Human Skin Collagenase: Isolation of Precursor and Active Forms from Both Fibroblast and Organ Cultures†

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ABSTRACT: Human skin procollagenase has been isolated, in pure form, from the medium of fibroblasts cultured in the presence or absence of added serum. Purification was achieved using a combination of cation-exchange (phosphocellulose or carboxymethylcellulose) and gel-filtration chromatography. Two forms (60 000 and 55 000 daltons) of the procollagenase were detected by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels and could be separated by chromatography on Ultrogel AcA-44. Each form was converted to active enzyme by trypsin, producing species of 50 000 and 45 000 daltons, respectively. An autoactivation process also occurred, which yielded active enzyme without a detectable change in molecular weight. Procollagenase also was found in organ cultures of human skin but only when serum was added to the medium. This suggests that a serum-inhibitable proteolytic system is present in these cultures which, like trypsin, converts procollagenase to the active enzyme forms that can be isolated from serum-free organ culture medium. The collagenase species obtained from either fibroblast or organ culture medium were chromatographically and electrophoretically identical.

The collagenases, with their unique ability to initiate collagen degradation, play a key role in both normal and pathological connective-tissue metabolism. These enzymes have been found in a variety of mammalian sources and several have been partially characterized (Harris and Krane, 1974; Eisen et al., 1970). A feature of many systems has been the presence of latent collagenolytic activity. A number of workers, using kinetic and chromatographic techniques, have suggested that this latency is due to the existence of a proenzyme (Kruze and Wojtecka, 1972; Vaes, 1972; Robertson et al., 1973; Oronsky et al., 1973; Hook et al., 1973; Bauer et al., 1975; Harris et al., 1975; Birkedal-Hansen et al., 1975, 1976a,b). However, the isolation of such a proenzyme has only been reported for the amphibian (tadpole tailfin) collagenase (Harper et al., 1971). In contrast, Nagai and co-workers have produced active collagenase by exposing latent collagenase to chaotropic agents, such as SCN^- (Abe and Nagai, 1972; Nagai, 1973). Thus, they suggest that the latency is a property of a complex composed of collagenase and another, as yet undefined, molecule. Although the data supporting either view are highly suggestive, direct proof has not been obtained by the analysis of a pure enzyme protein.

As reported earlier from this laboratory (Bauer et al., 1975), human skin fibroblasts in culture secrete a collagenase in inactive form. Large scale culture of these cells has now provided us with quantities of medium relatively rich in enzyme, thus permitting the development of purification methods capable of efficiently producing milligram quantities of pure collagenase. The studies described in this paper indicate that, following its secretion in precursor form, fibroblast collagenase can be converted into active enzyme by either tryptic cleavage or by an autoactivation process. In addition, human skin in organ culture also secretes procollagenase which is apparently converted to active enzyme by an extracellular proteolytic system.

Materials and Methods

Reagents. Acrylamide and bisacrylamide were purchased from Eastman Kodak. Sodium dodecyl sulfate, 99% pure, was obtained from Gallard-Schlesinger Chemical Manufacturing Co. Trizma base, bovine pancreatic trypsin, bovine serum albumin, ovalbumin, beef blood hemoglobin, and soybean trypsin inhibitor were purchased from Sigma Chemical Co. Chymotrypsinogen A and pepsin were purchased from Worthington Biochemical Corp. All other chemicals used were reagent grade.

Culture Methods. Normal human skin fibroblasts (CRL 1187) were purchased from the American Type Culture Collection. The cells were grown at 37 °C in glass roller bottles (1585 cm^2, Bellco) in 50 mL of Dulbecco's modified Eagle's medium-HG + glutamine (Microbiological Associates) containing 0.03 M Hepes[1] buffer (pH 7.6), 20% fetal calf serum, and 200 units of penicillin and 200 μg of streptomycin per mL. At visual confluence, the cells were put through several cycles of serum-free medium for 48–72 h as described by Bauer et al. (1975). In a typical purification, the pooled medium from 100–200 roller bottles, equivalent to $1-2 \times 10^{10}$ cells at confluence (Bauer et al., 1976), was used as starting material. The harvested serum-free medium was made 0.05 M Tris-HCl, pH 7.5, and concentrated by vacuum dialysis using a hollow fiber device (MDA Scientific) to a volume of 5 mL per roller bottle. The serum containing medium was buffered with 0.05 M Tris-HCl, pH 7.5, and stored without concentration. All harvested medium was stored at −20 °C.

Normal human skin obtained at surgery was prepared for organ culture in serum-free medium as previously reported

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DTT, dithiothreitol; SBTI, soybean trypsin inhibitor; BSA, bovine serum albumin; iPr_2FP, diisopropyl fluorophosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; CM, carboxymethyl; FCS, fetal calf serum.

(Eisen et al., 1968). The medium was changed daily and the harvests from days 3–10 of culture were pooled, dialyzed against distilled water at 4 °C, lyophilized, and stored at −20 °C. Skin explants were also grown in medium containing 20% FCS, and the harvests from days 3–10 of culture were pooled, buffered with 0.05 M Tris-HCl, pH 7.5, and stored at −20 °C.

Activation of Procollagenase. The activation of procollagenase was done proteolytically at 25 °C by the addition of trypsin for 10 min. At least a fourfold molar excess of SBTI was then added to inhibit any further tryptic action. A range of trypsin concentrations, usually from 0.1 to 5.0 μg per 50 μL sample, was used to ensure that maximal collagenase activity was achieved (Bauer et al., 1975). When determining collagenase activity, this procedure was done in the presence of gelled collagen substrate to prevent losses incurred in transferring the solution. However, the activation was performed in a separate tube when activity against gelatin was to be assayed to prevent the degradation of this substrate by the trypsin. No activation was required for collagenase derived from serum-free organ culture medium (Bauer et al., 1972a). The activation of the neutral protease found in cell culture medium was accomplished with this same procedure.

Assay Procedures. Collagenase activity was measured at 37 °C using native reconstituted [^{14}C]glycine-labeled guinea pig skin collagen as substrate (Nagai et al., 1966). The collagen was allowed to gel at 37 °C for at least 24 h to allow completion of the aggregation process. Fifty microliters of a 0.4% collagen solution having a specific activity of approximately 22 000 cpm/mg was used for each assay. To complete the assay, the tubes were centrifuged at 12 000*g* and the supernatant fraction was counted in a liquid scintillation spectrometer.

Gelatinolytic activity was measured at 37 °C by incubating the sample with 50 μL of gelatin, produced by heating the above [^{14}C]collagen solution to 55 °C for 20 min (Harris and Krane, 1972). The reaction was terminated by the addition of a 45% Cl_3CCOOH solution to give a final concentration of 15%. After chilling on ice for 15 min, the tubes were centrifuged at 12 000*g* and the supernatant fraction was counted in a liquid scintillation spectrometer.

