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Proteolytic Activities of Human Fibroblast Collagenase: Hydrolysis of a Broad Range of Substrates at a Single Active Site†

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ABSTRACT: The action of human fibroblast collagenase (HFC) on six substrates of markedly different size, sequence, and conformation, including rat type I collagen, rat α1(I) gelatin, β-casein, and the three synthetic oligopeptides Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln, Asp-Val-Ala-Gln-Phe-Val-Leu-Thr-Pro-Gly, and Pro-Val-Gln-Pro-Ile-Gly-Pro-Gln, has been examined. The first peptide is a model for the collagenase cleavage site in the α1(I) chain of type I collagen, while the latter two peptides are models for the autolytic activation and degradation sites in pro-HFC, respectively. The goal of these studies was to assess whether HFC hydrolyzes all of these disparate substrates at the same active site. Individual kinetic parameters for the hydrolysis of all six substrates have been determined. Gel zymography experiments using collagen, gelatin, and casein as substrates show that all three activities are associated solely with HFC rather than impurities. Recombinant HFC expressed in *Escherichia coli* also exhibits caseinase activity, reinforcing the view that this activity is not due to a contaminating protease from fibroblasts. The ratios of these activities agree within experimental error for several independent HFC preparations and do not change when two additional affinity purification steps are employed. The inhibition of the hydrolysis of these substrates by both 1,10-phenanthroline and Boc-Pro-Leu-Gly-NHOH is identical within experimental error. A series of assays carried out in the presence of pairs of these substrates clearly shows that they compete for the same active site. On the basis of these kinetic experiments, it is concluded that HFC has a single active site that is capable of hydrolyzing a much wider variety of natural and synthetic substrates than previously believed.

The first vertebrate collagenase (EC 3.4.24.7) was discovered in tadpole tissue explants by Gross and Lapiere in 1962 (Gross & Lapiere, 1962). The enzyme was subsequently shown to hydrolyze all three α chains of native, triple-helical type I calf skin collagen at Gly-[Leu or Ile] bonds at a single locus located approximately three-fourths from the NH_2 terminus to give two collagen fragments referred to as TC^A, TC^B fragments (Gross & Nagai, 1965; Gross et al., 1974). At temperatures at which these fragments remain triple-helical, subsequent proteolysis was minimal. This characteristic pattern of hydrolysis is now accepted as the key distinguishing feature of vertebrate collagenases (Harris & Vater, 1982; Cawston & Murphy, 1981; Harris et al., 1984; Woolley, 1984; Birked-

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al-Hansen, 1987). The discovery of tadpole collagenase was soon followed by the identification of collagenases from a variety of mammalian and human cells (Woolley, 1984) that exhibited the same mode of attack on not only type I but also type II and III collagens (Miller et al., 1976). Because of the remarkably specific mode of attack on the interstitial collagens by these collagenases, these enzymes gained the reputation as being among the most specific proteinases known, and little attention was initially directed toward identifying other substrates.

Over the last 20 years, evidence has accumulated that vertebrate collagenases can hydrolyze substrates other than triple-helical interstitial collagens. Additional reported substrates include various gelatins (Gross et al., 1974; McCroskery et al., 1975; Woolley et al., 1975, 1978; Stricklin et al., 1977; Welgus et al., 1982, 1985a; Sakamoto et al., 1978; Hori & Nagai, 1979; Bicsak & Harper, 1984; Roswit et al., 1983), casein (Sakamoto et al., 1978; Nagai, & Hori, 1972; Gillet et al., 1977; Cawston & Tyler, 1979), five different α-macroglobulins (Sottrup-Jensen & Birkedal-Hansen, 1989; Enghild et al., 1989), ovostatin (Sottrup-Jensen & Birkedal-Hansen, 1989; Enghild et al., 1989), and α_1-protease inhibitor (Desrochers & Weiss, 1988). Human fibroblast collagenase (HFC),[1] the most intensively studied tissue collagenase, hydrolyzes the macromolecular inhibitors at a variety of bonds, many of which differ appreciably from those hydrolyzed in the collagens. HFC has also been shown to exhibit autolytic activities that result in the activation and degradation of the proenzyme. Treatment of pro-HFC with organomercurials leads to activation that is accompanied by a reduction in molecular weight by approximately 9K (Stricklin et al., 1983). This occurs by hydrolysis at either the Gln_{99}–Phe_{100}, the Phe_{100}–Val_{101} or the Val_{101}–Leu_{102} bonds (Grant et al., 1987; Goldberg et al., 1986; Whitham et al., 1986). Highly purified pro-HFC also undergoes an internal autocatalytic cleavage at the Pro_{269}–Ile_{270} bond (Whitham et al., 1986; Birkedal-Hansen et al., 1988) which marks the transition between the NH_2-terminal prototype metalloproteinase domain and the COOH-terminal hemopexin-like domain (Matrisian et al., 1986). Tissue collagenases have also been shown to hydrolyze a variety of peptides, peptolides, and esters (Nagai et al., 1976; Masui et al., 1977; Williams & Lin, 1984; Weingarten et al., 1985; Weingarten & Feder, 1986; Fields et al., 1987).

In considering the action of collagenases on the substrates enumerated above, two features are distinctive. The first is that the bonds that are hydrolyzed in the interstitial collagens are different from those that are hydrolyzed in pro-HFC, macromolecular inhibitors, and certain oligopeptides, particularly with regard to the residue in subsite P_1. A second observation is that the kinetic parameters for the hydrolysis of interstitial collagens by collagenases (k_{cat} = 1–400 h^{-1}, K_M ~ 1 μM) vary markedly from those for other substrates, such as oligopeptides (k_{cat} = 300–1200 h^{-1}, K_M ~ 1 mM) (Fields et al., 1987). These observations raise the question of whether all of these activities are accommmodated at the same active site. A related concern is the possibility that even the purest collagenase preparations may contain traces of contaminating proteinases that are responsible for noncollagenolytic activities. For example, it is difficult to completely remove stromelysin from collagenase preparations, particularly those isolated from fibroblast tissue cultures. Since casein is a good substrate for stromelysin, it could be responsible for the caseinase activity reported for HFC.

