Matrix Metalloproteinase Triple-Helical Peptidase Activities Are Differentially Regulated by Substrate Stability†

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ABSTRACT: Matrix metalloproteinases (MMPs) are involved in physiological remodeling as well as pathological destruction of tissues. The turnover of the collagen triple-helical structure has been ascribed to several members of the MMP family, but the determinants for collagenolytic specificity have not been identified. The present study has compared the triple-helical peptidase activities of MMP-1 and MMP-14 (membrane-type 1 MMP; MT1-MMP). The ability of each enzyme to efficiently hydrolyze the triple helix was quantified using chemically synthesized fluorogenic triple-helical substrates that, via addition of N-terminal alkyl chains, differ in their thermal stabilities. One series of substrates was modeled after a collagenolytic MMP consensus cleavage site from types I−III collagen, while the other series had a single substitution in the P_1' subsite of the consensus sequence. The substitution of Cys(4-methoxybenzyl) for Leu in the P_1' subsite was greatly favored by MMP-14 but disfavored by MMP-1. An increase in substrate triple-helical thermal stability led to the decreased ability of the enzyme to cleave such substrates, but with a much more pronounced effect for MMP-1. Increased thermal stability was detrimental to enzyme turnover of substrate (k_{cat}), but not binding (K_M). Activation energies were considerably lower for MMP-14 hydrolysis of triple-helical substrates compared with MMP-1. Overall, MMP-1 was found to be less efficient at processing triple-helical structures than MMP-14. These results demonstrate that collagenolytic MMPs have subtle differences in their abilities to hydrolyze triple helices and may explain the relative collagen specificity of MMP-1.

Diseases such as cancer, atherosclerosis, rheumatoid arthritis, and osteoarthritis often involve pathological destruction of tissues. This process requires unbalanced turnover of a variety of extracellular matrix molecules, including collagen, fibronectin, and proteoglycans. One of the most intriguing enzymatic activities is the catabolism of intact triple-helical regions of collagens. Collagen is an important physiological barrier between tissues, and once destroyed or damaged it can allow tumor cell invasion or atherosclerotic plaque formation and rupture. Thus, proteases that can cleave intact collagen are of great interest as possible therapeutic targets.

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) is hydrolyzed within their triple-helical domain by MMP-1, -2, -8, -13, -14, and -18 (*1*, *2*). Other zinc metalloenzymes, such as *Clostridium histolyticum* collagenase, are collagenolytic (*3*). The intact triple helix of collagen is also cleaved by the cysteine protease cathepsin K and a variety of serine proteases, such as *Uca pugilator* (fiddler crab) collagenase 1, *Hypoderma lineatum* (cattle grub) collagenase, and *Penaeus vanameii* (shrimp) chymotrypsin (*4*). The collagenolytic activity of these proteases varies greatly; for example, the rate of type I collagen hydrolysis (i.e., k_{cat}/K_M value) is considerably lower for fiddler crab collagenase 1 compared with MMP-1 (*5*). Within the MMP family distinct preferences for collagen types are seen (*6*). MMP-1 hydrolyzes type III collagen more rapidly than type I, while MMP-8 and MMP-14 show a slight preference for type I collagen compared to type III (*6*, *7*). Neither MMP-1 nor MMP-8 hydrolyzes type II collagen efficiently (*6*). Conversely, MMP-13 prefers type II collagen and hydrolyzes this collagen much more rapidly than MMP-1 or MMP-8 (*6*). The determinants that may guide these specificities, such as subtle variations in sequence or triple-helix local stability, are unknown.

In order to develop collagenolytic-based inhibitors, we need to examine the interactions occurring between MMPs and collagen on a molecular level. MMP-1 is often considered the "classic" collagenase, and its activity has been previously quantified using type I collagen fibrils and linear and triple-helical collagen models (*7*−*14*). Interest in MMP-

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[1] Abbreviations: CD, circular dichroism; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMSO, dimethyl sulfoxide; 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; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; THP, triple-helical peptide.

