Matrix Metalloproteinase-1 Takes Advantage of the Induced Fit Mechanism To Cleave the Triple-Helical Type I Collagen Molecule†

Thomas J. O'Farrell, Rong Guo, Hisashi Hasegawa, and Tayebeh Pourmotabbed*

Department of Molecular Sciences, The University of Tennessee Health Science Center, Memphis, Tennessee 38163

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ABSTRACT: The collagenases are members of the matrix metalloproteinase family (MMP) that degrade native triple-helical type I collagen. To understand the mechanism by which these enzymes recognize and cleave this substrate, we studied the substrate specificity of a modified form of MMP-1 (FC) in which its active site region (amino acids 212−254) had been replaced with that of MMP-9 (amino acids 395−437). Although this substitution increased the activity of the enzyme toward gelatin and the peptide substrate Mca-PLGL(Dpa)AR-NH_2 by ∼3- and ∼11-fold, respectively, it decreased the type I collagenolytic activity of the enzyme to 0.13%. The replacement of Gly^{233}, the only amino acid in this region of FC that is conserved in all collagenase family members, with the corresponding Glu residue in MMP-9 resulted in a substantial decrease in the type I collagenolytic activity of the enzyme without affecting its general proteolytic activities. The kinetic parameters of the FC/G233E mutant for the collagen substrate were similar to those of the chimeric enzyme. In addition, substituting Gly^{233} for Glu in the chimera increased the collagenolytic activity of the enzyme by 12-fold. Interestingly, replacing Glu^{415} in MMP-9 with Gly, its corresponding residue in FC, endowed the enzyme with type I collagenolytic activity. The catalytic activity of the MMP-9 mutant toward triple-helical type I collagen was 2-fold higher than that of the collagenase chimera. These data in conjunction with the X-ray crystal structure of FC indicate that Gly^{233} provides the flexibility necessary for the enzyme active site to change conformation upon substrate binding. The flexibility provided by the Gly residue is essential for type I collagenolytic activity.

Turnover of extracellular matrix collagens by matrix metalloproteinases (MMPs)[1] is considered to be an important part of several normal physiological and pathological processes, such as skeletal growth and remodeling (*1*, *2*), wound healing (*3*, *4*), periodontal disease (*5*, *6*), cancer (*7*−*12*), and arthritis (*13*−*16*). There are four subclasses of MMPs: collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), and membrane-type MMPs (MMP-14−17). Most MMPs are secreted from cells as catalytically latent species and activated by proteolytic processing of their amino termini. With few exceptions, MMPs are composed of three domains: an amino-terminal pro-domain, a catalytic domain containing the catalytic zinc-binding active site region (AS), and a carboxyl-terminal domain that consists of a proline-rich hinge region followed by a hemopexin-like domain (reviewed in refs *17*−*21*).

The collagenases are some of the well-characterized MMPs. They are able to digest several extracellular matrix proteins but are best known for their ability to cleave fibrillar type I collagen. Since turnover of type I collagen is an important part of connective tissue remodeling, understanding the mechanism by which collagenases recognize and cleave this substrate has been of great interest. Collagenases cleave the α1 and α2 chains of triple-helical type I collagen at a unique bond, generating characteristic three-quarter and one-quarter length fragments. However, according to peptide substrate specificity studies and the primary amino acid sequence of the collagen chains, collagenases should be able to cleave many of the peptide bonds within the collagen molecule (*22*, *23*). This suggests that the availability of the potential cleavage site and the conformation of the triple helix in the vicinity of the cleavage site play important roles in scissile bond specificity. Several studies have been conducted with the purpose of identifying structural elements that play a role in the triple helicase and/or collagenolytic activity of the collagenases. We, as well as other investigators, have demonstrated that the carboxyl-terminal domain of the collagenases plays an essential role in their type I collagenolytic activity (*24*−*26*). If this domain is removed or replaced with that of stromelysin-1 (MMP-3) (*24*, *26*) or stromelysin-2 (MMP-10) (*25*), the resulting chimeric enzymes, although still catalytically competent, cannot cleave type I collagen. Further analysis of the carboxyl-terminal domain demonstrated that a 62-amino acid sequence consisting of the "hinge" region and the first hemopexin-like repeat

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* To whom correspondence should be addressed: Department of Molecular Sciences, The University of Tennessee Health Science Center, 858 Madison Ave., Suite G01, Memphis, TN 38163. Telephone: (901)448-4361. Fax: (901)448-7360. E-mail: TPourmotabbe@utmem.edu.

[1] Abbreviations: MMP, matrix metalloproteinase; MT, membrane-type; AS, catalytic zinc-binding active site region; FC, fibroblast collagenase, MMP-1; APMA, *p*-aminophenylmercuric acetate; Mca, (7-methoxycoumarin-4-yl)acetyl; Dpa, 3-(2′,4′-dinitrophenyl)-L-2,3-diaminopropionyl; Gel.B/AS, MMP-9 active site; FC/AS, MMP-1 active site; Gel.B/E415G, MMP-9 mutant.

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of the carboxyl-terminal domain of neutrophil collagenase (MMP-8) are essential for its type I collagenolytic activity (*24*). The carboxyl-terminal domain apparently binds the collagen and, with the use of the "hinge" region, moves it into the AS. Our recently published data demonstrate that Gly^{272} is responsible for the hinge bending motion (*27*). During this process, by means that are not thoroughly understood, the enzyme with the aid of the carboxyl-terminal domain partially unfolds the helix to allow cleavage (*28*, *29*). Studies have also suggested that the catalytic domain of fibroblast collagenase (MMP-1, FC) contributes to the substrate specificity of the enzyme. It has been shown that chimeric enzymes containing the entire catalytic domains of MMP-10 (*25*) or MMP-3 (*26*) attached to the carboxyl-terminal domain of FC, although retaining the ability to digest casein, had no type I collagenolytic activity. This suggests that the catalytic domain of FC contains structural elements that are required for the type I collagenolytic activity of the enzyme. Chung and co-workers have demonstrated that the ^{183}RWTNNFREY amino acid sequence in the catalytic domain of FC is critical for the collagenolytic activity of the enzyme (*30*). This sequence, however, was not sufficient to endow MMP-3 with type I collagenolytic activity, suggesting that other elements in the catalytic domain of FC are also required for the expression of this activity. Recently, Pelman and co-workers (*31*) reported that one of the important components of collagenolysis in MMP-8 is the interaction between the side chains of Asn^{188} that is located in the S3′ subsite and Tyr^{189} with the collagen substrate. In this report, we have discovered, for the first time, that the ability of FC to cleave type I collagen is dependent on the presence of a Gly residue at position 233 located in the AS region of the enzyme. The presence of a Gly residue at this position is sufficient to endow any MMP with type I collagenolytic activity. Gly^{233} provides the flexibility necessary for the enzyme AS to change conformation upon binding collagen, leading to catalysis.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Purification of Recombinant Proteins. The construction of expression plasmids pET/FC, pET/NG, and pET/FC+Gel.B/AS containing the cDNA for MMP-1 (FC), MMP-9, and the MMP-1 chimera containing the MMP-9 active site (FC+Gel.B/AS), respectively, and purification of their corresponding proteins have been described previously (*33*, *34*). A single-site mutation was introduced into pET/FC, pET/NG, and pET/FC+Gel.B/AS by the polymerase chain reaction (PCR) procedure.

