Characterization of 58-Kilodalton Human Neutrophil Collagenase: Comparison with Human Fibroblast Collagenase†

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ABSTRACT: A series of experiments has been carried out to characterize 58-kDa human neutrophil collagenase (HNC) and compare it with human fibroblast collagenase (HFC). N-Terminal sequencing of latent and spontaneously activated HNC shows that it is a distinct collagenase that is homologous to HFC and other members of the matrix metalloproteinase gene family. Activation occurs autolytically by hydrolysis of an M–L bond at a locus homologous to the Q_{80}-F_{81}-V_{82}-L_{83} autolytic activation site of HFC. This releases a 16-residue propeptide believed to contain the "cysteine switch" residue required for latency. Polyclonal antibody raised against HNC cross-reacts with HFC but with none of the other major human matrix metalloproteinases examined. Treatment of HNC with endoglycosidase F or *N*-glycosidase F indicates that it is glycosylated at multiple sites. The deglycosylated latent and spontaneously activated enzymes have molecular weights of approximately 44K and 42K, respectively. Differences in the carbohydrate processing of HFC and HNC may determine why HFC is a secreted protein while HNC is stored in intracellular granules. The kinetic parameters k_{cat} and K_M for the hydrolysis of the interstitial collagen types I, II, and III in solution by both collagenases have been determined. The strong preferences of HNC for type I collagen and of HFC for type III collagen found in earlier studies have been confirmed. The preference of HNC for type I over type III collagen is almost abolished when fibrillar collagens are used as substrates, but the preference of HFC for type III over type I collagen is only partially decreased. The k_{cat}/K_M values for the hydrolysis of synthetic octapeptides with sequences modeled after those of the α chains at the cleavage site of type I and III collagens have also been measured. The rates of hydrolysis of these peptides vary very little, indicating that it is the collagen conformation at the cleavage site and not the sequence specificity of the collagenases that determines their collagen specificities.

In the preceding paper (Mookhtiar & Van Wart, 1990), the purification to homogeneity of active and latent 58-kDa forms of human neutrophil collagenase (HNC)[1] was described. The availability of purified HNC has made it possible to characterize this enzyme so that it can be compared with human fibroblast collagenase (HFC), the only other distinct human collagenase that has been described to date. There have been a number of conflicting reports concerning the basic properties of HNC. For example, the molecular weight of latent HNC has been reported to vary from 57K (Hasty et al., 1986) to 105K (Williams & Lin, 1984) and to either decrease markedly (Macartney & Tschesche, 1983; Gillet et al., 1977; Sorsa et al., 1985; Callaway et al., 1986; Sorsa, 1987) or remain the same (Hasty et al., 1986; Williams & Lin, 1984) on activation. With regard to its collagen specificity, HNC has been reported to hydrolyze type I and III collagens equally efficiently in solution (Murphy et al., 1982) or to strongly prefer type I over type III (Horwitz et al., 1977; Hasty et al., 1987a).

In addition to the disagreements about the properties of HNC that have been examined, no information regarding the sequence of the protein chain or the extent or nature of its carbohydrate processing have been obtained to date. Such information is needed to establish definitively whether HNC is a distinct collagenase. In this paper, a number of properties of 58-kDa HNC have been investigated and compared with HFC. This includes a comparison with HFC of partial primary sequence (Goldberg et al., 1986), collagen specificity (Horwitz et al., 1977; Welgus et al., 1981; Hasty et al., 1987a), glycoprotein processing (Wilhelm et al., 1986), and immunological cross-reactivity (Hasty et al., 1984, 1987b). These studies clearly show that HNC and HFC are homologous members of the matrix metalloproteinase (MMP) gene family. The two collagenases share many similarities yet exhibit distinctive differences which may be related to their respective roles in vivo.

Materials and Methods

Materials. Rat type I collagen was isolated from tail tendon, bovine type II from nasal septum, and human types I and III from placenta. These collagens were extracted and purified,

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[1] Abbreviations: HNC, human neutrophil collagenase; HNG, human neutrophil gelatinase (92-kDa type IV collagenase); HFC, human fibroblast collagenase; HFS, human fibroblast stromelysin; HFG, human fibroblast gelatinase (72-kDa type IV collagenase); PUMP-1, putative metalloproteinase 1; MMP, matrix metalloproteinase; PCMB, *p*-(chloromercuri)benzoate; APMA, *p*-aminophenylmercuric acetate; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Fmoc, 9-fluorenylmethoxycarbonyl; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Brij-35, polyoxyethylene(23) lauryl ether; EDTA, ethylenediaminetetraacetic acid; A_{sp}, specific activity.