In this study, three possibilities are considered to explain the action of HFC on the substrates enumerated above. The first is that HFC contains a contaminating proteinase that is responsible for some or all of the noncollagenolytic activities. The second possibility is that HFC contains two active sites, one which hydrolyzes collagen and possibly collagen-like sequences in other substrates, while the second accounts for the other proteolytic activities. The third possibility is that HFC contains a single active site with a much broader range of specificities than previously recognized and that it is capable of hydrolyzing a variety of diverse substrates. The hydrolysis of type I collagen, α1(I) gelatin, β-casein, and three synthetic octapeptides has been investigated. The sequences of the peptides are patterned after the collagenase cleavage site in the native chick collagen α1(I) chains, and the autolytic activation and degradation sites in pro-HFC. The effects of the inhibitors 1,10-phenanthroline and Boc-Pro-Leu-Gly-NHOH have been quantitatively compared for all activities, and competition assays with pairs of the individual substrates have been carried out to distinguish between hydrolysis at the same or different active sites. The action of recombinant HFC isolated from a source containing no stromelysin on selected substrates has also been examined. The results show that HFC has a single active site with a broader specificity than presently believed. This indicates that HFC may be capable of initiating proteolytic events of physiological significance beyond collagenolysis.

Materials and Methods

Materials. Pro-HFC was purified from the harvest media of human gingival fibroblasts as described previously (Birkedal-Hansen, 1987; Birkedal-Hansen et al., 1988) using a modification of the procedure of Stricklin and associates (Stricklin et al., 1977). The yield was 0.7–1.0 mg/L of culture medium. The pro-HFC obtained was homogenous by SDS–PAGE, and Western blots carried out with antibodies toward stromelysin and 72K gelatinase showed that the preparation was free of these proteinases. The specific activity on activation with trypsin was in the range of 500–900 units/mg, where 1 unit cleaves 1 μg of reconstituted collagen fibrils using the radiofibril assay (Birkedal-Hansen, 1987; Birkedal-Hansen & Dano, 1981). Some of the purified native pro-HFC samples were chromatographed further on either a Sepharose-CH–Pro-Leu-Gly-NHOH affinity column (Moore & Spilburg, 1986) or a column consisting of an immobilized anti-HFC monoclonal antibody (Birkedal-Hansen et al., 1988).

Human recombinant pro-HFC (r-pro-HFC) was obtained by expression of human pro-HFC in *Escherichia coli* as follows (Windsor et al., 1990). The *Sal*I–*Bgl*II restriction fragment of pX7, a pSP64 plasmid vector containing the human pro-HFC cDNA, was ligated to the *Bam*HI–*Xho*I fragment of the plasmid Bluescript M13. Oligonucleotide site directed mutagenesis (Carter et al., 1985) was carried out to introduce a Shine–Dalgarno sequence and an AUG sequence in front of the Phe residue which constitutes the first amino acid residue of the pro-HFC sequence. *E. coli* (strain DE3, in which the T7 polymerase gene is under lac control) was transformed with the construct. Expression was induced with isopropyl β-D-thiogalactopyranoside. Pelleted broken cells, after passage through the french press, were extracted with

[1] Abbreviations: HFC, human fibroblast collagenase; pro-HFC, human fibroblast procollagenase; r-pro-HFC, recombinant pro-HFC; Fmoc, 9-fluorenylmethoxycarbonyl (Fmoc-amino acid denotes N^{α}-Fmoc, where all amino acids are of the L configuration); Boc, *tert*-butyloxycarbonyl; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; PCMB, *p*-(chloromercuri)benzoate; APMA, (*p*-aminophenyl)mercuric acetate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

2 M guanidine hydrochloride, diluted 10-fold, and purified in a single step by passage over an affinity column that consisted of monoclonal antibody (VI-3) against pro-HFC coupled to Sepharose 4B as described previously (Birkedal-Hansen et al., 1988). After being washed with 0.5 M NaCl, the adsorbed pro-HFC was eluted with 50 mM acetate, 0.2 M NaCl, and 5 mM $CaCl_2$, pH 4.0, and immediately neutralized by addition of 2.0 M Tris-HCl, pH 8.0. Activation resulted in generation of catalytic activity and in a reduction in molecular weight of approximately 10K in a manner similar to the enzyme isolated from fibroblast cultures (Windsor et al., 1990).

N^{α}-Fmoc-Amino acids and alkoxybenzyl alcohol resin were purchased from Bachem (Torrance, CA). Fluorescamine, *tert*-butyl carbonate, and 1,10-phenanthroline were obtained from Lancaster, and Tricine was obtained from Behring Diagnostics. Dicyclohexylcarbodiimide was purchased from Aldrich, and 1-hydroxybenzotriazole, dansyl chloride, and β-casein were from Sigma. High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were obtained from Fisher, and constant-boiling HCl and Sequanal-grade trifluoroacetic acid were from Pierce. Type I collagen was obtained from rat tail tendons (Chandrakasan et al., 1976). The α1(I) chain of rat tendon collagen was isolated by CM-cellulose chromatography (Piez et al., 1963), while β-casein was purified by chromatography over DEAE-cellulose (Thompson & Kiddy, 1964; Thompson & Pepper, 1964; Thompson, 1966).