To substitute a Glu for Gly^{233} in the FC molecule, a 5′ mutagenic primer (substituted nucleotide underlined), 5′-CTCGGCCATTCTCTTGGACTCTCCCATTC-TACTGATATCGAGGCTT-3′, complementary to nucleotides 724−770 in FC, containing a *Bst*XI site 5′ of the substituted nucleotide was used. The 3′ primer, 5′-TTTG-GACTCACACCATGTGTTTTCCATTCAAATTAG-3′, was complementary to the 3′ untranslated region of the cDNA. The 0.8 kb PCR product was digested with *Bst*XI and *Dra*III and ligated with a 5.1 kb *Bst*XI−*Dra*III fragment from pET/FC to produce the final expression vector, pET/FC/G233E.

pETFC+Gel.B/AS/E415G and pETNG/E415G mutants were generated using the mutagenic primer, 5′-CAT-GAGCGCTCCCGGCACTGAGGAATG-3′ (substituted nucleotides underlined). The expected mutations were confirmed, and all of the PCR-derived DNA was found to be free of secondary mutations by dideoxy sequencing. Expression of these plasmids in *Escherichia coli* strain BL21(DE3) and BL21(DE3)pLysS cells generated FC+Gel.B/AS chimeric mutant (FC+Gel.B/AS/E415G, where the number refers to that of the MMP-9 molecule; Glu^{415} in MMP-9 corresponds to Gly^{233} in FC) and MMP-9 mutant (Gel.B/E415G) proteins, respectively.

The recombinant FC/G233E and FC+Gel.B/AS/E415G enzymes were activated with 1 mM APMA and purified on a Zn-chelating columns (*34*), and the Gel.B/E415G mutant was purified as a latent enzyme via gelatin agarose column chromatography as described previously (*33*). Purified enzymes were dialyzed against 50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 150 mM NaCl, and 2 μM $ZnCl_2$ and stored at −80 °C.

Determination of k_{cat}/K_m Values for the Fluorogenic Peptide Substrate (7-Methoxycoumarin-4-yl)acetyl(Mca)-Pro-Leu-Gly-Leu-[3-(2′,4′-dinitrophenyl)-L-2,3-diaminopropionyl](Dpa)Ala-Arg-NH_2. The k_{cat}/K_m values for the fluorogenic peptide substrate were obtained by assaying the enzymes at 23 °C using 2 μM substrate as previously described (*34*). Duplicate assays were performed and the results averaged. Concentrations of active enzymes, $[E]_T$, were found by active site titration either with the amino-terminal, 14 kDa, inhibitory domain of the recombinant tissue inhibitor of metalloproteinase-2 (TIMP-2-ID, a kind gift from H. Tschesche, Lerstuhl für Biochemie, Universitat Bielefeld, Bielefeld, Germany) (*34*) or with GM6001 as described previously (*35*). These methods were used to determine the concentration of active enzyme used in every assay in this report. Concentrations of the active enzyme found by TIMP-2-ID titration correlated with those determined by the Bradford dye binding technique (standard Bio-Rad Laboratories assay), using bovine serum albumin (BSA) as a standard.

Analysis of Gelatinolytic Activity. Gelatinolytic activity was determined using ^{14}C-labeled gelatin essentially as described previously (*36*, *37*). Specific activity values were calculated by dividing the amount of gelatin degraded per hour (micrograms) by the nanomoles of enzyme used in the reaction. Each assay was performed in duplicate, and the results were averaged.

Type I Collagenolytic Activity. The type I collagenolytic activity of the enzymes was initially detected by SDS−PAGE. Enzymes (0.05−4 pmol) were incubated with 0.2 μg/μL bovine type I collagen (Fluka Biochemika, Milwaukee, WI) in assay buffer containing 50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 150 mM NaCl, 2 μM $ZnCl_2$, and 0.2 μg/μL BSA at 27 °C for 20 h. The reactions were then quenched with 10 mM EDTA, and the mixtures were boiled for 5 min and loaded onto a 6% SDS−PAGE gel. Protein bands were visualized by staining the gel with Coomassie Blue.

The kinetic parameters, k_{cat} and K_m, for soluble and FITC-labeled fibril type I collagen were determined as described previously (*27*, *38*) with the following modifications. The soluble type I collagen assay was performed at 35 °C for 1 h in assay buffer that contained 50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 150 mM NaCl, and various concentrations of bovine type I collagen (0.1−2.5 μM). After the incubation

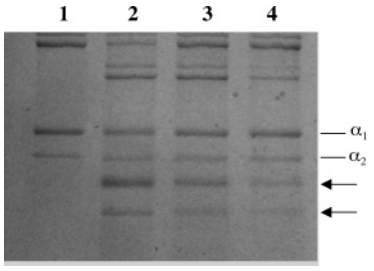

FIGURE 1: SDS−PAGE analysis of the type I collagenolytic activity of FC, FC+Gel.B/AS, and FC/G233E. The purified enzymes were incubated with 0.2 μg/μL bovine type I collagen in an assay buffer containing 50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 150 mM NaCl, 2 μM $ZnCl_2$, and 0.2 μg/μL BSA at 27 °C for 20 h. The reactions were then quenched with 10 nM EDTA, and the mixtures were boiled and separated on a 6% SDS−PAGE gel as described in Experimental Procedures. Protein bands were visualized by Coomassie Blue staining. The arrowheads indicate three-quarter length degradation products of α1 and α2 chains: lane 1, type I collagen alone (0.4 μg); lane 2, FC (0.06 pmol); lane 3, FC+Gel.B/AS (0.3 pmol); and lane 4, FC/G233E (0.3 pmol).

period, reactions were quenched with 10 mM EDTA and mixtures were boiled for 5 min and separated on a 6% SDS−PAGE gel. Protein bands were visualized by Coomassie Blue staining and quantified with an Alpha Imager 2000 Documentation and Analysis System (Alpha Innotech Corp., San Leandro, CA). Duplicate assays were performed and the results were averaged.

