STUDIES ON THE ADDITIONAL PEPTIDE OF PROCOLLAGEN EXTRACTED FROM CHICK EMBRYO CARTILAGE

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ABSTRACT

The additional peptide of procollagen is thought to play an important role in its synthesis, and attempts were made to elucidate the chemical properties of the additional peptide, particularly in regard to the possible existence of sugar substituents.

Labeled procollagens were obtained with 4 M guanidine-HCl from chick embryo epiphyseal cartilages which had been incubated with [^{14}C] proline, [^{14}C] tryptophan or [^{14}C] glucose. Each of the procollagen samples was digested with bacterial collagenase, and then chromatographed on 0.1% SDS-Sephadex G–150. The [^{14}C] glucose-labeled sample yielded a fraction with the size of additional peptide (molecular weight, 13,200), plus collagenase-digestible small peptides. The peptide was distinct from the collagenase-sensitive region (α-region) in having [^{14}C] tryptophan. On acid hydrolysis of the additional peptide samples, radioactive glucose, galactose, mannose and a small amount of glucosamine were recovered on paper chromatography and paper electrophoresis. Mannose and glucosamine are the components not found in the authentic α-chain of type II collagen.

The results demonstrate that the additional peptide of cartilage procollagen is different from the α1(II) chain in amino acid composition and also in the type of sugars attached.

INTRODUCTION

Studies on biosynthesis of type I collagen, $[\alpha 1(I)]_2\alpha 2$, have advanced rapidly in recent years (see ref. 1 for a review). The biosynthesis of interstitial collagen involves the initial elaboration of a precursor molecule, procollagen, which contains the NH_2- and the COOH-terminal non-herical additional peptides.[2~4] After its completion on rough ER, the procollagen molecule is modified by several steps. Some examples of such post-translational modifications are: the hydroxylation of prolyl and lysyl residues in the polypeptide chain;[5] the glycosylation of certain hydroxylysyl residues;[6,7] and the formation of interchain disulfide bonds and triple helix.[2~4,8,9] The triple-stranded procollagen is converted into the collagen building up the extracellular fibrils by cleavage of the additional peptides of procollagen molecule with the specific enzyme, procollagen peptidase, in the extracellular spaces.[10,11]

It is thought that cartilage collagen, $[\alpha 1(II)]_3$, is synthesized through a series of processes similar to those for type I collagen. Isolation of the precursor forms of type II collagen, pro-α1(II), has been reported from two laboratories using different methods. Thus, Dehm and Prockop[12] reported that matrix-free cells isolated by enzymatic digestion of embryonic chick sterna secreted a collagen-like molecule which was somewhat higher in molecular weight than α1(II) and contained cystine and interchain disulfide bonds. On

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the other hand, Oohira *et al.*[13] demonstrated the presence in chick embryo epiphyses of a guanidine-HCl extractable collagen which can be converted to α1(II) with a reduction of molecular weight as judged by pulse-chase experiments.

Functions postulated for a procollagen include initiation of triple helix formation,[2~4,8,9,14] inhibition of intracellular fibrogenesis,[15] facilitation of transmembrane movement of the protein,[16,17] and participation in the appropriate lateral aggregation and crosslinking of collagen during extracellular fibrogenesis.[18] The additional peptides of procollagen are thought therefore to play an important role in these processes, and it is certainly desirable to elucidate the chemical properties of additional peptides.

In this paper, some chemical properties of the additional peptide of cartilage procollagen are described.[19,20]

MATERIALS

The following commercial materials were used: L-[U-^{14}C] proline, 205 mCi per mmole, from New England Nuclear, Boston; D-[U-^{14}C] glucose, 180 mCi per mmole, from International Chemical and Nuclear Corp., Irvine; L-[3-^{14}C] tryptophan, 58 mCi per mmole, from Radiochemical Center, Amersham; carboxymethyl (CM) cellulose, Whatman CM-32, microgranular, from H. Reeve Angel & Co., London; Bio-Gel A-5m, 200-400 mesh, from Bio-Rad Laboratories; Sephadex G-150, from Pharmacia, Uppsala; and Diaflo membrane PM-10 from Amicon Corp., Lexington. All of the other chemicals were of the highest purity of commercially available reagents.

A highly purified preparation of collagenase from *Clostridium histolyticum* was kindly offered by Dr. Ohya, Amano Pharmaceutical Co., Nagoya.

