Collagen serves as a structural scaffold and a barrier between tissues, and thus collagen catabolism (collagenolysis) is required to be a tightly regulated process in normal physiology. In turn, the destruction or damage of collagen during pathological states plays a role in tumor growth and invasion, cartilage degradation, or atherosclerotic plaque formation and rupture. Several members of the matrix metalloproteinase (MMP) family catalyze the hydrolysis of collagen triple helical structure. This study has utilized triple helical peptide (THP) substrates and inhibitors to dissect MMP-1 collagenolytic behavior. Analysis of MMP-1/THP interactions by hydrogen/deuterium exchange mass spectrometry followed by evaluation of wild type and mutant MMP-1 kinetics led to the identification of three noncatalytic regions in MMP-1 (residues 285–295, 302–316, and 437–457) and two specific residues (Ile-290 and Arg-291) that participate in collagenolysis. Ile-290 and Arg-291 contribute to recognition of triple helical structure and facilitate both the binding and catalysis of the triple helix. Evidence from this study and prior studies indicates that the MMP-1 catalytic and hemopexin-like domains collaborate in collagen catabolism by properly aligning the triple helix and coupling conformational states to facilitate hydrolysis. This study is the first to document the roles of specific residues within the MMP-1 hemopexin-like domain in substrate binding and turnover. Noncatalytic sites, such as those identified here, can ultimately be utilized to create THP inhibitors that target MMPs implicated in disease progression while sparing proteases with host-beneficial functions.

The mechanism of collagenolysis, by which proteases catalyze the hydrolysis of amide bonds within triple helical structures, has been investigated for over 30 years. Despite this lengthy period, few inroads have been made in the identification of specific enzyme residues that facilitate collagenolysis. The primary mammalian collagenases have been identified as cathepsin K and several members of the matrix metalloproteinase (MMP) family. Most of the early work on MMP collagenolysis focused on analysis of the sites of hydrolysis, and how unique features within these sites may direct collagen catabolism (1). More recent work has evaluated the active sites and domains of MMPs to better understand the dynamic role that the enzyme plays in collagen hydrolysis (2–4).

Collagenolytic members of the MMP family possess similar domain organizations, including propeptide, catalytic (CAT), linker, and hemopexin-like (HPX) domains (5). Several of these domains and/or regions within them have been implicated in collagenolysis. For example, MMP-1 residues 183–191, which are on the V-B loop between the fifth β-strand and the second α-helix in the CAT domain, as well as the active site cleft itself, have substantial roles in collagenolysis (6, 7). MMP-1 residue Gly-233 has been implicated as necessary for conformational flexibility of the active site (8). Within the MMP-1 linker domain, residues 262–276 were proposed to form a polyproline type II helix and interact with and destabilize the MMP cleavage site in collagen (9), whereas Gly-272 may allow bending of the linker domain to aid in interaction between the CAT and HPX domains (10).

The HPX domain has a critical role in collagenolysis, as removal of the MMP-1, MMP-8, MMP-13, or MMP-14 (MT1-MMP) HPX domain results in a loss of collagenolytic activity (11–16). However, no information has been obtained as to the identity of specific residues within the HPX domain that participate in collagenolysis. Secondary binding sites (exosites) may promote interaction of proteases with large, macromolecular substrates, such as collagen. The identification of exosites involved in collagenolysis may aid in the design of selective MMP inhibitors (17–20). Ultimately, as exosites are identified, the manner in which the CAT, linker, and HPX domains work together to facilitate collagenolysis can be revealed.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Fig. S2.

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3 The abbreviations used are: MMP, matrix metalloproteinase; THP, triple helical peptide; fTHP, fluorogenic THP; HDX, hydrogen/deuterium exchange; MS, mass spectrometry; CAT, catalytic; HPX, hemopexin-like; HPLC, high pressure liquid chromatography; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; THPI, THP inhibitor; Dnp, 2,4-dinitrophenyl; Mca, 7-methoxycoumarin.
Hemopexin-like Domain Residues in Collagenolysis