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and a portion of each was labeled with [^{3}H]acetic anhydride (New England Nuclear) as described earlier (Mookhtiar et al., 1986). HFC was purified from serum-free cultures of human skin fibroblasts as described previously (Birkedal-Hansen, 1987). Human fibroblast stromelysin (HFS) was donated by Dr. Hideaki Nagase of the University of Kansas Medical Center and 72-kDa human fibroblast gelatinase (type IV collagenase) (HFG) provided by Dr. William G. I. Moore of the University of Alabama at Birmingham. Human neutrophil gelatinase (HNG) and latent and active 58-kDa HNC were purified to homogeneity by the procedures presented in the preceding paper (Mookhtiar & Van Wart, 1990). Trypsin (TPCK grade) was obtained from Cooper Biochemicals; soybean trypsin inhibitor, fluorescamine, *p*-(chloromercuri)-benzoate (PCMB), and pepsatin A were from Sigma Chemical Co.; Brij-35 and *N*-glycosidase F (EC 3.5.1.52) were from Boehringer Mannheim; endoglycosidase F (EC 3.2.1.96) was from New England Nuclear; bicinchoninic acid was from Pierce; 1,10-phenanthroline was from Lancaster; an Immun-Blot assay kit, goat anti-rabbit IgG antibody conjugated to alkaline phosphatase, biotinylated molecular weight standards, and avidin–alkaline phosphatase were from Bio-Rad; and alkoxybenzyl alcohol resin and N^{α}-Fmoc-amino acids were from Bachem (Torrance, CA).

Peptide Synthesis. All synthetic peptides were synthesized by the solid-phase method using N^{α}-Fmoc-amino acids (Stewart & Young, 1984) and their compositions verified by amino acid analysis, as described earlier (Fields et al., 1987).

Protein Sequencing. A sample of latent 58-kDa HNC that had undergone a significant amount of spontaneous activation on storage at −10 °C was applied to a Sepharose-CH–Pro-Leu-Gly-NHOH affinity resin (Moore & Spilburg, 1986) *without* pretreatment with PCMB. The residual latent HNC and autolytic fragments eluted in fraction A. Pure active 58-kDa HNC was desorbed in fraction B as described in the preceding paper (Mookhtiar & Van Wart, 1990), except that Brij-35 was omitted from the elution buffer and the sample was eluted directly into a dialysis bag containing 1 mL of 100 mM 1,10-phenanthroline. The sample was dialyzed vs three changes of 25 mM acetic acid and recovered by lyophilization. Pure latent 58-kDa was also prepared for sequencing by the same treatment, except that it was activated with PCMB immediately prior to application to the affinity resin. Automatic sequence analyses were performed with an Applied Biosystems 470A protein sequencer equipped with a 120A on-line PTH analyzer and a 900A data analysis system.

Immunological Methods and Electrophoresis. Antiserum was raised against latent 58-kDa HNC by subcutaneous, intramuscular, and intradermal injections of a total of 200 μg of enzyme in Freund's complete adjuvant. Booster injections of a total of 200 μg of enzyme in Freund's incomplete adjuvant were given 4 weeks later. Starting 12 days after the booster injections, 30 mL of blood was collected weekly and allowed to clot overnight. The serum obtained after centrifugation was used as the primary antibody for all Western blots. These blots utilized a goat anti-rabbit IgG alkaline phosphatase conjugate for color development and were carried out as described in the Bio-Rad Immun-Blot assay kit. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) experiments were carried out as described in the preceding paper (Mookhtiar & Van Wart, 1990).

Endoglycosidase Digestions. The digestion of HNC by endoglycosidase F was carried out in 50 mM Mes, pH 6, containing 40 mM EDTA, 0.1% SDS, 2% 2-mercaptoethanol, 10 mM PMSF, and 0.5% Brij-35 at 37°C, while the reaction with *N*-glycosidase F was carried out in 100 mM phosphate, pH 8.0, containing 50 mM EDTA, 0.1% SDS, 0.1% 2-mercaptoethanol, 10 mM PMSF, 1% Brij-35, and 10 μg/mL pepstatin A at 37 °C. Samples of HNC were boiled in the digestion buffers for 2 min before addition of the endoglycosidases. The reactions were terminated at various time intervals by addition of SDS–PAGE denaturing buffer and boiling for 2 min.

