Purification and Characterization of a Peptide from the Carboxy-Terminal Region of Chick Tendon Procollagen Type I†

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ABSTRACT: A disulfide-bonded peptide with a molecular weight of about 100 000 was isolated from the medium of cultured chick embryo tendons. It was shown to be a trimer with two types of subunits in a 2:1 ratio, and tryptic fingerprinting and immunological evidence indicated that it was derived from the carboxy-terminal-precursor-specific region of procollagen. Amino acid analysis after reduction and alkylation indicated that the trimer contains about 30 residues of half-cystine involved in intrachain as well as interchain disulfide bonding. The interchain bonds could be reduced and alkylated under nondenaturing conditions. Carbohydrate analysis showed that each of the three peptide chains in the trimer contains about two residues of *N*-acetylglucosamine and about ten residues of mannose. This suggests the presence of one or two oligosaccharide units per chain.

Collagen is synthesized as a precursor, procollagen, which is larger than collagen because of additional peptides at both the amino and carboxyl ends of the constituent proα chains (for recent reviews, see Bornstein, 1974; Martin et al., 1975; Veis and Brownell, 1975; Hance and Crystal, 1975; Prockop et al., 1976). During the physiological conversion of procollagen to collagen, the peptide extensions are thought to be removed by specific enzymes. The conversion proceeds in a stepwise fashion and recent studies using chick bone and tendon fibroblasts have indicated that the first step is proteolytic removal of the amino-terminal-precursor region to yield an intermediate with the carboxy-terminal-precursor region still present (Morris et al., 1975; Davidson et al., 1975; Fessler et al., 1975; Uitto and Lichtenstein, 1976).

In certain inherited disorders of connective tissue such as dermatosparaxis there is a defect in the normal conversion of type I procollagen to collagen and precursor molecules with amino-terminal precursor peptides accumulate in the tissues (Lenaers et al., 1971; Fjølstad and Helle, 1974). Studies on cattle (Furthmayr et al., 1972; Fietzek et al., in preparation) and sheep (Becker et al., 1976) with such a defect have allowed a chemical characterization of the amino-terminal-precursor peptides of type I procollagen. The studies have shown that the pα1 precursor peptide[1] contains three distinct structural domains (Becker et al., 1976). The amino-terminal portion of the pα1(I) peptide contains a globular domain which is resistant to bacterial collagenase. A collagen-like domain which is sensitive to bacterial collagenase occupies the central portion, and this region is linked to the α1(I) chain by a short nonhelical sequence. Evidence for a similar structural organization of the amino-terminal portion of the pα2(I) chain (Becker et al., 1977) and of pα(III) chains (Nowack et al., 1976) has recently been obtained.

A detailed chemical characterization of the carboxy-terminal-precursor region of procollagen has not been possible because of a lack of a good source of large quantities of intact procollagen. In preliminary studies, disulfide-linked peptides that cross-react immunologically with procollagen have been isolated from collagenase digests of procollagen (Sherr et al., 1973; Dehm et al., 1974; Olsen et al., 1976), from the medium of cultured bone (Murphy et al., 1975), and from extracts of cultured bone (Fessler et al., 1975). Although these peptides originally were thought to be derived from the amino-terminal portion of procollagen (Sherr et al., 1973; Dehm et al., 1974), it is now clear that they originate from the carboxy-terminal end of procollagen molecules (Byers et al., 1975; Fessler et al., 1975; Olsen et al., 1976). Recent data from our laboratory on the disulfide-linked carboxy-terminal peptide isolated from a bacterial collagenase digest of procollagen type I (Hoffmann et al., 1976) indicate that the primary structure of the carboxy-terminal extensions on proα1(I) is different from the carboxy-terminal extension on proα2(I). However, the amounts of available peptide material have not allowed a further chemical characterization of the extensions.

In the present report we describe an organ culture system which allows the isolation of large quantities of a disulfide-linked procollagen extension peptide. The peptide is a trimer and originates from the carboxy-terminal region of procollagen type I presumably as a result of the physiological conversion of procollagen to collagen.

Materials and Methods

Preparation of Procollagen. Cells were isolated by enzymic digestion of tendons from 17-day old chick embryos as described previously (Dehm and Prockop, 1972, 1973; Olsen et al., 1975). About 2×10^9 cells from 120 embryos were used for most experiments. The tendon cells were incubated at a concentration of 7.5×10^6 cells/mL with 0.38 μCi/mL of a synthetic mixture of ^{14}C-labeled amino acids for 4 h at 37 °C in modified Krebs medium (Dehm and Prockop, 1972) without fetal calf serum.

After incubation, the cells were removed by centrifugation. The medium was cooled on ice and enzyme inhibitors were added as described previously (Olsen et al., 1976). The medium procollagen was precipitated with 176 mg/mL of ammonium sulfate (Baker Chemical Co.) as described (Olsen et al., 1976). The precipitate was then dissolved in 5 mL of 0.4 M NaCl and

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[1] In the present paper we use the terms pα1(I), pα2(I), and pα(III) to describe procollagen peptides which contain an amino-terminal extension, but which lack the carboxy-terminal extension. The intact chains will be referred to as proα chains.

