MATURE COLLAGEN CROSSLINKS

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SUMMARY: During incubation with physiological buffers at 37°, as well as during in vivo maturation, native collagen fibers display a progressive increase in tensile strength and insolubility. This is paralleled by a progressive loss of reducible, intermolecular crosslinks. The experiments described in this paper indicate that nucleophilic addition of lysine and/or hydroxylysine residues to the electrophilic double bond of the reducible crosslinks transforms them into more stable, non-reducible crosslinks. Indeed, modification of lysine/hydroxylysine residues completely blocks this transformation, while modification of his, arg, glu and asp is without effect. On the basis of these and other experiments, tentative structures are proposed for the stable crosslinks.

INTRODUCTION: Formation of stable intermolecular crosslinks between tropocollagen molecules is a critical step in the maturation of collagen fibers, since these crosslinks endow the fibers with the mechanical strength and insolubility essential to their biological function. The initially formed intermolecular crosslinks have been shown (1, 2, 3, 4) to be aldimines produced by reaction between lysine or hydroxylysine residues and lysine-derived aldehydes, as indicated in figure 1. One of these crosslinks, dehydro-hydroxylysinohydroxynorleucine (II), which is an α-hydroxy-aldimine, can isomerize to the α-amino-ketone (III, figure 1) via an enaminol intermediate (5, 6, 7). These reducible, electrophilic crosslinks form rather rapidly in native collagen fibers. However, during maturation and in vitro aging, they are progressively lost (5). Bailey therefore deduced that they are transformed into more stable, non-reducible crosslinks of undetermined structure (8). The

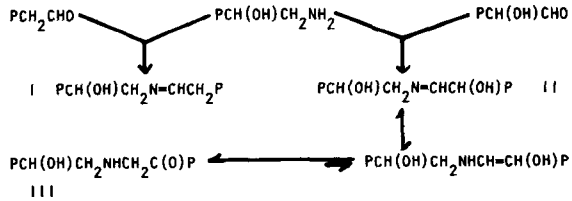

FIGURE 1 : FORMATION OF REDUCIBLE COLLAGEN CROSSLINKS.

present experiments were performed in order to test the proposal (Davis, 1973) that crosslink transformation involves addition of nucleophilic collagen residues to the electrophilic double bond of the reducible crosslinks to yield more stable, non-reducible crosslinks.

METHODS: Calf bone and dentin were decalcified by repeated extraction with 0.5 M EDTA at pH 7.4 and then extracted with 1.0 M NaCl (pH 7.4, 0.05 M tris). The insoluble collagens thereby obtained were stored at 4^{o} in the tris NaCl buffer under a layer of toluene and used in subsequent modification and *in vitro* "aging" studies.

The glu and asp residues of collagen were modified by treatment with N-ethyl-N'-dimethylaminopropyl carbodiimide (EDC) and gly ethyl ester (GEE), or with the above carbodiimide and NH_4Cl, as described previously (Davis, 1972).

Lys and hyl residues were completely acetylated by the following procedure. Collagen (600 mg, blotted dry) was equilibrated in 0.5 M borate buffer (pH 8.5) and the buffer was removed. Then, while the collagen was stirred in 30 ml of fresh, ice-cold borate buffer on an ice bath, 1 ml of acetic anhydride was added all at once. The pH was held at 8.5 $\pm$ 0.1 for one hour by addition of 5N NaOH. Four additional one-hour treatments with 1 ml of acetic anhydride at 0^{o}, pH 8.5 $\pm$ 0.1 were performed, and the acetylated collagen filtered off and washed repeatedly with 0.15 M NaCl (pH 7.4 tris).

Lys and hyl residues were also blocked by treatment of collagen (600 mg, blotted dry) with 10 ml of 0.1 M diethylpyrocarbonate (10) at pH 7.0 in 5 mM acetate buffer containing 10 percent ethanol for eighteen hours at 20^{o}. Since this procedure also reversibly blocks his, the his was regenerated by treatment with 1 M hydroxylamine (pH 7.0) for one hour at 20^{o} (10). This procedure leaves the ethoxycarbonyl derivatives of lys and hyl intact.