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14 collagenolytic activity has arisen because of the proposed role of MMP-14 in tumor cell invasion of type I collagen and metastasis (*15*−*20*). Quantification of MMP-14 collagenolytic activity has, however, been limited (*7*, *21*), as most prior studies have been qualitative in nature (*16*, *18*, *22*−*24*). The present study has used fluorescent resonance energy transfer (FRET) triple-helical substrates to compare the triple-helical peptidase activities of MMP-1 and MMP-14. 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 (*12*, *14*, *25*). Triple-helical peptidase activity has been examined herein as a function of triple-helical thermal stability. This has required the use of "peptide-amphiphiles", in which the thermal stability of the triple helix is modulated by pseudolipids attached to the *N*-terminus of the peptide (*26*). We have also addressed triple-helical sequence specificity by comparing substrates that differ by one amino acid residue in the P_1' subsite position.

MATERIALS AND METHODS

All standard chemicals were peptide synthesis or molecular biology grade and purchased from Fisher Scientific. 2-(1*H*-Benzotriazol-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 L-configuration (except for Gly).

Peptide Synthesis. Peptide-resin assembly of fluorogenic triple-helical substrates (fTHPs) was performed by Fmoc solid-phase methodology on an ABI 433A Peptide Synthesizer (*12*). All peptides were synthesized as C-terminal amides to prevent diketopiperazine formation (*27*). Peptide-resins were lipidated with decanoic acid [$CH_3(CH_2)_8CO_2H$, designated C_{10}] and palmitic acid [$CH_3(CH_2)_{14}CO_2H$, designated C_{16}] as described (*26*, *28*). Cleavage and side-chain deprotection of peptide-resins and peptide-amphiphile-resins proceeded for at least 2 h using thioanisole−water−TFA (5:5:90) (*29*). Peptide-amphiphile cleavage solutions were extracted with methyl *tert*-butyl ether prior to purification.

Peptide Purification. 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 5.0 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 (see below) was used to combine fractions of homogeneous product. C_{16}-Lipidated peptides were purified by biphasic liquid−liquid extraction (water:methyl *tert*-butyl ether).

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 Hewlett-Packard G2025A LD-TOF mass spectrometer using either α-cyano-4-hydroxycinnamic acid or a 2,5-hydroxybenzoic acid/2-hydroxy-5-methoxybenzoic acid (9:1, v/v) matrix (*30*, *31*). fTHP mass values were as follows: fTHP-4, $[M + H]^+$ 4595.2 Da (theoretical 4588.9 Da); C_{10}-fTHP-4, $[M + H]^+$ 4744.5 Da (theoretical 4743.2 Da); C_{16}-fTHP-4, $[M + H]^+$ 4822.6 Da (theoretical 4823.2 Da); fTHP-9, $[M + H]^+$ 4697.9 Da (theoretical 4698.9 Da); C_{10}-fTHP-9, $[M + H]^+$ 4846.2 Da (theoretical 4853.2 Da); and C_{16}-fTHP-9, $[M + H]^+$ 4925.6 Da (theoretical 4933.3 Da). All mass values were within 0.16% of theoretical.

Circular Dichroism Spectroscopy. CD spectra were recorded over the range $\lambda = 190$−250 nm on a JASCO J-600 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. Temperature was controlled using a JASCO PTC-348WI 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 was expressed in *E. coli* and folded from the inclusion bodies as described previously (*32*). ProMMP-1 was activated by reacting with 1 mM 4-aminophenylmercuric acetate and an equimolar amount of MMP-3 at 37 °C for 6 h. After activation, MMP-3 was completely removed from MMP-1 by affinity chromatography using an anti-MMP-3 IgG Affi-Gel 10 column. The amount of active MMP-1 was determined by titration with recombinant tissue inhibitor of metalloproteinases-1 (TIMP-1) over a concentration range of 0.1−3 μg/mL (*33*). ProMMP-1 with the C-terminal hemopexin-like domain deleted [residues 243−450; designated proMMP-1($\Delta_{243-450}$)] was expressed in *E. coli* using the expression vector pET3a (Novagen), folded from inclusion bodies and purified as described previously (*34*). The zymogen was activated as described above for the full-length proMMP-1. Recombinant MMP-14 with the linker and C-terminal hemopexin-like domains deleted [residues 279−523; designated MMP-14($\Delta_{279-523}$)] was purchased from Chemicon International (Temecula, CA). MMP-14($\Delta_{279-523}$) was expressed in the active form with Tyr112 the *N*-terminus. MMP-14($\Delta_{279-523}$), which, in contrast to MMP-14, does not undergo rapid autoproteolysis, was used in the present studies due to the relatively small differences in MMP-14($\Delta_{279-523}$) and MMP-14 triple-helical peptidase activities noted previously (*24*).

