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Evidence for an Amino-Terminal Extension in High-Molecular-Weight Collagens from Mature Bovine Skin

(procollagen/biosynthetic precursors/fibril formation/renaturation/electron microscopy)

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ABSTRACT **Insoluble, mature collagen fibers from bovine skin have been partially solubilized by mild, denaturing, but nonhydrolytic means. The soluble denatured collagen was fractionated by alcohol coacervation, and a fraction rich in high-molecular-weight α-chains was obtained. The heavy α-chains were isolated by carboxymethylcellulose chromatography. Renaturation, followed by measurements of optical rotation at 365 nm, showed that stable, in-register renaturation was more readily accomplished in mixtures of heavy α-chains than in $\alpha 1$-β_{11}-chain mixtures. Renatured heavy α-chain preparations were precipitated in SLS form, negatively stained, and examined by electron microscopy. The SLS precipitates were compared with SLS segments from native soluble collagen and were found to match in band pattern and spacing along their entire length from the COOH-terminal region, except for an NH_2-terminal extension of 170 $\pm$ 30 Å in the heavy α-chain SLS. The heavy α-chains correspond chromatographically with those previously reported to be intermediates in the conversion of procollagen to collagen, on the basis of their molecular weight and of labeling studies. The presence of NH_2-terminal extensions, and their existence in mature insoluble collagen, suggest that these intermediates may have a special role in fibril formation.**

Procollagen, the intracellular precursor of extracellular collagen (1), has a molecular weight about 20% higher than the usual extractable collagen (2, 3). *In vitro* experiments (4, 5) indicate that procollagen is rapidly reduced to the collagen form during or shortly after secretion. In contrast to these *in vitro* results, Clark and Veis (6) found that both bovine skin and rat skin from mature animals contained extractable collagens that yielded high-molecular-weight α-chains, $(\alpha)_h$, when denatured. Veis *et al.* (7), using [^{3}H]proline as a label for skin collagen from actively growing rats, found that the fractions corresponding chromatographically to the $(\alpha)_h$ from mature skins were labeled in such a fashion as to suggest a precursor–product relationship between $(\alpha)_h$ and the standard α-chains, $(\alpha)_l$. These data were interpreted as indicating that procollagen is not reduced in size in a single step to collagen $(C)_l$ *in vivo*, and that the intermediate form, $(C)_h$, has a relatively long persistence time in the tissue.

Dehm *et al.* (8) were able to precipitate procollagen from *in vitro* culture systems in the SLS form and to demonstrate that the extra procollagen peptide was at the NH_2-terminus. In order to show that the $(C)_h$ intermediate also has a peptide extension at the NH_2-terminus, we have isolated the $(\alpha)_h$-components, renatured them, and prepared SLS precipitates. As demonstrated below, the renatured $(C)_h$ molecules are identical to $(C)_l$ in electron microscopic appearance, except that the $(C)_h$ have an additional NH_2-terminal peptide sequence that is 170-Å long.

EXPERIMENTAL PROCEDURES

Isolation of $(\alpha)_h$. Skin from cattle about 2 years of age was purified by the method of Veis *et al.* (9). The insoluble collagen remaining after extraction of neutral salt and acid-soluble collagens was extracted by the 60° dissociation technique of Veis and Anesey (10). The high-molecular-weight gelatin extract was fractionated by alcohol–NaCl coacervation (10). The fraction precipitating in the alcohol–water ratio range of 2.2–2.5/1.0 was chromatographed on Whatman CM-52 carboxymethylcellulose by the 40° procedure of Clark and Veis (6). The fraction of interest was that designated U-1 (6), the first peak that emerged upon elution of the column with 6 M urea, after the standard α and β components were eluted with a salt gradient according to Piez *et al.* (11). Urea was removed from U-1 by dialysis against water at 4° and the gelatin was recovered by lyophilization.

Characterization of U-1. U-1 was dissolved at 1 mg/ml in 0.15 M sodium acetate buffer at pH 4.8 and sedimentation velocity runs were made in the analytical ultracentrifuge at 40°. Similar runs were made on $\alpha 1$-β_{11} mixtures obtained from the same carboxymethylcellulose fractionation. Acrylamide gel electrophoresis was performed by the procedure of Clark and Veis (12).

