrole cross-links and significance of the link observed between the microscopic character of bone trabeculae and the ratio of pyridinoline to pyrrole cross-links need further study.

The functional significance in general of the trivalent cross-links, which can link three adjacent-collagen molecules, two in register and a third staggered by a 4D-overlap (where $D=1/4.4$ of the molecular length), is not clear. These permanent, end-products of hydroxylysine-aldehyde cross-linking are associated with tough connective tissues that bear high loads and are subject to minimal turnover. Whether they add mechanical strength of a particular quality, for example preventing lateral slippage between sub-elements of fibrils or offer a mechanism for interfibrillar bonding, is not known.

Perhaps the most important questions driving current research are aimed at understanding the cellular mechanisms that control tissue-specific differences in collagen cross-linking chemistry. Pivotal enzymes clearly include the three known lysyl hydroxylases, one of which (PLOD2) is believed to act on telopeptide lysines and so adapt the nascent collagen to cross-link via the hydroxylysine aldehyde pathway. This pathway is associated with normally tough connective tissues and with pathological fibrotic conditions. Molecular mechanisms that control the expression and activities of these regulatory gene products in different cell types will be important to define.

References

1. Exposito JY, Cluzel C, Garrone R, Lethias C (2002) *Anat Rec* 268:302
2. Boot-Handford RP, Tuckwell DS (2003) *Bioessays* 25:142
3. Bailey AJ, Paul RG, Knott L (1998) *Mech Ageing Dev* 106:1
4. Eyre DR, Paz MA, Gallop PM (1984) *Annu Rev Biochem* 53:717
5. Robins SP (1999) Fibrillogenesis and maturation of collagens. In: Seibel MJ, Robins SP, Bilezikian JP (eds) *Dynamics of bone and cartilage metabolism*. Academic Press, London, p 31
6. Eyre DR, Glimcher MJ (1971) *Biochim Biophys Acta* 243:525
7. Van Ness KP, Koob TJ, Eyre DR (1988) *Comp Biochem Physiol B* 91:531
8. Grandhee SK, Monnier VM (1991) *Biol Chem* 266:11649