Fertile eggs were obtained from a local supplier on day 9 and were incubated in a moist atmosphere at 38° until they were used on day 12 or 13.

METHODS

Incubation Method

The cartilagenous portions of the tibias and femurs were prepared as previously reported.[13] Each incubation mixture consisted of about 300 mg of cartilage pieces. The tissues were preincubated at 37° for 20 min with gentle shaking in 1 ml of Eagle's MEM (medium A) or Krebs medium B (medium B) containing 10 % fetal calf serum and 0.005 % ascorbic acid in order to decrease the intracellular amino acid and glucose pools. Following this preincubation, 5 μCi of [^{14}C] proline were added to the suspensions in medium A, and 25 μCi of [^{14}C] glucose or 5 μCi of [^{14}C] tryptophan to the suspensions in medium B. 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.

Extraction Method

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. After these pretreatments, the cartilages were suspended in 15 vol. of 4 M guanidine-HCl (containing 50 mM Tris-HCl, pH 7.5) and then heated in a boiling water bath for 5 min. The suspensions were then stirred for 40 hours at room temperature. The cartilage residues were removed by centrifugation, and the supernatant

was collected for gel filtration.

Gel Filtration and CM Cellulose Column Chromatography

About 5 ml (3×10^5 cpm) of the guanidine-HCl extracts were chromatographed on a column (2.1 × 140 cm) of Bio-Gel A-5m (200-400 mesh) in 2 M guanidine-HCl containing 50 mM Tris-HCl, pH 7.5, at room temperature. Fractions of 5 ml were collected and assayed for radioactivity.

Radioactive material eluting in the α-fraction was pooled, concentrated to 5 ml on a Diaflo PM-10 membrane, and, after mixing with 3 mg of acid-soluble collagen from rat skin (internal marker), dialyzed at 4° against five 1-liter changes of 4 M urea containing 0.04 M sodium acetate buffer, pH 4.8. The sample was then heated to 40° for 10 min and chromatographed on a CM-cellulose column (1.6 × 3 cm) that had been equilibrated at 40° by use of a linear gradient of NaCl from 0 to 0.08 M in the equilibrating buffer.[13] Fractions of 2 ml were collected and assayed for both radio-activity and absorbance at 230 nm.

Collagenase Digestion

About 2×10^4 cpm each of the pro-α1(II) fractions obtained by CM-cellulose chromatography 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° for 1 hour, the precipitate was collected by centrifugation. The precipitation with ethanol was carried out for two additional times to ensure elimination of urea and 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° for 2 hours. The collagenase digestion was terminated by addition of solid sodium dodecyl sulfate (SDS) to give a final concentration of 2 %.

For gel filtration of collagenase digests, the mixture was adjusted to 2 % 2-mercaptoethanol and, after incubation at 37° 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 same SDS-Tris-HCl buffer at room temperature. Fractions of 1.2 ml were collected and assayed for radioactivity.

Acid Hydrolysis of Procollagen for Sugar Analysis

Fractions corresponding to the [^{14}C]glucose-labeled additional peptide were collected, lyophilized, and redissolved in water. To a 1×10^4 cpm portion of the sample, 0.2 μmole each of glucose, galactose, mannose, glucosamine and galactosamine were added as carrier, and the mixture was subjected to hydrolysis with 2 M HCl at 100° for 4 hours. The hydrolyzate was evaporated to dryness under reduced pressure, dissolved in water and passed successively through columns of Dowex 50-H^+ and Dowex 1-HCO_3^- (each bed vol., about 1 ml).

Count of Radioactivity

Radioactivity was counted in a Horiba liquid scintillation spectrometer, model LS-500 (Horiba Seisakusho, Kyoto) with 10 ml of a solution of 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)benzene in a liter of toluene.

