Biochemical Characterization of Human Collagenase-3*

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Vera Knäuper§, Carlos López-Otin*, Bryan Smith, Graham Knight**, and Gillian Murphy‡

From the Strangeways Research Laboratory, *Department of Cell and Molecular Biology and **Department of Cell Adhesion and Signalling, Worts' Causeway, Cambridge CB1 4RN, United Kingdom, the ‡Univua de Oviedo, Departamento de Biología Funcional, 33006 Oviedo, Spain, and ||Ceitech Ltd., 216 Bath Rd., Slough SL1 4EN, United Kingdom

The cDNA of a novel matrix metalloproteinase, collagenase-3 (MMP-13) has been isolated from a breast tumor library (Freije, J. M. P., Diez-Itza, I., Balbin, M., Sanchez, L. M., Blasco, R., Tovila, J., and López-Otin, C. (1994) J. Biol. Chem. 269, 16766–16773), and a potential role in tumor progression has been proposed for this enzyme. In order to establish the possible role of collagenase-3 in connective tissue turnover, we have expressed and purified recombinant human procollagenase-3 and characterized the enzyme biochemically. The purified procollagenase-3 was shown to be glycosylated and displayed a Mₐ of 60,000, the N-terminal sequence being LPLPSGKD, which is consistent with the cDNA-predicted sequence. The proenzyme was activated by p-aminophenylmercuric acetate or stromelysin, yielding an intermediate form of M₁, 50,000, which displayed the N-terminal sequence LGKDVVTGK. Further processing resulted in cleavage of the Glu⁴⁸-Tyr⁵⁰ peptide bond to the final active enzyme (M₂, 48,000). Trypsin activation of procollagenase-3 also generated a Tyr⁵⁰ N terminus, but it was evident that the C-terminal domain was rapidly lost, and hence the collagenolytic activity diminished. Analysis of the substrate specificity of collagenase-3 revealed that soluble type II collagen was preferentially hydrolyzed, while the enzyme was 5 or 6 times less efficient at cleaving type I or III collagen. Fibillar type I collagen was cleaved with comparable efficiency to the fibroblast and neutrophil collagenases (MMP-1 and MMP-8), respectively. Unlike these collagenases, gelatin and the peptide substrates Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ and Mca-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH₂ were efficiently hydrolyzed as well, as would be predicted from the similarities between the active site sequence of collagenase-3 (MMP-13) and the gelatinases A and B. Active collagenase-3 was inhibited in a 1:1 stoichiometric fashion by the tissue inhibitors of metalloproteinases, TIMP-1, TIMP-2, and TIMP-3. These results suggest that in vivo collagenase-3 could play a significant role in the turnover of connective tissue matrix constituents.

The human matrix metalloproteinases (MMPs)¹ comprise a family of at least 11 homologous zinc-dependent endopeptidases that degrade the macromolecular components of extracellular matrices. They have been implicated in matrix remodeling processes associated with normal mammalian development and growth and in the degenerative processes accompanying arthritis and tumor invasion. The MMPs can be divided into three main subfamilies, collagenases, stromelysins, and gelatinases, and other enzymes that do not belong to these groupings. Three highly homologous human collagenases, fibroblast (MMP-1), neutrophil (MMP-8), and collagenase-3 (MMP-13) have been identified by analysis of their respective cDNAs (Goldberg et al., 1986; Whitham et al., 1986; Hasty et al., 1990; Freije et al., 1994). Sequence comparison revealed that they share more than 50% sequence identity and three functionally important domains, namely the propeptide, catalytic, and C-terminal domains. Procollagenase latency is due to the propeptide domain, which consists of about 80 amino acids including a free cysteine residue within the highly conserved PRCGVPD sequence motif. The catalytic domain of about 180 amino acids contains two or one calcium and two zinc binding sites as revealed by x-ray crystallographic analysis of the catalytic domains of fibroblast and neutrophil collagenases in the presence of synthetic inhibitors (Borkakoti et al., 1994; Bode et al., 1994; Lovejoy et al., 1994). The structure comprises a five-stranded β-sheet, two bridging loops, and two α-helices. The C-terminal domain is linked via a short hinge sequence motif to the catalytic domain and shares sequence homology with vitronectin, being essential for the triple helicase activity of fibroblast and neutrophil collagenases (Murphy et al., 1992; Clark and Cawston, 1989; Sanchez-Lopez et al., 1993; Hirose et al., 1993; Knäuper et al., 1993a). The active enzymes form tight binding noncovalent complexes with their natural inhibitors, referred to as tissue inhibitors of metalloproteinases (TIMPs), in a 1:1 stoichiometric fashion. The interaction of the collagenases with TIMPs is mainly regulated by the catalytic domain (Murphy et al., 1992), but C-terminal domain interactions increase the association rates of complex formation.