Reversible modification of lys and hyl residues was achieved by treatment with citraconic anhydride at pH 8.5 (11). Samples of collagen (600 mg, blotted dry) were titrated to pH 8.5 in 7 ml of water and stirred rapidly while 0.7 ml of citraconic anhydride were added, all at once. The pH of the

vigorously stirred mixture was maintained at pH 8.5 ± 0.5 by addition of 5N NaOH. Within ten to fifteen minutes, all the anhydride had hydrolyzed or reacted. For more extensive modification, treatment with 0.7 ml of citraconic anhydride at pH 8.5 ± 0.5 was repeated once, twice or thrice. All the lys and hyl residues could be regenerated by treatment with 0.1 M formate (pH 3.5) at 37° overnight (11).

The content of free hyl and lys residues in acetylated, citraconylated, diethylpyrocarbonate treated, and untreated collagens was determined by stirring these collagens with 1.04 M acrylonitrile (12) in 0.075 M borate (pH 10.5) for four days (1 ml/30 mg of collagen). This completely and irreversibly cyanoethylates all unmodified lysine and hydroxylysine residues.

His residues of collagen were modified by stirring 200 mg samples of collagen (pre-treated with citraconic anhydride) with 10 ml of 0.3 M iodoacetamide in pH 7 phosphate buffer at 5°. Every three or four days the 0.3 M iodoacetamide solution was replaced with fresh 0.3 M iodoacetamide, and the treatment continued until amino acid analysis revealed a maximal carboxymethylation of his. At this point, the citraconyl protecting groups were removed at 37° in pH 3.5 formate (11).

Arg was modified by stirring 600 mg of citraconic anhydride treated collagen with 560 mg of 1,2-cyclohexanedione in 0.1 M triethylamine (pH 11) for one to six days in the dark (13). When all the arg was blocked, the citraconyl protecting groups were removed from lys/hyl by treatment at 37° overnight with pH 3.5 formate (11).

The rate and extent of reducible crosslink transformation in modified collagens, unmodified collagens and controls was determined after incubation at 37° in 0.15 M NaCl (pH 7.4 in 0.05 M tris or phosphate buffer). Samples were removed at intervals for reduction with $NaBH_4$ and reducible crosslink determination, as previously described (14).

RESULTS AND DISCUSSION: As indicated in figure 2, modification of his, arg, glu and asp residues of collagen has no discernible effect on reducible cross-

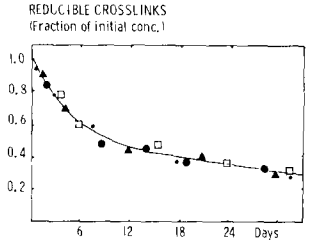

FIGURE 2 REDUCIBLE CROSSLINK TRANSFORMATION RATES

—— Collagen modified at histidine▲, at aspartate and glutamate□, at arginine●, and unmodified•

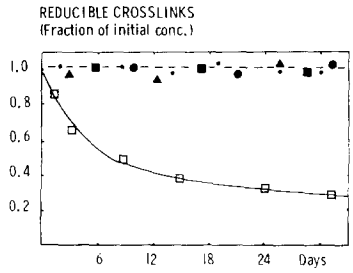

FIGURE 3 REDUCIBLE CROSSLINK TRANSFORMATION RATES

--- Collagens modified with acrylonitrile●, acetic anhydride•, ethoxyformic anhydride▲, and citraconic anhydride□ to block lysine and hydroxylysine residues.
—⊟— Collagens modified with citraconic anhydride, tested for blockage of transformation, and then de-modified at pH 3.5

link transformation since these collagens age at the same rate and to the same extent as unmodified collagens.

In contrast, specific modification of lys and hyl groups with acetic anhydride, diethylpyrocarbonate or acrylonitrile completely blocks the transformation of reducible crosslinks (figure 3). Control experiments in which collagen samples were held at the pH, solvent composition and temperature of the above modifications, but without modification reagents, all yielded collagens in which the reducible crosslinks were transformed at the same rate as in untreated collagen.