Fibroblast-derived collagenase inhibitor (Bauer et al., 1975) was assessed by the addition of 50–100 μL of sample to 25 μL of a stock solution of collagenase, prepared by redissolving the precipitate at 60% saturation of $(NH_4)_2SO_4$ from serum-free organ culture medium to a volume which gave approximately 1000 cpm/25 μL of enzyme per h in the collagenase assay. This mixture was incubated for 1 h at 37 °C with collagen substrate as described for the collagenase assay.

The determination of protein concentration was performed spectrophotometrically by the method of Groves et al. (1968). BSA was used to establish a standard curve.

Preparation of Culture Medium for Ion-Exchange Chromatography. Serum-free fibroblast culture medium was dialyzed against 40 volumes of 0.05 M Tris-HCl, pH 7.5 (buffer A), overnight at 4 °C and filtered through Whatman No. 1 filter paper prior to being applied to a phosphocellulose column. For chromatography on CM-cellulose, the serum-free medium was prepared by adding BSA to a final concentration of 0.5 mg/mL in order to reduce the tendency of the medium proteins to precipitate when dialyzed against 40 volumes of 0.01 M Tris-HCl, pH 7.5, containing 0.0001 M $CaCl_2$ (buffer B) at 4 °C for 24 h. Whatman No. 1 filter paper was used to remove any precipitate prior to application to the CM-cellulose column.

Serum-containing fibroblast culture medium was brought to a final saturation of 55% at 4 °C by the addition of solid $(NH_4)_2SO_4$. The precipitate was centrifuged at 9000*g* and redissolved in 0.05 M Tris-HCl, pH 7.5, containing 0.01 M $CaCl_2$. This solution was then dialyzed against two changes of 52 L each using either buffer A or B, as described above, depending upon the ion-exchanger to be used. Any precipitate formed during dialysis was removed by centrifugation and filtration through Whatman No. 1 filter paper.

Lyophilized organ culture medium was redissolved in 0.05 M Tris-HCl, pH 7.5, containing 0.01 M $CaCl_2$ to a concentration of 40-mg dry weight/mL. This solution was then dialyzed against 17 L of either buffer A or B at 4 °C for 24 h. Any precipitate was removed by centrifugation and filtration through Whatman No. 1 paper.

Serum-containing organ culture medium was prepared as described for serum-containing cell culture medium.

Chromatography. All chromatographic procedures were performed in jacketed columns maintained at 4 °C. Flow rates were regulated with a Gilson peristaltic pump. The effluent was monitored with a Gilson MUVRP recording spectrophotometer and fractions were collected with an LKB Ultrorac.

Ion-Exchange Chromatography. Phosphocellulose (Whatman, P11) was equilibrated with buffer A and packed into columns under 8 psi. Following application of the sample, buffer A was used to return the optical density to baseline values. A linear gradient of 0.0 to 0.4 M $(NH_4)_2SO_4$ in 0.05 M Tris-HCl, pH 7.5, was used to elute the bound proteins. For sample volumes under 200 mL, a 1.6 × 20 cm column was used at a flow rate of 25 mL/h and with a total gradient volume of 300 mL. For larger sample volumes, a 2.5 × 20 cm column was used at a flow rate of 60 mL/h with a total gradient volume of 600 mL. The use of samples derived from serum-containing medium required the use of batch techniques. The ammonium sulfate prepared samples obtained from 10 L of serum-containing medium were diluted to a volume of 1.0 L. Approximately 600 mL of phosphocellulose was added and the slurry was mixed gently at 4 °C for 30 min. The exchanger was allowed to settle and the supernate was decanted. The exchanger was washed twice with 2-L volumes of cold buffer A, poured into a large (5-cm diameter) column, and washed with buffer A for an additional 18 h at a flow rate of 100 mL/h. Elution was performed with a 2.5-L gradient of 0.0 to 0.5 M $(NH_4)_2SO_4$ in 0.05 M Tris-HCl, pH 7.5. To assay for collagenase, 50-μL aliquots of the column fractions were diluted with 50 μL of 0.05 M Tris-HCl, pH 7.5, containing 0.01 M $CaCl_2$ and activated with 0.5 μg of trypsin for 10 min followed by 20 μg of SBTI. Enzyme-containing fractions were pooled and concentrated when necessary with a single hollow-fiber device (MDA Scientific) under a mild vacuum and placed in a Ca^{2+}-containing buffer immediately by either gel filtration or dialysis techniques.

Carboxymethylcellulose (Whatman, CM-52) was equilibrated with 0.01 M Tris-HCl, pH 7.5, and packed into columns under 8 psi. Following sample application, starting buffer was used to return the optical density to baseline levels. A linear gradient of 0.0 to 0.3 M NaCl in 0.01 M Tris-HCl, pH 7.5, containing 0.001 M $CaCl_2$ was used to elute the bound proteins. For sample volumes up to 300 mL, a 1.6 × 35 cm column was used at a flow rate of 30 mL/h and with a total gradient volume of 300 mL. For larger samples, a 2.5 × 35 cm column was used at a flow rate of 60 mL/h and with a total gradient volume of 600 mL. For samples derived from serum-containing medium, an alternative procedure was followed. The ammonium sulfate prepared samples obtained from 10 L of serum-containing medium were applied in a volume of 750 mL to a

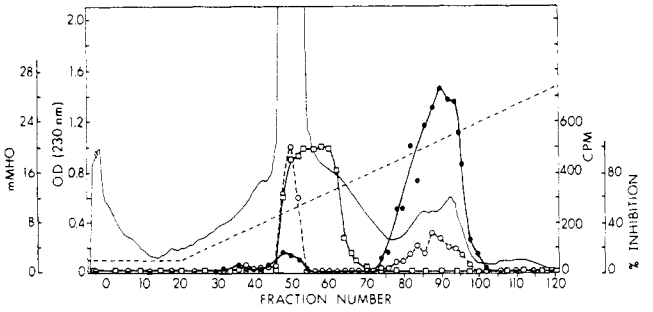

FIGURE 1: Phosphocellulose chromatography. Concentrated serum-free fibroblast culture medium, 555 mL, containing 99 mg of protein, was prepared as described under Methods and applied at a flow rate of 60 mL/h to a 2.5 × 14 cm column of phosphocellulose equilibrated with 0.05 M Tris-HCl, pH 7.5. The bound proteins were eluted with a 600-mL linear gradient of 0.0 to 0.4 M $(NH_4)_2SO_4$ in 0.05 M Tris-HCl, pH 7.5, starting with tube 1. Seven-milliliter fractions were collected. Optical density 230 nm (—); conductivity, $m\Omega^{-1}$ (- - -); collagenase activity, cpm (●); gelatinolytic activity, cpm (○); % inhibition of collagenase (□).