Biochemical studies on fibroblast and neutrophil collagenases describing their activation mechanism, substrate specificity, and inhibitor interaction in relation to their domain organization are well advanced (Murphy et al., 1987, 1992; Clark and Cawston, 1989; Hirose et al., 1993; Sanchez-Lopez et al., 1993; Knäuper et al., 1990a, 1990b, 1993a, 1993b), but there are currently no data available regarding the activation

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§To whom correspondence should be addressed. Tel.: 44-1223-243231; Fax: 44-1223-411609.

¹The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; Mca, (7-methoxycoumarin-4-yl)-acetyl; Cha, 3-cyclohexylalanine; Nva, norvalyl; Dpa, N-3-(2, 4-dinitrophenyl)-l-2,3-diaminopropionyl; Dnp, 2,4-dinitropheeryl; Nma, N-methylanthranilyl; APMA, p-aminophenylmercuric acetate; PAGE, polyacrylamide gel electrophoresis; CT398, N⁴-hydroxy-N⁴-(1-(S)-methylaminoacarboxyl-2-methylthiopropyl)-2-(R)-(4-chlorophenyl)propionic sucinamide; CT1847, N⁴-hydroxy-N⁴-(1-(S)-methylaminoacarboxyl-2-methylthiopropyl)-2-(R)-(2-methylpropyl)succinamide; HPLC, high performance liquid chromatography; NSO, non-secretor zero; TPCK, L-tosylamide-2-phenylethyl chloromethyl ketone.
human collagenase-3. We have therefore expressed the human collagenase-3 cDNA in a mammalian expression system and characterized the purified recombinant enzyme in comparison to the fibroblast and neutrophil collagenases.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Recombinant Procollagenase-3—The procollagenase-3 cDNA (Freje et al., 1994) was subcloned as a HindII fragment into the mammalian expression vector pEE12. The Naelinearized plasmid DNA (50 μg) was transfected into NSO mouse myeloma cells in suspension following selection of stably transfected cell lines in glutamine-free defined medium (Bebington et al., 1992). Serum-free culture medium from cells expressing high levels of procollagenase-3 was dialyzed against 20 mM Tris-HCl, pH 7.2, 5 mM CaCl₂, 0.05% NaN₃ and loaded onto a S-Sepharose fast flow (Pharmacia Biotech Inc.) column (3.2 x 10 cm). The matrix was washed in the above buffer to background A₂₈₀ readings followed by a wash step with buffer supplemented with 250 mM NaCl to remove impurities. Final elution of procollagenase-3 was achieved using 20 mM Tris-HCl, pH 7.2, 5 mM CaCl₂, 500 mM NaCl, 0.05% NaN₃ yielding 98% pure procollagenase-3 as judged by silver-stained SDS-PAGE (Laemmli and Favre, 1973; Heukeshoven and Dernick, 1985). Final purification was achieved by gel filtration chromatography using Sephacryl S-200 equilibrated in 20 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 300 mM NaCl, 0.05% NaN₃. About 6 mg purified procollagenase-3 was isolated from 400 ml of culture medium.

Activation of Procollagenases and Determination of Their Concentration—Procollagenase-3 and neutrophil procollagenase were routinely activated by incubation with 1 mM APMA at 37 °C. Procollagenase-3 (20 μg) was activated with either 1.4 or 2.8 μg of active stromelysin at 37 °C for up to 7 h. In order to achieve “superactivation” of both neutrophil and fibroblast procollagenase (purified according to Knauper et al., 1990a and Murphy et al., 1992), the enzymes were activated by combined treatment with trypsin and stromelysin (Knauper et al., 1993b; Murphy et al., 1992). The concentrations of the three active collagenases were determined by titration against the synthetic hydroxamic acid based metalloproteinase inhibitors CT1399 and CT1847 (kindly provided by Celltech Ltd., Slough, United Kingdom).

