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Human Skin Fibroblast Collagenase: Chemical Properties of Precursor and Active Forms†

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ABSTRACT: Human skin fibroblast procollagenase and the trypsin-activated enzyme species were isolated in pure form and subjected to chemical analysis. Ultracentrifugation confirmed the molecular weight of the zymogens to be 55 000–60 000 and of the active enzyme forms to be 45 000–50 000. Molecular weights determined by sodium dodecyl sulfate gel electrophoresis were in close agreement with these values. Molecular weights estimated from gel filtration on Sephadex G-100 or Ultragel AcA-44 are, therefore, apparently spurious for reasons not presently known. Amino acid analysis of each of the two zymogens revealed essentially no significant compositional differences between the two molecules; only the values for histidine appeared to vary significantly. Similarly, the two tryptically activated molecules appeared to be nearly identical in amino acid composition. Clear differences existed, however, between the composition of zymogen and activated molecules. Trypsin activation of mixed proenzyme species resulted in the loss of a peptide of approximately 10 000 daltons in mass and markedly altered the binding characteristics of these molecules to fibrillar collagen substrates. The procollagenases failed to bind to collagen, whereas the trypsin-activated enzymes bound tightly to the substrate. Cyanogen bromide cleavage of isolated procollagenase indicated that minor differences, possibly involving only one peptide, exist between the two molecular forms. Differences between proenzyme and activated enzyme species were greater, although still limited to a small number of peptides. The similar nature of all the forms of the human skin fibroblast collagenase is further emphasized by the fact that antibody to pure proenzyme reacts with identity to pure activated enzyme species as well.

Recent studies from this laboratory have demonstrated that human skin fibroblasts produce collagenase as a proenzyme and methods have been developed which have allowed the purification of the procollagenase to homogeneity (Stricklin et al., 1977). The resultant pure proteins were shown to comprise a set of two zymogens, each of which could be converted proteolytically to a corresponding active form. Furthermore, an autoactivation process occurred in which, by incubation at 37 °C or by freeze–thawing, each zymogen yielded an active enzyme without a detectable change in molecular weight. Serum-free explant cultures of human skin were shown to produce only enzyme species identical to the trypsin-activated forms, whereas if the explants were grown in the presence of serum only the zymogen forms of collagenase were present in the culture medium.

The proteins of this collagenolytic system behaved anomalously on gel filtration and, depending upon the matrix, a variety of apparent molecular weights from 25 000 to 42 000 could be obtained. These enzymes also displayed unusual electrophoretic properties. Neither the collagenase zymogens nor the active enzyme forms migrated in the standard basic polyacrylamide electrophoresis system of Davis (1974). Although the pure collagenases would migrate in an acidic polyacrylamide electrophoretic system (Reisfield et al., 1962), sharp bands were obtained only by the addition of 8 M urea. Electrophoresis of the zymogen and active enzymes could be obtained on sodium dodecyl sulfate–polyacrylamide gels employing a continuous buffer system (Fairbanks et al., 1971) but gave rise to still a different set of molecular weights. Two forms of the procollagenase were detected whose apparent molecular weights were 60 000 and 55 000. Each form could be converted to active enzyme by trypsin, producing species of 50 000 and 45 000, respectively.

An interesting characteristic of all of the fibroblast collagenase species has been their ability to bind to both anion- and cation-exchange resins under similar conditions. The purification employed (Stricklin et al., 1977) takes advantage of the fact that these proteins bind well to the cation-exchange resins, phosphocellulose, and CM[1]-cellulose, at neutral pH. Woolley and co-workers (1973) have shown that human skin collagenase also binds to QAE-Sephadex at similar pH and salt concentrations.

In order to ascertain the reasons for the differences between the two zymogen forms as well as the unusual behavior of the various enzyme species identified, it is essential to define some of the physical and chemical characteristics of the fibroblast collagenase system. This report presents the results of initial

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[1] Abbreviations used are: CM, carboxymethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl.

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studies on the zymogens and trypsin-activated forms of the enzyme isolated from cultured fibroblasts.

Experimental Procedure

Materials

Acrylamide and bisacrylamide were purchased from Eastman Chemical Co. Ampholyte solutions for isoelectric focusing were obtained from LKB Produkter. Urea and guanidine hydrochloride were purchased from Schwarz-Mann Co. Blue Dextran was purchased from Pharmacia Co. All other reagents were of analytical grade.

Methods

Preparation of Human Skin Fibroblast Collagenase. Pure preparations of a mixture of the two zymogen species of human fibroblast collagenase were prepared according to methods previously described (Stricklin et al., 1977), utilizing either serum-free or serum-containing culture medium from normal human fibroblasts (CRL 1187, American Type Culture Collection) as a starting material. The proenzyme mixture from CM-cellulose was stored in plastic at −80 °C until used.