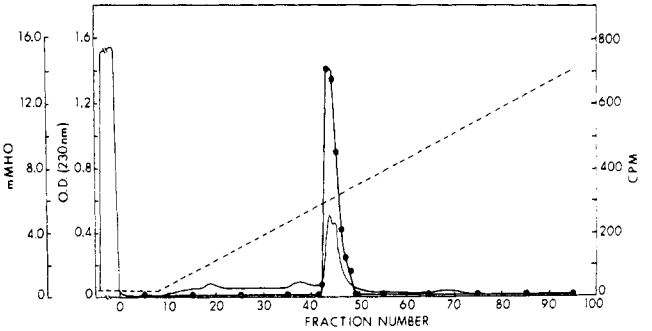

FIGURE 2: CM-cellulose chromatography. Concentrated serum-free fibroblast culture medium, 485 mL, containing 108 mg of protein, was prepared as described under Methods and applied at a flow rate of 60 mL/h to a 2.5 × 20 cm column of CM-cellulose equilibrated with 0.01 M Tris-HCl, pH 7.5. The bound proteins were eluted with a 600-mL linear gradient of 0.0 to 0.3 M NaCl in 0.01 M Tris-HCl, pH 7.5, containing 0.001 M $CaCl_2$ starting at tube 1. Six-milliliter fractions were collected. Optical density 230 nm (—); conductivity, $m\Omega^{-1}$ (- - -); collagenase activity, cpm (●).

5 × 25 cm column of CM-cellulose at a flow rate of 120 mL/h. The column was then washed for 18 h with 0.01 M Tris-HCl, pH 7.5, and eluted with a 1 L gradient of 0.0 to 0.4 M NaCl in a 0.01 M Tris-HCl, pH 7.5, buffer containing 0.001 M $CaCl_2$. To assay for collagenase, 50-μL aliquots of the column fractions were diluted and activated as described for the phosphocellulose procedures. When necessary, the pooled enzyme fractions were concentrated as previously described.

Gel Filtration. Gel filtration chromatography was performed in two 1.6 × 100 cm columns connected in series. With either Sephadex G-100 or Ultrogel AcA-44, a 0.05 M Tris-HCl, pH 7.5, buffer containing 0.01 M $CaCl_2$ was used at a flow rate of approximately 15 mL/h. The maximum sample volume applied was 15 mL and the effluent was monitored at 230 nm. For assay, 50-μL aliquots of the fractions were activated with 0.5 μg of trypsin for 10 min followed by 20 μg of SBTI. Calibration of the columns for molecular weight was performed as described by Andrews (1964, 1965) using Blue Dextran, BSA (66 000 daltons), ovalbumin (45 000 daltons), pepsin (34 500 daltons), chymotrypsinogen A (25 700 daltons), and phenol red as markers.

Electrophoretic Techniques. Basic (pH 8.6) polyacrylamide electrophoresis was performed by the method of Davis (1964) using 7.5 and 10.0% gels. Acidic (pH 4.3) polyacrylamide electrophoresis was performed as described by Reisfield et al. (1962) using 7.5% gels and incorporating 8 M urea into all components.

The continuous buffer sodium dodecyl sulfate electrophoresis system of Fairbanks et al. (1971) was used with 5.6% polyacrylamide gels cast in cylindrical or slab molds. This system uses a Tris-acetate-EDTA buffer at pH 7.4 containing 1% sodium dodecyl sulfate. Samples were dialyzed against 0.1 M acetic acid, lyophilized, and then dissolved in a 1:2 dilution of the sample solution as described by Fairbanks et al., modified to contain 2.5 mM EDTA and 2.5% sodium dodecyl sulfate. Reduction of disulfide linkages was accomplished by incorporating 6.25 g/mL of DTT into the sample solution. All dissolved samples were incubated in a boiling water bath for 10 min prior to electrophoresis.

Following electrophoresis in any of the above systems, the protein bands were fixed in a solution of 12.5% 2-propanol and 2.5% acetic acid and stained with 0.005% Coomassie brilliant blue.

Isoelectric Focusing. Electrofocusing was performed with pH 3.5–10.0 Ampholine using polyacrylamide gels (7.5%) as a stabilizing medium. Following the establishment of a pH gradient as determined by the use of a marker protein (hemoglobin), the location of protein bands in the sample gels was determined by staining with Coomassie brilliant blue as described above. The pH gradient was determined by slicing the gels and eluting each slice with 1 mL of distilled water prior to measurement with a Corning pH meter.

Results

Human skin collagenase, in either latent or active form, was purified from fibroblast and organ culture medium. The isolation of inactive collagenase was most easily accomplished using serum-free fibroblast culture medium, although significant quantities of latent enzyme were also obtained from serum-containing fibroblast and serum-containing organ culture medium. In addition, a highly purified active collagenase was obtained from serum-free organ culture medium.

The use of cation-exchange chromatography was the essential step in the purification of human skin fibroblast collagenase from serum-free medium. As seen in Figure 1, phosphocellulose bound several proteins of interest while allowing most of the medium proteins to pass through the column. Two of these proteins, a specific collagenase inhibitor and a noncollagenolytic neutral protease, were coeluted with the majority of the bound proteins. The collagenase eluted at approximately 0.2 M $(NH_4)_2SO_4$ and was usually well separated from the other protein peaks (Figure 1).

Although excellent results were obtained with the use of phosphocellulose, the tight binding of the collagenase to this matrix indicated that the weaker cation exchanger, CM-cellulose, might also be useful. In contrast to the phosphocellulose system, equilibration of the CM-cellulose with a more dilute Tris-HCl buffer at pH 7.5 as well as a much more complete equilibration of the sample with its buffer was required to permit the binding of collagenase to CM-cellulose. However, as can be seen in Figure 2, collagenase was virtually the only medium protein bound and, at approximately 0.15 M NaCl, the enzyme eluted as a sharp peak.

In the selection and use of these exchangers, two parameters were set by the stability requirements of the collagenase. First, the pH had to be maintained at approximately 7.5 (Eisen et al., 1968). Second, the enzyme is stabilized by Ca^{2+} (Seltzer et al., 1976). Even though phosphocellulose precludes the use

