Differentiation of Secreted and Membrane-Type Matrix Metalloproteinase Activities Based on Substitutions and Interruptions of Triple-Helical Sequences†

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ABSTRACT: The turnover of the collagen triple-helical structure (collagenolysis) is a tightly regulated process in normal physiology and has been ascribed to a small number of proteases. Several members of the matrix metalloproteinase (MMPs) family possess collagenolytic activity, and the mechanisms by which these enzymes process triple helices are beginning to be unraveled. The present study has utilized two triple-helical sequences to compare the cleavage-site specificities of 10 MMPs. One substrate featured a continuous Gly-Xxx-Yyy sequence (Pro-Leu-Gly∼Met-Arg-Gly), while the other incorporated an interruption in the Gly-Xxx-Yyy repeat (Pro-Val-Asn∼Phe-Arg-Gly). Both sequences were selectively cleaved by MMP-13 while in linear form, but neither proved to be selective within a triple helix. This suggests that the conformational presentation of substrate sequences to a MMP active site is critical for enzyme specificity, in that activities differ when sequences are presented from an unwound triple helix versus an independent single strand. Differences in specificity between secreted and membrane-type (MT) MMPs were also observed for both sequences, where MMP-2 and MT-MMPs showed an ability to hydrolyze a triple helix at an additional site (Gly-Gln bond). Interruption of the triple helix had different effects on secreted MMPs and MT-MMPs, because MT-MMPs could not hydrolyze the Asn-Phe bond but instead cleaved the triple helix closer to the C terminus at a Gly-Gln bond. It is possible that MT-MMPs have a requirement for Gly in the P_1 subsite to be able to efficiently process a triple-helical molecule. Analysis of individual kinetic parameters and activation energies indicated different substrate preferences within secreted MMPs, because MMP-13 preferred the interrupted sequence, while MMP-8 showed little discrimination between non-interrupted and interrupted triple helices. On the basis of the present and prior studies, we can assign unique triple-helical peptidase behaviors to the collagenolytic MMPs. Such differences may be significant for understanding MMP mechanisms of action and aid in the development of selective MMP inhibitors.

Collagen serves as a structural scaffold and a barrier between tissues, and thus, collagen catabolism (collagenolysis) is required to be a tightly regulated process in normal physiology. In turn, the destruction or damage of collagen during pathological states plays a role in tumor-cell invasion or atherosclerotic plaque formation and rupture. Only a small number of proteases have been identified capable of efficient processing of triple-helical regions of collagens. A mechanistic understanding of the cleavage of intact collagens has been pursued for many years. Several members of the zinc metalloenzyme family, specifically matrix metalloproteinases (MMPs),[1] possess collagenolytic activity. For example, one or more of the interstitial collagens (types I−III) are hydrolyzed within their triple-helical domain by MMP-1, MMP-2, MMP-8, MMP-13, MMP-18, MT1-MMP (MMP-14), and MT2-MMP (MMP-15) (*1*−*3*). The mechanism of

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[1] Abbreviations: APMA, 4-aminophenyl mercuric acetate; CD, circular dichroism; DMSO, dimethylsulfoxide; Dnp, 2,4-dinitrophenyl; Fmoc, 9-fluorenylmethoxycarbonyl; FRET, fluorescence resonance energy transfer; fTHP, fluorogenic triple-helical peptide; Hyp, 4-hydroxy-L-proline; MALDI−TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Mca, (7-methoxycoumarin-4-yl)acetyl; MMP, matrix metalloproteinase; NC, not cleaved; ND, not determined; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TIMP, tissue inhibitor of metalloproteinases.

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MMP-catalyzed collagenolysis is beginning to be unraveled, with the interactive roles of the enzyme and the substrate becoming better understood (*4*−*6*). For example, to access the individual strands of collagen, the MMP unwinds the substrate, and unwinding activity is present in multiple MMP domains (*4*, *6*). However, there are subtle differences in MMP collagenolytic activities. MMP-1 hydrolyzes type III collagen more rapidly than type I, while MMP-8 and MT1-MMP show a slight preference for type I collagen compared to type III (*7*, *8*). Neither MMP-1 nor MMP-8 hydrolyze type II collagen efficiently (*8*). Conversely, MMP-13 prefers type II collagen and hydrolyzes this collagen much more rapidly than MMP-1 or MMP-8 (*8*). Why MMPs exhibit collagen preferences is far from completely understood, although recent studies have suggested that the combination of substrate sequence and stability can play a role (*5*, *9*).

The sequence specificities of collagenolytic MMPs have been extensively examined utilizing single-stranded peptides, peptide libraries, and phage-display libraries. These include studies of MMP-1, MMP-2, and MMP-8 (*10*, *11*), MMP-13 (*12*, *13*), and MT1-MMP (*11*, *14*−*17*). However, there are only a few studies that described the effects of amino acid substitutions *within a triple-helical context* on MMP activity (*5*, *9*, *18*, *19*). The influence of the triple-helical structure on the interaction between MMP subsites and individual substrate residues has not been explored but may provide additional information for the mechanism of collagenolysis, the understanding of collagen specificity, and the design of selective inhibitors.

Collagenolytic activity may also be considered in light of an array of diseases caused by mutations occurring in collagen, such as osteogenesis imperfecta (*20*) or Ehlers−Danlos syndrome (*21*).[2] The majority of these mutations result in the replacment of Gly by another amino acid, thus causing an interruption of the Gly-Xxx-Yyy register found within the triple helix. Of direct relevance to collagenolysis is the mutation of Gly^{775} to Glu in the α1 chain of type III collagen, leading to Ehlers−Danlos syndrome IV and aortic aneurism (*22*). The α1(III) Gly^{775}-Leu^{776} bond is normally cleaved by collagenolytic MMPs (*23*). Collagenase (MMP-1, MMP-8, MMP-13, and MT1-MMP) cleavage of non-Gly-Xxx-Yyy repeating sequences has been examined but only with linear substrates (*10*−*17*). Within the triple helix, the enhancement or inhibition of collagenolysis because of Gly-Xxx-Yyy interruptions has not been explored.

