IDENTIFICATION OF STABLE INTERMOLECULAR CROSSLINKS PRESENT IN RECONSTITUTED NATIVE COLLAGEN FIBERS

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Received October 21, 1971

Summary: Soluble collagen extracted by 0.2 M cysteamine from rat skin contains 2.5 to 2.7 residues of peptide bound α-aminoadipic semi-aldehyde per α-chain. When reconstituted into fibers and maintained at 37° C these aldehydes become involved in the formation of stable non-reducible crosslinks. Using collagen biosynthetically labeled at the lysine and hydroxylysine positions, these stable crosslinks have been partially identified. Lysinonorleucine and its hydroxyderivative account for 50% of the initial aldehyde residues present on the collagen molecule while two unidentified peaks (post-lysine and post-histidine) account for the other 50%.

The participation of lysine-derived aldehydes in the formation of intra- and intermolecular crosslinks has been demonstrated in various types of collagen (1-5). Crosslinking intermediates formed between collagen molecules and involving Schiff bases derived from peptide bound lysine and hydroxylysine and their respective aldehydes have been identified after reduction of polymeric collagen with sodiumborohydride. The products of this reduction are hydroxylysinonorleucine, dihydroxylysinorleucine and lysinonorleucine. Intermediates of a similar nature occur in elastin, but whereas the structure and biosynthesis of these crosslinks has been determined (6-11), the structure of the stable crosslinks in collagen and the mechanism of their stabilization remains to be established.

The more mature fractions of collagen extracted from rat

skin by 0.2 M cysteamine, pH 7.0 or by 0.5 M acetic acid contain higher amounts of aldehydes and exhibit greater capacity to form covalent bonds in vitro than the more recently synthesized collagen (0.45 M salt soluble collagen) (12-14). Besides the aldehydes in the N-terminal region there are additional lysine-derived aldehydes in the helical region (15), which participate in intermolecular crosslinking (16, 17). The location of these aldehydes in the peptides originating from cleavage of collagen with cyanogen bromide has been established (15). Incubation of cysteamine soluble collagen at 37° C for various time periods revealed that stable crosslinks occur faster with this fraction than with salt soluble collagen (Deshmukh and Nimni, unpublished results). Because the stable intermolecular crosslinks in collagen are non-reducible (17) alternate methods became necessary to identify their nature. The present communication describes the use of collagen labeled with C^{14} at the lysine and hydroxylysine positions and of tritiated water to identify the stable intermolecular crosslinks.

MATERIALS AND METHODS

Preparation of the C^{14}-labeled substrate - 150 μc of L-lysine-UL-C^{14} (Sp. Act. 210 mC/mM) was injected to each of three male albino rats (70-80 g) in two divided doses. The animals were allowed to grow on a normal diet for four days and sacrificed. The dorsal skins were cleaned, homogenized and the 0.45 M salt soluble and 0.2 M cysteamine soluble collagens were successively extracted and purified as described earlier (14).

Identification of the C^{14}-labeled crosslinks:

Aliquots of C^{14}-labeled cysteamine soluble collagen were incubated at 37° C in sealed tubes in the presence of a drop

of toluene. At various time intervals, the polymeric collagen was hydrolyzed in 3 N HCl under nitrogen at 108° C for twenty four hours. The hydrolysate was diluted, placed on a 65 cm column of a JEOL amino acid analyzer and eluted with Na citrate buffer, pH 5.28. The flow rate was adjusted to 48 ml/H and the temperature to 60° C. The fractions were collected through the split stream device and counted using a Beckman liquid scintillation counter. Lysinonorleucine obtained from elastin, UC^{14}-lysine and a Beckman standard amino acid mixture were used to calibrate the run.

Incubation of non-radioactive cysteamine soluble collagen with tritiated water:

Cysteamine soluble collagen obtained from dorsal skins of normal untreated rats was purified and incubated (3 mg per tube) with 100 mC of H_2^3O (Sp. Act. after dilution=1.8 mC/mM) in sealed tubes at 37° C. After different incubation periods, the gels were dialyzed exhaustively against distilled water, hydrolyzed with 3 N HCl and analyzed on the amino acid analyzer as described earlier. The effluent fractions were counted for tritium activity.

RESULTS AND DISCUSSION

Figure 1 illustrates the elution pattern of an acid hydrolysate of reconstituted collagen fibers maintained at 37° C for four weeks.

Whereas the unincubated material exhibited C^{14}-radioactivity only under lysine and hydroxylysine, (incorporated biosynthetically *in vivo*) additional peaks became apparent during the course of the incubation. Lysinonorleucine (LNL) and its hydroxyderivative (HLNL) elute just prior to hydroxylysine and two unidentified peaks labeled post-lysine and post-histidine appear among the more basic amino acids. Recently