Preparation of Blue Dextran–Sepharose and Trypsin–Sepharose. Sepharose 4B was activated with CNBr as described by March et al., (1974). A solution of Blue Dextran, 0.8 g in 50 mL of 0.1 M $NaHCO_3$, pH 9.5, was added to an equal volume of packed, activated Sepharose and stirred gently at 4 °C for 18 h. Coupling was allowed to proceed for 18 h at 4 °C with stirring. The slurry was then washed with 2 L of 0.1 M $NaHCO_3$, pH 9.5, containing 1 M NaCl and then equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 0.01 M $CaCl_2$. Trypsin–Sepharose was obtained by adding 50 mg of trypsin in 50 mL of 0.1 M $NaHCO_3$, pH 9.5, to 25 mL of packed, activated gel and coupled as described above. The resultant material was washed with 2 L of 0.2 M $NaHCO_3$, 1 L of 0.05 M Tris-HCl, pH 7.5, containing 1 M NaCl and 0.01 M $CaCl_2$ and finally 1 L of 0.05 M Tris-HCl, pH 7.5.

Blue Dextran–Sepharose chromatography was performed in columns, 1.6 × 30 cm, with a flow rate of 35 mL/h. Samples were applied in 0.05 M Tris, pH 7.5, 0.01 M $CaCl_2$. Proteins were eluted with a 600-mL linear gradient of 0.05–0.25 M NaCl in the same buffer.

Chromatographic Techniques. Chromatography on phospho- and CM-cellulose as well as gel filtration on Sephadex G-100 and on Ultrogel AcA-44 was performed as described by Stricklin et al. (1977).

Preparation of CNBr Peptides. CNBr peptides were produced by the method of Nomoto et al. (1969). Protein samples, approximately 1 mg each, were dialyzed against 0.1 N CH_3COOH and lyophilized. The dried protein was redissolved in 250 μL of distilled H_2O and 750 μL of trifluoroacetic acid. One-hundred microliters of a 15 mg/mL solution of CNBr was then added, and the tubes were equilibrated with dry N_2, sealed, and incubated at 25 °C for 18 h. The solution was then evaporated to dryness under a stream of N_2 and redissolved in the appropriate sample buffer for electrophoresis.

Electrophoresis. Two electrophoretic systems were employed: the continuous buffer sodium dodecyl sulfate system of Fairbanks et al. (1971) and the discontinuous sodium dodecyl sulfate system of King and Laemmli (1971). In the continuous system, 5.6% acrylamide gels were employed, cast in slabs.

Isoelectric Focusing. Isoelectric focusing was performed using Ampholine (LKB, Inc.) with a pH range of 3.5–10. Two stabilizing media were employed: either 7.5% acrylamide in 4-mm glass tubes or a sucrose gradient (0–40%) in a commercial apparatus (LKB, Inc.). When acrylamide was used, protein bands were localized by staining with Coomassie brilliant blue after fixation of the proteins with a solution of 2.5% acetic acid and 12.5% 2-propanol. The shape of the pH gradient was determined by slicing the acrylamide gels into pieces 3 mm in length, eluting each slice with 2 mL of distilled water, and measuring the resultant pH. When sucrose was employed, the column was drained according to the manufacturer's instructions and the eluent monitored at 280 nm. The protein content of each fraction was determined spectrophotometrically (Groves et al., 1968).

Ultracentrifugation Analysis. Ultracentrifugation studies were performed in a Spinco Model E analytical ultracentrifuge. Sedimentation equilibrium studies were carried out at 20 °C, using an ultraviolet scanning system at 280 nm, and a speed of 24 000 rpm by the method of Yphantis (1964). Equilibrium was essentially attained when no further change in pattern was observed. The data were traced into a digital computer and the molecular weight was determined by the least-squares fit of the slope of log OD vs. distance squared (x^2) from the center of the rotor. Protein samples were dissolved at a concentration of 0.5 mg/mL in a solution of 6 M guanidine hydrochloride buffered with 0.05 M Tris-HCl, pH 7.5, and equilibrated by dialysis against several changes of the same solution.

Amino Acid Analysis. Analyses were performed by the method of Spackman al. (1958) with a Beckman Model C automated acid analyzer employing a single column. Samples were hydrolyzed in vacuo in 6 N HCl at 110 °C for 24, 48, or 72 h. Tryptophan was separately determined by the method of Edelhoch (1967) and half-cystine determined as cysteic acid as described by Moore (1963).

Carbohydrate Analysis. Total hexose was estimated by the phenol sulfuric acid method of Hodge et al. (1962).

Assay for Collagenase. Collagenase activity was assayed on native collagen fibrils, reconstituted at 37 °C from solutions of [^{14}C]glycine-labeled guinea pig skin collagen (Nagai et al., 1964). Twenty-five microliters of substrate gel contained 100 μg of collagen and approximately 3000 cpm. Enzyme solutions containing from 2 to 25 μg of protein in 100 μL were added to the substrate and incubations carried out at 37 °C in a shaking water bath. The reactions were terminated by centrifuging the residual collagen fibrils at 4 °C. The entire supernatant was removed and radioactivity determined in a liquid scintillation spectrometer.

