Biosynthesis of Type II Collagen. Removal of Amino- and Carboxy-Terminal Extensions from Procollagen Synthesized by Chick Embryo Cartilage Cells†

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ABSTRACT: The synthesis of cartilage procollagen was studied by incubating intact sterna or matrix-free cells prepared from sterna of 17-day-old chick embryos with [^{14}C]proline. The [^{14}C]proline-labeled procollagen isolated by diethylaminoethylcellulose chromatography was shown on the basis of cyanogen bromide peptide mapping to be a precursor of type II collagen. Cleavage with purified human collagenase demonstrated that type II procollagen contained peptide extensions at both ends of the molecule. When the cells were incubated with [^{35}S]cystine or [^{3}H]tryptophan, the extension peptides of the newly synthesized procollagen incorporated these two amino acids which are not found in the collagen portion of the molecule. The conversion of type II procollagen to collagen was studied by pulse labeling the intact sterna with [^{14}C]proline for 6 min, and further incorporation of the radioactivity was then stopped by the addition of [^{12}C]proline and cycloheximide. Examination of the ^{14}C-labeled protein by polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate demonstrated that most of the ^{14}C at the end of the pulse was in procollagen. If the incubation was continued for 2 h in the presence of [^{12}C]proline and cycloheximide, most of the radioactivity was recovered in ^{14}C-labeled α chains. In addition, two intermediate polypeptides, tentatively identified as p_c and p_n α chains, were also noted. The conversion of type II procollagen to collagen was completely inhibited by 1 mM disodium ethylenediaminetetraacetate, and the inhibition could be reversed by the addition of 10 mM Ca^{2+}. A serine protease inhibitor, α-toluenesulfonyl fluoride, had no effect on the conversion. The results demonstrate that processing of type II procollagen to collagen involves at least two separate cleavages, the amino- and carboxy-terminal extensions being removed separately by proteases which may require Ca^{2+} or another metal ion for their activity.

The intracellular biosynthesis of collagen involves translation of messenger RNA molecules coding for the precursor polypeptides, pro-α chains (for recent reviews on collagen biosynthesis, see Bornstein, 1974; Miller and Matukas, 1974; Gross, 1974; Martin et al., 1975; Veis and Brownell, 1975; Kivirikko and Risteli, 1976; Uitto and Lichtenstein, 1976a; Prockop et al., 1976). The initially synthesized pro-α chains are larger than collagen α chains because they contain additional peptide extensions at both the amino- and carboxy-terminal ends of the polypeptide chains (Tanzer et al., 1974; Fessler et al., 1975; Byers et al., 1975; Olsen et al., 1976; Uitto et al., 1976; Hoffmann et al., 1976; Lichtenstein et al., 1976). The extension peptides of type I procollagen[1] are different from the collagen portion of the molecule in that they contain amino acids such as cystine and tryptophan, which are not found in type I collagen. After translation, three pro-α chains associate and become linked by interchain disulfide bonds, and the collagen portions of the polypeptide chains fold into a triple-helical conformation (see Prockop et al., 1976; Schofield et al., 1974). The triple-helical procollagen molecules containing the nonhelical extension peptides at both ends of the molecule are subsequently converted to collagen by limited proteolysis which removes the amino- and carboxy-terminal extensions by at least two separate cleavages (Bornstein et al., 1975; Davidson et al., 1975; Fessler et al., 1975; Uitto and Lichtenstein, 1976b).

Cartilage contains a genetically distinct type of collagen, type II, which consists of three identical α chains (Miller and Matukas, 1969; Trelstad et al., 1970; Miller, 1971, 1972). Sternal cartilages and matrix-free cells prepared from sterna of 17-day-old chick embryos have previously been shown to synthesize procollagen which consists of a single type of pro-α chain and which contains peptide extensions at the amino- and carboxy-terminal ends of the molecule (Dehm and Prockop, 1973; Uitto and Prockop, 1974a; Müller and Jamhawi, 1974; Harwood et al., 1975; Olsen et al., 1976; Merry et al., 1976; Uitto et al., 1977). In the present work, we have studied the conversion of type II procollagen to collagen in sternal cartilages of chick embryos employing pulse-chase techniques in vitro. The results demonstrate that removal of the amino- and carboxy-terminal extensions involves two separate cleavages catalyzed by proteases requiring a metal ion, possibly Ca^{2+}. The results also demonstrate that the processing of type II procollagen is different from the processing of type I procollagen in that intermediates containing the amino-terminal extensions as well as intermediate polypeptides containing the carboxy-terminal extensions could be detected.

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[1] The term procollagen is used here to signify a collagen precursor molecule which contains intact extension peptides at both ends of the molecule. The designation p_n collagen refers to a precursor molecule which contains the amino-terminal extensions but is devoid of carboxy-terminal extension peptides. Correspondingly, p_c collagen refers to a precursor molecule which has the carboxy-terminal extensions present but which has lost the amino-terminal extensions. The p_c collagen and p_n collagen molecules consist of p_c α chains and p_n α chains, respectively.

Materials and Methods

Materials. Unless otherwise indicated, all the materials were purchased from the same suppliers as indicated previously (Dehm and Prockop, 1973; Uitto et al., 1977).

Preparation and Incubation of Embryonic Sterna. Sternal cartilages were dissected from 17-day-old chick embryos, the surrounding perichondrium was carefully removed, and the sterna were rinsed with a phosphate-free buffer solution, consisting of 1.5 mM KCl, 120 mM NaCl, 4 mM $NaHCO_3$, 1.4 mM $MgCl_2 \cdot 6H_2O$, and 13 mM dextrose, all in 20 mM Hepes[2] buffer, pH 7.4 (Krebs, 1950; Uitto 1970). The sterna were then incubated in the same medium supplemented with 20% fetal calf serum, 50 μg/mL ascorbic acid and 50 μg/mL β-aminopropionitrile hydrochloride at 37 °C. In some experiments, 1 mM $CaCl_2$ was added to the incubation medium (see Results).

For pulse-chase experiments, eight to ten sterna were incubated in 2.0 mL of the Hepes-buffered medium described above and containing 20% fetal calf serum, 50 μg/mL ascorbic acid and 50 μg/mL β-aminopropionitrile. After a 15-min preincubation at 37 °C, 3 μCi of [^{14}C]proline was added, and the incubation was continued for 6 min. Further incorporation of [^{14}C]proline was stopped by adding 0.1 mL of medium solution containing 2.2 mg/mL cycloheximide and 2.2 mg/mL [^{12}C]proline, and the incubation was continued for varying time periods (see Results).

At the end of each incubation, 0.5 mL of boiling 20% sodium dodecyl sulfate, containing 0.25 M iodoacetamide, 3 mM α-toluenesulfonyl fluoride, 0.1 M *N*-ethylmaleimide, and 0.2 M Na_2EDTA, was added, and the sample was immediately heated at 100 °C for 5 min. The sample was homogenized with a Polytron high-speed mechanical homogenizer and then further incubated at 37 °C for 2 h. After incubation, the samples were centrifuged at 18 000*g* for 30 min at 25 °C, and aliquots of the supernatant were prepared for polyacrylamide gel electrophoresis in sodium dodecyl sulfate by dialyzing against a buffer containing 2% sodium dodecyl sulfate, 10% glycerol, and 0.01% bromophenol blue in 0.062 M Tris-HCl, pH 6.8, at 25 °C. Dialyzed samples were then taken for slab-gel electrophoresis in sodium dodecyl sulfate as described below.