9. Bailey AJ, Sims TJ, Avery NC, Miles CA (1993) *Biochem J* 296:489
10. Fu MX, Wells-Knecht KJ, Blackledge JA, Lyons TJ, Thorpe SR, Baynes JW (1994) *Diabetes* 43:676
11. Bailey AJ, Sims TJ, Avery NC, Halligan EP (1995) *Biochem J* 305:385
12. Sady C, Khosrof S, Nagaraj R (1995) *Biochem Biophys Res Commun* 214:793
13. Paul RG, Bailey AJ (1996) *Int J Biochem Cell Biol* 28:1297
14. Slatter DA, Paul RG, Murray M, Bailey AJ (1999) *J Biol Chem* 274:19,661
15. Slatter DA, Bolton CH, Bailey AJ (2000) *Diabetologia* 43:550
16. Piez KA (1968) *Annu Rev Biochem* 37:547
17. Tanzer ML (1973) *Science* 180:561
18. Tanzer ML, Waite JH (1982) *Coll Relat Res* 2:177
19. Bornstein P (2003) *Matrix Biol* 22:385
20. Hanson DA, Eyre DR (1996) *J Biol Chem* 271:26508
21. Wu JJ, Eyre DR (1984) *Biochem* 23:1850
22. Eyre DR, Apone S, Su JJ, Ericsson LH, Walsh KA (1987) *FEBS Lett* 220:337
23. Kuboki Y, Okuguchi M, Takita H, Kimura M, Tsuzaki M, Takakura A, Tsunazawa S, Sakiyama F, Hirano H (1993) *Connect Tissue Res* 29:99
24. Yamauchi M, London RE, Guenat C, Hashimoto F, Mechanic GL (1987) *J Biol Chem* 262:11,428
25. Yamauchi M, Chandler GS, Tanzawa H, Katz EP (1996) *Biochem Biophys Res Commun* 219:311
26. Mechanic GL, Katz EP, Henmi M, Noyes C, Yamauchi M (1987) *Biochemistry* 26:3500
27. Henkel W, Rauterberg J, Stirtz T (1976) *Eur J Biochem* 69:223
28. Henkel W, Glanville R (1982) *Eur J Biochem* 122:205
29. Henkel W (1996) *Biochem J* 318:497
30. Wu JJ, Knigge PE, Eyre DR (1994) *Trans Ortho Res Soc* 19:131
31. Eyre DR, Wu JJ (1987) Type XI or $1\alpha 2\alpha 3\alpha$ collagen. In: Mayne R, Burgeson RE (eds) *Structure and function of collagen*. Academic Press, Orlando, p 261
32. Niyibizi C, Eyre DR (1989) *FEBS Lett* 242:314
33. Mayne R, Brewton RG, Mayne PM, Baker JR (1993) *J Biol Chem* 268:9381
34. Wu JJ, Eyre DR (1995) *J Biol Chem* 270:18865
35. Niyibizi C, Eyre DR (1994) *Eur J Biochem* 224:943
36. Shaw LM, Olsen BR (1991) *Trends Biochem Sci* 16:191
37. Wu JJ, Woods PE, Eyre DR (1992) *J Biol Chem* 267:23007
38. Diab M, Wu JJ, Eyre DR (1996) *Biochem J* 314:327
39. Eyre DR, Wu JJ, Fernandes RJ, Pietka TA, Weis MA (2002) *Biochem Soc Trans* 30:844
40. Fernandes RJ, Schmid TM, Eyre DR (2003) *Eur J Biochem* 270:3243
41. Eyre DR, Pietka T, Weis MA, Wu JJ (2004) *J Biol Chem* 279:2568
42. Poole CA, Flint MH, Beaumont BW (1987) *J Orthop Res* 5:509
43. Mendler M, Eich-Bender SG, Vaughan L, Winterhalter KH, Bruckner P (1989) *J Cell Biol* 108:191
44. Vaughan-Thomas A, Young RD, Phillips AC, Duance VC (2001) *J Biol Chem* 276:5303
45. Hagg R, Hedbom E, Mollers U, Aszodi A, Fassler R, Bruckner P (1997) *J Biol Chem* 272:20,650
46. Fassler R, Schnegelsberg PN, Dausman J, Shinya T, Muragaki Y, McCarthy MT, Olsen BR, Jaenisch R (1994) *Proc Natl Acad Sci USA* 91:5070
47. Nakata K, Ono K, Miyazaki J, Olsen BR, Muragaki Y, Adachi E, Yamamura K, Kimura T (1993) *Proc Natl Acad Sci USA* 90:2870

