The Role of Collagen Charge Clusters in the Modulation of Matrix Metalloproteinase Activity

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Members of the matrix metalloproteinase (MMP) family selectively cleave collagens in vivo. Several substrate structural features that direct MMP collagenolysis have been identified. The present study evaluated the role of charged residue clusters in the regulation of MMP collagenolysis. A series of 10 triple-helical peptide (THP) substrates were constructed in which Gly-Pro-helical peptide (THP) substrates were replaced with Gly-Asp-Lys or Lys-Gly-Asp motifs. The kinetic parameters for substrate hydrolysis by six MMPs were analyzed, and significant differences were observed. THPs containing the two different motifs were analyzed, and kinetic parameters for substrate hydrolysis by six MMPs were determined. A general trend for virtually all enzymes was that, as Gly-Asp-Lys motifs were moved from the extreme N and C termini to the interior next to the cleavage site sequence, the magnitude of the difference in substrate cleavage was considerably less compared with the Gly-Asp-Lys series. Of the MMPs tested, MMP-2 and MMP-9 most greatly favored the presence of charged residues. The lack of Gly-Asp-Lys clusters in native collagens may diminish potential MMP-2 and MMP-9 collagenolytic activity.

Background: The role of charged clusters in modulating MMP hydrolysis of collagen is unknown.

Results: Triple-helical peptides containing charged motifs were as good or better substrates than the parent (non-charge-clustered) peptide.

Conclusion: MMP-2 and MMP-9 greatly favored the presence of charged residues.

Significance: The lack of Gly-Asp-Lys clusters in native collagens may diminish potential MMP-2 and MMP-9 collagenolytic activity.

The hydrolysis of collagen (collagenolysis) is regulated on multiple levels. Features of the substrate, including its primary, supersecondary, and quaternary structure, all contribute to the efficiency at which collagen is catabolized. For example, the triple-helical supersecondary structure of collagen, which stems from its repeating Gly-Xaa-Yaa primary structure, is present. Collagen quaternary structures, such as fibrils, offer more resistance to proteolytic attack than isolated triple helices (2–4).

Several members of the matrix metalloproteinase (MMP) family have been recognized for their collagenolytic activity. MMPs are known to catalyze the hydrolysis of interstitial (type I–III) collagens at primarily a single site, either a Gly-Ile or Gly-Leu bond, producing 3/4- and 1/4-length collagen fragments (1, 5). Features of the MMP cleavage site in interstitial collagens have been investigated through a variety of approaches, including (α) kinetic analyses of MMP hydrolysis of native and mutant collagens and triple-helical peptide (THP) models of collagen, (b) biophysical studies (NMR spectroscopy and x-ray crystallography) of THPs, and (c) molecular dynamics simulations of triple helices (6–30). It has been noted that, although collagen has a significant distribution of charged residues, relatively few are found at the site of MMP hydrolysis (5, 31). More specifically, the overall 25-amino acid residue region surrounding the site of hydrolysis (subsites P3(1)–P3(2)2) contains a maximum of two charged residues (Glu in the P9 subsite of the α1(II) chain, Arg in the P9′ subsite of the α1(I) and α1(II) chains, and Arg in the P9′′ subsite of the α1(III) chain). Sequence specificity studies show variable tolerance of charged residues by collagenolytic MMPs. Arg in the P9′ and P9′′ subsites and His in the P9′ and P9′′ subsites are favored, but any charged residue in the P9′′ subsite is strongly disfavored (17, 32, 33).

THP Studies suggest that charged residues regulate the stability of triple-helical structure (34). For example, Gly-Asp-Lys

The abbreviations used are: MMP, matrix metalloproteinase; Dnp, 2,4-dinitrophenyl; FN II, fibronectin type II; fTHP, fluorogenic THP; Hyp, 4-hydroxyproline; Mca, (7-methoxycoumarin-4-yl)-acetyl; MT, membrane type; THP, triple-helical peptide; RFU, relative fluorescence unit.

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is destabilizing for the triple helix compared with Gly-Pro-Hyp. (Gly-Pro-Hyp)$_3$-Gly-Asp-Lys-(Gly-Pro-Hyp)$_3$-Gly-Gly had a melting temperature ($T_m$) value of 30.9 °C, whereas (Gly-Pro-Hyp)$_3$-Gly-Pro-Hyp-(Gly-Pro-Hyp)$_4$ Gly-Gly had a $T_m$ value of 47.3 °C (35). In contrast, Lys-Gly-Asp offers triple-helix stability comparable with Hyp-Gly-Pro. The triple-helical peptide (Gly-Pro-Hyp)$_3$-Gly-Pro-Lys-Gly-Pro-Hyp-(Gly-Pro-Hyp)$_4$ had a $T_m$ of 47.1 °C, which is comparable with that of (Gly-Pro-Hyp)$_3$-Gly-Pro-Hyp-Gly-Pro-Hyp-(Gly-Pro-Hyp)$_4$ ($T_m$ = 47.3 °C) (36). Lys-Gly-Asp and Lys-Gly-Glu sequences are present in fibrillar collagens more frequently than would be expected (36). Thus, the greater frequency of Lys-Gly-(Asp/Glu)-Lys compared with Gly-(Asp/Glu)-Lys indicates an evolutionary selection of charged residues.

