IN VITRO FORMATION OF CROSSLINKS IN MATURE RAT SKIN COLLAGEN IN THE ABSENCE OF N-TERMINAL PEPTIDES

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Summary: Soluble collagen extracted by cysteamine from rat skin contains 1.7 to 2.0 moles of peptide bound α-amino adipic semi-aldehyde along the helical portion of the α-chains. Removal of the N-terminal peptides by limited CNBr cleavage yields a collagen that has native characteristics (viscosity and optical rotation) and forms regular 640 Å banded fibers. Incubation of this material at 37° C. gives rise to Schiff base type crosslinks involving these aldehydes and lysine or OH-lysine of neighboring molecules.

The presence of lysine derived aldehydes in the N-terminal non-helical region of the collagen molecule and their participation in the formation of intra- and intermolecular crosslinks has become well established (1,2,3,4,5). Collagen from mature rat skin extracted with cysteamine at neutral pH represents a mature form of collagen which contains higher amounts of aldehydes than the more recently synthesized forms of collagen (6,7,8). Selective removal of the N-terminal peptides with cyanogen bromide leaves 70% of the total aldehydes covalently bound to the helical region of cysteamine soluble collagen, whereas neutral salt soluble collagen becomes totally depleted of its aldehydes. The location of these aldehydes on the helical part of cysteamine soluble collagen was determined after complete cleavage with cyanogen bromide. Peptides α1-CB7, α1-CB8, α2-CB3, α2-CB4 and α2-CB5 contain varying amounts of aldehydes derived from lysine or hydroxylysine (5).

The present communication describes the participation of these aldehydes in the *in vitro* formation of crosslinks.

MATERIALS AND METHODS

The dorsal skin of rats, weighing 100-150 gm, was cleaned, homogenized and extracted successively with 0.15 M NaCl, 0.45 M NaCl in 0.02 M Na phosphate buffer, pH 7 and 0.5 M Na citrate buffer, pH 3.6. The residual skin was extracted with 0.2 M cysteamine in 0.45 M NaCl, pH 7 for 72 hours. All the operations were carried out at 4° C. The cysteamine soluble collagen was purified as described in an earlier communication (9) and centrifuged at 105,000 xg for 2 hours. The clear supernatent was used for further studies.

Limited Cleavage with Cyanogen Bromide

Cysteamine soluble collagen was precipitated by dialysis against several changes of water. The precipitate was dissolved in 0.1 N HCl at a concentration of 3 to 4 mg/ml. Nitrogen was bubbled through the solution and 100 fold molar excess of cyanogen bromide (Eastman Organic) over methionine residues was added. The mixture was incubated at 15-20° C. overnight. Excess cyanogen bromide was removed by extensive dialysis against water. The contents were finally dialyzed against 0.45 M NaCl, pH 7 and centrifuged at 105,000 xg for 2 hours.

Acrylamide gel electrophoresis was performed using the method of Nagai, *et al.* (10) and the stained gels were scanned using a Gilford spectrophotometer. Viscosity measurements of treated and untreated collagens were carried out using a Zimm-Crothers low-shear viscometer and optical rotation with a Zeiss spectropolarimeter.

In Vitro Incubation

After removal of the N-terminal peptide cysteamine soluble collagen was incubated at 37° C. in 0.45 M NaCl, pH 7, at a concentration of 3.5 to 4 mg/ml for various time periods. At the end of each incubation period, the gels were broken to form a suspension and reduced with 100 fold molar excess of H^3-$NaBH_4$ (Sp. Act. 9.4 mc/mM).

The reaction was carried out at 4° C. for 2 hours, maintaining the pH between 7 and 8. These conditions were selected so as to avoid any hydrolysis of the peptide bond during reduction. Excess $NaBH_4$ was removed by dialysis against water. The samples were divided into two batches. One was hydrolyzed with 3 N HCl and the other with 2 N KOH in sealed ampules under N_2 atmosphere at 108° C. for 24 hours. Acid hydrolysates were dried to remove excess HCl, while base hydrolysates were adjusted to pH 4 with perchloric acid, chilled and centrifuged to remove the precipitated salt (5). Amino acid analysis of the hydrolysates was carried out using a Jeol amino acid analyzer. The fractions of the effluate were collected using a split stream device and the H^3 activity of the fractions was measured with a Beckman liquid scintillation counter.

RESULTS AND DISCUSSION

The untreated cysteamine soluble collagen contains 60-65% β-chains and 30-40% α-chains. After limited cleavage with cyanogen bromide, 96% of the collagen migrated like α-chains (α^{CNBr}) while only 3 to 4% remained as β-chains, reflecting the almost complete removal of the N-terminal peptide containing the intramolecular crosslink (Figure 1A). The limited cleavage procedure using CNBr has been previously shown to remove only the N-terminal peptides α1-CB1 and α2-CB1, as well as the dimeric peptides β11-CB1 and β12-CB1 (5). Similar cleavage of β-chains was shown to occur after incubation with chymotrypsin, however, cyanogen bromide was found to be less effective in the hands of these investigators (11).

