Matrix metalloproteinase (MMP-12) (or metalloelastase) efficiently hydrolyzed the gelatinase-selective α1(V)436–447 fluorescent triple helical peptide (THP) when the substrate was submicromolar. The sequence of this THP was derived from collagen V, a component of collagen I fibrils. The hemopexin domains of MMP-12 and -9 each increased $k_{cat}/K_m$ toward this substrate by decreasing $K_m$, just as the hemopexin domain of MMP-1 enhances its triple helical peptidase activity. Non-fluorescent α1(V) THP subtly perturbed amide NMR chemical shifts of MMP-12 not only in the active site cleft but also at remote sites of the β-sheet and adjoining loops. The α1(V) THP protected MMP-12 from the NMR line broadening effects of Gd-EDTA in the active site cleft and more dramatically in the V-B loop next to the primed subsites. Mutagenesis of the exosite in the V–B loop at Thr-205 and His-206 that vary among MMP sequences established that this site supports the high specific activity toward α1(V) fluorescent THP without affecting general MMP activity. Surprisingly the α1(V) THP also protected novel surfaces in the S-shaped metal-binding loop and β-strands III and V that together form a pocket on the remote side of the zinc binding site. The patterns of protection suggest bending of the triple helical peptide partly around the catalytic domain to reach novel exosites. Partial unwinding or underwinding of the triple helix could accomplish this to facilitate its hydrolysis.

Collagens are principal constituents of connective tissues. They are hydrolyzed during development, wound repair, and remodeling of the extracellular matrix. Dysregulated collagenolysis is active in inflammatory diseases such as atherosclerosis, cancer, rheumatoid arthritis, periodontitis, and liver and kidney pathologies. Matrix metalloproteinases (MMPs) play key roles in these processes (1–3). Mutagenesis has established the importance of the V–B loop (joining β-strand V and helix B) in collagenolysis by MMP-1 (4). Residues at the C-terminal end of this loop in MMP-8 were also implicated in collagenolysis (5) and triple helical peptidase activity (6). Binding of an inactivated MMP-1 to an intact collagen fibril likely unwinds the triple helix at the cleavage site to provide access for an active MMP-1 catalytic domain to hydrolyze individual chains (7). The direction of collagen triple helices at the active site is unknown, but the orientation can be hypothesized to be the same as in linear peptide substrates that run antiparallel to β-strand IV (8, 9). Single chain peptides from the α1 chain of type I/III collagens were soaked into crystals of MMP-12 and MMP-8 catalytic domains, and snapshots of bound hydrolysis products were obtained (8). However, neither crystallography nor NMR data representative of interactions with collagen have been reported because of technical limitations. More generally, structural studies of complexes of enzymes with their substrates have been rare presumably because of catalytic turnover and affinities lower than those for transition state analogues.

Several biologically active, self-assembling triple helical peptide (THP) mimics of collagens have been developed. These THPs have facilitated numerous biochemical and cell biological studies otherwise infeasible with insoluble, intact collagen fibrils. THPs are successful in reproducing the cleavage sites of MMPs in collagens I, II, III, V, and XI and in reproducing rate dependences on intact triple helical structure (10, 11). Fluorescent labeling of THPs has facilitated quantitative comparisons of the triple helical peptidase activities of a variety of MMPs in solution and in cell culture assay (6, 11, 12). The affinities of MMP catalytic domains for triple helices range from 1 to 80 μM (6, 11–13). Collagenolysis involves several MMP functions that include initial nonspecific binding and orientation of collagen fibrils (14, 15), manipulation of collagen fibrils with the C-terminal hemopexin domain (16) and catalytic domain (7), unwinding of triple helices within fibrils, and ensuing hydrolysis of single chains from the melted triple helix (7). THPs are not suitable for modeling the initial binding, orientation, and manipulation of full-length collagen assembled into large, insoluble fibrils (6, 11). They are too small to participate in these higher order events (7). Nonetheless THPs have proven themselves to be outstanding substrates for detailed characterization of the triple helical peptidase activities of MMPs and other metalloproteinases (6, 11, 17, 18). This activity includes localized unwinding of the collagen triple helix and hydrolysis of the exposed single strands (6, 11).
MMP-dependent hydrolysis of collagens is a hallmark of inflamed and vulnerable atheromatous plaques (19–21), which are rich in macrophages (21). Macrophages secrete MMP-12 at sites of inflammation (22–24). MMP-12 has emerged as a drug target for atherosclerosis because specific inhibition of it is expected to stabilize atherosclerotic plaques and prevent ruptures that precipitate heart attack and stroke (23, 25, 26). This protease also exacerbates inflammatory arthritis (27) where collagenolysis is well known. The active form of MMP-12 (also called macrophage metalloelastase) observed in situ consists of its catalytic domain apparently after autolytic loss of the C-terminal hemopexin domain (28). Numerous x-ray and NMR structures of complexes of MMPs with small inhibitors are available (29), including several of MMP-12 (9, 30–35). Attempts at crystallizing an MMP with THP bound may have been stymied by moderate affinities of MMPs for THPs and complexities of the binding modes of the THPs. The solution structure of MMP-12 with the active site vacant but inactivated by the E219A mutation was recently determined for supporting studies of substrate interactions (36). The moderate $K_m$ values of MMPs for THPs suggest that NMR methods accommodate moderate affinities and transient associations (37).

Type I collagen fibrils in most connective tissues are copolymerized with fibrillar type V collagen that is buried within the fibrils and present, for example, at 15–20% in cornea (38–41). Increasing collagen V content decreases the diameter of collagen I fibrils (38). Type V and XI collagens resist collagenases (42) but are sensitive to gelatinases (43–45). The gelatinase MMP-9 cleaves collagen chains $\alpha$1(V) between Gly-439 and Val-440 and the $\alpha$2(V) chain between Gly-445 and Leu-446 (46). The $\alpha1(V)436–447$ fTHP substrate mimics the former cleavage site and is selectively hydrolyzed by the gelatinases MMP-2 and MMP-9. MMP-1, -3, -13, and -14 fail to accommodate moderate affinities and transient associations (37).

