Exosite Interactions Impact Matrix Metalloproteinase Collagen Specificity

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Members of the matrix metalloproteinase (MMP) family selectively cleave collagens in vivo. However, the substrate structural determinants that facilitate interaction with specific MMPs are not well defined. We hypothesized that type I–III collagen sequences located N- or C-terminal to the physiological cleavage site mediate substrate selectivity among MMP-1, MMP-2, MMP-8, MMP-13, and MMP-14/membrane-type 1 (MT1)-MMP. The enzyme kinetics for hydrolysis of three fluorogenic triple-helical peptides (fTHPs) was evaluated herein. The first fTHP contained consensus residues 769–783 from type I–III collagens, the second inserted α1(II) collagen residues 763–768 N-terminal to the consensus sequence, and the third inserted α1(II) collagen residues 784–792 C-terminal to the consensus sequence. Our analyses showed that insertion of the C-terminal residues significantly increased kcat/Km and kcat for MMP-1. MMP-13 showed the opposite behavior with a decreased kcat/Km and kcat and a greatly improved Km in response to the C-terminal residues. Insertion of the N-terminal residues enhanced kcat/Km and kcat for MMP-8 and MT1-MMP. For MMP-2, the C-terminal residues enhanced Km and dramatically decreased kcat, resulting in a decrease in the overall activity. These changes in activities and kinetic parameters represented the collagen preferences of MMP-8, MMP-13, and MT1-MMP well. Thus, interactions with secondary binding sites (exosites) helped direct the specificity of these enzymes. However, MMP-1 collagen preferences were not recapitulated by the fTHP studies. The preference of MMP-1 for type III collagen appears to be primarily based on the flexibility of the hydrolysis site of type III collagen compared with types I and II. Further characterization of exosite determinants that govern interactions of MMPs with collagenous substrates should aid the development of pharmacotherapeutics that target individual MMPs.

Collagen is a scaffold and support network for cells in tissues. Normal collagen turnover facilitates growth, morphogenesis, angiogenesis, and wound healing. Altered collagen remodeling is involved in disease states such as arthritis, periodontitis, atherosclerosis, and tumor metastasis (1–4). Of the 29 known collagens, types I–III are the most abundant (5, 6). Although these interstitial fibrillar collagens possess a similar length supersecondary triple-helical structure, differences in sequence, tissue distribution, and glycosylation patterns have been well documented (5, 7).

Triple-helical structures are resistant to most proteases. However, several members of the matrix metalloproteinase (MMP)2 subfamily of zinc-dependent endopeptidases catalyze the hydrolysis of triple helices (5). Although highly homologous, MMPs have distinct preferences among collagenous substrates (5, 8). MMP-1, MMP-8, MMP-13, and MMP-14/membrane-type 1 MMP (MT1-MMP) are collagenases that hydrolyze the triple helix of fibrillar type I–III collagens with varying efficiencies at a single Gly–Ile/Leu site. Although not a classic “collagenase,” MMP-2 cleaves type I collagen (9). Type III collagen is the preferred collagen substrate for MMP-1, whereas MMP-8 and MT1-MMP preferentially cleave type I collagen (10, 11). MMP-13 is more selective for type II collagen (11). Although mechanistic reasons for collagen preferences have been postulated (8), collagen selectivity determinants among MMPs are not well understood.

The major structural domains shared among MMPs include the prodomain, the catalytic (CAT) domain, a flexible hinge region, and the C-terminal hemopexin-like (HPX) domain (5, 7). For the aforementioned collagenases, efficient catalytic activities toward collagens require both the CAT and HPX domains (11–17). Taking into account the importance of the CAT and HPX domains, the collagenolytic process has been proposed to involve discrete, sequential steps (17). First, an MMP binds a triple helix utilizing secondary binding sites (exosites) with prominent exosites identified in the HPX domain (18). Second, the MMP orients its other domains (such as the CAT domain) into the active configuration. Third, the MMP unwinds or destabilizes the collagen triple helix, moving a single collagen strand into the CAT domain active site. Fourth, there is rapid sequential cleavage of each strand.

