INTRAMOLECULAR CROSS-LINK OF CHICK SKIN COLLAGEN*

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Summary

Purified chick skin collagen constituent chains were examined for the content of the aldol condensation product of two residues of α-aminoadipic δ-semialdehyde after reduction with $NaBT_4$ and alkali hydrolysis. The single chains, α1 and α2, contained none whereas their cross-linked dimer, β_{12}, contained one equivalent. These findings were confirmed by the similar studies performed on the peptides derived from the cross-link region of collagen by CNBr cleavage. Data presented here strongly indicate that the aldol condensation product is the intramolecular cross-link.

Definitive understanding of physiologic maturation of collagen requires full knowledge of time dependent changes that occur in the collagen molecule after the synthesis of polypeptide chains is complete and the molecule is extruded into extracellular connective tissue. One of these is the formation of intramolecular, interchain covalent cross-links. Recent studies on rat skin collagen (Bornstein and Piez, 1966; Rojkind et al., 1968) and on chick skin collagen (Kang et al., 1969b) indicate that two specific lysyl residues located near the NH_2-termini of adjacent polypeptide chains become oxidatively deaminated to form α-aminoadipic δ-semialdehyde residues and that they then condense to form the crosslink. Direct evidence, however, for the chemistry of the cross-link was not obtained. Recently, Lent et al. (1969) have described the presence of the aldol condensation product (ACP) of two residues of α-aminoadipic δ-semialdehyde in elastin. This was achieved by reducing elastin with sodium borohydride and hydrolyzing the protein in 2 N NaOH. The same ACP has also been shown to be present in small amount in calf skin collagen (Paz et al., 1969). Using these procedures, we examined the purified α1 and α2 chains and the cross-linked dimer of these, the β_{12} component, as well as the cyanogen bromide (CNBr) peptides derived from the cross-link

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region of chick skin collagen. In this paper, we report evidence that the intramolecular cross-link in chick skin collagen is indeed the ACP of α-amino-adipic δ-semialdehyde.

Materials and Methods

Chick skin collagen was isolated and purified according to the procedure of Kang et al. (1969a). Purified α1, α2, and β_{12} chains were obtained by chromatography of gently heat-denatured, solubilized collagen on CM-cellulose as described elsewhere (Kang et al., 1969b). Preparation of the CNBr peptides from the cross-link region, α1-CB1^{Ald} and α2-CB1^{Ald}, and their precursors, α1-CB1 and α2-CB1, and the cross-linked peptide, β_{12}-CB1, was performed as described previously (Kang et al., 1969b). Reduction of the α1, α2 and β_{12} chains as well as the peptide fragments with $NaBT_4$ was carried out in 0.001 M EDTA, pH 9.0, as described by Gallop et al., (1968).

Amino acid analyses were performed on a Technicon amino acid analyzer employing the gradient described by Burns et al. (1965). In all cases a split stream device was employed. That portion of the eluent which had not been analyzed for ninhydrin reactivity was collected in fractions of 1.3 ml. Radioactivity in the individual fractions was determined in a liquid scintilation counter employing Bray's solution (Bray, 1960).

Results

The α1 and α2 chains and the β_{12} component. The α1 and α2 chains were obtained from lathyritic chick skin collagen to minimize possible contamination of the preparation by the β components. The β_{12} component was obtained from normal chick skin collagen as described above.

Each was reduced separately and hydrolyzed in 2 N NaOH at 110° for 22 hours in a sealed alkali-resistent vial. The radioactivity in the fractions corresponding to ε-hydroxynorleucine and the reduced ACP are given in Table I. It is clear from the data that the reduced ACP is present only in β_{12} and not in α1 or α2. The specific activity of the reduced ACP was 2.1×10^6 dpm per μmole. Based on the ninhydrin reaction, there was 0.4 residue of reduced ACP per two tyrosyl residues in β_{12}. Since there are two tyrosyl residues per α chain (both α1 and α2, see Kang et al., 1969b) and four tyrosyl residues per β_{12} component, there is 0.8 residue of reduced ACP per β_{12}. If the alkali-hydrolyzed reduced β_{12} is then hydrolyzed in 6 N HCl, the counts which occur in the reduced ACP region disappear. Similar acid lability of the reduced ACP has been also observed in elastin (Lent et al., 1969). The amino acid chromatogram for the reduced β_{12} component in the region between isoleucine and phenylalanine, is shown in Figure 1. Both α1 and α2 did not contain any ACP upon amino acid analysis.

The presence of the ACP in β_{12} and its absence in α1 and α2 suggests

TABLE I. Total Counts in ε-Hydroxynorleucine and the Reduced ACP[a]

	ε-Hydroxynorleucine	Reduced ACP
α1	22,000	260
α2	16,850	200
β_{12}	<500	26,800
β_{12} acid[b]	<200	<200

a. Total counts per 10 mg of protein. The amount of protein was calculated from amino acid analyses assuming four residues of tyrosine, eighteen residues of valine and twenty-seven lysine residues per α chain.
b. Hydrolysis in 6 N HCl was preceded by hydrolysis in 2N NaOH.

