THE IN VITRO FORMATION OF INTERMOLECULAR CROSS-LINKS IN CHICK SKIN COLLAGEN

Andrew H. Kang[1], Barbara Faris and Carl Franzblau[2]

Developmental Biology Laboratory, Departments of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, and Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts.

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Summary

Purified chick skin collagen was aggregated into native type fibrils in vitro and the intermolecular cross-links were stabilized by reduction with $NaBT_4$. Ion exchange chromatography and scintillation spectrometry of the resulting insoluble collagen after acid or alkaline hydrolysis indicated the presence of e-hydroxylysinonorleucine, lysinonorleucine and a third radioactive component of unknown structure eluting near histidine. The intramolecular cross-link, the aldol condensation product of two residues of α-aminoadipic δ-semialdehyde was no longer detectable in these reconstituted fibrils. Data presented here suggest that the intermolecular cross-link formation closely involves the intramolecular cross-link.

It has been clearly established that the intramolecular cross-link of soluble collagen is the aldol condensation product (ACP) of two residues of α-aminoadipic δ-semialdehyde (Kang et al., 1969a; Rojkind et al., 1969). Evidence to date indicates that this cross-link occurs near the NH_2-terminal region of the collagen molecule (Bornstein et al., 1966; Kang et al., 1969b; Rojkind et al., 1968). Recently, evidence has been presented by Bailey and Peach (1968) suggesting that the Schiff

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[1]Senior Investigator of the Arthritis Foundation at the Massachusetts General Hospital

[2]Established Investigator of the American Health Association.

base form of e-hydroxylysinonorleucine participates as an intermolecular cross-link in rat tail tendon collagen. Further studies on chick bone and bovine dentine by Bailey et al. (1969) revealed still another cross-link which was characterized as the aldol condensation product of one residue each of α-aminoadipic δ-semialdehyde and α-aminoadipic-δ-hydroxy-δ-semialdehyde. The latter compound arises from deamination of a residue of hydroxylysine. Since the above authors, working with insoluble collagenous tissue, did not study the integrity of the intramolecular cross-link, it was of interest to examine the possible relationship between the intramolecular and intermolecular cross-links. Our data suggest that, in vitro, native type fibril formation involves the disappearance of the intramolecular cross-link, described previously, with the concomitant appearance of hydroxylysinonorleucine, lysinonorleucine and another reducible component whose structure is as yet unknown. In a similar approach, Tanzer and Mechanic (1968) earlier showed the appearance of two new unidentified cross-links when native soluble collagen was converted to an insoluble fibril.

Materials and Methods

Purified, acid-extracted collagen was prepared from the skins of 3-week-old white leghorn chicks according to the procedure of Kang et al. (1969b). Native type fibrils were formed from the soluble collagen by the methods described by Gross and Kirk (1958). Briefly, acid-extracted collagen is dissolved in 0.1N acetic acid and dialyzed versus 0.05M Tris buffer (pH 8.5) containing 0.15M NaCl. The dialyzate is centrifuged and aliquots of the supernatant are incubated at 37°C. Fibril formation usually begins within one half hour after incubation. In a separate experiment, nonstriated fibrils were formed from the collagen

solution by the addition of NaCl to the final concentration of 20%. The ultrastructure of the fibril preparations was examined in each instance by the use of an RCA EMU 3G electron microscope after staining with 0.5% solution of uranyl acetate.

Reduction was carried out with $NaBT_4$ (200 mc/mM) as described previously (Kang et al., 1969a). After removal of the excess reagents by dialysis versus 0.1N acetic acid, the proteins were hydrolyzed in 2N NaOH or constant boiling HCl for 24 hours, and analyzed on an automatic amino acid analyzer equipped with a split stream device as described previously (Kang et al., 1969a). A portion of the effluent was continuously monitored for ninhydrin reactivity and the remaining portion was collected in fractions of 1.3ml. Radioactivity of the individual fractions was determined in a liquid scintillation counter employing Bray's solution (Bray, 1960).

