Extracellular Modifications to Muscle Collagen: Implications for Meat Quality

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ABSTRACT The extracellular matrix (EMC) of muscle is composed mostly of the protein collagen with lesser quantities of other constituents such as proteoglycans also present. The focus of this brief review is the extracellular modification of collagen, critical to forming a stable matrix, called crosslinking. Enzyme-mediated covalent collagen crosslinks are largely lysine-derived. Their formation is absolutely essential for stabilization of the EMC and a functional muscle. In cooked meat, the presence of crosslinks contribute to the shrinkage and tension development of collagen as it denatures with a subsequent increase in the toughness of meat. Both crosslink and collagen concentrations vary with differing muscle type, producing a wide range of textural differences among muscles. Furthermore, within a given muscle type, a wide range of conditions, often dependent on management choices, influence crosslinking patterns. Although information regarding the chemical structure, specific location, and quantity of collagen crosslinks is available, mechanisms that control and regulate their formation remain elusive. Recent studies, however, suggest a potential role for the proteoglycan decorin in regulating collagen fibrillogenesis, ordering the spatial arrangement of collagen molecules and, thus, influencing crosslinking patterns.

(*Key words*: collagen, crosslinking, muscle, decorin, pyridinium)

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INTRODUCTION

Collagen, the most abundant mammalian and avian protein, is a connective tissue constituent that is present in all tissues. To date, 19 different forms of collagen have been identified constituting a family of proteins that have a wide variety of roles in biological systems. The structure of collagens also varies, from the mesh-like network of Type IV collagen to the long, filamentous fibers of Types I, II, and III collagen (Nimni and Harkness, 1988). Types I and III collagen are the primary proteins of the muscle extracellular matrix (ECM), with lesser amounts of Type V and Type IV collagens associated with the perimysium and basement membranes, respectively (Bailey and Light, 1989).

Morphologically, there are three discrete collagen depots in muscle: the epi-, peri-, and endomysiums. The connective tissue sheath surrounding individual muscles and continuous with the tendon joining other muscles or bones is the epimysium. The epimysium is often thick and tough and resistant to both shear and solubilization. However, it is easily (and usually) separated from cuts of meat and is generally not considered to be a factor in meat quality. The three-dimensional collagen network that surrounds large and small bundles of muscle fiber and contains intramuscular lipid deposits and vasculature is the perimysium. The layer of connective tissue encircling each muscle fiber and overlying the basement membrane is the endomysium. The intramuscular connective tissue (IMCT) is, thus, the combined peri- and endomysium depots. The vast bulk of the IMCT, about 90%, is perimysium. The perimysium is thought to play the major role in determining meat texture differences that are related to connective tissue (Light *et al*., 1985). The role of endomysium in meat texture is less well understood, although the importance of perimysial-endomysial interactions (Lewis and Purslow, 1990) and endomysial shrinkage and subsequent changes in muscle cell diameter with heating (Bendall and Restall, 1983) have been noted.

The fibrillar collagen Types I and III are responsible for the transmission of force from the myofibers to the tendons of skeletal muscle (Purslow and Duance, 1990). Further, the epi-, peri-, and endomysial sheaths determine the architecture of muscle and protect muscle from overstretch (Borg and Caulfield, 1980). The high tensile strength of collagen as well as its mechanical stability are conferred by the amount and type of covalent

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Abbreviation Key: BF = *Biceps femoris*; EMC = extracellular matrix; GM = *Gluteus medius*; HP = 3-hydroxy-lysalpyridinium; IMCT = intracellular connective tissue; LD = *Longissimus dorsi*; LSN = Low Score Normal; PM = *Psoas major*; SM = *Semimembranosus*.