As described above, within the collagen cleavage site, MMPs have little tolerance for charged residues. However, the interaction of collagen with MMPs extends over a significant length of the triple helix as modulation of MMP-1, MMP-8, MMP-13, and MT1-MMP activity was observed in THP substrates spanning subsites P$_{1-3}$P$_{17-19}$ (17, 18, 22, 26). X-ray crystallographic and NMR spectroscopic studies of MMP-1-THP complexes also revealed extended interactions between enzyme and substrate (28, 29). Although it was noted over 3 decades ago that charged residue clusters have a unique distribution near the MMP cleavage site in type I collagen and that this distribution could influence collagenolysis (37), there are no experimental studies that have specifically addressed which charged residue clusters impact collagen hydrolysis when present in regions surrounding the cleavage site. The present study used Gly-Asp-Lys or Lys-Gly-Asp motifs at various distances from the MMP cleavage site to examine the effects of charge clusters on MMP collagenolytic activity. A total of 11 fluorogenic THP (fTHP) substrates were assembled. The stabilities of peptides containing the two different motifs were analyzed, and kinetic parameters for substrate hydrolysis by six MMPs were determined.

**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-NH$_2$) was synthesized by methods described previously (38, 39). Full-length pro-MMP-2 was provided by Dr. Hideaki Nagase. MMP-9 catalytic domain was expressed as described previously (40, 42). In brief, a 100 μM stock peptide solution was diluted 1:1 12 times. Peptide solution (76 μl) was mixed with 50 μl of 0.1 M Tris, pH 7.5, and diluted to 20–100 nM in ice-cold Tris salt buffer and equilibrated at 4 °C. The solvent phase was decanted.

**Pep tide purity was evaluated using an Agilent 1200 series analytical HPLC system (Santa Clara, CA) equipped with a 150 × 4.6-mm Vydac C$_{18}$ column. Solvent A was 0.1% TFA, H$_2$O; 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 C$_{18}$ column (218TP152022) on a Varian ProStar HPLC system (Agilent). Peak fractions were analyzed via analytical HPLC and reflectance MALDI-TOF mass spectra (Voyager DE-PRO Biospectrometry Workstation, Applied Biosystems, Carlsbad, CA). Peptide standards were cleaved from the resin using thioanisole-water-TFA (1:1:18), precipitated in methyl tert-butyl ether, and dried at −20 °C in amber vials. MALDI-TOF MS analysis indicated a mass of 4593.1 Da for fTHP-15 (theoretical, 4589.0 Da), 4653.0 Da for GDK A (theoretical, 4655.2 Da), 4653.0 Da for GDK B (theoretical, 4655.2 Da), 4655.0 Da for GDK C (theoretical, 4655.2 Da), 4656.0 Da for GDK D (theoretical, 4655.2 Da), 4656.0 Da for GDK E (theoretical, 4655.2 Da), 4677.0 Da for KGD A (theoretical, 4767.3 Da), 4658.6 Da for KGD B (theoretical, 4655.1 Da), 4658.0 Da for KGD C (theoretical, 4655.1 Da), 4655.5 Da for KGD D (theoretical, 4655.1 Da), 4616.0 Da for KGD E (theoretical, 4612.0 Da), 5122.8 Da for α1(III)748–798 KGD (theoretical, 5123.5 Da), and 5165.7 Da for α1(III)748–798 KGD (theoretical, 5157.6 Da).

**Circular Dichroism Spectroscopy**—Peptides were dissolved in Tris salt buffer and equilibrated at 4 °C (pH 7.5) to facilitate triple-helix formation. Peptide concentrations were determined using a Thermo Scientific NanoDrop 1000 (Waltham, MA) via a wavelength scan at λ = 363 nm, ε$_{DNP}$ = 15,900 M$^{-1}$ cm$^{-1}$. Triple-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. Thermal transition curves were obtained by recording the molar ellipticity ([θ]) at λ = 225 nm with temperature increasing by 20 °C/h from 5 to 80 °C. Temperature was controlled by a Jasco PTC-348W1 temperature control unit. The peptide $T_m$ was defined as the inflection point in the transition region (first derivative). The spectra were normalized by designating the highest [θ]$_{225}$ nm as 100% folded and the lowest [θ]$_{225}$ nm as 0% folded.

**MMP Assays**—Enzyme kinetics was determined in a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT) at λ$_{Excitation}$ = 324 nm and λ$_{Emission}$ = 393 nm as described previously (40, 42). In brief, a 100 μM stock peptide solution was diluted 1:1 12 times. Peptide solution (76 μl) was loaded onto a
Nunc 384 white polystyrene well plate (Sigma-Aldrich), the plate was read, and 4 μl of 10X enzyme stock solution was added. The plate was shaken at 25 °C every 30 s, and each well was read every 8 s (for 600 s) to determine initial reaction rates. Plates were stored at ambient temperature (≤24 h) before a final reading. Peptide (25 μM) was analyzed by analytical HPLC to determine the percentage of reaction completeness with 100% cleavage RFU = [(24-h RFU) − (0-h RFU)] × 100/(% peptide cleaved) where RFU is relative fluorescence unit. Enzyme activity was determined as follows: \( V_{corr} \) = [peptide] × (initial rate)/(100% cleavage RFU). Analysis of substrate concentration versus reaction velocity was achieved by Lineweaver-Burke, Hanes-Woolf, and Eadie-Hofstee plots as well as non-linear regression by GraphPad Prism. When the four modes of analysis correlated well, the experiment was considered a success.