Despite significant clinical interest, it has proven difficult to achieve selective inhibition of MMPs due to identical active site chemistry and flexible peptide binding sites among the MMP family members (19, 20). Small molecule hydroxamic acid-based inhibitors are highly effective in inhibiting the catalytically active zinc at low substrate concentration but have traditionally exhibited poor selectivity between MMPs (4, 21–23). Hydroxamic acid-based inhibitors failed in clinical trials par-

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Matrix Metalloproteinase Exosites

Matrix Metalloproteinase Exosites quantitatively due to high incidences of confounding and uncomfortable patient side effects (1, 19, 23–26). It is not uncommon for imbalances in the expression or activity of a single MMP to result in human disease; successful targeted MMP inhibition is attractive in drug development. Understanding the association of collagens with MMPs may provide insight into binding site selectivity within the MMP family. High throughput approaches have been utilized for designing single-stranded peptide inhibitors against the MMP catalytic sites but as yet have not produced inhibitors that sufficiently distinguish between target and "antitarget" MMPs (27). To evaluate substrate selectivity, an alternative and novel approach is to examine how MMPs interact with customized triple-helical peptides (THPs) through secondary binding sites (exosites)/allosteric sites (28).

THP substrates have been powerful tools for elucidating MMP kinetic behavior and substrate cleavage selectivity (17, 18, 29, 30). In a prior study, we recently have found that by varying the composition of collagen model sequences within THPs we can explore unique binding interactions in the CAT and HPX domains of MMP-1 (18). Therefore, in the present study, we hypothesized that probing the CAT and HPX domains of collagenolytic MMPs via THPs with varying contents of collagen model sequences will reveal different binding site interactions. To test this hypothesis, we utilized three fluorogenic THP substrates (Table 1). The first (fluorogenic triple-helical peptide (fTHP)-15) incorporated a consensus sequence from the MMP hydrolysis sites in type I–III collagens. The second (fTHP-16) had a Gly-Pro-Hyp-Gly-Pro-Ser-Gly-Ala-Glu sequence inserted N-terminal to the consensus sequence, whereas the third (fTHP-17) had a Gly-Leu-Hyp-Gly-Gln-Gly-Glu-Arg sequence inserted C-terminal to the consensus sequence. Biophysical properties of the fTHPs were determined, and modeling of their structures was undertaken. Overall activities and individual kinetic parameters were quantified for MMP-1, MMP-2, MMP-8, MMP-13, and MMP-14 catalysis of each substrate.

MATERIALS AND METHODS

All chemicals were molecular biology or peptide synthesis grade and purchased from Fisher. The Knight single-stranded peptide (Mca-Lys-Pro-Leu-Gly-Leu-Lys(Dnp)-Ala-Arg-NH₂) was synthesized by methods described previously (31, 32). MMP-1 overexpressed in Escherichia coli was refolded from 8 M urea-dissolved inclusion bodies as described (33). Purified pro-MMP-2 was isolated from human uterine cervical fibroblasts (34). Recombinant, full-length MMP-8, MMP-13, and MMP-14 (ectodomain only; no transmembrane domain) were purchased from Millipore (Danvers, MA).

Metalloproteinase Activation

All MMPs were activated (34) by mixing equal volumes of stock and activator to a final concentration of 1 mM p-amino-mercuric acetate, incubated for 45 min in a 37 °C water bath, and diluted to 20–100 nM in ice-cold TSB*Zn buffer (50 mM Tris, 150 mM NaCl, 0.02% NaN₃, 0.01% Brij-35, 10 mM CaCl₂, 1 μM ZnCl₂, pH 7.5) to prevent autoproteolysis. Enzyme aliquots were kept on wet ice and used the same day. MMP activity was initially evaluated using the Knight single-stranded peptide and compared with prior data (17, 18). In this way, Knight single-stranded peptide activity was used as an indicator of enzyme integrity rather than tissue inhibitor of metalloproteinase titration as performed previously (8).