Peptide Synthesis and Characterization. All peptides were synthesized by using the solid-phase method with N^{α}-Fmoc-amino acids according to the procedures described by Stewart and Young (1984). For Asp-Val-Ala-Gln-Phe-Val-Leu-Thr-Pro-Gly (**2**), all N^{α}-Fmoc-amino acid preformed symmetrical anhydrides were double coupled for 4 h in either CH_2Cl_2/trifluoroethanol (80:20) or CH_2Cl_2/*N*,*N*-dimethylformamide/trifluoroethanol (50:30:20). Fmoc-Gln was coupled in situ in *N*,*N*-dimethylformamide in the presence of 1-hydroxybenzotriazole. Boc-Pro-Leu-Gly-NHOH was synthesized as described by Moore and Spilburg (1986a). All peptides were purified by HPLC using a Beckman instrument equipped with a semipreparative Beckman Ultrasphere ODS 5-μm reverse-phase column (10 × 250 mm). Peptides were eluted isocratically with 15% acetonitrile (Baker, HPLC grade)/H_2O containing 0.1% Sequanal-grade trifluoroacetic acid and recovered by lyophilization. Amino acid compositions were determined with a Dionex Model D-300 analyzer after hydrolysis in constant-boiling 6 N HCl at 100 °C for 22 h. Prior to hydrolysis, the samples were repeatedly freeze-thawed and degassed under high vacuum. The amino acid compositions of all peptides were within experimental error of the theoretical values.

Kinetic Measurements. All buffers were freed from adventitious metal ions by extraction with dithizone in carbon tetrachloride. Prior to all assays, pro-HFC was activated by incubation with 1 mM *p*-(chloromercuri)benzoate (PCMB) in assay buffer (50 mM Tricine, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5) for 1 h at 30 °C. Since this treatment results in the ultimate conversion of all of the 57K/52K pro-HFC to the 46K/42K form with no significant conversion to lower molecular mass species, it was assumed that all of the zymogen was activated and that the concentration of active sites equalled the pro-HFC concentration, which was measured spectrophotometrically using $\epsilon_{280} = 6.8 \times 10^4\ M^{-1}\ cm^{-1}$. All reactions were carried out at 30 °C in microfuge tubes. The intial rate of hydrolysis of β-casein was determined by measuring the appearance of amino groups. The reaction was initiated by addition of 30 μL of 1.64 mM β-casein dissolved in 0.05 N NaOH to 95 μL of the activated enzyme to give an enzyme concentration of 0.37 μM and a substrate concentration of 390 μM. At various time intervals, 12.5-μL aliquots of the reaction mixture were withdrawn and added to Microfuge tubes containing 37.5 μL of 20 mM 1,10-phenanthroline to quench the reaction. After dilution to 1 mL with assay buffer, a 100-μL aliquot was withdrawn from each tube and added to culture tubes containing 1.9 mL of assay buffer. Each tube was then agitated while 200 μL of 22 mM fluorescamine (in acetone) was added. The relative concentration of amino groups was measured fluorometrically as described earlier (Fields et al., 1987). Initial rates (v) in units of micromolar per hour were obtained from plots of fluorescence versus time using only data points corresponding to less than 40% of full hydrolysis. Examination of the reaction by SDS–PAGE showed that the hydrolysis of β-casein by HFC occurred rapidly at a single site and was followed by slower cleavages at secondary sites. Thus, the rate measured in this fluorescamine assay corresponds solely to that of the initial cleavage. K_M and k_{cat} values for the reaction were determined from double-reciprocal plots.

The assay procedures for type I collagen and the synthetic peptides have been described previously (Mallya et al., 1986; Fields et al., 1987) and were carried out at substrate concentrations of 0.33 and 200 μM, respectively. Assays carried out with Asp-Val-Ala-Gln-Phe-Val-Leu-Thr-Pro-Gly (**2**) contained 0.6% methanol to increase solubility. The rates were corrected for the slight inhibition of HFC by the methanol. Because of the very slow rate of hydrolysis of this peptide and the background fluorescence produced in the fluorescamine assay from the N-terminal α-amino group, the double-reciprocal plots needed to determine k_{cat} and K_M could not be obtained. Acetylation of the N-terminus in an attempt to reduce the background resulted in such poor solubility that kinetic measurements were also not possible. The site of hydrolysis of all peptides was determined by dansylation of the reaction products followed by HPLC analysis. Aliquots (20 μL) of the reaction mixtures were removed at 0 and 48 h and reacted with 20 μL of 0.5 M $NaHCO_3$ and 20 μL of an 8 mM solution of dansyl chloride in acetonitrile. After standing in the dark for 24 h, the samples were applied to a Brownlee Aquapore C8 7-μm column (4.6 × 250 nm) and eluted with a linear gradient prepared by mixing 50 mM sodium phosphate, pH 6.5, and 80% methanol in water. The site of hydrolysis was determined by comparing the retention times of the peptide products with that of a dansylated peptide of known sequence that corresponded to either the N- or the C-terminal portion of the substrate. The rate of hydrolysis of α1(I) gelatin was monitored by SDS–PAGE at a substrate concentration of 5 μM as described by Welgus and co-workers (Welgus et al., 1982), except using the silver staining method (Merril et al., 1981) to visualize protein on the gels. Quantitation of protein bands in the gels was carried out with a Hoefer Scientific Instruments Model GS300 densitometer. Collagen zymograms were performed by the procedure of Birkedal-Hansen and Taylor (1982). Gelatin and casein zymograms were performed essentially as described by Heusen and Dowdle (1980) using 1 mg/mL substrate in the running gel.