The present study has considered MMP sequence specificities *in a triple-helical context* in a further attempt to evaluate the role of substrate characteristics on collagenolysis. More precisely, we have utilized prior studies on MMP-13 selectivity for single-stranded sequences (*12*, *13*) to evaluate the specificity of such sequences in a triple helix. We have examined changes in MMP activity for both continuous Gly-Xxx-Yyy sequences and as a result of interruption in the Gly-Xxx-Yyy motif. MMP triple-helical peptidase activities were compared using fluorescent resonance energy transfer (FRET) triple-helical substrates. The general considerations for the design and synthesis of such substrates, including (7-methoxycoumarin-4-yl)acetyl (Mca) as a fluorophore and 2,4-dinitrophenyl (Dnp) as a quencher, were reported previously (*24*−*26*). The effects of enhanced substrate thermal stability on MMP activity has also been evaluated on the basis of prior work, showing that collagenolytic MMPs have different tolerances for more thermally stable substrates (*5*, *9*).

[2] A list of known mutations can be found at the Database of Human Collagen Mutations (www.le.ac.uk/genetics/collagen).

MATERIALS AND METHODS

All standard chemicals were peptide-synthesis- or molecular-biology-grade and purchased from Fisher Scientific. 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, 1-hydroxybenzotriazole, and 9-fluorenylmethoxycarbonyl (Fmoc)-amino acid derivatives [including Fmoc-Lys(Mca) and Fmoc-Lys(Dnp)] were obtained from Novabiochem (San-Diego, CA). Amino acids are of the L configuration (except for Gly). Mca-Lys-Pro-Leu-Gly-Leu-Lys(Dnp)-Ala-Arg-NH_2 and NFF-3 were synthesized by methods described previously (*27*, *28*). The synthesis and characterization of fTHP-4 was also described previously (*5*).

Peptide Synthesis. Peptide-resin assembly of fluorogenic triple-helical peptides (fTHPs) was performed by Fmoc solid-phase methodology on an ABI 433A peptide synthesizer (*24*). All peptides were synthesized as C-terminal amides to prevent diketopiperazine formation (*29*). Peptide resins were lipidated with hexanoic acid [$CH_3(CH_2)_4CO_2H$, designated C_6] as described (*30*, *31*). Cleavage and side-chain deprotection of peptide resins proceeded for at least 3 h using thioanisole−water−trifluoroacetic acid (TFA) (5:5:90) (*32*). Cleavage solutions were extracted with methyl *t*Bu ether prior to purification.

Peptide Purification. Reversed-phase high-performance liquid chromatography (RP-HPLC) purification was performed on a Rainin AutoPrep System with a Vydac 218TP152022 C_{18} column (15−20 μm particle size, 300 Å pore size, 250 × 22 mm) at a flow rate of 10.0 mL/min. Eluants were 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The elution gradient was adjusted as required. Detection was at $\lambda = 220$ nm. Analytical RP-HPLC and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI−TOF MS) (see below) were used to identify fractions of the homogeneous product.

Peptide Analyses. Analytical RP-HPLC was performed on a Hewlett-Packard 1100 liquid chromatograph equipped with a Vydac 218TP5415 C_{18} RP column (5 μm particle size, 300 Å pore size, 150 × 4.6 mm). Eluants were 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The elution gradient was 0−100% B in 20 min with a flow rate of 1.0 mL/min. Detection was at $\lambda = 220$, 324, and 363 nm. MALDI−TOF MS was performed on a Applied Biosystems Voyager MALDI−TOF mass spectrometer using an α-cyano-4-hydroxycinnamic acid matrix (*33*). fTHP mass values were as follows: fTHP-12 $[M + H]^+$, 4594 Da (theoretical, 4592.0 Da); C_6-fTHP-12 $[M + H]^+$, 4697 Da (theoretical, 4690.2 Da); fTHP-13 $[M + H]^+$, 4651 Da (theoretical, 4648.0 Da); C_6-fTHP-13 $[M + H]^+$, 4748 Da (theoretical, 4749.2 Da); and C_6-fTHP-14 $[M + H]^+$, 4722.4 Da (theoretical, 4718.2 Da). All mass values were within 0.14% of the theoretical.

Circular Dichroism (CD) Spectroscopy. CD spectra were recorded over the range $\lambda = 190$−250 nm on a JASCO J-810 spectropolarimeter using a 1.0 cm path-length quartz cell. Thermal transition curves were obtained by recording the

molar ellipticity ([Θ]) at $\lambda = 222$ nm, while the temperature was continuously increased in the range of 5−95 °C at a rate of 0.2 °C/min. The temperature was controlled using a JASCO PFD-425S temperature control unit. For samples exhibiting sigmoidal melting curves, the inflection point in the transition region (first derivative) is defined as the melting temperature (T_m).