Triple-helical Substrates

All fTHPs were based on a consensus sequence derived from the collagenolytic MMP cleavage sites in human type I–III collagens (35). fTHP-15, fTHP-16, and fTHP-17 (see Table 1 for sequences) were synthesized by Fmoc (N-(9-fluorenyl)me-thoxy carbonyl) solid-phase chemistry as described previously (8, 17, 18, 30). Peptide synthesis was carried out on a Protein Technologies PS3 peptide synthesizer (Tucson, AZ). Peptides were cleaved from the resin using thioanisole-water-TFA (1:1:18), precipitated in methyl-tert-butyl ether, and sedimented at 4 °C. The solvent phase was decanted. Dry pellets were dissolved in water, frozen, and lyophilized under vacuum.

Peptide purity was evaluated using an Agilent 1200 series analytical HPLC (Santa Clara, CA) equipped with a 150 × 4.6-mm Vydac C₁₈ column. Solvent A was 0.1% TFA, H₂O; solvent B was 0.1% TFA, acetonitrile; the gradient was 0–70% over 14 min; and the flow rate was 1 ml/min. Analytical results were used to determine the optimal preparatory gradient where 4 ml of water-dissolved peptide was injected into a Vydac C₁₈ column (218TP152022) on a Varian ProStar HPLC (Agilent, Santa Clara CA). Peak fractions were analyzed via analytical HPLC and reflectance MALDI-TOF mass spectra (Applied Biosystems Voyager DE-PRO Biospectrometry work station, Carlsbad, CA). Pure fractions were pooled, frozen, lyophilized, and stored at −20 °C in amber vials until use. MALDI-TOF analysis confirmed masses of 4593.1 Da for fTHP-15 (theoretical, 4589.0 Da), 5087.7 Da for fTHP-16 (theoretical, 5087.1 Da), and 5022.6 Da for fTHP-17 (theoretical, 5021.4 Da).

Circular Dichroism Spectroscopy

Fluorogenic peptides were dissolved in TSB and equilibrated at 4 °C (8 h) to facilitate triple-helix formation. Peptide concentrations were determined using a Thermo Scientific NanoDrop 1000 (Waltham, MA) via wavelength scan at λ = 363 nm, ε₁₀₀₀ = 15,900 M⁻¹ cm⁻¹. Tripe-helical structure was evaluated by near-UV circular dichroism (CD) spectroscopy using a Jasco J-810 spectropolarimeter (Easton, MD) with a path length of 1 mm.

Peptide Kinetic Testing

Enzyme kinetics were determined in a BioTek Synergy 4 plate reader (Winooski, VT) running Gen5 1.07 software as described previously (17, 36). In brief, a range of peptide concentrations was created by diluting a 100 μM stock solution of peptide 1:1 12 times. A 76-μl volume of sample was loaded, the plate was read, and 4 μl of 20× enzyme stock solution (100 nm) was added. The kinetic protocol at 27 °C had 30 s of shaking followed by reading each well every 8 s (for 600 s) to determine initial reaction rates. Plates were stored at ambient temperature (>24 h) before a final reading. The 25 μM peptide concentration was analyzed by HPLC to determine the percentage of reaction completeness with 100% cleavage RFU = ([24-h
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RESULTS

Three fTHPs were utilized in the present study (Fig. 1 and Table 1). fTHP-15 incorporates a consensus sequence from type I–III collagens (Table 2) and provides for convenient monitoring of triple-helical peptidase activity by collagenolytic MMP family members. To examine the subtleties of individual MMP family members. To examine the subtleties of individual MMP family members.

The consensus cleavage site is in bold, whereas the inserted sequences are italicized.