Kinetics and Sites of Proteolysis—For accuracy, the concentration $[E_0]$ of intact protease active sites was measured (52) using galdarlin (GM6001) (53). The observed rate $k_{obs}$ was measured from a single progress curve under first order conditions where $[S_0] \ll K_m$ (54, 55). These two measurements then provided the second order rate constant from the relationship $k_{cat}/K_m = k_{obs}/[E_0]$ (54, 55). The preferred way to obtain $k_{cat}$ and $K_m$ was to use a new procedure of globally fitting two or more progress curves, saving time, substrate, and concern about fluorescence attenuation by the inner filter effect (51). The plateau in relative fluorescence units (RFU) reached within the 1st h of the reaction was used to convert between ΔRFU and substrate concentration. In cases where part of the $\alpha1(V) fTHP$ remains unhydrolyzed, the concentration of $\alpha1(V) fTHP$ hydrolyzed and apparent $k_{cat}$ may be overestimated compared with explicit evaluation of total hydrolysis. MMP substrate cleavage sites were established by MALDI-TOF mass spectrometry with an Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer using an α-cyano-4-hydroxycinnamic acid matrix. The substrate used to monitor general metalloproutease activity is FS-6 (Mca-Lys-Pro-Leu-Gly-Leu-Lys(Dnp)-Ala-Arg-NH$_2$), which possesses greater solubility than its predecessor, Knight’s substrate (55).

Samples for NMR Spectroscopy—MMP-12 was expressed with the inactivating alanine substitution of the glutamate general base (9, 56, 57), i.e. E219A. This mutation enables NMR studies of MMP-12 without turnover of substrate, without inhibitor, and without autolysis (36, 58). The lack of structural perturbation by the mutation beyond the side chain itself is evident from comparison of crystal structures (9, 30) and from the minimal and localized chemical shift perturbations of NMR spectra (36). Recombinant E219A-inactivated catalytic domain was prepared as described previously (36, 58, 59). Uniformly $^{15}$N-labeled MMP-12(E219A) was expressed (51) in an Escherichia coli BL21(DE3) RIL host using $^{15}$NH$_4$Cl-based M9 medium supplemented with Celtone (Spectral Stable Isotopes) at 20% (v/v). Samples of $^{15}$N-labeled MMP-12 were prepared for NMR typically at 0.6 mM with 20 mM imidazole at pH 6.6, 10 mM CaCl$_2$, 20 μM ZnCl$_2$, 1 mM sodium azide, and 90% H$_2$O, 10% D$_2$O.

EXPERIMENTAL PROCEDURES

Sample Preparation for Kinetic Analysis—The catalytic domain of human MMP-12 was prepared as a 164-residue construct as described previously (51) and used for kinetic analyses. All kinetic experiments using MMP-12 were performed in an assay buffer of 50 mM Tricine (pH 7.5) containing 100 mM NaCl, 10 mM CaCl$_2$, 0.1 mM ZnCl$_2$, 0.05% Brij-35, and 0.02% sodium azide. $\alpha1(V)436–447$ fTHP, with a C$_6$ (hexanoate) substituent at each N terminus, was first dissolved to 10 mM in high purity, anhydrous DMSO. Stock solutions of $\alpha1(V)436–447$ fTHP were prepared at 210 μM in assay buffer. The working stock of $\alpha1(V)436–447$ fTHP was serially diluted from 20- to 1000-fold further in the assay buffer. DMSO always remained less than 0.1% in the assay itself. The wt MMP-12 catalytic domain was kept as a storage stock solution of 9.59 μM in 50% glycerol, frozen at −80 °C. The working stock solution of 500 nM wt MMP-12 catalytic domain was prepared in assay buffer just prior to analysis. Assays typically used wt MMP-12 at 100 nM. Site-directed mutations were introduced to wt MMP-12 using the QuikChange mutagenesis kit (Stratagene). DNA sequencing confirmed the mutations and the absence of stray mutations elsewhere.
NMR Spectroscopy—NMR spectra and titrations were acquired at 26 °C on a Varian Inova 600-MHz spectrometer equipped with a high sensitivity 5-mm cryogenic HCN triple resonance probe with an actively shielded z-gradient coil. NMRPipe (60) was used to process NMR spectra. Sparky (61) was used to analyze spectra. The fast heteronuclear single quantum correlation approach (62) was used to minimize saturation of the water resonance. Spectral acquisitions used TROSY for its $^{15}$N line sharpening and decreased overlap (63). To recognize the affinity for $\alpha_1(V)436-450$ THP and sites it affects in $^{15}$N-labeled MMP-12(E219A), the perturbations of amide NMR chemical shifts by THP were monitored. For more accurate mapping of the residues in the interface, $\alpha_1(V)$ THP protection of MMP-12(E219A) residues from paramagnetic line broadening by inert EDTA-chelated Gd(III) was monitored (47, 48, 50).

NMR Titrations for Affinity and Perturbations of $\alpha_1(V)$ THP—Stock solutions of $\alpha_1(V)436-450$ THP were dissolved in the NMR buffer, adjusted to pH 6.6, and determined to be 7–8 mM by quantitative amino acid analysis. For identifying amide groups of MMP-12(E219A) that respond to the binding of $\alpha_1(V)436-450$ THP, $^{15}$N TROSY spectra in the absence and in the presence of a 1.5-fold molar excess of $\alpha_1(V)$ THP were compared for changes in amide $^1$H and $^{15}$N chemical shifts. To measure the affinity of the association, a series of $^{15}$N TROSY spectra were collected with progressive additions of this $\alpha_1(V)$ THP to attain molar ratios of $^{15}$N-labeled MMP-12 to the $\alpha_1(V)$ THP of 1:0, 1:0.1, 1:0.2, 1:0.3, 1:0.4, 1:0.6, 1:1, and 1:1.5. The chemical shift changes of affected MMP-12 residues were plotted against total $[\alpha_1(V)\text{THP}]$ and fitted by non-linear regression to the following equation (64, 65) to solve for $K_{\text{fp}}$:

$$\delta_{\text{obs}} - \delta_p = (\delta_{\text{pl}} - \delta_p)(K_d + L_t + P_t) - \frac{(K_d + L_t + P_t)^2 - 4P_t L_t}{2P_t}$$

(Eq. 1)

where $P_t$ and $L_t$ are the total concentrations of the protein $^{15}$N-labeled MMP-12 and $\alpha_1(V)$ THP ligand, respectively. $\delta_{\text{obs}}$ is the observed NMR peak position or chemical shift. $\delta_p$ and $\delta_{\text{pl}}$ are the chemical shifts of the free MMP-12 and the complex. Origin 7.5 (Micralc) was used to globally fit the binding isotherms from the $^1$H peak positions of all affected residues simultaneously.