48. Chapman KL, Briggs MD, Mortier GR (2003) *Pediatr Pathol Mol Med* 22:53
49. Fitzgerald J, Bateman JF (2001) *FEBS Lett* 505:275
50. Boute N, Exposito JY, Boury-Esnault N, Vacelet J, Noro N, Miyazaki K, Yoshizato K, Garrone R (1996) *Biol Cell* 88:37
51. Netzer KO, Suzuki K, Itoh Y, Hudson BG, Khalifah RG (1998) *Protein Sci* 7:1340
52. Fowler SJ, Jose S, Zhang X, Deutzmann R, Sarras MP Jr, Boot-Handford RP (2000) *J Biol Chem* 275:39,589
53. Ozbek S, Pertz O, Schwager M, Lustig A, Holstein T, Engel J (2002) *J Biol Chem* 277:49,200
54. Timpl R, Wiedemann H, van Delden V, Furthmayr H, Kuhn K (1981) *Eur J Biochem* 120:203
55. Hudson BG, Tryggvason K, Sundaramoorthy M, Neilson EG (2003) *N Engl J Med* 348:2543
56. Bailey AJ, Sims TJ, Light N (1984) *Biochem J* 218:713
57. Reddy GK, Hudson BG, Bailey AJ, Noelken ME (1993). *Biochem Biophys Res Commun* 190:277
58. Than ME, Henrich S, Huber R, Ries A, Mann K, Kuhn K, Timpl R, Bourenkov GP, Bartunik HD, Bode W (2002) *Proc Natl Acad Sci USA* 99:6607
59. Scott JE, Qian R, Henkel W, Glanville RW (1983) *Biochem J* 209:263
60. Bank RA, Robins SP, Wijmenga C, Breslau-Siderius LJ, Bardoel AF, van der Sluijs HA, Pruijs HE, te Koppele JM (1999) *Proc Natl Acad Sci USA* 96:1054
61. van der Slot AJ, Zuurmond AM, Bardoel AF, Wijmenga C, Pruijs HE, Sillence DO, Brinckmann J, Abraham DJ, Black CM, Verzijl N, DeGroot J, Hanemaaijer R, te Koppele JM, Huizinga TW, Bank RA (2003) *J Biol Chem* 278:40,967
62. Hautala T, Byers MG, Eddy RL, Shows TB, Kivirikko KI, Myllyla R (1992) *Genomics* 13:62
63. Szpirer C, Szpirer J, Riviere M, Vanvooren P, Valtavaara M, Myllyla R (1997) 8:707
64. Passoja K, Rautavuoma K, Ala-Kokko L, Kosonen T, Kivirikko KI (1998) *Proc Natl Acad Sci USA* 95:10,482
65. Ruotsalainen H, Sipila L, Kerkela E, Pospiech H, Myllyla R (1999) *Matrix Biol* 18:325
66. Yeowell HN, Walker L (1999) *Matrix Biol* 18:179
67. Hyland J, Ala-Kokko L, Royce P, Steinmann B, Kivirikko KI, Myllyla R (1992) *Nat Genet* 2:228
68. Yeowell HN, Walker LC (1997) *Proc Assoc Am Phys* 109:383
69. Yeowell HN, Walker LC (2000) *Mol Genet Metab* 71:212
70. Steinmann B, Eyre DR, Shao P (1995) *Am J Hum Genet* 57:1505
71. Eyre D, Shao P, Weis MA, Steinmann B (2002) *Mol Genet Metab* 76:211
72. Heikkinen J, Risteli M, Wang C, Latvala J, Rossi M, Valtavaara M, Myllyla R (2000) *J Biol Chem* 275:36,158
73. Wang C, Luosujarvi H, Heikkinen J, Risteli M, Uitto L, Myllyla R (2002) *Matrix Biol* 21:559
74. Wang C, Risteli M, Heikkinen J, Hussa AK, Uitto L, Myllyla R (2002) *J Biol Chem* 277:18,568
75. Uzawa K, Grzesik WJ, Nishiura T, Kuznetsov SA, Robey PG, Brenner DA, Yamauchi M (1999) *J Bone Miner Res* 14:1272
76. Mercer DK, Nicol PE, Kimbembe C, Robins SP (2003) *Biochem Biophys Res Commun* 307:803
77. Knott L, Bailey AJ (1998) *Bone* 22:181
78. Yamauchi M, Katz EP (1993) *Connect Tissue Res* 29:81
79. Banse X, Devogelaer JP, Lafosse A, Sims TJ, Grynpas M, Bailey AJ (2002) *Bone* 31:70
80. Knott L, Tarlton JF, Bailey AJ (1997) *Biochem J* 322:535

