THE NATURE OF THE INTRAMOLECULAR CROSS-LINK IN COLLAGEN*

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The proposed biosynthetic scheme for the formation of intramolecular cross-links in collagen includes oxidative deamination or transamination of the ε-amino group of specific lysine residues to yield residues of α-aminoadipic acid-δ-semialdehyde. α-Aminoadipic acid-δ-semialdehyde was shown to be present in collagen (Gallop et al., 1968) and in peptides obtained from collagen after treatment with CNBr (Bornstein and Piez, 1966). Bornstein and Piez (1966) proposed that intramolecular cross-links in collagen are formed by an aldol condensation of two residues of this aldehyde positioned properly in adjacent α chains. More recently, Rojkind et al. (1968), by means of metabolic studies using ^{14}C- and ^{3}H-lysine, demonstrated that intramolecular cross-links indeed are derived from incorporated lysine by the mechanism proposed. The presence of the aldol condensation product of two residues of α-aminoadipic acid-δ-semialdehyde has recently been described in calf skin collagen (Paz et al., 1969) and in elastin (Franzblau and Lent, 1968).

This communication describes the occurrence of the aldol condensation product in a peptide, known to have the intramolecular cross-links, derived from collagenase digests of rat skin collagen. This compound has also been shown to be present in peptides obtained

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from purified β chains of chick skin collagen known to contain the intramolecular cross-link by Kang, Faris and Franzblau (manuscript submitted for publication; personal communication).

EXPERIMENTAL

Approximately 10 gm of citrate soluble rat skin collagen were suspended in water and heat denatured at 60° for 30 minutes. Undissolved material was removed by centrifugation at 100,000 x g for 30 minutes, 10 ml of 1% EDTA were added to the supernate and the pH adjusted to 8.0. Approximately 600 mg of NaB^3H_4 (prepared and standardized by the method of Gallop et al., 1968) were added, and the pH maintained at 8.0 by addition of 1 N HCl. After 30 minutes, the reaction was terminated by addition of HCl to bring the pH to 3.0. The solution was then dialyzed against distilled water for 48 hours. From the specific activity of the reduced protein, a value of 4 moles of reduced compounds per 100,000 molecular weight was estimated.

The protein solution was made 0.005 M in $CaCl_2$, adjusted to pH 8.0 and digested with 2 mg of purified bacterial collagenase (gift of Drs. E. Harper and S. Seifter) at 40° for 48 hours. An aliquot of the digested protein containing 500 mg of protein was chromatographed on DEAE cellulose as previously described (Rojkind et al., 1968). The absorbance of each tube at 230 mμ was determined. Using a Packard Tricarb Liquid Scintillation counter, aliquots of every fourth tube (0.05 ml) were assayed for radioactivity employing 0.5 ml Hyamine hydroxide and 15 ml of scintillation fluid composed of toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis 2-(5-phenyloxazolyl) benzene. The efficiency of counting was 22 to 25%. The distribution of radioactivity and absorbance at 230 mμ are given in Figure 1. The last peak contained 10% of the material absorbing at 230 mμ and 4% of the total counts; this was dried and then desalted on Bio-Gel P-2. The peptide fraction thus obtained from several runs was pooled, lyophilized and dissolved in 10 ml of distilled water.

Aliquots (1 ml) of the crude fraction were applied to Whatman 3 MM paper and electrophoresis carried out at 2,000 V and 110 ma for 90 minutes in a pyridine-acetate