As indicated in the Methods, cartilage pieces were incubated with [^{14}C]proline, [^{14}C]

RESULTS

tryptophan or [^{14}C]glucose. The labeled tissues in each mixtures were then extracted by stirring in 4 M guanidine-HCl (pH 7.5). The yields of 4 M guanidine-HCl soluble radioactivity, expressed as percentage of the total radioactivity incorporated into cartilages, were about 85 % from [^{14}C]proline-labeled sample, and about 55 % from the [^{14}C] tryptophan- or [^{14}C]glucose-labeled sample. The extracts were chromatographed on Bio-Gel A-5m (Fig. 1). The [^{14}C]proline-labeled sample was eluted mostly in a large peak (No. 53) corresponding to the single type of α-chain (called "α-fraction") (Fig. 1A). The [^{14}C]tryptophan-labeled sample showed a more disperse pattern, plus a small peak corresponding to α-fraction (Fig 1B). The [14C] glucose-labeled sample also gave a small peak corresponding to α-fraction. In this case, however, about 70 % of the incorporated ^{14}C was eluted in the void volume (No. 34) (Fig. 1C). It has been reported that most of the ^{14}C in the void volume represents proteochondroitin sulfate.[21]

Each of the radioactive fraction corresponding to α-fraction was subjected to CM-cellulose chromatography as described under "Methods" (Fig. 2). The [^{14}C]proline- and [^{14}C]

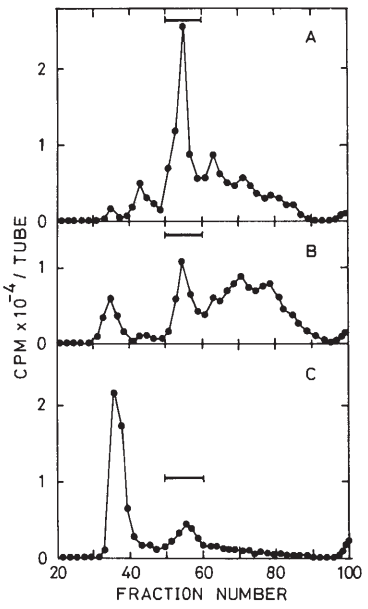

Fig. 1

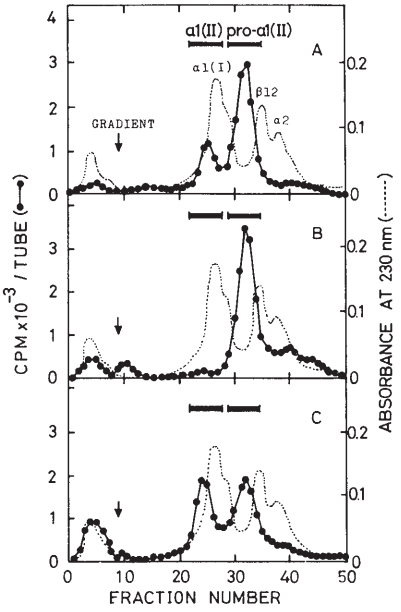

Fig. 2

Fig. 1. Chromatography on Bio-Gel A-5m of the extract from epiphyseal cartilage incubated for 30 min with [^{14}C]proline (panel A), for 60 min with [^{14}C]tryptophan (panel B), or for 120 min with [^{14}C]glucose (panel C).
Bar above curves indicates the fractions corresponding to α-chains. The Vo was Fraction 34 and the Vt was Fraction 98.

Fig. 2. Chromatography on CM-cellulose of the ^{14}C-labeled α-fractions from cartilage incubated with [^{14}C]proline (panel A), [^{14}C]tryptophan (panel B) or [^{14}C]glucose (panel C) as described in Methods. Radioactivity (●——●) and absorbance at 230 nm (---------) are shown. α1(I), β12 and α2 indicate the elution position of rat skin collagen.
Bar above curves, α1(II) and pro-α1(II), indicates the elution position of cartilage collagen and its procollagen, respectively.

glucose-labeled samples were eluted as two major peaks in the regions previously identified containing α1(II) and pro-α1(II), respectively (Fig. 2A and 2C).

In contrast, the [^{14}C] tryptophan-labeled sample was eluted almost exclusively as pro-α1(II) (Fig. 2B). Tryptophan is known to be an amino acid present in the additional peptides of the cartilage procollagen but not in the α1(II) (3, 12). Therefore, the presence of tryptophan can be taken as evidence that the fraction represents the additional peptide.