Preparation of Antisera. Adult male white rabbits weighing 2–3 kg were immunized initially with approximately 0.5 mg of electrophoretically homogeneous human fibroblast procollagenase emulsified in complete Freund's adjuvant. Rabbits were given booster injections of 0.25 mg of collagenase after 4 weeks and bled 1 week later. Similar booster injections were given at 3-week intervals.

Antisera which showed precipitating antibodies were pooled and taken to 33% saturation with ammonium sulfate, at 0 °C, to obtain the γ-globulin fraction. The ammonium sulfate precipitate was dissolved in 0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl, in a volume equal to the initial serum volume. The solution was dialyzed against the same buffer to remove residual ammonium sulfate.

Immunodiffusion and Enzyme Inhibition. Gel diffusion was performed in 0.75% Ionagar as described by Ouchterlony (1958). Experiments to assess inhibition of collagenase activity by antibody were performed by preincubating anti-collagenase γ-globulin in dilutions ranging from $^1/_3$ to $^1/_{120}$ with a constant amount of collagenase and assaying residual enzyme activity. Preincubations were performed in two ways. In one kind of

experiment preincubation was carried out for 2 h at 37 °C and the mixture assayed directly for collagenase activity. In a second experimental approach, enzyme and antibody were preincubated for 18 h at 4 °C after which residual enzyme activity was measured. Controls were always included, which contained an equal dilution of nonimmune rabbit γ-globulin.

Results

Preparation of Protein Components. In order to characterize the collagenase derived from human skin fibroblasts, adequate quantities of the various enzyme species had to be obtained. Processing batches of 10–12 L of serum-containing fibroblast medium on CM-cellulose as previously described yielded sufficient quantities of the two procollagenase species. The volume of the enzyme pool from ion-exchange chromatography usually required three gel-filtration runs on a 1.2 × 200 cm column of Ultrogel AcA-44 (Stricklin et al., 1977). Each run yielded approximately 1 mg of each species, as well as several milligrams of mixed forms in the overlap region. Attempts to concentrate and rechromatograph the large volumes of this mixed region invariably resulted in substantial losses of protein and enzyme activity.

Proteolytically activated collagenase was obtained by incubating partially purified procollagenase with trypsin–Sepharose followed by gel filtration using Sephadex G-100. Proenzyme solution containing both species and purified through the CM-cellulose step was used as starting material. Usually, 7.5 mg of protein in 15 mL was exposed to 2 mL of trypsin–Sepharose for 15 min at 25 °C. Attempts to subsequently isolate individual components using Ultrogel AcA-44 were unsuccessful due to overlap of the enzymes with leached trypsin and other degradation products. Separation could, however, be achieved by chromatography on a column of Blue Dextran–Sepharose. Collagenase bound to this matrix when the enzyme was applied in a buffer of 50 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$. Separation of the light active collagenase forms is illustrated in Figure 1. Following the application of a gradient of NaCl, the enzymes eluted in a pattern similar to that obtained with chromatography on Ultrogel AcA-44; i.e., the heavier active collagenase was present in the first peak and the lighter enzyme in the second peak. The gradient required to separate the species adequately resulted in relatively large elution volumes. Since it was difficult to concentrate the pure collagenases and maintain activity, the eluates were dialyzed, lyophilized, and then redissolved in 8 M urea buffered with 0.005 M Tris-HCl, pH 7.5. In this way, a small amount of each pure form could be obtained for amino acid analysis or ultracentrifugation.

Sedimentation Equilibrium Analysis of Components of the Collagenase System. The techniques employed in our initial study of the human skin fibroblast collagenase system resulted in conflicting values for the molecular weights of the various components of this complex system. Gel filtration on calibrated columns of Sephadex G-100 yielded a single molecular weight of 42 000 for all four components. Gel filtration on calibrated columns of Ultrogel AcA-44 yielded two apparent molecular weights, 30 000 and 25 000, respectively. These data were particularly confusing, since one zymogen and its respective lighter activation product appeared to chromatograph at the 30 000 position, whereas the second zymogen cochromatographed with its lighter active component at an apparent weight of 25 000. However, when appropriate mixtures of the components were electrophoresed in calibrated sodium dodecyl sulfate–polyacrylamide gels, the procollagenase forms displayed molecular weights of 60 000 and 55 000, respectively,