**TABLE 1**

| Peptide                | Sequence                        | $\theta_{222}$ | $^\circ$C | $T_m$ °C |
|------------------------|---------------------------------|----------------|----------|---------|
| fTHP-15                | (Gly-Pro-Hyp)$_{17}$            | 10.4           | 55       |
| fTHP-16                | (Gly-Pro-Hyp)$_{20}$            | 13.3           | 49       |
| fTHP-17                | (Gly-Pro-Hyp)$_{17}$            | 16.6           | 51       |

Ala–Glu sequence inserted N-terminal to the consensus sequence in fTHP-15 where the insertion exactly matches residues 763–768 from the $\alpha 1$(II) chain (Table 2). fTHP-17 has a Gly-Leu-Hyp-Gly-Gln-Arg-Gly-Glu-Arg sequence inserted C-terminal to the consensus sequence in fTHP-15 where the insertion exactly matches residues 784–792 from the $\alpha 1$(I) and $\alpha 1$(II) chains (Table 2). Two Gly-Pro-Hyp repeats, one from each end, were omitted in fTHP-17 compared with fTHP-15. Based on our prior studies with MMP-1 and MMP-9 (17, 18), we can presume that the inserted N-terminal sequence will interact with the MMP CAT domain, whereas the inserted C-terminal sequence will interact with either the HPX domain (for MMP-1, MMP-8, MMP-13, and MMP-14) or the fibronectin type II inserts (for MMP-2). Overall, the insertions offered significant residue diversification (Fig. 1). For example, the N-terminal insertion replaced two Hyp residues with Glu and Ser, whereas the C-terminal insertion replaced one Pro with Gln, one Pro with Glu, one Pro with Leu, and two Hyp residues with Arg residues (Table 2). The Gly-Pro-Hyp sequences may be modeled as a smooth triple helix in three dimensions (Fig. 1).
Sequence alterations introduce more varied surfaces for enzyme-substrate interaction (compare Fig. 1 and Table 1). In addition, fTHP-17 includes Leu in subsite P10/H11032, which has been suggested to contribute to MMP-1 recognition of collagen (39, 40).3

All three fTHPs exhibited CD spectra characteristic of triple-helical structures with positive molar ellipticities ([θ]/H225 nm) and strongly negative [θ] values at λ = 195 nm (data not shown). Monitoring of [θ] as a function of temperature resulted in sigmoidal melting curves for all fTHPs (Fig. 2A), indicative of transitions from triple helices to monomeric species. The melting points (Tm values) for fTHP-15, fTHP-16, and fTHP-17 were 55, 49, and 51 °C, respectively (Fig. 2B). These melting temperatures indicate fTHP thermal stabilities suitable for MMP kinetic analyses. The three fTHPs had similar stabilities, an important consideration as prior studies have shown that MMP hydrolytic activity toward both THPs and collagens can vary greatly due to differences in substrate thermal stability (35, 41). The close similarity of thermal stability among the three fTHPs warranted our continued analysis of the effects of sequence variations. Although the maintenance of similar thermal stabilities resulted in slightly different sequence lengths (45, 51, and 48 residues for fTHP-15, fTHP-16, and fTHP-17, respectively), this is not deemed to significantly confound the results as the present study investigated the relative effects of altered substrate sequences for a particular MMP. Moreover, prior studies using THPs of differing lengths (28–32 residues) indicated that peptide length per se was not a determining factor in relative activity (42–44).

Five MMPs were examined in the present study: MMP-1, MMP-2, MMP-8, MMP-13, and MMP-14/MT1-MMP. The hydrolysis of fTHP-15 by each of these MMPs has been reported previously (8, 17, 18, 35, 45) and occurs at the Gly/Leu bond. Thus, fTHP-15 served as a well established point of reference. For each MMP, kcat/Km values were determined for fTHP-15, fTHP-16, and fTHP-17 (Table 3). Relative activities were compared with fTHP-15 for each enzyme; and thus, fTHP-15 served to calibrate enzyme triple-helical peptidase activity.

