ALDEHYDE CONTENT AND CROSS-LINKING OF TYPE III COLLAGEN

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Received January 6,1976

SUMMARY: The aldehyde content and crosslink characteristics of Type III collagen, extracted from fetal calf skin, have been examined by employing NaB^3H_4 reduction. Direct comparison was made with Type I collagen from the same preparation of fetal calf skin. Collagen molecules and reconstituted collagen fibrils were assayed for tritium incorporation and for their content of reduced aldehydes and crosslinks. Type III collagen incorporated about 45% of the tritium found in Type I collagen and lysinonorleucine was the major crosslink in reconstituted Type III collagen fibrils. In contrast, reconstituted fibrils of Type I collagen primarily formed hydroxylysinonorleucine. These results demonstrate that Type III collagen resembles all other collagen isotypes by containing crosslinking aldehydes but that it differs from them by the predominance of lysinonorleucine. This difference may be related to the turnover and progressive loss of Type III collagen in developing tissues.

Four genetically distinct collagen isotypes have been described, comprising a total of five unique polypeptide chains (1). One of these collagen isotypes, Type III collagen, has been isolated from skin and other tissues, either by direct extraction (2-4) or by limited proteolysis and extraction (5,6). Type III collagen is more abundant in fetal skin (5) and appears to progressively decrease as Type I collagen increase (7,8). Another feature of Type III collagen is its content of cysteine residues which form intramolecular disulfide bonds (9); it has been inferred that intermolecular disulfide bonds may also occur in this protein *in situ* (10). Since all the other collagen isotypes contain aldehydes which undergo crosslinking we have examined Type III collagen for its content of

aldehyde and their conversion into crosslinks. We report our findings below, in comparison with parallel studies on Type I collagen.

MATERIALS AND METHODS

Fetal calf skin, from animals at 2-3 months gestation, was extracted and purified as previously described (2). The purified protein was fractionated by differential salt precipitation at 1.8 M NaCl (5), prior to DEAE ion-exchange cellulose chromatography (2,4). Type I collagen was also passed through the DEAE cellulose column, at low ionic strength. The protein peaks were dialyzed versus dilute acetic acid and lyophilized.

The collagen samples were dissolved in potassium phosphate buffer, $\mu = 0.4$, pH 7.6, by shaking at 4°C for 24 hours. The slightly cloudy solution of Type III collagen was centrifuged at 10,000 x g for 1 hour and the supernatant fluid was used for further studies. Reconstituted collagen fibrils were formed from solutions of Type I and Type III collagens as previously described (11).

The collagen solutions and reconstituted collagen fibrils were reduced with NaB^3H_4 (11); they were then dialyzed extensively against 0.1 N acetic acid and lyophilized. The tritiated proteins were hydrolyzed in 3 N HCl at 107°C for 48 hours, and the hydrolysates were dried. A portion of each hydrolysate was measured for specific radioactivity, and the data were expressed in terms of the measured hydroxyproline content of the hydrolysates. Chromatographic fractionation of the radioactive components of each hydrolysate was carried out on two different columns (12).

RESULTS AND DISCUSSION

The amino acid composition of extracted Type III collagen is shown in Table I; it is similar to earlier preparations (2). Fig. 1 shows the densitometric tracings of stained polyacrylamide gels following electrophoresis of a mixture of Type I and Type III collagens, with and without DTT reduction (13). α1 (III) and pro α1 (III) chains migrated into the α chain region of the gel only in the presence of DTT and migrated in positions different than α1 (I) and α2. The samples of isolated Type I and Type III collagens, prior to

TABLE I: AMINO ACID COMPOSITION OF TYPE III COLLAGEN EXTRACTED FROM FETAL CALF SKIN.