Matrix Metalloproteinases. ProMMP-1 and proMMP-3 were expressed in *Escherichia coli* and folded from the inclusion bodies as described previously (*34*, *35*). ProMMP-1 was activated by reacting with 1 mM 4-aminophenyl mercuric acetate (APMA) and 0.1 equiv of MMP-3($\Delta_{248-460}$) at 37 °C for 6 h. After activation, MMP-3($\Delta_{248-460}$) was completely removed from MMP-1 by affinity chromatography using an anti-MMP-3 IgG Affi-Gel 10 column. ProMMP-3 was activated by reacting with 5 μg/mL chymotrypsin at 37 °C for 2 h. Chymotrypsin was inactivated with 2 mM diisopropylfluorophosphate. ProMMP-2 was purified from the culture medium of human uterine cervical fibroblasts (*36*) and activated by incubating with 1 mM APMA for 2 h at 37 °C. ProMMP-8 was expressed in CHO-K1 cells as described previously (*37*). ProMMP-8 was activated by incubating with 1 mM APMA for 2 h at 37 °C. Recombinant MMP-9 with the linker and the C-terminal hemopexin-like domain deleted [residues 444−707, designated MMP-9($\Delta_{444-707}$)] was expressed in *E. coli* in the active form, with Phe107 at the N terminus as described elsewhere.[3] The resulting MMP-9($\Delta_{444-707}$) was similar to that previously documented (*38*). ProMMP-13 was purchased from R&D Systems (Minneapolis, MN) and activated by incubating with 1 mM APMA for 2 h at 37 °C. The concentrations of active MMP-1, MMP-2, MMP-3, MMP-8, MMP-9($\Delta_{444-707}$), and MMP-13 were determined by titration with a recombinant tissue inhibitor of metalloproteinases 1 (TIMP-1) or N-TIMP-1 over a concentration range of 0.1−3 μg/mL (*39*). Recombinant MT1-MMP with the linker and C-terminal hemopexin-like domains deleted [residues 279−523, designated MT1-MMP($\Delta_{279-523}$)] was purchased from Chemicon International (Temecula, CA). MT1-MMP($\Delta_{279-523}$) was expressed and activated, resulting in Tyr112 at the N terminus. MT1-MMP($\Delta_{279-523}$), which, in contrast to MT1-MMP, does not undergo rapid autoproteolysis, was used in the present studies because of the relatively small differences in MT1-MMP($\Delta_{279-523}$) and MT1-MMP triple-helical peptidase activities noted previously (*40*). Recombinant MT2-MMP with the linker and C-terminal hemopexin-like domains deleted [residues 268−628, designated MT2-MMP-($\Delta_{268-628}$)] was also purchased from Chemicon International. MT2-MMP($\Delta_{268-628}$) was expressed and activated, resulting in Tyr91 at the N terminus. Recombinant MT5-MMP and MT6-MMP were expressed in MDCK cells and *E. coli*, respectively, as described previously (*41*, *42*). The concentrations of active MT1-MMP($\Delta_{279-523}$), MT2-MMP-($\Delta_{268-628}$), MT5-MMP, and MT6-MMP were determined by titration with recombinant TIMP-2, N-TIMP-2, or N-TIMP-3 (*40*, *42*−*45*). ProMMP-3($\Delta_{248-460}$) was expressed in *E. coli* using the expression vector pET3a (Novagen), folded from inclusion bodies and purified as described previously (*46*). ProMMP-3($\Delta_{248-460}$) was activated by reacting with 5 μg/mL chymotrypsin at 37 °C for 2 h. Chymotrypsin was inactivated with 2 mM diisopropylfluorophosphate. Active-site titrations utilized either Mca-Lys-Pro-Leu-Gly-Leu-Lys-(Dnp)-Ala-Arg-NH_2 or NFF-3 as the substrate (*27*, *28*, *47*).

[3] Wei et al., manuscript in preparation.

Table 1: Sequences and Stabilities of (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-P_2-P_1∼P_1'-P_2'-P_3'-P_4'-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_5 fTHPs[a]

fTHP	P_2-P_1∼P_1'-P_2'-P_3'-P_4 sequence	peptide T_m (°C)	C_6 peptide T_m (°C)
fTHP-4	Gln-Gly∼Leu-Arg-Gly-Gln	36.5	ND[b]
fTHP-12	**Leu**-Gly∼**Met**-Arg-Gly-Gln	45.0	51.0
fTHP-13	**Val**-**Asn**∼**Phe**-Arg-Gly-Gln	39.0	50.0
fTHP-14	**Val**-**Asn**∼**Phe**-Arg-Gly-**Pro**	ND	46.0

[a] Substitutions relative to fTHP-4 are indicated in bold font. [b] ND = not determined.

Assays. Substrate stock solutions were prepared at various concentrations with 0.25−1.0% dimethylsulfoxide (DMSO) in EAB buffer (50 mM tricine, 50 mM NaCl, 10 mM $CaCl_2$, and 0.005% Brij-35 at pH 7.5). MMP assays were conducted in EAB buffer by incubating a range of substrate concentrations with 10 nM enzyme at 30 °C. Fluorescence was measured on a Molecular Devices SPECTRAmax Gemini EM dual-scanning microplate spectrofluoremeter using $\lambda_{excitation} = 324$ nm and $\lambda_{emission} = 393$ nm. Initial velocities were obtained from plots of fluorescence versus time, using data points from only the linear portion of the hydrolysis curve. The slope from these plots was divided by the fluorescence change corresponding to complete hydrolysis and then multiplied by the substrate concentration to obtain the initial velocity in units of micromolars per second. Kinetic parameters were evaluated by Lineweaver−Burk, Eadie−Hofstee, and Hanes−Woolf analyses. To determine activation energies (E_a), kinetic parameters were determined over the range of 24−37 °C (on the basis of the T_m of the substrate) and an Arrhenius plot of log k_{cat} versus 1/temperature (in kelvin) was constructed, where the slope $= -E_a/2.3R$ and R is the molar gas constant. MMP substrate cleavage sites were established by MALDI−TOF MS. Cleavage sites were not altered by peptide lipidation with hexanoic acid, as previously observed (*24*, *33*).

RESULTS AND DISCUSSION

Design and Structural Characterization of fTHP Substrates. fTHP-4 (Table 1) is a model of the MMP consensus cleavage site in human types I−III collagens (*5*). Important features of fTHP-4 include the Gly∼Leu cleavage site and Gly-Pro-Hyp repeats to enhance triple-helical stability. To serve as the FRET fluorophore-quencher pair, Mca and Dnp are linked to Lys side chains in the P_5 and P_5' subsites, respectively. The fTHP-4 sequence was modified subsequently in the P_2, P_1, and/or P_1' subsites to create fTHP-12 and fTHP-13. For fTHP-12, the P_2 subsite Gln and the P_1' subsite Leu from fTHP-4 were replaced with Leu and Met, respectively (Table 1). Deng et al. utilized the phage display to identify selective MMP-13 substrates and found that the sequence Pro-Leu-Gly∼Met-Arg-Gly was hydrolyzed by MMP-13 820 times more rapidly than by MMP-1, 1300 times more rapidly than by MMP-3, and 11 times more rapidly than by MMP-9 (*12*). Thus, fTHP-12 was constructed to determine if selectivity observed by MMP subsite interactions with single-stranded substrates was retained in triple-helical substrates. A similar prior study showed that Pro-