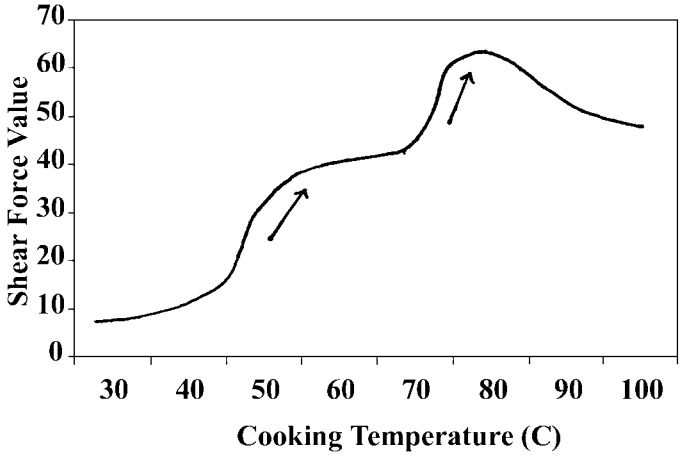

FIGURE 1. Cooking-dependent meat toughening. As temperature increases past 40 C meat exhibits increased shear force first, (42 to 52 C), as myofibrillar proteins denature and, second (64 to 70 C), as fibrillar collagen denatures and shrinks. The decrease in shear force when temperature exceeds 80 C reflects solubilization of collagen. Redrawn from Davey and Gilbert (1974).

crosslinks linking individual collagen molecules and fibrils together. The phenomenon of cooking-dependent textural changes in meat is also closely related to the crosslink characteristic of its constituent collagen. This brief review focuses on enzyme-mediated covalent crosslinking of collagen, obligatorily an extracellular event, the role collagen plays in cooked meat texture, and mechanisms by which crosslinking may be regulated.

HEAT-DEPENDENT TEXTURAL CHANGES IN MEAT

The higher quality, more tender cuts of meat cooked with dry heat for a relatively short time to an endpoint temperature are most tender in the raw state. Davey and Gilbert (1974) initially described the temperature-dependent increases in shear force (toughening) that occur with cooking and further supplied a basis for the role that collagen plays in determining cooked meat texture. Using beef *Sternomandibularis* muscle, they documented a biphasic increase in the toughening (increase shear force) of meat as temperature increased (Figure 1). The first sharp increase in shear force, occurring between 40 to 50 C, corresponds to the denaturation of the myofibrillar proteins actin and myosin. Before a temperature of 60 C is reached, both these proteins have undergone a transition from the gel state, initially posing little resistance to shear, to a hardened dehydrated form, although the water is still associated with the myofiber.

Beginning at 64 to 68 C, shear force again increases sharply, with the second increase in toughening corresponding to the thermal denaturation of collagen. Collagen is a unique molecule because, when it is assembled into a fibril, thermal denaturation results in shrinkage of the fibril accompanied by force or tension development. The degree of tension or shrinkage developed is a function of how heavily the collagen is crosslinked with mature, heat-stable crosslinks (Bailey and Light, 1989). As temperature increases to 80 to 90 C, the collagen will eventually gelatinize and shear force diminishes.

Extending and expanding these observations, Bendall and Restall (1983) demonstrated step-wise, temperature-dependent increases in tension development and shrinkage occurring in both single muscle fibers (endomysium) and in muscle fiber bundles using mechanical and histochemical techniques. The initial shrinkage, which is minimal and occurs around 58 C, probably corresponds to shrinkage of Type IV basement membrane collagen surrounding the myofibers. However, when temperature exceeds about 64 C, the critical shrink temperature of the perimysial collagen (Types I and III), tension development, shrinkage, and fluid expression rapidly increase.

An interesting corollary to the above observation is the extraordinary dependence of muscle length on temperature. As temperature is raised incrementally past the critical temperature (> 64 C), tension also increases stepwise. The tension development corresponds to the unraveling and contraction of the collagen triple helix. Remarkably, when temperature decreases to 38 C, the collagen molecules assume their native, rigid rod-like conformation and tension is relieved. In the case of beef *Sternomandibularis* muscle, tension development continues to increase up to 94 C. Significantly, when *Psoas major* (PM), is subjected to a similar heating regimen, PM can develop only about one third of the force of the *Sternomandibularis* muscle. The difference in tension development between the two muscles is a function of collagen content and how heavily crosslinked the collagen is (Bailey and Light, 1989).