Results

The substrates chosen for examination in this study vary widely in size, structure, and sequence. They include triple-helical rat type I collagen, rat α1(I) gelatin, β-casein, and three synthetic oligopeptides. The peptide Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln (**1**) was patterned after the collagenase cleavage

Table I: Kinetic Parameters for the Hydrolysis of Several Substrates by HFC[a]

substrate	k_{cat} (h^{-1})	K_M (μM)	k_{cat}/K_M (μM^{-1} h^{-1})
type I collagen (rat tendon)	16	0.83	19
α1(I) gelatin (rat tendon)	24	9.8	2.4
β-casein (bovine)	160	710	0.23
Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln (**1**)	730	3300	0.22
Pro-Val-Gln-Pro-Ile-Gly-Pro-Gln (**3**)	2.4	380	0.0063
Asp-Val-Ala-Gln-Phe-Val-Leu-Thr-Pro-Gly (**2**)	11[b]	1900[b]	0.0061[b]

[a] All assays were carried out in 50 mM Tricine, 10 mM $CaCl_2$, and 0.2 M NaCl, pH 7.5 at 30 °C. For Asp-Val-Ala-Gln-Phe-Val-Leu-Thr-Pro-Gly, 0.6% v/v methanol was included in the assay to increase solubility. [b] The value of k_{cat}/K_M was measured, the value of K_M estimated from substrate competition experiments, and the value of k_{cat} calculated from these two values.

site in the α1 chain of chick type I collagen, while Asp-Val-Ala-Gln-Phe-Val-Leu-Thr-Pro-Gly (**2**) and Pro-Val-Gln-Pro-Ile-Gly-Pro-Gln (**3**) were models for the two autolytic cleavage sites in pro-HFC. The kinetic parameters k_{cat} and K_M for the hydrolysis of all of these substrates by PCMB-activated HFC have been measured in assay buffer at 30 °C. These parameters are listed in Table I in order of decreasing specificity, as indicated by the value of k_{cat}/K_M. Rat tendon type I collagen is hydrolyzed with k_{cat} = 16 h^{-1} and K_M = 0.83 μM, and peptide **1** with k_{cat} = 730 h^{-1} and K_M = 3300 μM, as reported earlier (Fields et al., 1987). The rat α1(I) chain is hydrolyzed with k_{cat} = 24 h^{-1}, similar to the value for rat collagen, but the K_M value of 9.8 μM is significantly higher than for type I collagen. This trend is accentuated for β-casein which has k_{cat} = 160 h^{-1} and K_M = 710 μM. Thus, among these four substrates, there is a moderate increase in k_{cat} and a large increase in K_M compared to collagen that leads to a marked net decrease in k_{cat}/K_M. Peptides **2** and **3** were both hydrolyzed slowly at the Phe–Val and Pro–Ile bonds with k_{cat}/K_M values of 0.0061 and 0.0063 μM^{-1} h^{-1}, respectively. Peptide **2** is extremely hydrophobic and has a poor solubility in water. Thus, the value of k_{cat}/K_M is for the reaction carried out in assay buffer containing 0.6% v/v methanol. The kinetic parameters for its hydrolysis could not be determined directly, by the values in Table I were estimated by a method described later in this section. The k_{cat} values for the hydrolysis of these two peptides are considerably lower (2.4–11 h^{-1}) and the K_M values (380–1900 μM) lower than for other short peptides that have been examined (Fields et al., 1987).

One means of assessing whether an enzyme has multiple proteinase activities is to compare zymograms prepared with different protein substrates. If a contaminant were responsible for one of the activities and the contaminant had a different electrophoretic mobility than HFC, this would be evident from the zymograms. Zymograms for caseinase, collagenase, and gelatinase activities are shown in Figure 1 for different samples of pro-HFC. A silver-stained SDS gel of the pro-HFC sample used in these experiments is shown in frame A, lane 1. A freshly prepared sample of HFC (frame A, lane 2) shows caseinolytic activity associated with the enzyme 57K/52K doublet and with the two minor bands of autolytically generated 46K/42K HFC. As shown in previous studies, exposure to SDS activates pro-HFC without any apparent reduction of molecular mass (Birkedal-Hansen, 1987; Birkedal-Hansen & Taylor, 1982). After storage of this sample (frame A, lane 3), a greater proportion of pro-HFC is converted to the 46K/42K form, and caseinolytic activity is also associated with a 33K degradation band which constitutes 250 amino acids from the pump-1 like (Muller et al., 1988) NH_2-terminal