Renaturation and Electron Microscopy. U-1 was dissolved in pH 3.7, 0.25 M citrate buffer at 40° at a concentration of 1 mg/ml. After 1 hr at 40°, the samples were cooled to 4° for 2 hr to initiate collagen-fold formation. After 2 hr, the temperature was raised to 25° to melt out random out-of-register hydrogen bonds and to permit tempering to perfect in-register renatured segments. After about 20 hr at 25° in citrate, the renaturing solutions were dialyzed at 25° against 0.25 M acetate at pH 3.7. The reason for this change was that U-1 did not dissolve as readily in the acetate buffer as in the citrate, but acetate was preferred in the subsequent preparation for electron microscopy. The optical rotation at 365 nm was measured during the early stages of renaturation in the citrate buffer system with a Rudolph photoelectric polarimeter and jacketed 2-dm polarimeter cells.

After transfer of the solutions to the acetate buffer, the solutions were divided into several portions; one portion was

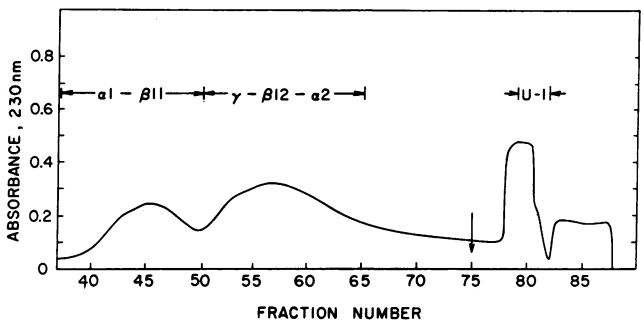

FIG. 1. Carboxymethylcellulose chromatogram of the alcohol coacervate fraction of the 60° extract of insoluble bovine-skin collagen. Chromatography at 40°. Region I, $\alpha 1$–β_{11} mixture; II, β_{12}; III, $\alpha 2$; IV, U-1; V, U-2. *Vertical arrow* indicates change to elution with 6 M urea–1.0 M NaCl.

dialyzed against H_2O, one portion was mixed with 1% acetic acid and the collagen was precipitated with ATP, and a final portion was treated for 18 hr at 25° with pepsin in a 10:1 collagen to pepsin ratio. The renatured, pepsin-treated collagen was precipitated by dialysis against water, dissolved in 1% acetic acid, and precipitated in SLS form with ATP.

The precipitate fractions were stained positively by exposure for 7 min to 1.5% phosphotungstic acid (pH 2.5), followed by 10 min exposure to saturated, unbuffered uranyl acetate. Negative staining was with 1% phosphotungstic acid at pH 7 for 2 min. The stained samples were examined in an Hitachi HU-11A electron microscope.

RESULTS AND DISCUSSION

The bovine-skin collagen extracted at 60° was selected because prior experience (10) had shown that the extract had substantial U-1 content. The alcohol coacervate fraction used was enriched in the U-1 component. As indicated in the chromatogram in Fig. 1, about 34% of the fraction was collected in the U-1 peak. Acrylamide gel electrophoresis showed U-1 to contain the expected mixture of $\alpha 1$- and $\alpha 2$-like components, along with some higher polymers (Fig. 2). Sedimentation velocity measurements also showed that U-1 contained mainly α-like components but, as previously shown (6, 10), these components had higher sedimentation coefficients than the standard $\alpha 1$ or $\alpha 2$ components under equivalent conditions. The U-1 examined was thus a mixture of essentially $(\alpha 1)_h$ and $(\alpha 2)_h$, as defined by Veis *et al.* (7).

Optical rotation data, Fig. 3, show that collagen-fold formation is more rapid in U-1 than in an $\alpha 1$–β_{11} mixture upon

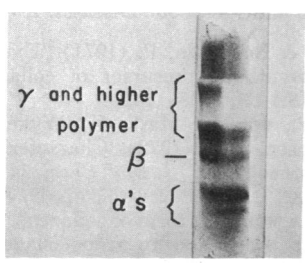

FIG. 2. Acrylamide gel electrophoresis of U-1 and unfractionated sample. Split-gel technique of Clark and Veis (12). *Left,* coacervate fraction before chromatography. *Right,* U-1 fraction.

