ISOLATION OF A LARGE GLYCOPEPTIDE FROM CARTILAGE PROCOLLAGEN BY COLLAGENASE DIGESTION AND EVIDENCE INDICATING THE PRESENCE OF GLUCOSE, GALACTOSE AND MANNOSE IN THE PEPTIDE*

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SUMMARY: Digestion of cartilage procollagen, pro-α1(II), with bacterial collagenase followed by fractionation of Sephadex G-150 yielded a large glycopeptide (molecular weight 13,200) which could not be demonstrated in a similarly prepared digest of α1(II) chain. Isotopic studies suggested that this glycopeptide contained, in addition to glucose and galactose, mannose, a sugar that is not found in the authentic α-chain of cartilage. The results imply that in pro-α1(II) there is a glycopeptide region differing from the α1(II) chain in amino acid composition and also in the type of carbohydrates attached.

Dehm and Prockop (1) demonstrated that cells isolated by enzymatic digestion of embryonic chick sterna secreted a collagen-like protein into the culture medium which, after reduction with mercaptoethanol and chromatography on an SDS-agarose column, yielded a single type of polypeptide chain with a molecular weight somewhat higher than that of α1(II) chain. The medium protein, considered to be a triple helical molecule, contained cystine and interchain disulfide bonds in the NH_2-terminal region (2). Müller and Jamhawi (3) presented evidence that the triple strands linked by disulfide bonds represent a state of the precursor collagen.

We previously demonstrated (4) the existence in embryonic chick epiphyses of a guanidine HCl-extractable collagen which, on the basis of pulse-chase experiments, was shown to convert to α1(II) with a reduction of molecular size. This precursor form, unlike the medium protein secreted by the matrix-

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Abbreviation: SDS, sodium dodecylsulfate.

free cells (see above), was obtained mainly as a single type of polypeptide chain without any treatments for reduction, and was readily separable from α1(II) by CM-cellulose chromatography in a urea-containing buffer. We here report studies with the isolated precursor form indicating that the molecule contains a characteristic peptide region in which at least three kinds of sugars are present as an integral part.

MATERIALS AND METHODS

Fertile eggs were obtained from a local supplier on day 9 and were incubated in a moist atmosphere at 38°C until they were used on day 12. L-[U-^{14}C]proline (205 Ci/mole), L-[3-^{14}C]tryptophan (58 Ci/mole) and D-[U-^{14}C]glucose (180 Ci/mole) were purchased from New England Nuclear, Boston, Radiochemical Center, Amersham, and International Chemical and Nuclear Corp., Irvine, respectively. A highly purified preparation of collagenase from Clostridium histolyticum was kindly given by Dr. T. Ohya, Amano Pharmaceutical Co., Nagoya. A criterion of the specificity of the collagenase was given previously (4).

Preparation of radioactive collagen samples: Tibias and femurs were removed from 12-day-old chick embryos and immediately placed in cold Tyrode's medium (5). They were dissected free of adherent connective tissue and muscle, and the cartilagenous portions of the bones were separated from the bony shaft. Each cartilage was cut into pieces about 1.5 mg wet weight with a razor.

Each incubation mixture consisted of about 300 mg of cartilage pieces. The tissues were preincubated at 37°C for 20 min with gentle shaking in A) 1 ml of Krebs medium II (6) supplimented with 50 μg of sodium ascorbate or B) 2.5 ml of glucose-free Krebs medium II with 125 μg of sodium ascorbate. Following this preincubation, 1 μCi of [^{14}C]proline or 5 μCi of [^{14}C]tryptophan was added to the suspensions in medium A, and 25 μCi of [^{14}C]glucose to the suspension in medium B, and incubation was allowed to continue for 30 min with [^{14}C]proline, for 60 min with [^{14}C]tryptophan or for 120 min with [^{14}C]glucose. At the end of the incubation periods, the cartilages were placed in 20 vol. of cold 2 % perchloric acid. After 30 min, the tissues were transferred to 20 vol. of cold 50 % ethanol and allowed to stand for 60 min. The tissues were then taken for extraction with 4 M guanidine HCl as described previously (4); yields of the radioactive materials solubilized with 4 M guanidine HCl (expressed as percentage of the total radioactivity incorporated into cartilage) were about 85 from the [^{14}C]proline-labeled sample, and about 55 from the [^{14}C]tryptophan- or [^{14}C]glucose-labeled sample.