Kinetic Measurements. The assay procedures used to quantitate the initial rate of hydrolysis of all soluble collagens and to determine the kinetic parameters k_{cat} and K_M for these reactions have been described earlier (Mallya et al., 1986). The activation procedures for HFC and HNC for these assays are described in the text. Assays for the hydrolysis of type I and III collagen fibrils were carried out by a modification of the procedure of Hasty et al. (1987b). Rat type I and human type III collagens were incubated at 1.5 mg/mL in a total volume of 50 μL of 50 mM Tricine, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$ at 35 °C for 16 h to form fibrillar gels. Samples of HNC and HFC were activated separately by incubation with 0.1 mM PCMB and 50 μg/mL trypsin, respectively, at 23 °C for 15 min. A 4-fold excess of soybean trypsin inhibitor was added to the HFC before addition to the assay. To start the assays, 50 μL of enzyme dissolved in the same buffer used for fibril formation was added to each tube. The tubes were then capped tightly and submerged in a 37 °C water bath. At various times, tubes were removed and centrifuged (12000*g*) at 23 °C for 4 min, and half of the supernatant (50 μL) was transferred to an empty microfuge tube. The collagen fragments in these tubes were denatured by heating to 70 °C for 30 min, after which a large excess of partially purified *Clostridium histolyticum* collagenase was added and the tubes were incubated at 37 °C for another 2 h to maximally hydrolyze the collagen fragments. Finally, fluorescamine (50 μL, 0.6 mg/mL in acetone) was added, the mixture was diluted 300-fold with assay buffer, and the fluorescence at 485 nm was recorded upon excitation at 385 nm. Each assay consisted of eight separate measurements, each corresponding to a different reaction time. Plots of fluorescence vs reaction time were used to obtain initial rates, which were converted into specific activities (A_{sp}) in units of micrograms of collagen degraded per minute per milligram of enzyme. Enzyme concentrations were determined spectrophotometrically using $\epsilon_{280} = 6.8 \times 10^4$ and $\epsilon_{277} = 5.6 \times 10^4$ M^{-1} cm^{-1} for HFC (Birkedal-Hansen et al., 1988) and HNC, respectively. The k_{cat}/K_M values for the hydrolysis of all peptides were measured by fluorometric analysis of the reaction mixtures after treatment with fluorescamine (Fields et al., 1987).

Results

Several physicochemical and kinetic properties of latent and active 58-kDa HNC have been studied and contrasted with those of HFC. This has included an examination of the N-terminal sequence, molar extinction coefficient, and carbohydrate processing of the protein chain. In addition, a polyclonal antibody has been raised against HNC and its cross-reactivity toward other members of the MMP gene family examined. Last, the collagen and peptide specificities of HNC have been examined and compared with HFC.

N-Terminal Sequencing. Of the first 20 amino acids of a sample of latent HNC that had undergone spontaneous activation on storage at −10 °C, 18 have been identified. They are listed in Table I where they are aligned and compared with the sequences of six other human matrix metalloproteinases (MMP) (Goldberg et al., 1986; Whitham et al., 1986; Wilhelm

Table I: Comparison of Partial Sequences of HNC and Other Human MMP

Enzyme	Sequence*	Reference
HNC-Active	XTPG NPKWERT X L T YRI RNY	This work
HNC-Latent	X MK XPRXGVPDS GX FMLTP	This work
HFC	L_{65} K V MKQPRCGVPDV AQ FVLTEG NPRWEQTH LT/RYRI ENY	a
HFS	L EVMRKPRCGVPDV GH FRT FPG I PKWRKTH L T YR IVNY	b
HFS-2	L EVMRKPRCGVPDV GH FS S FPGMPKWRKTH L T YR IVNY	c
72 kDa HFG	I ET MRKPRCGNPDV ANYNF FPR KPKWDKNQ I T YRI I GY	d
92 kDa HFG	L KAMRTPRCGVPDL GR FQT FEG DLKWHHHN I T YWIQNY	e
PUMP-1	L KVMKQPRCGVPDVAEY SL FPN S PKW T SKV V T YR IVSY	c

*X means that no amino acid was identified unambiguously in this sequencing cycle. [a]The numbering of residues for HFC assumes that F_{20} of the preproenzyme is the first residue of the secreted form (Goldberg et al., 1986). [b]Whitham et al. (1986), Wilhelm et al. (1987), and Saus et al. (1988). [c]Muller et al. (1988). [d]Collier et al. (1988). [e]Wilhelm et al. (1989).

et al., 1987, 1989; Saus et al., 1988; Muller et al., 1988; Collier et al., 1988). The N-terminal sequence of this active form of HNC corresponds to residues 83–102 of HFC, where the numbering of residues assumes that F_{20} of prepro-HFC sequenced by Goldberg et al. (1986) is the first residue of the secreted form. No amino acids were identified unambiguously in cycles 1 and 12, but the latter may be N, since this position is a possible N-linked glycosylation site (N-X-T) and the presence of a glycosylated N residue would explain the failure to detect any amino acid at this position. The N-terminal sequence of latent 58-kDa HNC has also been examined. Each cycle of the Edman degradation gave two amino acids, one of which corresponded exactly to the sequence of the spontaneously activated enzyme. Thus, part of the latent sample autoactivated on purification. The other sequence, which corresponds to the latent enzyme, is also listed in Table I and corresponds to residues 67–85 of HFC. Unambiguous amino acid assignments were made in 15 of the 19 cycles with agreement for residues 84 and 85 of the active and latent enzymes. Thus, 31 of the 36 residues corresponding to positions 67–102 in HFC have been assigned.