Furthermore, reversible modification of lys and hyl with citraconic anhydride (or maleic anhydride) indicates that when lys and hyl residues are blocked, transformation is blocked; but when the citraconyl (or maleyl) groups are removed by exposure to pH 3.5 formate, the de-modified collagens transform normally (figure 3). Modification of as few as eight lys and three hyl completely blocks the transformation of reducible crosslinks. Acrylonitrile treatment of collagen in which eleven lys/hyl residues per 1000 were previously blocked with citraconic anhydride yields a product with the remaining twenty-one lys/hyl residues irreversibly cyanoethylated. However, removal of the citraconyl group at pH 3.7, with regeneration of the eleven lys/hyl residues, produces a collagen which displays the same kinetics of crosslink transform-

ation as does unmodified collagen—despite the presence of twenty-one cyanoethylated lys/hyl residues. It is therefore unlikely that steric perturbation by blocking groups is responsible for the total inhibition of crosslink transformation resulting from modification of eleven lys/hyl residues. Indeed, adjacent to the N-terminal crosslink and the C-terminal crosslink are all three his residues of the α_1 chain, three arg residues, and three acidic residues. If collagen crosslink transformation were sensitive to steric perturbations, one would expect blockage of these accessible residues with the bulky groups employed here to inhibit crosslink transformation. Such modifications have no effect on transformation. It would therefore appear that lys/hyl modification blocks transformation by preventing nucleophilic attack of lys and/or hyl on the reducible crosslinks, and not by sterically perturbing the collagen structure.

These results strongly suggest that transformation of reducible crosslinks <u>in vitro</u> at 37°, as well as during normal maturation, involves nucleophilic addition of lys and/or hyl residues to the electrophilic double bond as outlined in figure 4. The only reactive centre in reducible crosslinks I and II is the electrophilic carbon of the **C=N** bond, while the only reactive centre in reducible crosslink III is the electrophilic carbon in the C=O bond. Hence, nucleophilic addition of lys or hyl to these reducible crosslinks must necessarily yield products IV and V. These crosslinks, which are not reducible with $NaBH_4$, can join together up to three different tropocollagen molecules. Crosslink V may also react further with another nucleophilic lys/hyl residue,

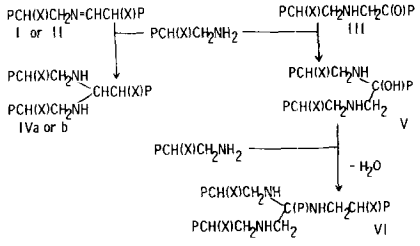

FIGURE 4: FORMATION OF STABLE COLLAGEN CROSSLINKS.
X = H or OH.

expelling water to form VI, a gem-diamine which could act as a tetrafunctional crosslink. Crosslinks IV and VI would resist thermal, hydrolytic and tensile disruption better than reducible crosslinks simply because they are more stable. Furthermore, formation of crosslinks IV and V could stabilize collagen fibers by forcing any equilibrium between crosslink precursors and crosslinks to favour the crosslinks. Finally, if crosslinks IV, V and VI are derived from lys residues on three different tropocollagen molecules, the more extensive crosslinking resulting from transformation of the bifunctional crosslinks I, II and III would give additional stability to the fiber.

The structures of IV, V and VI suggest that they should be cleaved back to I, II and III by hot acids. Indeed, we have found that when in vitro aged bone, which contains little II/III, is heated at 70^{o} in 0.1 M HCl for thirty minutes, up to 0.19 residues of II/III per 1000 residues are regenerated. Even thermal treatment at pH 7.4 regenerates some reducible crosslinks. However, the crosslinks appear to be stable to cold, dilute acids and to 70 percent HCOOH at room temperature. Hence, CNBr cleavage of mature $NaBH_4$ reduced collagens in 70 percent HCOOH could yield soluble peptides crosslinked by IV, V or VI. Identification of peptides containing these mature crosslinks should be possible because these, and only these, peptides will yield smaller peptides containing reducible crosslinks after thermal treatment to cleave the mature crosslinks. Furthermore, blockage of all free lys/hyl prior to thermal treatment should permit identification of the nucleophilic lys/hyl residues released from the stable crosslinks by thermal treatment. The lys/hyl residues, which should be released in amounts equivalent to the reducible crosslinks released, could then be labelled by treatment with an appropriate radioactive reagent specific for lys/hyl. We are currently investigating these possibilities in order to provide more direct evidence for the structure of stable collagen crosslinks.

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