Table 2: Cleavage-Site Specificity for MMP Hydrolysis of fTHP-4, fTHP-12, fTHP-13, and fTHP-14 at 30 °C

enzyme	fTHP-4	fTHP-12	fTHP-13	C_6-fTHP-14
MMP-1	Gly-Leu	ND	ND	NC
MMP-2	Gly-Leu, Gly-Gln	Gly-Met	Asn-Phe	NC
MMP-3	NC	ND	ND	ND
MMP-8	Gly-Leu	Gly-Met	Asn-Phe	Asn-Phe
MMP-9($\Delta_{444-707}$)	Gly-Leu	Gly-Met	ND	NC
MMP-13	Gly-Leu	Gly-Met	Asn-Phe	Asn-Phe
MT1-MMP($\Delta_{279-523}$)	Gly-Leu, Gly-Gln	Gly-Met, Gly-Gln	Gly-Gln	Asn-Phe
MT2-MMP($\Delta_{268-628}$)	Gly-Leu	Gly-Met, Gly-Gln	Gly-Gln	ND
MT5-MMP	NC	Gly-Met, Gly-Gln	NC	NC
MT6-MMP	NC	NC	NC	NC

Val-Asn-Phe-Arg was hydrolyzed by MMP-13 >10700 times more rapidly than by MMP-1, 400 times more rapidly than by MMP-3, 220 times more rapidly than by MMP-9, and 88 times more rapidly than by MMP-2 (*13*). Thus, replacement of the P_2-P_1' subsite Gln-Gly-Leu triplet from fTHP-4 with Val-Asn-Phe resulted in fTHP-13 (Table 1). fTHP-13 was further modified in the P_4' subsite, where Gln was replaced by Pro to remove the MT1-MMP($\Delta_{279-523}$) and MT2-MMP($\Delta_{268-628}$) Gly-Gln cleavage site (see below). The modified sequence was designated fTHP-14 (Table 1).

fTHP-12, fTHP-13, and fTHP-14 were acylated with hexanoic (C_6) acid to provide a second series of substrates with differing thermal stability, because the addition of hexanoic acid to the N terminus of triple-helical peptides was found previously to increase the triple-helix thermostability by 6.6−8.5 °C (*5*, *24*, *31*). MMP-1 and MT1-MMP-($\Delta_{279-523}$) triple-helical peptidase activities have been shown to be dependent upon substrate thermal stability (*5*), and thus, we wished to evaluate the role of substrate thermal stability on activity for a large number of MMPs. fTHP-14 could not be obtained in high purity, but C_6-fTHP-14 could; thus, only C_6-fTHP-14 was used in the present studies. The purified, lipidated substrates exhibited decreased solubility in the assay buffer compared with the nonlipidated substrates, and thus, DMSO was utilized for improved solubility.

CD spectra were obtained for all THPs and were found to be characteristic of triple helices (data not shown). To quantify the thermal stability of potential substrates, [Θ] at $\lambda = 222$ nm was monitored as a function of increasing temperature. All structures exhibited cooperative transitions, indicative of the melting of a triple helix to a single-stranded structure [data not shown but comparable to previously published fTHP melting curves (*24*−*26*)]. Melting temperatures ranged from 36.5 to 51.0 °C (Table 1). The decrease in triple-helical thermal stability of 6 °C going from a continuous triple-helical sequence (fTHP-12) to an interrupted one (fTHP-13) was not substantial. As shown previously, the use of triple-helical stabilizing regions [such as $(Gly\text{-}Pro\text{-}4\text{-}Hyp)_n$] on both the N and C termini can fold and order a central non-Gly-Xxx-Yyy region, minimizing the relative thermal destabilization (*48*, *49*). All fTHPs had appropriate thermal stabilities for the examination of substrate hydrolysis at 30 °C.

Cleavage-Site Specificity and Selectivity. All fTHPs were initially examined for their MMP cleavage-site specificities (Table 2) and rates of hydrolysis (Figures 1 and 2). MMP-1, MMP-8, MMP-13, and MT1-MMP were chosen on the basis of their ability to cleave types I−III collagen (*7*, *50*−*54*). Of the gelatinase family members, MMP-2 is also known to cleave types I and III collagen (*55*, *56*), in contrast to MMP-9, which does not process types I−III collagens (*57*). It is also of interest to compare the capabilities of gelatinases and collagenases to process triple-helical substrates, and thus, both gelatinases were included in these studies. MT2-MMP was included because, while the full range of activity is unknown, it has been reported to cleave type I collagen (*3*, *58*). MMP-3 was chosen to provide a negative control for secreted MMPs, because it was previously shown to have poor or no triple-helical peptidase activities (*25*, *59*). MT5-MMP and MT6-MMP were used as noncollagenolytic controls for transmembrane-type and GPI-anchored MMPs, respectively. The 10 MMPs were of either mammalian (MMP-2, MMP-8, MMP-13, MT1-MMP, MT2-MMP, and MT5-MMP) or bacterial (MMP-1, MMP-3, MMP-9, and MT6-MMP) origin. Prior studies had shown no difference in proteolytic activities based on MMP glycosylation (*60*, *61*), and thus, convenient and well-characterized expression systems for each MMP were used here.

Three MMPs lacking their hemopexin-like C-terminal domains were studied: MT1-MMP($\Delta_{279-523}$), MT2-MMP-($\Delta_{268-628}$), and MMP-9($\Delta_{444-707}$). A prior comparison of the MT1-MMP catalytic domain [MT1-MMP($\Delta_{319-523}$)] with the MT1-MMP ectodomain (residues 112−523, designated ΔTM-MT1-MMP) showed that MT1-MMP($\Delta_{319-523}$) was more efficient at single-stranded substrate hydrolysis than ΔTM-MT1-MMP, but differences in triple-helical peptidase activity were only slight (*40*). One may assume that MT2-MMP behaves in a similar fashion as MT1-MMP in terms of the role of the C-terminal domain. The C-terminal domain of MMP-9 does not modulate proteolytic activities of the enzyme (*61*, *62*). The C-terminal domain is not critical for triple-helical peptidase activity, as noted previously (*5*, *24*, *40*, *59*, *63*−*65*).