Significant changes were observed in kcat/Km values in response to the sequence insertion. Interestingly, these effects differed for the individual MMPs. For example, when the N-terminal sequence was inserted in fTHP-16, kcat/Km values were similar to fTHP-15 for MMP-1 and MMP-2 and increased to 170–230% for MMP-8, MMP-13, and MMP-14 (Table 3). The C-terminal sequence insertion in fTHP-17 resulted in even more diverse effects. The kcat/Km values for MMP-8 and MMP-14 showed little change compared with fTHP-15, MMP-2 exhibited a 73% reduction in activity, MMP-13 exhib-

3 L. H. Arnold, L. Butt, S. H. Prior, C. Read, G. B. Fields, and A. R. Pickford, manuscript submitted for publication.
MMP-1 resulted in a modest 6.5-fold increase in $k_{\text{cat}}/K_m$. Individual kinetic parameters ($k_{\text{cat}}$ and $K_m$) were also examined to determine the origins of effects on overall MMP activities (Table 3). Compared with fTHP-15, fTHP-16 hydrolysis by MMP-1 resulted in a modest 6.5-fold increase in $k_{\text{cat}}$. In contrast, comparison of fTHP-15 and fTHP-17 hydrolysis by MMP-1 indicated a significant increase in $k_{\text{cat}}$ from 0.0071 to 0.105 s$^{-1}$. MMP-2 showed similar $k_{\text{cat}}$ values for hydrolysis of fTHP-15 and fTHP-16. fTHP-17 showed a slight decrease in $k_{\text{cat}}$ compared with fTHP-15. For MMP-8 processing of fTHP-17, an approximate 3-fold increase in $k_{\text{cat}}$ was seen compared with fTHP-15. fTHP-17 had a 2-fold decrease in $k_{\text{cat}}$ (0.017 s$^{-1}$) compared with fTHP-15. Overall, the N-terminal inserted sequence increased $k_{\text{cat}}$ for MMP-1, MMP-8, and MMP-14 and decreased $k_{\text{cat}}$ for MMP-13, whereas the C-terminal inserted sequence enhanced $k_{\text{cat}}$ for MMP-1 and MMP-8 and decreased $k_{\text{cat}}$ for MMP-2, MMP-13, and MMP-14.

The magnitudes of $k_{\text{cat}}$ (based on the present study) and enzyme-substrate off-rate ($k_0/2K_m$) (based on a prior study of MMP-1 binding to a THP) (43) are of a similar order. Considering the proposed collagenolysis pathway (41), $K_m$ cannot be approximated here as $K_m$ and thus $K_m$ values are not independent of $k_{\text{cat}}$. However, one can still examine the relatedness of $K_m$ to $k_{\text{cat}}$ as an examination of exosite effects for a particular MMP and also use that relatedness to evaluate the relative strength of interaction between enzyme and substrate.

fTHP-15 hydrolysis by MMP-1 resulted in a $K_m$ value of 1.8 $\mu$M. $K_m$ increased to 13 $\mu$M for MMP-1 hydrolysis of fTHP-16 and slightly increased (2.9 $\mu$M) for hydrolysis of fTHP-17. For MMP-2, hydrolysis of fTHP-15 and fTHP-16 resulted in similar $K_m$ values (5.9 and 5.2 $\mu$M, respectively), whereas hydrolysis of fTHP-17 had a significantly decreased $K_m$ value (0.73 $\mu$M). For MMP-8, hydrolysis of fTHP-15 and fTHP-16 resulted in similar $K_m$ values (9.2 and 11 $\mu$M, respectively), whereas hydrolysis of fTHP-17 had a significantly increased $K_m$ value (29 $\mu$M). MMP-13 exhibited a slightly improved $K_m$ value for hydrolysis of fTHP-16 compared with fTHP-15 (25 versus 67 $\mu$M, respectively) that then decreased significantly for hydrolysis of fTHP-17 ($K_m = 0.15$ $\mu$M). For MMP-14, hydrolysis of fTHP-15

