THE ISOLATION OF A SOLUBLE TYPE III COLLAGEN PRECURSOR FROM RAT SKIN.*

Joan Anesey, Paul G. Scott, Arthur Veis and Douglas Chyatte
Department of Biochemistry, Northwestern University Medical School
Chicago, Illinois 60611

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SUMMARY: A soluble form of type III collagen has been isolated from the 1.0 M NaCl neutral salt soluble extract of rat skin. This component has a molecular weight of 350,000 and is converted by reduction and alkylation to three identical α-chains with molecular weight 118,000. Segment-long-spacing precipitates produced from the renatured disulfide-linked component are about 300 Å longer than collagen with extensions at both the amino and carboxyl terminal ends. Pepsin treatment removes both amino and carboxyl terminal extensions. These and radioisotope incorporation data lead to the conclusion that this component is a precursor of the [α1(III)$_3$] collagen.

Type III collagen, containing α1(III) chains, has been isolated from skin and other tissues following solubilization of the tissue by pepsin digestion (1,2). Acid soluble collagen does not appear to contain appreciable amounts of type III (3). The distinguishing characteristics of type III collagen are a high content of glycine, a high content of hydroxyproline, an hydroxyproline-proline ratio greater than 1, and the presence of cysteine in the pepsin digested molecule (1).

In the course of studies on the biosynthesis of rat skin collagen, ^{35}S-cystine and ^{3}H-proline were used to label the procollagen and any intermediates produced. CM-cellulose chromatography by the procedure of Veis et al. (4) of neutral salt-soluble collagen showed procollagen in the expected position labeled with both ^{3}H and ^{35}S. In addition, however, a second ^{35}S-labeled component was detected in the chromatograph in the region of the α2 component in this system. We report here the isolation and partial characterization of this ^{35}S-labeled collagen component. As shown below, we believe this component to be a soluble precursor of type III collagen.

MATERIALS AND METHODS: Sprague-Dawley rats, 120-180 g weight, were given

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intracardiac injections of ∿0.8 millicurie ^{3}H-proline (G) (1.9 Ci/mM) and 0.8 millicurie ^{35}S-cystine (1.4 Ci/mM). Thirty minutes after injection the rats were decapitated and the skins removed and chilled in a 0.005 M Tris, 0.01 M EDTA, 0.02 M NaCl, pH 7.4 buffer at crushed ice temperature. The skins were frozen in liquid N_2, shattered, then extracted three times with twice their weight of 0.15 M NaCl-Tris buffer, pH 7.4 for 16-24 hour periods. The residual skin was then extracted three times with 1.0 M NaCl-Tris buffer, pH 7.4. Collagen was salted out of both the 0.15 M and 1.0 M extracts by making the solutions 17% in NaCl. Each precipitated collagen was redissolved and reprecipitated twice to achieve further purification.

The purified 1.0 M extract collagen was redissolved in the 1.0 M NaCl buffer and dialyzed into 0.06 M sodium acetate-acetic acid, pH 4.8 starting chromatography buffer, at 4°C. The collagen was denatured at 45°C for 1/2 hour, then chromatogramed on Whatman CM-52 carboxymethyl cellulose by the procedure of Veis et al. (4). The effluent was monitored for the presence of ^{3}H and ^{35}S by removing aliquots of each fraction and counting in an Isocap 300 scintillation counter.

The material recovered in the region of $(\alpha 2)_h$ (4) was taken for chromatography on Agarose A5 in 1.0 M $CaCl_2$ - 0.05 M Tris-HCl, pH 7.5 (5).

Reduction and alkylation was carried out following the procedure of Monson and Bornstein (6).

Pepsin digestion was carried out at 4°C for 16 hours at a collagen/pepsin ratio of 100/1 in 0.05% HAc, following renaturation of the collagen. For renaturation, the appropriate fractions from Agarose chromatography were dialyzed against 0.5 M acetic acid at 4°C. The acetic acid solution was warmed briefly (10 minutes) to room temperature, then cooled to 4°C before pepsin addition. SLS* precipitates were formed by the addition of ATP to the renatured acid solutions.

SDS-gel** electrophoresis was carried out by the method of Furthmayr and

*SLS - Segment-long-spacing form of collagen precipitate.

** SDS-gel - Sodium dodecyl sulfate, 0.10% in 5% polyacrylamide.

TABLE I

AMINO ACID COMPOSITION OF γ-LIKE COMPONENT
ISOLATED FROM AGAROSE A5 GEL FILTRATION.
Residues/1000 Amino Acid Residues

4-Hydroxyproline	118
Aspartic acid	55
Threonine	13
Serine	39
Glutamic acid	76
Proline	105
Glycine	362
Alanine	87
Valine	14
Methionine[a]	(5)
Isoleucine	12
Leucine	16
Tyrosine	0.4
Phenylalanine	7.5
Hydroxylysine	7.8
Lysine	29
Histidine	9.7
Arginine	47
1/2 Cys	1.6

[a] Methionine plus methionine sulfoxide.