Secreted MMPs (MMP-1, MMP-2, MMP-8, and MMP-13) typically cleaved only one bond in fTHP-4, fTHP-12, fTHP-13, and fTHP-14 (Table 2). In some cases, cleavage sites could not be determined because of slow rates of hydrolysis. MMP-3 was not efficient at cleaving triple-helical substrates, consistent with prior results for MMP-3 hydrolysis of fTHP-4 (*9*). The Pro-Leu-Gly-Met-Arg-Gly-Gln sequence (fTHP-12), which proved to be MMP-13-selective as a phage-display-derived linear sequence, was neither specific nor selective when contained in a triple helix. fTHP-12 was cleaved by almost all MMPs at significant rates (Figure 1). The Pro-Leu-Gly∼Met-Arg-Gly motif was previously shown to be greatly favored by MMP-13 over MMP-1 and MMP-9 in a linear context (*13*). We found the same pattern to exist for this motif in a triple-helical context (Figure 1). However, MMP-2 favored Pro-Leu-Gly∼Met-Arg-Gly over MMP-13 in the triple helix, the opposite of the behavior observed for

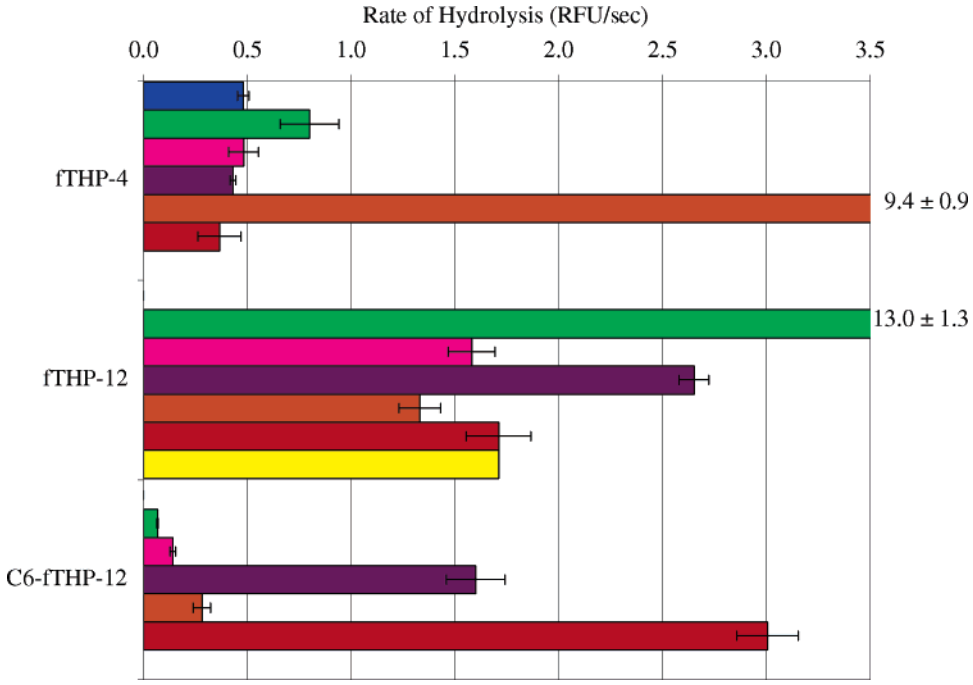

FIGURE 1: Rates of MMP hydrolysis for fTHP-4 and fTHP-12. Hydrolysis was examined using 10 nM of each MMP and 4 μM substrate (fTHP-4, fTHP-12, or C_6-fTHP-12). Fluorescence was measured using $\lambda_{excitation} = 324$ nm and $\lambda_{emission} = 393$ nm. Rates of hydrolysis were obtained from plots of fluorescence versus time, using data points from only the linear portion of the hydrolysis curve. The MMPs examined were MMP-1 (blue), MMP-2 (green), MMP-8 (pink), MMP-13 (purple), MT1-MMP (orange), MT2-MMP (red), and MT5-MMP (yellow). Numbers at the far right indicate off-scale hydrolysis values and standard deviations. Data are not shown in cases where hydrolysis rates were negligible.

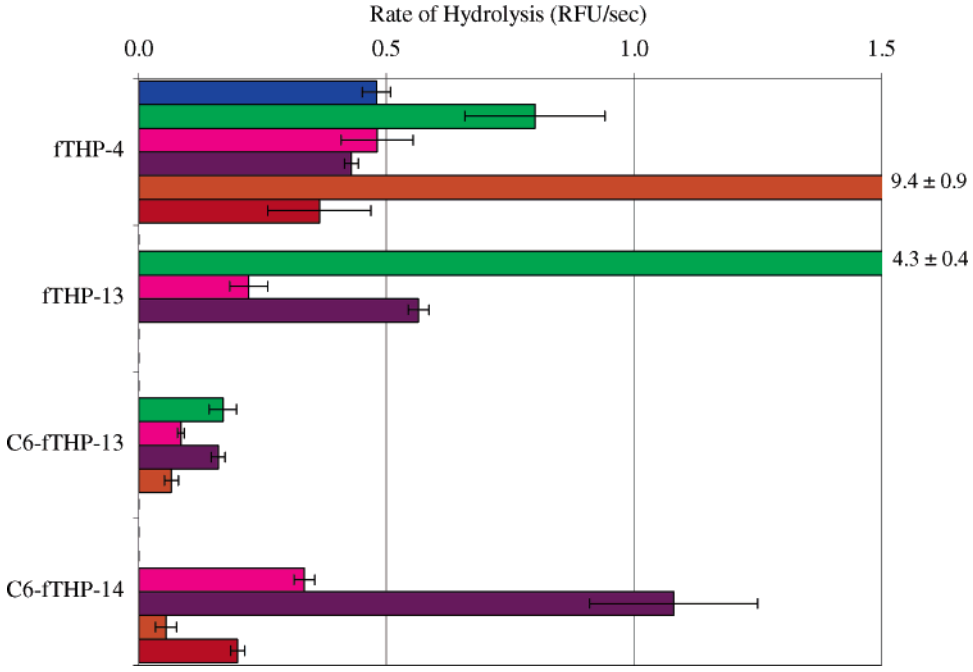

FIGURE 2: Rates of MMP hydrolysis for fTHP-4, fTHP-13, and fTHP-14. Hydrolysis was examined using 10 nM of each MMP and 4 μM substrate (fTHP-4, fTHP-13, C_6-fTHP-13, or C_6-fTHP-14). Fluorescence was measured using $\lambda_{excitation} = 324$ nm and $\lambda_{emission} = 393$ nm. Rates of hydrolysis were obtained from plots of fluorescence versus time, using data points from only the linear portion of the hydrolysis curve. The MMPs examined were MMP-1 (blue), MMP-2 (green), MMP-8 (pink), MMP-13 (purple), MT1-MMP (orange), MT2-MMP (red), and MT5-MMP (yellow). Numbers at the far right indicate off-scale hydrolysis values and standard deviations. Data are not shown in cases where hydrolysis rates were negligible.