For the separation of the additional peptide from the other peptide portions of procollagen, the pro-α1(II) samples obtained by these procedures were digested with highly purified bacterial collagenase and the digests were chromatographed on SDS-Sephadex G-150 as described under "Methods" (Fig. 3). In the case of [^{14}C] proline-labeling, over 80 % of the incorporated [^{14}C] proline became eluted near the end of the column (Fig. 3A). The incorporated [^{14}C] tryptophan, in contrast, became eluted almost exclusively in a peak slightly ahead of the elution position of a cytochrome c marker (molecular weight, 12, 384) (Fig. 3B). As above mentioned, this peak must correspond to the additional peptide of cartilage procollagen. In order to estimate its molecular weight, the additional peptide fraction from [^{14}C] tryptophan-labeled procollagen was submitted to an SDS-10 % polyacrylamide gel electrophoresis[22] 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 [^{14}C] glucose-labeled procollagen (2×10^4 cpm) was subjected to SDS-gel filtration, the ^{14}C became eluted mainly as two peaks. About one third of ^{14}C was eluted in a peak corresponding to the additional peptide and about two third of the ^{14}C near the end of the column (Fig. 3C). This result suggests that the additional peptide must bear considerable [^{14}C] sugar residues. To confirm this suggestion, the [^{14}C] glucose-labeled additional peptide was hydrolyzed with 2 M HCl as described under "Methods". About 45 % of the

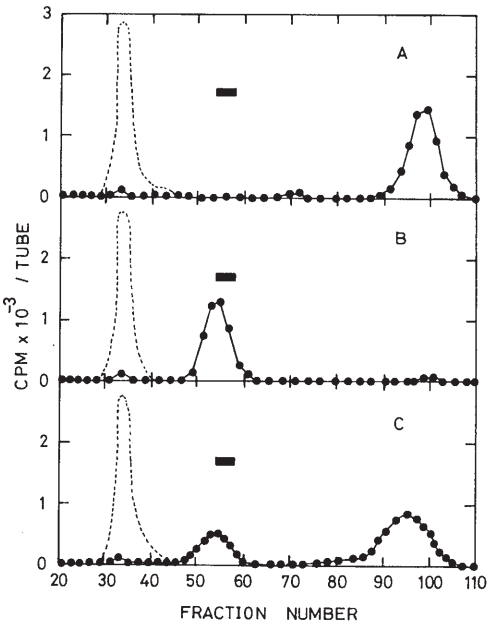

Fig. 3. Chromatography on Sephadex G-150 of the [^{14}C] labeled pro-α1(II) samples before (------) and after (●—●) treatment with bacterial collagenase. Elution patterns in experiments with [^{14}C] proline-, [^{14}C] tryptophan- and [^{14}C] glucose-labeled pro-α1(II) are shown in panel A, B and C, respectively. Bar above curves the position of a cytochrome c marker. The Vo was Fraction 33 and the Vt was Fraction 100.

^{14}C was recovered in the water washings of Dowex 50-H^+ and Dowex 1-HCO_3^- columns. The water washings were concentrated and then analyzed by paper chromatography in 1-butanol-pyridine-water (6:4:3). The ^{14}C was recovered, on paper chromatography, in three fractions corresponding to glucose, galactose and mannose (approximate radioactivity ratio, 3:2:2), respectively (Fig. 4). The zones corresponding to glucose, galactose and mannose were separately eluted from paper strips with water, lyophilyzed and then subjected to

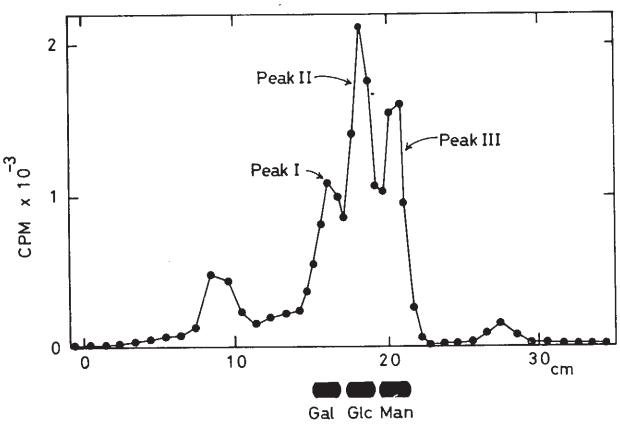

Fig. 4. Paper chromatography of neutral sugar fractions obtained by acid hydrolysis of the [^{14}C] glucose-labeled additional peptide. The following solvent system was used by descending chromatography on Toyo 51A filter paper (35 cm long): 1-butanol-pyridine-water (6:4:3). Radioactivity was detected by counting 0.5 cm strips of paper chromatogram. The peak corrospoinding to galactose, glucose and mannose marker was called peak I, II and III, respectively.