NMR Mapping of Protection from Paramagnetic Gd-EDTA—TROSY spectra of $^{15}$N-labeled MMP-12(E219A) were adjusted to pH 6.6 before use. TROSY spectra of $^{15}$N-labeled MMP-12(E219A), both in the absence and presence of $\alpha_1(V)436-450$ THP, were collected with additions of 0, 1, 2, and 3 mM Gd-EDTA. The line broadening effect from each addition of Gd-EDTA was quantified by dividing the amide peak heights of each MMP-12 residue by the peak height without the addition. This normalized the NMR peak height with paramagnetic probe present to a scale from 0 (complete broadening and high surface exposure) to 1 (absence of broadening and considerable burial). The normalized peak heights could then be easily compared as the difference between the free state and that bound to $\alpha_1(V)$ THP without complication by differences in sample concentrations or spectral acquisition parameters. The uncertainties in peak heights were estimated as the reciprocal of the signal to noise reported by the Sparky peak picking software (61) and then scaled up by 1.48-fold to represent ± 1 S.D. These uncertainties were incorporated into the normalized peak heights and their differences between bound and free states using the standard rules of error propagation.

Modeling of Complex of MMP-12 with THP—The triple helical peptide was initially represented by the crystal structure of the triple helical peptide from the collagenase cleavage site in collagen III (Protein Data Bank code 1BKV) (66) comprising 30-residue chains. This structure was docked by hand with the NMR structure of ligand-free MMP-12 (Protein Data Bank code 2P0J) (36) in MolMol (67). Using DeepView (68), the individual chains of the THP were rotated, one backbone torsion angle at a time, to bend the chains over surfaces of MMP-12 protected by $\alpha_1(V)436-450$ THP from Gd-EDTA. Each structural figure was rendered with PyMol (69).

RESULTS AND DISCUSSION

Activity of MMP-12 upon THP Mimic of Collagen V—The ability of MMP-12 to hydrolyze THP substrates of other MMPs was investigated. $\alpha_1(V)436-447$ fTHP, derived from the site of MMP-9 cleavage in fibrillar collagen V (11, 46), was efficiently hydrolyzed by MMP-12. (Because the mature form of active MMP-12 observed physiologically is autolytically truncated to the 22-kDa catalytic domain (28), we refer to the catalytic domain as MMP-12.) MMP-12 activity toward $\alpha_1(V)436-447$ fTHP at 30 °C exhibited an apparent $k_{\text{cat}}/K_m$ of 97,600 m$^{-1}$ s$^{-1}$, a $K_m$ of 24.8 µM, and an apparent $k_{\text{cat}}$ of 2.35 s$^{-1}$ (Fig. 1A and Table 1). Progress curves fitted to steady-state kinetics parameters using a new expanded analysis (51) are drawn in Fig. 1B for reactions at 25 °C. Arrhenius plotting of the temperature dependence of the second order rate constant $k_{\text{cat}}/K_m$ (Table 1) suggested an activation barrier of 13.7 kcal/mol (Fig. 1C). This is comparable to the activation energies of collagens with TTHP substrates (70, 71). Fits of steady-state kinetics parameters to progress curves for the digestion of $\alpha_1(V)436-447$ fTHP by full-length MMP-12, -9, or -2 or by MMP-9 catalytic domain are depicted in supplemental Fig. S1. The apparent $k_{\text{cat}}/K_m$ of MMP-12 toward $\alpha_1(V)436-447$ fTHP was 60% of that of the MMP-9 catalytic domain (Table 1). The $k_{\text{cat}}/K_m$ of full-length MMP-12 of 389,500 m$^{-1}$ s$^{-1}$ was 4.0-fold higher than that of the catalytic domain mainly because the $K_m$ dropped almost 3-fold to 8.4 µM, although the apparent $k_{\text{cat}}$ was also enhanced to 3.3 s$^{-1}$ (Table 1). The $k_{\text{cat}}/K_m$ of full-length MMP-12 was 85 and 103% that of full-length MMP-2 and MMP-9, respectively (Table 1). The gelatinases apparently have better affinity for the collagen V-derived THP than does MMP-9, respectively (Table 1).
hydrolysis of triple helical substrates in the cases of MMP-1, -9, and -12 that have been tested.

A lag phase of around 50 s took place before MMP-12 hydrolyzes α1(V)436–447 fTHP with the linear kinetics phase. The lag phase was around 4 s for MMP-9 constructs, and there was almost no lag phase for MMP-2. Each lag phase was removed prior to fitting steady-state kinetics (Table 1, Fig. 1, supplemental Fig. S1, and Fig. S6). The lengths of the lag phases appear to be independent of the concentration of either the MMP or the α1(V) fTHP. The longer lag phase of MMP-12 might reflect slower melting of the triple helix in advance of hydrolysis of the scissile bond. Perhaps interactions with fibronectin-like modules of the gelatinase hasten unwinding of the triple helix.