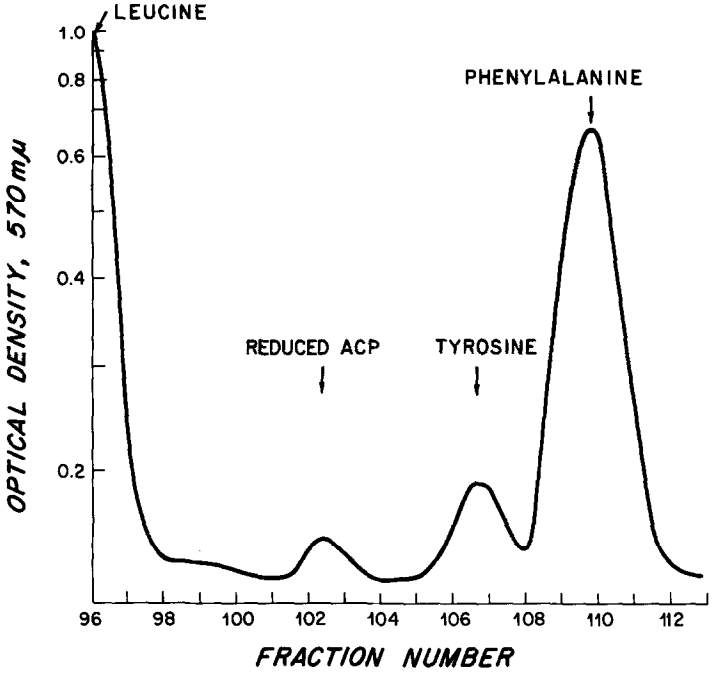

Figure 1. The amino acid chromatogram of reduced β_{12} in the tyrosine region where the reduced ACP is eluted.

strongly that ACP is the cross-linking amino acid. This conclusion is further supported by the data obtained from studies on the CNBr peptides derived from the cross-link region of the collagen molecule.

The CNBr Peptides from the Cross-link Region, α1-CB1^{Ald}, α2-CB1^{Ald} and β_{12}-CB1. Examination of these peptides, derived from the NH_2-terminal region

of collagen chains by CNBr cleavage, gave the following results. α1-CB1^{Ald} and α2-CB1^{Ald} contained a radioactive peak for ϵ-hydroxynorleucine indicating the presence of α-aminoadipic δ-semialdehyde in these peptides. There were no counts or ninhydrin peaks in the region where the reduced ACP normally eluted. On the other hand, β_{12}-CB1 showed a relatively large peak for the reduced ACP. Both the amino acid chromatogram and radioactivity profile of reduced β_{12}-CB1 is given in Figure 2. Based on the fact that there are three tyrosyl residues in β_{12}-CB1 (Kang et al., 1969b), there is 0.4 residue of the reduced ACP. This is only 40% of the theoretical value. However, decreased amounts of the reduced ACP have been observed after reduction of an elastase digest of elastin. The values obtained for reduced ACP were significantly lower than one normally finds after reduction of elastin (C. Franzblau, unpublished data).

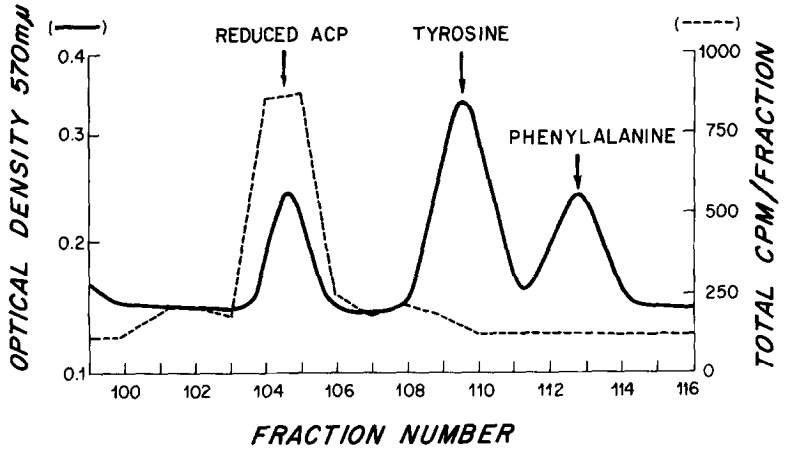

Figure 2. The tyrosine region of the amino acid chromatogram and radioactivity profile of reduced β_{12}-CB1.

Discussion

Spectral analyses of the N-Methyl benzothiazolone hydrazone derivative of the cross-link containing peptide, β_{12}-CB1, from rat skin collagen (Bornstein and Piez, 1966; Rojkind et al., 1968) and chick skin collagen (Kang et al, 1969b) suggested the presence of an α,β-unsaturated aldehyde. However, the exact structure of the cross-linking amino acid was not established then. The isolation and characterization of the ACP of two residues of α-aminoadipic δ-semialdehyde from elastin (Lent et al., 1969), and its presence in small amounts in calf skin collagen (Paz et al., 1969) stimulated us to explore the intramolecular cross-link of collagen.

The presence of stoichiometric amounts of the ACP in the cross-linked β_{12}

component but its absence in the single chains, $\alpha 1$ and $\alpha 2$, provide strong evidence that the ACP is the cross-link. This is further supported by the data obtained from the studies of the CNBr peptides derived from the cross-link region. The acid lability of the ACP as described previously (Lent et al., 1969) and observed in the present study explains the failure to identify the cross-link in the previous studies on rat skin and chick skin collagens.

Although only chick skin collagen was examined in this study, available evidence indicates that the mechanism of intramolecular cross-link formation is common to rat skin and chick skin collagens (Kang et al., 1969b; Rojkind, M., manuscript in preparation). Hence we suggest that the ACP is the intramolecular cross-linking amino acid in vertebrate collagens.

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