Timpl (7) using an ORTEC pulsed current slab gel apparatus.

Amino acid analyses were carried out following hydrolysis in sealed tubes in 6 N HCl, 22 hours, using a JEOL 6AH analyzer and a single column procedure.

RESULTS: The 30 minute labeling procedure was sufficient to permit incorporation of both ^{35}S-cysteine and ^{3}H-proline into the neutral salt soluble collagen. CM-cellulose chromatography of the purified 1.0 M extract collagen showed ^{3}H-labeling in the regions previously identified as containing the components $\alpha 1_h$ and $\alpha 2_h$. The α_h components have molecular weights about 10% greater than the α_ℓ components and it has been suggested (4) that these arise as intermediates between procollagen and collagen. However, in the present study the ^{35}S-cysteine appeared in the region of the $\alpha 2_h$ as well as in the urea eluted fraction, U-1, previously described by Clark and Veis (8).

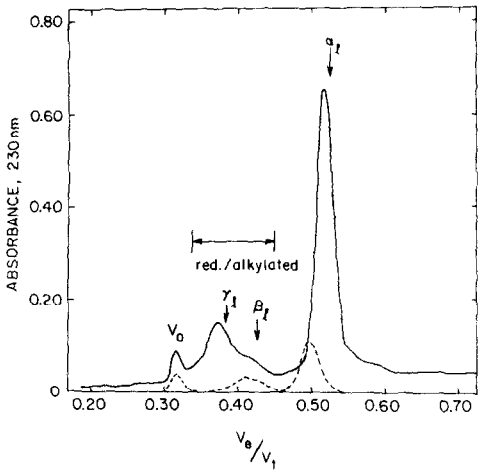

Figure 1 Gel filtration of $(\alpha 2)_h$ fraction from CM-cellulose chromatography on Agarose A5. The solid line represents the optical density of the $(\alpha 2)_h$ fraction. The major component is $(\alpha 2)_h$ and this fraction was free of ^{35}S-cysteine. The ^{35}S was exclusively included in the γ-component region. The fractions under the horizontal bar in the diagram were collected, reduced and alkylated and rechromatogramed, yielding the trace indicated by the dashed line. The ^{35}S was transferred to the major fraction eluting in the region just ahead of $(\alpha 2)_h$. Note that the small amount of β-component was not altered by the reduction and alkylation. The elution positions of $(\alpha)_\ell$, $(\beta)_\ell$ and $(\gamma)_\ell$ (weights 95,000; 190,000; and 285,000 daltons) on this column are indicated by vertical arrows.

Gel filtration of the material eluting from CM-cellulose in the $\alpha 2_h$ region revealed that the ^{35}S-label was not associated with the $\alpha 2_h$-chain, but instead with a component having a molecular weight near 350,000. This component was clearly collagenous (Table I). The component α-chains of this collagen were held together by disulfide linkages since, as shown in Figure 1, reduction followed by alkylation converted the γ-like component into individual α-like chains. On columns calibrated using known cyanogen bromide peptides from type I collagen and intact $\alpha 2_\ell$ and $\alpha 2_h$ components, the separate chains from the ^{35}S-containing Agarose fraction were determined to have a molecular weight of $\sim$118,000. The reduced and alkylated material migrated as a single component on SDS acrylamide gel electrophoresis in a system which cleanly separates $\alpha 1$- and $\alpha 2$-chains, demonstrating that the initial γ-like component was composed of three identical chains.