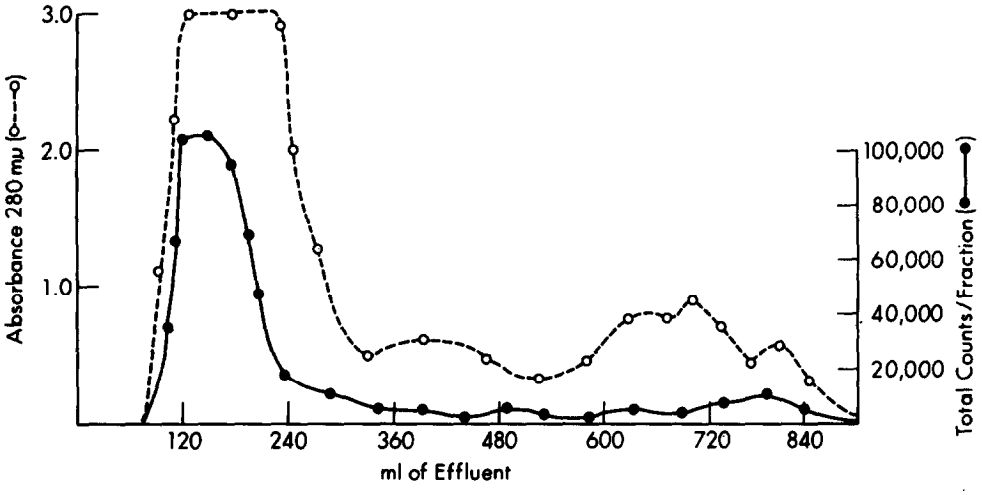

Figure 1. Absorbance at 230 mμ (o--- o) and distribution of radioactivity (●—●) on DEAE cellulose of a collagenase digest of rat skin collagen reduced with $NaBT_4$.

TABLE I

AMINO ACID COMPOSITION OF THE ALDEHYDE CROSS-LINKED PEPTIDE FROM RAT SKIN COLLAGEN

Amino Acid	Preparation 1	Preparation 2
Hydroxyproline	0.0	Present
Aspartic Acid	2.0	2.0
Threonine	0.0	0.0
Serine*	4.0	3.5
Glutamic Acid	2.0	3.0
Proline	4.0	5.0
Glycine	7.0	6.0
Alanine	2.0	2.0
Valine	3.0	3.0
Methionine + Sulfoxides	2.0	T
Isoleucine	0.0	0.0
Leucine	0.0	0.0
Tyrosine*	1.0	1.0
Phenylalanine	0.0	0.0

T Present but not determined.
* Not corrected for losses during hydrolysis.

buffer, pH 6.0. A strip from the center was cut into pieces of 1 x 2 cm which were assayed for radioactivity. The material from the radioactive zone was eluted from the paper with water and evaporated to dryness. One ml aliquots were hydrolyzed and the amino acid composition determined on a Beckman Amino Acid analyzer. The results obtained (Table I) clearly indicate that this peptide is indeed identical or similar to that known to contain the intramolecular cross-link (Bornstein and Piez, 1966; Rojkind et al., 1968).

A sample of the peptide containing 120,000 cpm was concentrated to dryness, dissolved in 0.5 ml of 2 N NaOH, sealed in an alkaline resistant tube and hydrolyzed at 110° for 20 hrs. The hydrolysate was acidified to pH 2.0 and the volume brought to 5 ml. A 2.5 ml aliquot was chromatographed on the Technicon Amino Acid Analyzer using the gradient of Burns et al. (1965). The eluant was divided so that 50% was collected in fractions of 1.3 ml and 50% analyzed directly by reaction with ninhydrin. One ml of each fraction was assayed for radioactivity in Bray's solution (1960) using a Packard Model 3310 Liquid Scintillation Spectrometer. The distributions of ninhydrin and tritium in the chromatogram are depicted in Figure 2A. Another aliquot of the peptide was subjected to oxidation by performic acid by the method of Moore (1963). The excess performate was removed by lyophilization after diluting the sample with water. The oxidized material was dissolved in 1.0 ml of water and 0.1 ml of a 0.1 M solution of sodium periodate added. The reaction was kept in the dark for one hour and 0.2 ml of glycerol added to stop the reaction. The sample was evaporated to dryness several times and redissolved in water. Radioactivity in the original and treated samples was determined as previously described.

RESULTS AND DISCUSSION

Different preparations of reduced collagen contained a total of 2 to 6 molar equivalents of compounds reduced by $NaBT_4$ per α chain. Samples treated at pH 8.0 for 30 minutes or

less contained the smallest values and those treated at pH 9.0 for more than 1 hour contained the higher values for reduced aldehydes.

The chromatography of the collagenase digest on DEAE showed a similar pattern to that previously reported for a collagenase digest of collagen treated with 2,4-dinitrophenylhydrazine (Rojkind et al., 1968).