Results

Soluble Collagen. A solution of collagen was reduced with $NaBT_4$ at 5°C for two hours. For each mg. of protein present, 0.1 mg. of $NaBT_4$ was used. Alkaline hydrolysis revealed the presence of ε-hydroxynorleucine and the ACP of two residues of α-aminoadipic δ-semialdehyde described previously (Kang et al., 1969a; Rojkind et al., 1969; Lent et al., 1969). The radioactivity profile of a reduced soluble collagen is presented in Figure 1A.

Native Fibrils. In a separate experiment the soluble collagen was aggregated into native type fibrils by incubation at 37°C. The fibrils were reduced in suspension with $NaBT_4$. It was observed that the reduced native type fibrils were completely insoluble upon dialysis versus 0.1N acetic acid. A portion of the reduced fibrils was subjected to

alkaline hydrolysis. Figure 1B depicts the radioactivity profile. Reduced ACP was not detected in the reduced fibrils. Instead, three new radioactive peaks were present. One eluting near hydroxylysine was shown to be e-hydroxylysinonorleucine by cochromatography with synthetic hydroxylysinonorleucine (Bailey and Peach, 1968). When this

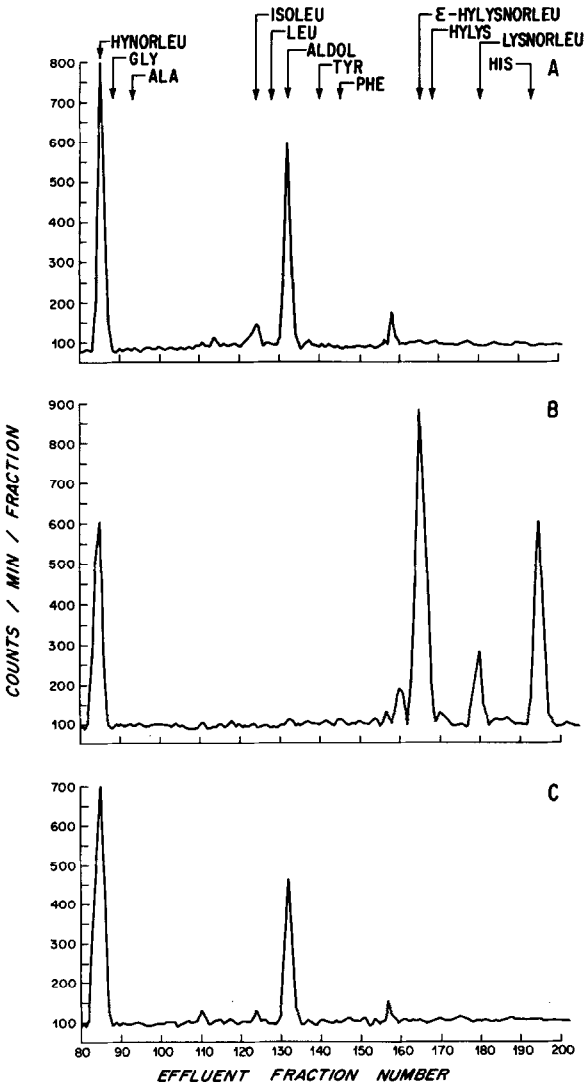

Figure 1. Elution patterns of alkaline hydrolyzates of reduced collagens. A) Collagen reduced in solution. B) Collagen reduced in the form of native type fibrils. C) Collagen reduced in the form of nonstriated fibrils.

compound obtained by pooling the appropriate fractions from several runs was desalted and treated with $NaIO_4$, subsequent chromatography indicated the presence of radioactive lysine as predicted. Another radioactive fraction eluting near ammonia cochromatographed with synthetic lysinonorleucine (Franzblau et al., 1969). A third radioactive component eluting just after histidine was always present but its structure has not yet been determined. Radioactivity profiles of HCl hydrolyzates were similar to that of alkali hydrolyzates except for the presence in the former of e-chloronorleucine and the concomitant decrease in the size of the e-hydroxynorleucine peak (Lent et al., 1969).

