Kinetic Analysis of Matrix Metalloproteinase Activity Using Fluorogenic Triple-Helical Substrates†

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ABSTRACT: Matrix metalloproteinase (MMP) family members are involved in the physiological remodeling of tissues and embryonic development as well as pathological destruction of extracellular matrix components. To study the mechanisms of MMP action on collagenous substrates, we have constructed homotrimeric, fluorogenic triple-helical peptide (THP) models of the MMP-1 cleavage site in type II collagen. The substrates were designed to incorporate the fluorophore/quencher pair of (7-methoxycoumarin-4-yl)acetyl (Mca) and *N*-2,4-dinitrophenyl (Dnp) in the P_5 and P_5' positions, respectively. In addition, Arg was incorporated in the P_2' and P_8' positions to enhance enzyme activity and improve substrate solubility. The desired sequences were Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly∼Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Ile/Val-Arg. Two fluorogenic substrates were prepared, one using a covalent branching protocol (fTHP-1) and one using a peptide self-assembly approach (fTHP-3). An analogous single-stranded substrate (fSSP-3) was also synthesized. Both THPs were hydrolyzed by MMP-1 at the Gly∼Leu bond, analogous to the bond cleaved in the native collagen. The individual kinetic parameters for MMP-1 hydrolysis of fTHP-3 were $k_{cat} = 0.080\ s^{-1}$ and $K_M = 61.2\ \mu M$. Subsequent investigations showed fTHP-3 hydrolysis by MMP-2, MMP-3, MMP-13, a C-terminal domain-deleted MMP-1 [MMP-1($\Delta_{243-450}$)], and a C-terminal domain-deleted MMP-3 [MMP-3($\Delta_{248-460}$)]. The order of k_{cat}/K_M values was MMP-13 > MMP-1 ∼ MMP-1($\Delta_{243-450}$) ∼ MMP-2 ≫ MMP-3 ∼ MMP-3($\Delta_{248-460}$). Studies on the effect of temperature on fTHP-3 and fSSP-3 hydrolysis by MMP-1 showed that the activation energies between these two substrates differed by 3.4-fold, similar to the difference in activation energies for MMP-1 hydrolysis of type I collagen and gelatin. This indicates that fluorogenic triple-helical substrates mimic the behavior of the native collagen substrate and may be useful for the investigation of collagenase triple-helical activity.

Matrix metalloproteinase (MMP)[1] family members are involved in the physiological remodeling of tissues and embryonic development as well as pathological conditions such as arthritis, cardiovascular diseases, and cancer cell metastasis (*1, 2*). One of the "committed" steps in extracellular matrix turnover is the hydrolysis of intact triple-helical regions found in native collagens. Several of the MMP family members possess collagenolytic activity (*2*). For example, interstitial collagens (types I−III) are hydrolyzed by MMP-1, -2, -8, -13, -14, and -18 (*3*−*9*), while basement membrane collagen (type IV) is hydrolyzed by MMP-2, -3, -7, -9, -10, and -12 (*2*). Conversely, MMP-3 and -9 bind to type I collagen, but do not cleave the triple-helical domain (*10*−*12*). The further elucidation of specific MMP collagenolytic activities may prove extremely valuable in the design of selective inhibitors.

Several approaches have emerged by which the collagenolytic activity of MMPs can be evaluated. Site-specific mutagenesis of the MMP-1 catalytic domain has been used to define residues important for catabolism of native collagen (*13*). In addition, chemically synthesized triple-helical substrates have been used to examine binding by discrete MMP domains (*14*−*17*) and for evaluating MMP kinetic parameters (*17*). Those studies have used rather laborious discontinuous assay methods. A continuous assay method, such as one that utilizes an increase in fluorescence upon triple-helix hydrolysis, would allow for rapid and convenient kinetic evaluation of MMPs and thus greatly aid mechanistic studies of these enzymes.

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[1] Abbreviations: CD, circular dichroism; Cha, 3-cyclohexylalanyl; DCM, dichloromethane; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; DIEA, *N*,*N*-diisopropylethylamine; DMSO, dimethyl sulfoxide; Dnp, 2,4-dinitrophenyl; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Fmoc, 9-fluorenylmethoxycarbonyl; fTHP, fluorogenic triple-helical peptide; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; 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; Mtt, 4-methyltrityl; ND, not determined; Nva, norvalyl; PTH, phenylthiohydantoin; RP-HPLC, reversed-phase high-performance liquid chromatography; SSP, single-stranded peptide; TFA, trifluoroacetic acid; THP, triple-helical peptide; TIMP, tissue inhibitor of metalloproteinases.

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The design of fluorogenic substrates requires several considerations. First, the fluorophore should have a high quantum yield, yet be efficiently quenched by a resonance energy transfer mechanism. Substantial quantum yields are found for 7-methoxycoumarin (Mca) derivatives, which can be readily incorporated into peptides (*18*). Mca fluorescence is also efficiently quenched by the 2,4-dinitrophenyl (Dnp) group, as described by Knight et al. (*19*). The Mca/Dnp pair has been utilized to create a substrate selective for MMP-3 (*20*). Second, one would like to incorporate the fluorophore and quencher internally in the peptide sequence. Since efficient quenching of the Mca fluorescence occurs when the Dnp group is within eight residues, internal incorporation of these groups would allow for quenching regardless of substrate size. A recently described fluorogenic triple-helical substrate incorporated the fluorophore/quencher pair at the N-terminus, which unfortunately resulted in destabilization of the triple helix and somewhat inefficient quenching (*21*). Third, the substrate should be sufficiently soluble. Since most fluorophores and quenchers are hydrophobic moieties, fluorogenic substrates typically have poor solubilities in aqueous environments. However, flexibility in MMP substrate specificities (*22*) can be utilized to construct fluorogenic substrates that contain hydrophilic residues. Fourth, the triple-helical structure of the substrate must be sufficiently stable under assay conditions. Thus, the overall goal of the present work is to create soluble, thermally stable triple-helical fluorogenic MMP substrates that incorporate Mca and Dnp moieties internally. To present a site for potential MMP binding and hydrolysis, the substrates will incorporate a modified version of the collagenase (MMP-1, MMP-8, and MMP-13) cleavage site in type II collagen.