COLLAGEN AND COLLAGEN CROSSLINK CHEMISTRY

The fibrillar collagen molecule consists of three polypeptide subunits, called α-chains, which associate via hydrogen bonding to form a superhelix. Both I and III phenotypes possess a large, central triple helical domain consisting of a repeating (GLY-X-Y) triplet and small, nonhelical regions at the carboxyl and amino termini called telopeptides. Interchain hydrogen bonding is enhanced by the large proportions of glycine, proline, alanine, and hydroxyproline amino acids and constitutional water present in collagen α-chains (Nimni and Harkness, 1988).

Collagen molecules undergo extensive post-translational modifications. Intracellularly, selected proline and lysine residues are enzymatically hydroxylated and some lysines are then glycosylated. Extracellularly, telopeptide regions, which promote α-chain assembly

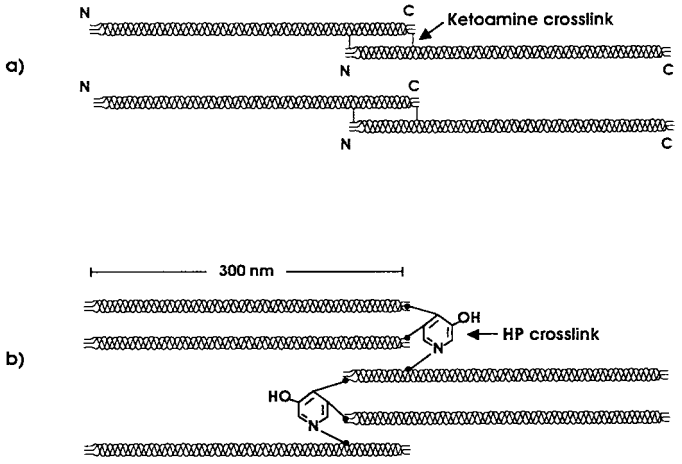

FIGURE 2. Divalent and trivalent crosslinking of fibrillar collagen. a). Two sets of collagen molecules in quarter-stagger (4-D) alignment. Divalent ketoamine crosslink links two molecules together as the head of one molecule overlaps the tail of the other. Four crosslinking sites occur per collagen molecule, two toward the N-terminus, two toward the C-terminus. b) With the condensation of two ketoamine crosslinks a trivalent pyridinium residue is formed with the potential to link three collagen molecules together, one of which may be associated with another microfibril. Redrawn from Eyre *et al.* (1984).

into the helix, are proteolytically processed. Collagen molecules assemble via hydrophobic and electrostatic interactions into a head-to-tail array, forming microfibrils. Molecules aggregate laterally into nascent fibrils with five molecules to the row (pentafibril), with each molecule overlapping the adjacent by approximately one-quarter of its length. Such an arrangement produces the quarter-staggered array (Nimni and Harkness, 1988) typical of newly formed fibrils.

Several comprehensive reviews of collagen crosslink biosynthesis have been published (Eyre *et al.*, 1984; Eyre, 1987; Bailey, 1989; Reiser *et al.*, 1992). Because the primary collagen phenotypes in perimysial collagen (the main IMCT depot) are Types I and III, we address here only the mechanisms of crosslink formation known to occur with these forms. Initial fibril orientation is unstable because collagen molecules associate only via noncovalent interactions in the immature fibril. Collagen molecules can slide past one another and the immature fiber is more subject to disruption by collagenolysis, variations in ionic strength, and temperature. Tensile strength and functionality of the collagen fibril are due primarily to the formation of intermolecular crosslinks. Crosslinking is initiated immediately upon fibril aggregation by the oxidative deamination of specific lysine or hydroxylysine residues by the enzyme lysyl oxidase. Lysine- or hydroxylysine-derived aldehydes (allysine and hydroxyallysine, respectively) result. In the fibrillar collagens (Types I and III), four crosslinking sites have been identified. Two sites occur toward the amino terminus, one in the telopeptide region, the other in the helical region. The second pair of sites occur toward the carboxyl terminus, one in the helical region, the other in the telopeptide region. The head-to-tail lateral alignment of collagen molecules in quarter-stagger array allows allysine or hydroxyallysine residues to approach and interact with other peptidyl aldehydes or unmodified lysine or hydroxylysine residues on adjacent α-chains. Because only four crosslinking sites may be present in the bonded, fibrillar collagens, the maximum number of crosslinks per molecule that can form is limited (Figure 2).