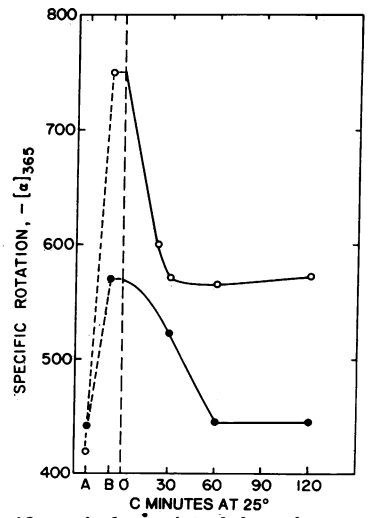

FIG. 3. Specific optical rotation, $[\alpha]_{365}$, of renaturing solutions as a function of time. Point *A*, initial denatured solution at 1 mg/ml in pH 3.7, 0.25 M citrate buffer at 40°; Point *B*, value after 2 hr at 4°C; Region *C*, rotation after warming to 25°. O, U-1, ●, $\alpha 1$–β_{11} mixture.

quenching to 4°, and that more stable fold regions are established. The long 25° tempering or annealing process does lead to complete renaturation of some collagen molecules in the $\alpha 1$–β_{11} mixture, but much higher yields of completely renatured molecules are obtained from the U-1 fraction.

Direct precipitation of renatured U-1 by dialysis of the solutions against salt-free water shows the typical formation of many fine filaments that tend to the native registration (Fig. 4). The addition of ATP to acid solutions of renatured U-1 leads to the formation of precipitates, but one sees only an occasional SLS-type aggregate in a predominantly filamentous network of native registration fibrils. Treatment of the renatured solutions with pepsin at 25° in acid alters the aggregation properties of the system, without much appreciable degradation of the renatured molecules, and allows the formation of SLS upon the addition of ATP. Fig. 5 shows positively-stained SLS from pepsin-treated renatured U-1. The band pattern in the interior of each molecular aggregate is entirely normal for SLS, and it does not appear that any

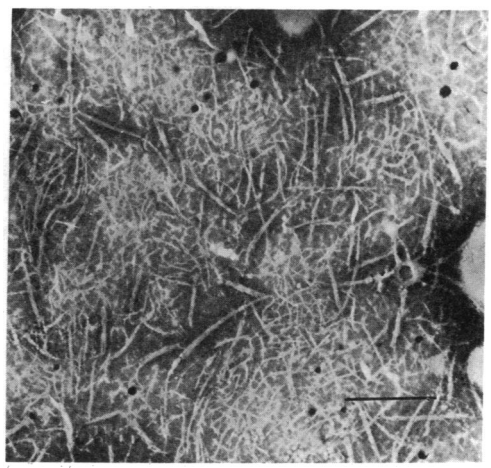

FIG. 4. Electron micrograph of dialysis-precipitated, renatured U-1 components. Negatively stained. *Bar* represents 3000 Å.

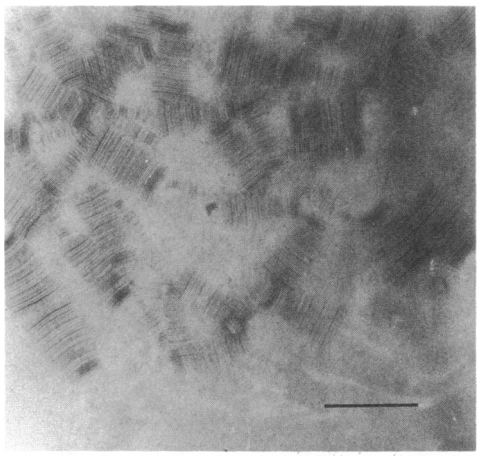

FIG. 5. Electron micrograph of ATP precipitation of pepsin-treated, renatured U-1. Positive stain. *Bar* represents 3000 Å.

normally staining bands have been removed from either end of the SLS aggregates. Moreover, the high yields show that SLS formation is not a rare or isolated phenomenon in this system.