RESULTS

Eleven fTHPs were utilized in the present study (Fig. 1 and Table 1). The first (fTHP-15) incorporated a 15-residue consensus sequence from type I–III collagens (subsites P7–P8) and provided for convenient monitoring of triple-helical peptidase activity by collagenolytic MMP family members (16–18, 22). To examine the impact of charged residue clusters on MMP activity, one of two tripeptides, Gly-Asp-Lys or Lys-Gly-Asp, replaced a Gly-Pro-Hyp or Hyp-Gly-Pro tripeptide, respectively, within the Gly-Pro-Hyp repeat regions of fTHP-15 (Fig. 1). For example, in peptide GDK A, the Gly-Pro-Hyp tripeptides at the far N and C termini were replaced by Gly-Asp-Lys (Fig. 1). In peptide GDK B, the Gly-Asp-Lys tripeptides were located 3 residues closer to the cleavage site compared with GDK A (Fig. 1). Ultimately, peptide GDK E possessed Gly-Asp-Lys tripeptides bordering the 15-residue cleavage site (Fig. 1). In a similar fashion, peptide KGD A had the Lys-Gly-Asp tripeptides at the far N and C termini, whereas KGD E had the Lys-Gly-Asp tripeptides bordering the cleavage site (Fig. 1). To accommodate Lys-Gly-Asp while keeping the Yaa-Gly-Xaa repeating motif in the KGD E triple helix, the cleavage site was extended by 1 residue in the N-terminal direction (Hyp in subsite P8) and reduced by 1 residue in the C-terminal direction (Arg was replaced by Lys in subsite P8).

All 11 fTHPs exhibited CD spectra characteristic of triple-helical structures with positive [\( \theta \)] values at \( \lambda = 225 \) nm and strongly negative [\( \theta \)] values at \( \lambda = 195 \) nm (Fig. 2). The magnitudes of the [\( \theta \)]_{225nm} values were similar for all peptides, indicating that the fTHPs had similar fractions of triple-helical content. Monitoring of [\( \theta \)]_{225nm} as a function of temperature resulted in sigmoidal melting curves for all fTHPs (Figs. 3 and 4) indicative of transitions from triple helices to monomeric species (43). The \( T_m \) values for GDK A, GDK B, GDK C, GDK D, and GDK E were 47, 37, 40, 37, and 27 °C, respectively (Table 1). Thus, all of the Gly-Asp-Lys-containing peptides were less thermally stable than the parent peptide, fTHP-15 (\( T_m = 55 \) °C), as expected based on prior studies (44). The stability was affected by the position of the Gly-Asp-Lys triplets. When a Gly-Asp-Lys triplet was substituted for a Gly-Pro-Hyp triplet at the extreme N and C termini (residues 1–3 and 43–45; GDK A), an 8 °C loss in stability was observed. Moving the Gly-Asp-Lys triplet closer to the cleavage site on each side (GDK B, GDK C,}

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**FIGURE 1. Modular structure of fTHPs used in the present study.** The parent, fTHP, contains an MMP cleavage site (denoted in yellow; sequence given in Table 1) in between (Gly-Pro-Hyp), repeats (denoted in blue). For the GDK series of peptides, single Gly-Pro-Hyp repeats were replaced by Gly-Asp-Lys (denoted in red). For the KGD series of peptides, single Hyp-Gly-Pro repeats were replaced by Lys-Gly-Asp (denoted in green). To properly accommodate Lys-Gly-Asp, KGD E required modification of the cleavage site sequence (see Table 1).
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### TABLE 1
Sequences and thermal stabilities of peptides used in this study

| Peptide         | Sequence                                                                 | $T_m$ (°C) |
|-----------------|--------------------------------------------------------------------------|------------|
| Knight SSP      | Lys(Mca)-Pro-Leu-Gly ↔ Leu-Lys(Dnp)-Ala-Arg-NH$_2$                      | NA         |
| fTHP-15         | (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ↔ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_2$-NH$_2$ | 55         |
| GKD A           | Gly-Asp-Lys-(Gly-Pro-Hyp)$_3$↔ Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ↔ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg | 47         |
| GKD B           | Gly-Pro-Hyp-Gly-Asp-Lys(Gly-Pro-Hyp)$_2$↔ Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ↔ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg | 37         |
| GKD C           | Gly-Val-Arg-(Gly-Pro-Hyp)$_3$↔ Gly-Asp-Lys-Gly-Pro-Hyp-NH$_2$            | 40         |
| GKD D           | Gly-Val-Arg-Gly-Pro-Hyp-Gly-Asp-Lys(Gly-Pro-Hyp)$_2$↔ Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ↔ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg | 37         |
| GKD E           | Gly-Val-Arg-Gly-Pro-Hyp-Gly-Asp-Lys(Gly-Pro-Hyp)$_2$↔ Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ↔ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg | 27         |
| KGD A           | Gly-Asp-Lys-(Gly-Pro-Hyp)$_2$↔ Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ↔ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg | 52         |
| KGD B           | Gly-Pro-Lys-Gly-Asp-Hyp(Gly-Pro-Hyp)$_2$↔ Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ↔ Leu-Arg-Gly-Gln-Lys(Dnp) | 51         |
| KGD C           | Gly-Val-Arg-(Gly-Pro-Hyp)$_2$↔ Gly-Pro-Lys-Gly-Asp-Hyp-Gly-Pro-Hyp-NH$_2$ | 51         |
| KGD D           | Gly-Val-Arg-Gly-Pro-Hyp-Gly-Asp-Hyp(Gly-Pro-Hyp)$_2$↔ Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ↔ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg | 51         |
| KGD E           | Gly-Val-Arg-Gly-Pro-Hyp-Gly-Asp-Hyp(Gly-Pro-Hyp)$_2$↔ Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ↔ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg | 51         |
| a1(III)748–798 fTHP | Gly-Val-Arg-Gly-Pro-Hyp-Gly-Asp-Hyp(Gly-Pro-Hyp)$_2$↔ Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ↔ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg | 64         |
| a1(III)748–798 KGD fTHP | Gly-Val-Arg-Gly-Pro-Hyp-Gly-Asp-Hyp(Gly-Pro-Hyp)$_2$↔ Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ↔ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg | 60         |

