Isolation of a Disulfide-stabilized, Three-Chain Polypeptide Fragment Unique to the Precursor of Human Collagen*

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SUMMARY

Cultured human diploid fibroblasts were labeled with [3H]-tryptophan, and the three-chain precursor of collagen in the medium was identified by immunoprecipitation and gel electrophoresis. [3H]Tryptophan was incorporated into the nonhelical, NH2-terminal extensions of the pro-α1 and pro-α2 chains of the precursor. Collagenase digestion of the helical segment of the precursor molecule left intact a disulfide-assembled protein fragment which contained all of the incorporated radioactivity. Reduction and alkylation of the fragment confirmed that it consisted of three chains of similar molecular weight. Large scale recovery of the labeled fragment was performed using gel filtration, collagenase digestion, and ion exchange chromatography. Immune precipitation showed that the isolated fragment was still antigenically reactive. The molecular weights of the fragment and its constituent chains were determined from their electrophoretic mobilities in sodium dodecyl sulfate-polyacrylamide gels, from their elution positions in Sephadex gels, and by analytical ultracentrifugation. The latter two methods gave a molecular weight of 75,000 to 80,000 for the intact fragment and 25,000 for the component chains released by reduction. The electrophoretic data gave somewhat higher molecular weights, which were judged to be anomalous. The fragment had an amino acid composition quite different from that of collagen: it was poorer in glycine and proline, it contained half-cystine residues, and 23% of its residues were derived from aspartate and glutamate.

Collagen α chains are synthesized as precursors (pro-α1 and pro-α2) with nonhelical, NH2-terminal peptide extensions (propeptides) (1–9). Using cultured human diploid fibroblasts, we have presented biochemical (10, 11) and immunological (12) evidence that two pro-α1 chains and one pro-α2 chain assemble intracellularly to form a molecule with a molecular weight of approximately 360,000, which we have called pro-tropocollagen. The molecule is stabilized by disulfide bonds between the pro-peptides of all three chains and by noncovalent interactions between the helical, α chain segments. After secretion from the cell, native tropocollagen is generated by sequential, enzymatic excision of propeptide sequences from the precursor.

Tryptophan is not present in tropocollagen but has been identified in pro-α1 chains (13, 14). We report the use of [3H]tryptophan as a marker for pro-α1 and pro-α2 chains and describe a method for the isolation and purification of the disulfide-assembled, three-chain propeptide fragment. The method employs collagenase to digest the helical portion of pro-tropocollagen (14), and the undigested propeptide fragment is then isolated by gel filtration and ion exchange chromatography. Molecular weights are calculated for the fragment and the component chains released by reduction, and the amino acid composition for the intact fragment is given.

MATERIALS AND METHODS

Cells, Culture Conditions, and Processing of Medium—Experiments were performed with confluent cultures of normal human fibroblast strains CRL-1121 and CRL-1106 (American Type Culture Collection). Both strains gave identical results, and only the data from CRL-1121 fibroblasts are presented. Cells in the 8th to 10th passage were grown at 37°C in plastic Petri dishes (80 mm diameter) in Dulbecco-Vogt medium (Grand Island Biological), supplemented with 10% fetal calf serum and sodium ascorbate (75 μg per ml). For purification of pro-tropocollagen, the standard medium was replaced for 24 hours with serum-free medium containing ascorbate. This had no significant effect on the secretion of pro-tropocollagen. After the serum-free medium was collected, standard medium was added back to the cultures for at least 48 hours. Collected medium was chilled to 4°C and acetic acid was added to 0.5 M. The medium was then dialyzed exhaustively against cold water and lyophilized. For large scale isolations, serum-free medium was serially collected from 50 plates, so that the starting material contained the equivalent of the 24-hour pro-tropocollagen production of 106 cells. To label pro-tropocollagen, L-[3H]tryptophan (2.3 Ci per mmole, New England Nuclear, Boston, Mass., 100 μCi per plate) was added to the cultures with the serum-free medium.

Gel Filtration—A Sephadex G-100 column (2.5 × 40 cm) was equilibrated and eluted with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.001 M CaCl2. A Sephadex G-200 column (2.5 × 60 cm) was equilibrated and eluted with 0.05 M…

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were acrylamide gels polymerized in 0.05 M Tris-HCl buffer, pH 9.4. Immunoglobulin A heavy (mol wt 56,000) and light (mol wt 22,500) chains (gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on α1 chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine).