The sequence of HNC is unique and clearly indicates that it is a distinct gene product; however, it is also clearly homologous to the other MMP. Of the 31 residues identified for HNC, 24 are identical with those in HFC,[2] 22 to HFS and HFS-2, 18 to PUMP-1, 17 to 92-kDa HFG, and 15 to 72-kDa HFG. Latency in the MMP gene family is believed to be the result of a complex between a C residue in the propeptide (C_{73} for HFC, Table I) and the active-site zinc atom in the adjoining catalytic domain (Springman et al., 1990; Van Wart & Birkedal-Hansen, 1990). While we have not been able to unambiguously identify C in this position for latent HNC, the strong homology in this region (P-R-C-G-V/N-P-D) strongly implies that residue 73 is C. Latent HFC is known to activate autolytically following treatment with trypsin or organomercurials by hydrolysis at one of the three bonds at the Q_{80}-F_{81}-V_{82}-L_{83} locus that separates the propeptide from the active collagenase (Goldberg et al., 1986; Whitham et al., 1986; Grant et al., 1987). The N-terminus of active HNC indicates that it has undergone a similar autolytic activation that involves hydrolysis of the M_{82}–L_{83} bond.

Molar Extinction Coefficient of Latent 58-kDa HNC. The optical spectrum of a sample of latent HNC dissolved in 50 mM Tricine, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$ was scanned in a Varian Model 219 spectrophotometer with the identical buffer in the reference cell. The spectrum (not shown) exhibited a maximum in the aromatic amino acid region at 277 nm. The protein concentration of the HNC solution was determined by the bicinchoninic acid assay as described in the preceding paper (Mookhtiar & Van Wart, 1990). From these data, the molar extinction coefficient ϵ_{277} was calculated to be 5.6×10^4 M^{-1} cm^{-1}. This compares with the ϵ_{280} value of 6.8×10^4 M^{-1} cm^{-1} for 52-kDa HFC (Birkedal-Hansen et al., 1988).

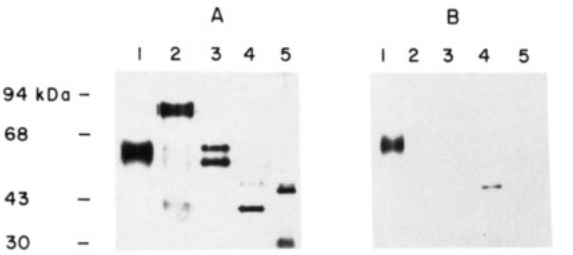

FIGURE 1: (A) SDS–PAGE gel showing 58-kDa HNC (lane 1), 92-kDa HNG (lane 2), (72 + 63)-kDa HFG (lane 3), 43-kDa HFC (lane 4), and 45-kDa HFS (lane 5; a degradation fragment is shown at the bottom of this lane). (B) Western blot of the same samples from (A) performed with antiserum against 58-kDa HNC.

Immunological Relationship between 58-kDa HNC and Other Human MMP. Polyclonal rabbit antiserum against 58-kDa latent HNC was prepared as described under Materials and Methods. Wester blots using this as the primary antibody and goat anti-rabbit IgG alkaline phosphatase conjugate as the second antibody were performed to assess whether this antibody cross-reacted with other human MMP. An SDS gel showing samples (~20 ng) of HNC (58 kDa, latent), HNG (92 kDa, latent), HFG (72 kDa, latent, + 63 kDa, active), HFC (43 kDa, active), and HFS (45 kDa, active) in lanes 1–5, respectively, is shown in Figure 1A. A Western blot from a parallel gel containing these same samples is shown in Figure 1B. This blot demonstrates that the antibody raised against 58-kDa latent HNC (lane 1) cross-reacts with HFC (lane 4), as reported by other workers (Hasty et al., 1987b). However, there is no significant cross-reactivity with HNG, HFS, or 72-kDa HFG.

Carbohydrate Processing of HNC. HFC is secreted as a zymogen with a molecular weight of 52K. Some of the zymogen molecules are glycosylated to give a form of the enzyme that has an apparent molecular weight in SDS–PAGE experiments that is approximately 5K higher. Wilhelm and associates (Wilhelm et al., 1986) have shown that the glycosylated enzyme contains N-linked complex oligosaccharides and that treatment with endoglycosidase F converts the 57-kDa

[2] There is a disagreement regarding the identity of the residue at position 96 between the studies of Goldberg et al. (1986) that indicate that it is R and those of Whitham et al. (1986) that indicate that it is T. This statement assumes that this residue is T.

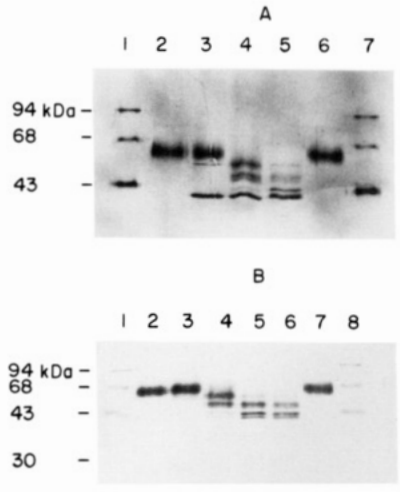

FIGURE 2: (A) SDS–PAGE gel showing molecular weight standards (lanes 1 and 7), 58-kDa HNC without treatment (lane 2), this same HNC sample incubated with endoglycosidase F for 0.1, 1, and 12 h (lanes 3–5, respectively), and the same sample incubated in digestion buffer for 12 h (lane 6). (B) Western blot of the same samples from (A) performed with antiserum against 58-kDa HNC except that a 6-h reaction time point has been inserted in lane 5.