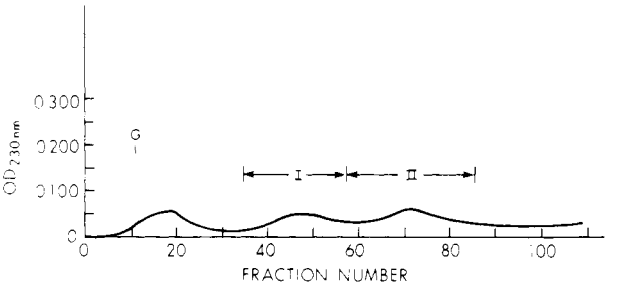

FIGURE 1: Chromatography of active collagenase species on Blue Dextran–Sepharose. Blue Dextran–Sepharose was coupled to Sepharose 4B as described under Methods. Approximately 3 mg of trypsin-activated collagenase was applied to a 1.6 × 30 cm column of the matrix. At G, a 600-mL gradient of 0.05–0.25 M NaCl in 0.05 M Tris-HCl, pH 7.5, containing 0.01 M $CaCl_2$, was applied at a flow rate of 36 mL/h. Fractions (6 mL) were collected and pooled as indicated. Pool I contained 50 000-dalton active collagenase and pool II contained 45 000-dalton enzyme.

TABLE I: Comparison of the Amino Acid Composition of Procollagenase and Active Collagenase Forms.[a]

Amino acid	Proenzyme I (60 000 daltons)	Proenzyme II (55 000 daltons)	Enzyme I (50 000 daltons)	Enzyme II (45 000 daltons)
Lys	65.9	65.4	53.4	52.6
His	34.9	40.1	41.9	37.0
Arg	54.7	56.2	46.2	51.3
Asp	119.8	119.4	130.7	128.0
Thr	52.9	49.2	62.6	64.2
Ser	55.2	55.0	64.2	62.6
Glu	110.9	110.3	105.6	103.4
Pro	63.3	62.0	63.0	64.1
Gly	86.8	89.2	90.8	88.9
Ala	74.5	71.7	70.6	69.1
½-Cys[b]				
Val	52.7	52.8	54.9	54.4
Met	15.7	16.7	14.2	13.3
Ile	34.2	36.5	44.3	42.6
Leu	68.4	66.2	59.5	61.0
Tyr	39.1	40.5	36.6	40.6
Phe	70.9	69.2	61.5	66.8
Trp[b]				

[a] Samples were hydrolyzed at 110 °C for 24 h; see Methods. No corrections for losses or incomplete hydrolysis were attempted. Values are residues/1000. [b] Not determined; half-cystine.

while the active collagenase migrated at positions corresponding to 50 000 and 45 000, respectively.

In order to resolve these conflicting values, the molecular weight was determined by equilibrium sedimentation. A plot of log C vs. x^2 was linear for the individual proenzyme components providing further evidence for the homogeneity of the preparations. A value of 0.725 for the partial specific volume was obtained from the amino acid analysis. Computer analysis of the plots yielded molecular weight values for the two procollagenase species of 55 300 and 53 100, respectively. Additionally, ultracentrifugation analysis of a mixture of the two proenzyme molecules yielded an average molecular weight value of 54 200. Lastly, the apparent molecular weight for a mixture of the two trypsin-activated collagenase species was 42 500. Insufficient material was available for individual determinations using isolated trypsin-activated components.

Amino Acid Composition. Table I presents compositional data for the proenzyme and active forms of collagenase. Analyses were performed on each separated form, as well as a mixture of the two for reference. The isolated zymogens are relatively rich in acidic amino acids (Asp + Glu = 231/1000)

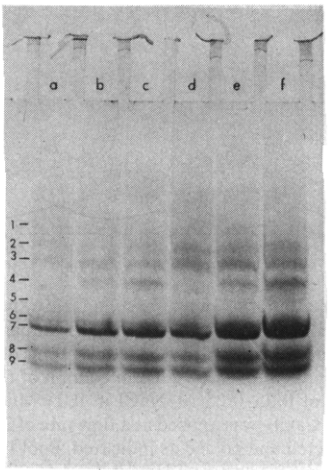

FIGURE 2: Cyanogen bromide digests of heavy and light proenzyme species. Approximately 1 mg each of 60 000-dalton proenzyme species (slots a and d), 50 000-dalton proenzyme form (slots c and f), and mixed species (slots b and e) were cleaved with cyanogen bromide. Approximately 60 μg of each digest was electrophoresed in the system of Fairbanks et al. (1971).

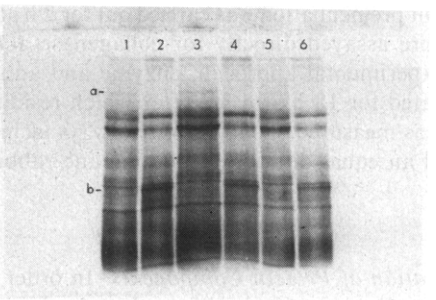

FIGURE 3: Cyanogen bromide digests of proenzyme and active enzyme species. Approximately 1 mg each of mixed zymogen forms (slots 1, 3, and 5) and mixed trypsin-activated species (slots 2, 4, and 6) was cleaved with cyanogen bromide. Approximately 75-μg samples were electrophoresed in the system of King and Laemmli (1971).