RESULTS

To assess contributions of the active site (AS) of MMP-1 (FC) to the type I collagenolytic activity of the enzyme, the ability of the FC+Gel.B/AS chimera (*34*) to cleave type I collagen was examined. The FC+Gel.B/AS chimera consists of a full-length FC with its AS residues (amino acids 212−254) replaced with that of MMP-9 (Gel.B/AS; amino acids 395−437) (*34*). We have recently used this chimera to identify structural elements that determine the substrate specificity of MMP-9 and have shown that the 42 kDa active FC+Gel.B/AS is properly folded and, like the FC, has a mixture of amino-terminal sequences, V^{101}LTEG and L^{102}-TEGN (*34*). As shown in Table 1 and previously reported (*34*), the substitution of the AS of FC with that of MMP-9 increased the catalytic efficiencies of the enzyme toward the peptide substrate, Mca-PLGL(Dpa)AR-NH_2, and gelatin by approximately 11 times and 2.5-fold, respectively. The presence of amino acid residues L^{397}, A^{410}, and A^{406} in Gel.B/AS is apparently responsible for the observed differences in the proteolytic activity of FC and FC+Gel.B/AS (*34*).

Analysis of Type I Collagenolytic Activity of FC+Gel.B/AS. To determine the effect of the AS substitution on the type I collagenolytic activity of FC, the ability of FC+Gel.B/AS chimera to cleave native triple-helical type I collagen was examined. As shown in Figure 1, FC+Gel.B/AS (lane 3), like FC (lane 2), was able to digest type I collagen into three-quarter and one-quarter length fragments. However, the K_m and k_{cat} values of FC+Gel.B/AS for soluble type I collagen were 6 times higher and ∼2 orders of magnitude lower than those of FC, respectively (Table 1). These results indicated that the substitution of AS of FC with Gel.B/AS adversely affected the binding of the enzyme to type I collagen and more notably resulted in a large decrease in the turnover number, k_{cat}, of the enzyme for this substrate. Considering that FC+Gel.B/AS was able to cleave gelatin and the peptide substrate with efficiencies higher than that of FC, it can be concluded that the substitution of the AS of FC with Gel.B/AS resulted in a specific decrease in the type I collagenolytic activity of FC. These observations suggest that the AS of FC contains unique amino acid residues that are particularly responsible for enhancing the type I collagenolytic activity of the enzyme.

The Conservation of Gly^{233} Is Crucial for the Type I Collagenolytic Activity of FC. To identify specific amino acid residues in the active site of FC (FC/AS) that may be important for enhancing its type I collagenolytic activity, a partial amino acid sequence alignment from this region of several collagenases and the gelatinases was made and is shown in Figure 2. This alignment revealed that all members of the collagenase family, including gelatinase A, contain a conserved Gly residue at a position corresponding to amino acid 233 in FC that is substituted with a Glu residue (Glu^{417}) in MMP-9. This observation raised the possibility that the conservation of this Gly residue is responsible for efficient utilization of type I collagen by the collagenases. To test this hypothesis, Gly^{233} in FC was mutated to the corresponding Glu residue of MMP-9 (FC/G233E). The FC/G233E mutant was expressed as a latent enzyme in *E. coli*, activated with APMA, and purified as a 42 kDa active species as described in Experimental Procedures. Like the wild-type enzyme, FC/G233E exhibited altered electrophoretic mobility under reducing versus nonreducing conditions (data not shown), which indicated that the disulfide bond in the carboxyl-terminal domain is intact. The specific activity of FC/G233E toward gelatin was found to be essentially equal to that of FC [255 and 272 μg h^{-1} $nmol^{-1}$, respectively (Table 1)]. These data suggested that FC/G233E was properly folded and that the general proteolytic activity of the enzyme was not compromised by this substitution. Interestingly, as shown in Table 1, the effects of the G233E mutation on the kinetic parameters of FC for the peptide substrate and type I collagen were quite similar to that of replacing the entire AS of FC with that of MMP-9 (FC+Gel.B/AS). The Gly^{233} to Glu mutation increased the catalytic activity of FC toward the peptide substrate by 6.5 times and reduced its catalytic efficiency (k_{cat}/K_m) toward type I collagen by more than 99% (Table 1). The k_{cat} value of FC/G233E for type I collagen was 27 times lower and its K_m value 7 times higher than that of the wild type enzyme. Like the FC+Gel.B/AS chimera, the Gly^{233} to Glu mutation did not affect the cleavage pattern of type I collagen (Figure 1, lane 4). These results suggest that Gly^{233} is responsible for the efficient utilization of type I collagen by FC.

To confirm this observation, Glu^{415} in the active site of the FC+Gel.B/AS chimera (the number refers to the amino acid residue in Gel.B/AS) was replaced with Gly, its amino acid counterpart in FC, and catalytic activities of the FC+Gel.B/AS/E415G mutant toward peptide substrate, gelatin, and native type I collagen were determined. If Gly^{233} is responsible for the efficient utilization of type I collagen by FC, then replacing Glu^{415} in the FC+Gel.B/AS chimera with Gly should in principle increase the type I collagenolytic activity of the chimera. As predicted, the mutation increased the catalytic activity of the FC+Gel.B/AS chimera toward type I collagen by 12-fold without affecting its affinity for the collagen substrate (Table 1). The K_m and k_{cat} values of

	214 236	Type I collagenolytic Activity
Human MMP-1 (FC)	– RVAAHELGHSLGLSHSSDI**G**ALM	+
Rabbit collagenase	– RVAAHELGHSLGLSHSTDI**G**ALM	+
Pig collagenase	– RVAAHELGHSLGLSHSTDI**G**ALM	+
Bovine collagenase	– RVAAHEFGHSLGLSHSTDI**G**ALM	+
Rat collagenase	– IVAAHELGHSLGLDHSKDP**G**ALM	+
Mouse collagenase	– IVAAHELGHSLGLDHSKDP**G**ALM	+
Human MMP-13	– LVAAHEFGHSLGLDHSKDP**G**ALM	+
Human MMP-8	– LVAAHEFGHSLGLAHSSDP**G**ALM	+
Catfish MMP-8	– LVAAHEFGHSLGLEHSNVP**G**ALM	+
Human MMP-2	– LVAAHEFGHAMGLEHSQDP**G**ALM	+
Human MMP-9	– LVAAHEFGHALGLDHSSVP**E**ALM	–

FIGURE 2: Sequence alignment of amino acids in the AS of collagenases from various species and gelatinases. Numbers refer to the amino acid residues of FC starting from the initial Met. The Gly residue that is conserved in all type I collagenases is shown in bold and underlined.