Activity Assays—The specific activities of the active collagenases were determined using 10°C-labeled type I collagen in a diffuse fibrill assay at 35 °C essentially as described by Cawston et al. (1981). Correspondingly, the gelatinolytic activity was determined at 37 °C using the fluorogenic substrate Mca-Pro-Cha-Gly-Nva-His-Ala-Dpa-(9-fluorenyl)methoxycarbonyl) on a Perceptive Biosystems 9050 Plus PepSynthesizer (Millipore) using standard procedures (Fields and Noble, 1990), characterized by time-of-flight mass spectrometry.

N-Terminal Amino Acid Sequencing—N-terminal sequence determinations of purified procollagenase-3 or active collagenase-3 were performed by automated Edman degradation using an Applied Biosystems 470A protein sequencer with on-line 190A HPLC for phenylthiodyanin-derivative analysis.

**RESULTS**

Expression and Purification of Human Procollagenase-3—Human procollagenase-3 was expressed by stable transfected NSO mouse myeloma cells and purified using S-Sepharose fast flow and Sephacyr S-200. The procollagenase-3 preparation was free of other matrix metalloproteinases as assessed by gelatin and casein zymographic analysis (results not shown). The final purified procollagenase-3 displayed a Mᵦ of 60,000 when analyzed by SDS-PAGE under reducing conditions (Fig. 1, lane 1). The proenzyme was shown to be glycosylated as demonstrated by N-glycosidase F treatment (Fig. 1, lane 2). This reduced the Mᵦ to 53,600, which is in excellent agreement with the Mᵦ predicted from the cDNA sequence. Thus 10% of the Mᵦ of the proenzyme corresponds to N-linked sugars. N-terminal amino acid sequencing of procollagenase-3 revealed the sequence LPLPSGD, which is consistent with the cDNA predicted sequence. The Asn98 residue was clearly glycosylated due to the lack of a signal during amino acid sequencing.

**Purification of Recombinant Tissue Inhibitors of Metalloproteinases TIMP-1, TIMP-2, and TIMP-3 and Determination of Their Concentrations—**The human TIMP-1, TIMP-2, and TIMP-3 cDNAs were expressed in transfected NSO mouse myeloma cells (Murphy et al., 1991; Willenbrock et al., 1993; Apte et al., 1995). TIMP concentrations were determined by titration against human recombinant stromelysin, the concentration of which had been determined by titration with a standard preparation of TIMP-1 (concentration determined by amino acid analysis) (Murphy and Willenbrock, 1995). Inhibiting Studies Using Synthetic Hydroxamic Acid-based Inhibitors CT1399 and CT1847—The rate constants for the association of 50 pm active collagenase-3 with the synthetic inhibitors CT1847 (2–8 mM) and CT1399 (100–300 mM) were determined by analysis of the progress curves of Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Ala-Arg-NH₂ hydrolysis (Willenbrock et al., 1993). The apparent Kᵢ values for both inhibitors were determined using the following equations:

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Kᵢ = Kᵦ (Vᵢ / Vᵦ)Kᵦ /
\]

**Expression and Purification of Recombinant Procollagenase-3**

Human procollagenase-3 was expressed by stable transfected NSO mouse myeloma cells and purified using S-Sepharose fast flow and Sephacyr S-200. The procollagenase-3 preparation was free of other matrix metalloproteinases as assessed by gelatin and casein zymographic analysis (results not shown). The final purified procollagenase-3 displayed a Mᵦ of 60,000 when analyzed by SDS-PAGE under reducing conditions (Fig. 1, lane 1). The proenzyme was shown to be glycosylated as demonstrated by N-glycosidase F treatment (Fig. 1, lane 2). This reduced the Mᵦ to 53,600, which is in excellent agreement with the Mᵦ predicted from the cDNA sequence. Thus 10% of the Mᵦ of the proenzyme corresponds to N-linked sugars. N-terminal amino acid sequencing of procollagenase-3 revealed the sequence LPLPSGD, which is consistent with the cDNA predicted sequence (Fig. 2). A minor portion of the secreted procollagenase-3 displayed the N-terminal amino acid sequence LPLPSGD. The loss of Leu³ in a part of the enzyme preparation may be due to the activity of a leucine aminopeptidase produced by the NSO mouse myeloma cells during the average culture period of 2 weeks. The procollagenase-3 preparation was >98% latent and displayed barely detectable levels of enzymatic activity prior to activation, and it can be concluded that the loss of Leu³ did not affect the latency of the proenzyme.