Non-fluorogenic α1(V)436–450 THP (C6-(GPO)4GPPG-VVGE ↓ QGEQ(GPO)4-NH2 where O represented was 4-hydroxyproline) was hydrolyzed by MMP-12 at the scissile bond indicated by the arrow. MALDI mass spectrometry supported this by way of the agreement of the measured mass of the N-terminal [M + Na]+ ion of 1922.5 Da with the theoretical mass of 1922.1 Da. Both MMP-12 and full-length MMP-12 mainly hydrolyze fluorogenic α1(V)436–447 fTHP at the analogous peptide bond marked by the underlined arrow in the following: C6-(GPO)4GEK(Mca)GPPG ↓ VVG ↓ E↓K(Dnp)GEQ(GPO)4-NH2. The additional arrows indicate linkages susceptible to MMP-2 or MMP-9 cleavage. The Glu ↓ Lys(Dnp) peptide bond appears to be the main site of hydrolysis by MMP-12 because the predominant peaks observed are (i) the N-terminal [M + Na]+ ion at 2665.8 Da coinciding with the theoretical mass of 2665.9 Da and (ii) the C-terminal [M + Na]+ ion of 1983.7 Da coinciding with the theoretical mass of 1984.0 Da. MMP-2 hydrolyzed this same site as evident from the C-terminal [M + Na]+ ion of 1984.8 Da observed. Yet MMP-2 also hydrolyzed the Gly ↓ Glu bond as indicated by the C-terminal fragment ion of 2113.9 Da (2113.1 Da theoretical) as well as the Gly-Val bond as indicated by both the C-terminal fragment ion of 2368.9 Da (2368.5 Da theoretical) and N-terminal fragment ion of 2281.8 Da (2281.5 Da theoretical). MMP-9 cleaved only the Gly ↓ Val bond in α1(V)436–447 fTHP that was equivalent to its cleavage site in type V collagen (46).

MMP-12 hydrolyzed fTHP-4, derived from a consensus of MMP cleavage sites in collagens I, II, and III (1); at 30 °C the apparent second-order rate constant kcat/Km of 3.20 ± 0.2 s−1, and Kcat is 73 ± 3 μM. For comparison, MMP-9 catalytic domain much more rapidly hydrolyzed fTHP-4 with apparent kcat/Km of 6.4 × 105 M−1 s−1.

### Table 1

| Protease | Temperature | kcat/Km | kcat | Km |
|----------|-------------|---------|------|-----|
| MMP-12 cat | 25 | 55,400 ± 4,700 | 1.23 ± 0.10 | 22.2 ± 5.6 |
| MMP-12 cat | 30 | 97,600 ± 3,200 | 2.35 ± 0.2 | 24.8 ± 5.6 |
| MMP-12 cat | 37 | 145,300 ± 1,900 | ND | ND |
| MMP-12 full | 30 | 389,500 ± 500 | 3.27 ± 0.08 | 8.4 ± 0.2 |
| MMP-9 cat | 30 | 156,400 ± 200 | 1.41 ± 0.05 | 9.0 ± 0.4 |
| MMP-9 full | 30 | 378,100 ± 490 | 1.67 ± 0.003 | 4.41 ± 0.01 |
| MMP-2 full | 30 | 459,700 ± 2,500 | 0.98 ± 0.01 | 2.13 ± 0.02 |

* Cat refers to catalytic domain; full refers to full length.
* Derived from individual progress curves at low [substrate] according to Refs. 54 and 55.
* Derived from a few progress curves by the rapid method of Ref. 51.
* Derived from non-linear fit of initial velocities (Fig. 1 A).
* Derived from expedited analysis of progress curves (51) at 25 °C with 10 nM MMP-12 (B).
* ND, not determined.

### FIGURE 1

Analysis of MMP-12 activity toward α1(V)436–447 fTHP. Steady-state kinetics results were obtained from initial velocities at 30 °C with 7.8 nM MMP-12 (A) and using expedited analysis of progress curves (S1) at 25 °C with 10 nM MMP-12 (B). The shorter progress curve in B, marked with triangles, used a different preparation of the α1(V) fTHP. C shows an Arrhenius plot of the temperature dependence. RFU, relative fluorescence units.

### TABLE 1

Kinetic parameters for hydrolysis of α1(V)436–447 fTHP

| Protease | Temperature | kcat/Km | kcat | Km |
|----------|-------------|---------|------|-----|
| MMP-12 cat | 25 | 55,400 ± 4,700 | 1.23 ± 0.10 | 22.2 ± 5.6 |
| MMP-12 cat | 30 | 97,600 ± 3,200 | 2.35 ± 0.2 | 24.8 ± 5.6 |
| MMP-12 cat | 37 | 145,300 ± 1,900 | ND | ND |
| MMP-12 full | 30 | 389,500 ± 500 | 3.27 ± 0.08 | 8.4 ± 0.2 |
| MMP-9 cat | 30 | 156,400 ± 200 | 1.41 ± 0.05 | 9.0 ± 0.4 |
| MMP-9 full | 30 | 378,100 ± 490 | 1.67 ± 0.003 | 4.41 ± 0.01 |
| MMP-2 full | 30 | 459,700 ± 2,500 | 0.98 ± 0.01 | 2.13 ± 0.02 |

* Cat refers to catalytic domain; full refers to full length.
* Derived from individual progress curves at low [substrate] according to Refs. 54 and 55.
* Derived from a few progress curves by the rapid method of Ref. 51.
* Derived from non-linear fit of initial velocities (Fig. 1 A).
* Derived from expedited analysis of progress curves (51) at 25 °C with 10 nM MMP-12 (B).
* ND, not determined.

M. O. Palmier, R. Bhaskaran, and S. R. Van Doren, manuscript in preparation.
Affinity of α(V) THP for MMP-12(E219A) by NMR—The affinity of α(V)436–450 THP for MMP-12(E219A) was investigated using the dependence of amide proton chemical shift $\Delta\omega_{NH}$ of MMP-12(E219A) on $\alpha$-helix THP concentration (Fig. 3). $\Delta\omega_{NH}$ values of 30 ± 6 $\mu$m (Fig. 3). This $K_i$ agrees within uncertainties with the $K_{i}$ values of steady-state turnover (Fig. 1). To further evaluate the significance of the $\alpha$-helix THP binding-induced changes in NMR chemical shifts inside and outside the active site cleft, the surface of MMP-12(E219A) occluded at its interfaces with the THP was probed.