Assays. Substrate stock solutions were prepared at various concentrations with or without 0.25% DMSO in enzyme assay buffer (50 mM Tricine, 50 mM NaCl, 10 mM $CaCl_2$, 0.005% Brij-35, pH 7.5). MMP assays were conducted in enzyme assay buffer by incubating the range of substrate concentrations (∼1−20 μM) with 10 nM enzyme at 30 °C. Fluorescence was measured on a Molecular Devices SPECTRAmax Gemini EM dual-scanning microplate spectrofluorometer 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 initial velocity in units of micromolar per second. To determine activation energies (E_a), an Arrhenius plot of

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α1(I)      Gly-Thr-Hyp-Gly-Pro-Gln-Gly~Ile-Ala-Gly-Gln-Arg-Gly-Val-Val
α2(I)      Gly-Thr-Hyp-Gly-Pro-Gln-Gly~Leu-Leu-Gly-Ala-Hyp-Gly-Ile-Leu
α1(II)     Gly-Pro-Hyp-Gly-Pro-Gln-Gly~Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val
α1(III)    Gly-Ala-Hyp-Gly-Pro-Leu-Gly~Ile-Ala-Gly-Ile-Thr-Gly-Ala-Arg
Consensus  Gly-Pro-Mca-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Dnp-Gly-Val-Arg
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FIGURE 1: Sequences of collagenolytic MMP cleavage sites in types I−III collagen (residues 769−783) and the consensus sequence used for fTHP-4.

A

(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH$_2$

(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Cys(Mob)-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH$_2$

B

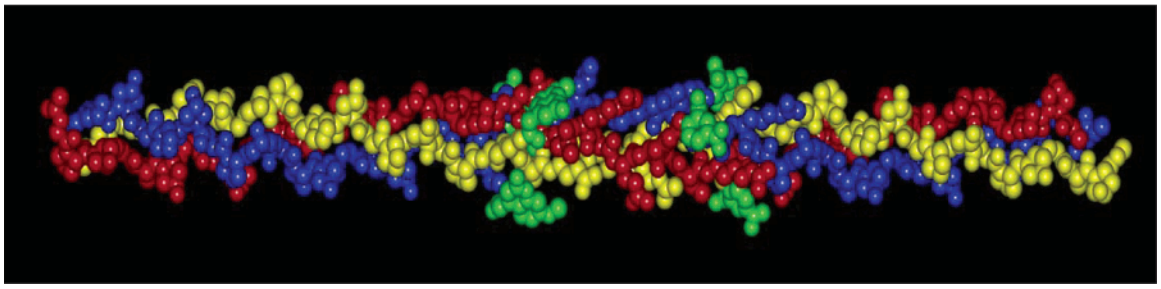

FIGURE 2: (A) Sequences of (top) fTHP-4 and (bottom) fTHP-9. (B) Space-filling computer generated model of fTHP-4, where the individual peptide strands are red, blue, and yellow, while the fluorophore (Mca) and quencher (Dnp) are green. The model was prepared as described previously (*4*).

log k_{cat} versus 1/temperature (K) was constructed where the slope $= -E_a/2.3R$ and R is the molar gas constant.