One approach for the rapid analysis of protein structure and identification of binding sites within proteins involves hydrogen/deuterium exchange (HDX) of protein backbone amide hydrogens with detection by mass spectrometry (MS) (21–23). A protein or protein/ligand pair is incubated for defined intervals in a deuterated environment. After rapid quenching of the HDX reaction, the partially deuterated protein is digested, and the resulting peptide fragments are analyzed by LC-MS. The deuterium buildup curve measured for each fragment yields an average amide exchange rate that reflects the environment of the peptide in the intact protein. HDX MS has been used previously to monitor the interaction between doxycycline and MMP-7 (24). The interaction sites identified were consistent with other biophysical studies mapping doxycycline binding outside of the catalytic Zn²⁺ (24). This present study has utilized HDX MS with a triple helical peptide (THP) substrate to identify nonactive site MMP-1 regions involved in collagenolysis. Subsequently, site-specific mutagenesis of MMP-1 in combination with THP inhibitors and substrates was utilized to identify, for the first time, specific HPX domain residues that participate in collagenolysis and to provide insight as to how these residues function mechanistically.

MATERIALS AND METHODS

All standard chemicals were peptide synthesis or molecular biology grade and purchased from Fisher. O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate, (7-azabenzo-triazol-1-yl)oxytripyrrolidinophosphonium hexafluorophosphate, 1-hydroxybenzotriazole, Fmoc (N-(9-fluorenylethoxycarbonyl)-amino acid derivatives, and MMP inhibitor III (a hydroxamic acid-Leu-homo-Phe dipeptide) were obtained from EMD Biosciences (San Diego). Amino acids were of L-configuration (except for Gly). Knight SSP (Mca-Lys-Pro-Leu-Gly-Lys(Dnp)-Ala-Arg-NH₂) was synthesized by methods described previously (25, 26). The α(I)772–786 THP ((Gly-Pro-Hyp)₅gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-Gly-Pro-Hyp-Hyp-)₅NH₂, fTHP-15 ((Gly-Pro-Hyp)₅gly-Pro-Lys(Mca)-Gly-Pro-Lys(Dnp)-Gly-Pro-Gln-Gly-Glu-Arg-Gly-Pro-Hyp-Hyp-)₅NH₂, and fTHP-16 ((Gly-Pro-Hyp)₅gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly-Val-Gly-Leu-Hyp-(Gly-Pro-Hyp)-₅NH₂) were expressed in Escherichia coli and folded from the inclusion bodies as described previously (6, 34). Pro-MMP-1 was activated by reacting with 1 mM 4-aminophenylmercuric acetate and 0.1 eq of MMP-3 (Δ248–460) at 37 °C for 6 h. After activation, MMP-3 (Δ248–460) was completely removed from MMP-1 by affinity chromatography using an anti-MMP-3 IgG Affi-Gel 10 column. Pro-MMP-3 was activated by reacting with 5 μg/ml chymotrypsin at 37 °C for 2 h. Chymotrypsin was activated with 2 mM diisopropyl fluorophospho-phate. Pro-MMP-1 (Δ243–450) was expressed in E. coli using the expression vector pET3α (Novagen), folded from inclusion bodies, and purified as described previously (35). The zymogen was activated as described above for the full-length pro-MMP-1. MMP-1(E200A) was also expressed in E. coli using pET3α as described (36). MMP-1(R291A) and MMP-1 (I290A,R291A) were generated according the methods described by Chung et al. (6) by Robert Visse and Hideaki Nagase (Imperial College London). Folding of these mutants was initially evaluated by SDS-PAGE (for disulfide bond formation in the HPX domain) and trypsin susceptibility as described (6). These results, together with the general proteolytic activities of the mutants (Table 1 and Fig. 4), indicate that the proteins are properly folded. The concentration of active MMP-1 was determined by titration with recombinant tissue inhibitor of metalloproteinase 1 or amino-terminal domain of tissue inhibitor of metalloproteinase 1 over a concentration range of 0.1–3 μg/ml. Active site titrations utilized Knight SSP as substrate (26).