$\alpha$-V THP Protection of MMP-12(E219A) from Paramagnetic Probe—Binding sites on a protein can be mapped accurately using the protection of the binding site by its binding partner from the NMR line broadening effects of an inert paramagnetic probe (47, 48, 74). Protection from the paramagnetic probe avoids the misdirection of chemical shift perturbations occurring at a distance from the binding site or resulting from binding-linked conformational change (47, 75). Protection from the paramagnetic probe is cheaper and easier to implement than cross-saturation for accurate mapping of binding sites (74). Chelated Gd(III) is a most convenient paramagnetic probe that avoids the apparatus needed to use oxygen as the probe. Chelation makes Gd(III) a global probe of biomacromolecular surfaces. The Gd(III) causes strong $^1$H NMR line broadening amount (1.5 molar eq) of the $\alpha$-V THP were measured as the radius of the changes, $\Delta\omega_{NH}$ from $^15$N TROSY spectra (Fig. 2A). The largest chemical shift perturbations from $\alpha$-V THP binding are 1–2 orders of magnitude smaller than the largest perturbations from inhibitor binding (see Ref. 36). This qualitatively correlates with the affinity of $\alpha$-V THP for MMP-12 being at least 3 orders of magnitude weaker than the affinity of inhibitors of MMPs. The residues sensitive to $\alpha$-V THP THP binding span the full breadth of the active site cleft and extend well beyond the active site. A threshold of significant $\Delta\omega_{NH}$ of 0.029 ppm captures several residues encompassing the heart of the active site of MMP-12 (Ala-182, Thr-210, Gly-221, His-222, Leu-224, Gly-225, Tyr-240, and Lys-241) and several residues further from the active site cleft (His-112, Tyr-113, Leu-146, Lys-148, Thr-205, Arg-244, and Asp-254) (Fig. 2). Lowering the threshold of significant $\Delta\omega_{NH}$ to 0.022 ppm adds the following residues around the active site cleft: Gly-106 and Val-108 at the N terminus, Ala-184 and Phe-185 next to sIV, Gly-209 of the V-B loop, Phe-213 of hB, and Val-243 of the S1 specificity loop or B-C loop (Fig. 2). (The nomenclature uses “s” for $\beta$-strand and “h” for $\alpha$-helix (29). Loops are named by the flanking elements of secondary structures.) The residues outside the catalytic cleft with 0.029 ppm > $\Delta\omega_{NH}$ ≥ 0.022 ppm are Ile-114 of sl, Val-161 and Val-162 of sIII, Gly-166 and His-172 of the III-IV loop, Asp-198 of sV, Thr-204 and His-206 of the V-B loop, Ile-245 of the S1 specificity loop, and Ser-260 of hC (Fig. 2). The affected residues prior to sl mark one extreme of the active site cleft and its “unprimed” subsites (Fig. 2B, left). The affected residues of the V-B loop mark the other extreme of the cleft and “primed” subsites (Fig. 2B, right). $\Delta\omega_{NH}$ perturbations from binding of $\alpha$-V THP surprisingly were distributed to all of the secondary structures of MMP-12 except perhaps hA. The cluster of spectral perturbations from binding of $\alpha$-V THP mapped to the N-terminal ends of sl and sII (His-112 to Leu-114 and Leu-147 to Lys-148), distant from the active site (Fig. 2B). This cluster coincides with inhibitor-induced chemical shift changes of His-112 and Lys-147 and an accompanying adjustment in the position of Arg-110 through Tyr-113 (36). Other THP-perturbed NMR peaks also mapped outside the active site to the III-IV loop that binds zinc and calcium, the IV-V loop, sV, and hC. Because inhibitors perturb equivalent remote sites in MMP-12, MMP-1, or MMP-3 (36), the subtle effects of binding of the $\alpha$-V THP substrate could be similarly propagating through the same internal network proposed within MMP catalytic domains (36). The chemical shift perturbations of Val-161 and Val-162 in sIII by $\alpha$-V THP also coincide with the chemical shift perturbations of Leu-160 through Phe-163 by inhibitors that may be related to the internal conforma-
en of groups near the surface. Dividing the NMR peak height with Gd(III) chelate to the line broadening probe according to highest 

\[ I \] 

Asp-131 of hA, Lys-151 of sII, and Ala-252, Asp-253, and Gly-

\[ \text{Thr-239, Tyr-240} \] at the active site cleft as expected (Figs. 4C and 5). (This lower threshold is conservative considering that a number of residues had base-line values of \( I_{\text{mim}} / I_0 \) and \( I_0 \), free < 0, beyond the uncertainties in Figs. 4C.) The negative values are consistent with the greater broadening expected for a complex because its slower tumbling enhances the paramagnetic line broadening (47).) Results with 2 or 3 mM Gd-EDTA probe manifested similar patterns of protection as at 1 mM. Increasing [Gd-EDTA] caused more extensive line broadening deeper within the protein but with the lower signal to noise of the lower peak heights. The data at 2 mM are shown in supplemental Fig. S3. The distance from the active site of the sites most protected by \( \alpha(V) \) THP is surprising and suggestive of exosites and/or nonspecific remote sites of contact.

Mutations That Decrease Specific Activity for \( \alpha(V) \) THP while Preserving General Activity—We sought to identify sequence determinants in the catalytic domain that may contribute to the activity of MMP-12 toward \( \alpha(V) \) THP. NMR

FIGURE 3. Binding isotherms for the association of MMP-12(E219A) with \( \alpha(V) \) THP. The \( ^1H \) component of the amide NMR chemical shift perturbations by binding of \( \alpha(V) \) 436 – 450 THP were measured from \( ^1H \) TROSY spectra and fitted to the ligand saturation expression of Equation 1.