RESULTS

Design and Structural Characterization of Fluorogenic Triple-Helical (fTHP) Substrates. All fTHPs were based on a consensus sequence derived from the collagenolytic MMP cleavage sites in human types I−III collagen (Figure 1) (*6*). The initial substrate, fTHP-4 (Figure 2A, top), was reported previously (*12*, *35*). 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. Arg residues in the P_2' and P_8' subsites are favored by collagenolytic MMPs (*10*) while serving to enhance fTHP solubilities (*12*). The consensus sequence was subsequently modified in the P_1' subsite, where Leu was replaced with Cys(Mob), to create fTHP-9 (Figure 2A, bottom). The Cys(Mob) residue in the P_1' subsite was reported to enhance MMP-14 activity and specificity (*36*). It should be noted, however, that the substrates from the prior MMP-14 study were neither triple-helical nor linear collagen models (*36*). Both fTHP-4 and fTHP-9 were acylated with decanoic (C_{10}) and palmitic (C_{16}) acids to confer differential thermal stability and allow the correlation of substrate structural stability to enzyme activity. The purified, lipidated substrates exhibited decreased solubility in assay buffer compared with the nonlipidated substrates, and thus DMSO (0.25−1.0% in stock solutions) was utilized for improved solubility.

CD spectra were obtained for all substrates and were found to be characteristic of triple helices (data not shown). To examine the thermal stability of potential substrates, the molar ellipticity ([Θ]) at $\lambda = 222$ nm was monitored as a function of increasing temperature. All structures exhibited sigmoidal transitions, indicative of the melting of a triple helix to a single-stranded structure (data not shown). For fTHP-4, C_{10}-fTHP-4, and C_{16}-fTHP-4, T_m values were 36.3, 43.0, and 69.0 °C, respectively. The analogous series of P_1'-substituted substrates, fTHP-9, C_{10}-fTHP-9, and C_{16}-fTHP-9, exhibited higher T_m values of 47.2, 60.0, and 80.0 °C, respectively.

Triple-Helicase Activities of MMP-1 and MMP-14. Initial studies were directed to the effects of DMSO on MMP-1 and MMP-14($\Delta_{279-523}$) hydrolysis of fTHP-4 and fTHP-9. Assays were performed over a range of fTHP concentrations, and individual kinetic parameters were calculated by Lineweaver−Burk, Hanes−Woolf, and Eadie−Hoftsee analyses (Table 1). The addition of DMSO slightly decreases MMP-1 activity toward fTHP-4 and increases activity toward fTHP-9. In contrast, DMSO enhances MMP-14($\Delta_{279-523}$) activity toward both fTHP-4 and fTHP-9, with k_{cat} being the kinetic parameter most significantly affected. Overall, DMSO has a greater influence on MMP-14($\Delta_{279-523}$) activity than MMP-1. Hydrolysis of fTHP-4 was also examined with MMP-1($\Delta_{243-450}$) to evaluate the role of the C-terminal hemopexin-like domain in MMP-1 triple-helical peptidase activity (Table 1). MMP-1($\Delta_{243-450}$) activity toward fTHP-4 was only half that of MMP-1, with the primary difference being the respective k_{cat} values.

The effect of the single substitution of Cys(Mob) for Leu in the P_1' subsite was substantially different for MMP-1 and MMP-14($\Delta_{279-523}$). MMP-1 preferred fTHP-4; the replace-

Table 1: Kinetic Parameters for fTHP-4 and fTHP-9 Hydrolysis by MMP-1 and MMP-14 at 30 °C[a,b]

enzyme	substrate	k_{cat}/K_M (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_M (μM)
MMP-1	fTHP-4	6400	0.07 ± 0.006	11.0 ± 6.7
MMP-1	fTHP-4 + 0.25% DMSO	3900	0.06 ± 0.01	15.2 ± 1.5
MMP-1 ($\Delta_{243-450}$)	fTHP-4 + 0.25% DMSO	1500	0.02 ± 0.01	13.3 ± 1.9
MMP-1	fTHP-9 + 0.25% DMSO	3000	0.06 ± 0.03	20.3 ± 2.3
MMP-14 ($\Delta_{279-523}$)	fTHP-4	32000	0.48 ± 0.10	15.1 ± 2.1
MMP-14 ($\Delta_{279-523}$)	fTHP-4 + 0.25% DMSO	59000	1.37 ± 0.08	23.1 ± 2.1
MMP-14 ($\Delta_{279-523}$)	fTHP-9	99000	1.15 ± 0.02	11.6 ± 3.1
MMP-14 ($\Delta_{279-523}$)	fTHP-9 + 0.25% DMSO	168000	1.34 ± 0.01	8.0 ± 0.3