There are two major pathways by which enzyme-mediated lysine-aldehyde crosslinks form in Type I and III collagen. The allysine pathway produces aldimine crosslinks formed from lysine aldehydes; the hydroxyallysine path results in ketoamine crosslinks arising from hydroxyaldehydes. The initial condensation products form reducible crosslinks because they contain Schiff base double bonds, which can be reduced. These reducible crosslinks are divalent, capable of linking only two collagen molecules together. Both crosslinks vary in their stability, with ketoamine crosslinks being less heat labile than aldimine. Furthermore, the reducible crosslinks occur transiently and in many tissues can be considered as intermediate products.

Divalent crosslinks tend to disappear from many tissues with time and are replaced by mature, nonreducible crosslinks. The crosslinking pathway that apparently predominates in skeletal muscle as well as myocardium is hydroxyallysine based. The known mature crosslinks on the hydroxyallysine pathway are trivalent, hydroxylysylpyridinium (HP), and lysyl pyridinium residues, with the latter present in negligible amounts in most tissues except bone (Figure 3). Hydroxylysylpyridinium is almost certainly formed in a precursor-product manner from the condensation of two reducible ketoamine crosslinks, a mechanism that is

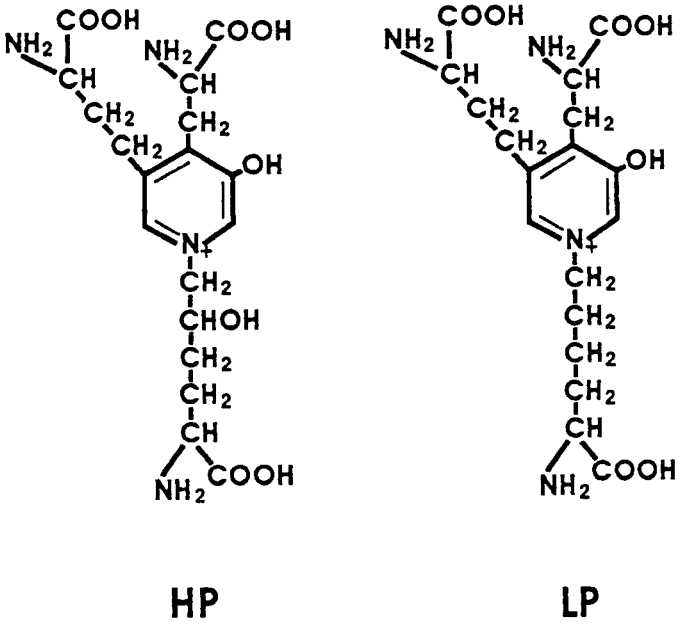

FIGURE 3. Pyridinium crosslinks. Hydroxylysylpyridinoline (HP) and its dehydroxy analog lysylpyridinoline (LP).

confirmed by the stoichiometric relationship between the disappearance of the reducible crosslink and the accumulation of the trivalent crosslink in tissues. The progression of crosslinks from divalent to trivalent forms is significant because multivalent crosslinks can link adjacent fibrils as well as individual molecules together, thus markedly increasing the strength of the IMCT matrix. Furthermore, HP crosslinks are heat stable; their introduction into muscle collagen would be expected to increase force development (shrinkage) upon denaturation and extend both time and ultimate temperature required for gelatinization of collagen to occur.