 Ionic Exchange Chromatography—A DEAE-Sephadex column (A-50, 1.5 × 30 cm) was equilibrated with 0.025 M Tris-HCl buffer, pH 8.5. Samples were applied and eluted in the same buffer with a linear gradient of NaCl from 0 to 0.5 M over a total volume of 270 ml.

Acrylamide Gel Electrophoresis—Electrophoresis on 5% acrylamide gels containing 0.1% sodium dodecyl sulfate and 0.5 M urea was performed as previously described (10). Authentic 14C-labeled α chains were added as internal markers. In some experiments, parallel gels were calibrated with cytochrome c and 125I-labeled immunoglobulin G (mol wt 157,000) and immunoglobulin A heavy (mol wt 56,000) and light (mol wt 22,500) chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine). Electrophoresis was also performed on 7S chains (a gift of Dr. Michael Lamm, New York University School of Medicine).

Immunochemical Procedures—Preparation of rabbit antisera against the antigen-free culture medium has been described (12). This antigen forms three precipitin lines in double immunodiffusion plates when tested against the immunizing antigen. The antigen contains antibodies specific for the NH₂-terminal propeptide determinants of pro-tropocollagen and does not recognize native tropocollagen. Titration of the antisera against [3H]labeled culture medium or against solutions of radiolabeled propeptide were performed as described previously (12). Prior to gel electrophoresis, immune precipitates were dissolved in 0.01 M phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate and 0.5 M urea.

Collagenase Digestion—Pooled fractions from Sephadex G-100 columns were digested for 16 hours at 37°C with purified collagenase (Worthington, Freehold, N.J.; CILSPA, 150 mg per ml) before reapplication to columns. In some experiments, immune precipitates of radiolabeled medium were suspended in 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.005 M CaCl₂, and were digested with collagenase as above. Digests were made 1% in sodium dodecyl sulfate and dialyzed against the running buffer for sodium dodecyl sulfate-acrylamide gels.

Collagenase Assay—Fractions from Sephadex G-100 columns were assayed for collagenase activity by a modification of the method of Grant and Alburn (15). Protein in 0.1-ml aliquots from each column fraction was estimated by the method of Lowry et al. (10) using a bovine serum albumin standard. Other aliquots of column fractions were incubated at 37°C for 60 min with 20 mg per ml of bovine achilles tendon collagen. After incubation, insoluble collagen was removed by centrifugation, and the protein in the supernatant fluid was measured. The amount of protein solubilized in 60 min was taken as an index of collagenase activity.

Reduction and Alkylation—Immune precipitates were dissolved in 0.5 M Tris-HCl buffer, pH 8.5, containing 1% sodium dodecyl sulfate and were reduced with 0.1 M dithiothreitol for 1 hour at 22°C. Iodoacetamide (recrystallized twice from petroleum ether) was added to give a final concentration of 0.24 M, and incubation was continued for 1 hour. The samples were then dialyzed against the running buffer for sodium dodecyl sulfate-acrylamide gels.

Desalted, lyophilized propeptide from DEAE-Sephadex columns was dissolved in 0.5 M Tris-HCl buffer, pH 8.5, and reduced and alkylated as above. Samples then were dialyzed against the running buffer for sodium dodecyl sulfate-acrylamide gels or against the eluting buffer for Sephadex G-100 gel filtration.

Amino Acid Analyses—Amino acid analyses were performed with a Spinco model 120C amino acid analyzer equipped for high sensitivity. Protein was hydrolyzed in constant boiling HCl for 18 hours at 110°C in vacuo. One aliquot was oxidized with performic acid (17) prior to hydrolysis.

Equilibrium Ultracentrifugation—Sedimentation equilibrium measurements were performed by the meniscus depletion method of Yphantis (18) using a Spinco model E ultracentrifuge equipped with interference optics. Centrifugation was performed for 18 hours at 16,000 rpm (20°C) using 0.4 mg per ml of protein (Lowry method (16)) dissolved in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl. Photographic plates were read on a Nikon comparator. A partial specific volume of 0.72 cc per g was calculated from the amino acid composition (19), and data were fitted by computer using a least squares method.