In contrast, autoactivated collagenase-3 displayed a Mᵦ of 48,000 when analyzed by SDS-PAGE, and its proteolytic activity could not be enhanced by APMA treatment. N-terminal amino acid analysis revealed the sequence YNVF PRTLK WSK-MXL demonstrating the complete loss of the propeptide domain and assigning Tyr₁⁸⁵ as the first amino acid of the active enzyme. The Asn⁹⁸ residue was clearly glycosylated due to the lack of a signal during amino acid sequencing.

**Activation of Procollagenase-3 by APMA—**Purified procollagenase-3 was activated by treatment with 1 mM APMA in a
Human Collagenase-3 (MMP-13)

![Diagram of Collagenase-3 structure](image)

**Fig. 2.** N-terminal sequence determination of procollagenase-3 and activated collagenase-3. N termini of APMA or stromelysin activated collagenase-3 are indicated by arrows.

**time-dependent fashion (Fig. 3A).** The activity generated was monitored using Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ described by Knight et al. (1992). Full activation was achieved after a time interval of 30 min. Parallel analysis of the Mr of the enzyme by SDS-PAGE revealed that the Mr of the proenzyme was reduced through at least one short-lived intermediate form (Mr, 50,000) to the final active collagenase-3 displaying a Mr of 48,000 (Fig. 1, lanes 3–5). Preincubation of procollagenase-3 with a two molar excess of recombinant TIMP-1 prior to APMA activation prevented the formation of the low Mr, final active enzyme form (Mr, 48,000), clearly demonstrating that this process was autoproteolytic (Fig. 1, lane 7). Under these conditions, two intermediate enzyme forms were demonstrated displaying apparent Mr values of 56,000 and 50,000. The Mr, 56,000 intermediate was not detectable in the absence of TIMP-1, which might indicate that it was extremely unstable being rapidly converted to the Mr, 50,000 species. This might indicate that procollagenase-3 activation by APMA is a three-step process.

N-terminal amino acid sequencing showed the initial activation of the sequence LEVTGKL after a 4-min activation of procollagenase-3 by APMA, which is due to the cleavage of the Gly⁵⁷–Leu⁵⁸ peptide bond (Fig. 2). Furthermore, the active enzyme was not stable in the presence of TPCK-treated trypsin during the start of the reaction. During the activation progress, the proenzyme was converted to the active collagenase-3 displaying a molecular mass of 48,000, which showed the new Mr terminus Tyr⁸⁵ as a result of the hydrolysis of the Glu⁸⁴–Tyr⁸⁵ peptide bond. Thus it was not the result of tryptic cleavage that should occur after Lys or Arg residues in position P₁. It has therefore to be concluded that the initial tryptic cleavage in the propeptide domain lead to the autoproteolytic loss of the rest of the propeptide by an autoproteolytic event. Furthermore, the active enzyme was not stable in the presence of TPCK-treated trypsin and was further hydrolyzed into smaller sized fragments, which might be due to tryptic cleavage at the Lys⁴¹⁴–His⁴¹⁸ or Lys⁴⁴⁴–Thr⁴⁵⁰ peptide bonds within the hinge region of collagenase-3. Identical results were obtained when a mixture of TPCK-treated trypsin, and stromelysin was used to activate procollagenase-3. As the collagenticolytic activity of collagenase-3 is dependent on the presence of the C-terminal domain, we did not determine the specific activity of trypsin activated collagenase-3 since high amounts of the catalytic and C-terminal domain were present in the reaction mixture even after only short incubation times (Fig. 1, lane 13). In addition, the activity versus the peptide substrate declined during prolonged incubation such that after 3 h only 60% of the initial maximal activity was retained, while the active enzyme was completely converted to the catalytic and C-terminal domain respectively (Fig. 1, lane 14).