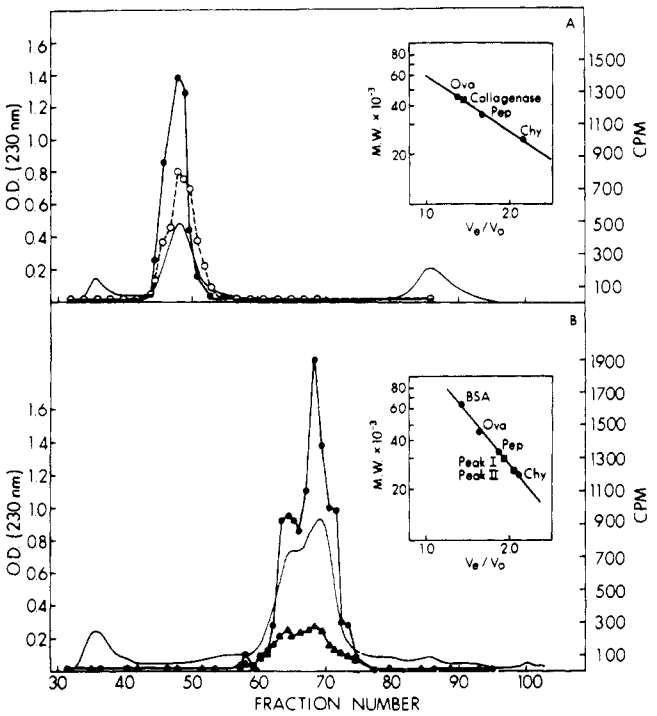

FIGURE 3: (A) Sephadex G-100 chromatography. Approximately 2 mg of collagenase purified by chromatography on phosphocellulose was applied in a volume of 2 mL to two 1.6 × 100 cm columns of Sephadex G-100 connected in a series and equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 0.01 M $CaCl_2$. A flow rate of 11.7 mL/h was maintained and 3.9-mL fractions were collected. The proteins used for calibration of molecular weights (insert) were ovalbumin (Ova), pepsin (Pep), and chymotrypsinogen A (Chy). Optical density 230 nm (—); trypsin-activated collagenase, cpm (●); unactivated collagenase, cpm (○). (B) Ultrogel AcA-44 chromatography. Approximately 2.2 mg of collagenase purified by chromatography on phosphocellulose was applied in a volume of 15 mL to two 1.6 × 100 columns of Ultrogel AcA-44, connected in series and equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 0.01 M $CaCl_2$. A flow rate of 12.7 mL/h was maintained and 4.2-mL fractions were collected. The proteins used for calibration of molecular weight (insert) were bovine serum albumin (BSA), ovalbumin (Ova), pepsin (Pep), and chymotrypsin A (Chy). Optical density 230 nm (—); collagenase activity, cpm (●), gelatinolytic activity, cpm (▲).

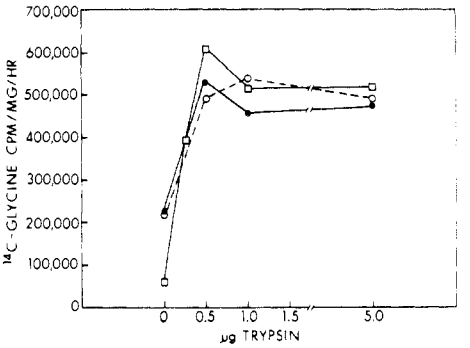

FIGURE 4: Purified collagenase obtained following gel filtration was activated with increasing amounts of trypsin as described under Methods for 10 min. Activation was terminated by adding at least a fourfold excess of SBTI, and the resulting mixture was assayed for collagenolytic activity as under Methods. Enzyme obtained from Sephadex G-100 (□), Ultrogel AcA-44 peak I (○), and Peak II (●).

of Ca^{2+} and CM-cellulose is a very weak exchanger at pH 7.5, conditions were found which allowed excellent purification of enzyme protein along with satisfactory retention of activity. Phosphocellulose, with its strong exchange properties and high capacity at this pH, permitted the sample to be applied after minimal dialysis while still producing good chromatography. Thus, the enzyme was not in a totally Ca^{2+}-free environment until it was bound to the column and washed with the Tris-HCl buffer. Subsequently, the time required for elution and assay was short enough that no undue loss of activity was incurred. The other exchanger, CM-cellulose, required the use of a dilute Tris-HCl buffer. However, a small amount of calcium (10^{-4}M) could be included in the sample buffer and, once the enzyme was bound, the calcium concentration of the gradient buffers could be raised to 0.001 M without eluting the enzyme.

Following either of these ion-exchange steps, the active fractions were pooled, concentrated when necessary, and subjected to gel filtration using Sephadex G-100 or Ultrogel AcA-44. When a column of Sephadex G-100 was used, the enzyme eluted as a single well-separated peak with an apparent molecular weight of approximately 42 000 daltons (Figure 3A). As can be seen in Figure 3B, the polyacrylamide–agarose gel-filtration medium, Ultrogel AcA-44, produced quite different results. When the same enzyme pool was applied to a column of this material and run under the same conditions as the Sephadex G-100 column, the enzyme eluted as two partially overlapping peaks whose apparent molecular weights were 30 000 and 25 000, respectively. In spite of the improved resolution with Ultrogel AcA-44, enzymatic assays with and without trypsin activation indicated that column fractions from either the single collagenase peak from Sephadex G-100 or the two enzyme peaks from Ultrogel AcA-44 required trypsin activation to obtain maximal collagenase activity. Specific activity measurements, using fully trypsin activated enzyme, indicated that the collagenase found in these three peaks exhibited identical catalytic efficiencies (Figure 4). Furthermore, as shown in Figure 3A, even though some collagenase activity may be present without trypsin activation, this activity was found to essentially coelute with the inactive enzyme on either Sephadex G-100 (Figure 3A) or Ultrogel AcA-44 (not shown). Thus, each column fraction under these peaks appeared to contain inactive collagenase as well as a smaller amount of the active enzyme.

As reported earlier (Bauer et al., 1975), collagenase from serum-free fibroblast culture medium produced the typical collagen cleavage products. Similar studies (not shown) using collagenase purified through the gel filtration step and then trypsin activated showed that it too cleaved the native collagen at a locus approximately 75% of the distance from the N-terminus of the molecule as originally described for human skin collagenase from organ culture (Eisen et al., 1968).

As indicated in Figures 1 and 3B, a low level of proteolytic activity was detected against gelatin when fractions under the collagenase peaks were assayed. This activity was found to cochromatograph with the collagenase in all procedures. Further, as indicated in Figure 3B, the specific activity measured against gelatin appeared to be constant across both of the collagenase peaks eluted from Ultrogel AcA-44. Lastly, the activity of this preparation against gelatin appeared coincidently with the collagenase activity when the fractions were trypsin activated.