glycosylated form directly to the unglycosylated form without detectable intermediates. In order to assess whether HNC is also a glycoprotein, a series of endoglycosidase digestions have been carried out. A sample of HNC that was 60% latent was digested with endoglycosidase F and the time course of the reaction monitored by SDS–PAGE (Figure 2A). Lanes 1 and 7 contain molecular weight standards, lane 2 contains HNC, and lanes 3–5 are samples from the reaction of HNC with endoglycosidase F for 0.1, 1, and 12 h, respectively. Lane 6 contains HNC that had been incubated in the reaction buffer without endoglycosidase F for 12 h. The reaction converts all of the 58-kDa HNC first to intermediates with molecular weights of approximately 53K and 48K, and ultimately to a sharply focused doublet with a molecular weight of approximately 43K. There is a band due to endoglycosidase F that migrates with a molecular weight of approximately 43K that makes it difficult to observe the lowest HNC band. Western blot analyses of these same samples (Figure 2B), however, clearly show that the lowest HNC band is a doublet with molecular weights of approximately 44K and 42K. These experiments strongly suggest that HNC contains complex N-linked carbohydrate at three sites and that endoglycosidase F sequentially removed this carbohydrate by hydrolyzing the 4GlcNAcβ1–4GlcNAc glycosidic bonds of the chitobiose core that is adjacent to the Asn residue of the protein chain (Maley et al., 1989). Furthermore, since the HNC sample was 60% latent, the doublet observed for the deglycosylated enzyme suggests that the two bands correspond to the latent and active enzymes.

To confirm that the changes in molecular weight observed in the endoglycosidase F digestion were not due to contaminating protease activity, a similar digestion was carried out with *N*-glycosidase F. This enzyme is an amidase that hydrolyzes the GlcNAc–Asn bond of glycoproteins (Maley et al.,1989) and, thus, should give results similar to those found for endoglycosidase F. This digestion was carried out with HNC samples that were 80% and 50% latent. Both digestions produced the same intermediate species that were observed for the digestion with endoglycosidase F (Figure 3), confirming that these species result from deglycosylation of HNC. As expected, the predominantly latent sample gave a much higher percentage of the 44-kDa band and the predominantly active sample more of the 42-kDa band (Figure 3).

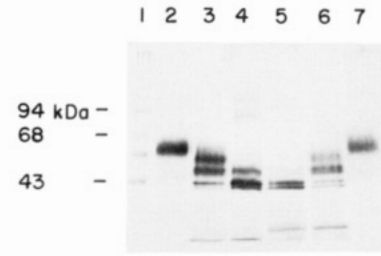

FIGURE 3: Western blot showing molecular weight standards (lane 1) and the digestion of samples of 58-kDa HNC that were 80% (lane 2) and 50% (lane 7) latent with *N*-glycosidase F for 50 min (lanes 3 and 6) and 13 h (lanes 4 and 5).

Comparative Collagen and Peptide Specificities of HNC and HFC. The kinetic parameters for the hydrolysis of rat and human type I, bovine type II, and human type III collagens by trypsin-activated HFC and PCMB-activated HNC have been determined at 30 °C to compare the collagen specificities of these two collagenases. The choice of different activators for these two enzymes was made so as to give the highest activities possible. There are a variety of methods by which latent collagenases can be activated (Springman et al., 1990), including treatment with proteolytic enzymes such as trypsin or organomercurials such as PCMB or APMA. Trypsin is a poor activator of latent 58-kDa HNC, but PCMB is quite effective. Thus, latent HNC was activated by incubation with 0.1 mM PCMB in 900 μL of assay buffer (50 mM Tricine, 10 mM $CaCl_2$, and 0.2 M NaCl, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$) for 15 min at 23 °C and the reaction initiated by addition of 100 μL of collagen. Trypsin is a better activator of latent HFC than organomercurials (Stricklin et al., 1983). Thus, activation was carried out by incubating latent HFC with 50 μg/mL trypsin in 100 μL of assay buffer which was diluted to 1 mL at the start of the assay. The final trypsin concentration in the assays of 5 μg/mL does not hydrolyze any of these collagen at an appreciable rate at the assay temperature of 30 °C (Mallya et al., 1986). Most workers quench the trypsin activity with a 5-fold excess of soybean trypsin inhibitor. However, this was found to lower activity, and this procedure was not used for the solution-phase assays. Interestingly, soybean trypsin inhibitor itself had no effect on PCMB-activated HFC, and, at present, the reason for its deactivating effect on the trypsin-activated enzymes is not known.