Several methods have been described for the synthesis of triple-helical peptides (THPs) incorporating collagen-like sequences and with T_m values greater than 30 °C. Our laboratory has developed a solid-phase THP synthetic method which features a C-terminal Lys covalent branch (*23*−*25*). One THP that incorporated the collagenase cleavage site in type I collagen was found to be hydrolzyed efficiently by MMP-1, MMP-2, and MMP-13, but not by MMP-3 (*17*). Ottl et al. have created heterotrimeric THPs using a C-terminal disulfide linkage strategy (*14*−*16*). The heterotrimeric THPs incorporate the [α1(I)]$_2$α2(I)772−785 region, and were hydrolyzed efficiently by MMP-8 (*14, 16*). We have also described an approach by which the noncovalent self-assembly of lipophilic molecules, N-terminally linked to a peptide, can be used to form stable triple helices (*26*−*28*). In this study, the branching and self-assembly methods are used to construct potential fluorogenic THP substrates. The THPs have been modeled after the human α1(II)769−783 sequence Gly-Pro-Pro-Gly-Pro-Gln-Gly∼Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val (*29*), and modified to allow for the incorporation of a fluorophore and quencher. The susceptibilities of fluorogenic THPs are compared for the MMP family members MMP-1, MMP-2, MMP-3, and MMP-13. To further elucide the role of the MMP C-terminal hemopexin-like domain, we have utilized C-terminal deletions of MMP-1 (residues 243−450) and MMP-3 (residues 248−460) in our kinetic studies. Finally, temperature-dependent assays have been performed to determine the activation energy for MMP-1 triple-helical peptidase activity.

MATERIALS AND METHODS

All standard chemicals were peptide synthesis grade and purchased from Fisher. HBTU, HOBt, and Fmoc-amino acid derivatives were obtained from Novabiochem (San Diego, CA). Amino acids are of the L-configuration (except for Gly). Hexanoic acid [$CH_3(CH_2)_4CO_2H$, designated C_6] was purchased from Aldrich.

Peptide Synthesis. Peptide-resin assembly was performed by Fmoc solid-phase methodology on an ABI 433A Peptide Synthesizer. Branched THPs were synthesized by methods previously described in our laboratory (*25, 30*). Boc-Gly was coupled as the N-terminal amino acid in the branched peptide syntheses. The 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) group was removed with two 10 min treatments of hydrazine−DMF (1:49). The 4-methyltrityl (Mtt) group was removed with three 10 min treatments of TFA−1,2-ethanedithiol−DCM (1:1:98). (7-Methoxycoumarin-4-yl)acetic acid (Mca) was coupled overnight using 4-fold excesses of Mca, HBTU, and HOBt and an 8-fold excess of DIEA. When necessary, Mca coupling was repeated overnight, or a 4-fold excess of 4-(dimethyl)-aminopyridine was added and coupling proceeded for 4 h.

All peptide-amphiphiles were synthesized as C-terminal amides to prevent diketopiperazine formation (*31*). Peptide-resins were characterized by Edman degradation sequence analysis as described previously for "embedded" (noncovalent) sequencing (*32*). Peptide-resins were lipidated (*26*) with the C_6 group. Removal of the Mtt group and coupling of the Mca group proceeded as described above.

Cleavage and side-chain deprotection of peptide-resins and peptide-amphiphile-resins proceeded for 2 h using thioanisole−water−TFA (5:5:90) (*33*). Peptide-amphiphile cleavage solutions were extracted with methyl *t*Bu 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. The elution gradient was 0−75% B in 75 min where A was 0.1% TFA in water and B was 0.1% TFA in acetonitrile. Detection was at 229 nm.

Peptide Analyses. Analytical RP-HPLC was performed on a Hewlett-Packard 1100 Liquid Chromatograph equipped with a Hypersil small-pore, narrow-bore C_{18} RP column (5 μm particle size, 120 Å pore size, 100 × 2.1 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 0.3 mL/min. Detection was at 229, 324, and 363 nm. Edman degradation sequence analysis was performed on an Applied Biosystems 477A Protein Sequencer/120A Analyzer as described (*32*) for "embedded" (noncovalent) sequencing. MALDI-TOF-MS was performed on a Hewlett-Packard G2025A LD-TOF mass spectrometer using either a sinapinic acid or a 2,5-dihydroxybenzoic acid/2-hydroxy-5-methoxybenzoic acid (9:1, v/v) matrix (*34*).

Circular Dichroism Spectroscopy. CD spectra were recorded over the range $\lambda = 190-250$ nm on a JASCO J-600 using a 1.0 cm path-length quartz cell. Thermal transition curves were obtained by recording the molar ellipticity ([Θ]) at $\lambda = 225$ nm while the temperature was continuously increased in the range of 5−80 °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 reflection point in the transition region (first derivative) is defined as the melting temperature (T_m). Alternatively, T_m was evaluated from the midpoint of the transition.

Matrix Metalloproteinases. ProMMP-1 (*35*) and proMMP-3 (*36*) were purified from the culture medium of human rheumatoid synovial cells stimulated with rabbit macrophage-conditioned medium. ProMMP-2 was purified from the culture medium of human uterine cervical fibroblasts (*37*). ProMMP-2 was activated by reacting with 1 mM 4-aminophenylmercuric acetate at 25 °C for 2 h. 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. ProMMP-3 was activated to the 45 kDa MMP-3 by reacting with 5 μg/mL chymotrypsin at 37 °C for 2 h. Chymotrypsin was inactivated with 2 mM diisopropyl fluorophosphate. ProMMP-13 was a generous gift from Dr. P. G. Mitchell, Pfizer, Inc. ProMMP-13 was activated with 1 mM 4-aminophenylmercuric acetate. ProMMP-1($\Delta_{243-450}$) and ProMMP-3($\Delta_{248-460}$) were expressed in *E. coli* using the expression vector pET3a (Novagen), folded from inclusion bodies and purified as described previously (*38*). Both zymogens were activated as described above for the full-length proMMP-1 and proMMP-3. The amounts of active MMP-1, MMP-2, MMP-3, and MMP-13 were determined by titration with recombinant TIMP-1 (*39*) over a concentration range of 0.1−3 μg/mL.