At this stage, obvious complexity existed within this preparation from serum-free fibroblast culture medium. Not only were active and inactive forms of collagenase detectable, but the chromatography using Ultrogel AcA-44 indicated the presence of two species of each. Therefore, an analytical system of high resolving power was sought in order to assess purity as well as to aid in the determination of the exact composition of this preparation. When electrophoresis of purified enzyme was

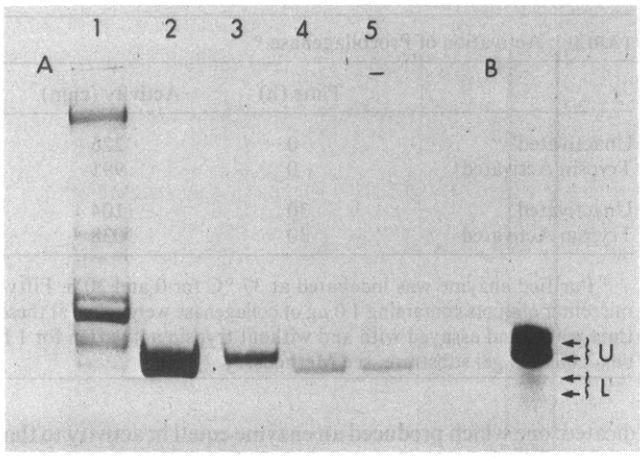

FIGURE 5: (A) Aliquots from the various stages of purification of collagenase from serum-free fibroblast culture medium were electrophoresed on a slab sodium dodecyl sulfate–polyacrylamide gel. One-hundred-micrograms of crude medium protein (slot 1), 50 μg of the collagenase peak from CM-cellulose (slot 2). Proteins from the two overlapping enzyme peaks from Ultrogel AcA-44 chromatography, Figure 3B, are seen in slots 3–5. Slot 3 represents 20 μg of Peak I enzyme, fractions 62–65. Slot 4 represents 20 μg of fractions 66–69 from the middle region. Slot 5 represents 20 μg of Peak II enzyme, fractions 70–73. (B) Sodium dodecyl sulfate–disc polyacrylamide electrophoresis of 40 μg of pure collagenase. "U" indicates upper doublet forms, "L" indicates lower doublet forms.

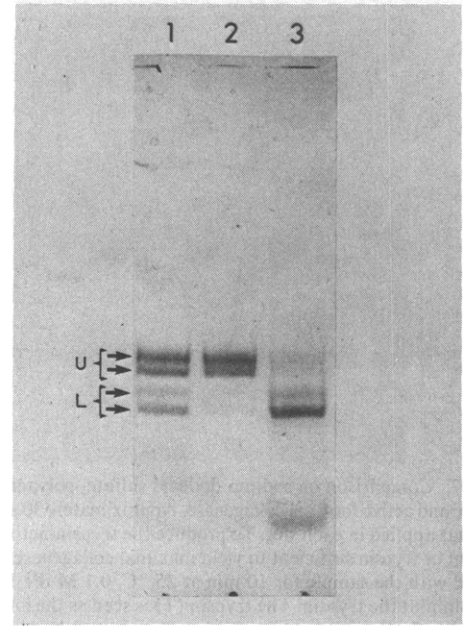

FIGURE 6: Comparison of collagenase obtained from fibroblast culture medium with that obtained from the medium of human skin cultured in the presence or absence of 20% FCS by sodium dodecyl sulfate slab gel electrophoresis. Slot 1, 30 μg of a pure collagenase preparation obtained from fibroblast culture medium which contained all four components, upper doublet (U), lower doublet (L). Slot 2, 30 μg of pure collagenase obtained from the medium of human skin cultured in the presence of 20% FCS. Slot 3, 30 μg of a purified collagenase preparation obtained from the medium of human skin cultured in the absence of FCS.

performed with a basic polyacrylamide system (Davis, 1964), no bands were detected in the separating gel using several concentrations of acrylamide. Similarly, no protein bands were detected when electrofocusing was performed using broad-range Ampholine (pH 3.5–10.0) in polyacrylamide gels. These results confirmed the highly cationic nature of collagenase, as revealed by its ion-exchange behavior. Although the enzyme would migrate in an acidic polyacrylamide electrophoretic system (Reisfield et al., 1962) containing 8 M urea, the resolution was poor.

The continuous buffer sodium dodecyl sulfate–polyacrylamide system of Fairbanks et al., (1971) was found to be the most convenient analytical procedure. Discontinuous buffer sodium dodecyl sulfate–polyacrylamide systems tended to give rise to artifacts in the form of apparent multimeric aggregates. All preparations purified through the gel-filtration step revealed a set of two characteristic protein doublets when electrophoresed using the continuous sodium dodecyl sulfate system (Figure 5; Figure 6, slot 1). In Figure 5B, a large quantity (40 μg) of such a collagenase preparation was electrophoresed in a sodium dodecyl sulfate disc gel in an effort to detect any contaminating proteins. None was observed. The upper doublet (U) has components whose apparent molecular weights are 60 000 and 55 000, respectively. The lower doublet (L) has components whose apparent molecular weights are 50 000 and 45 000, respectively. The upper doublet predominated in all preparations, while the lower doublet was usually present in only trace amounts, although rarely it comprised as much as 30–40% of the total protein (Figure 6, slot 1). Within any doublet, the components were usually present in roughly a 1:1 ratio. This distinctive pattern of doublets was not altered by variations in sample preparation, such as omitting reduction with DTT or changes in the time or temperature of incubation of the sample with sodium dodecyl sulfate prior to electrophoresis.

The significance of each of these bands was determined by analysis of the two enzyme peaks obtained from chromatography on Ultrogel AcA-44. Tubes comprising the leading half of the first peak (fractions 62–65) were pooled as peak I. Likewise, tubes comprising the trailing half of the second peak (fractions 70–73) were pooled as peak II. The middle tubes (fractions 66–69), which comprised the overlapping portions of both peaks, were also pooled. Sodium dodecyl sulfate electrophoresis of these fractions revealed that peak I consisted of the heavier component of the upper doublet, i.e., the 60 000-dalton band (Figure 5A, slot 3). Peak II consisted of the lighter component of the upper doublet, i.e., the 55 000 dalton band (Figure 5A, slot 5). Both of these components were seen in the middle region (Figure 5A, slot 4).

Trypsin activation of these peaks to the point of maximal collagenase activity was performed, followed by analysis of the protein on sodium dodecyl sulfate–polyacrylamide gels (Figure 7). Concomitant with activation, peak I showed a conversion of the 60 000-dalton band (Figure 7, slot 1) to a 50 000-dalton band (Figure 7, slot 2). Similarly, peak II showed a shift from the 55 000-dalton band (Figure 7, slot 7) to a 45 000-dalton band (Figure 7, slot 8). When the middle region containing both bands was trypsin activated, the upper doublet (Figure 7, slot 3) disappeared while the amount of protein in the lower doublet (Figure 7, slot 4) increased. Thus, trypsin treatment resulted in the specific loss of an apparent 10 000-dalton unit from each of the components of the upper doublet, leading to the formation of the lower doublet. Efforts to isolate and characterize the cleaved peptide are now in progress.