Hydrogen/Deuterium Exchange Mass Spectrometry (MS)—Solution phase amide HDX experiments were performed with a LEAP technologies (Carroboro, NC) CTC HTS twin PAL autosampler interfaced with an electrospray ionization linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) (37). 4 μl of the 10 μM MMP-1 stock solution (50 mM Tris-HCl, pH 8.6, containing 0.15 M NaCl, 5 mM CaCl₂, 100 μM MnCl₂, 5 mM β-mercaptoethanol, 1 mM 2-hydroxyethylsulfide, and 0.02% NaN₃) were diluted with 16 μl of an equivalent composition D₂O buffer and incubated for 1, 30, 60, 900, or 3600 s to allow HDX to occur. Following the allotted time period, the sample was diluted to 50 μl with cold (1.5 °C) 2 M urea containing 0.1% trifluoroacetic acid. Protein was then passed through an immobilized pepsin column (2 mm × 2 cm made in-house) held at 1.5 °C, and the resultant peptides were captured on a C₅ peptide micro trap (Michrom Bioresources, Inc., Auburn, CA). Peptides were
then eluted across an HPLC column (Betasil C$18 \times 2.1 \text{ mm, } 5 \mu\text{m}) into the electrospray ionization source with a gradient of 2% CH$_3$CN increasing to 50% CH$_3$CN over 18 min. For the MMP plus ligand HDX data, 10 $\mu$M MMP-1 stock solution was incubated with 1 mM ligand overnight at 25 °C prior to mixing with D$_2$O buffer and subsequent analysis. Data represent the average of three replicates and were processed with in-house developed software. Results were plotted with Microsoft Excel and visualized with Jmol (available on line). It should be noted that for each MMP-1 peptide identified in the experiment, we removed the two N-terminal residues when discussing (and/or displaying) the HDX MS data. For example, peptide 285–295 becomes region 287–295. This change is to account for the loss of the N-terminal amide during digestion and the resulting compromised exchange rate of the $n + 1$ amide, as the exchange rate is influenced by proximity to the N-terminal amino acid (38).

**Peptide Fluorescence Energy Transfer Assays**—Substrate stock solutions were prepared at various concentrations in EAB buffer (50 mm Tricine, 50 mm NaCl, 10 mm CaCl$_2$, 0.05% Brij-35, pH 7.5). MMP assays were conducted in EAB buffer by incubating a range of substrate concentrations (0.7–90 $\mu$M) with 2–5 nM enzyme at 25 °C. Fluorescence was measured on a Molecular Devices SPECTRAmax Gemini EM Dual Scanning Microplate Spectrofluorimeter using $\lambda_{\text{excitation}} = 324$ nm and $\lambda_{\text{emission}} = 393$ nm. Rates of hydrolysis were obtained from plots of fluorescence versus time, using data points from only the linear portion of the hydrolysis curve. The slope from these plots was divided by the fluorescence change corresponding to complete hydrolysis and then multiplied by the substrate concentration to obtain rates of hydrolysis in units of micromolar/s. Kinetic parameters were evaluated by Lineweaver-Burk, Eadie-Hofstee, and Hanes-Woolf analyses, as well as by a nonlinear regression, one-site hyperbolic binding model with SigmaPlot software.

**Inhibition Assays**—Peptide substrates and inhibitors were dissolved in TSB buffer (50 mm Tris-HCl, pH 7.5, containing 100 mm NaCl, 10 mm CaCl$_2$, 0.05% Brij-35, pH 7.5). 1–2 nM enzyme was incubated with varying concentrations of inhibitors for 2 h at room temperature. Residual enzyme activity was monitored by adding 0.1 volume of Knight SSP to produce a final concentration of <0.1 $K_M$. Initial velocity rates were determined from the first 20 min of hydrolysis when product release is linear with time. Fluorescence was measured as described above. Apparent $K_i$ values were calculated using Equations 1 and 2,

$$\frac{v_i}{v_o} = \left\{ E_i - I_i - K^{\text{app}}_i \right\} + \frac{\left( (E_i - I_i - K^{\text{app}}_i)^2 + 4E_iK^{\text{app}}_i \right)^{1/2}}{2E_i}$$