[a] Hydrolysis occurs at the Gly−Leu or Gly−Cys(Mob) bond in fTHP-4 or fTHP-9, respectively, as monitored by MALDI-TOF-MS. [b] Kinetic parameters were not determined for the enzyme/substrate combination MMP-1/fTHP-9.

Table 2: Kinetic Parameters for fTHP-4 and fTHP-9 Hydrolysis in 0.25% DMSO by MMP-14 at 30 °C[a]

substrate	substrate T_m (°C)	k_{cat}/K_M (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_M (μM)
fTHP-4	36.3	59000	1.37 ± 0.08	23.1 ± 2.1
C_{10}-fTHP-4	43.0	4100	0.0033 ± 0.0005	0.80 ± 0.31
C_{16}-fTHP-4	69.0	1200	0.0021 ± 0.0002	1.75 ± 0.05
fTHP-9	47.2	168000	1.34 ± 0.01	8.0 ± 0.3
C_{10}-fTHP-9	60.0	29000	0.080 ± 0.012	2.8 ± 0.4
C_{16}-fTHP-9	80.0	30000	0.196 ± 0.008	6.6 ± 0.4

[a] Hydrolysis occurs at the Gly−Leu or Gly−Cys(Mob) bond in fTHP-4 or fTHP-9, respectively, as monitored by MALDI-TOF-MS.

ment of Leu by Cys(Mob) increased K_M, resulting in a lower k_{cat}/K_M value. In contrast, MMP-14($\Delta_{279-523}$) preferred fTHP-9, with Cys(Mob) causing decreased K_M values resulting in higher k_{cat}/K_M values. This indicates that the P_1' subsite *in a triple-helical context* provides selectivity within these collagenolytic MMPs.

Subsequent assays with C_{10}-fTHP-4, C_{16}-fTHP-4, C_{10}-fTHP-9, and C_{16}-fTHP-9 were performed in 0.25% DMSO to enhance substrate solubility. MMP-1 showed little ability to cleave these substrates. MMP-14($\Delta_{279-523}$) was able to cleave all four substrates. The kinetic parameters were subsequently determined (Table 2). For both substrate series, a correlation was seen between fTHP T_m values and MMP-14 kinetic parameters. As T_m increases, k_{cat}/K_M values decrease or level off. In most cases, both K_M and k_{cat} decrease. Generally, the more thermally stable the triple helix, the slower the rate of hydrolysis.

Several interesting trends are observed for MMP-14($\Delta_{279-523}$) hydrolysis of fTHP-4 and fTHP-9 variants (Table 2). First, although fTHP-9 has a higher T_m value than fTHP-4, MMP-14($\Delta_{279-523}$) prefers the former substrate. If we compare similarly modified substrates (such as C_{10}-fTHP-4 to C_{10}-fTHP-9, etc.), the Cys(Mob) substitution consistently results in better kinetic parameters despite higher T_m values. Second, increasing the substrate thermal stability initially decreases K_M (compare fTHP-4 to C_{10}-fTHP-4 or fTHP-9 to C_{10}-fTHP-9), but a further increase in thermal stability (C_{10}-fTHP-2 or -9 to C_{16}-fTHP-4 or -9) increases K_M.