As can be seen in Figure 6, slot 1, the proteins of the lower-doublet forms appeared to cochromatograph with those of the upper-doublet forms on gel-filtration columns. When preparations having significant amounts of the lower-doublet protein or preparations which had been fully trypsin activated prior to gel filtration were run on an Ultrogel AcA-44 column, each of the lower-doublet components eluted at the same position as its respective upper-doublet form (not shown). Thus, even

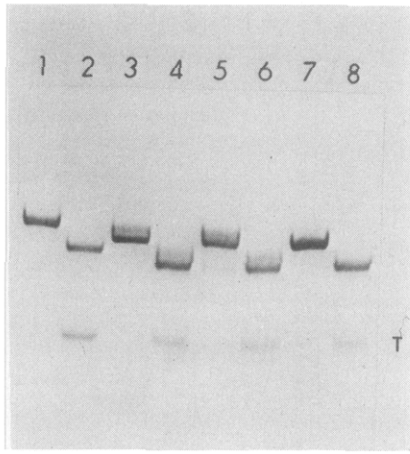

FIGURE 7: Comparison on sodium dodecyl sulfate–polyacrylamide gel of inactive and active forms of collagenase. Approximately 30 μg of enzyme protein was applied in each slot. To produce the trypsin-activated forms, an amount of trypsin sufficient to yield maximal collagenase activity was incubated with the sample for 10 min at 25 °C. 0.1 M iPr_2FP was then added to inhibit the trypsin. The trypsin (T) is seen as the lowest band in slots 2, 4, 6, 8. Autoactivated enzyme was obtained by incubating the sample for 24 h at 37 °C. Slot 1 represents the 60 000-dalton procollagenase. Slot 2 demonstrates the conversion of this 60 000-dalton procollagenase by trypsin to a 50 000-dalton active enzyme. Slot 3 depicts a mixture of 60 000- and 55 000-dalton procollagenases. In Slot 4, this mixture has been converted to trypsin to active enzyme forms of 50 000 and 45 000 daltons. Slot 5 represents fully autoactivated 60 000- and 55 000-dalton collagenases. In slot 6, these autoactivated forms have been cleaved by trypsin to produce 50 000- and 45 000-dalton enzymes. Slot 7 represents 55 000-dalton procollagenase. Slot 8 demonstrates the conversion of this 55 000-dalton procollagenase by trypsin to a 45 000-dalton active enzyme.

though a peptide of approximately 10 000 daltons had been removed, the decrease in molecular weight of the collagenase was not apparent using conventional gel-filtration chromatography with either Sephadex G-100 or Ultrogel AcA-44.

The proteolytic cleavage of the upper-doublet components occurred readily at 25 °C. The process appeared to be specific in that no intermediate forms were seen between the upper- and lower-doublet components. Furthermore, if a tenfold excess of trypsin over that just required to produce maximal levels of collagenolytic activity was incubated with the enzyme preparation, no products other than the lower-doublet bands were seen on sodium dodecyl sulfate–polyacrylamide gels. Also, as seen in Figure 4, no loss of enzyme activity occurred under these conditions.

From these results, it appeared that the upper doublet represented zymogen forms, while the lower doublet consisted of enzyme which had been activated by some tryptic-like protease during culture. However, on several occasions preparations were obtained which contained essentially none of the lower doublet but which, nevertheless, possessed a significant fraction of their maximum activity, even without trypsin activation. In addition, storage of the purest inactive collagenase preparations at −20 °C for several weeks or incubation of these preparations at 37 °C for 24 h was often sufficient for the attainment of maximal collagenase activity. When these autoactivated preparations were subjected to sodium dodecyl sulfate electrophoresis, all of the protein remained in the upper doublet (Figure 7, slot 5); there was little or no conversion to the lower doublet. It should be noted that, regardless of whether the preparation was activated with trypsin or allowed to autoactivate completely, the same level of collagenase activity was reached (Table I). Thus, a second mode of activation was indicated: one which produced an enzyme equal in activity to that produced by trypsin cleavage but which resulted in little, or possibly no, alteration in molecular weight. The resulting heavy active enzyme (60 000 and 55 000 daltons) may then be cleaved by trypsin to form the light active enzyme (50 000 and 45 000 daltons), as seen in the lower doublet (Figure 7, slot 6), without any loss in collagenase activity (Table I).

TABLE I: Activation of Procollagenase.[a]

	Time (h)	Activity (cpm)
Unactivated	0	226
Trypsin Activated	0	991
Unactivated	30	1104
Trypsin Activated	30	1038

[a] Purified enzyme was incubated at 37 °C for 0 and 30 h. Fifty-microliter aliquots containing 1.0 μg of collagenase were taken at these time points and assayed with and without trypsin activation for 1 h on a collagen gel substrate, see Methods.

Collagenase obtained from human skin organ culture medium was found to be chromatographically and electrophoretically identical to the fibroblast collagenase. When highly purified collagenase from serum-free organ culture medium was analyzed by sodium dodecyl sulfate electrophoresis (Figure 6, slot 3), two bands were seen which corresponded to the lower doublet bands of fibroblast collagenase (Figure 6, slot 1). However, only a trace amount of protein was seen which corresponded to the upper doublet of the fibroblast enzyme. This result is consistent with previous findings that no latent collagenase activity is present in serum-free organ culture medium (Bauer et al., 1972a). In contrast, however, collagenase obtained from serum-containing organ culture medium was primarily in the proenzyme state, as assessed by sodium dodecyl sulfate electrophoresis (Figure 6, slot 2) and by enzymatic assays with and without activation by trypsin.

Table II contains representative data obtained with the purification procedures reported above. The ability of these methods to isolate collagenase is best seen by the recoveries and fold purification data obtained using serum-containing fibroblast culture medium (11.7 mg of enzyme from 25 g of protein) or medium from organ culture (65% recovery and 135-fold purification). The recovery of activity from serum-free fibroblast medium following the ion-exchange step seemed low compared with that using organ culture medium, approximately 30% for the serum-free fibroblast medium vs. 65% using organ culture medium. Interestingly, the fold purification using serum-free fibroblast medium (10×) was also low compared with the ratio of pure enzyme protein recovered to total starting protein (2.6%). However, initial measurements utilizing a monospecific anticollagenase antiserum (Bauer et al., 1972b) indicated that practically 100% of the enzyme protein was recovered in the collagenase peak following ion-exchange chromatography (data not shown). Preliminary observations indicate that this disparity may be the result of an apparent enhancement of collagenase activity when assayed on collagen fibrils due to the presence of a noncollagenolytic neutral protease (in preparation) in the crude serum-free fibroblast culture medium.

Discussion

This paper reports the isolation, in pure form, of a human procollagenase. Since the initial report by Harper et al. (1971) of an amphibian procollagenase, a number of highly suggestive

TABLE II: Representative Recovery Data from Three Collagenase Sources.