Preparation of Type II Procollagen from Matrix-Free Cartilage Cells. Cells from sterna of 17-day-old chick embryos were isolated by enzymatic digestion using trypsin and bacterial collagenase as described previously (Dehm and Prockop, 1973; Uitto and Prockop, 1974a). The cells were washed three times with modified Krebs medium (Dehm and Prockop, 1971, 1972) containing 10% fetal calf serum, and the cells were then incubated at 37 °C in a concentration of 10^7 cells per mL in modified Krebs medium containing 20% fetal calf serum, 50 μg/mL ascorbic acid, and 50 μg/mL β-aminopropionitrile hydrochloride. After a 15-min preincubation, the radioactive amino acids were added, and the incubations were continued for 2 h. At the end of each incubation, 10 mM *N*-ethylmaleimide, 0.3 mM α-toluenesulfonyl fluoride, and 20 mM Na_2EDTA was added (Uitto and Lichtenstein, 1976b; Uitto et al., 1976), the incubation mixture was cooled to 0 °C, and the medium was then separated from the cells by centrifugation at 1200*g* for 3 min. The radioactive proteins in the medium were precipitated by the addition of 176 mg/mL ammonium sulfate (30% of saturation), and the precipitate was recovered by centrifugation at 18 000*g* for 30 min at 4 °C. The radioactively labeled procollagen was then isolated by DEAE-cellulose chromatography as described below.

Chromatographic Procedures. To chromatograph the radioactive proteins on DEAE-cellulose, the ammonium sulfate precipitates were dissolved in 5 mL of starting buffer consisting of 2 M urea in 0.025 M Tris-HCl, pH 7.5, and containing 1 mM Na_2EDTA. The samples were dialyzed against the same buffer for 3 h, changing the dialysis buffer three times. The ^{14}C-labeled protein was then chromatographed on a 2.5 × 10 cm column of DEAE-cellulose, as described previously (Smith et al., 1972; Uitto and Lichtenstein, 1976b; Uitto et al., 1976). The sample was eluted with a linear gradient prepared with 300 mL of starting buffer containing 2 M urea in 0.025 M Tris-HCl, pH 7.5, and 300 mL of limit buffer containing 0.3 M NaCl and 2 M urea in 0.025 M Tris-HCl, pH 7.5, at 8 °C. The chromatography was performed at a flow rate of 180 mL/h. Fractions of 8 mL were collected, and 0.4-mL aliquots were taken for assay of radioactivity. The peaks of radioactive protein were then pooled, 0.3 mM α-toluenesulfonyl fluoride, 10 mM *N*-ethylmaleimide, and 20 mM Na_2EDTA were added, and the samples were dialyzed against 0.4 M NaCl in 0.1 M Tris-HCl, pH 7.5, containing 1 mM Na_2EDTA. The radioactive proteins were then precipitated by adding 176 mg/mL ammonium sulfate, as indicated above.

Polyacrylamide Slab-Gel Electrophoresis in Sodium Dodecyl Sulfate. For electrophoresis on polyacrylamide slab gels in sodium dodecyl sulfate, the ammonium sulfate precipitates of radioactive proteins were dissolved in 0.2 mL of 2% sodium dodecyl sulfate and 0.05 M iodoacetamide in 0.062 M Tris-HCl, pH 7.4. The samples were heated for 3 min at 100 °C, incubated at 37 °C for an additional 2 h, and dialyzed against buffer consisting of 2% sodium dodecyl sulfate, 10% glycerol, and 0.01% bromophenol blue in 0.062 M Tris-HCl, pH 6.8. In some experiments, reduction was carried out by adding 2% 2-mercaptoethanol and heating for 10 min at 37 °C just prior to the electrophoretic run. The samples were electrophoresed on 10 × 14 cm slab gels using a system described elsewhere (King and Laemmli, 1971; Studier, 1973). After electrophoresis, the gels were soaked in 400 mL of Me_2SO for 30 min, and the procedure was repeated four times using fresh Me_2SO. The gels were then submerged in 4 volumes of 20% (w/w) PPO in Me_2SO (22.2%, w/w) for 3 h, rinsed in distilled water for 1 h, and then dried under vacuum. The dried gels were exposed on x-ray film (RP Royal X-Omat; Eastman Kodak) at −70 °C for 24 to 76 h (Bonner and Laskey, 1974). The radioautographs were scanned using a double-beam densitometer (Joyce-Loeb, England).

Enzymatic and Chemical Treatments of Proteins. To digest radioactive proteins with bacterial collagenase, the ammonium sulfate precipitates of the proteins were dissolved in 1 mL of 0.15 M NaCl and 0.01 M $CaCl_2$ in 0.05 M Tris-HCl buffer, pH 7.6, at 4 °C, and then dialyzed against the same buffer. Purified bacterial collagenase (Advanced Biofactors, N.Y.), 50 μg/mL, and 2.5 mM *N*-ethylmaleimide were added, and the samples were incubated for 15 h at 4 °C. The incubation was stopped by adding 1/10 volume of 0.25 M Na_2EDTA, and the samples were extensively dialyzed against 0.15 M NaCl and 1 mM Na_2EDTA, in 0.05 M Tris-HCl buffer, pH 7.6, at 4 °C. The release of radioactive peptides by dialysis or formation of peptides which were not precipitable by 10% Cl_3-AcOH (Peterkofsky and Diegelmann, 1971) were taken as a measure of collagenous proteins in the sample.

[2] Abbreviations used are: DEAE, diethylaminoethyl; PPO, 2,5-diphenyloxazole; Na_2EDTA, disodium ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Cl_3AcOH, trichloroacetic acid.

To prepare radioactive proteins isolated by DEAE-cellulose chromatography for digestion with a purified human skin collagenase, the ammonium sulfate precipitates of the proteins were dissolved in 1 mL of 0.15 M NaCl and 0.01 M $CaCl_2$ in 0.05 M Tris-HCl buffer, pH 7.5. Mammalian collagenase, purified from the medium of cultured human fibroblasts as described by Stricklin et al. (1977), 0.3 mM α-toluenesulfonyl fluoride, and 10 mM *N*-ethylmaleimide were added to the sample, and the mixture was incubated at 22 °C for 20 h. The incubation was stopped by the addition of $1/10$ volume of 0.25 M Na_2EDTA, and the contents of the incubate were treated with sodium dodecyl sulfate and iodoacetamide in a final concentration of 2% and 0.05 M, respectively, as described above.

To digest radioactive procollagen samples with pepsin, the ammonium sulfate precipitates of radioactive proteins were resuspended in 2 mL of 0.5 N acetic acid and then dialyzed against 0.5 N acetic acid. Pepsin, 100 μg/mL, was added and the sample was incubated at 4 °C for 15 h. After incubation, the pepsin was inactivated by dialyzing the samples against 0.4 M NaCl in 0.1 M Tris-HCl buffer, pH 7.5, at 4 °C. The samples were then treated with sodium dodecyl sulfate for electrophoresis on slab gels as described above.

To prepare radioactive proteins obtained from DEAE-cellulose chromatography or type II collagen isolated according to Miller (1971), for digestion with cyanogen bromide, the samples were dialyzed against distilled water, lyophilized, and then dissolved in 2 mL of 70% formic acid. The samples were flushed with N_2 for 20 min at room temperature, 200 mg of cyanogen bromide was added, and the samples were incubated at 30 °C for 3 h (Epstein et al., 1971). The samples were then diluted with 10 mL of cold distilled water and most of the cyanogen bromide was removed by aspiration under a water pump for 1 h. The samples were then lyophilized, redissolved in 5 mL of distilled water, and relyophilized. This procedure was repeated three times in order to completely remove cyanogen bromide from the sample. The cyanogen bromide peptides were treated with 2% sodium dodecyl sulfate and directly electrophoresed on slab gel without dialysis, as described above.