Assays. Substrate fTHP-1 was prepared as a 5 mM stock solution in DMSO. Substrate fTHP-3 was prepared as a 270 μM stock solution in "fluorometric assay" buffer (50 mM Tricine, pH 7.5, 50 mM NaCl, 10 mM $CaCl_2$, 0.005% Brij-35). MMP assays were carried out in assay buffer by incubating a range of substrate concentrations (1−50 μM) with 40 nM enzyme at 30 °C. For fTHP-1, a maximum of 0.3% DMSO was present in the enzyme assay. Fluorescence was measured on a Molecular Devices SPECTRAmax Gemini Dual-Scanning Microplate Spectrofluorometer using $\lambda_{excitation} = 325$ nm and $\lambda_{emission} = 393$ nm. Initial velocities were obtained from plots of fluorescence versus time, using only data points corresponding to less than 40% full hydrolysis. 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 log velocity versus 1/temperature (K) was constructed where the slope $= -E_a/2.3R$ and R is the molar gas constant.

RESULTS

Synthesis of Fluorogenic Triple-Helical Peptide (THP) Substrates. The design of the fluorogenic substrate proceeded as follows. First, a branching protocol was used, based on our recent success with a branched, nonfluorogenic triple-helical substrate (*17*). The sequence to be incorporated was based on the human type II collagen 769−783 region (Gly-Pro-Pro-Gly-Pro-Gln-Gly∼Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val) (*29*). This sequence is cleaved at the Gly∼Leu bond in native type II collagen by MMP-1, -8, and -13 (*40, 41*). To accommodate the quenching group, the Arg in the P_5' position was replaced by Lys(Dnp). To accommodate the fluorophore, the Pro in the P_5 position was replaced by Lys(Dde). The desired sequence was thus Gly-Pro-Lys(Dde)-Gly-Pro-Gln-Gly∼Leu-Ala-Gly-Gln-Lys(Dnp)-Gly-Ile-Val. Once the peptide was assembled, the Dde group could be selectively removed and the Mca group acylated to the Lys ϵ-amino group. The first substrate was thus constructed with this sequence incorporated between an N-terminal (Gly-Pro-Hyp)$_6$ repeat and the C-terminal branch. Following peptide assembly, the Dde group was removed with 2% hydrazine and the Mca group coupled with HBTU/HOBt. The peptide was cleaved from the resin, but was too insoluble to allow for efficient RP-HPLC purification. The sequence was then modified to improve substrate solubility. The Ala in the P_2' position and the Val in the P_8' position were both replaced by Arg. Prior studies have shown that MMP-1 and MMP-8 favor Arg in the P_2' position compared to Ala (*22*). The peptide was resynthesized with the sequence Gly-Pro-Lys(Dde)-Gly-Pro-Gln-Gly∼Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Ile-Arg. Assembly proceeded smoothly, but removal of the Dde group resulted in a shift in the Lys(Dnp) visible absorption spectrum. The Mca group was coupled, and the peptide cleaved from the resin. The Lys(Dnp) residue was believed to be damaged by the hydrazine treatment. A different protecting group was deemed necessary for selective Lys deprotection. The third version of the peptide used the Lys(Mtt) group, and thus contained the sequence Gly-Pro-Lys(Mtt)-Gly-Pro-Gln-Gly∼Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Ile-Arg. The Mtt group was removed with 1% TFA plus 1% 1,2-ethanedithiol in DCM, and Mca was coupled. The peptide (fTHP-1; Figure 1) was cleaved from the resin and purified.

Subsequent analysis of fTHP-1 revealed three problems. First, fTHP-1 was much less thermally stable than had been anticipated based on prior work with branched, triple-helical peptides (Figure 2). Although the T_m value was ∼36 °C, the thermal transition was broad. Second, the yield of fTHP-1 was very low. Third, treatment of the substrate with MMP-1 revealed two sites of hydrolysis by the enzyme. The desired Gly∼Leu bond was cleaved (N-terminal fragment $[M+H]^+ = 2462.3$ Da, theoretical 2459.6 Da), as well as the Gly∼Ile bond (N-terminal fragment $[M+H]^+ = 3260.7$ Da, theoretical 3265.5 Da). To alleviate these problems, several new design features were implemented. The nature of the triple-helical peptide was changed from the branched substrate to a self-assembling peptide-amphiphile. The thermal stabilities of triple-helical peptide-amphiphiles can be easily modulated by alkyl chain length (*27*), and thus the substrate stability could be increased by rational design. Also, because peptide-amphiphiles are purified as single-stranded species, they typically provide higher post-HPLC yields than branched triple-stranded species. Finally, the sequence itself was slightly modified to eliminate the second cleavage site; Ile in the P_7' position was replaced with Val.

Two peptide-amphiphile substrates were assembled, differing only in the number of Gly-Pro-Hyp repeats at the N- and C-termini. The sequences were (Gly-Pro-Hyp)$_4$-Gly-Pro-Lys(Mtt)-Gly-Pro-Gln-Gly∼Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_4$ and (Gly-Pro-Hyp)$_5$-Gly-Pro-