a linear construct (*13*). Thus, the ability of MMP-2 to process the Gly-Met bond was sensitive to the conformation of the substrate. We previously found that the α1(V) collagen-derived sequence, Gly-Pro−Pro-Gly∼Val−Val-Gly-Glu, was efficiently hydrolyzed by MMP-2 and MMP-9 in a triple-helical context but not as a single-stranded substrate (*25*).

There was a clear difference in fTHP-12 specificity between secreted MMPs and MT-MMPs. All MT-MMPs that cleaved fTHP-12 did so at two bonds, Gly-Met and Gly-Gln (Table 2). The secreted MMPs cleaved fTHP-12 only at the Gly-Met bond (Table 2). This substrate was also the only triple-helical sequence cleaved by MT5-MMP. The

addition of hexanoic acid (C_6) to fTHP-12 made this substrate more selective toward MMP-13 and MT2-MMP($\Delta_{268-628}$) (Figure 1). MT2-MMP($\Delta_{268-628}$) actually showed greater activity toward the C_6 variant of this substrate. MMP-2 was affected the most by the increase of thermal stability of the substrate, because activity was virtually lost in the C_6 variant (Figure 1).

It stands to reason that, because different MMPs are affected by triple-helical stability to a different degree, then interruption of the triple-helical sequence as a result of the Gly mutation might have similar effects. The introduction of a Gly interruption (Pro-Val-Asn-Phe-Arg-Gly-Gln; fTHP-13) did not seem to shift the "reading frame" of secreted MMPs, because cleavage still occurred at the same relative P_1-P_1' subsite location as in the uninterrupted sequences (Table 2). Selectivity, however, increased for MMP-13 and MMP-2 as compared to the consensus type I−III sequence (fTHP-4; see Figure 2). MT-MMPs were more effected than secreted MMPs by the introduction of a Gly interruption, which apparently resulted in an inability of MT-MMPs to cleave at the putative P_1-P_1' bond (Asn-Phe). A secondary cleavage site (Gly-Gln) found in fTHP-4 and fTHP-12 became the primary MT1-MMP($\Delta_{279-523}$) and MT2-MMP-($\Delta_{268-628}$) cleavage site in fTHP-13 (Table 2). This suggests either the necessity of Gly present in P_1 subsite or an intact Gly-Xxx-Yyy register for MT-MMPs to bind and process the triple-helical molecule. The addition of C_6 to the Gly interrupted sequence (C_6-fTHP-13) practically abolished the activities of MT1-MMP($\Delta_{279-523}$), MT2-MMP($\Delta_{268-628}$), and MMP-8 and significantly lowered MMP-2 and MMP-13 activities (Figure 2). The subsequent substitution of Gln in the P_4' subsite for Pro (fTHP-14) led to the more selective substrate (Figure 2). Interestingly, MT1-MMP($\Delta_{279-523}$) was able to cleave the Asn-Phe bond in this case but at a very insignificant rate.

On the basis of prior studies, it is not readily apparent why MT1-MMP($\Delta_{279-523}$) does not cleave the Asn-Phe bond in fTHP-13. MT1-MMP has activity toward a great variety of proteins beyond collagen (*66*, *67*). No significant differences are observed in the S_1 pocket of MT1-MMP compared to other MMPs (*68*), and thus, Asn should be as well-tolerated as in the secreted MMPs. A variety of linear substates containing Asn∼Phe-Arg motifs were previously shown to be hydrolyzed efficiently (k_{cat}/K_M = 5900−49 600 M^{-1} s^{-1}) by MT1-MMP (*16*). Triple-helical peptide, linear peptide, and phage-display peptide library studies indicate that MT1-MMP prefers long chain, hydrophobic residues in the P_1' subsite (*5*, *14*−*16*). More specifically, Phe in the P_1' subsite should also be well-tolerated, because both MT1-MMP and MT2-MMP have deep hydrophobic S_1' pockets as evidenced by their ability to cleave Gly-Cys(Mob) bonds (*5*, *9*). However, the above-mentioned linear peptides all contained Glu in P_2, Thr in P_3', and Ala in P_4', which are not seen in fTHP-13. Thus, the lack of MT-MMP hydrolysis of the fTHP-13 Asn-Phe bond may be due to the influence of neighboring residues, triple-helical context, or both. It is conceivable that triple-helical peptides incorporating the Gly-Pro-Val-Asn-Phe-Arg-Gly-Gln motif can be useful as an active MMP profiling tool because of the presence of two distinct cleavage sites, one of which is amenable to cleavage by secreted MMPs and another by MT-MMPs.

Table 3: Kinetic Parameters for fTHP-4, fTHP-12, and fTHP-13 Hydrolysis by MMP-8 and MMP-13 at 30 °C

enzyme	substrate	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (μM)
MMP-8	fTHP-4	4500[a]	0.035 ± 0.016[a]	7.60 ± 2.40[a]
MMP-8	C_6-fTHP-12	5090	0.070 ± 0.020	14.50 ± 4.80
MMP-8	fTHP-13	3010	0.028 ± 0.004	9.30 ± 1.84
MMP-8	C_6-fTHP-13	3870	0.020 ± 0.010	3.90 ± 2.30
MMP-13	fTHP-4	1600[a]	0.015 ± 0.005[a]	8.60 ± 1.70[a]
MMP-13	C_6-fTHP-12	9710	0.060 ± 0.030	6.35 ± 3.0
MMP-13	fTHP-13	6116	0.127 ± 0.046	20.50 ± 6.22
MMP-13	C_6-fTHP-13	5800	0.060 ± 0.040	9.95 ± 0.35

[a] From ref *9*.