Amino acid	Residues per 1000 total residues
4-Hydroxyproline	108
Aspartic acid	57
Threonine	16
Serine	45
Glutamic acid	79
Proline	112
Glycine	339
Alanine	87
Valine	19
Methionine	6
Cysteine	5
Isoleucine	14
Leucine	15
Tyrosine	4
Phenylalanine	9
Hydroxylysine	5
Lysine	28
Histidine	8
Arginine	44

No corrections have been made for destruction or incomplete release during hydrolysis. The values are the mean of three separate analyses of one preparation (200 mg) of Type III collagen.

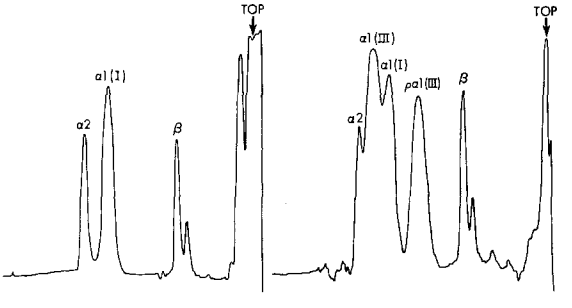

Fig. 1 Densitometric tracings of polyacrylamide gels (6.3%, pH 5.8), following electrophoresis of a mixture of Type I and III collagens. DTT was included in the sample shown in the right panel.

TABLE II: SPECIFIC RADIOACTIVITY

	Collagen in solution	Reconstituted collagen fibrils
Type I	1633 (1581-1674)	2196 (1860-2393)
Type III	699 (685-715)	1000 (940-1067)

Data are expressed as cpm/μg hydroxyproline and are mean values obtained from three individual experiments. Figures in parentheses are the minimum and maximum values.

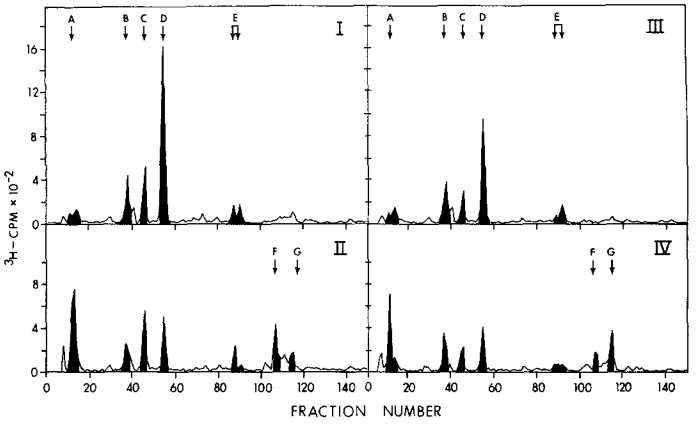

Fig. 2 Chromatographic patterns of the radioactive components in acid hydrolysates of NaB^{3}H$_4$-reduced collagen solutions and reconstituted collagen fibrils. I: Type I collagen in solution, II: Reconstituted Type I collagen fibrils, III: Type III collagen in solution, IV: Reconstituted Type III collagen fibrils. The peaks are: A, unknown (fall through); B, dihydroxynorleucine; C, unknown; D, hydroxynorleucine; E, N$^{\epsilon}$-hexosylhydroxylysine; F, hydroxylysinonorleucine; G, lysinonorleucine.

mixing, appeared to be free of any cross-contamination when electrophoresed on separate gels. Moreover, SDS polyacrylamide gels confirmed these results and those in Fig. 1 as well as showing that there were no contaminating proteins in the collagen preparations.