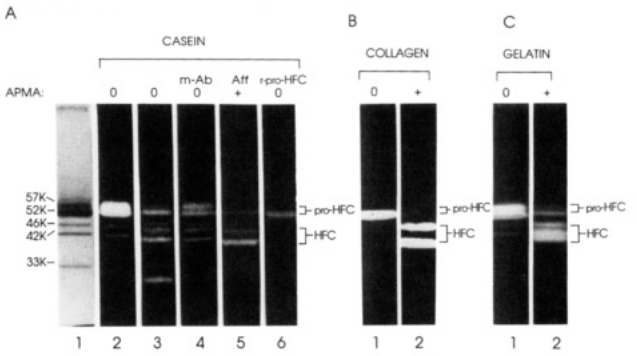

FIGURE 1: Proteolytic activity of (0) pro-HFC and (+) APMA-activated pro-HFC against (A) casein, (B) type I collagen, and (C) type I gelatin. (A) Casein zymogram: Lane 1 shows a silver-stained SDS gel containing a sample of pro-HFC (2.2 μg) for reference in reading the zymograms. There is a pro-HFC doublet at 57K/52K, the "active" 46K/42K form, and some formation of the NH_2-terminal 33K pro-HFC fragment. Samples in lanes 2–6 were incubated with 2% SDS-containing sample buffer under nondenaturing conditions and then resolved by an 11% polyacrylamide gel copolymerized with 1 mg/mL casein. The figure is a composite of several zymograms. To adjust for minor differences in running conditions, the lanes have been aligned by using the major 52K pro-HFC band. Lane 2 is freshly isolated pro-HFC (2.4 μg). Lane 3 is a pro-HFC (2.0 μg) sample after storage at −70 °C showing extensive "autocatalytic" conversion to the active HFC species and to the 33K fragment, all of which possess caseinolytic activity (compare with lane 1). Lane 4 is pro-HFC after passage over a monoclonal antibody (VI-3) affinity column. Lane 5 is APMA-activated human HFC after passage over a Sepharose–CH-Pro-Leu-Gly-NHOH affinity column. Lane 6 is pure r-pro-HFC. The incubation time was 4 h for all zymograms. (B) Type I collagen zymograms: Samples were prepared as described under frame A and resolved by SDS–PAGE under nondenaturing conditions. The Triton X-100 washed gel was then overlaid with a trypsin-resistant, air-dried film of reconstituted rat tail tendon type I collagen fibrils for 16 h. Lane 1 is pro-HFC (3.2 μg), and lane 2 is APMA-activated pro-HFC (3.4 μg). (C) Type I gelatin zymogram: the samples were prepared as described for the collagen zymogram, except that the incubation time was 16 h.

portion of pro-HFC (Whitham et al., 1986). The caseinolytic activity was retained after passage of this sample of pro-HFC over an affinity column consisting of a Sepharose-bound monoclonal antibody, VI-3, which specifically recognizes pro-HFC but not either stromelysin or gelatinase (frame A, lane 4). Similarly, the caseinolytic activity is not removed after affinity chromatography over a column prepared by coupling Pro-Leu-Gly-NHOH to Sepharose 4B, as described by Moore and Spilburg (1986b). Frame A, lane 6, shows that the 52K r-pro-HFC displays this same caseinolytic activity. Since this enzyme was isolated from *E. coli*, it does not contain stromelysin. Only a single pro-HFC band is seen because *E. coli* fails to glycosylate the enzyme. The activity of pro-HFC and (*p*-aminophenyl)mercuric acetate (APMA)-activated pro-HFC against reconstituted type I collagen fibrils (frame B) and gelatin (frame C) is also shown in Figure 1. The SDS-treated proenzyme and APMA-activated enzyme both dissolve collagen fibrils and give rise to lytic zones. On extended incubation, similar zones of lysis are seen in gelatin zymograms.

If the activities toward the substrates listed in Table I are all truly attributable to pro-HFC and none are due to a contaminant, then the ratios of these activities to each other should be constant for independent preparations. Moreover, these ratios should not change if additional purification steps are employed. The activities ($v/[E_0]$) of four independent preparations of PCMB-activated pro-HFC toward collagen, casein, gelatin, and peptides **1** and **3** were measured using standard assays carried out at a single substrate concentration, as specified under Materials and Methods. The average values of the ratios of the $v/[E_0]$ values for the hydrolysis of casein,

Table II: Ratio of Activities of HFC Preparations toward Different Substrates

purification method	activity ratio[a] casein/collagen ($\times 10^3$)	gelatin/collagen ($\times 10^1$)	peptide 1/collagen ($\times 10^3$)	peptide 3/collagen ($\times 10^4$)
conventional[b]	6.7 ± 0.4	1.3 ± 0.1	7.8 ± 0.6	1.8 ± 0.5
+monoclonal antibody[c]	6.1 ± 0.5	1.1 ± 0.1	7.8 ± 0.6	2.5 ± 0.6
+Sepharose-CH-Pro-Leu-Gly-NHOH	6.0 ± 0.5	1.1 ± 0.1	6.8 ± 1.0	2.2 ± 0.5

[a] Based on $v/[E_0]$ values measured by using standard assays carried out at the single substrate concentrations specified under Materials and Methods. [b] Average of single assays on four independent purifications. [c] Average of four assays on same preparation.

Table III: Inhibition of HFC Activities by 1,10-Phenanthroline and Boc-Pro-Leu-Gly-NHOH

substrate	1,10-phenanthroline IC_{50} (μM)	Boc-Pro-Leu-Gly-NHOH K_I (μM)
type I collagen	49 ± 5	110 ± 11
α1(I) gelatin	55 ± 5	110 ± 11
β-casein	48 ± 5	100 ± 10
Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln (**1**)	58 ± 5	110 ± 11
Pro-Val-Gln-Pro-Ile-Gly-Pro-Gln (**3**)	52 ± 5	

gelatin, and peptides **1** and **3** to that of collagen and the standard deviations of these values are 0.0067 ± 0.0004, 0.13 ± 0.01, 0.0078 ± 0.0006, and 0.00018 ± 0.00005, respectively (Table II). The standard deviations given are no greater than those found when the *same* sample of pro-HFC is subjected to four independent assays. This indicates that there is no statistically significant variation in these ratios between preparations and that these ratios reflect an intrinsic property of HFC. In support of this conclusion, further attempts to purify HFC by affinity chromatography over an anti-pro-HFC monoclonal antibody column or over Sepharose-CH-Pro-Leu-Gly-NHOH do not alter these ratios (Table II). Thus, the hydrolysis of all of these substrates is catalyzed by HFC, and there is no evidence that any of these activities is due to a contaminant.