81. Wassen MH, Lammens J, te Koppele JM, Sakkers RJ, Liu Z, Verbout AJ, Bank RA (2000) *J Bone Miner Res* 15:1776
82. Paschalis EP, Verdelis K, Doty SB, Boskey AL, Mendelsohn R, Yamauchi M (2001) *J Bone Miner Res* 16:1821
83. Paschalis EP, Recker R, DiCarlo E, Doty SB, Atti E, Boskey AL (2003) *J Bone Miner Res* 18:1942
84. Blank RD, Baldini TH, Kaufman M, Bailey S, Gupta R, Yershov Y, Boskey AL, Copper-smith SN, Demant P, Paschalis EP (2003) *Connect Tissue Res* 44:134
85. Brady JD, Robins SP (2001) *J Biol Chem* 276:18812
86. Adamczyk M, Johnson DD, Reddy RE (2000) *Bioconj Chem* 11:124
87. Kletter GA, Damen JJ, Kettenes-van den Bosch JJ, Bank RA, te Koppele JM, Veraart JR, ten Cate JM (1998) *Biochim Biophys Acta* 1381:179
88. Eriksen HA, Sharp CA, Robins SP, Sassi ML, Risteli L, Risteli J (2004) *Bone* 34:720
89. Bank RA, te Koppele JM, Janus GJ, Wassen MH, Pruijs HE, Van der Sluijs HA, Sakkers RJ (2000) *J Bone Miner Res* 2000 15:1330
90. Delmas PD (1993) *J Bone Miner Res* 8:S549
91. Eyre DR (1995) *Acta Orthop Scand Suppl* 266:266
92. Christenson RH (1997) *Clin Biochem* 30:573
93. Garnero P, Delmas PD (1998) *Endocrinol Metab Clin North Am* 27:303
94. Watts NB (1999) *Clin Chem* 45:1359
95. Beardsworth LJ, Eyre DR, Dickson IR (1990) *J Bone Min Res* 5:671
96. Uebelhart D, Gineyts E, Chapuy MC, Delmas PD (1990) *Bone Miner* 8:87
97. Robins SP, Black D, Paterson CR, Reid DM, Duncan A, Seibel MJ (1991) *Eur J Clin Invest* 21:310
98. Seibel MJ, Robins SP, Bilezikian JP (1992) *Trends Endocrinol Metab* 3:263
99. Eastell R, Colwell A, Hampton L, Reeve J (1997) *J Bone Miner Res* 12:59
100. Hanson DA, Weis MA, Bollen AM, Maslan SL, Singer FR, Eyre DR (1992) *J Bone Miner Res* 7:1251
101. Risteli J, Elomaa I, Niemi S, Novamo A, Risteli L (1993) *Clin Chem* 39:635
102. Bollen AM, Eyre DR (1994) *Bone* 15:31
103. Eyre DR, Atley LM, Wu JJ (2002) Collagen cross-links as markers of bone and cartilage degradation In: Hascall VC, Kuettner KE (eds) *The many faces of osteoarthritis*. Birkhäuser Verlag, Basel, Switzerland, p 275
104. Clemens JD, Herrick MV, Singer FR, Eyre DR (1997) *Clin Chem* 43:2058
105. Atley LM, Mort JS, Lalumiere M, Eyre DR (2000) *Bone* 26:241
106. Eyre DR (1992) *US Patent* 5,140,103
107. Lohmander LS, Atley LM, Pietka TA, Eyre DR (2003) *Arthritis Rheum* 48:3130
108. Eyre DR, Atley LM, Vosberg-Smith K, Shaffer K, Ochs V, Clemens D (2000) Biochemical markers of bone and cartilage degradation. In: Goldberg M, Boskey A, Robinson C (eds) *Chemistry and biology of mineralized tissues*. AAOS, p 347
109. Atley LM, Sharma L, Clemens JD, Shaffer K, Pietka TA, Riggins JA, Eyre DR (2000) *Trans Orthop Res Soc* 25:168
110. O'Kane JW, Atley L, Hawley C, Teitz C, Eyre DR (2001) *Orthop Res Rep*, UW, Dept of Orthopedics and Sports Medicine, 2001:3
111. Wu JJ, Eyre DR, Slayter HS (1987) *Biochem J* 248:373
112. Folk JE (1980) *Ann Rev Biochem* 49:517
113. Griffin M, Casadio R, Bergamini C (2002) *Biochem J* 368:377
114. Lorand L, Graham RM (2003) *Nat Rev Mol Cell Biol* 4:140
115. Aeschlimann D, Mosher D, Paulsson M (1996) *Semin Thromb Hemost* 22:437
116. Bowness JM, Folk JE, Timpl R (1987) *J Biol Chem* 262:1022

117. Kleman JP, Aeschlimann D, Paulsson M, van der Rest M (1995) *Biochemistry* 34:13,768
118. Akagi A, Tajima S, Ishibashi A, Matsubara Y, Takehana M, Kobayashi S, Yamaguchi N (2002) *J Invest Dermatol* 118:267
119. Fetterer RH, Rhoads ML, Urban JF Jr (1993) *J Parasitol* 79:160
120. Yang J, Kramer JM (1999) *J Biol Chem* 274:32,744
121. Edens WA, Sharling L, Cheng G, Shapira R, Kinkade JM, Lee T, Edens HA, Tang X, Sullards C, Flaherty DB, Benian GM, Lambeth JD (2001) *J Cell Biol* 154:879
122. Rucklidge GJ, Milne G, Robins SP (1996) *Matrix Biol* 15:73
123. Orth MW, Luchene LJ, Schmid TM (1996) *Biochem Biophys Res Commun* 219:301