Other Assays. ^{14}C was assayed with a liquid scintillation counter (Beckman LS 3155 P), and [^{14}C]hydroxyproline was assayed using a specific radiochemical method, as described previously (Juva and Prockop, 1966; Uitto and Prockop, 1974a).

Results

Isolation and Further Characterization of Procollagen Synthesized by Embryonic Cartilage Cells. Matrix-free cartilage cells isolated from sterna of chick embryos were incubated with [^{14}C]proline for 2 h, and a mixture of protease inhibitors was then added to the incubation medium in order to prevent any degradation of the proteins during subsequent processing of the material. [^{14}C]Proline-labeled protein recovered in the incubation medium was precipitated by ammonium sulfate and then chromatographed on DEAE-cellulose under nondenaturing conditions. Most of the ^{14}C-labeled protein was recovered from DEAE-cellulose as a sharp peak eluting in fractions 22–28 (Figure 1). Examination of the ^{14}C-labeled protein in this peak using slab-gel polyacrylamide electrophoresis in sodium dodecyl sulfate demonstrated that the ^{14}C-labeled protein consisted of molecules which had a mobility of less than γ chains of type II collagen if the samples were electrophoresed without reduction (Figure 2). If the

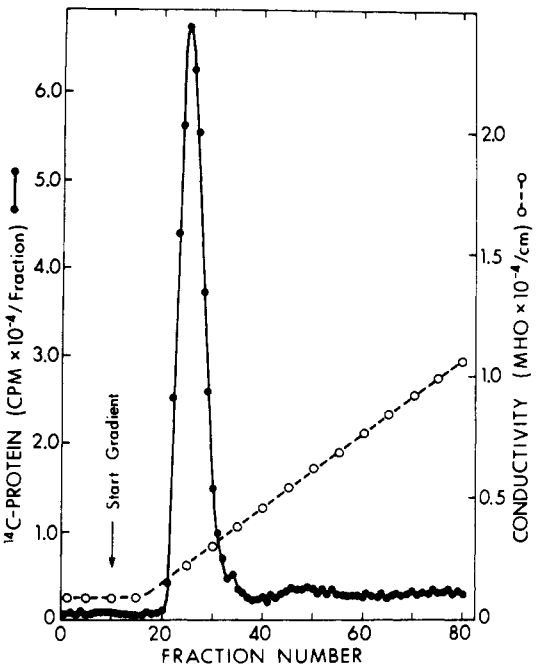

FIGURE 1: Isolation of [^{14}C]proline-labeled type II procollagen by DEAE-cellulose chromatography. Cartilage cells, 3.8×10^8, were incubated with 12 μCi of [^{14}C]proline for 120 min, and the [^{14}C]proline-labeled protein in the medium was then isolated and chromatographed on DEAE-cellulose, as described under Materials and Methods.

protein was reduced with 2-mercaptoethanol prior to electrophoresis, essentially all of the radioactive protein electrophoresed as a sharp band in the position between β and α chains of type II collagen (Figure 2). The isolated ^{14}C-labeled protein, therefore, consisted of polypeptide chains larger than α chains of collagen and these chains were linked by disulfide bonds.

The ^{14}C-labeled protein isolated by DEAE-cellulose chromatography was judged to be collagenous protein by the following criteria. First, the relative proportion of [^{14}C]hydroxyproline to the total ^{14}C incorporated into this protein was found to be 40%, a value which agrees with known proportions of hydroxyproline and proline in both type II procollagen and collagen (Miller, 1971; Uitto et al., 1977). Secondly, if the ^{14}C-labeled protein was incubated with highly purified bacterial collagenase, over 90% of the ^{14}C-labeled protein was converted into small peptides which were dialyzable and which were not precipitated by 10% Cl_3AcOH. Thirdly, if the ^{14}C-labeled protein was incubated with pepsin employing conditions under which the triple-helical portion of collagen molecule resists pepsin proteolysis essentially all ^{14}C-labeled protein was converted to ^{14}C-labeled peptides which in slab-gel electrophoresis in sodium dodecyl sulfate migrated in the same position as α chains of type II collagen (Figure 2). It is concluded, therefore, that the matrix-free cartilage cells synthesize and secrete a precursor form of collagen which is larger than collagen because of nonhelical extensions.

The synthesis of a similar precursor molecule of type II collagen was also demonstrated by incubating intact sterna from 17-day-old chick embryos in vitro. Sterna were incubated with [^{14}C]proline for 60 min, and the ^{14}C-labeled protein was then extracted and examined on DEAE-cellulose chromatography. A large amount of ^{14}C-labeled protein in the chromatogram eluted in the same position as the precursor molecules of collagen recovered from the medium of matrix-free cartilage cells. The ^{14}C-labeled protein in this peak had in sodium dodecyl sulfate slab-gel electrophoresis the same elu-

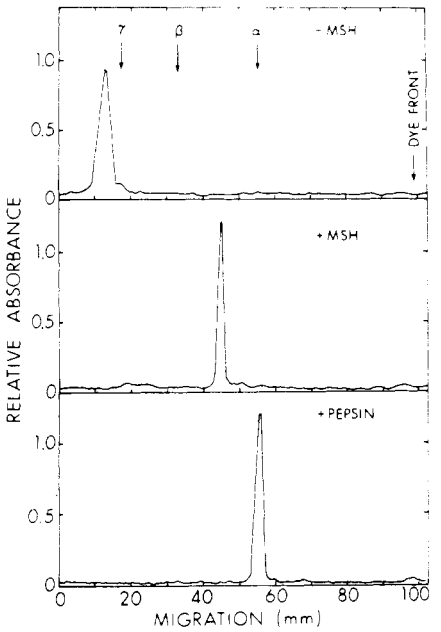

FIGURE 2: Polyacrylamide-sodium dodecyl sulfate slab-gel electrophoresis of [^{14}C]proline-labeled procollagen. The radioactive procollagen isolated by DEAE-cellulose chromatography (see Figure 1) was concentrated, and part of the sample was digested with pepsin, as described under Materials and Methods. The samples were then treated with sodium dodecyl sulfate and prepared for polyacrylamide gel electrophoresis. The slab gel was composed of a 2.5-cm stacking gel of 4.5% polyacrylamide and of a separating gel of 6% polyacrylamide. After electrophoresis, the ^{14}C-labeled peptides were detected by exposing the gels to x-ray films and the radioautographs were scanned by a densitometer, as described under Materials and Methods and by Uitto, et al. (1976). The samples were electrophoresed either without reduction (−MSH) or with reduction with 2-mercaptoethanol (+MSH). The migration position of the pepsin-treated ^{14}C-labeled protein was unaffected by the reduction with 2-mercaptoethanol. The migration positions of γ, β, and α chains of type II collagen isolated from 10-week-old chicken sterna according to Miller (1971, 1972) are indicated.