TABLE II: Amino Acid Composition of Human Skin Fibroblast Collagenase.

Amino acid	Procollagenase		Active collagenase		Putative cleavage peptide,
	Res/ 1000	Res/ molecule[a]	Res/ 1000	Res/ molecule[b]	res/ molecule[c]
Lys	63.5	32	48.6	20	12
His	36.2	18	34.2	14	4
Arg	53.5	27	50.8	21	6
Asx	117.2	59	122.2	51	8
Glx	105.0	53	98.7	42	11
Thr[b]	55.1	28	57.4	24	4
Ser[b]	54.0	27	60.0	25	2
Pro	60.5	31	63.8	27	4
Gly	77.5	39	84.9	36	3
Ala	65.9	33	66.0	28	5
½-Cys[d]	13.4	7	11.6	5	2
Val	56.0	28	51.1	22	6
Met	16.3	8	14.8	6	2
Ile	38.9	20	43.8	18	2
Leu	60.8	31	61.9	26	5
Tyr	39.4	20	43.0	18	2
Phe	70.7	36	68.8	29	7
Trp	16.3	8	18.5	8	0

[a] Residues per molecule calculated assuming an average molecular weight of 57 500 and expressed as integral numbers. [b] Residues per molecule calculated assuming an average molecular weight of 47 500 and expressed as integral numbers. [c] Residues per molecules obtained by subtraction. [d] Half-cystine.

as compared to basic residues (Lys + Arg + His = 160). Polar residues comprise approximately 57% of the total, and both molecules contain at least 10% aromatic amino acids. Very few differences could be detected between the two isolated molecules. Only histidine appeared to vary by a significant amount. The content of all other amino acids appeared identical, within the limits of accuracy of the analyzer. Similar data for the active enzyme species show a pattern qualitatively similar to the zymogen forms. Very few differences can be detected between the individual species. Again, histidine appears to vary between the two proteins.

Differences between the compositions of the zymogen and the activated enzyme, however, were more marked. Table II is a comparison of the amino acid analyses of mixed zymogen species, in roughly a 1:1 ratio, and a similar mixture of trypsin-activated enzyme species. The data indicate that the active enzyme is somewhat less basic than the zymogen due to a significant reduction in lysine content. Changes in the content of other amino acids are much smaller. Using the molecular weights indicated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and confirmed by sedimentation equilibrium (60 000 and 55 000 for proenzyme; 50 000 and 45 000 for activated enzyme), the number of amino acids per molecule was calculated (Table II). The values obtained indicate that tryptic activation of procollagenase is accompanied by the loss of a peptide, as yet not isolated, of approximately 85 amino acids. The predicted amino acid composition of this putative peptide is also presented in Table II. Relative to the parent molecules, it appears to be somewhat richer in basic amino acid residues and in phenylalanine and somewhat less rich in glycine and the hydroxyamino acids.

None of the species examined contained detectable amounts of hydroxyproline or hydroxylysine, amino acids characteristic of collagen itself. Additionally, examination of these proteins for total hexose, using the phenol–sulfuric acid technique (Hodge et al., 1962) indicated that all species were devoid of hexose, and amino acid analysis indicated the absence of the amino sugars, glucosamine and galactosamine.

CNBr Peptides. In an effort to illustrate differences between these apparently very similar proteins, cyanogen bromide cleavage was employed. Amino acid analysis indicated the existence of approximately eight methionine residues per molecule of protein. This suggested that a manageable number of peptides might be obtained by CNBr cleavage. The CNBr cleavage products of the two separated proenzyme species and a mixture of approximately equal amounts of the two are shown in Figure 2. Each species gives rise to nine observable peptides, almost all of which are common to the two proteins. Each, however, appears to possess a single unique peptide. Only the 60 000-dalton molecule contained peptide no. 1 (slots a and d), and only the 55 000-dalton proenzyme contained peptide no. 4 (slots c and f). The mixture (slots b and e) contains both peptides 1 and 4.

In a second type of experiment a mixture of both proenzyme forms and a similar mixture of both light enzyme forms were subjected to CNBr cleavage, and the resultant peptides were compared in Figure 3. Although electrophoresed in a different system from the peptides of Figure 2, a small number of peptides appear to be different in the two preparations. One in particular, peptide a (slots 1, 3, and 5), is present in the zymogen mixture and absent in the activated species (slots 2, 4,

TABLE III: Binding of Fibroblast Collagenase Zymogen and Active Forms to Collagen.[a]

Enzyme form	Preincub condit at 27 °C 1	2	Collagenase act. Remaining in supernatant	Bound to collagen
Proenzyme	Buffer	Buffer	1830	
Proenzyme	Collagen	Buffer	1853	0
Proenzyme	Collagen	Collagen	1798	0
Active enzyme	Buffer	Buffer	1881	
Active enzyme	Collagen	Buffer	1320	602
Active enzyme	Collagen	Collagen	601	1151