RESULTS AND DISCUSSION

As indicated in the Methods, cartilage pieces were incubated with [U-^{14}C]proline, [3-^{14}C]tryptophan or [U-^{14}C]glucose. The tissues in each mixture were then extracted with 4 M guanidine HCl and chromatographed on Bio-Gel A-5m (Fig. 1). As already reported (4), a large portion of the incorporated [^{14}C]proline was eluted as the single type of α-chains. The [^{14}C]tryptophan-labeled sample showed a more disperse pattern, plus a peak

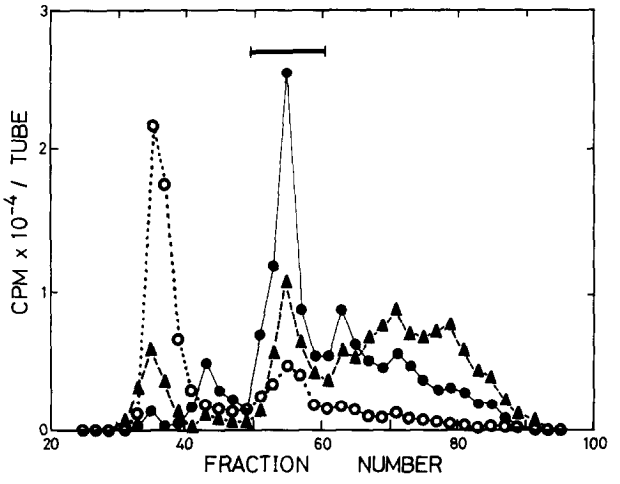

Fig. 1. Gel filtration of the extract from cartilage incubated for 30 min with [^{14}C]proline (●———●), for 60 min with [^{14}C]tryptophan (▲– – –▲), or for 120 min with [^{14}C]glucose (○·····○). About 5 ml (3 X 10^5 cpm) each of the concentrated guanidine-HCl extracts (see text) was fractionated in a Bio-Gel A-5m column (2.1 X 140 cm), equilibrated with 2 M guanidine HCl-20 mM Tris-HCl, pH 7.5, at 20°C. Fractions of 5 ml were collected and assayed for radioactivity. The Vo was Fraction 34 and the Vt was Fraction 98. *Bar above curves* indicates the fractions corresponding to α-chains, which were subsequently pooled and subjected to chromatography on CM-cellulose (Fig. 2).

corresponding to α-chains. The [^{14}C]glucose-labeled sample also yielded a small peak corresponding to α-chains. In this case, however, approx. 70 % of the incorporated ^{14}C was eluted in the void volume. Evidence that most of the ^{14}C in the void volume represents proteochondroitin sulfate has already been provided by previous studies (7).

Each of the radioactive fractions corresponding to α-chains was subjected to chromatography on CM-cellulose (Fig. 2). The [^{14}C]proline- and [^{14}C]glucose-labeled samples were eluted in each instance as two major peaks in the regions previously identified as containing α1(II) and pro-α1(II), respectively. The [^{14}C]tryptophan-labeled sample, in contrast, was eluted almost exclusively as pro-α1(II). The result is consistent with the findings of other workers (1) that tryptophan is an amino acid present in the NH_2-terminal extension but not in the α1(II) region of cartilage procollagen.

The pro-α1(II) samples obtained by these procedures were treated with bacterial collagenase. Chromatography of the digests on SDS-Sephadex G-150

