AGE-RELATED CHANGES IN ALDEHYDE LOCATION ON RAT TAIL TENDON COLLAGEN

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SUMMARY: Rat tail tendons were dissolved in denaturants and the α-chains were separated by chromatography and reduced with sodium [^{3}H] borohydride. The distribution of reduced allysine residues among the cyanogen bromide peptides from these chains was studied. In young tendons, 90% of the allysine residues are in the amino-terminal telopeptide region and 10% are elsewhere, probably mostly in the carboxy-terminal region. In older tendons these percentages change to approximately 60% amino-terminal and 30% elsewhere.

Many investigators have shown that the collagen in the connective tissues (particularly skin and tendon) of many animal species changes as the animal matures and ages. These changes, which include a decrease in the solubility and an increase in resistance to proteolysis, have been interpreted to reflect increased intermolecular cross-linking (1-3). Attempts have been made to measure these cross-links quantitatively by the tritiated borohydride method (4-6). In general it has been found that the number of reducible bonds decreases with age. Bailey and his coworkers (7-9) have concluded that the reducible aldehyde adducts in the course of time become modified to a non-reducible state. We have concluded that the decreased solubility of collagen with age is less a result of an accumulation of cross-links than of their conversion from an acid-labile to a stable state (10).

The tendons and skin of the rat show little evidence of this conversion from the labile to the non-reducible bonds (10). Thus rat tail tendons even from old animals can be extensively dissolved in dilute acetic acid, and completely dissolved in denaturants (e.g. 6M urea). In such preparations the only borohydride-reducible residues are α-aminoadipic δ-semialdehyde (allysine) and the aldol adduct that is formed between two allysines. We have studied the distri-

bution of the allysine residues along the α-chains isolated from these tendons and have found an age- or maturation-related change.

MATERIALS AND METHODS: Rat tails were taken from Sprague-Dawley or Fisher strain animals of known age, carefully cleaned and dissected, and immediately immersed in freshly cooled 8M urea in 0.01M EDTA, 0.05M hydrochloric acid adjusted to pH 6 with ethylenediamine. The urea solution was first acidified to destroy cyanate and the diamine buffer was included to react with any cyanate produced in the urea and thus minimize any carbamylation of the collagen (11). The urea was intended to denature the collagen and any proteolytic enzymes in the tendons. The solutions were maintained cool to minimize any proteolytic attack before the denaturation of any enzymes was complete, and the EDTA was included to inactivate any metal-dependent and denaturation-resistant enzymes (12).

After overnight treatment the solution was warmed to room temperature and then dialyzed against 0.05M acetic acid to eliminate the urea. Aliquots of 100 mg of the collagen chains were briefly warmed to 45° and applied to a carboxymethylcellulose (Whatman CM32) column at 42° for fractionation of the α and β components (13). The α1 and α2 chains were rerun on the same column to eliminate contaminating β components and they were then separately reduced in 0.1M phosphate pH 7.5 with 1% by weight [^{3}H]-sodium borohydride (New England Nuclear Corp.). After 3 hrs. reduction the samples were dialyzed against 0.05M acetic acid repeatedly to eliminate unbound tritium and the protein was lyophilized.

Weighed aliquots of the α-chains were digested with 500-fold molar excess resublimed cyanogen bromide in 70% formic acid under a nitrogen atmosphere (14, 15) and after 5 hrs. at 30° the samples were cooled to 0°, diluted 10-fold with cold water, and lyophilized. The cyanogen bromide peptides from the α1 and α2 chains were applied to ion-exchange (16) or gel filtration (17) columns and the distribution of radioactivity in the peptide chains resolved by these procedures was measured. Samples from the column effluents were mixed with Aquasol (New England Nuclear) and counted in a Beckman LS 230 scintillation counter. The profile of the eluted peptides was monitored by recording the absorbance at 206 nm on a Uvicord III chromatographic monitor (LKB). Appropriately pooled samples of the eluted peptides were concentrated by lyophilisation, desalted on a P-2 column (Biorad) in 0.2M acetic acid and again lyophilised.