0.1 M Tris-HCl buffer, pH 7.8, dialyzed against 2 M urea and 50 mM Tris-HCl, pH 8.6, and chromatographed (Smith et al., 1972) on a 1.6 × 10 cm column of DEAE-cellulose[2] (DE-52; Whatman). The column was equilibrated at 4 °C with 2 M urea and 50 mM Tris-HCl, pH 8.6, and it was eluted with a linear gradient of NaCl (Hoffmann et al., 1976). Fractions were assayed by liquid scintillation counting. The fractions containing procollagen were pooled and dialyzed against 0.4 M NaCl-0.1 M Tris-HCl buffer, pH 7.

Preparation of Procollagen Extension Peptides. A disulfide-bonded carboxy-terminal extension peptide was isolated from the medium of cultured tendons. Leg tendons from 120 17-day old chick embryos were dissected in modified Krebs medium II and then incubated in 100 mL of Dulbecco modified Eagle medium under 10% CO_2/90% air with moderate shaking at 37 °C. The medium contained 100 units/mL of penicillin (GIBCO), 100 μg/mL of streptomycin (GIBCO), 40 μg/mL of sodium ascorbate (Sigma Chemical Co.), 64 μg/mL of β-aminopropionitrile fumarate (General Biochemicals), and 1 μCi/mL of a synthetic mixture of 15 ^{14}C-labeled amino acids (New England Nuclear Corp.). The medium was replaced by fresh medium after 12 h and 24 h. After incubation for 36 h, the media were removed from the tendons, chilled on ice, and centrifuged at 30 000*g* for 30 min at 4 °C. Proteolysis in the supernates was inhibited by adding protease inhibitors (Olsen et al., 1976) and the media were dialyzed at 4 °C against 2 M urea and 50 mM Tris-HCl buffer, pH 8.6 (at 25 °C). After dialysis, the dialyzed medium was subjected to chromatography on DEAE-cellulose.

The carboxy-terminal collagenase-resistant extension peptide was prepared by digestion of procollagen with purified bacterial collagenase as described previously (Dehm et al., 1974; Olsen et al., 1976).

Procedures for Purification of Medium Peptides. The medium obtained from the tendon incubation was chromatographed on a 2.5 × 10 cm column of DEAE-cellulose. The column was equilibrated at 4 °C with 2 M urea and 50 mM Tris-HCl buffer, pH 8.6 (at 25 °C), and it was eluted with a linear gradient prepared with 500 mL of starting buffer and 500 mL of starting buffer with 0.3 M NaCl. The flow rate was 120 mL/h and 10-mL fractions were collected. The fractions were assayed by counting 200 μL from each fraction in a liquid scintillation counter. The fractions containing the carboxy-terminal peptide were pooled and the peptide was desalted on a column of polyacrylamide (Bio-Gel P-2) which was equilibrated and eluted with 0.2 M NH_4HCO_3. After lyophilization, the peptide was purified further by chromatography on CM-cellulose (CM 52; Whatman). The peptide was prepared for CM-cellulose chromatography by dissolving the lyophilized peptide in 50 mL of 6 M urea and 50 mM sodium acetate buffer, pH 3.6. The CM-cellulose chromatography was performed at room temperature with a 1.6 × 10 cm column equilibrated with 6 M urea and 50 mM sodium acetate buffer, pH 3.6. The column was eluted with a linear gradient prepared with 300 mL of starting buffer and 300 mL of starting buffer with 0.2 M NaCl. The flow rate was 120 mL/h and 6-mL fractions were collected. The eluate from the column was assayed by continuous monitoring at 230 nm using a Beckman Model 25 spectrophotometer equipped with a flow cell. The fractions containing the carboxy-terminal peptide were pooled and the peptide was desalted on a Bio-Gel P-2 column equilibrated with 0.2 M NH_4HCO_3. After lyophilization the peptide preparation was stored frozen at −20 °C.

In some experiments the carboxy-terminal peptide obtained after chromatography on CM-cellulose was purified further by chromatography on a 1.5 × 90 cm column of 8% agarose (Bio-Gel A-1.5m, 200–400 mesh; Bio-Rad). The column was equilibrated and eluted at 4 °C with 0.2 M NH_4HCO_3.

After reduction and alkylation in urea, the two types of subunits in the carboxy-terminal medium peptide were separated by chromatography on a 1.0 × 8 cm column of CM-cellulose. The column was equilibrated with 6 M urea and 50 mM sodium acetate buffer, pH 3.6, at room temperature. The elution was with a linear gradient prepared with 300 mL of starting buffer and 300 mL of starting buffer with 0.2 M NaCl. The flow rate was 80 mL/h and 5-mL fractions were collected. The eluate was monitored at 230 nm. After pooling appropriate fractions, the two peptides were desalted on P-2 in 0.2 M NH_4HCO_3 and lyophilized.