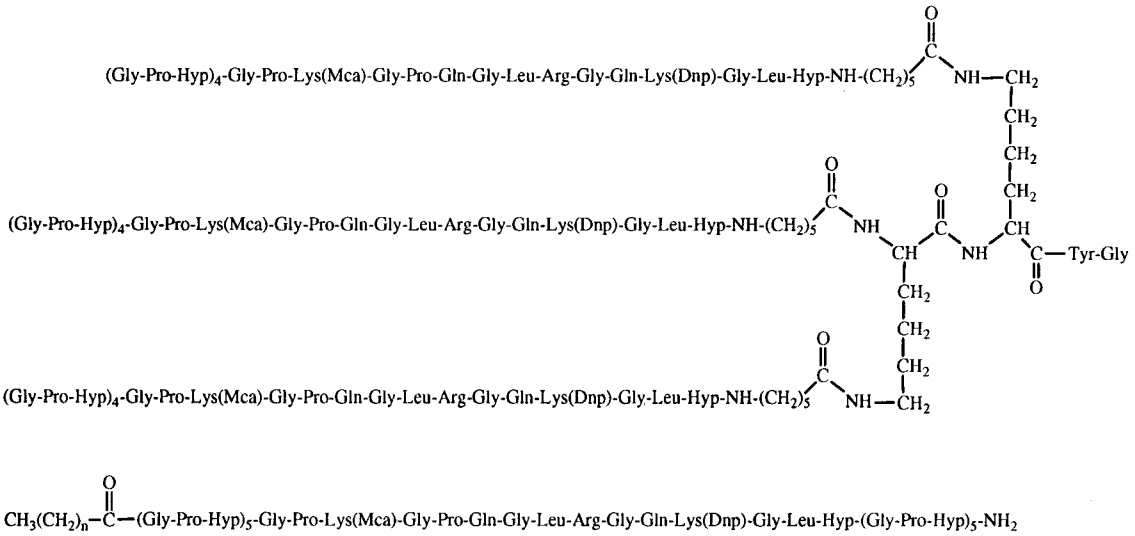

FIGURE 1: Structures of (top) fTHP-1 and (bottom) fTHP-3. Both triple-helical peptides incorporate models of the α1(II)769−783 sequence from type II collagen.

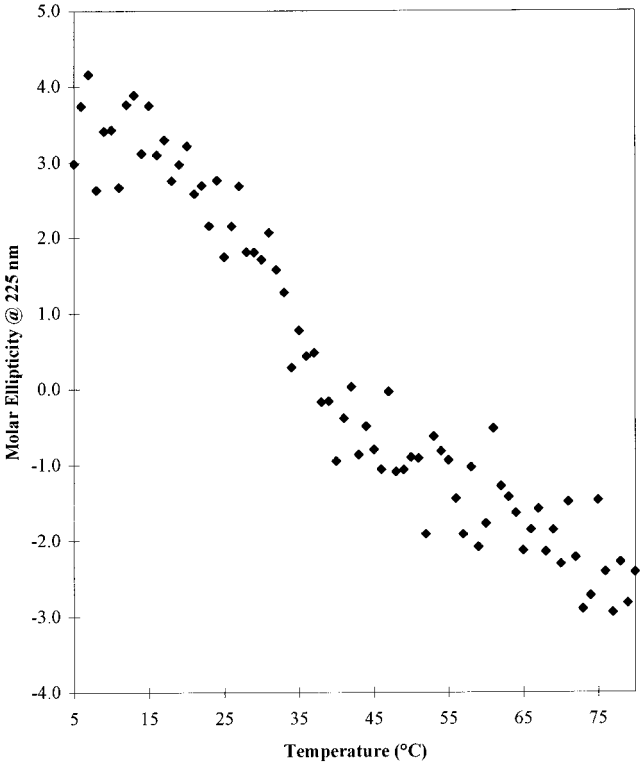

FIGURE 2: Thermal transition curve for purified fTHP-1 in 0.01% (v/v) fluorometric assay buffer at [fTHP-1] = 5−10 μM. Molar ellipticity ([θ], deg cm^2 dmol × 10^{-3}) was recorded at λ = 225 nm while the temperature was increased from 5 to 80 °C.

Lys(Mtt)-Gly-Pro-Gln-Gly∼Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$. In both cases, a C_6 alkyl chain was added to create the final peptide-amphiphile. After addition of the alkyl chain, the Mtt group was removed, and the Mca group was acylated. The peptides were cleaved from the resin and purified by RP-HPLC. MS analysis yielded $[M+H]^+$ values of 4147.7 Da (theoretical 4152.1 Da) for C_6-(Gly-Pro-Hyp)$_4$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly∼Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_4$-NH_2 (fTHP-2) and 4685.5 Da (theoretical 4687.0 Da) for C_6-(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 (fTHP-3; Figure 1). The substrate incorporating four Gly-Pro-Hyp repeats at both the N- and C-termini (fTHP-2) required DMSO for solubilization at concentrations greater than ∼50 μM, while the substrate incorporating five Gly-Pro-Hyp repeats at both the N- and C-termini (fTHP-3) was soluble in aqueous buffer up to a concentration of at least 100 μM. Thus, fTHP-3 was used for subsequent biophysical and kinetic studies.

Circular dichroism spectroscopy showed that fTHP-3 had a melting point of ∼45 °C (Figure 3). This T_m value is suitable for MMP kinetic analyses. The thermal stability of fTHP-3 was not affected substantially by either Brij-35 or DMSO (Figure 3).

Hydrolysis of Fluorogenic Triple-Helical Peptides by Matrix Metalloproteinases. Initial enzyme studies were performed with MMP-1 at 30 °C. Edman degradation sequence analysis of fTHP-3 cleavage products showed that MMP-1 hydrolysis occurred exclusively at the Gly∼Leu bond, as the only amino acid seen in the first cycle is PTH-Leu (emanating from the C-terminal fragment of the cleaved THP). MMP-1 hydrolysis of fTHP-3 was also examined by MALDI-TOF-MS. If the Gly∼Leu bond is cleaved, the triple helix denatures, and the two products generated are the single-stranded N-terminal peptide C_6-(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ($[M+H]^+$ = 2290.5 Da) and the single-stranded C-terminal peptide Leu-Arg-Gly-Gln-Lys-(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2 ($[M+H]^+$ = 2414.6 Da). Mass spectrometric analysis of MMP-1 hydrolysis showed a product of $[M+H]^+$ = 2411.0 Da, corresponding to the C-terminal fragment. The N-terminal peptide fragment would not be expected to ionize strongly due to the lack of a free amino group. Since Edman degradation analysis revealed the sequence of Leu-Arg-Gly, it is concluded that MMP-1 cleaved fTHP-3 exclusively at the Gly∼Leu bond. This is the analogous bond cleaved by MMP-1 in the native α1(II) collagen chain (*3*, *40*). Thus, initial studies with MMP-1 indicated that the incorporation of the fluorophore/quenching pair of Mca and Dnp did not affect the ability of the enzyme to recognize or cleave the substrate.