and GKD D) resulted in a 15–18 °C loss in stability. The greatest loss in stability (28 °C) was observed when Gly-Asp-Lys was substituted for Gly-Pro-Hyp at residues 13–15 and 31–33. This result is consistent with prior predictions in which the greatest perturbation of triple-helical stability occurs when Gly-Pro-Hyp triplets do not surround the substituted site (44).

The $T_m$ values for KGD A, KGD B, KGD C, KGD D, and KGD E were ~52, ~51, ~51, ~51, and ~43 °C, respectively (Table 1). In contrast to the Gly-Asp-Lys series, the majority of the Gly-Gly-Asp-containing peptides were of thermal stability comparable with the parent peptide, fTHP-15 ($T_m = 55 °C$), with the exception of KGD E, which was destabilized by ~12 °C. These results are consistent with prior studies indicating that Lys-Gly-Asp sequences offer stability comparable with Gly-Pro-Hyp (36, 44). The decreased stability for KGD E where Lys-Gly-Asp borders the cleavage site sequence may be due to unfavorable interactions between specific residues. For all 11 peptides, the melting temperatures indicated thermal stabilities suitable for MMP kinetic analyses.

The melts for the KGD THPs, the GKD-E THP, and fTHP-15 (Figs. 3 and 4) have a biphasic appearance. In the case of GKD-E, the high melting, small component ($T_m ~ 59 °C$) is likely from the Gly-Pro-Hyp repeats at the ends. In the cases of fTHP-15, KGD A, KGD B, and GKD C, there is a lower melting, less steep component of the curve ($T_m ~ 25–30 °C$) that might be the central region of the THP, which bears few Pro and Hyp residues (45). Melting in segments suggests that the “all or nothing” behavior (two-state model) attributed to triple-helical melts is oversimplified, which has been reported previously (46). Microunfoldings in THPs has been described (47, 48). The lower melting components could allow greater accessibility to the MMP than the more tightly wound triplets (see later discussion). Although a single $T_m$ may be an oversimplification of some of the curves presented herein, it nonetheless allows for the evaluation of relative stability and appropriate use of the substrates based on their stabilities.

Six MMPs were examined in the present study. Two of these (MMP-8 and MMP-13) are considered “classic collagenases” (5, 49–52), whereas MT1-MMP is a cell surface-bound MMP well recognized for its collagenolytic activity (53). Although also considered a classic collagenase, results with MMP-1 were not included in the present study as MMP-1 exhibited very low activity toward the GKD fTHPs and inconsistent behavior with the GKD fTHPs (data not shown). MMP-1 appears to be particularly sensitive to substrate charge residue clusters. Two of the other MMPs included here are the gelatinase members of the family (MMP-2 and MMP-9) shown previously to cleave interstitial collagens (54–56). The sixth enzyme, MMP-12, has been reported to hydrolyze type I and III collagens (57). The hydrolysis of fTHP-15 by each of these MMPs with the exception of MMP-12 has been reported previously (16–18, 22, 40) and occurs at the Gly $\downarrow$ Leu bond. MMP-12 hydrolysis of fTHP-15 was considerably slower than for other MMPs (Table 2) and much slower than for MMP-12 hydrolysis of an fTHP derived from type V collagen (58). The MMP-12 used herein lacks a C-terminal hemopexin-like domain, which enhances MMP-12 catalytic activity for the type V collagen fTHP (59), and fTHP-15 serves as a well established point of reference for all of the MMPs.

For each MMP and fTHP, $k_{cat}/K_m$ values were determined (Table 2). Significant changes were observed in $k_{cat} / K_m$ values in response to insertion of charge clusters. A general trend for virtually all enzymes was that, as Gly-Asp-Lys triplets were moved from the extreme N and C termini (GKD A) to the interior closer to the collagen model sequence (GKD D or GKD E), $k_{cat}/K_m$ values increased (Table 2). Additionally, all GKD pep-
tides were as good or better substrates than the parent, fTHP-15. Of particular interest was the trend for the GDK B, GDK C, and GDK D peptides. The thermal stabilities of these peptides were similar (Table 1), so the increase of $k_{cat}/K_m$ going from GDK B to GDK C to GDK D was due to the position of the charged residues, not triple-helix stability.