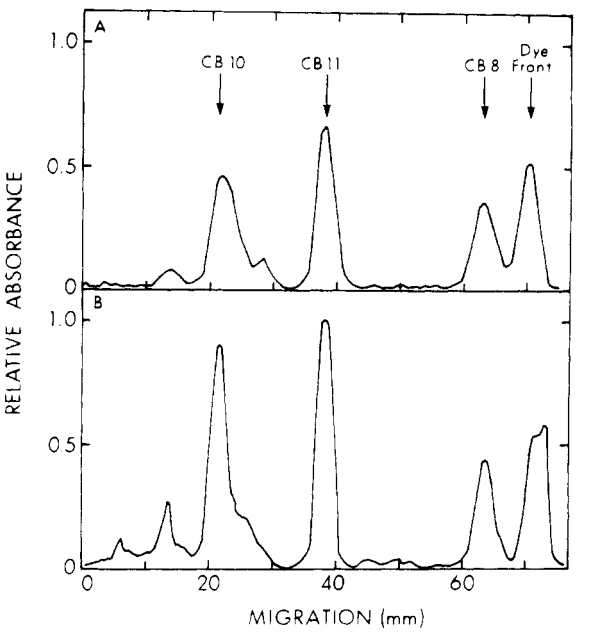

FIGURE 3: Cyanogen bromide peptide mapping of procollagen synthesized by matrix-free cartilage cells. The [^{14}C]proline-labeled procollagen isolated by DEAE-cellulose chromatography was digested with cyanogen bromide as described under Materials and Methods. The ^{14}C-labeled peptides were electrophoresed on an 8% polyacrylamide slab gel in sodium dodecyl sulfate. The radioactive peptides were then visualized by radioautography, and the radioautographs were quantitated by densitometric scanning (see Materials and Methods and Figure 2.). The cyanogen bromide peptide map was compared to a cyanogen bromide peptide pattern derived from authentic type II collagen isolated by pepsin digestion of 10-week-old chicken sterna, as described by Miller (1971, 1972). The cyanogen bromide peptides derived from quantitive amounts of type II collagen were visualized by staining the gels with Coomassie blue, and the stained bands were quantitated by scanning with a Gilford spectrophotometer 2400-S. Tentative identification of the cyanogen bromide peptides, based on previous publications (Miller, 1971; Smith et al., 1975), is indicated in the figure. Frame A: Peptides from ^{14}C-labeled procollagen. Frame B: Peptides derived from authentic type II collgen.

tion position as the procollagen isolated from medium of matrix-free cartilage cells, and limited proteolytic digestion by pepsin also converted essentially all ^{14}C-labeled protein in this peak to collagen α chains.

To identify the genetic type of the newly synthesized procollagen, the ^{14}C-labeled protein, isolated by DEAE-cellulose chromatography, was subjected to cleavage with cyanogen bromide and the peptide pattern was compared to the cyanogen bromide peptide pattern derived from type II collagen using slab-gel electrophoresis in sodium dodecyl sulfate (Figure 3). The pattern of radioactivity of ^{14}C-labeled peptides derived from the newly synthesized procollagen was similar to the cyanogen bromide peptide map derived from authentic type II collagen. On the basis of these experiments, it is concluded that the procollagen synthesized by cartilage cells and recovered in DEAE-cellulose in fractions 22–28 is a precursor of type II collagen.

In order to locate the noncollagenous extension peptides on the procollagen molecule, the ^{14}C-labeled protein from DEAE-cellulose chromatography was incubated with purified mammalian collagenase. Examination of the collagenase cleavage products on slab-gel electrophoresis in sodium dodecyl sulfate after reduction with 2-mercaptoethanol demonstrated that the cleavage by mammalian collagenase produced two fragments (upper frame in Figure 4). The larger ^{14}C-labeled fragment, migrating about 35 mm from the origin, had a mobility slightly less than α chains of collagen and clearly less than the α^{A} [3] produced by collagenase cleavage of carrier type II collagen. The smaller ^{14}C-labeled fragment, migrating about 65 mm from the origin, had an electrophoretic mobility intermediate between α^{A} and α^{B} markers of type II collagen. Incubation of the ^{14}C-labeled fragments with pepsin after collagenase digestion but before denaturation with sodium dodecyl sulfate converted the two ^{14}C-labeled collagenase cleavage fragments to α^{A} and α^{B} peptides. It can be concluded, therefore, that the collagenase cleavage fragment migrating at 35 mm, pro-α^{A}, is derived from the amino-terminal end of the procollagen molecule and consists of α^{A} plus an extension peptide. Correspondingly, the fragment migrating at 65 mm, pro-α^{B}, is derived from the carboxy-terminal portion of the procollagen molecule and consists of α^{B} and a nonhelical extension. Type II procollagen, therefore, contains nonhelical extension peptides at both ends of the molecule. Electrophoresis of the collagenase cleavage products without prior reduction demonstrated that treatment with 2-mercaptoethanol had no effect on the migration of pro-α^{A} (lower frame in Figure 4). For reasons unclear, a small amount of trailing was observed in this peak when the samples were electrophoresed without

[3] Designations α^{A} and α^{B} refer to fragments derived from the amino- and carboxy-terminal parts of the collagen molecule, respectively, when the molecule is cleaved by mammalian collagenase. The corresponding fragments derived from the cleavage of procollagen molecules and containing nonhelical peptide extensions are called pro-α^{A} and pro-α^{B}.

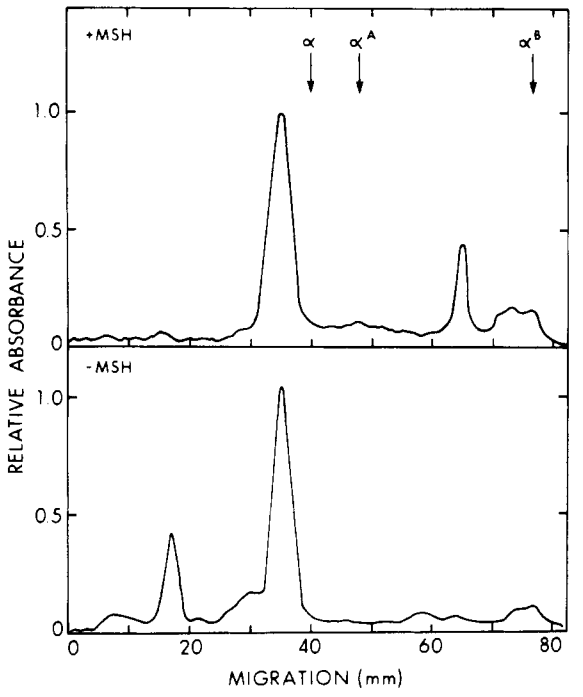

FIGURE 4: Cleavage of type II procollagen by human skin collagenase. [^{14}C]Proline-labeled procollagen isolated by DEAE-cellulose chromatography was prepared for digestion with purified human skin collagenase, and the cleavage products were electrophoresed on a slab gel of 8% polyacrylamide. The samples were electrophoresed either without reduction (−MSH) or with reduction with 2-mercaptoethanol (+MSH). The gels were exposed to x-ray films and the radioactive peptides were quantitated by scanning the radioautographs with a densitometer, as described under Materials and Methods. The migration positions of α^A and α^B fragments as well as of α chains of purified type II collagen are indicated in the chromatogram.