**Determination of the Substrate Specificity of Collagenase-3:** Physiologically Relevant Substrates—Active collagenase-3 degraded the interstitial collagens (I, II, III) at 25 °C into typical 3/4 and 1/4 fragments. Collagenase-3 cleaved type II collagen about 5 times faster than type I and 6 times faster than type III collagen. Attempts to quantitate the cleavage of soluble type I, II, and III collagen using the SDS-gel scanning protocol (Welig et al., 1981) were performed at varying enzyme to substrate ratios, but it proved impossible to establish linearity. In addition, we found that the 3/4 fragments were stained more efficiently than intact collagen, which made it impossible to accurately quantify collagenolysis. Quantitative comparison of the activity of collagenase-3 relative to those of fibroblast and neutrophil collagenase was therefore only possible by generating data simultaneously using the ³H-labeled type I collagen difuse fibril assay, and these are summarized in Table I. Collagenase-3 displayed a specific activity of 100 μg/min/nmol, which was comparable with those values obtained for “super-

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2 V. Knäuper and G. Murphy, unpublished results.
Activated fibroblast or APMA-activated neutrophil collagenase. In contrast, "superc Activated" neutrophil collagenase was 3 times as active and can be assigned as the most efficient type I collagenolytic enzyme in the human.

The gelatinolytic activity of collagenase-3 and its homologous counterparts were determined using \(^{14}C\)gelatin (Table I). Collagenase-3 displayed the highest specific activity, 90.7 μg/min/mmol, respectively. Thus the enzyme was 44 times more efficient than fibroblast and 3-8 times better than neutrophil collagenase.

The rapid proteolytic degradation of two different serpins (α-antichymotrypsin and plasminogen activator inhibitor 2) by highly purified active collagenase-3 was demonstrated by SDS-PAGE, while antithrombin III was resistant to degradation (not shown). Further analysis of the α1-antichymotrypsin cleavage products by N-terminal amino acid sequence determination revealed that collagenase-3 hydrolyzed the Ala^{362}-Leu^{363} peptide bond within the extended reactive site loop of the serpin, two amino acid residues downstream from the reactive site center. The cleavage of the Ala^{362}-Leu^{363} peptide bond of α1-antichymotrypsin coincides with its inactivation as recently demonstrated by Mast et al. (1991) for collagenase (MMP-1) and stromelysin (MMP-3).

Active site titrations of fully APMA-activated collagenase-3 were performed using the synthetic inhibitor CT1399 to determine the enzyme concentration. The initial rate of substrate hydrolysis showed linear dependence on substrate concentration in the concentration range 0.7-8 μM, demonstrating that kcat ≈ 8 μM. At substrate concentrations greater than 8 μM, estimates could not be made due to insolubility of the substrate and absorptive quenching. Therefore, individual values of kcat and Km could not be determined. The values of kcat/Km for the hydrolysis of both substrates were estimated at substrate concentrations of 0.7 and 1.4 μM, which fulfilled the requirements of [S] < Km allowing direct determination of kcat/Km. Simultaneously, kcat/Km values for fibroblast and neutrophil collagenase were determined under identical conditions and compared with the values obtained for collagenase-3 (Table II). Collagenase-3 hydrolyzed both synthetic peptide substrates 70-100 or 7-10 times more efficiently than fibroblast or neutrophil collagenase. Thus collagenase-3 is the most potent peptidolytic enzyme of all three homologous collagenases.

Inhibition of Active Collagenase-3 by TIMPs—Inhibition studies of active collagenase-3 with TIMP-1, TIMP-2, and TIMP-3 were performed by preincubation of collagenase-3 with TIMP concentrations (determined by active site titration with active stromelysin) up to 2 times the enzyme concentration (determined by active site titration with CT1399) using 2-h preincubations. Residual enzymic activities were determined by hydrolysis of Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ and plotted versus TIMP concentration. Analysis of the data revealed that all three TIMPs inhibited the enzyme in a 1:1 stoichiometric fashion (Fig. 4). Initial kinetic analysis of collagenase-3TIMP interaction demonstrated that TIMP-1 showed association rate constants in the region of 8 × 10⁶ M⁻¹ s⁻¹ and TIMP-3 -10 × 10⁷ M⁻¹ s⁻¹, while the value for TIMP-2 was -1.8 × 10⁷ M⁻¹ s⁻¹. Thus TIMP-3 reacted 12 times faster than TIMP-1 and 5.5 times faster than TIMP-2.