Table 4: Activation Energies for Substrate Hydrolysis by MMPs

enzyme	substrate	substrate T_m (°C)	E_a (kcal/mol)
MMP-8	fTHP-4	36.3	12.8 ± 2.1
MMP-8	fTHP-13	39.0	15.5 ± 1.0
MMP-13	fTHP-4	36.3	17.4 ± 2.0
MMP-13	fTHP-13	39.0	12.7 ± 0.6
MMP-1	fTHP-4	36.3	20.0 ± 1.6[a]
MMP-1($\Delta_{243-450}$)	fTHP-4	36.3	29.0 ± 0.6[a]
MMP-14($\Delta_{279-523}$)	fTHP-4	36.3	8.8 ± 0.1[a]

[a] From ref *5*.

Kinetic Parameters and Activation Energies. Kinetic parameters and activation energies for substrate hydrolysis were determined for two secreted enzymes, MMP-8 and MMP-13, because these MMPs exhibited reasonable rates of hydrolysis for all potential substrates (Figures 1 and 2). Initially, a comparison was made between the consensus triple-helical sequence (fTHP-4) and the interrupted triple-helical sequence (fTHP-13). MMP-8 showed a marginally higher k_{cat}/K_M with fTHP-4 than with fTHP-13 (4490 versus 3010 M^{-1} s^{-1}; Table 3). MMP-13 exhibited the reverse k_{cat}/K_M trends for fTHP-4 and fTHP-13, with values of 1735 and 6116 M^{-1} s^{-1}, respectively. This indicates that, among the secreted MMPs, differences exist in specificity toward triple-helical sequences with an interrupted Gly register. In the case of MMP-13, the difference in specificity is primarily because of an increase in k_{cat}.

A second comparison was made for the MMP-13-derived triple-helical sequence (fTHP-12) and the interrupted triple-helical sequence (fTHP-13). In this case, the C_6 variants of the substrates were used, because their thermal stabilities were very similar (Table 1). A comparison of kinetic parameters for MMP-8 and MMP-13 hydrolysis of C_6-fTHP-12 and C_6-fTHP-13 (Table 3) indicated similar activities toward both substrates, but MMP-8 had distinctly different individual k_{cat} and K_M values for each substrate. For example, C_6-fTHP-12 was turned over more rapidly than C_6-fTHP-13 by MMP-8. MMP-8 had a much worse K_M value for the uninterrupted sequence (C_6-fTHP-12), while MMP-13 had a worse K_M value for the interrupted sequence (C_6-fTHP-13).

The Gly interruption of fTHP-13 had opposite effects on MMP-8 and MMP-13 activation energies. MMP-8 showed a lower E_a for hydrolysis of an uninterrupted sequence (fTHP-4), whereas MMP-13 had a lower E_a with the interrupted fTHP-13 sequence (Table 4). These activation energy trends correlate with the sequence specificities of the enzymes. Overall, MMP-8 showed much closer E_a values

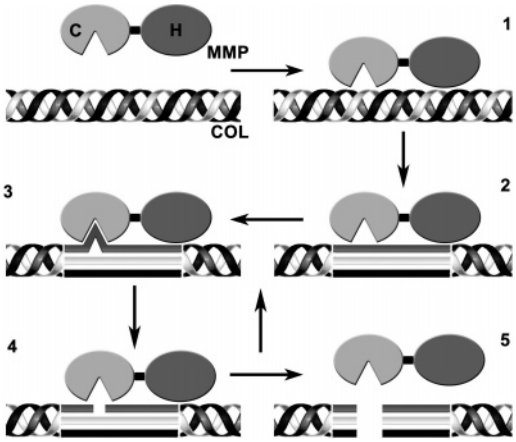

FIGURE 3: Putative stepwise mechanism for MMP-catalyzed triple-helix hydrolysis. Step 1 is the binding of the triple helix to the MMP. Step 2 is the unwinding of the triple helix, allowing access to individual strands. Step 3 is the binding of a single strand to the MMP active site. Step 4 is the hydrolysis of a single strand and release of the product. For step 5, in rapid succession, the other two strands are bound and cleaved and the products are released. COL = collagen, C = catalytic domain, and H = hemopexin-like domain.

and individual kinetic parameters for both sequences than MMP-13. Because activation energies reflect the difficulty in accessing water at the site of hydrolysis in native collagen, i.e., reaching the transition state (*69*, *70*), differences between MMP-8 and MMP-13 may well-reflect the collagenolytic potential of the two enzymes. Considering the present and prior studies (*5*), MT1-MMP exhibited the lowest activation energy among all tested collagenolytic MMPs for the hydrolysis of the consensus type I−III collagen sequence (fTHP-4). In light of this result, the inability of MT1-MMP to cleave an interrupted sequence is even more intriguing.

A putative mechanism by which MMP-catalyzed hydrolysis of a triple helix can be divided into several distinct steps (Figure 3). First is an initial binding of the MMP to a triple helix. Many MMPs are capable of binding to triple helices, including some that do not cleave collagen (*71*, *72*). Second, an unwinding of the triple helix allows access to single strands (*4*). The unwinding step appears to differentiate collagenolytic from noncollagenolytic MMPs (*4*). Third, a single strand binds to the active site. The binding of the single strand to the active site appears to be the last step in the triple-helix unwinding process (*4*). Fourth, a single strand is cleaved, and the product is released. Fifth, in rapid succession, the other two strands are bound and cleaved and the products are released. The results of the present study suggest that the third and fourth steps occur at a different rate than if the single strand is introduced independently (i.e., not part of an initial triple helix). For example, the Pro-Leu-Gly-Met-Arg-Gly-Gln sequence was not selective for MMP-13 when presented in a triple helix but was selective as a single-stranded sequence (*12*). In a similar fashion, the Asn-Phe-Arg motif, when part of a triple helix, was not hydrolyzed by MT1-MMP, while single-stranded sequences containing this motif were hydrolyzed (*16*). Although collagenases have different collagen specificities, K_M values for the hydrolysis of types I−III collagen are similar (*73*, *74*). Overall, this suggests that the conformational presentation of substrate sequences to a MMP active site is critical for enzyme specificity, in that activities differ when sequences are presented from an unwound triple helix versus an independent single strand.