Purification of the Collagenase-Resistant Peptide. The carboxy-terminal collagenase-resistant peptide prepared from procollagen was purified by chromatography on a 1.5 × 90 cm column of 8% agarose (Bio-Gel A-1.5m, 200–400 mesh; Bio-Rad) equilibrated and eluted with 0.1 M Tris-HCl buffer, pH 7.8 at 4 °C (Dehm et al., 1974; Olsen et al., 1976).

Treatment of Procollagen with Pepsin and Tadpole Collagenase. For digestion with pepsin [^{14}C]procollagen was incubated with 100 μg/mL of pepsin in 0.5 M acetic acid for 6 h at 4 °C (Olsen et al., 1976). The sample was then dialyzed against 0.4 M NaCl and 0.1 M Tris-HCl buffer, pH 7.8 at 4 °C.

For digestion with tadpole collagenase, [^{14}C]procollagen was incubated with the enzyme at 22 °C as described previously (Olsen et al., 1976). Purified tadpole collagenase was kindly provided by Dr. Yutaka Nagai, Tokyo Medical and Dental University, Tokyo, Japan. After digestion the fragments obtained by tadpole collagenase cleavage were separated by ammonium sulfate fractionation (Olsen et al., 1976).

Preparation of Antibodies and Radioimmunoassays. Antibodies to the carboxy-terminal medium peptide were prepared in rabbits by intradermal injections of purified medium peptide in Freund's complete adjuvant. The antisera were tested with direct binding immunoassays using a double-antibody method with goat anti-rabbit IgG antiserum in the second precipitation step (von der Mark et al., 1973; Nist et al., 1975). The assays were performed by adding the appropriate amount of antiserum to ^{14}C-labeled antigen (2000–5000 cpm) in 0.1 mL of 0.15 M NaCl-0.1 M Tris-HCl, pH 7.5, containing 0.1% ovalbumin and 0.01% sodium azide. The total amount of rabbit IgG was kept constant in all tubes by adding appropriate amounts of nonimmune rabbit serum. After incubation at room temperature for 1 h, 1 mL of goat anti-rabbit IgG antiserum was added, and the precipitation was allowed to occur for 24 h at 4 °C. The precipitate was separated by centrifugation, washed once with 5 mL of 0.15 M NaCl, dissolved in 1 mL of 0.2 N HCl, and counted in 15 mL of Aquasol (New England Nuclear Corp.). All assays were performed in duplicate.

Polyacrylamide Slab Gel Electrophoresis in Sodium Dodecyl Sulfate. Polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate was carried out on slab gels according to the procedure of King and Laemmli (1971). Separating gels of 1.5-mm thickness were prepared from 10 or 15% polyacrylamide, and stacking gels were 6% polyacrylamide. The electrophoresis was carried out with about 50 mA for 3 h at 15

[2] Abbreviations used: DEAE, diethylaminoethyl; CM, carboxymethyl; $NaDodSO_4$, sodium dodecyl sulfate; $TosPheCH_2Cl$, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

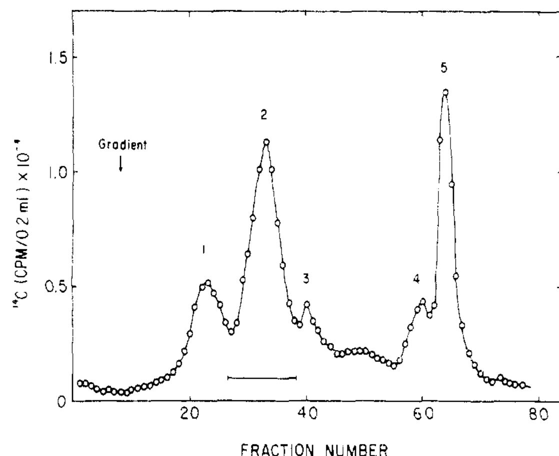

FIGURE 1: Chromatography on DEAE-cellulose of medium from cultured tendons. The chromatography was carried out in the presence of 2 M urea as described in Materials and Methods. The numbers indicate peaks referred to in the text. The horizontal bar indicates how fractions in peak 2 were pooled for further examination.

°C. The samples were prepared by dissolving lyophilized peptides in a "sample buffer" consisting of 0.125 M Tris-HCl, pH 6.8 (room temperature), containing 2% $NaDodSO_4$ (Bio-Rad Laboratories), 10% glycerol (Mallinckrodt Chemical Works), and 0.001% bromophenol blue (Eastman). The samples were stored at −20 °C. The gels were stained by incubation for 1 h at room temperature in a solution containing 0.25% Coomassie Brilliant Blue R (Sigma Chemical Co.) and 20% trichloroacetic acid (Mallinckrodt Chemical Works), and they were destained in 7.5% acetic acid and 15% methanol. For quantitation the gels were scanned in a Joyce-Loebl 3CS microdensitometer. In experiments where radioactive bands were located in the gels, the gels were impregnated with 2,5-diphenyloxazole (PPO; Eastman) and dried under vacuum (Bonner and Laskey, 1974). They were then exposed to RP Royal "X-OMAT" x-ray films which had been "preflashed" to make their fluorographic response linear (Laskey and Mills, 1975).