Inhibition of Active Collagenase-3 by Hydroxamic Acid-based Inhibitors and Kinetic Analysis of Their Interaction—The collagenase-3 concentration was determined by active site titration using the synthetic hydroxamic acid-based peptide inhibitors CT1399 and CT1847. These inhibitors are competitive and react with 1:1 stoichiometry as revealed from x-ray crystallographic analyses of structurally related inhibitors with the catalytic domains of fibroblast and neutrophil collagenase (Lovejoy et al., 1994; Borkakoti et al., 1994; Bode et al., 1994). Apparent kcat values for their interaction with collagenase-3 were determined as described under "Experimental Procedures." The enzyme (50 pmol) was added to the reaction mixture containing 0.7 μM substrate and 100-300 pmol CT1399 or 2-8 nm CT1847. Inhibition was observed as curvature in the progress of substrate hydrolysis and analyzed according to Willenbrock et al. (1993). Equivalent assays in the absence of inhibitor

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**TABLE I**

| Enzyme | N terminus | Collagen | Gelatin |
|--------|------------|----------|---------|
| MMP-1  | Phε³⁵      | 120.5    | 2.2     |
| MMP-8  | Met³⁴ or Leu³⁵ | 106.7    | 10.8    |
| MMP-13 | Tyr³⁵      | 338.5    | 25.2    |
|         |            | 100.6    | 90.7    |
Human collagenase-3 (MMP-13) was a novel member of the matrix metalloproteinase subfamily and has been cloned from a breast tumor cDNA library (Frei
er et al., 1994). The enzyme is expressed in the surrounding endothelia of the tumor and may be involved in tumor progression and metastasis. Consequently, biochemical analysis of the activation mechanism, substrate specificity, and inhibition profile of collagenase-3 is of vital importance in order to understand its possible role in vivo. We have, therefore, expressed and purified recombinant human procollagenase-3 and analyzed its biochemical properties in detail and compared these with the homologous human collagenases and gelatinase A.

Procollagenase-3 showed a high degree of N-linked glycosylation as demonstrated by enzymatic deglycosylation (11.7% of its M_r corresponds to N-linked sugars). Amino acid sequencing revealed a lack of signal for the Asn^98 residue, thus it can be deduced that the glycosylation site N^98LT carries N-linked sugars. This glycosylation site is conserved between collagenase-3, neutrophil collagenase (Knauper et al., 1990b), and gelatinase-B and is occupied in all three enzymes. The role of the high levels of glycosylation observed for these three enzymes is not quite clear to date. It has been speculated that glycosylation of neutrophil collagenase and gelatinase-B might be important for targeting these enzymes to the specific granules of neutrophils, where they are stored prior to exocytosis. However, in the case of collagenase-3 it is not clear where the enzyme might be produced in vivo and why it carries a relatively high amount of N-linked sugars. It is most unlikely that the glycosylation will cause any changes in the enzymatic properties, activation, or TIMP interaction of collagenase-3, since studies on the natural and recombinant catalytic domain of neutrophil collagenase have shown that the unglycosylated recombinant protein has indistinguishable enzymatic properties (Knauper et al., 1993a; Schnierer et al., 1993). Activation of matrix metalloproteinases is one of the control mechanisms regulating extracellular connective tissue turnover. We have therefore studied the mechanisms leading to procollagenase-3 activation. Stromelysin activated procollagenase-3 by a two-step mechanism, which is similar to that observed for gelatinase-B (Shapiro et al., 1995; Ogata et al., 1992). In addition, neutrophil procollagenase was activated by stromelysin by a single-step mechanism (Knauper et al., 1993b), while the fibroblast procollagenase cannot be directly activated by stromelysin (Murphy et al., 1987; Suzuki et al., 1990). The peptide bonds cleaved within procollagenase-3, neutrophil procollagenase, and gelatinase-B seem to be readily accessible to stromelysin, while fibroblast procollagenase is resistant until proteolysis of upstream regions of the propeptide have been affected by combined trypsin-stromelysin treatment leading to "superactivation" (Murphy et al., 1987; Suzuki et al., 1990). In contrast, procollagenase-3 was very susceptible to either trypsin alone or trypsin in combination with stromelysin, which lead to the rapid loss of the C-terminal domain, thereby destroying the collagenolytic activity of the enzyme. Although relatively high amounts of stromelysin were needed to activate procollagenase-3 efficiently over 6 h, this activation pathway may still be of relevance in vivo, since very high levels of stromelysin have been observed under certain pathological conditions (Walakovits et al., 1992; Matrisian and Bowden, 1990).