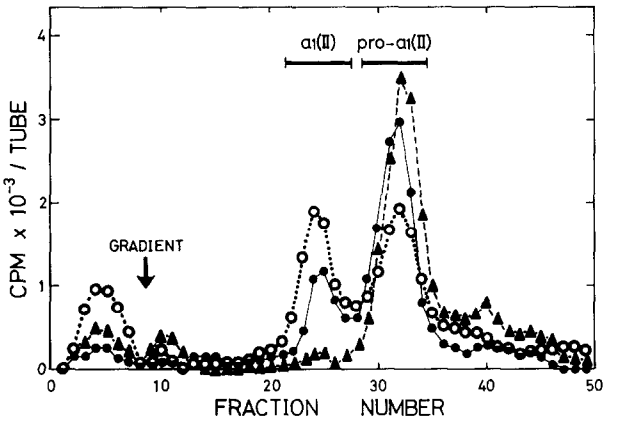

Fig. 2. Chromatography on CM-cellulose of the ^{14}C-labeled α-components from cartilage incubated with [^{14}C]proline (●——●), [^{14}C]tryptophan (▲- - -▲), or [^{14}C]glucose (O·····O). Each of the fractions obtained by Bio-Gel chromatography (indicated by the bar above curves in Fig. 1) was concentrated to 5 ml on a Diaflo PM-10 membrane. To this solution, 3 mg of acid-soluble collagen from rat skin (8) were added as carrier, and the sample was dialyzed exhaustively against 4 M urea-0.04 M sodium acetate buffer, pH 4.8, at 4°C. The sample was heated to 40°C for 10 min and immediately applied to a column (1.6 X 3 cm) of CM-cellulose that had been equilibrated at 40°C with the urea-acetate solution. The column was developed at 40°C by use of a linear salt gradient of NaCl from 0 to 0.08 M in the equilibrating buffer. Fractions of 2 ml were collected and assayed for both radioactivity and absorbance at 230 nm (the elution profile of the carrier is not shown).

indicated that over 80 % of the incorporated [^{14}C]proline became eluted near the end of the column (Fig. 2A). The incorporated [^{14}C]tryptophan, in contrast, became eluted almost exclusively in a peak slightly ahead of the elution position of a cytochrome c standard (mol wt 12,384) (Fig. 2B). The results can be interpreted as indicating that the α1(II) chain characterized by the presence of proline and hydroxyproline (9, 10) was degraded to give rise to the peptides of relatively small size while the NH_2-terminal extension characterized by the presence of tryptophan (1) remained undigested. In order to estimate molecular weight, the large peptide fraction from [^{14}C]tryptophan-labeled procollagen was submitted to electrophoresis on an SDS-10 % acrylamide gel column (11) which was calibrated using ovalbumin (mol wt 43,000), trypsin (mol wt 23,800) and cytochrome c (mol wt 12,384). A single band was obtained in the gel electrophoresis as monitored for the

presence of ^{14}C. Determination of the molecular weight by this method gave a value of 13,200.

When the collagenase digest of the [^{14}C]glucose-labeled procollagen (2×10^4 cpm) was subjected to SDS-gel filtration, about one third of the ^{14}C was eluted in the position corresponding to the large peptide (Fig. 3C), suggesting that the NH_2-terminal extension (from which the large peptide should originate) must bear considerable [^{14}C]sugar residues. To confirm

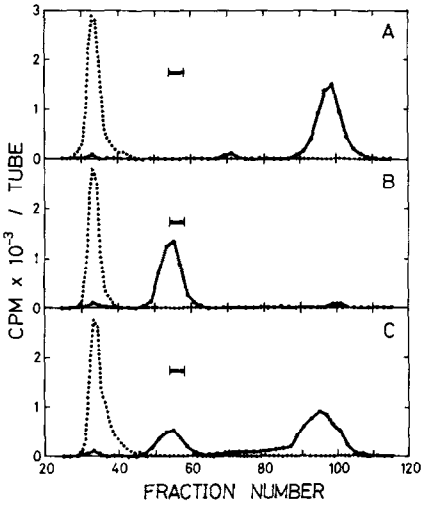

Fig. 3. Chromatography on SDS-Sephadex G-150 of the ^{14}C-labeled pro-α1(II) samples before (·····) and after (————) treatment with bacterial collagenase. Elution patterns in experiments with [^{14}C]proline-, [^{14}C]tryptophan- and [^{14}C]glucose-labeled pro-α1(II) are shown in Pannels A, B and C, respectively. For collagenase digestion, about 2×10^4 cpm each of the pro-α1(II) fractions obtained by CM-cellulose chromatography (Fig. 2) was mixed with solid guanidine HCl to give a concentration of 2 M, concentrated to 2 ml on a Diaflo PM-10 membrane, and then diluted with 4 ml of water. To this solution, 18 ml of 95 % ethanol containing 1.3 % potassium acetate were added and, after standing at 0°C for 1 hour, the precipitate was collected by centrifugation. The precipitation with ethanol was carried out two additional times to ensure elimination of guanidine HCl. The final precipitate was dissolved in 1 ml of a solution consisting of 20 mM Tris-HCl, pH 7.5, 10 mM KCl, 10 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 0.1 mg collagenase, and incubated at 37°C for 2 hours. The collagenase treatment was terminated by addition of solid SDS to give a final concentration of 2 %. For gel filtration, the mixture was adjusted to 2 % mercaptoethanol and, after incubating at 37°C overnight, loaded on a Sephadex G-150 column (1.5 × 65 cm) equilibrated with 0.1 % SDS-20 mM Tris-HCl, pH 7.5. The column was eluted with the SDS-buffer at room temperature. Fractions of 1.2 ml were collected and assayed for radioactivity. *Bar above curve* indicates the position of a cytochrome c standard. The Vo was Fraction 33 and the Vt was Fraction 100.