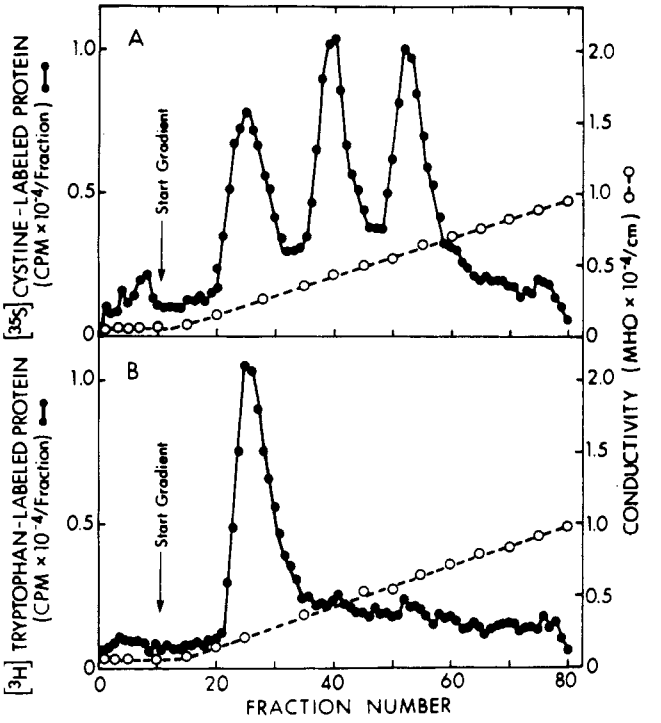

FIGURE 5: Incorporation of radioactive cystine and tryptophan into type II procollagen synthesized by matrix-free cartilage cells. Cells, 2.0×10^8, were incubated either with 100 μCi of [^{35}S]cystine or with 200 μCi of [^{3}H]tryptophan for 2 h in modified Krebs medium containing 20% fetal calf serum, 50 μg/mL ascorbic acid, and 50 μg/mL β-aminopropionitrile hydrochloride. The radioactive proteins in the medium were then isolated and chromatographed on DEAE-cellulose as indicated under Materials and Methods and in Figure 1. Frame A: Radioactive protein from cells incubated with [^{35}S]cystine. Frame B: Radioactive protein from cells incubated with [^{3}H]tryptophan.

reduction. The migration of pro-α^B, however, was affected so that these peptides now migrated about 17–18 mm from the origin. These results demonstrate that the interchain disulfide bonds in type II procollagen are located in the carboxy-terminal extensions.

In order to partially characterize the amino acid composition of extension peptides of type II procollagen, the matrix-free cartilage cells were incubated with [^{35}S]cystine or [^{3}H]tryptophan, two amino acids which are not found in the collagen portion of type II collagen. The radioactive protein recovered in the medium of the cells was then examined by DEAE-cellulose chromatography. When the cells were labeled with [^{3}H]tryptophan, most of the radioactivity was recovered in a peak eluting in the same position as the ^{14}C-labeled procollagen recovered from the medium when the cells were labeled with [^{14}C]proline (Figures 1 and 5). When the cells were incubated with [^{35}S]cystine, a peak in the same position as [^{14}C]proline-labeled procollagen was also observed, but other radioactive peaks eluting later in the chromatogram were also seen (Figure 5). Further examination of the [^{35}S]cystine- and [^{3}H]tryptophan-labeled protein eluting on DEAE-cellulose in the same position as [^{14}C]proline-labeled procollagen demonstrated that these polypeptides migrated in slab-gel electrophoresis in sodium dodecyl sulfate after reduction with 2-mercaptoethanol in the same position as [^{14}C]proline-labeled pro-α chains. In further studies, the [^{35}S]cystine- and [^{3}H]tryptophan-labeled procollagen was subjected to cleavage by mammalian collagenase as described above. The cleavage products of the collagenase were then examined on slab-gel electrophoresis employing radioautographic detection techniques. The results demonstrated that [^{35}S]cystine could be detected in both extension peptides of type II procollagen, whereas [^{3}H]tryptophan was detected only at the carboxy-terminal end of the molecule. Incubation of the cleavage products with pepsin at a temperature at which the α^A and α^B fragments of collagen retained their helical conformation completely removed the radioactivity from the cleavage products. The results indicate, therefore, that the nonhelical carboxy-terminal extensions contained both cystine and tryptophan, whereas the amino-terminal extensions contain only cystine.

Conversion of Type II Procollagen to Collagen. In order to study the removal of the amino- and carboxy-terminal extensions from type II procollagen, the intact sterna from 17-day-old chick embryos were incubated with [^{14}C]proline for 6 min, a time period estimated to correspond to the translation time of a type II pro-α chain (Miller et al., 1973). Further protein synthesis was stopped by adding cycloheximide and [^{12}C]proline into the incubation medium, and the incubation was then continued for 2 h at 37 °C. At the end of each incubation, boiling sodium dodecyl sulfate containing a mixture of protease inhibitors was immediately pipetted into the incubation tube, the total sample was first heated at 100 °C and then homogenized and extracted. The extraction procedure consistently solubilized 92–98% of the nondialyzable ^{14}C in the sample, and the ^{14}C-labeled protein could be recovered in the 18 000g supernatant when the extract was subsequently centrifuged at room temperature. The addition of cycloheximide and [^{12}C]proline into the medium also effectively stopped further incorporation of [^{14}C]proline into the protein in that,

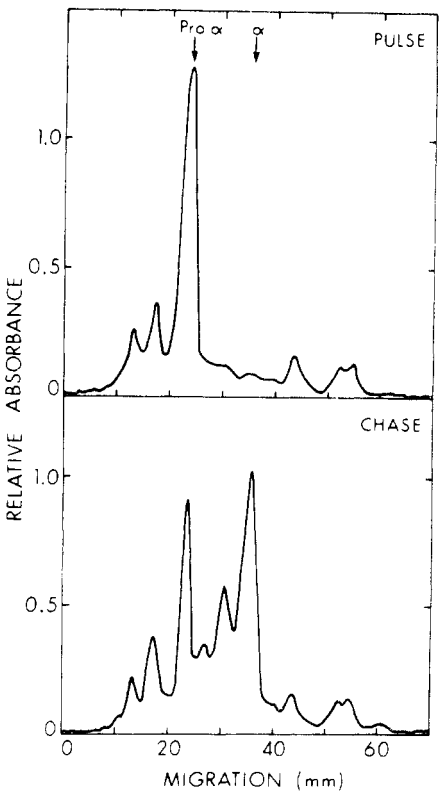

FIGURE 6: Conversion of type II procollagen to collagen. In each sample, eight sterna from 17-day-old chick embryos were incubated in 2 mL of phosphate-free Hepes-buffered medium (see Materials and Methods) containing 20% fetal calf serum, 50 μg/mL ascorbic acid, 50 μg/mL β-aminopropionitrile hydrochloride, and 1 mM $CaCl_2$. After a 15-min preincubation, 3 μCi of [^{14}C]proline was added and the cartilages were pulse labeled for 6 min. Further incorporation of ^{14}C was then stopped by chasing the radioactivity by the addition of 0.1 mL of medium containing 2.2 mg/mL cycloheximide and 2.2 mg/mL [^{12}C]proline. One of the samples (pulse) was immediately treated with sodium dodecyl sulfate, iodoacetamide, and protease inhibitors, as described under Materials and Methods. Another sample (chase) was incubated for an additional 2 h at 37 °C, and then treated with sodium dodecyl sulfate, as above. The sodium dodecyl sulfate-solubilized ^{14}C-labeled proteins were electrophoresed on polyacrylamide gel electrophoresis in sodium dodecyl sulfate, and the ^{14}C-labeled peptides were detected by scanning the radioautographs of the gels, as described under Materials and Methods and Figure 2. The migration positions of pro-α chains of procollagen and α-chains of type II collagen are indicated. Upper frame: Sample labeled for 6 min with [^{14}C]proline. Lower frame: Sample labeled for 6 min with [^{14}C]proline and then incubated for 2 h with cycloheximide and [^{12}C]proline.

in a typical experiment, shown in Figure 6, the total non-dialyzable ^{14}C at the end of a 6-min pulse in the sample was 1.81×10^5 cpm, while the corresponding value after a 2-h chase in the presence of cycloheximide and [^{12}C]proline was 1.68×10^5 cpm. Also, the amount of radioactive procollagen or collagen in the same samples was relatively unchanged during the chase, since the values for [^{14}C]hydroxyproline in the total samples at the end of 6-min pulse and after a 2-h chase period were 0.37×10^5 and 0.45×10^5 dpm, respectively.