FIGURE 4. Exposure of the backbone of MMP-12(E219A) to line broadening by Gd-EDTA probe in the absence and presence of \( \alpha(V) \) THP. The \( \alpha(V) \) 436 – 450 THP was added to 1.5-fold molar excess. Amide NMR peak heights of MMP-12 with 1 mM Gd-EDTA present, \( I_{\text{mim}} / I_0 \), are normalized by the peak heights without addition of the probe, \( I_0 \). The normalized ratios, \( I_{\text{mim}} / I_0 \), on a scale of 0 to 1, are readily compared between the free (A) and THP-bound states (B) as the difference in C. THP-bound minus free differences, \( I_{\text{mim}} / I_0 \), free < 0.4 are considered large protections from the probe by \( \alpha(V) \) THP. Differences where 0.4 < \( I_{\text{mim}} / I_0 \), free < 0.15 are considered medium-sized protections by \( \alpha(V) \) THP. Cylinders mark the helices, and arrows mark the \( \beta \)-strands.
mating of another protein-protein interface with the same methods had rationally guided our mutagenesis directly and fruitfully to an unexpected hot spot (50) and guided our choice of mutations here as well. Two issues regarding MMP-12-specific activity toward α1(V) THP substrate need to be considered. First, the best characterized central region of the active site cleft is nearly identical in sequence among MMPs with widely disparate rates of turnover of this THP substrate. Second, NMR footprinting protection from the line broadening probe suggested prospective exosites based on the THP-dependent contacts at sites outside the catalytic cleft but in the catalytic domain (Figs. 4 and 5). We decided to mutate the most likely of the prospective exosites selected by three criteria: (i) protection by α1(V) THP from the NMR line broadening probe, (ii) a sequence differentiating MMP-12 from close MMP homologues that are much less active on this THP, and (iii) relative proximity to the active site. Three loci have their backbone protected by α1(V) THP and differ in sequence from the closest MMPs: sII (Leu-162, Ala-164, and Phe-166), the V-B loop (Asp-200, His-206, and Thr-210), and Thr-239 of the B-C or S1 specificity loop. We ignored the latter two threonines located in the active site cleft, the most distant sites of sIII, and Asp-200. We selected for mutagenesis both His-206 (Fig. 5, arrow) and Thr-205 (Fig. 2, arrow) because both lie outside the active site cleft but are nearest among the prospective exosites with distinctive sequence. Because the THP-protected backbone amide group of His-206 lies between the side chains of Thr-205 and His-206, either side chain might contact the THP. If α1(V) THP contacts the Thr-205 side chain, that could account for THP perturbation of the backbone amide chemical shift of Thr-205 (Fig. 2B, arrow). We mutated both residues to their sequence counterparts from the MMP-3 close homologue that is inactive upon the α1(V) THP. The resulting T205K/H206D double mutation retained 88% of wild type activity toward the general MMP substrate FS-6 (Table 2). However, the T205K/H206D mutation was 3.3-fold lower than wt in the second order rate constant kcat/Km for α1(V) THP (Fig. 6 and Table 2). Nearly all of this drop in specific activity was from the 3.15-fold higher Km of the T205K/H206D mutant for α1(V) THP (Table 2). Adding mutations of the Ser-207 and Gly-208 neighbors at the apex of the loop to their MMP-3 counterparts failed to change kcat/Km further. Thus, Thr-205 and His-206 of MMP-12 appear sufficient to form an important exosite in the catalytic domain of MMP-12 for α1(V) THP substrate. This is a subset of the larger chimeric swap of the entire V-B loop of MMP-1 to that of MMP-3 that demonstrated the V-B loop to be essential to collagenolysis by MMP-1 (4). The kcat/Km of MMP-12(T205K/H206D) toward either DQ-collagen I or DQ-collagen IV was 1.8-fold lower than wild type, suggesting this exosite to participate in collagenolysis by MMP-12 as well.

Implications for Packing and Unwinding of Triple Helices—A viable explanation of the protection data and mutagenesis data is that the triple helix of α1(V) THP bends to reach and pack against the V-B loop, III-IV loop, sIII, and sV. The bending would likely be accompanied by underwinding and potentially partial separation of chains of the triple helix, placing at least two of the chains such that they cover the remote protected sites. An alternative explanation of the α1(V) THP protection of
surfaces outside the active site cleft would be additional but non-productive binding modes of rodlike triple helices. The reliance of MMP-12 on His-206 or Thr-205 at the remote exosite in the V-B loop for triple helical peptidase activity (Table 2) supports the bending of the triple helix rather than non-productive contacts with Thr-205/His-206. Thus, a model with bending of the triple helix seems more plausible. The thermal activation barrier of 13.7 kcal/mol that accompanies hydrolysis of α1(V)/436–447 fTHP (Fig. 1C) and the kinetic lag phase for triple helical peptidase activity may be invested in the melting of the triple helix.

Where the GAX repeats of the triple helix are enriched in imino acids, namely Pro at X and hydroxyproline at Y, the triple helix is optimal in stability and structural, cylindrical regularity (66, 76–78). Such enrichment in imino acids is characteristic of the four triplets on the N-terminal side of the scissile bonds of interstitial collagens. The N-terminal side of the cleavage sites feature relatively small side chain volumes, Pro always present at the P3 position, and Pro never present at the P2 position from the cleavage site (79). In contrast, the four triplets on the C-terminal side of the scissile bond are depleted of imino acids in the X and Y positions, loosening or destabilizing the triple helix (79). The molecules in collagen model peptides and collagen fibrils predominantly have helical 7-fold symmetry (80, 81). Depletion of imino acids may cause the triple helix to become locally less tightly wound (66, 80). Suppose that triple helical peptides lie along the catalytic cleft with N to C terminus running from unprimed to primed subsites. Linear peptide substrates are observed in this orientation (8, 9), which is left to right in standard displays such as in Figs. 2B and 5 and supplemental Fig. S4. This orientation would place the stable N-terminal end of the triple helix intact into the wide funnel-shaped cleft of the unprimed subsites at left, accounting for the protections and chemical shift perturbations (Figs. 2 and 5, left). This orientation would place the destabilized C-terminal side of the scissile bond on the primed (right) side of the MMP active site.

If this C-terminal region is destabilized enough to melt the triple helix at least locally, the partly melted chains may be able to “turn the corner” in the V-B loop to reach and protect many sites from Gd-EDTA.