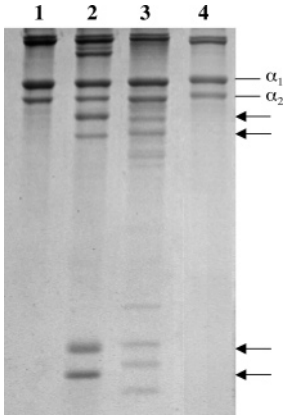

FIGURE 3: SDS−PAGE analysis of the type I collagenolytic activity of FC, MMP-9, and the Gel.B/E415G mutant. The purified enzymes were incubated with 0.2 μg/μL bovine type I collagen as described in the legend of Figure 1 except the reaction mixtures were incubated at 25 °C for 8 h. The arrowheads indicate three-quarter and one-quarter length degradation products of α1 and α2 chains, respectively: lane 1, type I collagen alone (0.4 μg); lane 2, FC (0.2 pmol); lane 3, Gel.B/E415G (3.0 pmol); and lane 4, MMP-9 (3.0 pmol).

the FC+Gel.B/AS/E415G mutant for type I collagen were 1.83 μM and 83 h^{-1}, respectively. The Glu^{415} to Gly mutation, however, reduced the general proteolytic activity of FC+Gel.B/AS to that of FC. As shown in Table 1, the catalytic activities of the FC+Gel.B/AS/E415G mutant toward synthetic substrate and gelatin were 6 times and 2-fold lower than that of FC+Gel.B/AS, respectively. These data clearly demonstrate that Gly^{233} plays a direct role in the type I collagenolytic activity of FC. These data also suggest that the presence of a Gly residue at this position may be sufficient to endow any MMP with the ability to utilize native triple-helical type I collagen as a substrate.

Substitution of Gly for Glu^{415} in MMP-9 Confers the Enzyme with Type I Collagenolytic Activity. To substantiate the hypothesis given above, the Glu residue at position 415 in full-length MMP-9 was substituted with Gly (Gel.B/E415G), its corresponding residue in FC. As shown in Table 1, the Gel.B/E415G mutant cleaved gelatin and synthetic substrate with an efficiency similar to that of wild-type MMP-9. However, contrary to MMP-9 (Figure 3, lane 4) but similar to FC (Figure 3, lane 2), the Gel.B/E415G mutant was able to cleave the α1 and α2 chains of triple-helical type I collagen at a unique bond, generating characteristic three-quarter and one-quarter length fragments (Figure 3, lane 3). Longer incubation of the enzyme with the substrate generated several smaller fragments (Figure 3, lane 3). This is consistent with Gel.B/E415G acting as a gelatinase. The catalytic efficiency of the Gel.B/E415G mutant toward type I collagen (k_{cat}/K_m = 2.92 h^{-1} μM^{-1}) was similar to that of the FC+Gel.B/AS chimera (k_{cat}/K_m = 3.7 h^{-1} μM^{-1}) with its k_{cat} value being 2-fold higher than that of the chimera. NH_2-terminal sequence analyses of the one-quarter length of α1(I) collagen fragments revealed that the cleavage of α2(I) chain of type I collagen by the Gel.B/E415G mutant occurred between Gly^{825} and Leu^{826}, three-fourths of the distance from the amino terminus. These data indicate that the presence of a Gly residue at this position of the MMP-9 molecule is sufficient to endow the enzyme with type I collagenolytic activity.

DISCUSSION

Turnover of type I collagen by mammalian collagenases is considered to be an important part of connective tissue remodeling. It has been noted that the collagen triple helix is too large to fit into the AS of the collagenases (*28*), suggesting that a partial unfolding of the triple helix by the collagenases and/or changes in the active site conformation, induced upon binding to the collagen molecule, are needed for the peptide chains to fit into the enzyme subsites. Chung and co-workers (*29*) have recently suggested that the carboxyl-terminal domain of MMP-1 (FC) helps to unwind the triple-helical structure of the collagen molecule prior to catalysis. In this report, we demonstrate, for the first time, that the ability of FC to cleave type I collagen is also dependent on its AS ability to adopt a conformation that mediates collagen binding and catalysis, and this process is dependent on the presence of a Gly residue at position 233 located in the AS region of the enzyme. Gly^{233} provides the flexibility necessary for the enzyme AS to change conformation upon binding collagen (induced fit; *32*), leading to catalysis. This finding is based upon the observations that the presence of a Gly residue in the MMP-9 active site is sufficient to endow the enzyme with type I collagenolytic activity and the FC chimera in which the AS of FC was replaced with that of MMP-9 (FC+Gel.B/AS), although had higher activity toward the peptide substrate and gelatin than that of FC, it was not able to efficiently cleave type I collagen. The specific decrease in the type I collagenolytic activity of the enzyme was due to a large decrease in the k_{cat} and a significant increase in the K_m value. FC+Gel.B/AS was by all indications stable, properly folded, and able to be processed by APMA to an active species

having the same M_r and amino termini as FC. An amino acid sequence alignment of the collagenase family revealed that the conserved Gly^{233} in collagenases (the number refers to the amino acid residue in FC) is substituted for a Glu residue in MMP-9 (Figure 2), suggesting that this substitution is responsible for the observed difference in the type I collagenolytic activity of FC+Gel.B/AS and FC. This was confirmed by the observation that the replacement of Gly^{233} in FC with Glu (FC/G233E), the corresponding residue in MMP-9, substantially decreased the catalytic efficiency of FC toward type I collagen (Table 1). However, the FC/G233E mutant retained full peptidolytic and gelatinolytic activity, indicating that the decrease in the collagenolytic activity of FC was specific and that its general proteolytic activity was not compromised by this substitution. The effect of the G233E mutation on kinetic parameters of FC for type I collagen was similar to the effect of the replacement of the entire AS of FC with that of MMP-9 (Table 1). These data indicated that the conservation of Gly^{233} in FC is crucial for the efficient cleavage of type I collagen by the enzyme. This was substantiated by substituting the Glu^{415} residue (the number refers to the position of the amino acid residue in the MMP-9 molecule) in FC+Gel.B/AS with Gly, the corresponding residue in FC. The catalytic activity of the FC+Gel.B/AS/E415G mutant toward type I collagen was 12- and 2.7-fold higher than that of FC+Gel.B/AS and the FC/G233E mutant, respectively (Table 1). The mutation, however, did not have any effect on the K_m value of FC+Gel.B/AS for the collagen substrate. These data demonstrated that the type I collagen binding capacity of FC+Gel.B/AS was not affected by the Gly substitution and suggested that the Gly residue contributes to catalysis by facilitating the interaction between the collagen substrate and the active site residues.