(Eq. 1)

$$K^{\text{app}}_i = K_i \left( A_i + K_M \right) / K_M$$

(Eq. 2)

where $I_i$ is the total inhibitor concentration; $E_i$ is the total enzyme concentration; $A_i$ is the total substrate concentration; $v_o$ is the activity in the absence of inhibitor; and $K_M$ is the Michaelis constant. The value of $E_i/K^{\text{app}}_i$ did not exceed 100 so that the inhibitor was distributed in both free and bound forms, and $K^{\text{app}}_i$ was calculated by fitting inhibition data to Equation 1. Because the substrate concentration was less than $K_M/10$, $K^{\text{app}}_i$ values were insignificantly different from true $K_i$ values. In cases where weak inhibition occurred, and $K^{\text{app}}_i$ values were calculated using $v_i = v_o / \left( 1 + I_i / K^{\text{app}}_i \right)$. It should be noted that the diastereomers present in $\alpha(1–III)\text{Gly}^\Psi(\text{PO}_2\text{H-CH}_3)\text{Leu}$ THP1 were not separable by RP-HPLC. Because the $S$ stereoisomer is likely a better inhibitor than the $R$ stereoisomer (17), the true $K_i$ values for the $S$-form are likely lower than illustrated here.

**Collagen and Gelatin Assays**—MMP-1 assays for collagenolytic and gelatinolytic activities were as described previously (36).
RESULTS

To initially identify regions within MMP-1 that interact with interstitial collagens, an active site mutant of MMP-1 (MMP-1(E200A)) and a THP model of the MMP-1 cleavage site in type I collagen (\(\alpha_1(I)772–786\)) were utilized. MMP-1(E200A) binds to the MMP-1 cleavage site but does not effectively catalyze hydrolysis (36). The \(\alpha_1(I)772–786\) THP is a substrate for MMP-1, hydrolyzed at the bond analogous to the MMP-1 cleavage site in type I collagen (27). HDX MS was utilized initially to examine the relative dynamics of MMP-1(E200A) (Fig. 1, left). Relatively rapid exchange (30–50%) was found to occur in several of the loops of the CAT domain and several of the \(\beta\)-sheet regions in the HPX domain, whereas regions surrounding the active site and one of the HPX domain \(\beta\)-sheets exhibited slow exchange rates (<10%).

Sites of interaction between MMP-1(E200A) and \(\alpha_1(I)772–786\) THP were evaluated using HDX MS following overnight association of the enzyme and substrate. The triple helix was found to exhibit protection to exchange by \(\sim 20\%\) at several MMP-1 regions outside of the active site, specifically residues 287–295, 304–316, and 439–457 (Fig. 2), all located in the HPX domain of the enzyme (Fig. 1, right). These regions were all found to exhibit rapid exchange in the enzyme alone (Fig. 1, left). The HDX MS changes in MMP-1 observed here appear to be specific for THP binding. Comparison of small molecule-inhibited MMP-1 to MMP-1 with no inhibitor present showed a 1.2 Å variation in HPX domain structures (39). This variation was evenly distributed throughout the HPX domain (see Fig. 5a in Ref. 39) rather than arising from the sequences identified here.

MMP-1 molecular modeling studies suggest that, of the three regions identified here, the 287–295 sequence is the most proximal to the triple helix when the substrate initially binds (36, 40), and thus site-specific mutagenesis was pursued within this sequence. Residues 287–295 (within peptide 285–295; Asp-Ala-Ile-Thr-Thr-Ile-Arg-Gly-Glu-Val-Met) are located in the first \(\beta\)-strand of the MMP-1 HPX domain blade I (Fig. 1). Asp-285 is the only exposed residue on the bottom of the HPX domain, whereas Thr-Ile-Arg-Gly is partly exposed on the top (Fig. 3). Based on structural analysis of MMP-1 (39, 41), it seemed more likely that residues exposed on the top of the HPX...
domain rather than on the bottom would participate in triple helical peptidase activity. Also, the amide hydrogen of Asp-285 was removed during digestion and therefore could not contribute to the exchange behavior of this region. Examining the Thr-Ile-Arg-Gly region in more detail, the hydroxyl group from the side chain of Thr-289 forms a hydrogen bond with Lys-432 from blade II, but it is also phobic interactions with residues from blade II, and it is also partially unwound. Interestingly, the MMP-1 mutant $K_i$ values were less sensitive to increased temperature, as only slightly worse $K_i$ values were observed at 37 °C compared with 10 °C (Table 1). In contrast, $K_i$ for MMP-1($243–450$) increased ~8 times at 37 °C (Table 1). To determine whether an increase in $K_i$ as a function of temperature is a general trend for inhibition of MMP-1, inhibition of MMP-1, MMP-1(R291A), MMP-1(I290A,R291A), and MMP-1($243–450$) by a small molecule (MMP inhibitor III) was examined. The $K_i$ values for MMP inhibitor III inhibition of all four enzymes increased ~2-fold from 10 to 37 °C (Table 1). Thus, for a small molecule inhibitor, an increase in temperature slightly decreased the affinity toward MMP-1. This suggests that the more substantial, 4-fold change in $K_i$ value for MMP-1 inhibition by THPI as a function of increasing temperature was because of unfolding of the inhibitor triple helical structure, whereas the two mutant enzymes (MMP-1(R291A) and MMP-1(I290A,R291A)) were less sensitive to the triple helical structure of the THPI. It is interesting to note that, of the four enzymes, MMP-1($243–450$) was the most sensitive to the effects of increasing temperature for inhibition by THPI. Based on the behavior of the other two mutant enzymes, the temperature effect for MMP-1($243–450$) was less likely because of the structure of the HPX domain deleted (MMP-1($243–450$)) (36) was obtained to provide a reference for the contribution of the HPX domain for interaction with THPs.