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**TABLE 3**

Kinetic parameters for hydrolysis of triple-helical substrates by MMPs

| Substrate | $k_{\text{cat}}/K_m$ | $K_m$ | $k_{\text{cat}}$ | Relative activity (compared with fTHP-15) |
|-----------|----------------------|-------|------------------|-----------------------------------------|
|           | $s^{-1} \mu$M | $\mu$M | $s^{-1}$ | % |
| **MMP-1** | | | | |
| fTHP-15  | 4.100 ± 800 | 1.8 ± 0.30 | 0.0071 ± 0.0011 | 100 |
| fTHP-16  | 3.500 ± 580 | 13 ± 2.2 | 0.046 ± 0.0093 | 86 |
| fTHP-17  | 37.000 ± 4.700 | 2.9 ± 0.077 | 0.105 ± 0.0046 | 900 |
| **MMP-2** | | | | |
| fTHP-15  | 210,000 ± 17,000 | 5.9 ± 0.57 | 1.2 ± 0.12 | 100 |
| fTHP-16  | 230,000 ± 43,000 | 5.2 ± 0.84 | 1.2 ± 0.05 | 110 |
| fTHP-17  | 57,000 ± 12,000 | 0.73 ± 0.062 | 0.042 ± 0.0022 | 27 |
| **MMP-8** | | | | |
| fTHP-15  | 9,600 ± 1,200 | 9.2 ± 2.1 | 0.088 ± 0.012 | 100 |
| fTHP-16  | 22,000 ± 1,800 | 11 ± 0.44 | 0.24 ± 0.009 | 230 |
| fTHP-17  | 10,000 ± 900 | 29 ± 2.4 | 0.30 ± 0.035 | 107 |
| **MMP-13** | | | | |
| fTHP-15  | 63,000 ± 1,200 | 67 ± 3.9 | 4.2 ± 0.25 | 100 |
| fTHP-16  | 107,000 ± 6,400 | 25 ± 2.7 | 2.7 ± 0.12 | 170 |
| fTHP-17  | 39,000 ± 12,000 | 0.15 ± 0.022 | 0.0058 ± 0.00054 | 60 |
| **MMP-14** | | | | |
| fTHP-15  | 3,000 ± 120 | 11 ± 1.9 | 0.034 ± 0.0036 | 100 |
| fTHP-16  | 5,200 ± 120 | 20 ± 5.8 | 0.102 ± 0.011 | 170 |
| fTHP-17  | 3,300 ± 510 | 5.2 ± 0.58 | 0.017 ± 0.00037 | 110 |
and fTHP-16 resulted in similar $K_m$ values (11 and 20 μM, respectively), whereas hydrolysis of fTHP-17 had a decreased $K_m$ value (5.2 μM). Overall, the N-terminal inserted sequence worsened $K_m$ for MMP-1 and improved $K_m$ for MMP-13, whereas the C-terminal extended sequence enhanced $K_m$ for MMP-2, MMP-13, and MMP-14 and worsened $K_m$ for MMP-8.

The relatedness of $k_{\text{cat}}$ and $K_m$ values was examined by plotting a comparison of the individual kinetic parameters for each substrate and enzyme (Fig. 3). In cases where hydrolysis of one substrate compared with another substrate resulted in no change in kinetic parameters, superimposable points would be observed. Such a result would indicate a lack of additional exosite interactions for one substrate compared with the other. Alternatively, data points far apart for two substrates would indicate significant differences in exosite interactions. Therefore, an examination of $k_{\text{cat}}$ versus $K_m$ served as a diagnostic for potential exosite interactions conferred by substrates of different collagen model sequences. MMP-13 hydrolysis of fTHP-17 resulted in a $k_{\text{cat}}$ versus $K_m$ value that was considerably shifted from the $k_{\text{cat}}$ versus $K_m$ values for hydrolysis of fTHP-15 and fTHP-16 (Fig. 3). Thus, fTHP-17 was subject to additional exosite interactions with MMP-13 compared with the other two fTHPs. In contrast, relatively modest differences in $k_{\text{cat}}$ versus $K_m$ values were observed for hydrolysis of the three fTHPs by MMP-8 (Fig. 3). Prominent exosite interactions did not occur with MMP-8 and fTHP-16 or fTHP-17 compared with fTHP-15. The overall susceptibility to modified exosite interactions among the analyzed MMPs was MMP-13 > MMP-1 > MMP-2 > MMP-14 > MMP-8 (Fig. 3).