Initial velocities, v_i, were measured at 30 °C over a 10-fold range of collagen concentrations that spanned the K_M value for each reaction. Normal Michaelis–Menten kinetics were observed, and double-reciprocal plots (not shown) for the hydrolysis of all four collagens by both collagenases were linear. The values of K_M and k_{cat} obtained from the x and y intercepts of these plots for HFC, respectively, and values of k_{cat}/K_M are listed in Table II together with those reported by Welgus et al. (1981) under similar, but not identical, conditions. There is generally good agreement between the two sets of values, especially considering the different methods used for the measurements. The K_M values for human and rat type I collagens are very similar, while the values for bovine type II and human type III collagens are somewhat higher. However, they all lie within the 0.82–2.4 μM range. In contrast, the k_{cat} values vary markedly from a value of 3.2 h^{-1} for bovine type II to 350 h^{-1} for human type III collagens. The k_{cat}/K_M values which reflect the specificity of the enzyme show that HFC prefers human type III collagen 11-fold over rat type I, 3.9-fold over human type I, and 160-fold over bovine type II collagens.

Table II: Kinetic Parameters for the Hydrolysis of Various Soluble Collagens by HFC

collagen	k_{cat} (h^{-1})		K_M (μM)		k_{cat}/K_M (μM^{-1} h^{-1})	
	this study[a]	literature[b]	this study[a]	literature[b]	this study[a]	literature[b]
rat type I	16	19.5	0.83	0.9	19	21.7
human type I	44	53.4	0.82	0.8	54	66.8
bovine type II	3.2	2.7	2.4	1.6	1.3	1.7
human type III	350	565.0	1.7	1.4	210	403.6

[a] Assays from this study were carried out in 50 mM Tricine, 10 mM $CaCl_2$, and 0.2 M NaCl, pH 7.5 containing 0.05% Brij-35 and 50 μM $ZnSO_4$ at 30 °C. [b] Data of Welgus et al. (1981) obtained from assays carried out in 50 mM Tris, 10 mM $CaCl_2$, and 0.2 M NaCl, pH 7.5 at 25 °C.

Table III: Kinetic Parameters for the Hydrolysis of Various Soluble Collagens by Active and PCMB-Activated Latent HNC[a]

collagen	k_{cat} (h^{-1})	K_M (μM)	k_{cat}/K_M (μM^{-1} h^{-1})
Active HNC			
rat type I	370	1.1	410
human type I	250	0.93	270
bovine type II	60	1.9	32
human type III	110	2.6	42
PCMB-Activated Latent HNC			
rat type I	690	1.0	690
human type I	460	1.0	460
bovine type II	130	2.3	57
human type III	200	2.5	80

[a] Assays were carried out in 50 mM Tricine, 10 mM $CaCl_2$, and 0.2 M NaCl, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$ at 30 °C.

Table IV: Comparison of k_{cat}/K_M Values for Hydrolysis of Different Soluble Collagens by Active HNC at 25 °C from This Study with Those Reported in the Literature

collagen	k_{cat}/K_M (μM^{-1} h^{-1})	
	this study[a]	literature[b]
rat type I	210	
human type I	120	9.14
bovine type II	11	
human type III	9.0	0.47
guinea pig type I		9.68
human type II		2.14

[a] Assays were carried out in 50 mM Tricine, 10 mM $CaCl_2$, and 0.2 M NaCl, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$ at 25 °C. [b] Data of Hasty et al. (1987b) obtained from assays carried out in 50 mM Tris, 10 mM $CaCl_2$, and 0.25 M NaCl, pH 7.5 at 25 °C.

The kinetic parameters for the hydrolysis of these four collagens by both active and PCMB-activated latent HNC at 30 °C have been obtained by the same procedure and are listed in Table III. The K_M values for the hydrolysis of each collagen by active and PCMB-activated latent HNC are all very similar and range from 0.93 to 2.6 μM. The k_{cat} and k_{cat}/K_M values for the hydrolysis of each collagen by active HNC are all approximately 60% of the values for the PCMB-activated latent enzyme. Thus, the two enzymes have the same collagen specificities, but the species isolated in an active form (and which had already undergone a presumably proteolytic activation) is catalytically less efficient than the PCMB-activated latent enzyme. The k_{cat} values for the hydrolysis of the four collagens vary from 60 to 370 h^{-1} for active HNC, and from 130 to 690 h^{-1} for PCMB-activated latent HNC. The specificity of both forms of HNC toward the different collagen types, as manifested by the k_{cat}/K_M values, clearly establishes a strong preference for type I compared to type II and III collagens. This is in marked contrast to HFC, which prefers type III over type I and II collagens. Human type I collagen is hydrolyzed approximately 6-fold faster than human type III collagen by both forms of HNC at 30 °C, which is in qualitative agreement with earlier reports (Horwitz et al., 1977; Hasty et al., 1987a).

Hasty et al. (1987b) have reported kinetic parameters for the hydrolysis of guinea pig type I and human type I, II, and III collagens at 25 °C by an active form of HNC. The K_M values for human type I and III collagens reported by these authors are in good agreement with those found here, even though they were measured at a slightly lower temperature. However, the k_{cat} values reported by these authors are 39- and 130-fold lower than the values found here at 30 °C for human type I and III collagens, respectively. To assess whether these lower values were due to the difference in assay temperature, the k_{cat}/K_M values for the hydrolysis of each type of collagen by active HNC were also measured at 25 °C (Table IV). The k_{cat}/K_M values for the hydrolysis of human type I and III collagens at 25 °C are 13- and 19-fold higher than reported by these workers, indicating that their samples had a fraction of the activity of those used here. However, the ratios of the k_{cat}/K_M values for the hydrolysis of human type I and III collagens at 25 °C reported earlier (Welgus et al., 1981) of approximately 19 (Hasty et al., 1987a) and 15 (Horwitz et al., 1977) are in good agreement with the value of 13 obtained here (Table IV).