Ultracentrifugation. A Spinco Model E ultracentrifuge with a scanner was applied. All measurements were performed in 0.1 M Tris-HCl buffer, pH 7. Sedimentation velocity runs were performed in double sector cells at 56 000 rev/min and 20 °C. For sedimentation equilibrium measurements, double sector cells and column heights of 2.5 or 5 mm were used. Establishment of equilibrium was accelerated by overspeeding (Teller et al., 1969; Roark, 1976). Each material was run at at least three rotor speeds in the range of 8000 to 18 000 rev/min for the unreduced peptide and of 14 000 to 20 000 rev/min for the reduced peptides. Baselines were determined by meniscus depletion at a rotor speed of 48 000 rev/min after the equilibrium experiment. Data were evaluated according to the equation $\ln c/c_m = K(r^2 - a^2)$ with $K = \omega^2 M(1 - \bar{v}\rho)/(2RT)$. Here c is the protein concentration which is proportional to the optical density displayed by the scanner, r and a are the distances from the rotor center to the point in the cell for which c is measured and to the meniscus, respectively, c_m is the concentration at the meniscus, $\omega = 2\pi f$ is the angular velocity where f is the rotor speed, $\bar{v}$ is the partial specific volume, ρ is the density of the buffer, R is the gas constant, and T the absolute temperature.

From the slope of linear $\ln c$ vs. r^2 plots, the molecular weight M of homogeneous materials was obtained. In the case of curved $\ln c$ vs. r^2 plots, the weight-average molecular weight was determined by low speed runs according to Van Holde and Baldwin (1958). In some cases the curved $\ln c$ vs. r^2 plots were fitted by a computer simulation for given molecular weight distributions for two species 1 and 2 according to $c = c_{m,1} \exp K_1(r^2 - a^2) + c_{m,2} \exp K_2(r^2 - a^2)$ and the equation of mass conservation. A simultaneous analysis of the data obtained at three rotor speeds reduced the inherent lack of uniqueness of such fits.

Other Assays and Procedures. For complete reduction and alkylation of protein samples, 20 mM dithiothreitol (Sigma Chemical Co.) and 80 mM sodium iodoacetate (Sigma Chemical Co.) in 8 M urea were employed as described by Gollwitzer et al. (1972). Partial reduction and alkylation under nondenaturing conditions were performed as described previously (Dehm et al., 1974).

For amino acid analysis samples were hydrolyzed under an atmosphere of nitrogen with 6 M HCl and 0.06% mercaptoethanol at 120 °C for 16 h. The analyses were performed with a JEOLCO JLC-6AH amino acid analyzer. The observed values for threonine and serine were corrected by factors of 1.08 and 1.21, respectively (Rauterberg and Kühn, 1971). Half-cystine residues were assayed as carboxymethylcysteine after reduction and alkylation of the peptides.

Neutral sugars were determined with a modification of the method used by Metz et al. (1971). The peptides were hydrolyzed in vacuo in 1 N HCl at 100 °C for 9 h and the neutral sugars were converted to alditol acetates by reduction with sodium borohydride and acetylation with acetic anhydride. The alditol acetate derivatives were analyzed with a Packard Model 421 gas-liquid chromatograph using a column packed with 3% SP-2340 on 100/120 Supelcoport (Supelco, Inc.). D-(−)-Arabinose was used as internal standard. Hexosamines were determined according to Hayes et al. (1975) with 8 N HCl.

For preparation of tryptic peptides, the reduced and alkylated medium peptide was digested with $TosPheCH_2Cl$-treated trypsin (Worthington Biochemical Corp.) in 0.1 M NH_4HCO_3 at 37 °C for 4 h. The enzyme:substrate ratio was 1:50 and the protein concentration was 1 mg/mL. After lyophilization, the peptides were fingerprinted according to the method of Bates et al. (1975). Chromatography was carried out with water-acetic acid-pyridine-1-butanol (302:76:378:344) followed by electrophoresis for 60 min at 450 V with water-acetic acid-pyridine (890:100:10), pH 3.5. The plates were treated with 0.03% fluorescamine (Fluram; Roche Diagnostics) in acetone and viewed under ultraviolet light. For the tryptic fingerprint of the radioactive collagenase-resistant carboxy-terminal region of procollagen, the plates were treated with scintillator and exposed to x-ray film as described (Hoffmann et al., 1976).