Hydrolysis of fTHP-3 was examined for MMP-2, MMP-3, and MMP-13. Sequence analysis indicated that MMP-2

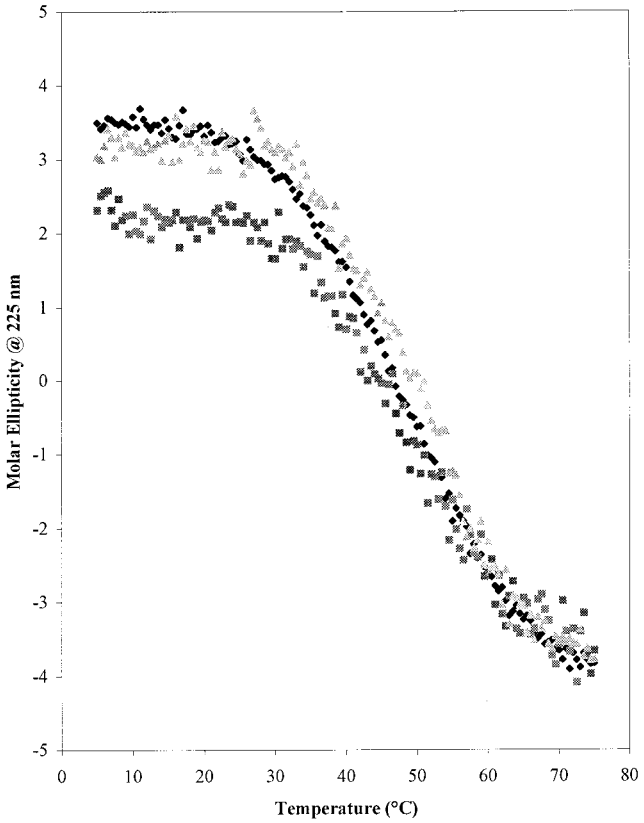

FIGURE 3: Thermal transition curves for purified fTHP-3 in 0.01% (v/v) fluorometric assay buffer (triangles), 0.01% (v/v) fluorometric assay buffer minus Brij-35 (closed diamonds), or 0.01% fluorometric assay buffer plus 0.25% DMSO (squares) at [fTHP-3] = 5−10 μM. Molar ellipticities ([θ], deg cm^2/dmol × 10^{-3}) were recorded at λ = 225 nm while the temperature was increased from 5 to 80 °C.

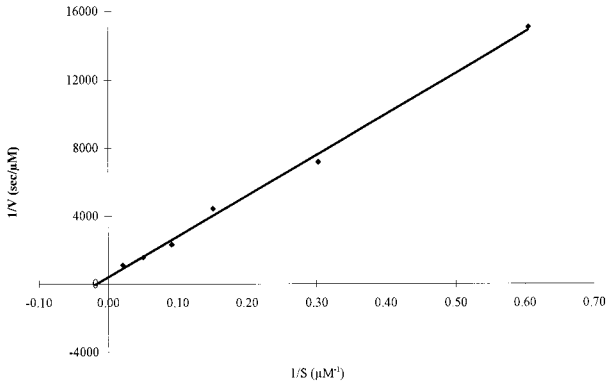

FIGURE 4: Lineweaver−Burk analysis for MMP-1 hydrolysis of fTHP-3 in fluorometric assay buffer at 30 °C. The substrate concentration range is 1−50 μM. For this analysis, R^2 = 0.9962.

and MMP-13 cleaved fTHP-3 at two loci, Gly∼Leu and Gly∼Gln, while MMP-3 cleaved fTHP-3 at the same Gly∼Leu locus as MMP-1. MMP-2 has been shown to cleave Gly∼Gln bonds in triple-helical substrates (*17*, *42*), while MMP-13 has been shown to cleave the Gly∼Gln bond three residues away from the Gly∼Leu bond in type II collagen (*41*). For either enzyme, it is not entirely clear if the Gly∼Gln bond hydrolysis occurs after an initial cleavage of the intact triple helix or as an independent event.

Individual kinetic parameters for MMP hydrolysis of fTHP-3 were next determined (Figure 4). Kinetic parameters were evaluated by Lineweaver−Burk, Eadie−Hofstee, and Hanes−Woolf analyses. The relative order of k_{cat}/K_M values is MMP-13 > MMP-1 ∼ MMP-2 ≫ MMP-3 (Table 1). MMP-13 has the highest k_{cat}/K_M value for hydrolysis of fTHP-3 primarily due to a lower K_M value (20.5 μM for MMP-13 versus 61.2 μM for MMP-1). Consistent with the fTHP-3 results, MMP-13 cleaves type II collagen more rapidly than MMP-1 (*41*). Interestingly, the K_M value for MMP-1 hydrolysis of fTHP-3 is approximately 75 times higher than that of type I collagen (*3*).

Table 1: Kinetic Parameters for fTHP-3 Hydrolysis by MMPs at 30 °C

enzyme	k_{cat}/K_M (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_M (μM)
MMP-1	1278	0.080	61.2
MMP-1($\Delta_{243-450}$)	1312	0.087	66.6
MMP-2	1082	0.017	17.2
MMP-3	503	0.031	65.0
MMP-3($\Delta_{248-460}$)	554	0.035	60.3
MMP-13	2273	0.045	20.5

Table 2: Activation Energies for Substrate Hydrolysis by MMP-1

substrate	E_a (kcal/mol)
fTHP-3 [α1(II)769−783]	11.6
fSSP-3 [α1(II)769−783]	3.4
type I collagen (guinea pig skin)[a]	49.2
α1(I) gelatin (guinea pig skin)[a]	13.9
α2(I) gelatin (guinea pig skin)[a]	13.3

[a] From reference (*55*).