[a] Equal aliquots (25 μL) of either procollagenase or trypsin-activated enzyme were subjected to successive preincubations for 20 min at 27 °C as indicated in the table. When preincubated with substrate, the appropriate solution was added to 50 μL of reconstituted native collagen fibrils. When buffer is indicated, 50 μL of 0.05 M Tris, pH 7.5, containing 0.01 M $CaCl_2$ was substituted for the collagen. After the first preincubation, all tubes were centrifuged at 6000*g* for 10 min and the entire supernatant carefully removed and transferred to a second aliquot of either buffer or collagen. After a second preincubation, the centrifugation was repeated. The resultant supernatants were assayed for residual collagenase, either directly in the case of previously activated collagenase or after trypsin activation in the case of proenzyme-containing supernatants. Assays were carried out for 45 min at 37 °C as described under Methods. The collagen pellets remaining after centrifugation were also examined for the presence of bound enzyme. Pellets were resuspended in 0.05 M Tris, pH 7.5, 0.01 M $CaCl_2$ (150 μL) and incubated at 37 °C for 90 min. The residual collagen was again centrifuged and the entire supernatants, containing collagenase-dependent [^{14}C]glycine labeled peptides, were removed and counted in a liquid scintillation spectrometer. In the case of collagen preincubated with proenzyme, trypsin activation was performed directly on the pellet prior to preincubation.

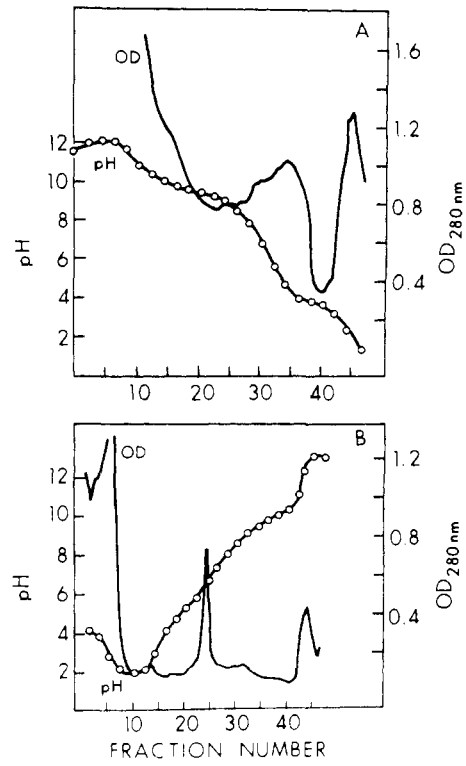

FIGURE 4: Isoelectric focusing of human skin collagenase. Mixed proenzyme (5 mg) was focused in a pH 3.5–10 gradient as described under Methods, either in the absence (A) or the presence (B) of 8 M urea in all reagents.

and 6). Conversely, peptide b is absent in the zymogen (slots 1, 3, and 5) and present in the active enzymes (slots 2, 4, and 6). Other minor variations may also exist between the mixtures.

Charge Properties. Isoelectric Point. Since human fibroblast collagenase behaves anomalously on both ion-exchange resins and in commonly used electrophoresis systems, experiments were designed to assess the p*I* of mixed procollagenase species by isoelectric focusing. Attempts to electrofocus the proenzyme in gradients stabilized by polyacrylamide were unsuccessful because the protein uniformly failed to enter the gel. When the focusing was performed in a liquid column stabilized by a sucrose density gradient, precipitated protein appeared over a wide area of the column, corresponding to pH values of 5 to 12 (Figure 4A). However, when 8 M urea was incorporated into all components of the same system, the results shown in Figure 4B were obtained. The protein migrated as a single peak at a position corresponding to a pH of approximately 6.7. Thus, in its native state the procollagenase appears to be a highly charged molecule, whereas in 8 M urea the protein possesses a more neutral net charge.

Binding of Enzyme Forms to Collagen. Experiments were performed to determine whether differences existed in the binding of the various forms of collagenase to collagen, and the results of a typical experiment are presented in Table III. When aliquots of a solution of mixed proenzyme species were trypsin activated, a significant fraction of the resultant enzyme activity could be readily shown to bind to collagen fibrils at 27 °C. Incubation with a second aliquot of substrate resulted in a further removal of collagenase from solution. The bound enzyme remained complexed with the substrate during subsequent centrifugation and resuspension of the fibrils. Upon incubation of the resuspended fibrils at 37 °C, the enzyme continued to act upon the substrate. In direct contrast, no proenzyme was removed from solution when incubated with the same substrate (Table III). Additionally, no activity could be detected on the collagen fibrils which had been incubated with proenzyme. Variations in the concentrations of the enzyme species or of the substrate did not qualitatively alter these binding properties. Identical results were obtained when the binding steps were performed at 37 °C. In this case, however, degradation of collagen by activated enzyme during the binding period resulted in very high blank values.