To evaluate the differences in binding triple helices and the sensitivity to triple helical conformation, inhibition of MMP-1 and the mutant enzymes was compared using THPI. THPI mimics an α1(I–III)$769–783$ collagen consensus sequence (42), where the P$_1$–P$_4$ subsites were substituted by a nonhydrolyzable Gly$\Psi$[PO$_2$H$\cdot$H$_2$]Leu transition state analog (19). The modest $T_m$ of THPI (30 °C) was allowed for $K_i$ values to be obtained below and above its melting temperature (at 10 and 37 °C, respectively) to document changes in binding affinity due to the presence or absence of triple helical structure. At 10 °C, low nanomolar $K_i$ values were observed for inhibition of MMP-1 peptidase activity by THPI (Table 1). The $K_i$ values for MMP-1(R291A) and MMP-1(I290A,R291A) were ~2 and ~4 times higher, respectively, than wild type MMP-1 (Table 1). $K_i$ values comparable with MMP-1(I290A,R291A) resulted with MMP-1($243–450$), where the HPX domain is absent (Table 1). Thus, mutation of Ile-290 and Arg-291 decreased the affinity of MMP-1 for the THPI.

The MMP-1 $K_i$ increased ~4 times at 37 °C (Table 1), a tem-perature at which the inhibitor triple helix would be at least partially unwound. Interestingly, the MMP-1 mutant $K_i$ values were less sensitive to increased temperature, as only slightly worse $K_i$ values were observed at 37 °C compared with 10 °C (Table 1). In contrast, $K_i$ for MMP-1($243–450$) increased ~8 times at 37 °C (Table 1). To determine whether an increase in $K_i$ as a function of temperature is a general trend for inhibition of MMP-1, inhibition of MMP-1, MMP-1(R291A), MMP-1(I290A,R291A), and MMP-1($243–450$) by a small molecule (MMP inhibitor III) was examined. The $K_i$ values for MMP inhibitor III inhibition of all four enzymes increased ~2-fold from 10 to 37 °C (Table 1). Thus, for a small molecule inhibitor, an increase in temperature slightly decreased the affinity toward MMP-1. This suggests that the more substantial, 4-fold change in $K_i$ value for MMP-1 inhibition by THPI as a function of increasing temperature was because of unfolding of the inhibitor triple helical structure, whereas the two mutant enzymes (MMP-1(R291A) and MMP-1(I290A,R291A)) were less sensitive to the triple helical structure of the THPI. It is interesting to note that, of the four enzymes, MMP-1($243–450$) was the most sensitive to the effects of increasing temperature for inhibition by THPI. Based on the behavior of the other two mutant enzymes, the temperature effect for MMP-1($243–450$) was less likely because of the structure of the HPX domain and most probably because of the stability of MMP-1($243–450$) itself. It is possible that the fold of the MMP-1 CAT domain, particularly regions outside of the active site that contact triple helical structure, is stabilized by the presence of the HPX domain. THPI did not inhibit MMP-3(2428–460) up to an inhibitor concentration of 1 μM at either 10 or 37 °C.