The C-terminal hemopexin-like domain has been proposed to be essential for MMP collagenolytic activity (*43*) but not for cleavage of THPs (*17*). To test this hypothesis, we studied the hydrolysis of fTHP-3 by MMP-1 lacking the C-terminal domain [MMP-1($\Delta_{243-450}$)]. Sequence analysis indicated that MMP-1($\Delta_{243-450}$) cleaved the THP at the same Gly∼Leu bond as cleaved by MMP-1. Kinetic analysis revealed that MMP-1($\Delta_{243-450}$) hydrolyzed fTHP-3 with a k_{cat}/K_M value of 1312 s^{-1} M^{-1} (Table 1), which is similar to the k_{cat}/K_M value for MMP-1. The C-terminally deleted MMP-3 [MMP-3($\Delta_{248-460}$)] cleaved fTHP-3 at the same bond and with a similar k_{cat}/K_M value (Table 1) as MMP-3.

The temperature-dependent hydrolysis of fTHP-3 was evaluated for MMP-1. Kinetic parameters were determined at 25, 30, 35, and 40 °C, and the activation energy (E_a) was calculated (Table 2). E_a was 11.6 kcal/mol for MMP-1 hydrolysis (Table 2). For comparison, the activation energy was determined for MMP-1 hydrolysis of the non-triple-helical substrate Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly∼Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-NH_2 (fSSP-3). E_a was 3.4 kcal/mol (Table 2). Thus, the activation energies differed substantially for MMP-1 hydrolysis of the triple-helical substrate compared with the non-triple-helical substrate. In addition, we have found that the relative K_M values differ by ∼5-fold for MMP-1 binding to analogous single-stranded and triple-helical fluorogenic substrates.[2]

A limited number of kinetic analyses were performed with the branched fluorogenic THP (fTHP-1). Due to the relatively low melting temperature of fTHP-1, kinetic parameters were determined for MMP-1 hydrolysis at 25 and 30 °C. MMP-1 cleaved fTHP-1 at the Gly∼Leu and Gly∼Ile bonds (see

[2] T. Sritharan, J. L. Lauer-Fields, H. Nagase, and G. B. Fields, manuscript in preparation.

earlier discussion). The relative k_{cat}/K_M value increased 4-fold when the temperature was decreased from 30 to 25 °C (data not shown). This was partially due to an increased affinity for the branched THP at the lower temperature. A decrease from 30 to 25 °C shifts the conformational equilibrium of fTHP-1 in favor of triple-helical structure (Figure 2), and MMP-1 has a greater affinity toward an intact triple helix (*16*). MMP-2, MMP-3, and MMP-13 were all found to cleave fTHP-1 primarily at the Gly∼Leu bond (data not shown).

DISCUSSION

Fluorogenic substrates provide a particularly convenient enzyme assay method, as they can be monitored continuously and utilized at reasonably low concentration ranges. There are three types of fluorogenic substrates: (i) aromatic amines, (ii) contact quenched, and (iii) resonance energy transfer quenched (*44*). MMP fluorogenic substrates have been developed using resonance energy transfer quenching, which are sometimes referred to as intramolecular fluorescence energy transfer substrates (IFETS) (*45*). Knight and colleagues proposed the use of Mca as a fluorophore for MMP substrates (*19*) based on the high quantum yield of Mca ($\Phi_F = 0.49$). Mca is efficiently quenched by Dnp moieties (*19*, *20*), as the shoulder in the Dnp absorption spectrum overlaps the Mca fluorescence emission spectrum (*19*). To use the Mca/Dnp pair, a Dnp derivative such as *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionic acid (Dpa) (*19*) or Lys(Dnp) (*20*) can be synthesized and incorporated by solid-phase methods, while Mca is acylated to the peptide N-terminus. Alternatively, the Mca derivative L-2-amino-3-(7-methoxy-4-coumaryl)propionic acid (Amp) can be synthesized and incorporated by solid-phase methods (*46*, *47*). We sought out a slightly different approach, whereby the Mca group is acylated on-resin to a free Lys ϵ-amino group. Substrates containing the fluorophore *N*-methylanthranilic acid (Nma) have been synthesized using solid-phase reactions to create Lys(Nma) moieties (*48*).

The construction of a fluorogenic triple-helical substrate proved to be most challenging, based on numerous difficulties. First were undesired side-products. The modification of the Dnp group during hydrazine treatment fell into this category. The mechanism by which Dnp was modified was not determined, but modification could be minimized by including 2,4-dinitrophenol in the hydrazine−DMF (1:49) solution. However, we found it easier to simply use Mtt instead of Dde as the orthogonal protecting group for the side chain of Lys. The removal of Mtt with 1% TFA had no apparent detrimental effect on the Dnp group. Although subsequent Mca acylation was attainable, efficiency may be increased by using an Fmoc-L-Amp (*46*, *47*) or Fmoc-L-Lys(Mca) (*49*) derivative instead of postsynthesis Mca incorporation.

The second category of difficulties for the synthesis of fluorogenic substrates were physical properties of the constructs. THP solubility and stability in the aqueous environment were the major issues to be addressed. Solubility of the fluorogenic substrate could be improved by substituting hydrophilic amino acids in subsites where such substitutions are well tolerated by MMP family members (*22*). Improved solubility in the aqueous environment was also achieved by incorporating additional Gly-Pro-Hyp repeats. The stability of the triple helix was apparently decreased by the presence of the fluorophore and/or quencher, perhaps due to steric hindrance, negative inductive effects (*50*), and/or disruption of triple-helix-stabilizing hydrogen bonds (*51*). The proper balance of alkyl chain length (*27*) and number of Gly-Pro-Hyp repeats could be used to enhance the triple-helical stability and solubility of the substrate. Ultimately, the C_6-(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 fluorogenic THP (fTHP-3) was assembled and found to be compositionally correct and sufficiently stable for use as a substrate. The yield of this product was greatly improved compared to the branched fluorogenic substrate (data not shown).