Examination of the solubilized ^{14}C-labeled protein at the end of a 6-min pulse demonstrated that most of the radioactive protein after reduction with 2-mercaptoethanol migrated in slab-gel electrophoresis in sodium dodecyl sulfate in the same position as pro-α chains of type II procollagen (Figure 6). If the incubation after the addition of cycloheximide and [^{12}C]proline was continued in the same medium for 2 h at 37 °C, a large fraction of the ^{14}C-labeled protein electrophoresed now in the same position as α chains of type II collagen (Figure 6). Also two radioactive bands migrating between the pro-α and α chains could be noted (Figure 6).

Similar results were obtained in six additional experiments, and, in these studies, consistently about 60–70% of the newly synthesized [^{14}C]procollagen molecules were converted to [^{14}C]collagen during a 2-h chase.

To identify the ^{14}C-labeled polypeptides migrating between pro-α and α chains on slab gel electrophoresis after denaturation and reduction, these two bands as well as pro-α and α chains were cut out of the gels and then assayed for [^{14}C]hydroxyproline after hydrolysis in 6 M HCl. The bands migrating in the positions of pro-α and α chains and also the two bands migrating in the intermediate positions had the ratio of [^{14}C]hydroxyproline to total ^{14}C radioactivity 36–42%. Furthermore, incubation of the ^{14}C-labeled protein with highly purified bacterial collagenase before electrophoresis resulted in the disappearance of all four bands when examined by slab-gel electrophoresis before and after digestion with bacterial collagenase. In further studies, the samples were electrophoresed without prior reduction. Examination of the ^{14}C-labeled peptides in slab gels demonstrated that the polypeptides which after reduction migrated in the position of pro-α chains, and also the intermediate peptides migrating next to pro-α chains, were now recovered in a position with a mobility less than γ chains of type II collagen (see Figures 2 and 6). These polypeptides, therefore, appeared to contain interchain disulfide bonds which are located at the carboxy-terminal extensions. The migration positions of α chains and the ^{14}C-labeled intermediate peptides migrating next to α chains were unaffected by the reduction with 2-mercaptoethanol.

It should be noted that small amounts of radioactivity were also detected in two bands having a lower mobility than the pro-α chains of procollagen and in a few minor bands migrating faster than α chains (Figure 6). Since these ^{14}C-labeled bands did not contain significant amounts of [^{14}C]hydroxyproline and since the ^{14}C radioactivity in these bands was relatively unchanged during the 2-h chase period, these polypeptides were considered to be unrelated to pro-α or α chains, and they were not examined further.

Demonstration That Conversion of Procollagen to Collagen Requires a Metal Ion Which Is Possibly Ca^{2+}. In order to study the enzymatic nature of the conversion of procollagen to collagen, the effect of various protease inhibitors on the conversion was tested. If the cartilage tissues were incubated for 6 min with [^{14}C]proline, and 1 mM EDTA was then added to the incubation medium together with cycloheximide and [^{12}C]proline, the subsequent conversion of procollagen to collagen was completely abolished during the 2-h chase period (Figure 7). Similar results were obtained by using 10 mM Na_2EDTA. The results suggest that the conversion of procollagen to collagen is dependent on a metal ion which could be chelated by Na_2EDTA. In order to study the possibility that Ca^{2+} is involved in the conversion reaction, a pulse-chase experiment was performed in which 1 mM Na_2EDTA was added to the incubation medium at the end of a pulse-labeling period and 10 mM $CaCl_2$ was added to the medium 5 min later. The results demonstrated that the addition of excess of Ca^{2+} into the incubation medium reversed the inhibition produced by 1 mM Na_2EDTA (Figure 7).

Control experiments were performed to exclude the possibility that Na_2EDTA prevented the conversion by interfering with the secretion of procollagen into the extracellular space. In these studies, a pulse-chase experiment was performed as described above, and 10 mM Na_2EDTA was added into the incubation medium 60 min after the addition of cycloheximide

and [^{12}C]proline; such a delay would allow the [^{14}C]procollagen molecules to be secreted out of the cells before the addition of Na_2EDTA, since the secretion time for a procollagen molecule by embryonic cartilage cells is about 35 min (Dehm and Prockop, 1973). In these experiments, the addition of Na_2EDTA inhibited any further conversion of procollagen to collagen during the latter part of the chase, and, therefore, Na_2EDTA appeared to inhibit the conversion of procollagen to collagen in the extracellular space.

The effect of a serine protease inhibitor, α-toluenesulfonyl fluoride, was also studied in a pulse-chase experiment similar to the one shown in Figure 7. The addition of this inhibitor, in concentrations ranging from 0.1 to 3 mM, to the incubation medium at the end of the pulse-labeling period did not affect the subsequent conversion of procollagen to collagen.

Discussion

Matrix-free connective-tissue cell systems have been used extensively for studies on the biosynthesis of collagen (see Prockop et al., 1976). These cells are particularly useful for studies on the intracellular steps in procollagen synthesis, since a relatively large fraction of radioactive amino acids, such as proline and glycine, is incorporated into procollagen polypeptides. Also, these cell systems are highly convenient for studies on collagen secretion, because the secreted procollagen molecules remain soluble in the incubation medium, and the extracellular macromolecules can be separated from the intracellular proteins by a simple centrifugation. The matrix-free connective-tissue cell systems, however, are not useful for studies on the conversion of procollagen to collagen because the enzyme or enzymes cleaving the extension peptides from procollagen are removed or inactivated during isolation of the cells, and the relatively short incubation periods are not sufficient to accumulate the converting enzymes into the medium (Dehm and Prockop, 1971; Jimenez et al., 1971; Uitto and Lichtenstein, 1976b).

In this study, intact sternal cartilages from 17-day-old chick embryos were used to examine the conversion of type II procollagen to collagen. The sterna were pulse-labeled with radioactive proline for 6 min, a time period which corresponds to an approximate translation time of a pro-α chain of procollagen (Vuust and Piez, 1971, 1972; Miller et al., 1973). Addition of cycloheximide and [^{12}C]proline completely stopped further incorporation of [^{14}C]proline into the protein, and previous studies have clearly demonstrated that cycloheximide or [^{12}C]proline in the concentrations used do not interfere with posttranslational modifications or intracellular translocation of procollagen molecules (see Schofield et al., 1974; Uitto and Prockop, 1974b; Prockop et al., 1976). The results demonstrated that at the end of a 6-min pulse-labeling period most of the radioactive proteins were recovered, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as pro-α chains of procollagen. The newly synthesized procollagen molecules were then converted to collagen so that after a 120-min chase period most of the radioactive polypeptides were recovered as α chains.