Immunological Relationships. Antiserum was produced in rabbits using pure, mixed procollagenase species as antigen and was reacted with various enzyme preparations in Ouchterlony double-diffusion analysis (Figure 5). It is important to note that a single precipitin line was observed (Figure 5a) when the antiserum was reacted either with crude fibroblast medium or with the homogeneous proenzyme species. These findings provide further documentation of the high degree of purity of the enzyme immunogen. Furthermore, as shown in Figure 5b–d, regardless of the enzyme source (fibroblast or organ culture) or its state of activation (proenzyme, trypsin-activated collagenase, or organ culture enzyme), only a single precipitin line was ever observed. Relationships of immunologic identity were observed between all species thus far examined. There was no indication of a difference between the two isolated, pure zymogen species (Figure 5e) nor between enzyme obtained from fibroblasts or organ cultures (Figure 5d).

The specificity of the antibody utilized in these studies was further defined by examining the ability of the antibody globulin to inhibit the activity of collagenase in solution, as indicated in Table IV. With a brief (2 h) incubation of the antibody with the purified collagenase (expt. I), approximately 90% of the activity was abolished. Longer incubation at 4 °C (expt. II) produced the expected enhancement of inhibition

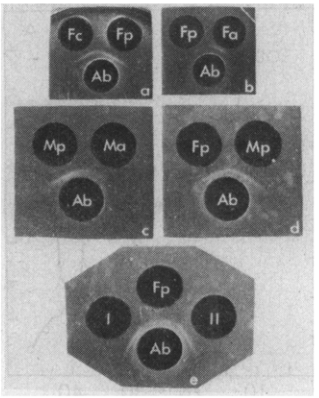

FIGURE 5: Gel diffusion analysis of various preparations of human skin collagenase with antiserum to purified human skin fibroblast collagenase. In all figures, Ab refers to anti-human fibroblast collagenase antiserum. (a) Fc, crude fibroblast culture medium; Fp, pure fibroblast procollagenase. (b) Fp, pure fibroblast procollagenase; Fa, trypsin-activated pure fibroblast collagenase. (c) Mp, highly purified procollagenase from serum containing human skin explant cultures; Ma, active collagenase from serum-free human skin explant cultures. (d) Fp, pure fibroblast procollagenase; Mp, highly purified procollagenase from serum-containing human skin explant cultures. (e) Fp, pure fibroblast procollagenase; I and II refer to the heavy and light molecular weight species of procollagenase.

TABLE IV: Inhibition of Purified Human Skin Fibroblast Collagenase by Anti-Collagenase γ-Globulin.

Preincubation conditions	Antibody globulin dilution	Cpm of [^{14}C]collagen	Inhibition (%)
Expt I			
2 h, 37 °C	Control	1906	
	1/120	1565	17.9
	1/60	983	48.4
	1/30	615	67.7
	1/15	310	83.7
	1/3	210	89.0
Expt II			
30 min, 37 °C,	Control	2019	
18 h, 4 °C	1/120	979	51.4
	1/60	851	57.9
	1/30	369	81.7
	1/15	248	87.7
	1/3	193	90.4

[a] Collagenase activity is expressed as cpm of [^{14}C]collagen solubilized above blank in 4 h at 37 °C. The control value represents collagenase preincubated under the same conditions in the presence of a one-third dilution of nonimmune rabbit γ-globulin.

at lower concentrations of antibody (1/15 to 1/120).

Discussion

The finding that human skin fibroblasts secrete collagenase as a set of two inactive zymogens has introduced additional complexity into the already complex area of collagenase biochemistry. The proteins of the collagenase system behave anomalously in a variety of analytical systems such that, even when pure, uncertainty existed regarding their true molecular weight and charge properties (Stricklin et al., 1977).

Gel filtration of zymogen mixtures on Sephadex G-100 invariably resulted in a single, nearly symmetrical peak at a molecular weight corresponding to 42 000. Surprisingly, tryptic activation of the mixtures prior to chromatography failed to alter the apparent molecular weight of the resultant species. In addition, collagenase from serum-free human skin organ cultures—always present as fully active enzyme—also displayed an apparent molecular weight of 42 000. When, however, Ultrogel AcA-44 was employed as the gel-filtration matrix, mixtures of zymogen and active enzyme resolved into two incompletely separated peaks with apparent molecular weights of 30 000 and 25 000, respectively. Again in this matrix, as was the case on Sephadex, trypsin-activated enzyme cochromatographed with the proenzyme forms.

Neither of these results was consistent with those derived from electrophoresis of the protein in sodium dodecyl sulfate–polyacrylamide slab gels, which indicated the molecular weights of the zymogens to be 60 000 and 55 000 and of the active species to be 50 000 and 45 000.