The fractionation of the cyanogen bromide peptides was checked by disc electrophoresis in a gradient 7.5 - 15% polyacrylamide gel in a gel slab apparatus under the buffer conditions described by Laemmli (18). The peptides after electrophoresis were fixed in trichloracetic acid, stained with Coomassie Blue, and then destained (19).

The reduced and tritium-labeled residues in the collagen and peptide samples were determined by column chromatography after alkaline hydrolysis as previously described (20).

RESULTS: The chromatographic profile of the polypeptide chains in the denatured tendon preparations from old and young rats differed (Fig. 1), but from all preparations better than 90% of the protein was recovered from the CMC columns.

By repeated chromatography it was possible to isolate α1 and α2 chains with less than 5% β-components contaminating them (Fig. 1). Analysis showed that the only significant reducible residue in the α-chains was hydroxynorleucine (reduced allysine).

Both hydroxynorleucine and the reduced aldol are destroyed by 6N acid

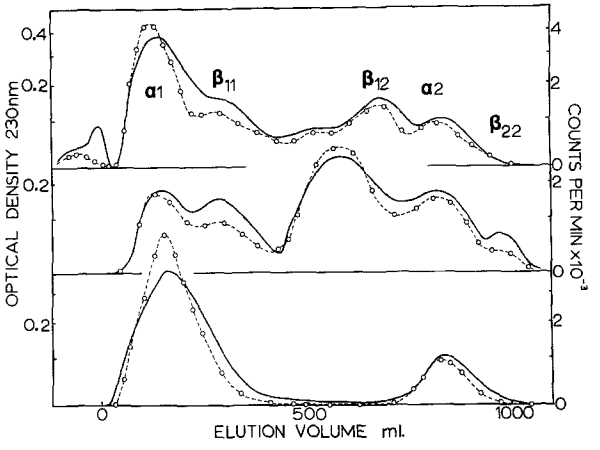

Fig. 1. Carboxymethylcellulose chromatography of reduced collagen chains from: (top) 6 week rat tail tendons; (middle) 26 month rat tail tendons; (bottom) α1 and α2 chains refractionated from the top sample. In this particular experiment the whole preparation was reduced prior to fractionation. The [^{3}H] label is noticeably skew in the lower diagram, reflecting the fact that some of the chains contain no allysine residues (31). The specific activity of the old tendons is not markedly different from the young (both preparations were reduced under the same conditions with the same solution of borohydride).

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hydrolysis (21) but measurements showed that more than 70% of both survive 5 hours in 70% formic acid at 30°; therefore the α1 and α2 chains were cleaved in this solvent with cyanogen bromide and the resulting peptides were fractionated to determine the distribution of the hydroxynorleucine between the peptides (Fig. 2). This distribution was confirmed by chromatography on carboxymethyl cellulose (not shown). Samples from each of these peaks were hydrolyzed in alkali and chromatographed. In each case hydroxynorleucine was the only significant reduced compound detected (Fig. 3).

Similar analyses on the reduced α2 chains revealed that while most allysine was in the amino-terminal region, 10% (in young rats) to 30% (in old) was in either α-2-CB4 or α2-CB5 (these peptides were not clearly resolved by gel filtration or on carboxymethycellulose). The latter is the carboxy-terminal peptide.

These analyses were conducted on four young (4-6 weeks) and five old (9-27 month) tendon preparations and the results confirmed 10-15% of the cross-linking

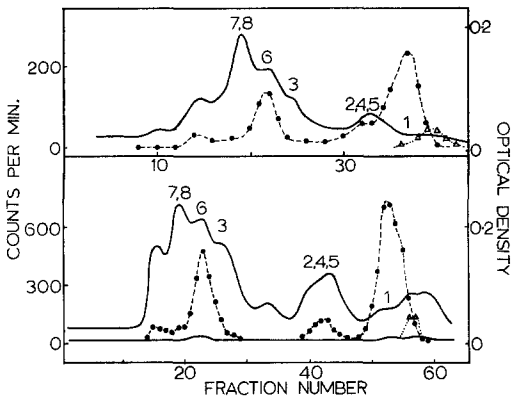

Fig. 2. Gel filtration of CNBr peptides from α1 chains of (top) 6 week rat tail tendon collagen on a G100 Sephadex column, and (bottom) 26 month old rat tendons on a P150 (Biorad) column. The absorbance (unbroken line) was measured at 206 nm. and (lower line, bottom) at 278 nm. The dashed line indicates the radioactivity in the samples of the effluent: -●- tritium, ··△·· [^{14}C] valine added as a void volume marker. The peptides are identified by number (see ref. 16)