Collagenase-3 can be assigned to the collagenase subfamily of matrix metalloproteinases, according to substrate specificity analysis, hydrolyzing the interstitial collagens I-III into 3/4 and 1/4 fragments preferentially cleaving type II collagen over type I and III. In contrast, fibroblast collagenase preferentially cleaves type III and neutrophil collagenase type I collagen.

| Enzyme | k_m/K_m | k_m/K_m |
|--------|---------|---------|
| MMP-1 (Phe^98 N terminus) | 1.21 x 10^4 | 2.5 x 10^4 |
| MMP-8 (Met^90 or Leu^91 N terminus) | 1.35 x 10^4 | 2.5 x 10^4 |
| MMP-8 (Phe^98 N terminus) | 1.93 x 10^4 | 2.5 x 10^4 |
| MMP-13 (Tyr^93 N terminus) | 7.57 x 10^4 | 1.09 x 10^5 |

**DISCUSSION**

Human collagenase-3 is a novel member of the matrix metalloproteinase superfamily and has been cloned from a breast tumor cDNA library (Frej et al., 1994). The enzyme is expressed in the surrounding endothelia of the tumor and may be involved in tumor progression and metastasis. Consequently, biochemical analysis of the activation mechanism, substrate specificity, and inhibition profile of collagenase-3 is of vital importance in order to understand its possible role in vivo. We have, therefore, expressed and purified recombinant human procollagenase-3 and analyzed its biochemical properties in detail and compared these with the homologous human collagenases and gelatinase A.

**TABLE II**

**Comparison of the proteolytic activities of collagenase-3 (MMP-13), fibroblast collagenase (MMP-1), and neutrophil collagenase (MMP-8) versus Mca-Pro-Leu-Gly-Leu-Dpa-Ala-NH2 and Mca-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH2**

The activated collagenases were incubated with the synthetic quenched peptide substrates at 25°C at 1.4 and 0.7 μM substrate concentration, which fulfilled the requirements of [S] < K_m allowing direct determination of k_m/K_m.

**FIG. 4. Inhibition of active collagenase-3 by the three homologous TIMPs.** Active collagenase-2 (2 nM) was incubated with increasing concentrations of either TIMP-1 (●), TIMP-2 (■) or TIMP-3 (▲).
Comparison of the ratios of collagenolytic activity over gelatinolytic activity of collagenase-3 (MMP-13), fibroblast collagenase (MMP-1), and neutrophil collagenase (MMP-8)

| Enzyme       | Ratio of collagenolytic/gelatinolytic activity |
|--------------|-----------------------------------------------|
| MMP-1 (Phe($^{1}$)) | 54.7                                          |
| MMP-8 (Met($^{20}$) or Leu($^{19}$)) | 98.8                                          |
| MMP-8 (Phe($^{29}$)) | 134                                          |
| MMP-13 (Tyr($^{45}$)) | 1.1                                          |