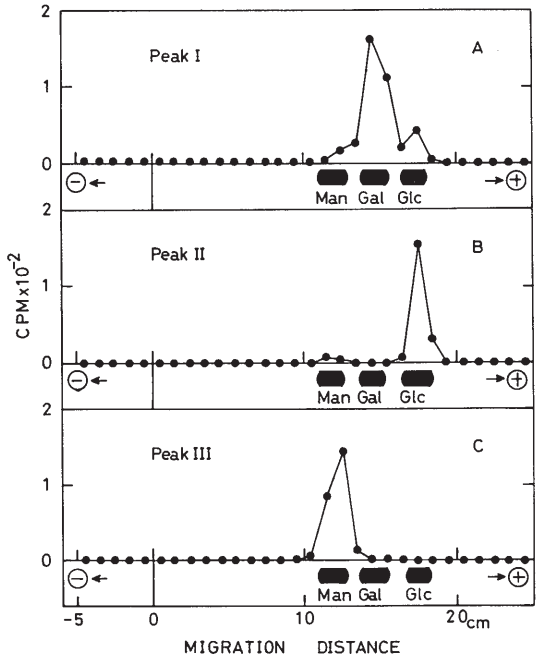

Fig. 5. Paper electrophoresis of Fractions I, II and III obtained by paper chromatography (see Fig. 4). Paper electrophoresis was carried out on a 60 cm strip of Toyo 50 filter paper in 0.05M sodium borate (pH 9.0) at a potential gradient of 25 volts per cm for 60 min. The electrophoretic profiles of peak I, II and III are shown in panel A, B and C, respectively. Radioactivity was detected by counting 1 cm strips of paper electrogram.

paper electrophoresis in 50 mM sodium borate (pH 9.0). The samples corresponding to glucose, galactose and mannose from paper chromatographic strips moved as glucose, galactose and mannose, respectively, on paper electrophoresis (Fig. 5).

An additional amount (35 %) 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°, 8 hours), N-acetylated[23] 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 system 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-acetylglucosamine 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 additional peptide and the α1(II) chain, the collagenase-digestible small peptides of [^{14}C]glucose-labeled procollagen (Fig. 3C) were analyzed in a similar way. Only glucose and galactose were obtained from this sanple in an approximate radioactivity ratio of 1 : 1 (Fig. 6).

DISCUSSION

Dermatosparactic calves, missing procollagen peptidase genetically, are characterized by marked cutaneous fragility due to a defect in intermolecular cross-linkage of collagen.[10,11] Therefore, the procollagen containing the NH_2-terminal additional peptide can be easily extracted from the calf skin. Furthmayer *et al.* reported that the additional peptide (molecular weight, 19,500) of dermatosparactic skin collagen exhibited a distinct amino acid composition and absence of hydroxyproline and hydroxylysine. Carbohydrate analyses revealed the presence of 2-3 % hexoses, less than 0.2 % sialic acid and hexosamines in the peptide.[24]

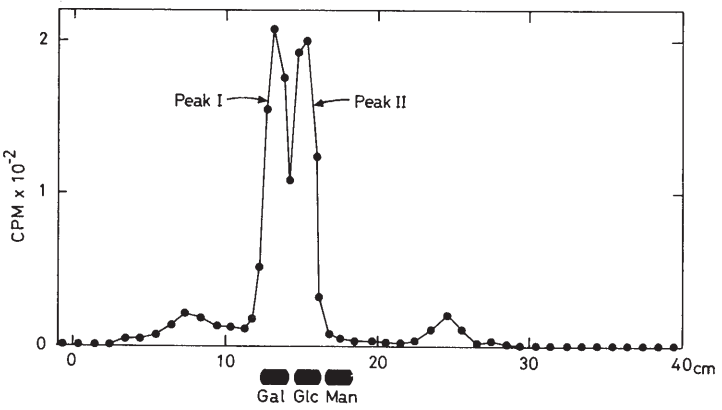

Fig. 6. Paper chromatography of neutral sugar fractions obtained by acid hydrolysis of the [^{14}C] glucose-labeled α1(II). Method was the same as in Fig. 4.

The additional peptide of the type I procollagen from healthy animals is also suggested to contain sugar residues as that from the dermatosparactic procollagen. It seems reasonable to assume that the sugar residues of the additional peptide may differ from those in the α-region, depending on the difference in amino acid composition.