Table 3: Activation Energies for Substrate Hydrolysis in 0.25% DMSO by MMP-1 and MMP-14

enzyme	substrate	substrate T_m (°C)	E_a (kcal/mol)
MMP-1	fTHP-4	36.3	20.0
MMP-1($\Delta_{243-450}$)	fTHP-4	36.3	29.0
MMP-14($\Delta_{279-523}$)	fTHP-4	36.3	8.8
MMP-14($\Delta_{279-523}$)	fTHP-9	47.2	11.4
MMP-14($\Delta_{279-523}$)	C_{16}-fTHP-9	80.0	14.3

The temperature-dependent hydrolysis of fTHP-4, fTHP-9, and C_{16}-fTHP-9 was evaluated for MMP-1, MMP-1($\Delta_{243-450}$), and MMP-14($\Delta_{279-523}$). Kinetic parameters were determined at 25, 30, 35, and 37 °C, and the activation energies (E_a) were calculated (Table 3). The activation energy could not be determined for MMP-1 with fTHP-9 or C_{16}-fTHP-9 since there was very little or no hydrolysis even at higher temperatures. Comparison of MMP-1 to MMP-14($\Delta_{279-523}$) demonstrates a higher activation energy for triple-helix hydrolysis by MMP-1. This is consistent with the lower k_{cat}/K_M values observed for MMP-1 hydrolysis of fTHPs compared with MMP-14($\Delta_{279-523}$) and the greater sensitivity to triple-helical thermal stability exhibited by MMP-1. The C-terminal hemopexin-like domain contributes to the ease of hydrolysis, as MMP-1($\Delta_{243-450}$) has a higher activation energy than MMP-1. Finally, an increase in substrate thermal stability (C_{16}-fTHP-9 compared with fTHP-9) results in a higher activation energy for MMP-14($\Delta_{279-523}$) hydrolysis.

DISCUSSION

Collagenolytic behavior has recently been examined using triple-helical peptide (THP) models of collagenase cleavage sites. Up to this point, THP models have been used to observe different behaviors between MMPs (*31*, *37*), quantify MMP triple-helical peptidase activity (*11*, *12*, *14*, *25*, *35*), or design MMP-2/MMP-9 selective substrates (*25*). In the present study, we have quantified (a) the effects of a single P_1' subsite substitution and (b) the consequence of increasing substrate thermal stability on MMP-1 and MMP-14 triple-helical peptidase activities. The template was a consensus sequence derived from the collagenolytic MMP cleavage sites in human types I−III collagen (*6*). The single substitution of Cys(Mob) for Leu in the P_1' subsite increased triple-helical thermal stability by ∼11−17 °C. Comparison to prior "host−guest" studies showed that replacement of Leu in the Xxx position of a Gly-Xxx-Yyy repeating sequence by Phe or Tyr destabilized the triple helix by 4.7−6.1 or 5.5 °C, respectively (*38*, *39*). The discrepancy between Cys(Mob) and Phe/Tyr structural consequences may be due to the longer side chain of Cys(Mob) prior to the benzyl group. In addition, the contribution of Arg in the Yyy position is unknown. Structural studies would provide further insights into understanding the ramifications of Cys(Mob) in a triple-helical environment.

Comparison of fTHP-4 and fTHP-9 hydrolysis by MMP-1 and MMP-14 indicated that (i) the P_1' subsite substitution of Cys(Mob) for Leu is disfavored by MMP-1 and greatly favored by MMP-14; and (ii) MMP-14 has much greater triple-helical peptidase activity than MMP-1. As previously shown, MMP-14 has a deeper S_1' subsite pocket than MMP-1 (*40*), and thus Cys(Mob) probably introduces additional favorable interactions in the S_1' subsite environment of MMP-14 while creating steric clashes in the MMP-1 S_1'

subsite. The favorable interactions of the Cys(Mob) side chain may well facilitate MMP-14 unwinding the triple helix. This feature, along with other unique sequence preferences (*36*, *41*, *42*), can be explored in subsequent design of MMP-14 specific substrates or inhibitors. Also, the selectivity of substrates can be modulated by using different substitutions in P_1' subsite position. It should be noted that the 3−3.5-fold difference seen for MMP-14 hydrolysis of fTHP-4 versus fTHP-9 is considerably less than the 38-fold difference reported for MMP-14 hydrolysis of dansyl-Pro-Leu-Ala−Cys(Mob)-Trp-Ala-Arg-NH_2 versus dansyl-Pro-Leu-Ala−Leu-Trp-Ala-Arg-NH_2 (*36*). Different effects of the P_1' subsite substitution on MMP-14 activity, based on different templates, further support the notion that subsite substitutions are not independent with respect to interaction with MMPs (*10*, *43*−*45*). In addition, the conformation of the triple helix offers a unique three-dimensional presentation of residues to the enzyme.