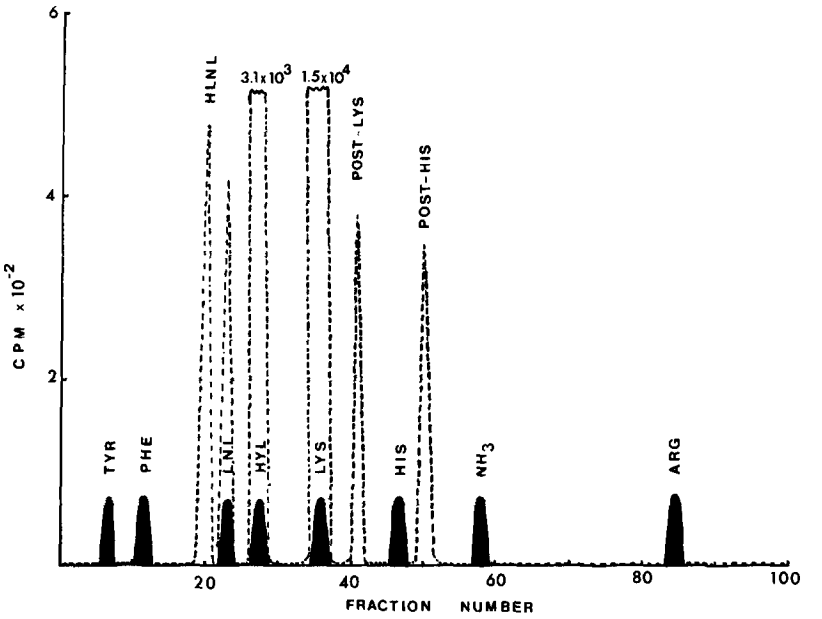

Figure 1. Collagen labeled with C^{14}-lysine and hydroxylysine was incubated at 37° C for 4 weeks, acid hydrolyzed and chromatographed. Dark areas represent markers for standard amino acids, dotted lines the location of radioactive peaks.

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it was shown that the in vitro incubation of chick skin collagen gave rise to an acid stable "post-histidine" peak and it was suggested that it may result from an intermolecular aldol condensation product (18).

The formation of these compounds as a function of time are recorded in Table I. Radioactivity in all the newly formed components appeared after one week of incubation, increasing by the second and fourth weeks.

From this data it becomes apparent that the Schiff-base compounds (LNL and HLNL) previously identified in elastin and collagen become stabilized in the absence of an exogenous reducing agent. It therefore became of interest to find out if water from the media is in equilibrium with the hydrogen used for this purpose.

Table I

Radioactivity eluted under the peaks indicated in Fig.1*

Component	Incubation Period (Weeks) cpm/mg Collagen			
	0	1	2	4
Hydroxylysine	1390	1330	1300	1350
Lysine	5720	5530	5540	5470
Hydroxylysinonorleucine	0	111	140	143
Lysinonorleucine	0	85	130	130
Post-lysine	0	79	125	135
Post-histidine	0	89	107	125

*Sample applied to column corresponded to 5-6 mg of hydrolyzed collagen fibers.

Therefore non-radioactive collagen was aggregated into fibers in the presence of tritiated water of a high specific activity and incubated at 37° C for various time intervals. The only location where radioactivity could be detected coincided with the post-lysine peak previously detected using C^{14}-lysine and C^{14}-hydroxylysine containing collagen. For purpose of comparison, the uptake of C^{14}-lysine (from data in Table I) is tabulated along with the uptake of tritium into this post-lysine peak (Table II).

The radioactivity in this fraction from both isotopes reached a maximum between the second and third week, consistent with the rapid rate of crosslinking exhibited by the soluble collagen extracted from skin by cysteamine.

Table III summarizes all our observations. Based on the specific activity of the C^{14}-lysine in the initial collagen (containing 28 lysine residues/1000) and that of the tritium in the water used as a solvent, the molar concentration of

Table II

Incorporation of radioactivity from peptide bound C^{14}-lysine and from H_2^3O added to the media, into the post-lysine peak.

Time of Incubation (weeks)	cpm/mg of Collagen	
	C^{14}	H^3
0	0	0*
0.5	30	875
1	79	2925
2	125	5225
3	135	5400
4	135	5450

*Placed in contact with H_2^3O and immediately dialyzed prior to hydrolysis.

Table III

Moles of peptide bound C^{14}-lysine and H^3 from H_2^3O present in various components isolated after 4 weeks of incubation. (*)

Component	Moles of C^{14}-lysine	Moles of H^3
Hydroxylysinonorleucine	0.73	0
Lysinonorleucine	0.67	0
Post-lysine	0.69	1.8
Post-histidine	0.64	0

(*) Values are expressed as Moles per mole of α-chain equivalent.

the various products were calculated and expressed per α-chain equivalent. (Molec. Weight 95,000)

A total of 2.73 lysine residues appears to become incorporated into crosslinks. This is in good agreement with the expected values since we have previously shown that collagen extracted by 0.2 M cysteamine from mature skin fibers contains

2.5 to 3.0 residues of aldehydes derived from lysine per α-chain, two thirds of which are located in the non-helical region of the molecule (15). Most of these residues, therefore seem to become involved in the formation of crosslinks, a process that occurs more rapidly (and probably more completely) *in vitro* than *in vivo*.

These findings enable us to explain the decline in tritium uptake into dehydro LNL and dehydro HLNL during aging (17). Our previous observations suggested that the final stable crosslink in collagen had to involve some process of stabilization, probably an addition to this double bond. Our present data indicates that the intermediate acid labile crosslinks become stabilized under physiological conditions by addition of hydrogen. This hydrogen does not seem to exchange with water from the media. Since even purified collagen preparations contain some amine-oxidase activity (17) which can only be inhibited by BAPN and chelating agents, one can speculate that a co-factor involved in this reaction may contribute the hydrogen molecules required for the reduction of the labile Schiff base intermediates.

Acknowledgement:

We wish to thank Dr. C. Franzblau for kindly supplying us lysinonorleucine from elastin; Sylvia Padilla and Maria Just for excellent technical assistance.

This research was supported by grants from the National Institutes of Health (AM-10358 and DE-02471) and from the Arthritis Foundation.

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