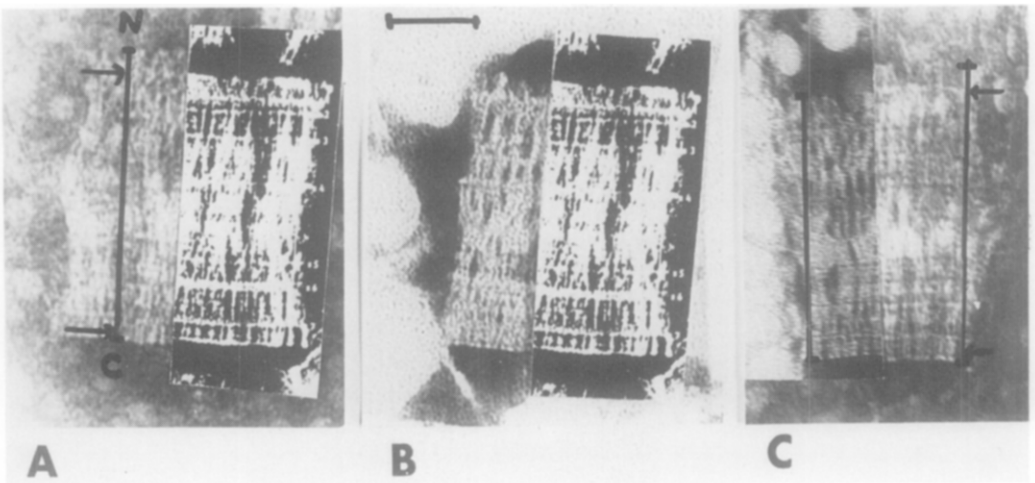

Figure 2 Electron micrographs of segment-long-spacing collagen, negatively stained with phosphotungstate. A. Comparison of renatured γ-component (left) with SLS from acid-soluble rat tail tendon collagen prepared by Olsen (10) (right). B. Comparison of pepsin digested renatured γ-component (left) and standard tendon preparation (right) showing correspondence in molecular lengths with only slight erosion at the γ-component NH_2-terminal end. C. Direct comparison of γ-component (right) and pepsin treated γ-component (left). The vertical line running through the renatured γ-component SLS in A delineates the length of the SLS segments. The arrows mark the positions of the amino terminal (marked N) and carboxyl terminal (marked C) ends of the standard SLS preparation on the right with all bands matched through the helical region. Likewise in C the vertical lines denote the lengths of the pepsin treated and untreated renatured γ-components. The arrows on the right hand show the positions of the ends of the pepsin treated SLS with all internal helical region bands matched. The horizontal bar in B denotes the length of 1000 Å in all three electron micrographs.

The denatured γ-like component isolated from the initial Agarose chromatography was renatured as described by the procedure of Veis *et al.* (9) and SLS collagen was produced by precipitation with ATP. The SLS, Figure 2a, show two special features when compared with type I collagen. First, there is an extra band approximately 50 Å in length at the carboxyl terminal end. In addition, there is an amino terminal extension approximately 250 Å in length.

When the renatured γ-like component was treated with pepsin, the amino- and carboxyl-terminal extensions were removed without destruction of the central region of the molecule (Figure 2b).

Kinetic studies on the incorporation and disappearance of ^{3}H-proline, to be reported elsewhere, indicate that the γ-like component in the skin is rapidly converted to the insoluble form. The specific activities of the γ-like component, $\alpha 2_h$ and $\alpha 2_\ell$ all isolated from the same tissue extract are shown in Table II. Although the question of relative pool size must be taken into account it seems evident that the specific activity of the γ-like component is higher than that of the type I intermediate $\alpha 2_h$ and of $\alpha 2_\ell$ in absolute terms of dpm per micromole of hydroxyproline or proline.

CONCLUSIONS: The amino acid analysis data presented in Table I identify the γ-like component as being related to type III collagen (1,2), for example, this component contains 362 residues of glycine, 1.6 residues of 1/2 cystine and 118 residues of hydroxyproline per 1000 total amino acid residues. The hydroxyproline to proline ratio is 1.12.

The observations that the γ-like component has a molecular weight on the order of 350,000, a length approximately 300 Å longer than normal type I collagen, and a higher specific activity in terms of incorporation of ^{3}H-

TABLE II

SPECIFIC ACTIVITIES OF HYDROXYPROLINE AND PROLINE
IN ISOLATED COLLAGEN COMPONENTS FROM
THE SAME COLLAGEN EXTRACT.
1.0 M NSC, 30 minute pulse label ^{3}H-proline.

Component[a]	DPM/μmole Hydroxyproline	DPM/μmole Proline
$\alpha 2_\ell$	1.8×10^3	2.2×10^3
$\alpha 2_h$	5.8×10^3	6.9×10^3
disulfide-linked γ	8.3×10^3	7.8×10^3

[a] All fractions purified by A5 gel filtration chromatography.

proline into both hydroxyproline and proline than the α2-chain of type I collagen all lead us to conclude that the soluble component is either pro-$\alpha 1(III)_3$ or a partially degraded high molecular weight intermediate, $[\alpha 1(III)_h]_3$, in the notation of Veis et al. (4). This interpretation is consistent with the observation that pepsin digestion of this γ-component in the native state reduces its SLS length to that of collagen SLS spools. Finally, it is evident that the extensions of the type III chains are on both amino- and carboxyl-terminal ends.

The precursor of $[\alpha 1(III)_3]$ is thus present in significant amounts in rat skin in vivo and is readily extractable in neutral salt solutions. These data provide another example demonstrating that procollagens may have a relatively long survival time within the extracellular compartment of normal skin, at least in rapidly growing animals.

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