Removal of peptide extensions from type I procollagen has been suggested to involve two separate endopeptidases. The evidence for separate enzymes for the removal of amino- and carboxy-terminal extensions comes from two different types of experiments. First, an endopeptidase which cleaves only the amino-terminal extensions from type I collagen has been purified from calf connective tissues (Lapière et al., 1971; Kohn et al., 1974). Similar or related peptidases have also been demonstrated in rat calvaria (Bornstein et al., 1972), in the medium of human cultured fibroblasts (Lichtenstein et al., 1973; Layman and Ross, 1973), and in mouse 3T3 fibroblast culture medium (Goldberg et al., 1975). Secondly, evidence for the existence of separate amino- and carboxy-terminal peptidases comes from the detection of discrete collagen precursor intermediates with only the carboxy- or amino-terminal extensions present. Such molecules have been isolated either from tissues of animals with a genetic connective-tissue defect, dermatosparaxis (Lenaers et al., 1971; Becker et al., 1976) or from incubations of tissues synthesizing type I collagen (Fessler et al., 1975; Byers et al., 1975; Uitto and Lichtenstein, 1976b; Uitto et al., 1976).

In the present study, the conversion of newly synthesized

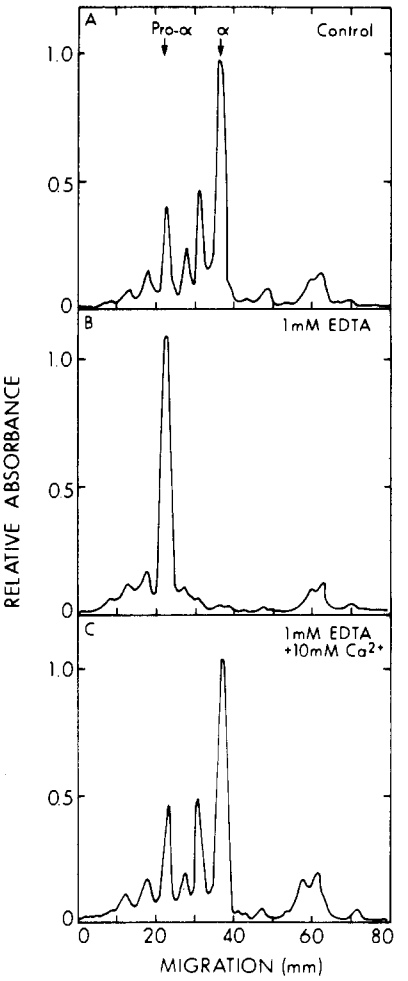

FIGURE 7: Inhibition of the conversion of procollagen to collagen by Na_2EDTA and reversal of the inhibition by Ca^{2+}. Sterna from 17-day-old chick embryos were pulse labeled for 6 min with [^{14}C]proline as described in Figure 6. Further incorporation of ^{14}C radioactivity was then stopped by the addition of cycloheximide and [^{12}C]proline, and the incubation was continued for 120 min. The control sample (frame A) contained 1 mM $CaCl_2$ in the incubation medium. One of the samples (frame B) was incubated in Hepes-buffered medium without $CaCl_2$; instead, 1 mM Na_2EDTA was added to the incubation medium together with cycloheximide and [^{12}C]proline, and the incubation was continued for 120 min, as indicated above. The third sample (frame C) was pulse labeled in a medium without $CaCl_2$, and 1 mM Na_2EDTA was then added at the end of the 6-min pulse, as above. After a 5-min chase, 10 mM $CaCl_2$ was added to the incubation medium, and the incubation was continued for an additional 115 min. At the end of each incubation, the ^{14}C-labeled proteins were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the ^{14}C-labeled peptides were detected by scanning the radioautographs of the gels, as described under Materials and Methods. The migration positions of pro-α chains of type II procollagen and α chains of type II collagen are indicated in the figure.

type II procollagen to collagen was shown to involve formation of two intermediate precursor molecules which could be detected on polyacrylamide gel electrophoresis as peptides migrating intermediate between pro-α and α chains. Since the carboxy-terminal extension of type II procollagen has been shown to be larger than the amino-terminal extension (Merry et al., 1976), it appears that the intermediate polypeptides migrating next to pro-α chains are derived from p_c collagen, while the intermediate polypeptide migrating next to α chains represents p_n collagen. This conclusion was further supported by the observation that the intermediate polypeptide migrating next to pro-α chains had the carboxy-terminal extensions containing the interchain disulfides present, whereas the intermediate polypeptides migrating next to α chains were devoid of interchain disulfide bonds. The results demonstrate, therefore, that processing of type II procollagen to collagen involves two separate cleavages, the amino- and carboxy-terminal extensions being removed separately. The existence of an intermediate containing the amino-terminal extensions also demonstrates that the removal of the amino-terminal extensions is not a necessary prerequisite for removal of the carboxy-terminal extensions, as has been suggested to be the case during processing of type I procollagen to collagen (Fessler et al., 1975).

The conversion of type II procollagen to collagen could be inhibited by Na_2EDTA and the inhibition could be reversed by the addition of Ca^{2+}. On the other hand, addition of a serine protease inhibitor α-toluenesulfonyl fluoride did not affect the conversion. The results suggest, therefore, that the converting enzyme or enzymes may require Ca^{2+} or another metal for its activity. Previously, the endopeptidase, which removes the amino-terminal extensions from type I procollagen and which has been purified from calf skin, was shown to require Ca^{2+} for its activity (Kohn et al., 1974). The results here do not conclusively resolve the question of whether the two cleavages removing the extension peptides from type II procollagen are catalyzed by the same peptidase or whether two separate enzymatic activities are required. Preliminary results from our laboratory, however, suggest that the two extensions are removed by separate amino- and carboxy-terminal endopeptidases (Lindy et al., 1977).

In the process of studying the conversion of procollagen to collagen, the type II procollagen used as a standard marker in slab-gel electrophoresis was isolated by DEAE-cellulose chromatography and then partially characterized. In accordance with previous publications, the procollagen synthesized by matrix-free cartilage cells was shown to consist of a single-type of pro-α chain linked by disulfide bonds (Dehm and Prockop, 1973; Uitto and Prockop, 1974a; Harwood et al., 1975). The collagen portion of the polypeptides was shown, on the basis of cyanogen bromide peptide mapping, to be structurally similar to type II collagen, and, therefore, the procollagen isolated by DEAE-cellulose chromatography from matrix-free cartilage cells was a precursor of type II collagen. In accordance with previous publications (Merry et al., 1976; Olsen et al., 1976; Uitto et al., 1977), the type II procollagen was shown to contain both amino-terminal and carboxy-terminal extension peptides, and the interchain disulfides were located at the carboxy-terminal end of the molecule. The nonhelical extension peptides were also shown to differ from the collagen portion of the molecule in that the extensions incorporated radioactive cystine and tryptophan. In this respect, type I and II procollagens appear similar, and further studies are now in progress to determine whether the precursors of all interstitial collagens have extensions with similar structures. Such similarities in structure, if existing, might contribute to our understanding of the functional role of the extension peptides during the biosynthesis of collagen.

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References

Becker, U., Timpl, R., Helle, O., and Prockop, D. J. (1976), *Biochemistry 15,* 2853.

Bonner, M. W., and Laskey, R. A. (1974), *Eur. J. Biochem. 46,* 83.

Bornstein, P. (1974), *Annu. Rev. Biochem. 43,* 567.

Bornstein, P., Davidson, J. M., and Monson, J. M. (1975), in Proteases and Biological Control, Reich, E., Rifkin, D. B., and Shaw, E., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory, pp 579–590.

Bornstein, P., Ehrlich, H. P., and Wyke, A. P. (1972), *Science 175,* 544.

Byers, P. H., Click, E. M., Harper, E., and Bornstein, P. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3009.

Davidson, J. M., McEneany, L. S. G., and Bornstein, P. (1975), *Biochemistry 14,* 5188.