We found that cleavage of fTHP-3 did occur with MMP-1, only at the analogous Gly_{775}−Leu_{776} bond in the native type II collagen. In similar fashion, the Gly∼Leu bond was primarily cleaved upon MMP-13 treatment of fTHP-3. Ottl et al. have demonstrated that a triple-helical peptide model of the $[\alpha1(I)]_2\alpha2(I)772-785$ region is cleaved by MMP-8 at a single bond (*14*, *16*). We previously reported that a α1-(I)772−786-branched THP is cleaved exclusively at the Gly∼Ile bond by MMP-1 (*17*, *42*). We can thus assume that THPs contain all the necessary information for efficient hydrolysis by MMPs, and that this information is contained within a 24 residue stretch of the substrate (*3*).

MMP-2 was found to cleave fTHP-3. Cleavage occurred at two sites, the Gly∼Leu bond and the Gly∼Gln bond. This result is consistent with the general proteolytic behavior reported for MMP-2 (*16*, *17*). Minor cleavage of the Gly∼Gln bond was also observed with MMP-13, consistent with MMP-13 behavior toward type II collagen (*41*). MMP-3, which does not cleave types I and II collagen (*10*, *11*), cleaved the fTHP-3 slowly at the Gly∼Leu bond. Since the fluorogenic THP is triple-helical at 30 °C (Figure 3), MMP-3 is not simply cleaving non-triple-helical substrate in equilibrium with the triple-helical form (also see comments below). MMP-3 is able to cleave this triple-helical substrate, and thus must (a) possess some triple-helical peptidase activity and/or (b) destabilize the hydrophobic interactions between the alkyl chains of the substrate, destabilizing the overall triple helix. However, MMP-3 can also cleave the branched fluorogenic THP (fTHP-1), even at 25 °C (data not shown). A branched, nonfluorogenic THP that was hydrolyzed by MMP-1, MMP-2, and MMP-13 was not cleaved by MMP-3 at an appreciable rate (*17*). These results suggest that MMP-3 may possess selective triple-helical peptidase activity. Future studies will examine MMP-3 and other noncollagenolytic protease activity using a variety of triple-helical constructs.

MMP-13 was found to hydrolyze fTHP-3 1.8 times more rapidly than MMP-1 (Table 1). This difference in rate is consistent with the ability of MMP-13 to more rapidly hydrolyze type II collagen compared to MMP-1 (*41*). Interestingly, prior studies have shown that the differences in relative rates of hydrolysis between MMP-13 and MMP-1 become greater when single-stranded substrates are considered. For example, MMP-13 cleaves gelatin 41.2 times more rapidly than MMP-1, while the single-stranded fluorogenic peptides Mca-Pro-Leu-Gly∼Leu-Dpa-Ala-Arg-NH_2 and Mca-Pro-Cha-Gly∼Nva-His-Ala-Dpa-NH_2 are cleaved 62.6 and 74.1 times more rapidly, respectively, by MMP-13 compared to MMP-1 (*8*). Such results are further indicative of the triple-

Table 3: Kinetic Parameters for MMP Binding to and/or Hydrolysis of Triple-Helical Peptides

enzyme	substrate	substrate T_m (°C)	K_M (μM)	K_D (μM)	k_{cat} (s^{-1})
MMP-1	fTHP-3 [α1(II)769−783]	45	61.2		0.080
MMP-1($\Delta_{243-450}$)	fTHP-3 [α1(II)769−783]	45	66.6		0.087
MMP-2	fTHP-3 [α1(II)769−783]	45	17.2		0.017
MMP-3	fTHP-3 [α1(II)769−783]	45	65.0		0.031
MMP-3($\Delta_{248-460}$)	fTHP-3 [α1(II)769−783]	45	60.3		0.035
MMP-13	fTHP-3 [α1(II)769−783]	45	20.5		0.045
MMP-1	α1(I)772−786 THP	32	63.0[a]		0.11
MMP-1($\Delta_{243-450}$)	α1(I)772−786 THP	32	207.6[a]		0.28
MMP-13	α1(I)772−786 THP	32	148.2[a]		0.49
MMP-8	[α1(I)]2α2(I)772−784 THP	33	5.0[b]		ND
MMP-1(E200A)	$[\alpha1(I)]_2\alpha2(I)$772−784 THP	41		3.7[c]	ND
MMP-2(E375A)	$[\alpha1(I)]_2\alpha2(I)$772−784 THP	41		0.67[c]	ND

[a] From reference (*17*). Assay performed at 30 °C. [b] From reference (*14*). Assay performed at 25 °C. [c] From reference (*16*). Assay performed at 22 °C.

helical nature of our substrate. In addition, cleavage of the fTHP-3 by MMP-1 occurs at 25 °C, where virtually no single-stranded species exists (Figure 3). Finally, the activation energy value differences for MMP-1 hydrolysis of fTHP-3 and fSSP-3 are of similar magnitude as for MMP-1 hydrolysis of collagen and gelatin (see discussion below).

It has been proposed that the ability of an MMP to cleave a triple helix is lost when the C-terminal domain or the hinge region between the catalytic and C-terminal domains is replaced with one from MMP-3 (*11, 52*). We thus examined the behavior of MMP-1($\Delta_{243-450}$) toward the fluorogenic THP. Cleavage of the fluorogenic THP occurred with MMP-1($\Delta_{243-450}$), at the same Gly∼Leu bond cleaved by MMP-1. Consistent with these results, both MMP-8 and MMP-8($\Delta_{243-467}$) hydrolyzed a heterotrimeric THP (*14*), while both MMP-1 and MMP-1($\Delta_{243-450}$) hydrolyzed a homotrimeric, nonfluorogenic THP (*17*). For triple-helical substrates, we have recently proposed that the C-terminal domain of MMP-1 is necessary for orienting the whole, native collagen molecule but not necessary for binding to and cleaving a triple helix (*17*). Thus, the C-terminal domain is necessary for collagenolytic activity but not triple-helical peptidase activity. This proposal is consistent with the large distance observed between the MMP-1 catalytic and C-terminal domains observed by X-ray structural analysis (*53*). Also, it was reported that the MMP-1 C-terminal domain alone could bind native collagen (*11*), while the MMP-1 C-terminal domain did not bind synthetic triple helices (*16*) and the MMP-8 C-terminal domain did not bind type I collagen (*54*). The MMP-1 and -8 catalytic domains alone can cleave THPs, suggesting that they unwind individual strands of the synthetic triple helix (*14, 17*).