Two types of experiments have been performed to investigate whether any of these proteolytic activities could be attributed to hydrolysis at distinct active sites on HFC. One means of distinguishing between such activities is to assess whether they are inhibited in the same manner. The chelating agent 1,10-phenanthroline is known to inhibit the collagenase activity of HFC (Seltzer et al., 1977). Thus, independent inhibition curves for the effect of 1,10-phenanthroline on all five activities have been obtained under identical conditions. IC_{50} values (the inhibitor concentration resulting in 50% inhibition of the enzyme at a fixed substrate concentration) obtained from these independent curves (Table III) agree within experimental error. In all cases, the average number of complexed ligands per mole of zinc ion, n, was calculated from these plots to be 2.0 ± 0.2 (Kistiakowsky & Shaw, 1953). This strongly implies that the active-site zinc ion has been removed by the 1,10-phenanthroline. A *cumulative* set of data points for the inhibition of all five activities by 1,10-phenanthroline are shown in Figure 2. A single inhibition curve can be drawn through these data points with IC_{50} = 52 μM.

The inhibition of four of these activities by Boc-Pro-Leu-Gly-NHOH was determined in a similar manner. Double-reciprocal plots for the inhibition of the hydrolysis of collagen and peptide **1** by HFC showed Boc-Pro-Leu-Gly-NHOH to be a competitive inhibitor of both. The K_I value was 110 ± 11 μM for both substrates (Table III). Values of K_I for the inhibition of β-casein and α1(I) gelatin hydrolysis were estimated from IC_{50} values by using the equation for competitive inhibitors (Segel, 1975):

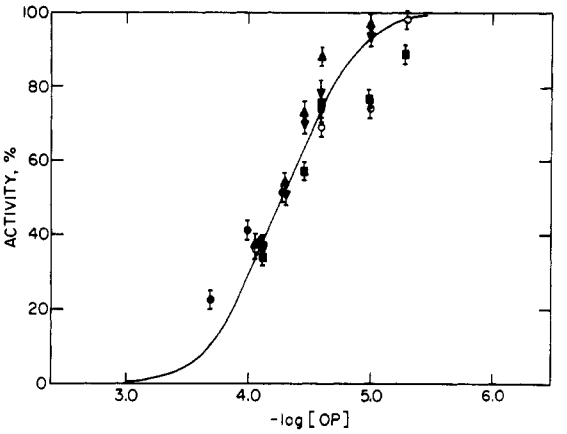

FIGURE 2: Inhibition by 1,10-phenanthroline (OP) of the hydrolysis of (○) type I collagen, (□) α1(I) gelatin, (■) β-casein, (△) Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln, and (▲) Pro-Val-Gln-Pro-Ile-Gly-Pro-Gln by HFC. Assays were performed at 30 °C in 50 mM Tricine, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Substrate concentrations are given in the text.

$$IC_{50} = K_I(1 + [S]/K_M) \quad (1)$$

The values of K_I were found to be 100 ± 10 and 110 ± 11 μM, respectively, which agree within experimental error with those for collagen and peptide **1** (Table III). Due to the insensitivity of the assay, the IC_{50} value for the inhibition of the hydrolysis of peptide **3** by the hydroxamate could not be accurately determined.

A second type of experiment to assess whether the hydrolysis of two substrates is taking place at the same or different active sites is a substrate competition experiment. In these experiments, the rate of hydrolysis of one substrate (at a fixed concentration) was monitored as a function of the concentration of a second substrate. The underlying principle behind such experiments is that a competing substrate will act as an inhibitor with respect to the hydrolysis of the other substrate. The rate of hydrolysis of substrate A in the presence of B, v_{A+B}, compared to the rate in the absence of B, v_A, is given by (Segel, 1975)

$$\frac{v_{A+B}}{v_A} = \frac{(K_M)_A + [A]}{(K_M)_A[1 + [B]/(K_M)_B] + [A]} \quad (2)$$

Competition experiments were carried out for all pairs of substrates for which the measurement of the rate of hydrolysis of one substrate in the presence of the other was possible. In some cases, the measurement was not possible because B was hydrolyzed so much faster than A that its concentration was depleted faster than the assay could be carried out (e.g., B = collagen). In other cases, the presence of B interferred with the mechanics of the assay (e.g., A = Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln and B = casein). For each pair of substrates, eq 2 can be used to calculated a theoretical inhibition curve using the values of K_M listed in Table I. The experimental data for the competition experiment were plotted on the same graph to see if they agreed within experimental error with the theoretical curves. A complete set of data for inhibition of the hydrolysis of A = collagen by HFC in the presence of increasing concentrations of B = β-casein, gelatin, and peptides **1** and **3**, v_{A+B}/v_A, is shown in Figure 3A–D, respectively. For all four sets of data, inhibition was observed that was in reasonable agreement with the theoretical curve. The same