Source	Stage	Protein (mg)	Sp Act.[a]	Yield (%)	Purification (fold)
Serum-Free Fibroblast	Crude	76.7	69.7	100.0	
	Phosphocellulose	2.3	824.5	34.7	11.8
	Ultrogel AcA-44	2.0	735.8	27.7	10.6
Serum-Containing Fibroblast	Crude[b]	25 000			
	$(NH_4)_2SO_4$[b] precipitate	8 000			
	CM-cellulose	18.4	703.5		
	Sephadex G-100	11.7	958.2	86.9[c]	
Serum-Free Organ Culture	Crude	1 659[d]	2.5	100.0	
	CM-cellulose	7.8	342.9	64.5	135.0

[a] Specific activity refers to μg of collagen solubilized per minute at 37 °C per mg of protein. [b] Reliable collagenase assays not obtained due to the large amounts of serum proteins present. [c] Percent recovery compared to preceding step. [d] Does not include added BSA.

but indirect reports have appeared describing latent forms of collagenase in a variety of mammalian organ and cell culture systems (Vaes, 1972; Abe and Nagai, 1972; Kruze and Wojtecka, 1972; Nagai, 1973; Robertson et al., 1973; Oronsky et al., 1973; Hook et al., 1973; Bauer et al., 1975; Harris et al., 1975; Birkedal-Hansen et al., 1976a,b 1975). In almost every such system, it has proven difficult to unequivocally distinguish between a true zymogen and an enzyme-inhibitor complex. Birkedal-Hansen et al. (1976a), in the most complete study to date, have used serum α-2-macroglobulin to bind and remove active collagenase from bovine gingival fibroblast culture medium. The resultant solution contained an inactive species which was then converted to active enzyme with small amounts of trypsin. These workers suggested, with some reservations, that their finding argues for the existence of a true zymogen, requiring the scission of a peptide bond for conversion to active enzyme. On the other hand, Kawamoto and Nagai (1976) have reported the existence, in embryonic chick skin organ culture medium, of a latent collagenase which can be activated by incubating with a chaotropic ion such as SCN^- at 4 °C, or by freezing and thawing. These workers suggest the possibility that this latency is due to the presence of inactive enzyme-inhibitor complexes. Similar activations have been shown (Nagai, 1973; Abe and Nagai, 1972) to occur when complexes between α-2-macroglobulin and collagenase are treated with SCN^-.

The results reported here present data from a single system which might explain both kinds of observations. The human skin fibroblasts appear to synthesize collagenase almost exclusively as a zymogen. The zymogen can then be converted to the active enzyme by two different pathways, which are schematically depicted in Figure 8. First, a classical proteolytic cleavage at a specific locus yields an active enzyme with an accompanying loss of 10 000 daltons. No intermediates appear to participate in this conversion, as indicated in Figure 7. However, the conversion is very rapid, even at 25 °C, and transient intermediates could exist without being visualized by our present techniques. Firm conclusions on this point must await isolation of the cleaved peptide as well as quantitative measurement of the stoichiometry involved.

The second mechanism of conversion of zymogen to active collagenase appears to be much less conventional and may be the result of a conformational change in the zymogen molecule, resulting in exposure to the active site without an accompanying peptide bond cleavage. This kind of conversion, which is observed during freeze-thawing, and other time-dependent increases in activity could be representative of that observed by Kawamoto and Nagai (1976) in embryonic chick skin.

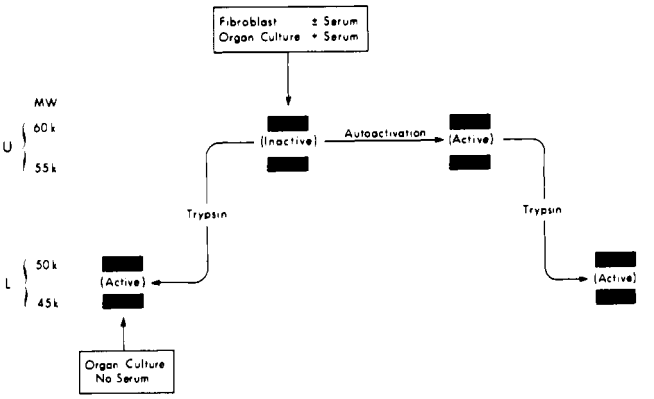

FIGURE 8: Schematic representation of the production and activation pathways of human skin collagenase from both fibroblast and organ cultures. Sodium dodecyl sulfate-polyacrylamide electrophoresis of pure collagenase revealed the presence of an upper (U) and lower (L) doublet. Fibroblasts cultured either in the presence or absence of serum, and human skin cultured in the presence of serum, yield the inactive procollagenases (U) of 60 000 and 55 000 daltons. These procollagenases may be trypsin activated, resulting in the formation of active enzymes (L) having molecular weights of 50 000 and 45 000 daltons. Alternatively, the procollagenases may be autoactivated to form active enzymes without an apparent loss in molecular weight. These heavier molecular weight autoactivated enzymes (U) may then be cleaved by trypsin to form the lower molecular weight (L) active enzymes. Human skin organ cultures maintained in the absence of serum yield only the active lower doublet (L) collagenases.

Alternatively, very small changes in molecular weight during activation could be responsible for the autoactivation process. Using the sodium dodecyl sulfate-polyacrylamide system, we feel that a change in molecular weight of approximately 2000 (about 20 amino acids) would have been detected, had it occurred. No evidence of such a change was seen. The physiologic significance of such an autoactivation pathway is unknown at this time.

It is noteworthy that precursor forms of collagenase, which appear to be identical to those produced by fibroblasts, are seen in human skin explant cultures only when serum is present (see Figure 8). Thus, some serum-inhibitable proteolytic system must exist extracellularly in organ culture which is capable of converting proenzyme to active collagenase. Since many proteases have been shown to activate latent collagenases (Vaes, 1972; Kruze and Wojtecka, 1972; Robertson et al., 1973; Oronsky et al., 1973; Hook et al., 1973; Birkedal-Hansen et al., 1975; Birkedal-Hansen et al., 1976b), the specificity and physiologic significance of this particular system remain to be established. It should be noted that, in bovine gingiva, the en-

zyme is found in the precursor state in serum-free cultures of both fibroblasts and explants (Birkedal-Hansen et al., 1976c). Hence, species differences may well exist and each system will require individual definition.

Thus, although significant quantities of enzyme can be obtained from both cell and organ cultures, the medium from fibroblast cultures is advantageous in that, since these cells continue to secrete enzyme under the serum-free conditions described, the resulting medium is extremely rich in collagenase (1–3%) as compared with serum-free medium from organ culture (approximately 0.05%). It is of interest, however, that collagenase may also be isolated from cell culture medium even when serum is present. Although this serum contains functional α-2-macroglobulin, a potent collagenase inhibitor (Eisen et al., 1970, 1971), the zymogen does not appear to complex with this protein, in agreement with the recent observations of Birkedal-Hansen et al. (1976a). Similarly, pure procollagenase may also be obtained from serum-containing organ culture medium. Serum-free organ culture medium, however, appears to be less amenable to purification by the methods described here. Nevertheless, a very highly purified active collagenase is obtainable from this source.