this, 0.2 μmole each of glucose, galactose, mannose, glucosamine and galactosamine (carriers) were added to a 1×10^4 cpm portion of the large peptide fraction, and the mixture was subjected to hydrolysis with 2 M HCl at 100°C for 4 hours. The hydrolyzate was evaporated to dryness under reduced pressure, dissolved in water and passed successively through small columns of Dowex 50 (H^+) and Dowex 1 (HCO_3^-). About 50 % of the ^{14}C was recovered in the water washings and analyzed by paper chromatography in 1-butanol-pyridine-water (6:4:3) and paper electrophoresis in 50 mM sodium borate (pH 9.0). The ^{14}C was recovered, in each case, in three fractions corresponding to glucose, galactose and mannose (approximate radioactivity ratio, 3:2:2), respectively.

An additional amount (32 %) of ^{14}C was recovered by subsequent elution of the Dowex 50 column with 0.3 M HCl ("hexosamine-like material"). When the eluate was successively acid treated (6 M HCl, 100°C, 8 hours), N-acetylated (12) and passed through the Dowex 50 (H^+) and Dowex 1 (HCO_3^-) columns, more than 85 % of the unlabeled hexosamine carriers were recovered in the water washings as N-acetylhexosamines (as judged by the Morgan-Elson assay and by paper chromatography in the solvent described above). However, the labeled materials were readily distinguished from the hexosamine carriers in that over 85 % of the ^{14}C was retained in the Dowex columns. Thus, paper chromatography of the water washings indicated that only 70 cpm of ^{14}C were present in the N-acetylhexosamine area. The behavior toward Dowex columns before and after N-acetylation suggests that most, if not all, of the hexosamine-like material may represent amino acids or peptides (or both).

To document clearly the difference in sugar composition between the NH_2-terminal extension and the α1(II) chain, the late peak fraction from the digest of [^{14}C]glucose-labeled procollagen (Fig. 3C) was analyzed in a similar way. Only glucose and galactose were obtained from this sample in an approximate radioactivity ratio of 1:1.

It remains to be determined whether the guanidine HCl-extracted

procollagen, i.e. single type of pro-α1(II), represents a physiological precursor in the conversion to a triple-stranded pro-α1(II) or results from exposure of the triple strands to tissue proteases during extraction. Regardless of the remaining uncertainties, our results indicate that the predominant peculiarity of cartilage procollagen is an additional peptide which differs from the rest of the molecule not only in amino acid composition but also in the type of bound sugars.

REFERENCES

1. Dehm, P., and Prockop, D.J. (1973) Eur. J. Biochem. 35, 159-166.
2. Uitto, J., and Prockop, D.J. (1974) Biochemistry 13, 4586-4591.
3. Müller, P.K., and Jamhawi, O. (1974) Biochim. Biophys. Acta 365, 158-168.
4. Oohira, A., Kimata, K., Suzuki, S., Takata, K., Suzuki, I., and Hoshino, M. (1974) J. Biol. Chem. 249, 1637-1645.
5. Tyrode, M.V. (1910) Arch. Int. Pharmacodyn. Ther. 20, 205-223.
6. Krebs, H.A. (1950) Biochim. Biophys. Acta 4, 249-269.
7. Kimata, K., Okayama, M., Oohira, A., and Suzuki, S. (1974) J. Biol. Chem. 249, 1646-1653.
8. Piez, K.A., Eigner, E.A., and Lewis, M.S. (1963) Biochemistry 2, 58-66.
9. Trelstad, R.L., Kang, A.H., Igarashi, S., and Gross, J. (1970) Biochemistry 9, 4993-4998.
10. Miller, E.J. (1971) Biochemistry 10, 1652-1658.
11. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
12. Roseman, S., and Dafner, I. (1956) Anal. Chem. 28, 1743-1746.