**DISCUSSION**

**Exosite Regulation of MMP Activity**—Collagenase action is a complex, carefully regulated, multistep process. Prior studies from this and other laboratories have found MMP secondary binding sites (exosites) in both the CAT and HPX domains that contribute to collagenolysis. For example, the 202–210 region in the CAT domain of MMP-1 participates in efficient collagen catabolism (33). MMP-1 residue 210 (and the corresponding residue in MMP-8) facilitates collagenolytic and triple-helical peptidase activities (8, 46). When studying macrophage elastase (MMP-12) interactions with triple-helical substrates by NMR spectroscopy, several CAT domain exosites were identified and localized (47, 48). For example, analyzing MMP-12 alone or in complex with the α1(V)436–450 THP showed interactions located far from the catalytic cleft; more than 30 residues total were altered from the uncomplexed enzyme, especially Asp-124, Asp-164, and Phe-197 (47). Kinetic analyses of triple-helical peptidase activity in combination with site-directed mutagenesis found that Ile-290 and Arg-291 in the HPX domain of MMP-1 were important exosites for collagenolysis (18).

It is interesting that MMPs would exploit transient exosite binding event(s) to refine selectivity of collagen targets. These interactions are possibly brief and occur with low affinity by nature although in combination with subsequent events may exert significant effects. Such observations encouraged the present analyses to look beyond “conventional” MMP active sites and peptide sequences to identify specificity elements. fTHP-16 could access exosites in the MMP CAT domain, whereas fTHP-17 could access exosites in the MMP HPX domain (except for MMP-2).

**Comparison of fTHP and Collagen Selectivities**—MMP-1 has greater catalytic activity on type III collagen as a substrate. At 25 °C, MMP-1 cleaves type I, II, and III collagens with $k_{\text{cat}}/K_m$ values of 18,500, 130, and 112,000 s⁻¹ M⁻¹, respectively (10). MMP-1 collagen preferences are manifested in $k_{\text{cat}}$ not $K_m$ values (49). The N-terminal region of fTHP-15 has more sequence similarity to type III collagen than fTHP-16 (Table 2), fTHP-17 could be a better substrate for MMP-1 than fTHP-15 even though the sequence extension is closer to type II than type III collagen. Other than repeating Gly residues, type II and III collagens share only a Leu at position 785. This Leu residue may be sufficient to confer the observed dramatic $k_{\text{cat}}$ increase by interactions with an MMP-1 HPX domain exosite. MMP-1 may also utilize C-terminal regions for initial collagen binding and orientation prior to cleavage. However, this does not explain the preference of MMP-1 for type III collagen. That preference may originate from the relative stabilities of the triple helices surrounding the cleavage sites as type III collagen is known to have greater susceptibility to general proteolysis than type I or III collagen and thus a more flexible (less thermally stable) cleavage site (8, 50, 51).

MMP-8 preferentially cleaves type I collagen over type II and III collagens at 25 ºC ($k_{\text{cat}}/K_m = 2,570, 590$, and $130 \text{ s}^{-1} \text{ M}^{-1}$, respectively) (10). As with MMP-1, MMP-8 collagen preferences are based on $k_{\text{cat}}$ values. The α1(I) collagen chain (763–792) is ~90% similar to the α1(II) chain (Table 2). The C-terminal insertion from fTHP-15 to fTHP-17 increases the sequence similarity (+4 amino acids; +56% similarity) compared with α1(I) but also results in fTHP-17 being only a slightly better substrate for MMP-8 than fTHP-15. Adding α1(I)-like sequence (+4 amino acids; +66% similarity) to the region N-terminal of the cleavage site more than doubled $k_{\text{cat}}/K_m$ for MMP-8 hydrolysis of fTHP-16 compared with...
fTHP-15 (Table 3). These effects argue that MMP-8 interacts with type I collagen at N-terminal regions adjacent to the active site and that residues inserted N-terminal to the catalytic site are critical for substrate orientation prior to catalysis. These results are consistent with MMP-8 having a preference for type I collagen.