Data for hydrolysis of the Lys-Gly-Asp series was considerably different from data for the Gly-Asp-Lys series (Table 2). First, the highest $k_{cat}/K_m$ values were observed when the Lys-Gly-Asp triplet was located in the middle of the Gly-Pro-Hyp repeats (KGD C) (Table 2), whereas for the Gly-Asp-Lys series, the highest $k_{cat}/K_m$ values were observed when Gly-Asp-Lys triplets were closest to the site of hydrolysis (GDK E) (Table 2). Second, the increase in $k_{cat}/K_m$ values for the Lys-Gly-Asp peptides compared with the parent peptide, fTHP-15 (Table 2), was considerably smaller than the increase in $k_{cat}/K_m$ values observed for the Gly-Asp-Lys series compared with fTHP-15 (Table 2). For example, KGD C had 4.5 times the activity of fTHP-15 for MMP-2, whereas GDK E had 9.7 times the activity of fTHP-15 for the same enzyme (Table 2). MT1-MMP exhibited 1.1 times the activity for KGD C compared with fTHP-15 but had 5.2 times the activity for GDK E compared with fTHP-15.

Individual kinetic parameters ($K_m$ and $k_{cat}$) were next examined to determine the origins of effects on overall MMP activities (Tables 3 and 4). Effects were very MMP-dependent, but some general trends emerged. For the gelatinases (MMP-2 and MMP-9), improved activity in the Gly-Asp-Lys series was due to both improved $K_m$ and $k_{cat}$ values (Table 3). In contrast, for MMP-8, MMP-13, and MT1-MMP, improved activity in the Gly-Asp-Lys series was due to increased $k_{cat}$ values as $K_m$ values were worse as Gly-Asp-Lys motifs moved closer to the cleavage site region (Table 3).

Lys-Gly-Asp motifs primarily enhanced $k_{cat}$ with little effect on $K_m$ for MMP-2 and MMP-9 (Table 4). Lys-Gly-Asp motifs resulted in worse $K_m$ values and much better $k_{cat}$ values for MMP-12, whereas for MMP-13, $K_m$ values became worse and $k_{cat}$ values became better as the Lys-Gly-Asp motifs were closer to the cleavage sequence (Table 4). The trade-off of $K_m$ and $k_{cat}$ values for MMP-8 and MT1-MMP was not particularly significant (Table 4).

**FIGURE 2.** CD spectra of fTHP-15 (black), GDK A (red), GDK B (yellow), GDK C (green), GDK D (blue), and GDK E (purple) (top) and fTHP-15 (black), KGD A (red), KGD B (yellow), KGD C (green), KGD D (blue), and KGD E (purple) (bottom). Scans were taken from $\lambda = 200–250$ nm.
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To examine the effects of a charge cluster that occurs in native collagen, α1(III)748−798 fTHP and α1(III)748−798 KGD fTHP were synthesized (Table 1). The α1(III)748−798 KGD fTHP incorporates the Lys-Gly-Asp sequence found at residues 750−752 in native type III collagen (Table 5). The sequence of fTHP-15 was extended in the N-terminal direction with Gly-Pro-Hyp repeats to allow for the incorporation of Lys-Gly-Asp. α1(III)748−798 fTHP and α1(III)748−798 KGD fTHP formed triple helices of high thermal stability ($T_m = 63.8$ °C and 60.4 °C, respectively), which was not surprising based on their length and high composition of triple helix-stabilizing motifs (Gly-Pro-Hyp and Lys-Gly-Asp).

Hydrolysis of α1(III)748−798 fTHP and α1(III)748−798 KGD fTHP was first examined with MMP-8. $k_{cat}/K_m$ values were 19,334 and 17,767 s$^{-1}$ M$^{-1}$, respectively. Thus, the presence of the Lys-Gly-Asp triplet had little effect on MMP-8 activity. Hydrolysis of α1(III)748−798 fTHP and α1(III)748−798 KGD fTHP was then examined with MMP-9. $k_{cat}/K_m$ values were 37,928 and 54,538 s$^{-1}$ M$^{-1}$, respectively. In contrast to MMP-8, the presence of Lys-Gly-Asp increased MMP-9 activity 1.4-fold.

**DISCUSSION**

Collagenolytic MMPs catalyze the hydrolysis of interstitial (types I, II, and III) collagen at a single site possessing a Gly ↓ Ile/Leu)-(Ala/Leu) motif (1). Several features of the cleavage site have been shown to contribute to MMP specificity, including the distribution of secondary amino acids (Pro and Hyp), dynamics of the peptide backbone, and overall lack of charged residues (with the primary exception of the P$_5$ and P$_9$ subsites) (1, 5, 24, 31). The interaction of MMPs with triple helices encompasses at least the P$_{13}$–P$_{17}$ subsites (17, 32, 33). The interaction of MMPs with triple helices with charged residues (with the primary exception of the P$_5$ and P$_9$ subsites) (1, 5, 24, 31). The interaction of MMPs with triple helices encompasses at least the P$_{13}$–P$_{17}$ subsites (26, 27, 29, 60). As interstitial collagens contain a significant number of charge clusters, the present study addressed the possible role of these charge clusters in regulating MMP activity. The substrate design featured clusters placed outside of the immediate cleavage site as (a) the cleavage site region in native collagens contain few charge residues and (b) prior studies have examined the effects of charged residues on MMP catalysis when inserted within the P$_{13}$–P$_{17}$ subsites (17, 32, 33).