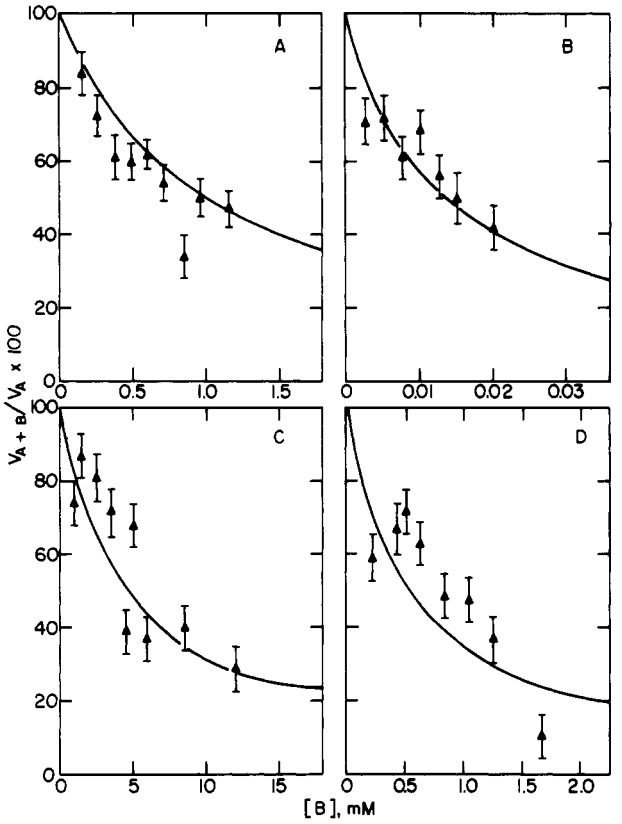

FIGURE 3: Inhibition of the hydrolysis of 0.33 μM type I collagen (substrate A) by HFC ($v_{A+B}/v_A \times 100$) by substrate B, where B was (A) β-casein, (B) α1(I) gelatin, (C) Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln, and (D) Pro-Val-Gln-Pro-Ile-Gly-Pro-Gln. Assays were performed at 30 °C in 50 mM Tricine, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Solid lines are theoretical curves for the inhibition that were calculated by using eq 2 and the kinetic parameters shown in Table I.

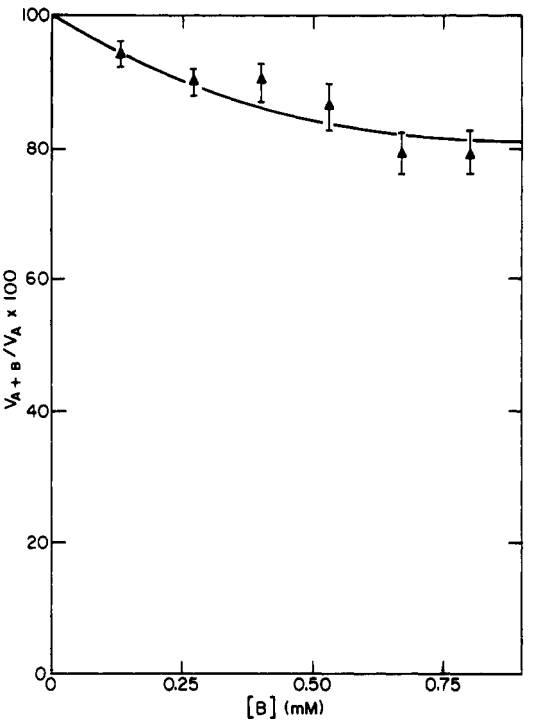

FIGURE 4: Inhibition of the hydrolysis of 0.33 μM type I collagen (substrate A) by HFC ($v_{A+B}/v_A \times 100$) by Asp-Val-Ala-Gln-Phe-Val-Leu-Thr-Pro-Gly (substrate B). Assays were performed at 30 °C in 50 mM Tricine, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5.

agreement with the theoretical curve was also found for A = peptide **1**, B = gelatin or peptide **3**, and A = β-casein, B = gelatin or peptide **3** (data not shown). Thus, it is clear that all of these substrates are hydrolyzed by HFC at the same active site. While we were unable to quantitate the K_M value for the hydrolysis of peptide **2** by HFC, this peptide inhibits the hydrolysis of collagen (Figure 4). The line drawn through the data corresponds to a K_M value of 1.9 mM. Using this value of K_M and the value of k_{cat}/K_M measured directly, we calculated k_{cat} for this peptide to be 11 h^{-1}.

Discussion

The literature contains numerous reports of the ability of vertebrate collagenases to hydrolyze noncollagenolytic substrates. The collagenases from rabbit muscle tumor (Gross et al., 1974; McCroskery et al., 1975), human rheumatoid synovium (Woolley et al., 1975), human skin fibroblasts (Stricklin et al., 1977; Woolley et al., 1978; Welgus et al., 1982), mouse bone (Sakamoto et al., 1978), tadpole back skin (Hori & Nagai, 1979; Bicsak & Harper, 1984), and rat uterus (Roswit et al., 1983; Welgus et al., 1985a) have all been reported to hydrolyze the α1(I) and α2(I) chains of type I gelatin. In addition, rabbit muscle tumor collagenase has been shown to cleave type II and III gelatins (McCroskery et al., 1975), and HFC to hydrolyze type II, III, IV, and V gelatins (Welgus et al., 1982). Tadpole back skin (Nagai & Hori, 1972), mouse bone (Sakamoto et al., 1978; Gillet et al., 1977), and pig synovium collagenases (Cawston & Tyler, 1979) have also been reported to hydrolyze casein. HFC has been shown to cleave human α_2-macroglobulin at the Gly_{679}–Leu_{680} bond (Sottrup-Jensen & Birkedal-Hansen, 1989; Enghild et al., 1989), human pregnancy zone protein at the Gly_{685}–Leu_{686}, Gly_{687}–Val_{688}, and Gly_{757}–Ile_{758} bonds, rat α_1-macroglobulin at the Ala_{683}–Leu_{684} and Ala_{685}–Met_{686} bonds, rat α_2-macroglobulin at the His_{681}–Leu_{682} and Phe_{691}–Leu_{692} bonds, rat α_1-inhibitor 3 (variant 2J) at the Pro_{683}–Val_{684} bond, and rat α_1-inhibitor 3 (Variant 27J) at the Ala_{666}–Val_{667} bond (Sottrup-Jensen & Birkedal-Hansen, 1989). Human neutrophil collagenase has been shown to hydrolyze α_1-protease inhibitor at the Phe_{352}–Leu_{353} bond (Mookhtiar, Weiss, and Van Wart, unpublished observations). Several tissue collagenases have also been shown to hydrolyze synthetic oligopeptides (Nagai et al., 1976; Masui et al., 1977; Williams & Lin, 1984; Weingarten et al., 1985; Weingarten & Feder, 1986; Fields et al., 1987). Nagai and associates have examined the hydrolysis of several peptides with sequences patterned after collagen sequences by tadpole back skin collagenase (Nagai et al., 1976; Masui et al., 1977). Human neutrophil collagenase has been reported to hydrolyze two peptides (Williams & Lin, 1984), HFC to hydrolyze a variety of peptides, peptolides, and esters (Weingarten et al., 1985; Fields et al., 1987), and six different collagenases to cleave a series of five hexapeptides (Weingarten & Feder, 1986).