The progressive nature of crosslink biosynthesis does not mean that there is always a steady, irreversible shift of immature to mature forms. Although there is generally an increase in mature IMCT crosslinks with chronological age, it is also clear that the rate of crosslink formation and directional shifts in the concentration of mature crosslinks, regardless of age, can be altered. Furthermore, wide differences in crosslink type and concentration occur between different tissues and muscle types (McCormick, 1994).

CROSSLINKING IN SKELETAL MUSCLES

In skeletal muscle, reducible crosslinks are rapidly replaced with more mature forms. For example, by 1 yr of age in steers, the concentration of HP (expressed as moles of crosslink per mole of collagen) in IMCT is more than twofold greater than its reducible precursor, the ketoamine crosslink, dihydroxylysinonorleucine (McCormick, 1994). Thus, we generally monitor crosslinking patterns in muscle by quantifying HP concentration.

In higher quality muscles [*Longissimus dorsi* (LD)] of domestic animals (cattle, sheep, pigs) of market age, HP concentrations generally range from somewhat less than 0.20 to somewhat more than 0.35 mol HP/mol of collagen. As age increases, HP values increase, with 0.62 mol HP/mol collagen in the LD muscle of white-tailed deer greater than 5 yr old being the highest documented value for mammalian skeletal muscle (McCormick, 1994). Interestingly, the progression of crosslinking occurs significantly faster in avian skeletal muscle than in the mammals examined. For example, in fowl (Leghorn chicken) pectoral muscle, HP values are in excess of 1.0 mol HP/mol collagen by 1 yr of age, the highest value yet noted for skeletal muscle (Velleman *et al.*, 1996).

Concentration of lysine aldehyde-derived crosslinks varies with (and within) muscle type and with a host of conditions related to time, growth, and adaptation. Data on muscle HP concentrations from various species including cattle, sheep, pigs, and rats suggest a relatively wide range of values for different muscles. In general, locomotor muscles possess more crosslinking than postural muscles (Zimmerman *et al.*, 1993), although there is clearly an interaction between level of crosslinking and muscle collagen concentration. Furthermore, within a muscle type, numerous treatments that apparently result in remodeling of the muscle connective tissue matrix will also influence crosslinking patterns. Examples include compensatory growth, somatotropin-mediated growth, and exercise (McCormick, 1989; Andersen *et al.*, 1992; Zimmerman *et al.*, 1993).

TABLE 1. Selected collagen characteristics of ovine *Longissimus* muscle[1]

Characteristic	Ram	Wether
Collagen, mg/100 mg	1.85[a]	1.67[a]
Soluble collagen, g/mg	2.26[a]	1.62[b]
Type III collagen, %	24.18[a]	20.43[b]
Pyridinoline, mol/mol collagen	0.25[a]	0.18[b]
Warner-Bratzler shear force, kg	2.45[a]	1.64[b]

[a,b]Means in the same row with no common superscript differ significantly ($P < 0.05$). Adapted from Maiorano *et al.* (1993).

We recently compared collagen concentration and crosslinking in five different bovine muscles that span a wide range of texture variation (McCormick, unpublished data). For each muscle, collagen (expressed as percentage of muscle dry weight) and HP crosslink concentration were determined. In muscles that are decidedly less tender, such as *Biceps femoris* (BF) and *Semimembranosus* (SM), collagen concentrations are high, approaching 3%. Levels of HP are also high at nearly 0.5 mol HP/mol collagen. Conversely, a muscle such as LD, the tenderness of which is much more acceptable than that of either BF or SM, possesses significantly less collagen (1.86%) and less crosslinked collagen (0.36 mol HP/mol collagen). *Gluteus medius* (GM) and PM muscle present an interesting contrast. The GM possesses a relatively high concentration of collagen (2.77%) but low concentrations of the HP crosslink (0.35 mol HP/mol collagen). The PM has little collagen (1.41%) but elevated crosslink levels (0.45 mol/mol collagen). Both muscles possess excellent textural characteristics despite the high connective tissue parameter, with PM acknowledged as the most tender muscle.