A more thermally stable triple helix translated into a decreased ability of either MMP-1 or MMP-14 to cleave the triple helix. For the Cys(Mob)-containing substrate (fTHP-9), an increase in thermal stability was detrimental to turnover by MMP-14 (k_{cat} decreased by ∼10-fold) but not binding affinity to MMP-14 (K_M slightly decreased). This may stem from an inability to access individual strands of the triple helix. For the Leu-containing substrate (fTHP-4), decreased MMP-14 activity was also due to diminished k_{cat} values, but the affinity of MMP-14 increased with respect to thermal stability. Thus, there is a careful balance of optimum MMP-14 affinity for a substrate with a triple-helical T_m value of 40−60 °C and turnover between 35 and 50 °C. At any given temperature, a triple helix with a lower T_m value has more backbone mobility than a triple helix with a higher T_m value (*46*). It can be hypothesized that the decreased backbone mobility of C_{10}-fTHP compared with fTHP may account for more favorable binding interactions with MMP-14. Although the molecular details of collagen/MMP associations are relatively unknown, recent X-ray crystallographic and NMR studies have found that triple-helical structure is retained during collagen binding to the α2 integrin subunit and von Willebrand factor A-domains (*47*, *48*). Application of such methods with THPs of varying stability may provide insight into the effects of subtle collagen backbone movement on MMP binding affinity.

MMP-1 showed much less activity toward all substrates compared with MMP-14, and also had higher triple-helical peptidase activation energies than MMP-14. Relative activation energies have a significant role in collagenolysis efficiency (*8*), and thus such differences between MMP-1 and MMP-14 may well reflect the collagenolytic potential of the two enzymes. These results could explain the greater levels of extracellular collagenolysis seen with MMP-14 compared to MMP-1 (*16*, *20*), and the profound skeletal defects observed in MMP-14 knockout mice due to lack of collagen turnover (*22*). In contrast to MMP-14, an increase in substrate thermal stability resulted in a complete loss of MMP-1 activity. Prior qualitative observations showed a general trend of increasing triple-helical peptidase activity as substrate T_m values increased to the 33−40 °C range, followed by a decrease in activity with substrates that were more thermally stable (*31*, *37*, *49*). The present studies have more precisely shown that MMPs clearly differ in their abilities to efficiently hydrolyze a triple helix, and differential thermal stability can be used to create MMP selective substrates. These studies need to be extended to other collagenolytic MMPs (MMP-8, MMP-13) to determine if the trends observed here are indeed general.

One does need to consider the potential aggregation of the substrates used in this study, and how aggregation might influence kinetic parameters. We have observed broader thermal transition curves when C_{10} and C_{16} alkyl chains are attached to triple-helical peptides, and attributed this behavior to aggregation (*26*). Due to the large headgroup of triple-helical peptide-amphiphiles, it was anticipated that micelles are formed by this species (*28*, *50*). Cryo transmission electron microscopy revealed that THPs containing N-terminal, C_{16} branched alkyl chains formed micelles (*51*). Size-exclusion chromatographic analysis of triple-helical peptide-amphiphiles demonstrated that C_{10} and C_{16} alkyl chains induce small aggregates of 18 and 22 triple helices, respectively (*52*). However, in all of these prior cases, the triple-helical peptide-amphiphile concentration was 0.5−1.5 mM (*26*, *52*). The present study has used C_{10} and C_{16} triple-helical peptide-amphiphiles at considerably lower concentrations (1−20 μM). Aggregates at these concentrations were not observed by dynamic light scattering (data not shown). This result is consistent with the high critical micellular concentrations (CMCs) of the potassium salts of octadecanoic acid (C_{18}·K^+), *cis*-Δ9-octadecanoic acid ($C_{18:1}$·K^+), and 12-hydroxy-*cis*-Δ9-octadecanoic acid ($C_{18:1-OH}$·K^+) at 0.5, 1.5, and 3.6 mM, respectively (*53*). We thus believe that aggregation was not a significant factor in directing MMP activity toward the substrates described herein. In addition, association of collagen molecules to form fibrils reduces, but does not eliminate, MMP-1 collagenolytic activity (*54*, *55*), and thus aggregation alone could not account for the complete loss of MMP-1 triple-helical peptidase activity observed here.