The amino acid composition of the pure peptide (Table I) is similar to the analysis already reported for the same peptide obtained as the 2,4-dinitrophenylhydrazone derivative (Rojkind et al., 1968) and the peptide ($\beta_{11}CB_1$, or $\beta_{12}CB_1$) isolated after cleavage of purified β chains with CNBr (Bornstein and Piez, 1966). Using two different preparations, the aldehyde content calculated from specific activity was one residue per peptide.

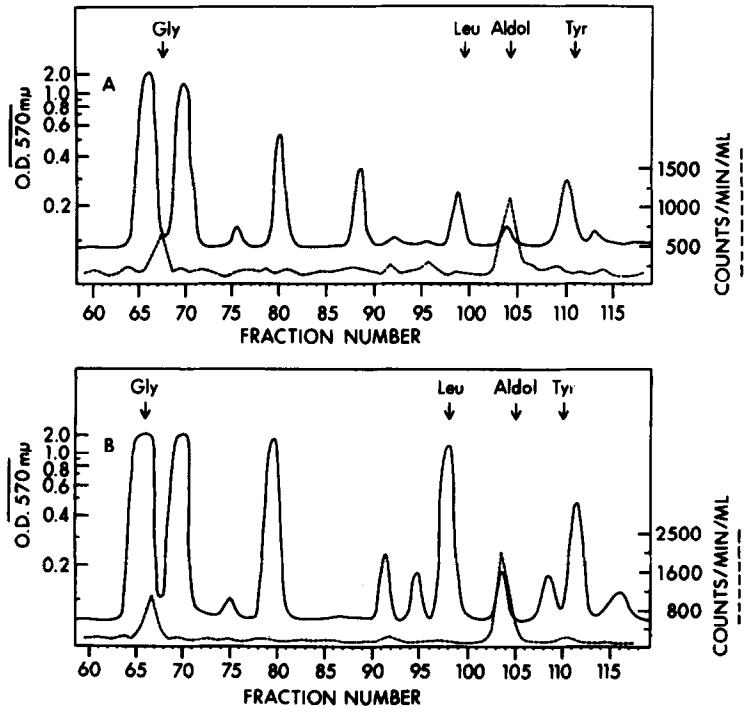

Figure 2. A. Distribution of ninhydrin (—) and tritium (---) of the peptide obtained from rat skin collagen, hydrolyzed in 2N NaOH.

B. Distribution of ninhydrin (—) and tritium (---) of elastin previously reduced with $NaBT_4$ and hydrolyzed in 2N NaOH.

Since the reduced forms of the aldehydes involved in intramolecular cross-linking are unstable to acid hydrolysis but are stable to hydrolysis in 2 N NaOH (Lent et al., 1969), base hydrolysis was used in these studies. As shown in Figure 2A, two radioactive compounds were obtained in the rat skin peptide hydrolysate, one eluting proximate to glycine (ε-hydroxynorleucine) and the second between leucine and tyrosine. As shown in Figure 2B, the second peak is identical with the aldol condensation product of two residues of α-aminoadipic acid-δ-semialdehyde already identified in elastin (Lent et al., 1969). In order to confirm that the peak eluting between leucine and tyrosine is indeed the aldol condensation product, the base-hydrolyzed peptide was further hydrolyzed in 6 N HCl. As was shown in the case of the aldol in elastin (Lent et al., 1969), this peak also disappeared after this treatment. The primary proof of structure of the aldol condensation product, as a derivative obtained after reduction with tritiated sodium borohydride, has already been carried out by the following procedures: mass spectral analysis of the ethyl ester derivative; characterization of products after its oxidation by periodate-permanganate; and identification of products after catalytic hydrogenation (Lent et al., 1969).

The presence of approximately 30% of the counts as ε-hydroxynorleucine suggests that the peptide from the β chains is contaminated with peptides deriving from α chains which contain the saturated precursor aldehyde.

These results and those of Kang, Faris and Franzblau (manuscript submitted) are strong evidence that the intramolecular cross-link in collagen is indeed the aldol condensate of two α-aminoadipic acid-δ-semialdehyde residues.

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