To further evaluate the effects of the MMP-1 mutations on processing triple helices, the activity of both mutants was compared with the wild type and HPX domain-deleted enzymes for single-stranded and triple helical substrates. MMP-1, MMP-1($243–450$), and the double mutant MMP-1(I290A,R291A) had very similar kinetic parameters toward a small, linear substrate (Knight substrate; Mca-Lys-Pro-Leu-Gly-Leu-Lys(Dnp)Ala-Arg-NH$_2$) (26, 43) (Table 2). The single mutant MMP-1(R291A) showed reduced activity toward the linear substrate compared with MMP-1. Activities of the four enzymes were then compared using fTHP-15, which is a fluorescence resonance energy transfer THP of similar sequence to α1(I)$772–
Hemopexin-like Domain Residues in Collagenolysis

FIGURE 4. Collagenolytic (lanes A–C) and gelatinolytic (lanes D–F) activities of MMP-1, MMP-1(R291A), and MMP-1(I290A,R291A). The enzyme concentrations are 10 nM MMP-1 (lanes A and D), 10 nM MMP-1(R291A) (lanes B and E), 100 nM MMP-1(I290A,R291A) (lane C), and 10 nM MMP-1(I290A,R291A) (lane F).

786 THP used for the HDX MS studies. In this case, both mutants had significantly reduced triple helical peptidase activity compared with wild type MMP-1 (Table 2). Complete absence of the HPX domain (MMP-1(Δ243–450)) resulted in a lower triple helical peptidase activity than observed with either mutant (Table 2).

To examine the subtleties of HPX/triple helix interactions, two longer triple helical peptides were constructed and tested as MMP substrates. One (fTHP-16) had a Gly-Pro-Hyp-Gly-Pro-Ser-Gly-Ala-Glu extension on the N-terminal compared with fTHP-15, whereas the other (fTHP-17) had a Gly-Leu-Hyp-Gly-Ala-Glu-Gly-Glu-Arg extension on the C-terminal compared with fTHP-15. Both peptides exhibited CD spectra characteristic of triple helices and had cooperative melts that indicated $T_m$ values of ~50 and ~48 °C, respectively (data not shown). MMP-1 exhibited greater activity toward both extended peptides compared with fTHP-15 (Table 2). Activity toward the C-terminally extended TTHP (fTHP-17) was ~3 times greater compared with the N-terminally extended TTHP (fTHP-16). The $k_{cat}$ values for the extended THPs were similar, and thus the greater activity toward fTHP-17 compared with fTHP-16 was because of a better $K_m$ value.

Interestingly, both MMP-1(R291A) and MMP-1(I290A,R291A) had lower relative activity toward fTHP-16 and fTHP-17 compared with MMP-1 (Table 2). In the case of the N-terminally extended substrate (fTHP-16), $K_m$ values were ~5 times worse for the mutant enzymes compared with MMP-1. However, for the C-terminally extended substrate (fTHP-17), the decreased relative activity was because of both a 2-fold increase in $K_m$ and an ~3-fold reduction in $k_{cat}$ values (Table 2). MMP-1(Δ243–450) exhibited a low level of relative activity toward fTHP-16 and virtually no activity toward fTHP-17 (Table 2). The low activity was because of both an increase in $K_m$ and a decrease in $k_{cat}$ values, but the effect on $k_{cat}$ values was more substantial with the C-terminally extended fTHP-17.

The activity of MMP-1, MMP-1(R291A), and MMP-1(I290A,R291A) toward collagen was then compared. Using 10 nM enzyme, MMP-1(R291A) exhibited an ~3–4-fold decrease in activity compared with MMP-1 (Fig. 4). MMP-1(I290A,R291A) had very little collagenolytic activity (~30–40-fold lower than MMP-1). Even at a 10-fold higher enzyme concentration (100 nM), MMP-1(I290A,R291A) had ~40% of the activity of MMP-1 (Fig. 4). For comparison, removal of the HPX domain (MMP-1(Δ243–450)) rendered the enzyme essentially inactive toward type I collagen under similar conditions (see Fig. 3A in Ref. 36). Activity toward denatured collagen (gelatin) was not affected by the mutations (Fig. 4). In a similar fashion, removal of the HPX domain (MMP-1(Δ243–450)) had no effect on MMP-1 gelatinolytic activity (compare Fig. 6A in Ref. 6 to Fig. 3C in Ref. 36).