The extensive protection of the V-B loop by α1(V) fTHP strongly suggests that one or more of its chains packs against one or more locations in this loop. For the fTHP to protect the N-terminal end of the V-B loop (Asp-200 through His-206), the fTHP must bend from the active site cleft at a right angle around the C-terminal end of the V-B loop. This proposed right angle turn would be inconsistent with a regular and rodlike triple helix. The protection of the S-shaped III-IV loop of MMP-12 by α1(V) fTHP (Figs. 4 and 5) suggests that a chain of the fTHP may also drape across the III-IV loop. Separation of chains of the fTHP may account for protections occurring on both the V-B and III-IV loops. Most surprising is the protection of a remote pocket formed among residues of the upper lobe of the III-IV loop (Gly-166 and His-168), sIII (Val-162 to Leu-164), sV (Ala-195, His-196, and Phe-197), and even sI (Asn-119) (Fig. 5C). (The wall of this pocket contains the two fTHP-protected histidine ligands of the structural zinc between the III-IV loop and sV.) If the α1(V) fTHP were to extend from the active site and wrap all the way around the III-IV loop, the fTHP might protect this remote pocket directly. Such extensive bending of a chain of the α1(V) fTHP could promote melting of the triple helix. A non-productive binding mode in which some intact triple helix covers this pocket is an alternative hypothesis for the protection observed.

One speculative model that accounts for the protections and orients the fTHP with bending as discussed above is depicted in supplemental Fig. S4. The model illustrates that bending of the triple helix around the catalytic domain could largely account for the protection of its surface by α1(V) fTHP. Such bending would be conducive to separating the strands of the triple helix (supplemental Fig. S4). Like bending of DNA promotes unwinding and may facilitate localized strand separation in initiation of transcription (82), bending of the collagen triple helix about the MMP catalytic domain might also facilitate unwinding and separation of its chains at less stable loci in the triple helix. A path that collagens might traverse from the active site of a gelatinase to the collagen and gelatin binding site identified in fibronectin module 2 of MMP-9 (83, 84) can be hypothesized to bend analogously around the primed end of the catalytic cleft as well as around fibronectin module 2.

**Comparison of Specificity and Collagenolysis of MMP-12 and the Gelatinases—**MMP-12 hydrolyzes collagens IV and V, α1(V) fTHP, gelatin, and elastin (28, 72, 85–87) as do the gelatinases (11, 88–91). Both MMP-12 and MMP-2 hydrolyze DQ-collagens I and IV. Full-length MMP-2, -9, and -12 are remarkably similar in efficiency of turnover of α1(V) fTHP with apparent k<sub>cat</sub>/K<sub>m</sub> spanning a range of only 22% among them (Table 1). The C-terminal hemepxin-like domains of MMP-9 and -12 clearly enhance K<sub>m</sub> and k<sub>cat</sub>/K<sub>m</sub> (Table 1). Stoichiometric binding of inhibited MMP-2 almost completely unwinds collagen I at 37 °C, apparently by way of the fibronectin-like inserts (92). In contrast, the catalytic domain of MMP-12 may be sufficient for partial unwinding of α1(V) fTHP at 26 °C in the absence of the fibronectin-like modules of the gelatinases. The
hydrolysis of α1(V)436–447 fTHP by MMP-12 differs from the gelatinases in having a longer lag phase and a shifted preference in site of cleavage. As the concentration of α1(V)436–447 fTHP is raised through the low micromolar range, the catalysis of MMP-12 results in smaller amplitudes of fluorescence increase than the gelatinases. Perhaps α1(V)436–447 fTHP might form small soluble aggregates that impede and complicate hydrolysis by MMP-12 but not the hydrolysis by gelatinases. Higher order self-association has been reported for some THPs (93). Alternatively MMP-12 might be more sensitive to product inhibition. Thus, in addition to its lack of fibronectin-TMPs (93). Alternatively MMP-12 might be more sensitive to product inhibition. Thus, in addition to its lack of fibronectin similar modules, MMP-12 exhibits altered kinetic behavior compared with gelatinases that may differentiate them in some respects of their mechanisms of unwinding and hydrolysis of the α1(V) THP substrate.

Given the differences, it need not be surprising that MMP-12 prefers to hydrolyze the α1(V) THP at a different site than MMP-9 does. The Glu↓Gln scissile bond preferred by MMP-12 is cleaved by MMP-2 also but is four residues closer to the C terminus than the Gly↓Val linkage attacked by both MMP-9 and MMP-2. The Glu↓Gln site lies in the center of the imino-deficient sequence that is more easily melted at the time of cleavage. The cleavage sites of the gelatinases tend to have Gly at P₄, Pro at P₃, Gly at P₁, Leu at P₁, and Gly at P₄ with frequencies of about 30% and greater according to the MEROPS database (94). This pattern corresponds to the collagen triple repeat sequence of Gly-Pro-Xaa except at P₁ where Leu is much preferred over Pro. Among these preferences, MMP-12 shares only the preferences of Pro at P₃ and Leu at P₁ as do all of the mammalian MMPs characterized (94). The distinctive hydrolysis of the VGE↓Q site in α1(V) THP and its VGE↓K(Dnp) counterpart in fluoroegenic α1(V) THP by MMP-12 is consistent with its known cleavage sites with Glu at P₁ in 16% of cases and Val at P₃ in 12% of cases; these are much higher frequencies than for the gelatinases (94). MMP-12 is more tolerant of bulky groups in P₁. Consequently MMP-12 exerts an order of magnitude higher $k_{cat}/K_m$ for the substrate NFF-2 with bulky norvaline in P₁ (95) than either of the gelatinases (96). Tolerance of bulky groups at P₁ (87) by MMP-12 may accommodate the Lys(Dnp) at P₁ of α1(V)436–447 fTHP.