Experimental Results

Purification of Medium Peptides. The media from each tendon incubation were chromatographed on DEAE-cellulose in the presence of 2 M urea. The elution profile of ^{14}C-labeled protein showed five distinct peaks (Figure 1). Analysis of the peak fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (not shown) indicated that peak 1 contained a mixture of $\alpha1$(I), $\alpha2$, p$\alpha1$(I), and p$\alpha2$ chains and that peak 3 contained intact procollagen. Peak 4 was not characterized further. Preliminary data indicated that peak 5 contained a peptide originating from the amino-terminal region of the pro$\alpha1$(I) chain.

The material eluting in peak 2 was purified further by chromatography on CM-cellulose in the presence of 6 M urea (Figure 2). The same profile was observed when the eluate was

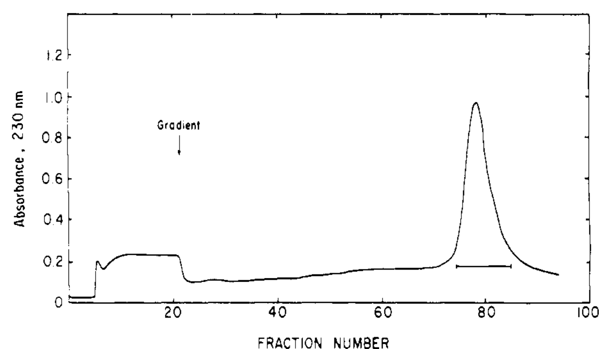

FIGURE 2: Chromatography on CM-cellulose of medium peptide (peak 2 in Figure 1). The chromatography was carried out in 6 M urea. The horizontal bar indicates how fractions were pooled for further examination.

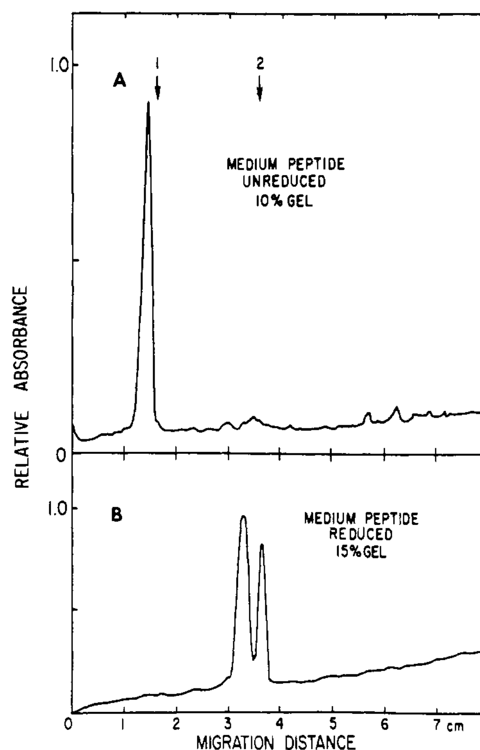

FIGURE 3: (A) Polyacrylamide gel electrophoresis of unreduced medium peptide in a 10% gel. The arrows indicate the positions of bovine serum albumin dimers (1) and monomers (2) in the same gel. The gel was scanned in a densitometer after staining with Coomassie blue as described in Materials and Methods. (B) Polyacrylamide gel electrophoresis of reduced medium peptide in a 15% gel. The gel was scanned in a densitometer after staining with Coomassie blue as described in Materials and Methods.

assayed for absorption at 230 nm and when fractions were assayed for ^{14}C by liquid scintillation counting. The elution profile showed that about 50% of the total radioactivity which was applied to the column was recovered in a single peak.

The peak fractions from the CM-cellulose column were pooled (Figure 2), desalted by gel filtration on P-2, and lyophilized. In a few experiments, the peptide was subjected to a final gel filtration step on a column which contained 8% agarose and was equilibrated with 0.2 M ammonium bicarbonate. The elution profile from this column (not shown) showed a single peak both when the fractions were assayed for ^{14}C and when they were read at 230 nm.

$NaDodSO_4$-polyacrylamide gel electrophoresis analysis of the peptide obtained after CM-cellulose chromatography showed one major band when the peptide was examined without reduction (Figure 3A). After reduction with 2-mercaptoethanol, two bands were observed (Figure 3B). The band with the lower mobility stained more intensely than the fast-moving band. The intensity ratio between the two bands was about 1.7:1.

Isolation of Two Different Peptide Chains from the Medium Peptide. Since $NaDodSO_4$ gel electrophoresis of the reduced medium peptide indicated the presence of two types of subunits, we attempted to separate these chains by ion-exchange chromatography. The purified medium peptide obtained after chromatography on CM-cellulose in 6 M urea was reduced and alkylated in 8 M urea and then chromatographed on CM-cellulose in the presence of 6 M urea. The elution profile showed two peaks (Figure 4). The recovery of protein from this column was 80–95%. The fractions in the two peaks were pooled as indicated in Figure 4, desalted by gel filtration on P-2, and lyophilized. Analysis of the two peaks by $NaDodSO_4$ gel electrophoresis showed that the peaks represented the two types of chains in the medium peptide.