The present study has delineated secreted MMP from MT-MMP triple-helical peptidase activity, in terms of sequence specificity (cleavage of Gly-Gln bonds) and hydrolysis of interrupted triple-helical sequences. In turn, among the secreted collagenolytic MMPs, MMP-8 and MMP-13 show different relative preferences for interrupted versus uninterrupted triple helices. On the basis of the present and prior

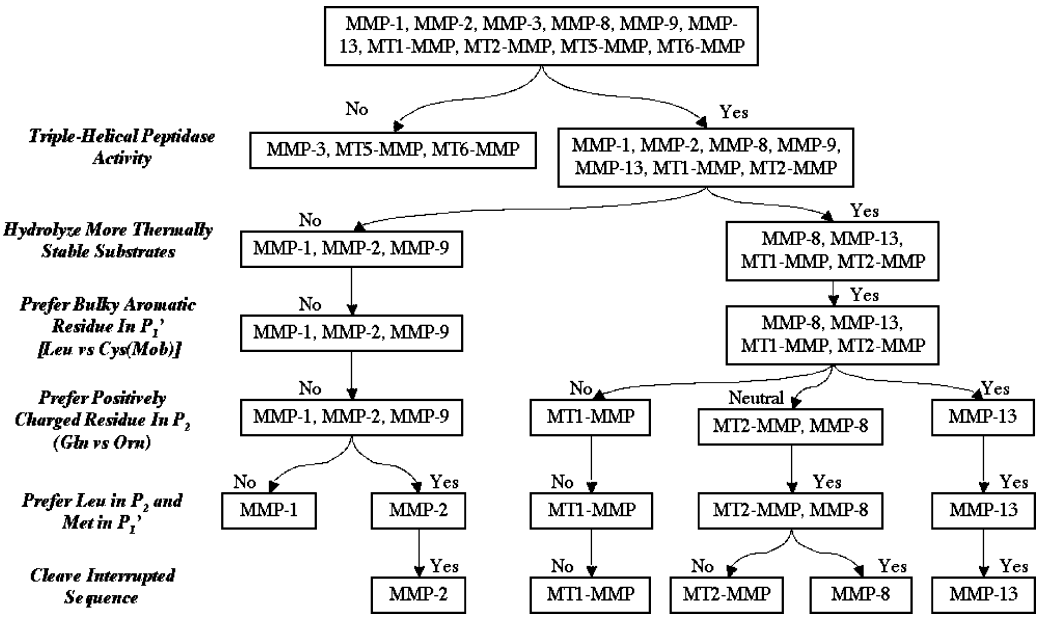

FIGURE 4: Flowchart depicting MMP triple-helical peptidase activities. The 10 MMPs examined in the present study are initially subdivided on the basis of their ability to cleave a triple-helical substrate. A subdivision within the triple-helical peptidase MMPs occurs upon their ability to process more thermally stable substrates. The sequence specificity of each MMP, initially based on individual substitutions within the P_1' and P_2 subsites (*9*), allows for the next subdivision. The sequence specificity of each MMP, based on combined substitutions within the P_1' and P_2 subsites, followed by preferences for an interrupted sequence, allows for a final subdivision. Ultimately, all "collagenolytic" MMPs (MMP-1, MMP-2, MMP-8, MMP-13, MT1-MMP, and MT2-MMP) display distinct behaviors.

studies (*5*, *9*, *24*, *25*, *33*, *59*), we can assign unique triple-helical peptidase behaviors to most of the collagenolytic MMPs (Figure 4). MMP-1 has the weakest triple-helical peptidase activity and has great difficulty in hydrolyzing more thermally stable substrates. MMP-1 does not tolerate long chain, hydrophobic residues interacting with its S_1' subsite nor positively charged residues interacting with the S_2 subsite. MMP-2 possesses many of the characteristics described for MMP-1 but additionally will cleave Gly-Gln bonds. Unlike MMP-1, MMP-2 prefers the combination of Leu in the substrate P_2 subsite and Met in the P_1' subsite compared to Gln and Leu in these respective subsites. MMP-8 is a robust triple-helical peptidase that favors long chain, hydrophobic residues interacting with its S_1' subsite. MMP-9 activity is similar to that of MMP-2, except that cleavage of Gly-Gln bonds is not observed. In contrast to MMP-2, MMP-9 does not cleave type I collagen, although it does bind to it (*72*, *75*). Because MMP-9 is active against other collagen types (*8*), specific features of type I collagen may prevent the enzyme from hydrolyzing it. Alternatively, the heavily glycosylated linker of MMP-9 may interfere with the hydrolysis of type I collagen, although MMP-9 glycosylation has not yet been shown to play a role in proteolytic activities (*61*). MMP-13 processes thermally stable sequences efficiently, with little effect on the sequence. In contrast to MMP-1, MMP-8, and MT1-MMP, MMP-13 prefers a positively charged residue interacting with the S_2 subsite and may favor certain interrupted triple-helical sequences over uninterrupted ones. MT1-MMP is the most robust triple-helical peptidase and is reasonably sensitive to substrate thermal stability. It disfavors positively charged residues interacting with the S_2 subsite and is also relatively ineffectual at processing interrupted triple-helical sequences that are cleaved by soluble MMPs. MT2-MMP behaves similarly to MT1-MMP, except that it is less active.

Phage-display and peptide libraries have demonstrated that many members of the MMP family not only cleave but may hydrolyze more rapidly non-Gly-Xxx-Yyy sequences (*11*). As discussed earlier, the Pro-Val-Asn∼Phe-Arg motif was hydrolyzed more rapidly by MMP-13 than by MMP-2 in linear form (*13*). However, this same motif was hydrolyzed more rapidly by MMP-2 than by MMP-13 when incorporated into a triple-helical construct. Thus, while phage display can be informative, it is not necessarily predictive of interactions between enzymes and proteins because of conformational influences. Such behaviors may be quite significant for understanding MMP mechanisms of action and aid in the development of selective MMP inhibitors.

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