We have concluded that mature crosslinks and collagen concentration have an additive effect on the toughening of meat. This observation is supported by a recent study that compared textural differences in porcine LD with both reducible and nonreducible IMCT crosslinking (Avery *et al.*, 1996). No relationships between texture or the collagen crosslinks (type or concentration) were found. A plausible explanation may reside in the low collagen concentration of the porcine LD (< 1.5% on average). When both collagen and crosslink concentrations are elevated, less tender meat results. However, if either factor (collagen or crosslink concentration) is diminished, the influence of the remaining component on meat texture is minimized, although it may be relatively abundant.

Although variation in collagen characteristics between muscle types is reasonably predictable, a more complex issue is the change that occurs in connective tissue of a muscle with the multiple factors that affect muscle growth and adaptation over time. One general observation that we have made is that treatment or conditions that result in connective tissue turnover, and usually muscle growth, also markedly influence collagen characteristics.

Using testosterone-mediated growth of lambs as the model, we examined collagen concentration, collagen heat solubility, collagen phenotype, crosslinking, and shear force of the LD of both rams and wethers (Maiorano *et al.*, 1993). The data is summarized in Table 1. Meat from intact males is tougher than that from similar-age castrates. These data suggest that textural differences are due largely to increased mature crosslinking, the effect of which may be exacerbated by elevated amounts of collagen in the muscle of rams compared to wethers. Previous studies have documented significantly increased metabolic activity of collagen (synthesis and turnover) in the presence of testosterone, which probably accounts for the somewhat elevated muscle collagen concentration in rams. Of greater interest with respect to connective tissue matrix remodeling that occurs in this model of rapid growth are the relationships between collagen phenotypes, solubility, and crosslinking.

Generally, greater fractions of both Type III collagen and heat-soluble collagen are indicative of more youthful, labile, less crosslinked collagen. Indeed, Type III collagen is considered to be the embryonic form of fibrillar collagen, with greatest amounts occurring in the fetus and neonate. With maturation, Type III collagen is replaced by both Type I collagen and a greater compliment of mature crosslinks (Kovanen and Suominen, 1989). Likewise, the primary determinant of heat lability of IMCT is the lack of mature, heat-stable crosslinks such has HP (Bailey, 1989).

In the present study, the IMCT from rams clearly possessed greater mature crosslinking (about 40%) than IMCT from wethers, but at the same time had proportionately more Type III collagen and heat-labile collage. Because rams under the influence of testosterone synthesized more collagen than wethers (Miller *et al.*, 1989), the youthful nature of their IMCT as typified by phenotype and solubility is understandable. Harder to explain is the association of these attributes with rapid and extensive crosslinking of the IMCT. Certainly our inability to relate some physical and biochemical properties of muscle tissue to crosslinking patterns stems from a lack of knowledge of the mechanisms that regulate crosslinking in muscle. Recently completed studies that included determination of the proteoglycan decorin, together with elucidation of the structure of decorin and its role in collagen fibrillogenesis, have suggested a possible mechanism by which covalent crosslinking may be regulated.

MECHANISMS REGULATING CROSSLINK FORMATION

Little is known of possible mechanisms regulating crosslink formation. Two enzymes of collagen metabolism that may play a regulatory role in crosslinking are lysyl hydroxylase and lysyl oxidase. Lysyl hydroxylase catalyzes the post-translational hydroxylation of selected lysyl residues prior to collagen helix formation. Levels of lysine hydroxylation affect crosslinking patterns in tissues, including proportions of HP to its ketoamine precursor and the ratio of allysine to hydroxyallysine crosslink (Last *et al.*, 1989). Tissues in which the HP crosslink is relatively scarce, for example, epimysium and tendon compared to IMCT, also possess fewer hydroxylysines. Levels or degree of variability in IMCT lysine hydroxylation have not been reported.