DISCUSSION

This study has identified regions within MMP-1 that interact with triple helical structures and mechanistically explored the contributions made by nonactive site residues to collagenolysis. The initial HDX MS studies showed a lack of widespread changes in MMP-1 upon THP binding. This result suggests similar overall conformational dynamics between the free and substrate-bound forms of MMP-1. Thus, binding of the THP may not “lock” the CAT and HPX domains in a closed form or otherwise create domain interactions not found in the free enzyme. Such a result is consistent with structural observations of MMP-1, MMP-9, and MMP-12, in which movement of the CAT and HPX domains toward and away from one another occurs in the absence of substrate (44–46). An HDX MS study of thermolysin also found similar conformational dynamics between the free and substrate-bound forms of the enzyme (47). Alternatively, the MMP-1 E200A mutant used in the HDX MS studies may have an equilibrium between “closed” and “open” forms, where such an equilibrium may not be found in the wild type enzyme or the MMP-1 E200A mutant may not fully proceed to the closed form.

Results from HDX MS and site-specific mutagenesis experiments have revealed Ile-290 and Arg-291 as two residues within the HPX domain that facilitate interactions between collagenolytic MMPs (MMP-1) and triple helical structures. These are the first HPX residues identified as participants in collagenolysis. The kinetic changes observed for the Ile-290 and Arg-291 MMP-1 mutants with the C-terminally extended THP are manifested in both $K_m$ and $k_{cat}$, suggesting effects on substrate binding and coupled motions for catalysis (48). Protein motions and catalytic activity can be coupled (48), and there could be an ensemble of coupled conformational states in collagenolytic MMPs, as proposed for enzymes in general (49).

The substrate extension toward the C terminus promoted further interactions with the HPX domain. The effects seen for the N-terminally extended substrate suggested proper alignment of the triple helix by the HPX domain into the CAT domain. Thus, the HPX domain may ultimately be involved in alignment and distortion of the triple helix to facilitate hydrolysis. The need for proper alignment could explain why some MMPs, such as MMP-3, can bind to collagen but cannot catalyze hydrolysis (14, 50). Although MMP-3 is known to bind to collagen via its HPX domain (50), chimeras containing the MMP-3 HPX domain and either the MMP-1 or MMP-8 CAT and linker domains have little or no collagenolytic activity (14,
Comparison with prior studies further supports roles of the 304–316 and 439–457 regions in collagenolysis. MMP-8/MMP-3 chimeras showed that substitution of MMP-8 residues 302–326 (equivalent to MMP-1 residues 301–325) with MMP-3 residues decreased collagenolytic activity 16 times (51). The interaction of MMP-1 Arg-291 and Arg-304 with carbonyl groups from a THP substrate was suggested based on molecular modeling studies (40). The 304–316 sequence contains a three-residue region (Phe-308–Tyr-309–Pro-310) that shows a large conformational change between pro-MMP-1 and active MMP-1 (39). This 308–310 loop forms a hydrophobic contact with the prodomain (63). It has been proposed that Arg-300 acts as the “pivot” around which the HPX domain undergoes displacement upon activation (39). Additional mutagenesis studies can be targeted for the 304–316 and 439–457 regions to evaluate their roles in triple helical peptidase and collagenolytic activities.