The data in Tables II and III point out some interesting differences between HNC and HFC. A comparison of the k_{cat}/K_M values shows that, with the exception of type III collagen, HNC is generally a more potent collagenase. PCMB-activated latent HNC hydrolyzes rat type I collagen 36-fold faster, human type I collagen 8.5-fold faster, and bovine type II collagen 44-fold faster than HFC. However, the k_{cat}/K_M value for the hydrolysis of type III collagen by HFC is 2.6-fold higher than that of HNC. The preference of each collagenase for the different collagens is illustrated more clearly from an internal comparison of the ratios of the k_{cat}/K_M values. This ratio for rat type I and human type III collagens is 0.090 for HFC, and 9.8 and 8.6 for active and PCMB-activated latent HNC, respectively. Thus, this ratio is 110- and 96-fold higher for active and PCMB-activated latent HNC than for HFC, respectively, clearly demonstrating the marked difference in the preferences of these two enzymes for type I and III collagens in solution. The ratios are only 25- and 22-fold higher when human type I and III collagens are compared. Interestingly, the ratios of k_{cat}/K_M values for bovine type II and human type III collagens are 120- and 110-fold higher for active and PCMB-activated latent HNC than for HFC, respectively.

It is of interest to assess whether these dramatic differences in collagen specificity are attributable to the sequence specificities of the two collagenases (since the collagens have different α-chain sequences at the cleavage sites). Thus, the rates of hydrolysis of three synthetic octapeptides with sequences matching those of the α chains at the cleavage sites of type I and III collagens have been measured. Peptides **1** and **2** are modeled after the α1(I) and α2(I) chains of chick type I collagen and have the sequences G-P-Q-G-I-A-G-Q and G-P-Q-G-L-A-G-Q (Gross et al., 1974), respectively. Peptide **3** is modeled after the human α1(III) chain with the sequence G-P-L-G-I-A-G-Q (Seyer & Kang, 1981). The k_{cat}/K_M values

Table V: Rates of Hydrolysis of Synthetic Peptides by PCMB-Activated Latent HNC and Trypsin-Activated HFC[a]

peptide	k_{cat}/K_M (μM^{-1} h^{-1}) HFC	HNC
1	0.60	7.2
2	0.72	13
3	1.4	18

[a] Assays were carried out in 50 mM Tricine, 10 mM $CaCl_2$, and 0.2 M NaCl, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$ at 30 °C.

for the hydrolysis of these peptides at the central G–(I or L) bond by both PCMB-activated latent HNC and trypsin-activated HFC are listed in Table V. A control assay with 5 μg/mL trypsin showed no hydrolysis of any of the peptides. It is clear from the data shown in Table V that HNC is a more potent peptidase than HFC and uniformly hydrolyzes **1**–**3** at significantly higher rates. More interesting, however, is the finding that the rates of hydrolysis of the three peptides by each collagenase vary very little, indicating that they do not discriminate appreciably between these sequences when they are not part of the collagen chains that they model. This lack of correlation is clearly illustrated by the ratios of the k_{cat}/K_M values for the hydrolysis of the type I collagen model peptides (**1** and **2**) to the type III model peptide (**3**) listed. If the sequences of these peptides accounted for the collagen specificities, then the ratios for the collagens (Table III) and the peptides (Table V) would be equal for both HFC and HNC. However, the ratio for the peptides varies from 0.40 to 0.72, while that for the collagens varies from 0.09 to 8.6. Thus, the rates for the peptides do not correlate with those for the collagens for either collagenase, and the collagen specificities of HFC and HNC are not directly attributable to the sequence specificities of the enzymes.

The A_{sp} values of trypsin-activated HFC and PCMB-activated latent HNC toward the fibrillar form of rat type I and human type III collagens have been measured at 37 °C (Table VI). Since type III collagen is sensitive to trypsin at this temperature, a 4-fold excess of soybean trypsin inhibitor was added before starting the assays with trypsin-activated HFC. The specificities of these collagenases toward these two fibrillar collagen types differ from those found in the solution-phase studies. The ratios of A_{sp} values for the hydrolysis of type I and III fibrils by HFC and HNC are 0.18 and 1.1, while the ratios of the k_{cat}/K_M values in solution at 30 °C are 0.090 and 8.6, respectively. Thus, the preferences of type III over type I by HFC and of type I over type III by HNC are both reduced for the fibrillar collagens, but the change is much greater for HNC. These results are somewhat different from those reported by Hasty et al. (1987b) which showed that there was a marked loss in specificity for both HFC and HNC.