Summary—MMP-12 joins the gelatinases in actively digesting the triple helical peptide derived from the collagen V site that they cleave (Fig. 1 and Table 1). The C-terminal domains of MMP-9 and -12 increase their second order rate constants ($k_{cat}/K_m$) toward this substrate by improving $K_m$ values. This implies that the C-terminal domains may contribute favorable contacts to this THP substrate or guide the binding of α1(V) THP. The hypothesis of bending and partial unwinding of the imino-depleted segment of α1(V)436–450 THP could account for the novel and complex distribution of MMP-12 surfaces it protects and for the effect of the T205K/H206D lesion. The exosite at Thr-205 and His-206 in the V-B loop is necessary for the full specific activity of MMP-12 for α1(V)436–447 fTHP but not for a general substrate of MMPs. This corroborates the need of MMP-1 for the V-B loop for collagenolysis (4) and pinpoints an exosite to the N-terminal end of the V-B loop. NMR footprinting identified prospective exosites in the catalytic domain that contact α1(V)436–450 THP. The novel exosites principally appear in the V-B loop, the S-shaped III-IV loop that binds zinc and calcium, and a pocket it forms together with β-strands sIII and sV (Fig. 5). These surfaces of MMPs may merit investigation as potential exosites for collagen interaction in addition to exosites on the C-terminal hemopexin domain and fibronectin-like insert domains.

REFERENCES

1. Woessner, J. F., Jr., and Nagase, H. (2000) Matrix Metalloproteinases and TIMPs, pp. 126–129, Oxford University Press, New York
2. Sernlicht, M. D., and Werb, Z. (2001) Annu. Rev. Cell Dev. Biol. 17, 463–516
3. Hu, J., Van den Steen, P. E., Sang, Q. X., and Opdenakker, G. (2007) Nat. Rev. Drug Discov. 6, 480–498
4. Chung, L., Shimokawa, K., Dinakarpandian, D., Grams, F., Fields, G. B., and Nagase, H. (2000) J. Biol. Chem. 275, 29610–29617
5. Pelman, G. R., Morrison, C. J., and Overall, C. M. (2005) J. Biol. Chem. 280, 2370–2377
6. Minond, D., Lauer-Fields, J. L., Cudic, M., Overall, C. M., Pei, D., Brew, K., Vise, R., Nagase, H., and Fields, G. B. (2006) J. Biol. Chem. 281, 38302–38313
7. Chung, L., Dinakarpandian, D., Yoshida, N., Lauer-Fields, J. L., Fields, G. B., Vise, R., and Nagase, H. (2004) EMBO J. 23, 3020–3030
8. Bertini, I., Calderone, V., Fragai, M., Luchinat, C., Maletta, M., and Yeo, K. J. (2006) Angew. Chem. Int. Ed. Engl. 45, 7952–7955
9. Lang, R., Kocourek, A., Braun, M., Tschesche, H., Huber, R., Bode, W., and Maskos, K. (2001) J. Mol. Biol. 312, 731–742
10. Lauer-Fields, J. L., Tuzinski, K. A., Shimokawa, K., Nagase, H., and Fields, G. B. (2000) J. Biol. Chem. 275, 13282–13290
11. Lauer-Fields, J. L., Sritharan, T., Stack, M. S., Nagase, H., and Fields, G. B. (2003) J. Biol. Chem. 278, 18140–18145
12. Lauer-Fields, J. L., Broder, T., Sritharan, T., Chung, L., Nagase, H., and Fields, G. B. (2001) Biochemistry 40, 5795–5803
13. Minond, D., Lauer-Fields, J. L., Nagase, H., and Fields, G. B. (2004) Biochemistry 43, 11474–11481
14. Sun, H. B., Smith, G. N., Jr., Hasty, K. A., and Yokota, H. (2000) Anal. Biochem. 283, 153–158
15. Saffarian, S., Colllier, I. E., Marmer, B. L., Elson, E. L., and Goldberg, G. (2004) Science 306, 108–111
16. Overall, C. M. (2002) Mol. Biotechnol. 22, 51–86
17. Lauer-Fields, J. L., Minond, D., Sritharan, T., Kashiwagi, M., Nagase, H., and Fields, G. B. (2007) J. Biol. Chem. 282, 142–150
18. Minond, D., Lauer-Fields, J. L., Cudic, M., Overall, C. M., Pei, D., Brew, K., Moss, M. L., and Fields, G. B. (2007) Biochemistry 46, 3724–3733
19. Galis, Z. S., Sukhova, G. K., Lark, M. W., and Libby, P. (1994) J. Clin. Invest. 94, 2493–2503
20. Sukhova, G. K., Schonbeck, U., Rabkin, E., Schoen, F. J., Poole, A. R.,Billinghurst, R. C., and Libby, P. (1999) Circulation 99, 2503–2509
21. Libby, P., and Aikawa, M. (2002) Nat. Med. 8, 1257–1262
22. Shapiro, S. D. (1999) Thromb. Haemostasis 82, 846–849
23. Matsusomo, S., Kobayashi, T., Katoh, M., Saito, I., Ikeda, Y., Kobori, M., Masuho, Y., and Watanabe, T. (1998) Am. J. Pathol. 153, 109–119
24. Liang, J., Liu, E., Yu, Y., Kitajima, S., Koike, T., Jin, Y., Morimoto, M., Hatakeyama, K., Asada, Y., Watanabe, T., Sassaguri, Y., Watanabe, S., and Fan, J. (2006) Circulation 113, 1993–2001
25. Johnson, J. L. (2007) Expert Rev. Cardiovasc. Ther. 5, 265–282
26. Johnson, J. L, George, S. J, Newby, A. C., and Jackson, C. L. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 15575–15580
27. Wang, X., Liang, J., Koike, T., Sun, H., Ichikawa, T., Kitajima, S., Mori- moto, M., Shikama, H., Watanabe, T., Sasaguri, Y., and Fan, J. (2004) Am. J. Pathol. 165, 1375–1383
28. Shapiro, S. D., Kobayashi, D. K., and Ley, T. J. (1993) J. Biol. Chem. 268, 23824–23829
29. Maskos, K. (2005) Biochimie (Paris) 87, 249–263
30. Nar, H., Werle, K., Bauer, M. D., Dollinger, L., and Jung, B. (2001) J. Mol. Biol. 312, 743–751
31. Bertini, I., Calderone, V., Cosenza, M., Fragai, M., Lee, Y. M., Luchinat, C.,...