It has been noted that the AS of many enzymes contains a recognizable Gly-containing sequence motif, G-X-Y or Y-X-G, where X and Y are polar and nonpolar residues, respectively, with Leu and Ala having the highest frequency (*40*). These Gly residues have been shown to be important for the catalytic hinge bending motions of several enzymes (*41*−*43*). Gly^{233} is located in the G-A-L oligopeptide motif in the active site of FC (Figure 2), suggesting that it directly and/or indirectly contributes to the local flexibility of the active site, which is generally considered to be important for catalysis. Thus, it is conceivable that any substitution at this position has a general effect on the mobility of the active site of FC. The observation that FC+Gel.B/AS and the FC/G233E mutant have significantly lower catalytic activity toward the collagen substrate than that of FC+Gel.B/AS/E415G supports this concept and suggests that the flexibility provided by Gly is essential for type I collagenolytic activity. This idea is in accord with the X-ray crystal structure of FC (*39*). In FC, Gly^{233} is located in a turn at the bottom of the AS where the polypeptide chain turns back toward the center of the enzyme (Figure 4A) (*39*). The distortion of the turn that is presumably needed to "open" the AS of the enzyme may be less easy to achieve when Gly^{233} has been replaced with Glu as in MMP-9, FC+Gel.B/AS, and FC/G233E. Thus, Glu may have "locked" the AS of these enzymes in a more closed state, resulting in reduced catalytic efficiency as demonstrated by either their lack of collagenolysis or the low catalytic efficiency toward the collagen substrate (Table 1). A molecular modeling study demonstrated that for the side chain of a Glu residue to fit at position 233 in FC without affecting the overall structure of the enzyme, it would point either toward the first β-propeller of the carboxyl-terminal domain or in the opposite direction toward the hinge region (Figure 4B). If it pointed toward the former, it would clash with Phe^{316}. To avoid this, Phe^{316} could rotate out into the collagen binding area of the carboxyl-terminal domain. If the substituted Glu residue pointed in the opposite direction, it would fill the space between the bottom of the AS and the hinge region and would clash with Asp^{231} and/

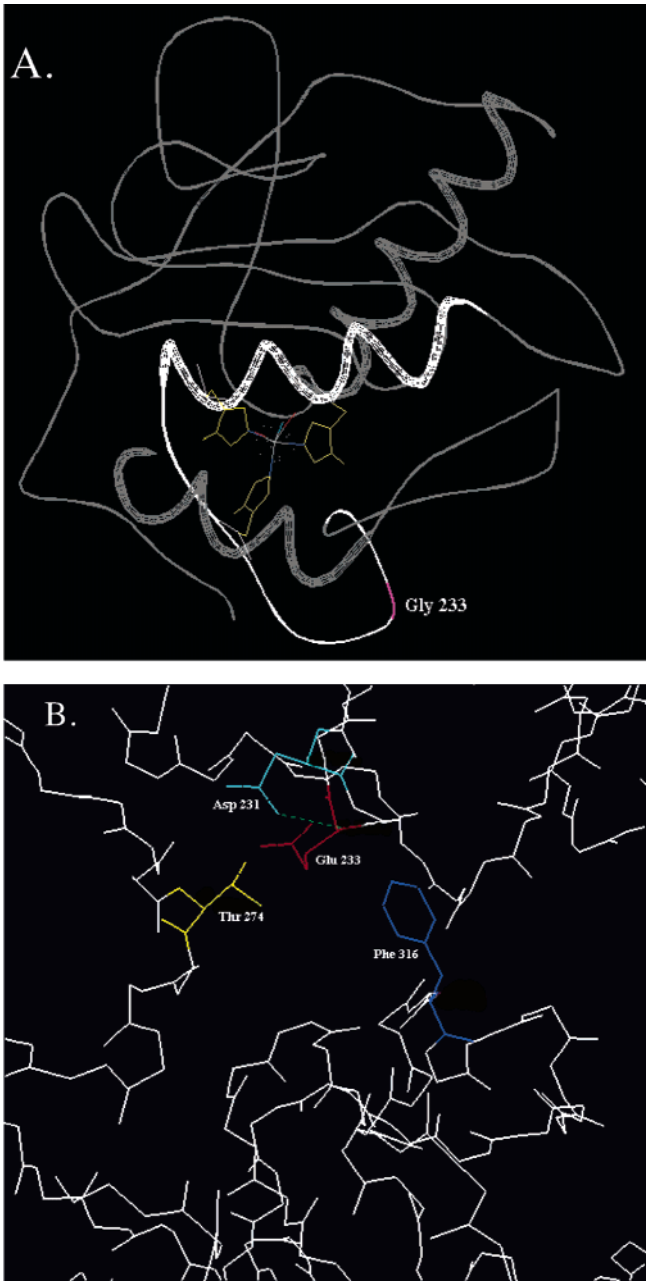

FIGURE 4: X-ray crystal structure of FC. (A) Catalytic domain of FC highlighting the active site of the enzyme. The conserved Gly residue is shown in magenta. The position of the catalytic zinc ion and three His residues shown in yellow are indicated. (B) Amino acids that surround Gly^{233}. Swiss Pdb viewer modeling program was used to substitute Gly^{233} of FC with Glu. The mutated amino acid (Glu) is shown in red. Asp^{231}, Thr^{274}, and Phe^{316}, residues that might interact with the substituted Glu in the FC/G233E mutant, are shown in green, yellow, and blue, respectively. This figure was adapted from the crystal structure of FC solved by Li et al. (*39*).