Discussion

The characterization of 58-kDa HNC described here clearly establishes that it is a distinct collagenase. The N-terminal sequences of the latent and spontaneously activated enzymes show that HNC is a member of the MMP gene family and that it is most homologous to HFC. This is supported by the observation that antiserum prepared against HNC cross-reacts with HFC. Latency in all of the MMP is thought to require a propeptide containing the "cysteine switch" residue that forms a complex with the catalytic zinc atom, thus blocking the active site (Springman et al., 1990; Van Wart & Birkedal-Hansen, 1990). HFC has a ~9-kDa propeptide in which the cysteine switch residue is found at position 73. The latent 58-kDa form of HNC is strongly homologous to HFC in the cysteine switch region and presumably has a homologous C residue at this position. The N-terminus of spontaneously activated HNC corresponds to that of the 43-kDa form of HFC formed autolytically after activation of the 52-kDa enzyme by trypsin or organomercurials (Stricklin & Welgus, 1983; Grant et al., 1987). Thus, both HFC and HNC undergo an autolytic cleavage that is associated with activation near residues 80–83. The active 58-kDa form that is isolated from neutrophils when PMSF is omitted probably became activated by a serine protease that cleaved the latent enzyme near this region. Thus, latent 58-kDa HNC has only a 16-residue propeptide, and the autolytic cleavage associated with activation results in a small (1K–2K) decrease in molecular weight that can only be detected in SDS–PAGE experiments after deglycosylation. This explains an earlier report that activation of 58-kDa HNC is not accompanied by a change in molecular weight (Hasty et al., 1986).

The size of the protein chain in HNC is difficult to assess because it is glycosylated. The deglycosylation of 58-kDa HNC performed here, however, shows that the molecular weight of the active protein chain is 42K, approximately the same value found for HFC. This and the sequence data discussed above suggest that HNC and HFC have the same domain organization and that the differences in their mobilities in SDS–PAGE experiments are attributable to differences in the size of their propeptides and in the type and/or extent of glycosylation. HFC appears to be glycosylated at a single site (N_{101} or N_{124}) that increases the apparent molecular weight by 5K (Wilhelm et al., 1986). In contrast, 58-kDa HNC appears to be glycosylated at three sites, each of which increases its apparent molecular weight by 5K. One of these additional sites in HNC may be N_{94} that is an H residue in HFC. The latent 75-kDa form of HNC probably contains a full size (~9 kDa) propeptide that is itself glycosylated, accounting for the additional 17K in apparent molecular weight. HNC is stored intracellularly after synthesis, while HFC is secreted. The differences in the carbohydrate processing of these two collagenases may be related to the targeting of HNC to the specific granules of the neutrophil. This could resemble the mannose 6-phosphate marker that directs lysosomal enzymes to their destination (Kornfeld, 1987). A similar carbohydrate tag might be involved in the targeting of HNC into its storage granules.

HFC and HNC differ with regard to their collagen specificities. The preferences of HNC for type I over type II and

Table VI: Comparison of the Specific Activities (A_{sp}) of Trypsin-Activated HFC and PCMB-Activated HNC toward Fibrillar Type I and Type III Collagens

enzyme	A_{sp} (μg min^{-1} mg^{-1}) type I: this study[a]	type I: literature[b]	type III: this study[c]	type III: literature[d]	A_{sp}(type I)/A_{sp}(type III): this study[a,c]	A_{sp}(type I)/A_{sp}(type III): literature[b,d]
HFC	1100	540	6000	970	0.18	0.56
HNC	7000	130	6500	90	1.1	1.4

[a] Rat type I collagen. [b] Guinea pig type I collagen (Hasty et al., 1987a). [c] Human type III collagen. [d] Human type III collagen (Hasty et al., 1987a).

III collagens (Hasty et al., 1987a), and of HFC for type III over type I and II collagens (Welgus et al., 1981) in solution, and the leveling of these specificities on going to fibrillar collagens reported in earlier studies (Welgus et al., 1981; Hasty et al., 1987a) have been confirmed. However, our results differ from previous studies in that we find HNC to be a more potent collagenase than HFC and that HFC retains a 5-fold preference for type III over type I collagen fibrils. The latter difference could be because the type I collagen used in the earlier study (Hasty et al., 1987a) was obtained from guinea pig while that used in this study was from rat tendon. The large differences in collagen specificity (k_{cat}/K_M) in solution are due primarily to different values of k_{cat}. Thus, the two collagenases discriminate between the collagen types in the catalytic rather than the binding step. These differences are remarkable given the similarity in the cleavage site sequence in these collagens. The k_{cat}/K_M values for the hydrolysis of peptides with these sequences do not reflect the dramatic differences found for the collagens. Thus, the collagen specificities of HFC and HNC cannot be explained by the sequence specificities of these collagenases. Apparently, the collagenases discriminate between these collagens on the basis of some presently unrecognized conformational feature of the cleavage site.

Registry No. **1**, 109053-05-6; **2**, 109053-14-7; **3**, 109053-09-0; collagenase, 9001-12-1; procollagenase, 39287-99-5.

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