Gel electrophoresis confirmed that the first, minor, tritium peak represents incompletely cleaved peptides. The tritium in the α1-CB6 peptides (identified by size and by the 278 nm. absorbance indicative of the tyrosine residues) comprised 15% (top) and 34% (bottom) of the total. The tritium in the peptides α1-CB2, 4, and 5 (in the bottom diagram) was 8% of the total.

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aldehydes are in the C-terminal peptides in young animals, 26-35% in the old.

DISCUSSION: These investigations have shown that the aldehyde residues generated by lysyl oxidase (22) on type I collagen are located predominantly in the amino-terminal telopeptide but 10-30% are found elsewhere, particularly in the carboxy-terminal, cyanoge bromide peptides. These experiments do not demonstrate that the label is in the carboxy-terminal telopeptide, but pepsin digestion studies to be reported elsewhere support that idea.

Earlier investigations demonstrated that aldehydes are formed in the amino-terminal regions of rat α-chains, and this is the site of the intramolecular cross-link in the β components (23,24). Kang (25) and Deshmukh (26) subsequently reported that aldehydic residues in rat tendons are restricted to the amino-terminal peptides; Eyre and Glimcher reported that aldehydes in chick bone collagen are largely restricted to the amino-terminal location (27). Deshmukh and Nimni (28) reported some aldehydes on internal cyanogen bromide

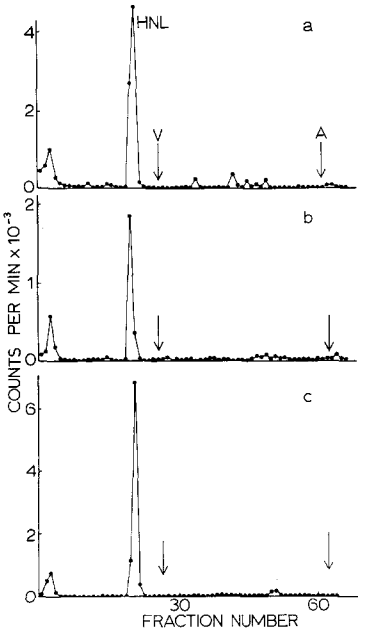

Fig. 3. Elution profile of reduced compounds from an alkaline hydrolyzate of aliquots from: a, α1-CB6; b, α1-CB2, -3, and -4; c, α1-CB1 peptides from the lower diagram in Fig. 2. The arrows show the elution positions of [^{14}C] valine (V) and arginine (A) added as markers. The only significant peaks are unretarded material (probably [^{3}H]-H_2O produced by hydrogen exchange or by hydrolytic destruction in the alkali) and hydroxynorleucine (HNL).

peptides from rat skin, including α1-CB7 and α1-CB8. We have found negligible levels in the large α1-CB7 and α1-CB8 peptides from tendon. The considerable amounts we find in α1-CB6, for example, seem to have been previously overlooked. While some label is present in α1-CB2, 4, or 5, this may arise from an incomplete cleavage of the methionine between α1-CB1 and α1-CB2. Kühn and his colleagues have demonstrated allysine in the C-terminal telopeptide in bovine collagen (29). In view of the recognition of type III collagen in skin (15,30), the assignment of aldehydes to intra-helical regions of the collagen chains on the basis of peptide chromatography (28) may require re-evaluation.

The age-related change in aldehyde distribution has not been reported previously. It remains to be determined if the relative increase in α1-CB6 label, for example, relates to the continued action of lysyl oxidase on the

molecules in pre-formed fibrils, or to replacement through turnover with molecules with more, or differently distributed aldehydes. The specific activity of α-chains from old animals is not obviously greater than in young (Fig. 1), so there is no indication of a large increase in aldehyde number. We deduce that the α-chains that can be isolated from these tendons for examination carry, on the average, more than one but less than two aldehyde residues.

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