MMP-1 and MMP-14 have different collagen preferences, with MMP-1 favoring type III collagen over type I, and vice versa for MMP-14 (*6*, *7*). It has been previously observed that type III collagen is more susceptible to general proteolysis than type I collagen (*56*, *57*), and the susceptibility of interstitial collagens to cleavage by trypsin and trypsin/chymotrypsin mixtures appears to be dependent upon the relative unwinding of the triple helix (*57*−*61*). Thus, there may be a more prevalent local instability in the type III molecule compared to type I. Local unwinding or enhanced backbone mobilities have been proposed to facilitate collagenolysis, as the MMP cleavage site in interstitial collagens is distinguished by being "loosely" triple helical (low imino acid content) following the cleavage site and "tightly" triple helical (high imino acid content) prior to the cleavage site, as well as possessing a low content (<10%) of charged residues (*4*, *9*). Recent work utilizing triple-helical peptide models and NMR hydrogen exchange spectroscopy experiments (*62*, *63*) or molecular dynamics trajectories (*64*) concluded that such flexibility exists at the site of MMP hydrolysis. MMP-1 may prefer type III collagen due to the more mobile triple helix at the cleavage site and MMP-1 having limited ability to hydrolyze more thermally stable local structures.

The role of MMP hemopexin-like C-terminal domains in collagenolysis is still undefined. Several studies have concluded that the MMP catalytic domain contains determinants

for collagenolysis (*13*, *32*, *65*). Even if the C-terminal hemopexin-like domain is deleted from MMP-1, MMP-8, or MMP-14, triple-helical peptidase activity is retained (*11*, *12*, *24*, *37*, *49*). More specifically, prior studies with the MMP-14 catalytic domain [MMP-14($\Delta_{319-523}$)] and the MMP-14 ectodomain (residues 112−523; designated ΔTM-MMP-14) showed that MMP-14($\Delta_{319-523}$) was more efficient at single-stranded substrate hydrolysis than ΔTM-MMP-14, but differences in triple-helical peptidase activity were only slight (*24*). Thus, the MMP-14 C-terminal domain offered some advantage for cleaving a triple helix. Similar results were obtained here with MMP-1 and MMP-1($\Delta_{243-450}$) for assays performed in DMSO, in that loss of the C-terminal domain resulted in a 50% decrease in triple-helical peptidase activity and an increase in activation energy. In contrast, deletion of the C-terminal hemopexin-like domain from MMP-1, MMP-8, MMP-13, or MMP-14 results in virtually a complete loss of collagenolytic activity (*7*, *24*, *66*−*69*). Pertinent to the present studies, the MMP-1 and MMP-14 C-terminal hemopexin-like domains bind type I collagen (*18*, *69*), and the MMP-1 C-terminal domain has been implicated in destabilizing and unwinding the collagen triple helix (*70*). The C-terminal domain of MMP-2 is responsible for MMP lateral diffusion on substrate (*71*). Lateral diffusion could be a general MMP modis operandi, whereby the C-terminal domain allows MMPs to function as ATP-independent molecular motors (*71*, *72*) moving in "inchworm" or "hand-over-hand" manners (*73*). Consistent with this hypothesis are atomic force microscopic images showing MMP-8 binding to many sites on type II collagen, yet cleaving only a single site (*74*). The C-terminal hemopexin-like domain may ultimately be involved in (i) movement of the MMP along collagen to the site of hydrolysis and (ii) distortion of the triple helix to facilitate hydrolysis.

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