Nonstriated Fibrils. Nonstriated fibrils prepared as described above were reduced with $NaBT_4$ in suspension in a manner similar to the native type fibrils. In contrast to the reduced native type fibrils, the reduced nonstriated fibrils became completely soluble upon dialysis versus 0.1N acetic acid as described by Tanzer (1968). The radioactivity profile of an alkaline hydrolyzate is shown in Figure 1C. It is essentially identical to that of reduced soluble collagen (Figure 1A); i.e., reduced ACP is present, however, no e-hydroxylysinonorleucine, lysinonorleucine or the radioactive component eluting near histidine could be detected. This further suggests Tanzer's earlier suggestion that a specific alignment of the collagen molecule is necessary for the formation of intermolecular cross-links and its insolubilization.

Fibril Formation From Reduced Soluble Collagen. Soluble collagen which had been reduced with $NaBT_4$ in solution could be aggregated into native type fibrils as judged by electron microscopy. These fibrils were then treated again in suspension with $NaBT_4$. Upon dialysis versus 0.1N acetic acid, these fibrils became readily solubilized as has been

shown by Tanzer (1968). The radioactivity profile of alkaline or HCL hydrolyzate was indistinguishable from that of reduced soluble collagen, indicating that a functional group or groups reducible by $NaBT_4$ is necessary for the formation of intermolecular cross-links.

Table 1. Total Counts in e-Hydroxynorleucine, the Reduced ACP, e-Hydroxylysinonorleucine, Lysinonorleucine and "Post-histidine" Peak[a]

	e-Hydroxy-norleucine	ACP	e-Hydroxylysino-norleucine	Lysino-norleucine	"Post-histidine" peak
Collagen Solution	7.8×10^3	4.8×10^3	---	---	---
Native Type Fibril	5.0×10^3	---	8.0×10^3	1.0×10^3	5.0×10^3
Nonstriated Fibril	7.5×10^3	3.1×10^3	---	---	---

a) Total counts per 10 mg. of protein. The amounts of protein were calculated from amino acid analyses assuming twelve residues of phenylalanine per 1000 residues of amino acid. A dash indicates a value less than 200 counts per minute.

Discussion

From the in vitro reconstituted native type fibrils of chick skin collagen we isolated three radioactive cross-link compounds after $NaBT_4$ reduction. Of these, e-hydroxylysinonorleucine and the compound eluting near histidine were also shown to be present in rat tail tendon by Bailey and Peach (1968). The occurrence of lysinonorleucine in collagen, however, has not been identified until the present[1], although it has been shown to serve as a cross-link in elastin (Franzblau et al., 1969). It has been suggested by Bailey and Peach (1968) that e-hydroxylysinonorleucine occurs by a reduction of a Schiff base formed by the carbonyl group of a residue of α-aminoadipic δ-semialdehyde and the

1. During the preparation of this manuscript, we learned that Tanzer also found lysinonorleucine in the hydrolyzate of the borotritide reduced calf skin collagen (Private communication).

e-amino group of a residue of hydroxylysine. Since it is known that the hydroxylation of the lysyl residues in collagen chains is incomplete (Butler, 1968; Lane and Miller, 1969), the occurrence of lysinonorleucine is not surprising. The distribution of radioactivity among various compounds obtained by reduction of various forms of collagen is summarized in Table I. The most striking data presented is that showing the formation of the intermolecular cross-link compounds is invariably associated with disappearance of the intramolecular cross-link. This is consistent with the observation that in the CNBr digests of reduced collagen fibrils β12-CB1, the peptide containing the intramolecular cross-link, is not present (A.H. Kang and J. Gross, unpublished data). Whether the ACP becomes incorporated into any of the intermolecular cross-links directly, or whether it undergoes reversible dissociation to the α-aminoadipic δ-semialdehyde to participate in the formation of intermolecular cross-links is not clear at present but is a subject for investigation.

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