The two motifs utilized here, Gly-Asp-Lys and Lys-Gly-Asp, have very different effects on the thermal stability of triple helices. The forces contributing to the lack of stabilization of triple helices by Gly-Asp-Lys and the stabilization of triple helices by Lys-Gly-Asp have been clarified. NMR spectroscopic studies have noted that axial and lateral interactions occur between charged residues with the axial interactions being the most stabilizing (34). Gly-Asp-Lys-type THPs possess two lateral salt bridges that can be formed between residues in the $i + 1$, $i$ positions in the leading and middle chains and middle and lagging chains (Lys → Asp) (Fig. 5) (34, 36). Although axial salt bridges are possible between residues in the $i$, $i + 1$ positions in the leading and middle chains and middle and lagging chains (Asp → Lys) (Fig. 5), they are not likely (36). A salt bridge is not possible between $i + 1$, $i$ (Lys → Asp) in the leading and lagging chains (Fig. 5) as the residues are too far apart (34, 36). The lateral Asp → Lys interactions expected in the Gly-Asp-Lys THPs evidently stabilize weakly compared with either Gly-Pro-Hyp triplets or Lys-Gly-Asp triplets.

Conversely, Lys-Gly-Asp-type THPs exhibit two axial salt bridges according to results of Hartgerink and co-workers (34). The first bridge has Lys in the $i$ position in the leading chain and Asp in the $i + 2$ position in the middle chain, whereas the second bridge repeats this pattern between the middle and trailing chains (Fig. 5) (34, 61). These THPs should also exhibit lateral contact between the lagging chain and the leading chain (34).
Table 2: Activities of MMPs toward Gly-Asp-Lys and Lys-Gly-Asp triple-helical substrates

| Peptide | MMP-2 | MMP-8 | MMP-9 | MMP-12 | MMP-13 | MT1-MMP |
|---------|-------|-------|-------|--------|--------|---------|
|         | $k_{cat}/K_m$ | Relative activity | $k_{cat}/K_m$ | Relative activity | $k_{cat}/K_m$ | Relative activity | $k_{cat}/K_m$ | Relative activity | $k_{cat}/K_m$ | Relative activity | $k_{cat}/K_m$ | Relative activity |
| fTHP-15 | 120,423 | 100 | 21,753 | 100 | 69,771 | 100 | 1,953 | 100 | 31,039 | 100 | 20,784 | 100 |
| GKD A   | 262,004 | 220 | 803,827 | 1150 | 5,791 | 300 | 34,067 | 110 | 19,779 | 100 |
| GKD B   | 314,950 | 220 | 1,171,153 | 1680 | 55,973 | 180 | 33,502 | 160 |
| GKD C   | 728,309 | 420 | 1,948,364 | 2790 | 106,861 | 340 | 71,005 | 340 |
| GKD D   | 986,155 | 600 | 2,443,634 | 3500 | 131,115 | 430 | 108,043 | 520 |
| GKD E   | 1,162,216 | 970 | 1,982,689 | 2840 | 211,231 | 680 | 107,577 | 520 |
| KGD A   | 337,988 | 280 | 511,077 | 730 | 4,467 | 230 | 54,890 | 180 | 24,077 | 116 |
| KGD B   | 324,215 | 270 | 994,009 | 1430 | 4,846 | 250 | 59,460 | 190 | 23,486 | 113 |
| KGD C   | 543,854 | 450 | 1,174,706 | 1680 | 7,827 | 410 | 98,946 | 320 | 23,012 | 111 |
| KGD D   | 227,927 | 190 | 588,329 | 840 | 4,077 | 210 | 48,172 | 160 | 24,300 | 117 |
| KGD E   | 279,035 | 230 | 729,451 | 1050 | 5,258 | 280 | 68,652 | 220 | 23,260 | 112 |

* Relative activity (%) compared with fTHP-15.

Table 3: Kinetic parameters for hydrolysis of Gly-Asp-Lys triple-helical substrates by MMPs

| Peptide | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ |
|---------|-------|----------|-------|----------|-------|----------|-------|----------|-------|----------|
| fTHP-15 | 5.4 ± 0.97 | 0.654 | 23.0 ± 9.4 | 0.500 | 6.4 ± 0.79 | 0.449 | 56.6 ± 0.50 | 0.111 | 21.8 ± 2.2 | 0.677 | 13.1 ± 1.6 | 0.300 |
| GKD A   | 5.7 ± 0.79 | 1.485 | 19.4 ± 0.75 | 0.559 | 2.9 ± 0.52 | 2.363 | 26.3 ± 2.2 | 0.153 | 6.2 ± 0.67 | 0.330 | 7.9 ± 0.86 | 0.305 |
| GKD B   | 8.5 ± 0.60 | 1.424 | 25.1 ± 1.1 | 1.180 | 2.3 ± 0.14 | 2.790 | 72.8 ± 6.3 | 0.554 | 9.5 ± 0.67 | 0.330 | 7.9 ± 0.86 | 0.305 |
| GKD C   | 3.3 ± 0.35 | 2.374 | 18.6 ± 0.74 | 1.681 | 1.3 ± 0.22 | 2.621 | 57.4 ± 2.4 | 0.892 | 11.4 ± 0.59 | 1.213 | 8.3 ± 0.86 | 0.589 |
| GKD D   | 2.7 ± 0.62 | 2.635 | 44.4 ± 2.4 | 5.758 | 1.7 ± 0.21 | 4.071 | 19.9 ± 3.4 | 0.361 | 25.6 ± 2.16 | 3.402 | 9.5 ± 0.29 | 1.026 |
| GKD E   | 2.4 ± 0.42 | 2.804 | 58.5 ± 6.7 | 8.233 | 1.4 ± 0.15 | 2.679 | 32.1 ± 4.3 | 1.047 | 19.9 ± 2.0 | 4.197 | 12.0 ± 0.97 | 1.291 |