Negative staining more sharply reveals the molecular ends; as shown in Fig. 6, the U-1 SLS end-regions are clearly demarcated. The SLS segments obtained from U-1, as in Fig. 6, can be divided into two groups based on overall length. The longer segments, denoted in Fig. 6, are 170 ± 30 Å longer than the shorter segments. The shorter segments correspond, band-for-band, and in length, with SLS prepared from native acid-soluble collagen from rat-tail tendon. Similarly, the longer segments can be matched band-for-band along their length from the COOH-terminal region with the rat-tail tendon collagen as prepared by Olsen (13). However, as shown in Fig. 7, there is an extra segment at the NH_2-terminal end of this group of SLS spools. Long spools were not found among the SLS prepared from renatured $\alpha 1$, β_{12}, or $\alpha 2$ fractions from the carboxymethylcellulose chromatography, as in Fig. 1, although many individual negatively-stained SLS forms were measured in each case.

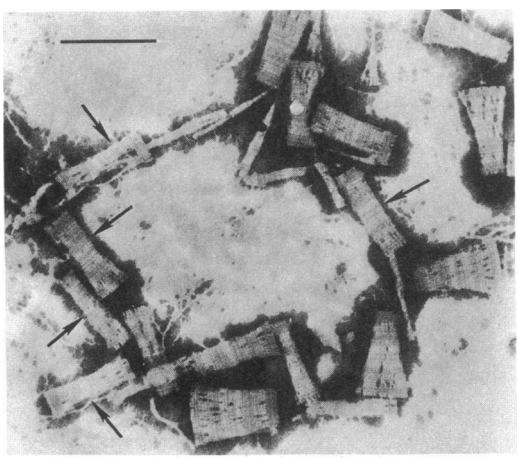

FIG. 6. Negative stain of ATP precipitate of pepsin-treated, renatured U-1. Segments denoted by *arrows* indicate longer aggregates. *Bar* represents 3000 Å.

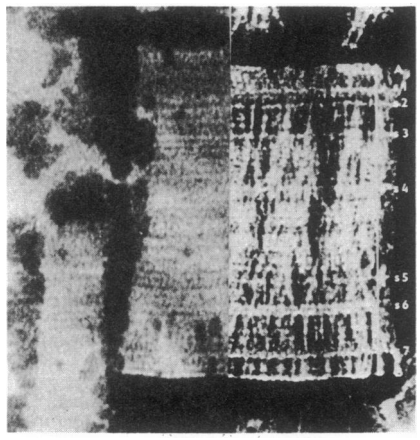

FIG. 7. Comparison of longer SLS (*left*) seen in Fig. 6 with negatively stained SLS from native rat-tail tendon (*right*) prepared by Olsen (13). The *arrow* represents the native molecular length of $(C)_l$, and the numbering system for the main bands is that of Olsen. *A* is the NH_2-terminal region. Note the extra segment at the *A*-end only of the renatured U-1.

CONCLUSIONS

High-molecular-weight α-chains, $(\alpha)_h$, have been isolated from mature, insoluble bovine-skin collagen by mild extraction procedures. The mixture of $(\alpha 1)_h$ and $(\alpha 2)_h$ obtained in the urea eluate from carboxymethylcellulose chromatography corresponds to the $(\alpha)_h$ components proposed (6, 7) as intermediates in the conversion of procollagen to collagen *in vivo*. The SLS prepared by renaturation and ATP precipitation from a fraction rich in the $(\alpha)_h$ components are 170 Å longer than corresponding SLS from the usual acid-soluble collagen or renatured $(\alpha)_l$-components. The extra segment is at the NH_2-terminal end of the renatured $(\alpha)_h$ SLS spools. The SLS are similar in all other respects to those of the shorter, native acid-soluble collagens.

These data show conclusively that an $(\alpha)_h$-containing collagen, $(C)_h$, is a component of mature collagen fibers, and that the C_h differ from the major collagen component, C_l, in having an additional short NH_2-terminal sequence. Radioisotope incorporation studies (7) in young, actively growing animals lead us to suggest that, while the C_h are intermediates in the $C_p \rightarrow C_l$ conversion, the intermediates are not immediately converted to the lower weight form. The present work suggests that the long-lived $(C)_h$ are incorporated directly into the insoluble fibers and may be important in the native registration of newly formed collagen fibrils.

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