Irrespective of the source, the gel-filtration characteristics of collagenase are clearly anomalous. Crude explant medium has been reported to give unusually broad activity profiles when chromatographed on Sephadex G-150, a finding which has been attributed to protein–protein interactions (Fiedler-Nagy et al., 1976). In contrast, when pure preparations of collagenase are gel filtered, well-defined peaks are observed whose apparent molecular weights seem to depend upon the column material used for chromatography (Figure 3). Consequently, it is impossible to assign molecular weights based on gel-filtration chromatography. On the other hand, chromatography on Ultrogel AcA-44 does provide a simple method of separating the various components of this enzyme preparation. Although the protein bands as seen on sodium dodecyl sulfate–polyacrylamide gels form a distinct and reproducible pattern, they generate additional apparent molecular weights for the inactive and active species. Since, for most proteins, sodium dodecyl sulfate electrophoretic techniques are capable of yielding accurate molecular weights, we feel that the electrophoretically derived molecular weights given for the various species of collagenase are the most reliable. The reason for the surprising variation in the apparent molecular weight, depending upon which gel-filtration material is employed, is not known. The enzyme behaves as a very basic protein and it is possible that weak, variable ionic interactions could be occurring between the enzyme and the two matrices. It is possible that these charge properties as well as the tendency of collagenase to interact with other proteins in crude solutions may explain the disparate molecular weights which have been reported for this enzyme in the past (Woolley et al., 1973; Bauer et al., 1970).

In addition to the anomalous gel-filtration behavior, widely varying electrophoretic properties of the collagenase have been reported (Woolley et al., 1973; Bauer et al., 1971; Bauer et al., 1970). Although collagenase from organ culture medium has been eluted from basic polyacrylamide gels (Woolley et al., 1973, 1975), we were able to detect such activity only if crude explant medium was applied (unpublished observations). When, however, fully purified fibroblast enzyme was used, no activity could be eluted from the separating gel and no protein bands were visualized. Since both the electrofocusing results and the ion-exchange behavior of collagenase are suggestive of a highly cationic protein, it is reasonable to assume that some modification of charge, possible by way of the protein–protein interactions as suggested by Fiedler-Nagy et al. (1976), may be required for the protein to enter a basic gel.

Sodium dodecyl sulfate gel electrophoresis provided the analytical tool necessary to resolve the components of this enzyme system (Fairbanks et al., 1971). Electrophoretic analysis of a pure preparation before and after trypsin treatment established that cleavage of both components of the upper doublet to form the components of the lower doublet was associated with activation. At this point, the significance of the two components in each doublet is unknown. Elucidation of differences must await the availability of large quantities of each component in pure form. Isolation of each of the upper-doublet proteins then allowed us to establish that each of these bands represented a zymogen form which was converted, with an apparent 10 000-dalton loss, to its respective lower-doublet form. In addition, preparations having little or none of the lower-doublet protein but which nevertheless became fully active without the use of trypsin established the existence of a heavy active form of collagenase. By specific-activity measurements, all of these active forms appear to have the same catalytic efficiency. Lastly, sodium dodecyl sulfate electrophoresis of inactive collagenase isolated from serum-containing organ culture medium indicated that the same proenzyme is secreted in both cell and organ culture systems.

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Hydroxylation of (Pro-Pro-Gly)$_5$ and (Pro-Pro-Gly)$_{10}$ by Prolyl Hydroxylase. Evidence for an Asymmetric Active Site in the Enzyme†

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ABSTRACT: Previous studies with ^{14}C-labeled synthetic peptides demonstrated that prolyl hydroxylase, which synthesizes the hydroxyproline in collagen, preferentially hydroxylates the fourth triplet from the NH-terminal end of the peptide (Pro-Pro-Gly)$_5$. In the experiments reported here, the prolyl hydroxylase reaction was investigated further by preparing chemically modified derivatives of (Pro-Pro-Gly)$_5$ and by synthesizing ^{14}C-labeled preparations of (Pro-Pro-Gly)$_{10}$. Essentially, the same k_{cat} value was found for the hydroxylation of (Pro-Pro-Gly)$_5$, *N*-acetyl-(Pro-Pro-Gly)$_5$, (Pro-Pro-Gly)$_5$ methyl ester, (Pro-Pro-Gly)$_{10}$, and for larger polypeptide substrates of the enzyme. It appeared therefore that preferential hydroxylation of specific triplets in peptides of the structure (Pro-Pro-Gly)$_n$ cannot be explained by differences in the kinetic constants for individual triplets. Hydroxylation of ^{14}C-labeled preparations of (Pro-Pro-Gly)$_{10}$ demonstrated that the ninth triplet was preferentially hydroxylated over any other triplet. The results were best explained by the hypothesis that prolyl hydroxylase has an asymmetric active site in which binding subsites are located adjacent to but not symmetrical with the catalytic subsite.

Prolyl hydroxylase synthesizes the hydroxyproline in collagen by hydroxylating prolyl residues in nascent or completed chains of the precursor known as procollagen.[1] Hydroxylation of prolyl residues in procollagen is essential for the protein to fold into a triple-helical conformation at 37 °C (Berg and Prockop, 1973a; Rosenbloom et al., 1973) and folding into the triple-helical conformation is apparently necessary for procollagen to be secreted from fibroblasts at a normal rate (for review, see Prockop et al., 1976).

Collagen consists of three polypeptide chains folded into a triple helix and each chain has 330 repeating -Gly-X-Y- tripeptide sequences in which 100 of the Y positions are hydroxyproline (for review, see Traub and Piez, 1971). Therefore, one of the interesting features of the reaction catalyzed by prolyl hydroxylase is that the enzyme must hydroxylate about 100 prolyl residues in each of the three polypeptide chains of procollagen. Studies with natural substrates and with synthetic substrates have shown that the enzyme hydroxylates prolyl residues in the Y position of -X-Y-Gly- sequences in which the X position is proline, alanine, arginine, leucine, glutamate, or, apparently, a variety of other amino acids (for reviews, see Cardinale and Udenfriend, 1974; Prockop et al., 1976; Fietzek and Kühn, 1976). It appears therefore that in hydroxylating the polypeptides of procollagen prolyl hydroxylase interacts with multiple equivalent sites and in this sense it is comparable to other enzymes which operate on polymeric substrates, such as nucleic acid polymerases (Sherman and Gefter, 1976; McClure and Jovin, 1975) and methylases (for review, see Kerr and Borek, 1973).

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[1] Procollagen is the precursor form of collagen which has been isolated from a variety of embryonic tissues; it differs from collagen in that it has peptide extensions on the NH_2- and COOH-terminal ends of each of the three polypeptide chains. Type I procollagen from embryonic tendon cells (Olsen et al., 1976) and several other systems (Tanzer et al., 1974; Fessler et al., 1975; Byers et al., 1975) has been shown to consist of three such pro-α chains linked by interchain disulfide bonds among the COOH-terminal extensions.