MMP-13 cleaves type II collagen 5 and 6 times faster than type I and type III collagens, respectively, at 25 °C (52). Overall, fTHP hydrolysis by MMP-13 was enhanced by the N-terminal insertion and decreased by the C-terminal insertion, a pattern that differs from MMP-8 (see above) and MMP-14 (see below). Examining individual kinetic parameters showed that the insertion of an α1(II)-like sequence either N- or C-terminal to the cleavage site enhanced $K_m$ and decreased $k_{cat}$ (Table 3). The improved $K_m$ values for both insertions would be expected for an enzyme that prefers type II collagen. Perhaps most significant is that altering the C-terminal substrate sequence by 5 amino acids caused an almost 350% enhancement in $K_m$ and 625% decrease in $k_{cat}$. These data suggest that substrate sequence C-terminal to the cleavage site plays a dominant role in substrate binding and orientation for MMP-13.

MMP-14 prefers type I collagen as activity against type I collagen and 6.5 times that of type III collagen (11). The better MMP-14 $k_{cat}/K_m$ value with fTHP-16 compared with fTHP-15 and fTHP-17 (Table 3) is caused mainly by an increase in $k_{cat}$ in a fashion similar to MMP-8 and consistent with the preference of MMP-14 for type I collagen. In comparison, integrating a C-terminal sequence that was more native collagen-like decreased $K_m$ and $k_{cat}$. These data suggest that the region C-terminal of the catalytic site affects substrate affinity, whereas the N-terminal region influences turnover.

MMP-2 has been found to cleave type I collagen at 25 °C with a $k_{cat}/K_m$ of 530 s$^{-1}$ M$^{-1}$ (53). Comparisons of hydrolysis rates for type II and III collagens have not been made previously. Although the N-terminal sequence extension failed to show significant change in $k_{cat}/K_m$, $K_m$ or $k_{cat}$ for MMP-2, the insertion of C-terminal residues corresponding to type II collagen (as seen in fTHP-17) decreased $k_{cat}/K_m$ through an improved $K_m$ and substantially decreased $k_{cat}$. In this regard, the behavior of MMP-2 is quite distinct from the other collagenolytic MMPs studied here. MMP-2 has been observed previously to utilize its fibronectin-like repeats rather than its HPX domain for efficient collagenolysis (8, 54, 55). Of particular importance to the present study is that the origin of interactions for the C-terminal extension is within the MMP-2 fibronectin-like repeats rather than the HPX domain (17).

**Summarizing Individual MMP Trends**—Each MMP was distinct when considering the behaviors of N-terminal and C-terminal sequence insertions. For example, the inserted N-terminal residues increased $K_m$ and increased $k_{cat}$ for MMP-1, whereas inserted C-terminal residues significantly increased $k_{cat}$ for this enzyme. The exact opposite trends were observed for MMP-13; although additionally, the inserted C-terminal residues enhanced $K_m$. The N-terminal insertion had no notable effect on MMP-2, whereas the C-terminal insertion enhanced $K_m$ and decreased $k_{cat}$ for this enzyme. MMP-8 $K_m$ was increased by inserted C-terminal residues, but $k_{cat}$ for this enzyme was enhanced by both the N- and C-terminal insertions. MMP-14 $K_m$ was decreased by inserted C-terminal residues, whereas $k_{cat}$ for this enzyme was enhanced by the N-terminal and decreased by the C-terminal insertions. These differences could be exploited for the future design of selective, triple-helical peptide substrates and inhibitors (17, 18, 56, 57).

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