Purification of Procollagen and the Carboxy-Terminal Collagenase-Resistant Extension Peptide. The carboxy-terminal collagenase-resistant peptide was prepared by first incubating matrix-free tendon fibroblasts in suspension culture and isolating procollagen from the medium. The [^{14}C]procollagen was then purified by DEAE-cellulose chromatography and digested with bacterial collagenase. The collagenase-resistant carboxy-terminal region of procollagen was isolated by chromatography of the digest on an 8% agarose column (data not shown).

Identification of the Medium Peptide. The identity of the medium peptide was established by two-dimensional analysis of tryptic peptides and by immunological criteria. For analysis of tryptic peptides, the carboxy-terminal collagenase-resistant

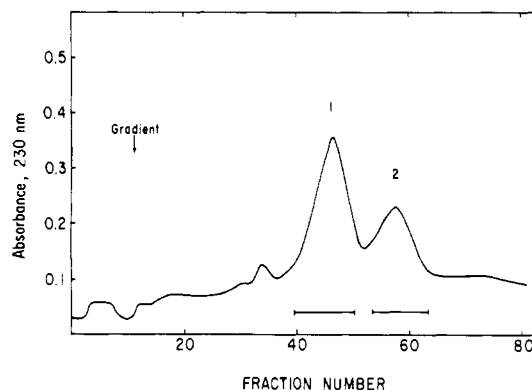

FIGURE 4: Chromatography of reduced and alkylated medium peptide on CM-cellulose in the presence of 6 M urea. The horizontal bars indicate how fractions in peaks 1 and 2 were pooled for further examination.

peptide derived from procollagen and the medium peptide isolated by DEAE- and CM-cellulose chromatography were reduced and alkylated, digested with trypsin, and analyzed by two-dimensional chromatography/electrophoresis. The tryptic peptides of the collagenase-resistant fragment were identified by fluorography, whereas the tryptic peptides of the medium peptide were identified by staining with fluorescamine. The similarity of the tryptic fingerprints (Figure 5) clearly indicated that the medium peptide and the carboxy-terminal collagenase-resistant peptide contained common sequences and that the medium peptide therefore was derived from the carboxy-terminal region of procollagen.

The identity of the medium peptide was also established by immunological criteria. For this purpose antibodies were

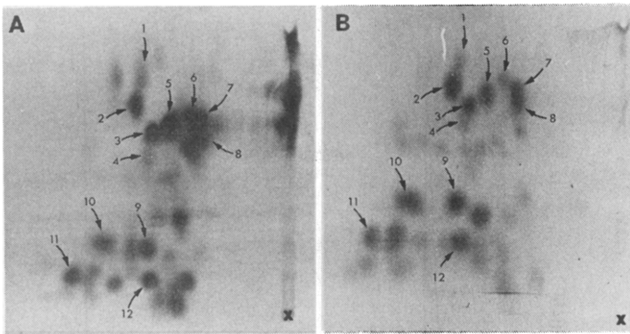

FIGURE 5: Tryptic peptides obtained from the collagenase-resistant carboxy-terminal precursor region of procollagen (A) and the medium peptide (B). The samples were applied in the lower right-hand corner of the plates (X). Chromatography was carried out in the vertical direction and electrophoresis in the horizontal direction. Plate A was treated with 7% diphenyloxazole in ether and exposed to x-ray film in order to visualize spots of ^{14}C-labeled peptides. Plate B was treated with fluorescamine and viewed under ultraviolet light. The arrows indicate major spots which are identical in the two peptides. In addition, the collagenase-resistant peptide contains tryptic peptides which are not observed in the medium peptide.

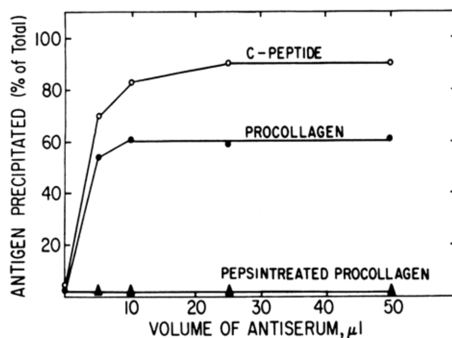

FIGURE 6: Titration curves using antiserum against the medium peptide. Antigens were: (○—○) medium peptide (C-peptide); (●—●) procollagen; (▲—▲) pepsin-treated procollagen. For precipitation of medium peptide, undiluted antiserum was used; for precipitation of procollagen and pepsin-treated procollagen, the antiserum was diluted 1:10 with 0.1% ovalbumin in 0.15 M NaCl-0.1 M Tris-HCl, pH 7.5, containing 0.01% sodium azide.

produced by injecting the purified medium peptide into rabbits. The antisera were then used to compare the medium peptide with procollagen and defined procollagen peptides in a direct binding radioimmunoassay (Figures 6 and 7). The antiserum reacted with the purified medium peptide and with procollagen isolated from the medium of tendon fibroblasts incubated in suspension, but it did not react with procollagen from which the amino-terminal and carboxy-terminal peptide extensions had been removed by treatment with pepsin (Figure 6). When the antiserum was tested against the amino-terminal and carboxy-terminal fragments obtained after cleavage of procollagen with tadpole collagenase, it was found to react only with the carboxy-terminal fragment proγ^B (Figure 7).