Table 4: Kinetic parameters for hydrolysis of Lys-Gly-Asp triple-helical substrates by MMPs

The lateral interaction shows several possible hydrogen bonds and salt bridges (34). In view of the lower stability of Gly-Asp-Lys THPs and the structural data (34), the lateral interactions probably contribute little to triple-helix stability, and the two axial interactions $(i, i + 2)$ are why Lys-Gly-Asp motifs are stabilizing for triple helices (34). In a similar fashion, Lys-Gly-Glu motifs are stabilizing, whereas Gly-Glu-Lys motifs are destabilizing (36, 62).

As expected, THP substrates where Gly-Asp-Lys motifs replaced Gly-Pro-Hyp repeats (the GKD series) were less stable than the parent THP (fTHP-15), whereas THPs with Lys-Gly-Asp motifs (the KGD series) had stability comparable with fTHP-15 (Table 1). In general, GDK A–E were better MMP substrates than KGD A–E (Table 2). Of the MMPs tested, MMP-2 and MMP-9 most greatly favored the presence of charged residues with preference for the GKD series. This preference for the GKD series could be due to the destabilization of the triple helix, sequence specificity that favors Gly-Asp-Lys over Lys-Gly-Asp, or both effects. The destabilizing Gly-Asp-Lys substitutions likely promote micromelting of the triple helix that exposes single strands for favorable binding by MMP-2 and MMP-9, which are known to utilize their fibronec-
The position of Lys-Gly-Asp or Gly-Asp-Lys clusters in the sequence influenced the MMP activity toward the substrate. For example, moving the Gly-Asp-Lys triplets closer to the cleavage site increased $k_{cat}/K_m$ values. As described above, the improved activity could be due to destabilized triple helices or sequence-specific preferences for charged residues. The present study suggests some sequence specificity as GDK B, GDK C, and GDK D have similar stabilities (Table 1), but GDK D or GDK E is better for all MMPs tested (Table 2). Comparison with the KGD series indicates that improvement in activity is dependent upon both the location and nature of the cluster. For example, KGD C is the best substrate in the KGD series for the MMPs tested except for MT1-MMP (Table 2). The most favored position for Lys-Gly-Asp motifs is not as close to the cleavage site as Gly-Asp-Lys motifs. Improvements in activity, regardless of the charged motif, were manifested in $k_{cat}$ values.

The MMP activity correlated with the destabilization by the Gly-Asp-Lys triplet, which increased with proximity to the central sequence having fewer Pro/Hyp residues. The activity of MMP-8, MMP-12, MMP-13, or MT1-MMP grew 5–6-fold in the progression from GDK-A through GDK-E (Table 2). This is consistent with the hypothesis of localized melting of sites (44, 71, 72) susceptible to proteolysis with collagenolytic MMPs, which has been suggested by molecular dynamics simulations of THPs that manifest localized separation of a chain from the triple helix with disruption of a few interchain hydrogen bonds around the scissile bond (14, 21, 73). The five triplets encompassing the scissile bond (in this study) could become more likely to melt and release a chain the closer the Gly-Asp-Lys triplet is placed. Conversely, the Lys-Gly-Asp motif, being much less destabilizing to the triple helix (Fig. 4), might account for the smaller effects of the position of the Lys-Gly-Asp triplet on catalytic efficiency, which are limited to less than 2.2-fold (Table 2). Thus, destabilization of the triple helix by Gly-Asp-Lys motifs could be a dominant contributor to triple-helical peptidase activity but a much smaller issue with the relatively stable Lys-Gly-Asp motifs.

Lys-Gly-(Asp/Glu) motifs are more common than Gly-(Asp/Glu)-Lys motifs in interstitial collagens (50 versus 19) (Fig. 6 and Table 5). A comparison of potential MMP cleavage site sequences in interstitial collagens, based on the Gly ↓ (Leu/Ile)- (Leu/Ala) motif (Table 6), with charge clustering (Table 5) reveals that Lys-Gly-(Asp/Glu) motifs are found closer to potential cleavage sites than Gly-(Asp/Glu)-Lys motifs (Fig. 6). For example, in homotrimeric (type II and III) collagens, Lys-Gly-(Asp/Glu) motifs are found within 30 residues or less of five potential MMP cleavage sites (Fig. 6). In contrast, Gly-(Asp/Glu)-Lys motifs are never found closer to potential cleavage sites then Gly-(Asp/Glu)-Lys motifs (Fig. 6).