Table 1: Kinetic Parameters of FC, FC+Gel.B/AS, MMP-9, and Their Mutants[a]

	Mca-PLGL(Dpa)AR-NH_2	gelatin	type I collagen		
enzyme	k_{cat}/K_m (mM^{-1} s^{-1})	specific activity (μg h^{-1} $nmol^{-1}$)	k_{cat} (h^{-1})	K_m (μM)	k_{cat}/K_m (h^{-1} μM^{-1})
FC	14.17 ± 0.51	272 ± 9	828 ± 120	0.31 ± 0.08	2760
FC+Gel.B/AS	160 ± 4	692 ± 25	6.7 ± 0.08	1.8 ± 0.4	3.7
FC/G233E	95.0 ± 4.2	255 ± 4	31 ± 3	2.2 ± 0.4	14
FC+Gel.B/AS/E415G	26.4 ± 2.3	335 ± 60	83 ± 8	1.83 ± 0.47	45.3
Gel.B/E415G	1336 ± 4	61850 ± 6570	12.95 ± 0.02	4.44 ± 0.09	2.92
MMP-9	2313 ± 123	83030 ± 2770	ND[b]	ND[b]	ND[b]

[a] Experimental details are given in Experimental Procedures. [b] Not determined.

or Thr^{274} of the hinge region (Figure 4B). This could cause Asp^{231} to rotate out into the collagen binding area and/or Thr^{274} to rotate toward the center of the enzyme or out toward solvent. The displacement of any one of the amino acid side chains, Phe^{316}, Asp^{231}, or Thr^{274}, not only makes the structure of the active site more rigid, hampering the ability of the AS to accommodate the collagen substrate, but also interferes with substrate binding. These result in a decrease in k_{cat} and an increase in K_m values. The observation that FC+Gel.B/AS and FC/G233E have a significantly lower k_{cat} and a higher K_m for type I collagen than the wild-type enzyme corroborates this hypothesis. In addition, the catalytic activities of FC+Gel.B/AS and the FC/G233E mutant toward the collagen substrate are significantly lower than that of FC+Gel.B/AS/E415G, suggesting that the flexibility provided by Gly is essential for type I collagenolytic activity. This concept is further supported by the fact that although the three-dimensional structure of the catalytic domain of MMP-9 (*44*) is highly homologous to that of FC (*39*), MMP-9 cannot cleave the native type I collagen substrate (Figure 4, lane 4). Glu^{415} is the corresponding Gly residue in the active site of MMP-9 (Figure 2). On the other hand, like FC, the MMP-9 mutant (Gel.B/E415G) in which Glu^{415} is substituted with Gly, its corresponding residue in FC, is able to cleave native triple-helical type I collagen at three-quarters of the distance from the amino terminus (Figure 3, lane 3). This observation indicates that the AS of the Gel.B/E415G mutant has acquired the conformation necessary for type I collagenolysis.

The replacement of the Gly residue at position 233 with a Glu as in FC+GelB/AS and FC/G233 may also affect collagenolysis by interfering with the ability of the carboxyl-terminal domain to present the collagen to the AS. Considering the different catalytic-hemopexin-domain configurations of latent and active MMP-1, Jozic and co-workers (*46*) have proposed that proper positioning of the hemopexin domain with respect to the catalytic domain may be necessary for the triple helicase activity of the enzyme. They speculate that the open form of the active MMP-1 interacts with native triple-helical collagen and this interaction triggers a change in the position of the hemopexin domain relative to the catalytic domain leading to a closed conformation, which in turn locally unwinds triple-helical collagen. In the wild-type enzyme, the presence of a Gly at position 233 provides enough space between the hinge region and the bottom of the AS (Figure 4) to allow the hinge region to flex and properly position the hemopexin domain during catalysis. However, as mentioned above, if the substituted Glu residue were oriented in the direction of the hinge region, it would fill this space, leaving little room for the hinge region to flex toward the catalytic domain. This would severely hamper the ability of the carboxyl-terminal domain to position the collagen sufficiently close to the AS for efficient catalysis, resulting in a decrease in k_{cat}. Consistent with this hypothesis, we have found that the k_{cat} of FC/G233E for type I collagen was much lower than that of the wild-type enzyme. However, contrary to this notion is the fact that both the MMP-9 mutant [Gel.B/E415G (Figure 3 and Table 1)] and MMP-2 (gelatinase A) (*45*) that contains a Gly residue at position 418 corresponding to Gly^{233} in FC (Figure 2, bold and underlined) are also able to cleave triple-helical type I collagen. Studies (*34*, *36*, *47*−*49*) suggest that the fibronectin-like domain of these enzymes is responsible for binding to and presenting macromolecular substrates, including type I collagen in the case of MMP-2 (*49*, *50*), to the AS of the enzyme for cleavage. This domain is unique to the gelatinases and is inserted into their catalytic domains, adjacent to their AS (*17*−*21*). Thus, it is reasonable to conclude that the presence of a small amino acid residue like Gly at this position provides the flexibility necessary for enzyme active site to change conformation upon substrate binding.

It is worth mentioning that not all MMP members with collagenolytic activity contain this Gly residue. The membrane-bound MMP (MT1-MMP, MMP-14) that also cleaves triple-helical type I collagen contains a Ser residue at this position in its active site. In comparison to the collagenase family, the MT1-MMP molecule contains a convertase cleavage site between the pro-peptide and the catalytic domain and also a transmembrane-1 and a cytoplasmic tail after the hemopexin-like domain (*18*−*21*). In contrast to MMP-1 (*29*), Tam and co-workers have recently shown that the isolated carboxyl-terminal domain of MT-1MMP (MT1-LCD) binds to the native type I collagen and induces structural perturbation in the molecule (*50*). In addition, they have demonstrated that MT1-LCD enhances the type I collagenolytic activity of MMP-1 and -8 in trans, while it inhibits the type I collagenolytic activity of MT1-MMP. MT1-LCD, however, was not able to endow MMP-7 and trypsin, the noncollagenolytic proteases, with type I collagenolytic activity, suggesting that unwinding of triple-helical collagen by MT1-LCD is localized and MT1-LCD does not completely denature the collagen molecule. These data also suggest that MT1-MMP binds collagen at a different site than MMP-1 and MMP-8, and triple helicase activity is independent of α-chain hydrolysis. Further studies are needed to identify the collagen binding sites and elucidate the mechanism of collagenolytic activity of MT1-MMP.