Molecular Weight of the Medium Peptide. The molecular weights of the medium peptide and the constituent chains were estimated by polyacrylamide slab gel electrophoresis and by ultracentrifugation. Since the molecular weight of unreduced proteins cannot be reliably estimated by $NaDodSO_4$ gel electrophoresis, the molecular weight of the unreduced peptide was determined by sedimentation equilibrium only. Using a value of 0.722 for the partial specific volume as calculated from the amino acid composition, the molecular weight was found to be 100 000 ± 10 000 for the native unreduced peptide. In sedimentation velocity experiments, the bulk of the material

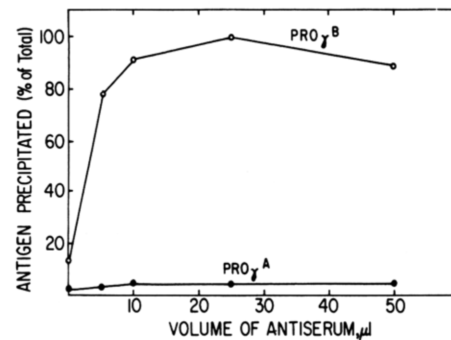

FIGURE 7: Titration curves using antiserum against the medium peptide. Antigens were: (○—○) carboxy-terminal fragment proγ^B obtained by cleavage of procollagen with tadpole collagenase (Olsen et al., 1976); (●—●) amino-terminal fragment proγ^A obtained by cleavage of procollagen with tadpole collagenase. The antiserum was diluted 1:10 with 0.1% ovalbumin as described in the legend for Figure 6.

sedimented with a sedimentation coefficient $s_{20,w} = 5.2 \times 10^{-13}$ s at $c = 0.5$ mg/mL.

Using globular proteins as standards, $NaDodSO_4$ gel electrophoresis gave values of 34 000 and 31 000 for the molecular weights of the two peptide subunits seen after reduction of the medium peptide (Figure 3B). Analysis of the medium peptides by sedimentation equilibrium after reduction and alkylation in urea or under nondenaturing conditions gave a weight-average molecular weight of 43 000 ± 5000 for the mixture of the peptides. The ln c vs. r^2 plots showed a higher curvature than expected for a 1:2 mixture of two chains, the molecular weights of which differ only by 10 to 20% according to $NaDodSO_4$ gel electrophoresis. The data were consistent with a molecular weight of 35 000 to 40 000 for 70 to 80% of the material (by weight) and the presence of 20 to 30% dimers with molecular weight 70 000 to 80 000. No significant dependence of the equilibrium data on total protein concentration was detectable in a concentration range of 0.2 to 0.6 mg/mL.

Amino Acid and Carbohydrate Compositions of the Medium Peptide. Amino acid and carbohydrate analysis of the medium peptide and its constituent chains (Table I) showed that there were about 30 residues of half-cystine per peptide trimer and about 10 residues of half-cystine per subunit, when the peptides were analyzed after complete reduction and alkylation in 8 M urea. After partial reduction and alkylation under nondenaturing conditions, only 50% of the total number of half-cystines could be alkylated (Table I). Other features of interest were the absence of hydroxyproline and hydroxylysine and the high content of tyrosine (34 residues per 1000 residues). The carbohydrate analysis showed that both types of subunits contained oligosaccharide side chains with about 2 residues of *N*-acetylglucosamine and about 10 residues of mannose per peptide chain (Table I).

Discussion

Incubation of 17-day old chick embryo tendons in these experiments showed the feasibility of obtaining large amounts of a peptide derived from the carboxy-terminal extension of procollagen type I. When tendons from 120 embryos were incubated for 36 h, about 5 mg (50 nmol) of purified medium peptide was obtained after chromatography on CM-cellulose. The system allows, therefore, the isolation of this peptide in the amounts which are required for detailed structural analysis. The purification procedure is rapid and simple; after chromatography on DEAE- and CM-cellulose, $NaDodSO_4$ gel electrophoresis showed that the isolated peptide was about 95%

TABLE I: Amino Acid and Carbohydrate Compositions of Medium Peptide and the Two Types of Subunits.