The specific radioactivity of NaB^{3}H$_4$-reduced Type I and Type III collagens

were compared (Table II). These data were not corrected for the small differences in hydroxyproline content of the two types of collagen (2-4). The results show that Type III collagen incorporated about 45% of the radioactivity of Type I collagen, both in solution and in reconstituted fibrils. The fibrils have a higher specific activity than the solutions, probably due to some non-specific tritium adsorbtion, which exchanges slowly. This is supported by the presence of the initial peaks in the chromatograms of the fibrils, as previously observed (14). Prior digestion of Type I and Type III collagen with pepsin, followed by NaB^3H_4 reduction of the repurified proteins showed specific activities of 883 cpm/μg (Type I) and 771 cpm/μg (Type III) respectively. Thus, pepsin had no effect on the aldehyde content of Type III collagen but diminished the aldehyde content of Type I collagen. Control studies, using the gel system of Fig. 1, showed that Type III collagen was converted by pepsin into a single polypeptide chain, migrating between α2 and α1 (I), as previously described (2). Thus, although pepsin decreased the mass of the molecules, it did not alter the aldehyde content of Type III collagen. This result implies that such aldehydes are interior to the pepsin digestable non-collagen extensions in Type III collagen, similar to some of the cysteine moieties in this protein (9).

The chromatographic patterns of the radioactive components in acid hydrolysates of NaB^3H_4-reduced collagen solutions and reconstituted fibrils are shown in Fig. 2. Both Type I and III collagens showed a change in their radioactive profiles when the respective solutions and fibrils were compared. In the case of Type I collagen, previous studies have shown that the changes reflect aldehyde incorporation into crosslinks in the fibrils (11). We assume that the same interpretation is valid for Type III collagen, particularly since we can identify the major radioactive components using two chromatographic systems. Thus, the reduced aldehyde, hydroxynorleucine, is prominent in the chromatographic patterns of both collagens and it decreases significantly in the fibril preparations (Fig. 2). The decrease in hydro-

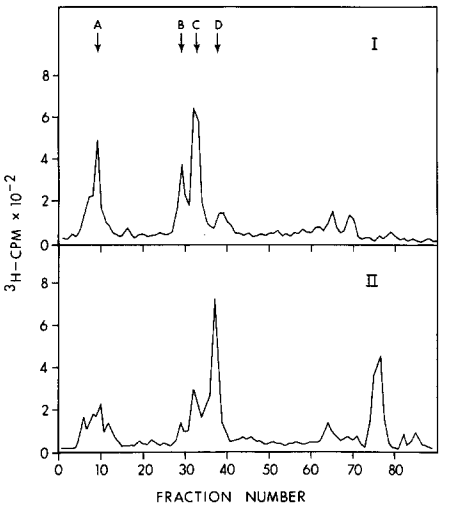

Fig. 3 Chromatographic patterns of the radioactive components which elute in the crosslink-containing region (aromatic and basic amino acids). I: Reconstituted Type I collagen fibrils. II: Reconstituted Type III collagen fibrils. The peaks are: A, unknown (fall through); B, unknown; C, hydroxylysinonorleucine; D, lysinonorleucine.

xynorleucine was from 30.5% to 9.7% of the eluted radioactivity for Type I collagen and from 24.7% to 8.5% for Type III collagen. These results show that the lysine-derived aldehyde, allysine, is the principal contributor to crosslink formation in both types of collagen. The identity of the crosslinks which have formed is shown in Fig. 2 and 3. These chromatograms show that lysinonorleucine was the major crosslink in reconstituted Type III collagen fibrils, whereas Type I collagen primarily contained hydroxylysinonorleucine. Since allysine is the major aldehyde precursor in both types of collagen, and since the hydroxylysine contents of both collagens are very similar, the differences in crosslinking probably reflect some fundamental differences in fibril organization. An analogous situation occurs in the case of Type I collagen itself, as shown by the unique crosslinking patterns of bone and tendon fibrils (15); the polymeric organization of Type I collagen is known to be quite different in these two tissues (16). Another implication of the present results may be a relationship between collagen

stability and its crosslinking pattern. Type III collagen progressively diminishes during skin development, being replaced by Type I collagen (5). Perhaps the relative stability of the crosslinks is a factor in the regulation of collagen turnover since the individual reducible crosslinks do vary in their ability to withstand environmental changes (17).

ACKNOWLEDGEMENT: This work was supported by a grant from NIH, AM-12683.

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