Dehm, P., and Prockop, D. J. (1971), *Biochim. Biophys. Acta 240,* 358.

Dehm, P., and Prockop, D. J. (1972), *Biochim. Biophys. Acta 264,* 375.

Dehm, P., and Prockop, D. J. (1973), *Eur. J. Biochem. 35,* 159.

Epstein, E. H., Jr., Scott, R. D., Miller, E. J., and Piez, K. A. (1971), *J. Biol. Chem. 246,* 1718.

Fessler, L. I., Morris, N. P., and Fessler, J. H. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4905.

Goldberg, B., Taubman, M. B., and Radin, B. (1975), *Cell 4,* 45.

Gross, J. (1974), *Harvey Lect. 68,* 351.

Harwood, R., Bhalla, A. K., Grant, M. E., and Jackson, D. J. (1975), *Biochem. J. 148,* 129.

Hoffmann, H.-P., Olsen, B. R., Chen, H.-T., and Prockop, D. J. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 4304.

Jimenez, S. A., Dehm, P., and Prockop, D. J. (1971), *FEBS Lett. 17,* 245.

Juva, K., and Prockop, D. J. (1966), *Anal. Biochem. 15,* 77.

King, J., and Laemmli, U. K. (1971), *J. Mol. Biol. 62,* 465.

Kivirikko, K. I., and Risteli, L. (1976), *Med. Biol. 54,* 159.

Kohn, L. D., Isersky, C., Zupnik, J., Lenaers, A., Lee, G., and Lapière, C. M. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 40.

Krebs, H. A. (1950), *Biochim. Biophys. Acta 4,* 249.

Lapière, C. M., Lenaers, A., and Kohn, L. D. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 3054.

Layman, D. L., and Ross, R. (1973), *Arch. Biochem. Biophys. 157,* 451.

Lenaers, A., Ansay, M., Nusgens, B. V., and Lapière, C. M. (1971), *Eur. J. Biochem. 23,* 533.

Lichtenstein, J. R., Bauer, E. A., and Uitto, J. (1976), *Biochem. Biophys. Res. Commun. 73,* 665.

Lichtenstein, J. R., Martin, G. R., Kohn, L. D., Byers, P. H., and McKusick, V. A. (1973), *Science 182,* 298.

Lindy, S., Uitto, J., and Lichtenstein, J. R. (1977), *Clin. Res. 25,* 362A.

Martin, G. R., Byers, P. H., and Piez, K. A. (1975), *Adv. Enzymol. 42,* 167.
Merry, A. H., Harwood, R., Woolley, D. E., Grant, M. E., and Jackson, D. J. (1976), *Biochem. Biophys. Res. Commun. 71,* 83.
Miller, E. J. (1971), *Biochemistry 10,* 1652.
Miller, E. J. (1972), *Biochemistry 11,* 4903.
Miller, E. J., and Matukas, V. J. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64,* 1264.
Miller, E. J., and Matukas, V. J. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1197.
Miller, E. J., Woodall, D. L., and Vail, M. S. (1973), *J. Biol. Chem. 248,* 1666.
Müller, P. K., and Jamhawi, O. (1974), *Biochim. Biophys. Acta 365,* 158.
Olsen, B. R., Hoffmann, H.-P., and Prockop, D. J. (1976), *Arch. Biochem. Biophys. 175,* 341.
Peterkofsky, B., and Diegelmann, R. (1971), *Biochemistry 10,* 988.
Prockop, D. J., Berg, R. A., Kivirikko, K. I., and Uitto, J. (1976), in Biochemistry of Collagen, Ramachandran, G. N., and Reddi, A. H., Ed., New York, N.Y., Plenum Press, pp 163–273.
Schofield, J. D., Uitto, J., and Prockop, D. J. (1974), *Biochemistry 13,* 1801.
Smith, B. D., Byers, P. H., and Martin, G. R. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3260.
Smith, B. D., Martin, G. R., Miller, E. J., Dorfman, A., and Swarm, R. (1975), *Arch. Biochem. Biophys. 166,* 181.
Stricklin, G., Bauer, E. A., Jeffrey, J. J., and Eisen, A. Z. (1977), *Biochemistry 16,* 1607.
Studier, F. W. (1973), *J. Mol. Biol. 79,* 237.
Tanzer, M. L., Church, R. L., Yaeger, J. A., Wampler, D. E., and Park, E. D. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3009.
Trelstad, R. L., Kang, A. H., Igarashi, S., and Gross, J. (1970), *Biochemistry 9,* 4993.
Uitto, J. (1970), *Biochim. Biophys. Acta 201,* 438.
Uitto, J. (1977), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 752.
Uitto, J., Hoffmann, H.-P., and Prockop, D. J. (1977), *Arch. Biochem. Biophys. 179,* 654.
Uitto, J., and Lichtenstein, J. R. (1976a), *J. Invest. Dermatol. 66,* 59.
Uitto, J., and Lichtenstein, J. R. (1976b), *Biochem. Biophys. Res. Commun. 71,* 60.
Uitto, J., Lichtenstein, J. R., and Bauer, E. A. (1976), *Biochemistry 15,* 4935.
Uitto, J., and Prockop, D. J. (1974a), *Biochemistry 13,* 4586.
Uitto, J., and Prockop, D. J. (1974b), *Eur. J. Biochem. 43,* 221.
Veis, A., and Brownell, A. G. (1975), *Crit. Rev. Biochem. 2,* 417.
Vuust, J., and Piez, K. A. (1971), *J. Biol. Chem. 245,* 6201.
Vuust, J., and Piez, K. A. (1972), *J. Biol. Chem. 247,* 856.

Stereochemistry of Internucleotidic Bond Formation by tRNA Nucleotidyltransferase from Baker's Yeast†

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ABSTRACT: Isomer A of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) is a substrate for tRNA nucleotidyltransferase from baker's yeast, whereas isomer B is a competitive inhibitor. The tRNA resulting from this reaction has a phosphorothioate instead of a phosphate diester linkage at the last internucleotidic linkage between cytidine and adenosine. On limited digestion of this tRNA with RNase A, one can isolate cytidine 2′,3′-cyclic phosphorothioate which can be deaminated to uridine 2′,3′-cyclic phosphorothioate. It can be shown that this compound is the endo isomer and that, therefore, the phosphorothioate diester bond in the tRNA must have had the *R* configuration. This result indicates that no racemization during the condensation of ATPαS, isomer A, onto the tRNA had occurred. Whether inversion or retention of configuration had taken place awaits elucidation of the absolute configuration of isomer A of ATPαS.

Transfer ribonucleic acid nucleotidyltransferase has been isolated from a variety of sources. It catalyzes the incorporation of CMP and AMP residues into the 3′ terminus of tRNAs, taking as substrates CTP, ATP, and tRNAs lacking the CCA end (Deutscher, 1974). The enzyme from baker's yeast has been used to incorporate a number of CTP and ATP analogues into tRNAs and it has been shown to possess an SH group essential for enzymatic activity (Sternbach et al., 1976). Its biological function is not as yet clearly established, although it is conceivable that it is responsible for the completion of the CCA end of tRNAs lacking this sequence partly or totally (Seidman and Mc Clain, 1975).

This enzyme belongs to the class of enzymes which is capable of forming phosphodiester bonds and thus resembles RNA and DNA polymerases. There is little known about the mechanism by which these enzymes condense the terminal 3′-hydroxyl group of a growing polynucleotide chain with the α-phosphate group of a nucleoside 5′-triphosphate. The

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