RESULTS

Experiments were performed to test whether [3H]tryptophan could be specifically incorporated into the propeptides of pro-α1 and pro-α2 chains. Cultures were labeled for 24 hours with [3H]tryptophan, and freeze-dried samples of the acidified medium were dissolved and subjected to electrophoresis on sodium dodecyl sulfate gels (Fig. 1A). Two major radioactive peaks were found. Peak A corresponds in electrophoretic mobility to intact pro-tropocollagen and Peak B, to a denatured digestion intermediate derived from the latter (10, 11). The other radioactive peaks are proteins of lower molecular weight than α chains and presumably are not collagenous molecules.

To confirm that Peak A was pro-tropocollagen, [3H]tryptophan-labeled medium was precipitated with serum containing antibodies to the NH₂-terminal propeptide determinants of pro-tropocollagen (12). Titration revealed that 45% of the radioactive material in the medium could be precipitated at antibody excess. When the dissolved immune precipitates were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, all of the radioactivity was found in Peak A (Fig. 1B). Gel electrophoresis of reduced and alkylated immune precipitates showed that both pro-α1 and pro-α2 chains were labeled (Fig. 1B).

To test if a three-chain, propeptide fragment could be derived from pro-tropocollagen by enzymatic digestion of the helical sequences, radiolabeled immune precipitates were digested with collagenase, dissolved, and dialyzed for gel electrophoresis. No radioactivity was lost during dialysis, indicating that all of the incorporated [3H]tryptophan was in a collagenase-insensitive segment of pro-tropocollagen. Electrophoresis of the digests on sodium dodecyl sulfate-acrylamide gels showed a single peak.
with a mobility greater than immunoglobulin A (Fig. 2). When collagenase-treated immune precipitates were reduced and alkylated prior to electrophoresis, all of the radioactivity was recovered in a new peak with a molecular weight one-third that of the unreduced fragment (Fig. 2, and see below). These experiments show that a three-chain, propeptide fragment assembled through disulfide bonds can be obtained by collagenase digestion of pro-tropocollagen.

**Large Scale Isolation and Purification of the Propeptide Fragment**—After dialysis and lyophilization of 1.5 liters of [3H]pro-tropocollagen, the material was subjected to electrophoresis on sodium dodecyl sulfate gels of an aliquot of the pooled fractions from the Sephadex G-100 column (Fig. 3A), and the material eluting in the void volume (brackets) was pooled. Rechromatography demonstrated that all of the material was redelivered in the void volume. A sodium dodecyl sulfate-acylamide gel of an aliquot of the excluded material confirmed that pro-tropocollagen was present in these fractions. When the pooled fractions were treated with collagenase and rerun on Sephadex G-100 columns, the pattern shown in Fig. 3B was obtained. Less radioactivity was delivered in the void volume, and a major radioactive peak (brackets) was now present which eluted just ahead of the bovine serum albumin marker. As the absorbance and radioactivity were not coincident in this region, it seemed likely that the radioactive species was contaminated with unlabeled protein(s). Assay of the column fractions showed that 35 to 50% of the collagenase activity applied to the column eluted with the major radioactive peak, and stained sodium dodecyl sulfate gels of an aliquot of the pooled fractions showed four protein bands, only one of which was labeled.

To purify further the presumptive propeptide fragment, the pooled fractions from the Sephadex G-100 column (Fig. 3B) were desalted, lyophilized, and chromatographed on DEAE-Sephadex with a linear salt gradient (Fig. 4). At least four major absorbing species were resolved, but only one major radioactive species was eluted between 150 and 180 ml. When the pooled fractions (brackets) were desalted and lyophilized, and the protein was subjected to electrophoresis on sodium dodecyl sulfate gels, a single symmetrical peak of radioactivity was obtained with a mobility identical with that of the propeptide fragment isolated by collagenase digestion of immune precipitates (see above). Reduction and alklylation of the sample prior to electrophoresis, as above, of dissolved immune precipitates prepared by precipitation of [3H]tryptophan-labeled culture medium with an antiserum to pro-tropocollagen. Precipitates were run before (---) or after (O--O) reduction and alklylation.