Components	Medium peptide		Residues/33 000 daltons	
	Residues/ 1000	Residues/100 000 daltons	Subunit 1	Subunit 2
Amino acid				
4-Hyp	0	0	0	0
Asp	126	115	40	41
Thr	89	81	28	29
Ser	62	57	20	22
Glu	113	103	35	30
Pro	52	48	13	11
Gly	80	73	27	25
Ala	67	61	19	21
½-Cystine[a]	34 (17[b])	31 (16[b])	11	11
Val	48	44	16	12
Met	7.2	6.6	2.5	0.7
Ile	52	48	16	18
Leu	71	65	20	24
Tyr	34	31	11	8.5
Phe	35	32	9.5	11
Hyl	0	0	0	0
Lys	69	63	19	20
His	21	19	5.8	7.6
Arg	44	40	13	13
Carbohydrate[c]				
Mannose		30	9	13
N-Acetylglucosamine		6.5	2	2
Protein (%)		93.2	93.7	91.6
Carbohydrate (%)		6.8	6.3	8.4

[a] Determined as carboxymethylcysteine after complete reduction and alkylation in urea. [b] Determined as carboxymethylcysteine after partial reduction and alkylation under nondenaturing conditions. [c] In addition to mannose and *N*-acetylglucosamine, trace amounts of glucose and unidentified components were observed.

pure. Also, sedimentation equilibrium runs of the isolated peptide demonstrated that it was relatively homogeneous.

The medium peptide isolated and purified here clearly originates from the carboxy-terminal region of procollagen. This conclusion is based on the immunological evidence and on the similarity of the tryptic fingerprints of the medium peptide and the collagenase-resistant carboxy-terminal trimeric peptide isolated from procollagen. The result of the immunoassays demonstrated that antibodies against the medium peptide also reacted with intact procollagen but not with pepsin-treated procollagen. The antigenic determinants with which the antibodies react are therefore located in the precursor-specific portions of procollagen which can be removed by pepsin. The additional finding that the antisera reacted with the carboxy-terminal tadpole collagenase fragment of procollagen and not with the amino-terminal fragment, restricts the location of the antigenic determinants to the carboxy-terminal precursor-specific portion of procollagen. The tryptic fingerprints (Figure 5) of the medium peptide and the collagenase-resistant procollagen peptide contained spots which were identical (arrows in Figure 5) as well as spots which were different. This indicates that the two peptides contain common sequences, but that they are not identical.

The molecular weight determined for the unreduced peptide is about three times the values estimated for the reduced subunits. This indicates that the peptide is a trimer. The finding of two bands in an approximate 2:1 ratio by $NaDodSO_4$ gel electrophoresis of the reduced peptide further indicates that the trimer is composed of two different types of subunits. This is in agreement with the separation of two peaks in a 2:1 ratio when the reduced and alkylated peptide is chromatographed on CM-cellulose (Figure 4).

We conclude, therefore, that the medium peptide purified here contains three chains connected by disulfide bridges. Two of the chains are derived from the proα1(I) carboxy-terminal extension and one chain is derived from the proα2 carboxy-terminal extension.

The amino acid composition and molecular weight of the peptide characterized here are similar to that of the peptide isolated from the medium of cultured cranial bone by Murphy et al. (1975). In particular, the cysteine content is the same in the two peptides, with 34 residues of half-cystine per 1000 residues. Also, the tyrosine content is high (Table I). The amino acid composition of the two peptide subunits reported here shows that they contain the same amount of half-cystine (Table I). Based on a molecular weight of 100 000 for the trimer, this gives about 10 half-cystines per peptide chain in the trimer.

Partial reduction and alkylation under nondenaturing conditions cleaved all the interchain disulfide bonds of the trimer since the molecular weight was found to be about 40 000 after partial reduction. The amino acid analysis demonstrated that only 50% of the total number of half-cystines were alkylated under these conditions. We conclude, therefore, that at least 50% of the half-cystines in the trimer form intrachain disulfide bonds. This limits the number of interchain disulfide bridges to a maximum of six per trimer and two per peptide subunit.

The finding of *N*-acetylglucosamine and mannose in the medium peptide is in agreement with recent radiolabeling data (Murphy et al., 1975; Clark and Kefalides, 1976), which showed incorporation of [^{3}H]mannose, [^{3}H]glucosamine, and [^{3}H]galactosamine into procollagen. We did not observe significant amounts of galactosamine in the medium peptide. It is likely, therefore, that *N*-acetylgalactosamine is not present in the region of procollagen from which the medium peptide is derived. The finding of about two residues of *N*-acetylglucosamine per chain indicates that there are one or two oligosaccharide units per chain in the peptide trimer. At the present, we can only speculate upon the functional significance of these oligosaccharide units in procollagen. A role for the carbohydrate in intracellular transport and secretion of procollagen has been suggested (Clark and Kefalides, 1976) but recent data on the effect of tunicamycin on procollagen secretion (Duksin and Bornstein, 1977) seem to invalidate such a hypothesis. It is possible that the oligosaccharide functions as a molecular recognition site on the carboxy-terminal extension in the extracellular matrix during fibrillogenesis.

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