For both MMP-2 and MMP-9, the Gly-Asp-Lys and Lys-Gly-Asp residues mostly likely interact with the FN II modules found in the catalytic domain. For example, the distance between Arg$^{307}$–Asp$^{323}$ of MMP-9 FN II module 2 (residues identified as important for gelatin binding (65)) and the enzyme active site is 40–45 Å. Modeling of MMP-2 revealed a 70-Å distance between the enzyme active site and the most distal collagen interactions with FN II module 3 (66). All three MMP-2 FN II modules contribute to collagen binding with the greatest effects observed for modules 2 and 3 (and specifically Arg$^{368}$ in module 3 and Phe$^{297}$ in module 2) (67–69). The FN II inserts would interact with residues on the prime side of the scissile bond or zinc-binding group (70). Based on the length of a triple helix being 0.286 nm/residue, the Gly-Asp-Lys triplets would range from 26 Å (for GDK E) to 60 Å (for GDK A) from the site of hydrolysis. 
Lys-Gly-Asp motif appears advantageous over the Gly-Asp-Lys motif not only for keeping the appropriate collagen stability but also by preventing unwanted MMP hydrolysis. More specifically, the lack of Gly-Asp-Lys clusters may diminish potential MMP-2 and MMP-9 activity. Although the /H92512(I) chain is the one case where Gly-(Asp/Glu)-Lys motifs are closer to potential cleavage sites than Lys-Gly-(Asp/Glu) motifs, there is only one copy of this chain in type I collagen, and the analogous potential cleavage sites are not found in the /H92511(I) chain.

To test whether charge clusters could influence MMP activity over the distances noted above in native collagens, two fTHPs were synthesized that modeled the 24-residue span between the Lys-Gly-Asp cluster observed at positions 750–752 in type III collagen and the MMP cleavage site at positions 775–776. MMP-8 hydrolysis was not affected by the presence of Lys-Gly-Asp in similar fashion to what was generally observed with the KGD series of peptides (Table 2). MMP-9 activity was enhanced by the presence of Lys-Gly-Asp in similar fashion to the KGD series (Table 2). Thus, the presence of Lys-Gly-Asp, even at a significant distance from the MMP cleavage site found in native collagens, modulated MMP-9 hydrolysis of triple helices.

MMP-12 behaves in a fashion similar to MMP-2 and MMP-9 in terms of regulation of activity by charge clustering (Table 2). However, MMP-12 cannot have the same extended interactions with substrate as MMP-2 and MMP-9 because MMP-12 does not possess the fibronectin type II inserts that MMP-2 and MMP-9 utilize to bind collagens (1). Prior studies showed that the MMP-12 catalytic domain possesses collagenolytic activity (57), and this domain provides contacts outside of the active site cleft (exosites) that participate in collagenolysis (58, 74). As one of the proposed contacts involves Lys241 of MMP-12, its interactions with charge clusters might differ from those of the collagenases (MMP-1, MMP-8, MMP-13, and MT1-MMP) and gelatinases (MMP-2 and MMP-9).

The present study indicates that MMPs have extensive long range interactions with collagenous substrates. Based on the positioning of the charged motifs, modulation of MMP activities spans the P23–P23 subsites. Collagenolytic MMPs have been shown to exhibit a range of conformations in solution, including those in which an “open” form of the enzyme (where the overall radius of gyration ($R_g$) is 28.5–29.0 Å) interacts with the triple helix (28, 60, 75). The predominant form of MMP-1 in solution is an open conformation positioned to interact with collagen (76). Thus, extensive interactions occur between an open MMP conformation and triple-helical residues.

Recombinant bacterial collagen has been utilized to examine the extent of MMP-1 interaction with the cleavage site in interstitial collagens (27). An extended cleavage site was found to participate in MMP-1 collagenolysis, consistent with prior

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**TABLE 6**

Potential MMP cleavage sites in interstitial collagens

| Cluster   | Location          |
|-----------|-------------------|
| Gly-Ile-Ala | a1(I)226–228, a1(II)226–228, a1(I)259–261, a1(I)559–561, a1(III)616–618, a1(I)775–777, a1(III)775–777, a2(II)808–810, a2(II)943–945 |
| Gly-Leu-Ala | a1(II)322–324, a1(III)337–339, a2(II)424–426, a1(III)514–516, a1(II)775–777, a1(III)784–786, a1(I)826–828, a2(II)829–831 |
| Gly-Leu-Leu | a2(I)775–777      |
| Gly-Ile-Leu | a2(I)781–783      |

* Numbering begins at the N terminus of the triple-helical region of each collagen. Bold indicates actual cleavage sites.
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observations based on fTHP substrates (26). The bacterial collagen was stabilized by charge clusters (as Pro was not modified to Hyp), and as presented herein, charge clustering can also modulate MMP activity.

MMP substrates have been developed for in vivo analysis of MMP activity (77, 78). One of the key considerations for these probes is the selectivity of the substrate (77, 78). Our group has previously described a THP fluorescence resonance energy transfer substrate that is selective for MMP-2, MMP-9, and MMP-12 (58, 79). This substrate has subsequently been utilized for optical imaging of MMP-2/MMP-9 activity in vivo (80). Modification of the imaging agent by substitution of Lys-Gly-Asp sequences as exemplified by KGDC could further enhance its activity and selectivity while not sacrificing thermal stability of the triple helix. In general, the Lys-Gly-Asp motif could be used to improve solubility of triple-helical peptide probes while still offering the stability of Hyp-Gly-Pro sequences.

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