**Fig. 1.** A, sodium dodecyl sulfate polyacrylamide gel electrophoresis of medium from cultured fibroblasts labeled for 24 hours with [3H]tryptophan. Peaks A and B have mobilities expected for pro-tropocollagen and a digestion intermediate, respectively. The positions of [3H]-labeled a chains run as internal markers are indicated. B, electrophoresis, as above, of dissolved immune precipitates prepared by precipitation of [3H]tryptophan-labeled culture medium with an antiserum to pro-tropocollagen. Precipitates were run before (---) or after (O--O) reduction and alklylation.

**Fig. 2.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis of dissolved immune precipitates after digestion with collagenase. Digested precipitates were run on gels before (○○○) and after (O--O) reduction and alklylation. The positions of protein markers run on a parallel gel are given: IgA, immunoglobulin A; H, immunoglobulin A heavy chains; L, immunoglobulin A light chains; Cyto C., horse heart cytochrome c.

**Fig. 3.** Gel filtration on Sephadex G-100. A, chromatography of [3H]tryptophan-labeled medium. The positions of globular protein markers used in calibrating the column prior to the run are indicated: DB, blue dextran 2000, excluded from the column; BSA, bovine serum albumin; OVA, ovalbumin; Cyto C., horse heart cytochrome c. O--O, B, 3H radioactivity; O--O, absorbance at 280 nm. Brackets indicate pooled V₆ fractions. B, chromatography of the V₆ fraction from A after treatment with collagenase. Symbols are the same as those used in A. Brackets indicate pooled fractions from major peak.
to electrophoresis gave a single radioactive peak with a mobility identical with that obtained for the reduced samples of Fig. 2. When the pooled DEAE-Sephadex fraction was titrated with anti-pro-tropocollagen serum, 100% of the radioactivity was precipitated at antibody excess. A double immunodiffusion assay with the same antigen and antiserum gave a single line of precipitation (Fig. 5A). These data show that a disulfide-assembled, three-chain propeptide fragment had been isolated and that it retained its antigenicity after purification.

To estimate the purity of the preparation, 40 µg (estimated by the Lowry method (16)) of the propeptide fragment were run on a Tris-HCl-buffered gel (pH 9.4) without denaturants. After staining, only a single band was detected (Fig. 5B) which contained all of the radioactive material applied to the gel (Fig. 5C). When 100 µg of the same material was run on a sodium dodecyl sulfate gel (Fig. 5D), staining revealed a major band in the predicted position, which contained all of the radioactive material, and a lighter stained band running just ahead of the major band. Densitometry showed that the minor band contained 6% of the total detectable protein in the gel. These results indicate that the propeptide fragment is contaminated by a small amount of unlabeled protein, possibly derived from the collagenase preparation.

The recoveries of radioactivity and absorbing species (280 nm) at each step in the purification are shown in Table I. After DEAE-Sephadex chromatography, 15.6% of the radioactivity and 5.5% of the absorbing species were recovered. Since immune precipitation showed that only 45% of the initial radioactivity in the medium was incorporated into the propeptide sequences of pro-tropocollagen, the final recovery of radioactive propeptide was approximately 35%.

Molecular Weight Measurements—The molecular weights of the propeptide fragment and its constituent chains were derived from calibration of the acrylamide and Sephadex gels with globular proteins. Plots of log molecular weight versus mobility or elution volumes were employed. For acrylamide gels (e.g. Fig. 2), the molecular weight of the intact propeptide fragment

![Figure 4](image-url) DEAE-Sephadex chromatography of pooled fractions from Sephadex G-100 (Fig. 3B). The arrow indicates the start of a linear gradient of NaCl from 0 to 0.5 M, over 270 ml. ■ ■ ■, radioactivity; ■ ■ ■, absorbance at 280 nm. Brackets indicate pooled fractions containing purified propeptide fragment.