In conclusion, triple-helical type I collagenolysis is a function of a multistep process. Initially, the carboxyl-terminal domain in the case of collagenases (*24*) or the

fibronectin-like domain in the case of gelatinase A (*49*, *50*) binds collagen, the hinge region then presents the substrate to the catalytic domain (*27*). This is followed by unwinding of triple-helical collagen by the enzyme (*29*) and a conformational change in the AS leading to catalysis.

REFERENCES

1. Sellers, A., Reynolds, J. J., and Meikle, M. C. (1978) Neutral metallo-proteinases of rabbit bone. Separation in latent forms of distinct enzymes that when activated degrade collagen, gelatin and proteoglycans, *Biochem. J. 171*, 493−496.
2. Vu, T. H., Shipley, J. M., Bergers, G., Berger, J. E., Helms, J. A., Hanahan, D., Shapiro, S. D., Senior, R. M., and Werb, Z. (1998) MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes, *Cell 93*, 411−422.
3. Wolf, C., Chernard, M. P., Durand de Grossouvre, P., Bellocq, J. P., Chambon, P., and Basset, P. (1992) Breast-cancer-associated stromelysin-3 gene is expressed in basal cell carcinoma and during cutaneous wound healing, *J. Invest. Dermatol. 99*, 870−872.
4. Wysocki, A. B., Staiano-Coico, L., and Grinnell, F. (1993) Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9, *J. Invest. Dermatol. 101*, 64−68.
5. Overall, C. M., Sodek, J., McCulloch, A. G., and Birek, P. (1991) Evidence for polymorphonuclear leukocyte collagenase and 92-kilodalton gelatinase in gingival crevicular fluid, *Infect. Immun. 59*, 4687−4692.
6. Page, R. C. (1991) The role of inflammatory mediators in the pathogenesis of periodontal disease, *J. Periodontal Res. 26*, 230−242.
7. Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M., and Shafie, S. (1980) Metastatic potential correlates with enzymatic degradation of basement membrane collagen, *Nature 284*, 67−68.
8. Liotta, L. A., Abe, S., Robey, P. G., and Martin, G. R. (1979) Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor, *Proc. Natl. Acad. Sci. U.S.A. 76*, 2268−2272.
9. Matrisian, L. M., Bowden, G. T., Krieg, P., Furstenberger, G., Briand, J.-P., Leroy, P., and Breathnach, R. (1986) The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumor, *Proc. Natl. Acad. Sci. U.S.A. 83*, 9413−9417.
10. Bernhard, E. J., Muschel, R. J., and Hughes, E. N. (1990) Mr 92,000 gelatinase release correlates with the metastatic phenotype in transformed rat embryo cells, *Cancer Res. 50*, 3872−3877.
11. Bernhard, E. J., Gruber, S. B., and Muschel, R. J. (1994) Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells, *Proc. Natl. Acad. Sci. U.S.A. 91*, 4293−4297.
12. Bafetti, L. M., Young, T. N., Itoh, Y., and Stack, M. S. (1998) Intact vitronectin induces matrix metalloproteinase-2 and tissue inhibitor of metalloproteinases-2 expression and enhanced cellular invasion by melanoma cells, *J. Biol. Chem. 273*, 143−149.
13. Hayakawa, T., Yamashita, S., Kodama, H., Iwata, H., and Iwata, K. (1991) Tissue inhibitor of metallo-proteinases and collagenase activity in synovial fluid of human rheumatoid arthritis, *Biomed. Res. 12*, 169−173.
14. Hirose, T., Reife, R. A., Smith, G. M., Jr., Stevens, R. M., Mainardi, C. L., and Hasty, K. A. (1992) Characterization of type V collagenase (gelatinase) in synovial fluid of patients with inflammatory arthritis, *J. Rheumatol. 19*, 593−599.
15. Yu, L., Smith, G. J., Jr., Brandt, K., and Capello, W. (1990) Type XI collagen-degrading activity in human osteoarthritic cartilage, *Arthritis Rheum. 33*, 1626−1633.
16. Harris, E. D., Jr. (1990) Rheumatoid arthritis: Pathophysiology and implications for therapy, *N. Engl. J. Med. 322*, 1277−1289.
17. Woessner, J. F., Jr. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling, *FASEB J. 5*, 2145−2154.
18. Matrisian, L. M. (1992) The matrix-degrading metalloproteinases, *BioEssays 14*, 455−463.
19. Birkedal-Hansen, H., Moore, W. G. I., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J. A. (1993) Matrix metalloproteinases: A review, *Crit. Rev. Oral Biol. Med. 4*, 197−250.
20. Massova, I., Kotra, L. P., Fridman, R., and Mobashery, S. (1998) Matrix metalloproteinases: Structures, evolution, and diversification, *FASEB J. 12*, 1075−1095.
21. Nagase, H., and Woessner, J. F., Jr. (1999) Matrix metalloproteinases, *J. Biol. Chem. 274*, 21491−21494.
22. Netzel-Arnett, S., Fields, G. B., Birkedal-Hansen, H., Van Wart, H. E., and Fields, G. (1991) Sequence specificities of human fibroblast and neutrophil collagenases, *J. Biol. Chem. 266*, 6747−6755.
23. Fields, G. B., Van Wart, H. E., and Birkedal-Hansen, H. (1987) Sequence specificity of human skin fibroblast collagenase. Evidence for the role of collagen structure in determining the collagenase cleavage site, *J. Biol. Chem. 262*, 6221−6226.
24. Hirose, T., Patterson, C. E., Pourmotabbed, T., Mainardi, C. L., and Hasty, K. A. (1993) Structure-function relationship of human neutrophil collagenase: Identification of regions responsible for substrate specificity and general proteinase activity, *Proc. Natl. Acad. Sci. U.S.A. 90*, 2569−2573.
25. Sanchez-Lopez, R., Alexander, C. M., Behrendsten, O., Breathnach, R., and Werb, Z. (1993) Role of zinc-binding- and hemopexin domain-encoded sequences in the substrate specificity of collagenase and stromelysin-2 as revealed by chimeric proteins, *J. Biol. Chem. 268*, 7238−7247.
26. Murphy, G., Allan, J. A., Willenbrock, F., Cockett, M. I., O'Connell, J. P., and Docherty, A. J. (1992) The role of the C-terminal domain in collagenase and stromelysin specificity, *J. Biol. Chem. 267*, 9612−9618.
27. Tsukada, H., and Pourmotabbed, T. (2002) Unexpected crucial role of residue 272 in substrate specificity of fibroblast collagenases, *J. Biol. Chem. 277*, 27378−27384.
28. Bode, W., Reinemer, P., Huber, R., Kleine, T., Schnierer, S., and Tschesche, H. (1994) The X-ray crystal structure of the catalytic domain of human neutrophil collagenase inhibited by a substrate analogue reveals the essentials for catalysis and specificity, *EMBO J. 13*, 1263−1269.
29. Chung, L., Dinakarpandian, D., Yoshida, N., Lauer-Fileds, J. L., Fileds, G. B., Visse, R., and Nagase, H. (2004) Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis, *EMBO J. 23*, 3020−3030.
30. Chung, L., Shimokawa, K., Dinakarpandian, D., Grams, F., Fields, G. B., and Nagase, H. (2000) Identification of the ^{183}RWTNN-FREY^{191} region as a critical segment of matrix metalloproteinase 1 for the expression of collagenolytic activity, *J. Biol. Chem. 275*, 29610−29617.
31. Pelman, G. R., Morrison, C. J., and Overall, C. M. (2005) Pivotal molecular determinants of peptidic and collagen triple helicase activities reside in the S3′ subsite of matrix metalloproteinase 8 (MMP-8): The role of hydrogen bonding potential of ASN188 and TYR189 and the connecting cis bond, *J. Biol. Chem. 280*, 2370−2377.
32. Koshland, D. E., Jr. (1958) Application of a theory of enzyme specificity to protein synthesis, *Proc. Natl. Acad. Sci. U.S.A. 44*, 98−104.
33. Pourmotabbed, T., Solomon, T. L., Hasty, K. A., and Mainardi, C. L. (1994) Characteristics of 92 kDa type IV collagenase/gelatinase produced by granulocytic leukemia cells: Structure, expression of cDNA in *E. coli* and enzymic properties, *Biochim. Biophys. Acta 1204*, 97−107.
34. O'Farrell, T. J., and Pourmotabbed, T. (2000) Identification of structural elements important for matrix metalloproteinase type V collagenolytic activity as revealed by chimeric enzymes. Role of fibronectin-like domain and active site of gelatinase B, *J. Biol. Chem. 275*, 27964−27972.
35. Pourmotabbed, T., Aelion, J. A., Tyrrell, D., Hasty, K. A., Bu, C. H., and Mainardi, C. L. (1995) Role of the conserved histidine and aspartic acid residues in activity and stabilization of human gelatinase B: An example of matrix metalloproteinases, *J. Protein Chem. 14*, 527−535.
36. O'Farrell, T. J., and Pourmotabbed, T. (1998) The fibronectin-like domain is required for the type V and XI collagenolytic activity of gelatinase B, *Arch. Biochem. Biophys. 354*, 24−30.