The present result indicates clearly that the additional peptide of type II procollagen contains a considerable amount of [^{14}C] carbohydrate residues. Furthermore, the carbohydrates in the additional peptide are characterized as glucose, galactose, mannose and a small amount of glucosamine.[19,20] Mannose and glucosamine are the components not found in the authentic α-chain of type II collagen.

The low content of [^{14}C] glucosamine may be interpreted as being due to the presence of a large glucosamine pool in cartilage cells which may exert an isotope dilution effect.

Clark and Kefalides[24] have recently reported that isotopically labeled mannose and glucosamine were incorporated into the additional peptide of the procollagen secreted by matrix-free chick embryo tendon cells, and that the peptide contained galactosamine, in addition to glucosamine and mannose. It seems likely therefore that the procollagens from cartilage and tendon are different in regard to the type and composition of sugars attached to the additional peptides. It remains to be determined, however, whether the sugars containing mannose and hexosamines are attached to the NH_2-terminus or COOH-terminus.

Early studies of procollagen suggested that there was a single noncollagenous domain at the NH_2-terminus of each precursor chains.[26] More recent studies, however, have indicated that there is a noncollagenous peptide extension of the COOH-terminus of the procollagen molecule, in addition to that at the NH_2-terminus.[2~4,9] Thus, Bornstein and colleagues[3] reported that the procollagen is consisted of three pro-α chains each containing nonhelical NH_2-terminal extensions of 20,000 molecular weight and COOH-terminal extensions of about 35,000 molecular weight, and that the interchain disulfide bonds occur exclusively in the COOH-terminal region. More recently, Prockop *et al.*[27] also reported that tendon and cartilage procollagens contain interchain disulfide bonds in the COOH-terminal additional peptide.

In the present study, only the single type of procollagen was obtained by extracting with 4 M guanidine-HCl as described in Methods (Method A). The single type of procollagen thus obtained contained only one additional peptide; thus, the question remains whether the single chain represents a physiological precursor or results from artificial degradation of the native form of procollagen. To obtain further information on this point, the extraction method has been modified as follows: [^{14}C] proline-labeled tissues were homogenized in 4 M guanidine-HCl (pH 7.5) containing 50 mM iodoacetamide and extracted with shaking at 37° for 2 hours (Method B).[28] By using Method B, a triple-stranded procollagen as well as a single-stranded procollagen could be obtained on a Bio-Gel A-5m column chromatography. The former has been shown to contain 2-mercaptoethanol-sensitive disulfide bonds. The 2-mercaptoethanol-reduced molecule of the triple-stranded procollagen was indistinguishable from the single type of procollagen obtained by Method A on SDS-gel electrophoresis. The results suggest that the single procollagen obtained by Method A should contain at least a part of the COOH-terminal additional peptide. Therefore, the glycopeptide with molecular weight of 13,200 is likely to be located in the COOH-terminal region. This is consistent with the observation of Duksin and Bornstein[29] that [^{3}H] mannose was incorporated to the COOH-terminal non-helical fragment of type I procollagen.

SUMMARY

Labeled procollagens were isolated from [^{14}C] proline-, [^{14}C] tryptophan- and [^{14}C] glucose-labeled cartilages by extraction with 4M guanidine-HCl followed by chromatography on Bio-Gel A-5m and CM-cellulose.

Each of the procollagen samples was digested with highly purified bacterial collagenase and then chromatographed on Sephadex G-150 (elution buffer, 0.1 % SDS-20 mM Tris-HCl, pH 7.5). Over 80 % of the incorporated [^{14}C] proline were recovered as small peptides eluted near the end of the column. In contrast, the incorporated [^{14}C] tryptophan was eluted almost exclusively in a peak corresponding to a relatively large peptide (molecular weight, 13,200), indicating that the procollagen had a collagenase-resistant region (i.e. additional peptide) characterized by the presence of tryptophan. The [^{14}C] glucose-labeled procollagen yielded a fraction corresponding to the additional peptide, plus collagenase-digestible small peptides. The results suggest that the additional peptide contains carbohydrate residues. On acid hydrolysis, the additional peptide samples from [^{14}C] glucose-labeled procollagen yielded radioactive glucose, galactose and mannose. Only a small proportion of ^{14}C was recovered in glucosamine fraction. Mannose and glucosamine are the components not found in the authentic α-chain of type II collagen.

It is concluded that the additional peptide of cartilage procollagen is different from the α1(II) chain in amino acid composition and also in the type of sugars attached.

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