The activation energies for MMP-1 hydrolysis of a triple-helical (fTHP-3) and single-stranded (fSSP-3) fluorogenic substrate were found to be 11.6 and 3.4 kcal/mol, respectively. Welgus et al. (*55*) have shown the activation energy for MMP-1 hydrolysis of type I collagen and gelatin in solution is 49.2 and 13.3−13.9 kcal/mol, respectively (Table 2). The higher activation energy was proposed to reflect the difficulty in accessing water at the site of hydrolysis in native collagen. Upon denaturation of the triple helix, the activation energy decreases. The activation energies reported here for triple-helical and single-stranded fluorogenic peptide hydrolysis are considerably lower than those for type I collagen and gelatin, respectively. However, the *trends* for MMP-1 hydrolysis of the triple-helical and non-triple-helical species are very similar. For example, the activation energy for hydrolysis of the non-triple-helical peptide is ∼3.4-fold lower than that for the triple-helical peptide, while the activation energies for hydrolysis of collagen and gelatin differ by ∼3.6-fold.

The kinetic parameters for MMP binding to and/or hydrolyzing a variety of THPs have been reported in several studies (Table 3). K_M values for MMP-1 or MMP-8 range from 5 to 63 μM. These K_M values are higher than those for MMP-1/MMP-8 binding to collagen (*3, 5*). For example, MMP-1 binds to fTHP-3 and type I collagen with K_M = 61.2 and 0.81−1.0 μM, respectively. The different K_M values indicate that a longer triple helix is bound with higher affinity by the enzyme, most likely with the help of the C-terminal domain of the enzyme. Interestingly, the K_M or K_D values for MMP-2 binding to triple-helical peptides are lower than for MMP-1. This is most likely the contribution of the fibronectin type II domain of MMP-2 (*16*). The opposite trend is exhibited when these enzymes bind to type I collagen, as MMP-2 exhibits a K_M = 8.5 μM, while MMP-1 has a K_M = 1.0 μM (*5*). These observations are further indicative of the different roles in collagen binding between the hemopexin-like C-terminal domain of MMP-1 and the fibronectin type II-like domain within the catalytic domain of MMP-2 (*16, 56*). Both the hemopexin-like and fibronectin type II-like domains do not contribute to the hydrolysis of THPs. It is, however, notable that the proteinase domain of MMP-1 alone cannot cleave triple-helical interstitial collagens, even at a very high concentration (1 μM) (*13*). MMP-3 also does not cleave triple-helical types I and II collagen in native conformation. Therefore, one must also consider that triple-helical peptides containing diverse sequences may have subtle structural variations (*57*) that differ from native collagens, which could have a significant affect on the individual kinetic parameters for MMP hydrolysis of triple helices.

Longer triple-helical peptides may need to be designed in order to ensure interaction with both the MMP-1 N- and C-terminal domains, in a fashion similar to collagen. The required length is not easy to determine, however, as the proximity of the collagenase (MMP-1, -8, and -13) catalytic (N-terminal) and hemopexin-like (C-terminal) domains is difficult to ascertain due to the flexible linker that connects these domains (*53, 58*). A prior modeling study utilized the

X-ray crystallographic structure of MMP-1 (*53*) to "dock" a heterotrimeric THP (*16*) and evaluate enzyme−substrate interactions. The heterotrimeric THP had 14−15 residues downstream from the MMP cleavage site. Since triple helices have a length of ∼3 Å per residue (*59*), the THP extended 42−45 Å from the cleavage site. The modeling studies suggested that the end of the C-terminal (downstream) region of the THP interacted with the MMP-1 C-terminal domain (*16*). However, our substrate (fTHP-3) has 24 residues downstream from the cleavage site, stretching 72 Å, yet apparently does not interact with the MMP-1 C-terminal domain (see Table 1). It is possible that the MMP-1 C-terminal domain recognizes specific sequences not present in fTHP-3. Alternatively, due to the flexible linker, the MMP-1 N- and C-terminal domains may be less proximal than previously believed. Additional THP constructs varying in length and sequence will be required to further evaluate substrate interactions with both the MMP-1 N- and C-terminal domains.

In an effort to better understand MMP kinetics and selectivity, we have successfully adapted the synthesis of fluorogenic substrates to include fluorogenic triple-helical substrates. To fully dissect collagenolytic activity of the native triple-helical interstitial collagens, three issues need to be considered: (a) the ability to bind collagen efficiently; (b) the ability to unwind the triple helix; and (c) the ability to cleave the individual strands of the triple helix. MMPs are believed to possess superior collagenolytic activity based on their ability to (a) bind collagen efficiently and (b) unwind the triple helix more efficiently than other proteases. However, virtually all previous studies have been performed with collagen, which does not allow for the discrimination of (a) and (b). It is possible that enzymes may differ greatly in the ability to bind collagen efficiently (a) but not greatly in the ability to unwind the triple helix (b) or the ability to cleave the individual strands of the triple helix (c). By creating small triple-helical models, it should be possible to separate out "collagenolytic" activity (the ability to efficiently bind collagen and unwind and cleave a triple helix) from "triple-helicase" activity (the ability to efficiently unwind and cleave the triple helix). Additional exploration of fluorogenic substrates that vary in T_m or the collagen sequence incorporated may allow us to investigate the detailed mechanism of both collagen-binding and triple-helicase activities of collagenase. This may also lead to the design of selective MMP fluorogenic substrates.

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