![Figure 5A](image-url) Double immunodiffusion assay and gel electrophoresis of propeptide fragment from DEAE-Sephadex (Fig. 4). A, double immunodiffusion assay using purified propeptide fragment (p) and antiserum (ab) against NH₂-terminal propeptide determinants of pro-tropocollagen. B, electrophoresis of propeptide fragment on Tris-HCl buffered gel at pH 9.4. O denotes the cathodal origin. C, the same gel shown in B was cut into 1-mm slices and radioactivity in the slices was determined. The position of the labeled band (■■■) coincides with the position of the stained band shown in B. D, electrophoresis of propeptide fragment on a sodium dodecyl sulfate gel. Tapered origin (O) and width of major band indicates overloading of the gel.
The data clearly show that the spectroscopic analysis (not shown) shows a low absorbance at the conditions used for the analyses, hydroxylysine and hydroxyproline levels. If present, would not have been uniquely identified. Under 25,000 molecular weight.

The purified material is relatively rich in aspartate and glutamate with the acidic behavior of the fragment on the DEAE-Sephadex column. As expected, half-cystine residues, not found in α chains, are present. Although tryptophan was not measured, it must be present in both pro-α1 and pro-α2 propeptide components (see Fig. 1B). However, spectroscopic analysis (not shown) shows a low absorbance at 280 nm relative to the absorbance at 235 nm, suggesting that the tryptophan content is low. These data clearly show that the composition of the propeptide fragment differs from that of tropocollagen.

**Table I**

Recoveries of radioactivity and absorbing species at 280 nm

| Sample                  | Radioactivity | Absorbance | Radioactivity recovered | Absorbance recovered |
|-------------------------|---------------|------------|------------------------|----------------------|
| Lyophilized culture medium | 7.31          | 27.3       | 100                    | 100                  |
| Excluded fraction, Sephadex G-100 column | 5.18          | 15.2       | 70.9                   | 55.7                 |
| Included major peak, Sephadex G-100 column | 2.52          | 9.50       | 34.5                   | 34.8 \(a\)           |
| Major peak, DEAE-Sephadex | 1.14          | 1.51       | 15.6                   | 5.5                  |

\(a\) Includes contribution of contaminating collagenase.

was calculated to be 105,000, and chains of 33,000 molecular weight were generated by reduction and alkylation. By contrast, data from Sephadex G-100 columns gave a value of 75,000 for the intact fragment and 26,000 for the individual chains released by reduction. Calibration of a Sephadex G-200 column in the presence of 0.1% sodium dodecyl sulfate gave a value of 80,000 for the intact fragment, in agreement with the results obtained without denaturant.

Since different results were obtained by electrophoresis and gel filtration, the molecular weight of the propeptide fragment was also determined by sedimentation equilibrium. No heterogeneity was detected in the sample by interference and Schlieren optics, and a value of 75,400 was obtained, in good agreement with the gel filtration data. We therefore believe that the mobilities of the intact and reduced propeptide fragments on sodium dodecyl sulfate gels are somewhat anomalous and consider the fragment to be composed of three chains, each of approximately 25,000 molecular weight.

**Amino Acid Composition**—The amino acid composition of the purified fragment is shown in Table II. We caution that errors might have been introduced by the presence of an unidentified contaminant at a level of approximately 6% (see above). Under the conditions used for the analyses, hydroxylysine and hydroxyproline, if present, would not have been uniquely identified. The purified material is relatively rich in aspartate and glutamate which comprise 25% of the total residues. This is consistent with the acidic behavior of the fragment on the DEAE-Sephadex column and in the Tris-HCl buffered gel. As expected, half-cystine residues, not found in α chains, are present. Although tryptophan was not measured, it must be present in both pro-α1 and pro-α2 propeptide components (see Fig. 1B). However, spectroscopic analysis (not shown) shows a low absorbance at 280 nm relative to the absorbance at 235 nm, suggesting that the tryptophan content is low. These data clearly show that the composition of the propeptide fragment differs from that of tropocollagen.