These reports appear to challenge the widely held hypothesis that these collagenases are among the most specific proteinases known. The present studies were carried out with highly purified pro-HFC and r-pro-HFC to establish the basis for the broad range of activities reported. In particular, it was of interest to establish whether all of these activities were the result of hydrolysis at a single active site. The different series of experiments carried out here provide convincing evidence that the six widely divergent substrates studied are hydrolyzed exclusively by HFC at the same active site. The zymograms employing type I collagen, gelatin, and casein as substrates show that all active bands in the gel are accounted for as HFC bands and show no evidence of contaminating proteases that could be contributing to the hydrolysis of these substrates. The

observation of caseinase activity for r-pro-HFC is particularly noteworthy, since it was not isolated from a fibroblast culture and cannot contain any stromelysin. The ratios of the activities toward type I collagen, α1(I) gelatin, β-casein, and peptides **1** and **3** do not vary appreciably from one conventional purification to another, and do not change when the samples are subjected to two additional affinity purification steps. These five activities are inhibited in an almost identical fashion by 1,10-phenanthroline and Boc-Pro-Leu-Gly-NHOH. Moreover, substrate competition experiments involving pairs of these five substrates give inhibition curves that agree within experimental error with those calculated by using the independently determined K_M values of these substrates.

The activity of HFC toward the interstitial collagens has been intensively studied (Welgus et al., 1980, 1981, 1985b; Narayanan et al., 1984), and the ability of the enzyme to hydrolyze such triple-helical substrates has long been widely acknowledged. The first careful study of the gelatinase activity of HFC was carried out by Welgus and associates (Welgus et al., 1982), who demonstrated that type I–V gelatins were hydrolyzed at multiple sites at rates that were significant compared to the interstitial collagens. The action of HFC on α1(I) gelatin is confirmed here. Similar results have also been obtained with rat uterus collagenase (Welgus et al., 1985a). Prior to those studies, the ability of tissue collagenases to hydrolyze gelatins at an appreciable rate was in question (McCroskery et al., 1973, 1975). The results presented here show that casein is also a substrate for HFC. Although the cleavage site in β-casein is not known, it must differ from the collagen Gly-[Ile or Leu]-[Ala or Leu] cleavage sequences in the interstitial collagens, since such sequences are not found in β-casein (Dumas et al., 1972). The hydrolysis of peptides **2** and **3** by HFC observed here is consistent with the view that the internal cleavages of pro-HFC that accompany its activation and degradation are autolytic.

The results presented here taken together with those showing that HFC hydrolyzes a variety of macromolecular inhibitors (Sottrup-Jensen & Birkedal-Hansen, 1989; Enghild et al., 1989) clearly demonstrate that the enzyme is capable of hydrolyzing a much wider variety of substrates than originally believed. Moreover, these studies show that HFC can hydrolyze bonds of very different sequence than those found at the cleavage site in collagens. This conclusion is consistent with an in-depth specificity study of the action of HFC on 60 octapeptides with different sequences which shows that the enzyme has an unexpectedly broad specificity (Fields, Birkedal-Hansen, and Van Wart, unpublished results). This broad range of activities may be of physiological significance. Clearly, the rapid rate of cleavage of the Gly_{679}–Leu_{680} bond of α_2-macroglobulin by HFC ($k_2/K_I = 2.8 \times 10^6$ M^{-1} s^{-1}) and resulting inactivation of the enzyme (Enghild et al., 1989) suggest that HFC can be effectively scavenged by this inhibitor in vivo. The hydrolysis of α_1-protease inhibitor by human neutrophil collagenase abolishes its ability to inhibit serine proteases (Desrochers & Weiss, 1988). Thus, this inactivation could perturb the protease/anti-protease balance of tissue under certain circumstances. These results suggest that HFC may be capable of hydrolyzing other as yet unknown substrates at physiologically significant rates.

Although HFC can now be acknowledged to hydrolyze substrates other than just interstitial collagens, it should be underscored that it is still its activity toward these collagens that distinguishes it from other enzymes. Among all known human enzymes, only the collagenase from human neutrophils shares with HFC the ability to hydrolyze interstitial collagens in the same fashion (Murphy et al., 1980; Horwitz et al., 1977; Hasty et al., 1987). Although fibroblast stromelysin and gelatinase are homologous to HFC, neither of these enzymes hydrolyzes interstitial collagens at an appreciable rate. The basis for this remarkable selectivity is not yet fully understood, but is not due solely to the sequence specificities of these enzymes. The collagenases are apparently unique in their ability to recognize and bind the collagen triple helix at the cleavage site. This ability is reflected in the low K_M values for hydrolysis of collagen (~1 μM) compared to peptides and other protein substrates. Further work designed to investigate the significance of the secondary structure of the substrate on recognition by HFC is in progress.

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