37. Bu, C. H., and Pourmotabbed, T. (1996) Mechanism of Ca^{2+}-dependent activity of human neutrophil gelatinase B, *J. Biol. Chem. 271*, 14308−14315.
38. Welgus, H. G., Jeffrey, J. J., and Eisen, A. Z. (1981) The collagen substrate specificity of human skin fibroblast collagenas, *J. Biol. Chem. 256*, 9511−9515.
39. Li, J., Brick, P., O' Hare, M. C., Skarzynski, T., Lloyd, L. F., Curry, V. A., Clark, I. M., Bigg, H. F., Hazleman, B. L., Cawston, T. E., and Blow, D. M. (1995) Structure of full-length porcine synovial collagenase reveals a C-terminal domain containing a calcium-linked, four-bladed β-propeller, *Structure 3*, 541−549.
40. Yan, B. X., and Sun, Y. Q. (1997) Glycine residues provide flexibility for enzyme active sites, *J. Biol. Chem. 272*, 3190−3194.
41. Veltman, O. R., Eijsink, V. G., Vriend, G., de Kreij, A., Venema, G., and Van den Burg, B. (1998) Probing catalytic hinge bending motions in thermolysin-like proteases by glycine → alanine mutations, *Biochemistry 37*, 5305−5311.
42. Bone, R., Silen, J. L., and Agard, A. A. (1989) Structural plasticity broadens the specificity of an engineered protease, *Nature 339*, 191−195.
43. Johnson, K. A. (1993) Conformational coupling in DNA polymerase fidelity, *Annu. Rev. Biochem. 62*, 685−713.
44. Rowsell, S., Hawtin, P., Minshull, C. A., Jepson, H., Brockbank, S. M., Barratt, D. G., Slater, A. M., McPheat, W. L., Waterson, D., Henney, A. M., and Pauptit, R. A. (2002) Crystal structure of human MMP-9 in complex with a reverse hydroxamate inhibitor, *J. Mol. Biol. 319*, 173−181.
45. Aimes, R. T., and Quigley, J. P. (1995) Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments, *J. Biol. Chem. 270*, 5872−5876.
46. Jozic, D., Bourenkov, G., Lim, N. H., Visse, R., Nagase, H., Bode, W., and Maskos, K. (2005) X-ray structure of human proMMP-1: New insights into procollagenase activation and collagen binding, *J. Biol. Chem. 280*, 9578−9585.
47. Allan, J. A., Docherty, A. J., Barker, P. J., Huskisson, N. S., Reynolds, J. J., and Murphy, G. (1995) Binding of gelatinases A and B to type-I collagen and other matrix components, *Biochem. J. 309*, 299−306.
48. Murphy, G., Nguyen, Q., Cockett, M. I., Atkinson, S. J., Allan, J. A., Knight, C. G., Willenbrock, F., and Docherty, A. J. (1994) Assessment of the role of the fibronectin-like domain of gelatinase A by analysis of a deletion mutant, *J. Biol. Chem. 269*, 6632−6636.
49. Steffensen, B., Wallon, U. M., and Overall, C. M. (1995) Extracellular matrix binding properties of recombinant fibronectin type II-like modules of human 72-kDa gelatinase/type IV collagenase. High affinity binding to native type I collagen but not native type IV collagen, *J. Biol. Chem. 270*, 11555−11566.
50. Tam, E. M., Moore, T. R., Butler, G. S., and Overall, C. M. (2004) Characterization of the distinct collagen binding, helicase and cleavage mechanisms of matrix metalloproteinase 2 and 14 (gelatinase A and MT1-MMP): The differential roles of the MMP hemopexin C domains and the MMP-2 fibronectin type II modules in collagen triple helicase activities, *J. Biol. Chem. 279*, 43336−43344.

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