Lysyl oxidase, which requires copper as a co-factor, is the only known enzyme involved in the actual formation of crosslinks. Severely copper-deficient rats and swine expressed depressed nonreducible crosslinking in the myocardium, although not in skeletal muscle (Farquharson *et al.*, 1989; Vadlamudi *et al.*, 1993). The influence of copper deficiency on the crosslinking of heart muscle may reflect its greater complement of copper-requiring enzymes. Generally, in the absence of severe copper deficiency or inactivation of lysyl oxidase by lathyrogens, it is unlikely that variations in lysyl oxidase activity influence crosslinking. In skin, for example, lysyl oxidase concentration greatly exceeds minimal requirements for crosslink formation (Romero-Chapman *et al.*, 1991).

The knowledge that crosslinking residues on adjacent collagen molecules or fibrils must be precisely aligned for crosslinking to occur indicates that spatial relationships between collagen molecules and fibrils may be a regulating factor in crosslink formation. A mechanism by which spatial relationships among collagen molecules and fibrils may be altered involves the binding of decorin to fibrillar collagen. Decorin is a small chondroitin sulfate / dermatan sulfate proteoglycan with a leucine-rich protein core that associates with collagen Types I, II, and III. Among its major functional roles is the regulation of collagen fibrillogenesis, growth factor activity, and cellular growth (Vogel and Trotter, 1987; Pringle and Dodd, 1990; Thieszen and Rosenquist, 1994; Danielson *et al.*, 1997). Interactions between the core protein of decorin and collagen govern the rate and extent of collagen fibril growth. The binding of decorin to collagen is axial and periodic along the fibril, with decorin binding sites corresponding to the lateral shift or stagger of one collagen molecule to another. Weber *et al.* (1996) recently modeled a three-dimensional structure of decorin that is arch shaped, with its concave inner surface wrapping about and interacting with triple helical collagen about 0.8 Da from the amino terminal end. A second putative binding site for decorin, approximately 1.6 Da from the amino terminus, was also

proposed. This model describing the collagen/decorin complex provides a clear basis for understanding the major role decorin plays in collagen fibrillogenesis. By preventing lateral fusion of collagen molecules, the initial staggered arrangement of molecules within the nascent microfibril is promoted.

We hypothesize that alterations in decorin expression and accumulation would be expected to influence the pattern of fibril formation and, thus, alignment of crosslinking sites. Given functionally reactive crosslinking residues, a key mechanism regulating crosslink formation is proximity of those sites. Recent studies in which we have examined both crosslink concentration and decorin expression suggest that such a decorin/crosslink relationship may exist. In the avian Low Score Normal (LSN) model of skeletal muscle weakness (Velleman *et al*., 1996) as well as in viable left ventricle after surgically induced myocardial infarction in rats (Zimmerman, 1997), the rapid accumulation of the HP crosslink in the IMCT is preceded by an increase in tissue decorin levels. In the case of LSN fowl, there was a 6-wk lag between markedly elevated decorin levels (at 1 d prior to hatch) and significantly elevated HP levels (at 6 wk posthatch). For infarcted myocardium, elevation of decorin preceded greatly elevated crosslinking in surviving myocardium by 7 to 8 wk. In both instances we surmised that alterations in decorin expression imposed a level of order on collagen molecule orientation that permitted increased crosslinking. The temporal gap between increased decorin expression and the measured levels of elevated crosslinking is consistent with the concept that development of crosslinking is a time-dependent process. As fibrillogenesis proceeds, crosslinks form as reactive residues align; initial reducible crosslinks condense, ultimately forming the mature trivalent HP crosslink.

CONCLUSION

Crosslinking is a primary extracellular modification to collagen that determines the *in vivo* functional properties of connective tissue. In the case of cooked meat, resulting textural properties are highly dependent on how heavily crosslinked the collagen fraction is. The contribution that increasing concentrations of collagen make to meat texture is largely determined by its crosslink profile.

Much progress has been made toward understanding the chemical nature of collagen crosslinks and the pathways that result in their formation. Furthermore, an increasing body of evidence indicates that crosslinking is important to meat texture and influenced by a myriad of practices and circumstances that accompany animal production. Unfortunately, little is known of mechanisms that actually regulate crosslink formation. Our research indicates that a mechanism by which crosslink development in muscle tissue is mediated is the decorin/collagen complex.

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