Collagenase-3 cleaved gelatin and the two synthetic peptide substrates with highly improved efficiency when compared with fibroblast or neutrophil collagenase. Thus, it appears that collagenase-3 not only efficiently degrades type I collagen, but it might also act as a gelatinase to further degrade the initial cleavage products of collagenolysis to small peptides suitable for further metabolism. This is in agreement with results obtained earlier for rat collagenase, which shows relatively high levels of gelatinolytic activity (Welgus et al., 1985) and shares the highest degree of homology with human collagenase-3, as does mouse collagenase (Henriet et al., 1992; Quinn et al., 1990). According to the high degree of functional and sequence homology between human collagenase-3 and the rodent collagenases, these enzymes belong to the collagenase-3 subfamily (MMP-13) of matrix metalloproteinases and are distinct from human fibroblast collagenase (MMP-1). We therefore propose to introduce a revised nomenclature for the rodent collagenases to prevent further confusion in the literature assigning them as MMP-13. Indeed, it may be concluded that rat and mouse cells express only collagenase-3 (MMP-13), there being no evidence to date for a homologous MMP-1 in either rat or mouse. The relative distribution of fibroblast collagenase (MMP-1) and collagenase-3 (MMP-13) in human tissues awaits detailed studies, but initial observations suggest that MMP-1 is predominant.

Comparison of the ratios of gelatinolytic over peptidolytic activity of collagenase-3 with those values obtained for human gelatinase A revealed that collagenase-3 is 10 times less efficient than wild-type gelatinase A (Murphy et al., 1994). The high efficiency of wild-type gelatinase A against gelatin as a substrate can be attributed to the fibronectin-like type II repeat, since a gelatinase A deletion mutant (Δ$_{193}$-238) of gelatinase A lacking these sequence motifs has a similar ratio of gelatinolytic over peptidolytic activity to collagenase-3 (Murphy et al., 1994). Thus collagenase-3 shares some proteolytic characteristics with the gelatinase subfamily of matrix metalloproteinases, which is reflected in common structural elements shared by collagenase-3 and the gelatinases being localized within the active site cleft as discussed below.

Sequence alignments of the active site residues of the collagenases with the gelatinases revealed that the Arg (Fig. 5, number 1) in fibroblast collagenase is changed to Ile or Leu in collagenase-3, the rodent collagenases, neutrophil collagenase, and in the gelatinases. It has been noted by Stams et al. (1994) that the S$_{3}$-pocket in neutrophil collagenase is significantly larger than the equivalent pocket in fibroblast collagenase and that we can deduce that due to the presence of Leu within collagenase-3 and the gelatinases that these have a similar enlarged S$_{3}$-pocket and structure. Hence these enzymes should be able to hydrolyze a broader range of substrates.

Second, collagenase-3, neutrophil collagenase, and the rodent homologues share a Pro residue (Fig. 5, number 3) with the gelatinases, while fibroblast collagenase has an Ile residue in this position. Furthermore, collagenase-3, the rodent enzymes, and the gelatinases contain negatively charged residues just preceding the third His residue of the catalytic zinc binding motif (either Asp or Glu; Fig. 5, number 2). In contrast, this residue corresponds to Ser or Ala in fibroblast or neutrophil collagenase. The presence of a negatively charged residue in collagenase-3 and the gelatinases might well have implications on the polarization of the zinc-bound water molecule within these enzymes, possibly increasing its nucleophilic nature (Fig. 6). This would certainly account for the increased proteolytic efficiency of collagenase-3 and the gelatinases, as indicated by our experimental results, but it remains to be confirmed by site-directed mutagenesis.

Analysis of the inhibition profile of collagenase-3 by the three homologous TIMPs revealed that all react in 1:1 stoichiometry by forming noncovalent tight-binding complexes, which is in agreement with earlier published data on other matrix metalloproteinases (for review see, Murphy and Willenbrock (1995)).
Comparison of the efficacy of two synthetic hydroxamate inhibitors against collagenase-3 confirmed the structural similarity to the gelatinases. CT1399, which has a $K_i$ of less than 10 pM for gelatinase A and 16 pM for gelatinase B, had an approximate $K_i$ of $\sim$4 pM for collagenase-3 and a $K_i$ of 385 nM for MMP-1. Similarly, CT1847, which has a $K_i$ of 1.55 nM against gelatinase A and 2.1 nM against gelatinase B had $K_i$ values of 0.54 nM against collagenase-3 and of 2.9 nM against MMP-1. It may be concluded that inhibitors directed against gelatinase-3 will also be efficient in the control of collagenase-3.

Our studies have indicated that human collagenase-3 is a potent proteinase with a broad spectrum of activity against extracellular matrix proteins (data not shown) as well as collagenolytic and high gelatinolytic activity. The regulation and location of its expression relative to the more specific fibroblast collagenase will be a matter of great importance for future study.

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