**Table II**

Amino acid composition of propeptide fragment

| Amino acid | Residues per mole \(a\) |
|------------|-------------------------|
| Lysine     | 44                      |
| Histidine  | 11                      |
| Arginine   | 25                      |
| Aspartic Acid | 85               |
| Threonine  | 43                      |
| Serine     | 54                      |
| Glutamic Acid | 79               |
| Proline    | 37                      |
| Glycine    | 63                      |
| Alanine    | 66                      |
| Half-cystine | 23                     |
| Valine     | 42                      |
| Methionine | 11                      |
| Isoleucine | 28                      |
| Leucine    | 38                      |
| Tyrosine   | 24                      |
| Phenylalanine | 25          |
| Tryptophan | Not determined          |

\(a\) Residues per mole were calculated using 75,400 as the molecular weight of the fragment, assuming no carbohydrate and neglecting tryptophan. Half-cystine was determined as cysteic acid following performic acid oxidation. Methionine was determined as such, or as the sulfone.

sequences of the pro-α1 and pro-α2 chains. Collagenase treatment of the immune precipitate digested the helical α chain segments of the precursor and generated a protein fragment which, upon reduction, gave polypeptide chains with one-third the molecular weight of the assembled fragment.

Given these observations, tryptophan-labeled protein from a large volume of culture medium was subjected to gel filtration, collagenase digestion, and ion exchange chromatography. After these procedures, a radioactive protein was isolated in reasonable yield which, by immunologic and biochemical criteria, was identified as the three-chain, disulfide-assembled propeptide fragment of the precursor molecule.

It is generally agreed that tropocollagen has a molecular weight of 285,000, and we have calculated a molecular weight of 360,000 for pro-tropocollagen using sodium dodecyl sulfate gels calibrated with collagen components (10). The propeptide fragment should account for the difference between these two molecular weights. By the gel filtration and centrifugation analyses reported here, the molecular weight of the purified fragment was found to be 75,000 to 80,000, in good agreement with the expected value. However, the molecular weights of the fragment and its component chains in sodium dodecyl sulfate gels were significantly greater than those determined by the other two techniques. Others have reported anomalous electrophoretic mobilities in sodium dodecyl sulfate gels for propeptide derived from pro-α1 chains, particularly in 5% gels, where the sieving effect is reduced (13, 14). Our results also suggest that the mobilities of the fragment and its component chains are anomalous. Glycoproteins are known to behave anomalously in sodium dodecyl sulfate gels (20), and carbohydrate has been found in a propeptide derived from pro-α1 chains (14). We therefore suggest that the presence of carbohydrate could account for the observed anomalous mobilities.

Compared to tropocollagen, the propeptide fragment is rela-
tively rich in aspartate and glutamate and poor in glycine and proline. Moreover, it contains cysteine and tryptophan which are not present in tropocollagen. In these respects, the data are in agreement with the results obtained for pro-α1 propeptides from embryonic cranial bone (13) and the skin of dermatosparaxic cattle (14). It must be emphasized, however, that our analyses were performed on a fragment containing propeptide sequences of both pro-α1 and pro-α2 chains; therefore, critical comparison with the published data for pro-α1 propeptides is not possible. Additional problems arising in such comparisons are: tissue and species differences, the varying lengths of pro-α chains analyzed in different instances, and the demonstrated presence of a small quantity of contaminating protein in our analyses.

Most of the collagen precursors isolated from the culture medium are disulfide-assembled molecules, but a smaller quantity of nonequivalently assembled three-chain precursors is also present (10–12). The latter arise through the action of an extracellular enzyme which sequentially excises the propeptides of pro-tropocollagen. The enzymatically shortened pro-α chains have been shown to lack some or all of their cysteine residues and to migrate just behind the marker α1 and α2 chains in sodium dodecyl sulfate gels (10, 11). The experiment of Fig. 1A shows that there is only background radioactivity in the region of the marker chains and so, shortened pro-α chains also lack tryptophan. These data demonstrate that pro-α chains released by denaturation of digestion intermediates can be expected to differ in molecular weight and amino acid composition from pro-α chains released by reduction and denaturation of unprocessed pro-tropocollagen.

Studies of pro-α chains isolated from embryonic cranial bones initially give no evidence for disulfide assembly (1, 2, 7). However, Monson and Bornstein (21) have recently reported that a disulfide-assembled collagen precursor can be isolated from this system with a different method of extraction. This suggests that the earlier analyses may have been performed on shortened pro-α chains released by denaturation of a partially digested precursor molecule. Given the accumulating evidence for disulfide assembly of pro-α chains in a variety of culture systems (see also Refs. 5, 8, 22), it appears that the manner of assembly and extracellular processing of the collagen precursor molecule is similar for most tissues.

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