Using cysteamine soluble collagen we find that 70% of the initial amount of aldehydes is retained by the α^{CNBr} chains. The treated collagen exhibits the same optical rotation at 365 mμ as its untreated control, affording proof for nativity of the remaining molecule. The intrinsic viscosity at 15° C. in 0.05 M acetic

acid dropped from 22 dl/gm to 13 dl/gm, a value close to that of neutral salt soluble collagen from normal or lathyritic rat skin.

Cysteamine soluble collagen (2 to 2.5 mg/ml) forms a very firm gel at 37° C. After a few hours of incubation these gels become completely resistant to redissolution when cooled at 4° C. It was necessary to use a higher concentration (3.5 to 4 mg/ml) of treated collagen to obtain similar gels. Also, the time factors required for stabilization were longer since after 4 days of incubation at 37° C., these gels still partially dissolved when cooled and only became stable when incubated for longer time periods. Fibers made out of cysteamine soluble collagen after limited cleavage, showed the same 640 Å periodicity under the electron microscope as the

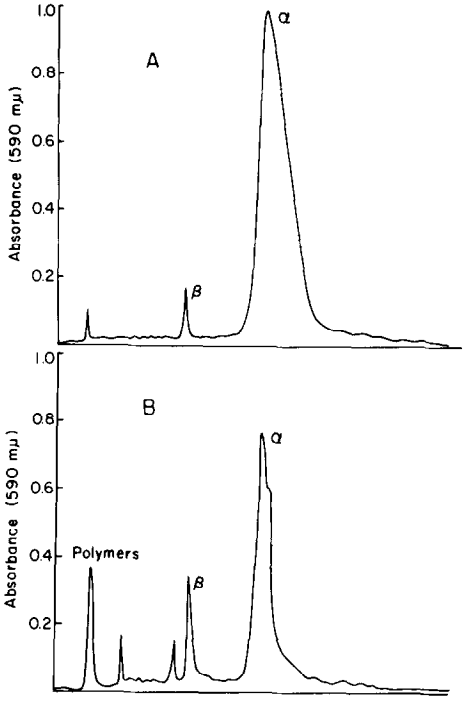

Figure 1. Densitometric tracing of collagen subunits separated by acrylamide disc electrophoresis after removal of the N-terminal peptides by limited cleavage with CNBr.

A. Before incubation
B. After incubation for 1 week at 37° C., pH 7.0

untreated controls or those arising from neutral salt soluble collagen. This is an indication that cysteamine soluble collagen retains its capacity to align properly even in the absence of the N-terminal non-helical region. It should be mentioned that neutral salt soluble collagen, which is depleted of all its aldehydes by limited cleavage, almost completely loses its capacity to form stable gels at 37° C. after removal of its N-terminal peptides.

The capacity of the CNBr treated collagen to form crosslinks is reflected by the change in electrophoretic pattern seen after 1 week of incubation at 37° C. (Figure 1B). There is a drop in α^{CNBr} components and a relative increase in β-like and other polymeric forms of large molecular size.

Reduction of the limited-cleaved collagen with NaB^3H_4 followed by amino acid analysis shows most of the radioactivity appearing as ε-hydroxynorleucine, a product of α-amino adipic semialdehyde (Figure 2A). A trace of radioactivity which can be attributed to the small amount of uncleaved β-chains eluted as a reduced aldol condensation product. After incubation at 37° C. for 1 week, two radioactive peaks appeared in the region where reduced Schiff base products derived from α-amino adipic semialdehyde and the ε-amino groups of lysine and hydroxylysine elute (12) with a concurrent decrease in ε-hydroxynorleucine (Figure 2B). There was no change seen in the aldol condensation product. These observations clearly reflect the participation of aldehyde groups in the helical region of collagen in the formation of Schiff bases and support the concept that the aldol condensation product between aldehyde groups derived from lysine is restricted mainly to the N-terminal region of the collagen molecule.

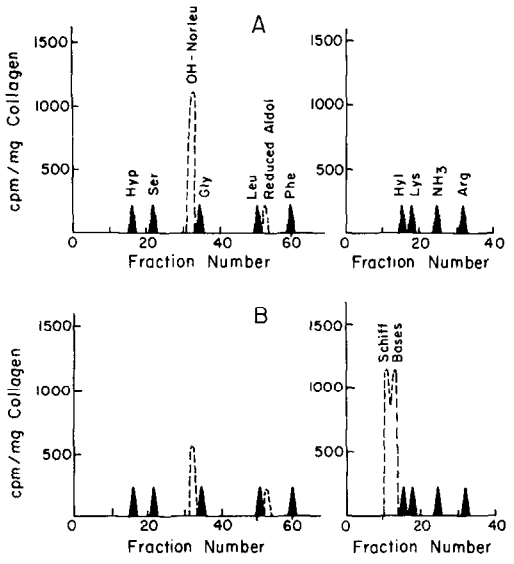

Figure 2. Radiochromatograms obtained after reduction of collagen with NaB^3H_4 followed by hydrolysis. The dotted lines indicate elution of radioactivity, the black inserts are position markers for various amino acids. This figure represents a composite of separate acid and base hydrolyzed samples (5).

A. Collagen after removal of the N-terminal peptides by limited cleavage.
B. Same material incubated at 37° C. for 1 week.

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