The studies presented here indicate that, by ultracentrifugation, the molecular weight of the collagenase zymogens is approximately 55 000. The data obtained from the ultracentrifuge do not allow the determination of the exact difference in molecular weight between the two zymogen species. Clearly, however, these experiments indicate generally close agreement with the values obtained by sodium dodecyl sulfate–polyacrylamide electrophoresis.

Ultracentrifugation also confirms the loss in molecular weight of approximately 10 000 daltons from the proenzyme molecules during tryptic activation to give rise to active enzyme species whose molecular weights are about 45 000. Hence, for purposes of convenience, and based on the values provided by carefully calibrated sodium dodecyl sulfate–polyacrylamide gel electrophoresis experiments, we have assigned molecular weights of 60 000 and 55 000 to the proenzyme molecules. Similarly, we have assigned weights of 50 000 and 45 000 to the tryptically activated collagenase species.

It should be noted that in fibroblasts lower molecular weight species possessing collagenase activity have not been observed. One such species, termed "human skin collagenase-fast (HSC_f)", previously reported by this laboratory (Bauer et al., 1970), was occasionally obtained from the medium of human skin organ cultures. The present studies indicate that the majority of the collagenase present in organ culture medium is identical, chemically and immunologically, with the fibroblast-derived enzyme described herein. Thus, at present, the nature of "HSC_f" is unknown.

The nature of the difference between the two proteins secreted by the fibroblasts remains to be defined more clearly. Within the limits of the analytical methods employed in this study, the two proenzyme molecules are essentially identical except for an apparent difference in molecular weight. Amino acid analyses indicate that any differences in overall composition are too small to be assessed by this technique. The presence of a level of carbohydrate in either species which could affect its behavior appears unlikely. Cyanogen bromide peptide analysis, however, does suggest that there is a difference in the composition of some part of the two molecules. Clearly, firm identification of the unique peptides and their placement within the molecules will be required to provide ultimate answers. Whatever the fundamental basis for any differences between the two proteins, however, it seems clear that the practical results of such differences are small, both with respect to the molecular characteristics described here and enzymatic behavior. The two species have essentially identical specific activities against collagen substrates, and it is unlikely that major enzymologic differences exist between the two molecules.

The immunologic behavior of the two species also serves to emphasize the apparently minor differences between them. Reactions other than of identity between the species of the

collagenase system have never been observed. It is possible that, when individual antibodies to each pure species are available, minor differences will manifest themselves. The present immunologic evidence, taken together with the available chemical and physical evidence, cannot distinguish whether the two species are products of the same gene in which differences may be introduced only after translation or whether two entirely separate gene products are formed.

The results of the substrate-binding experiments indicate that the 10 000-dalton fragments present in the proenzyme species prevent the human skin fibroblast zymogen from forming complexes with collagen. This inability of the procollagenase to bind to substrate has also been suggested by Birkedahl-Hansen et al. (1976) in a bovine gingival fibroblast system. It must be emphasized, however, that the human skin fibroblast zymogen can also undergo an autoactivation process which converts the proenzyme to active collagenase without a detectable loss of molecular weight (Stricklin et al., 1977). There was no evidence for the dissociation of a possible enzyme–inhibitor complex during this process. This form of active enzyme, then, still contains the 10 000-dalton peptide which is removed during trypsin activation. Thus, the presence, per se, of the 10 000-dalton peptide does not determine binding properties. More probably, the conformation of the peptide within the molecule is the crucial determinant of substrate binding. On the other hand, it appears that major species differences may exist, since both Gillet et al. (1977), working with mouse bone collagenase, and Woessner (1977), in a rat uterus system, have suggested that collagenase zymogens can bind tightly to tissue collagen.

Of continuing interest is the anomalous behavior of these proteins on gel-filtration matrices, ion-exchange resins, and in electrophoretic systems. The reasons for the aberrant molecular weights obtained from G-100 and AcA-44 are still unclear. Recently Fiedler-Nagy et al. (1977) have suggested that collagenase from organ culture medium displays a wide variation in molecular weights on gel-filtration matrices due to the formation of complexes with small collagenous peptides. In the case of the pure enzyme species studied here, however, such complexes cannot exist. In addition to these anomalies, there is no obvious explanation for the failure of the native enzyme proteins to electrophorese normally in either acidic or basic acrylamide gels. Only in the presence of high urea concentrations or in sodium dodecyl sulfate–polyacrylamide gels does electrophoresis occur. Furthermore, although the components of the collagenase system behave as strongly cationic proteins on phosphocellulose, they also behave as anions under essentially identical conditions, since they also bind, although less well, to DEAE-cellulose (unpublished observations) and exhibit a p*I* of approximately 6.7. It is not unreasonable to suggest that these human collagenases possess a peculiar three-dimensional structure which, in large measure, is responsible for their behavior. Information on the structure of these proteins should be of great help in understanding not only their anomalous interactions with the reagents of protein purification but also their associations with the components of the extracellular matrix.

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