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REFERENCES

1. Fields, G. B. (1991) J. Theor. Biol. 153, 585–602
2. Overall, C. M. (2002) Mol. Biotechnol. 22, 51–86
3. Overall, C. M., and Butler, G. S. (2007) Structure 15, 1159–1161
4. Murphy, G., and Nagase, H. (2008) Mol. Aspects Med. 29, 290–308
5. Woessner, J. F., and Nagase, H. (2000) Matrix Metalloproteinases and TIMPs, pp. 1–10, Oxford University Press, Oxford, UK
6. Chung, L., Shimokawa, K., Dinakarandian, D., Grams, F., Fields, G. B., and Nagase, H. (2000) J. Biol. Chem. 275, 29610–29617
7. Kñaüer, V., Patterson, M. L., Gomis-Rüth, F. X., Smith, B., Lyons, A., Docherty, A. J., and Murphy, G. (2001) Eur. J. Biochem. 268, 1888–1896
8. O’Farrell, T. J., Guo, R., Hasegawa, H., and Pourmotabbed, T. (2006) Biochemistry 45, 15411–15418
9. De Souza, S. J., Pereira, H. M., Jacchieri, S., and Brentani, R. R. (1996) FASEB J. 10, 927–930
10. Tsukada, H., and Pourmotabbed, T. (2002) J. Biol. Chem. 277, 27378–27384
11. Clark, I. M., and Cawston, T. E. (1989) Biochem. J. 263, 201–206
12. Kñaüer, V., Cowell, S., Smith, B., López-Otín, C., O’Shea, M., Morris, H., Zardi, L., and Murphy, G. (1997) J. Biol. Chem. 272, 7608–7616
13. Kñaüer, V., Osthues, A., De Clerck, Y. A., Langley, K. E., Blaser, J., and Tschesche, H. (1993) Biochem. J. 291, 847–854
14. Murphy, G., Allan, J. A., Willenbrock, F., Cockett, M. L., O’Connell, J. P., and Docherty, A. J. (1992) J. Biol. Chem. 267, 9612–9618
15. Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seki, M., and Okada, Y. (1997) J. Biol. Chem. 272, 2446–2451
16. Hurst, D. R., Schwartz, M. A., Ghaffari, M. A., Jin, Y., Tschesche, H., Fields, G. B., and Sang, Q. X. (2004) Biochem. J. 377, 775–779
17. Lauer-Fields, J., Brew, K., Whitehead, J. K., Li, S., Hammer, R. P., and Fields, G. B. (2007) J. Am. Chem. Soc. 129, 10408–10417
18. Murphy, G., and Nagase, H. (2008) Nat. Clin. Pract. Rheumatol. 4, 128–135
19. Lauer-Fields, J. L., Whitehead, J. K., Li, S., Hammer, R. P., Brew, K., and Fields, G. B. (2008) J. Biol. Chem. 283, 20087–20095
20. Lauer-Fields, J. L., Minond, D., Chase, P. S., Baillargeon, P. E., Saldana, S. A., Stawikowska, R., Hodder, P., and Fields, G. B. (2009) Bioorg. Med. Chem. 17, 990–1005
21. Hamuro, Y., Coales, S. J., Southern, M. R., Nemeth-Cawley, J. F., Stranz, D. D., and Griffin, P. R. (2003) J. Biomat. Sci. 14, 171–182
22. Englander, S. W. (2006) J. Am. Soc. Mass Spectrom. 17, 1481–1489
23. Dai, S. Y., Chalmers, M. J., Bruning, J., Bramlett, K. S., Osborne, H. E., Montrose-Rafizadeh, C., Barr, R. J., Wang, Y., Wang, M., Burris, T. P.,
Hemopexin-like Domain Residues in Collagenolysis

Dodge, J. A., and Griffin, P. R. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105**, 7171–7176

24. Garcia, R. A., Pantazatos, D. P., Gessner, C. R., Go, K. V., Woods, V. L., Jr., and Villarreal, F. J. (2005) *Mol. Pharmacol.* **67**, 1128–1136

25. Nagase, H., Fields, C. G., and Fields, G. B. (1994) *J. Biol. Chem.* **269**, 20952–20957

26. Neumann, U., Kubota, H., Frei, K., Ganu, V., and Leppert, D. (2004) *Anal. Biochem.* **328**, 166–173

27. Lauer-Fields, J. L., Nagase, H., and Fields, G. B. (2000) *J. Chromatogr. A* **890**, 117–125

28. Lauer-Fields, J. L., Broder, T., Sritharan, T., Chung, L., Nagase, H., and Fields, G. B. (2001) *Biochemistry* **40**, 5795–5803

29. Minond, D., Lauer-Fields, J. L., Cudic, M., Overall, C. M., Pei, D., Brew, K., Visse, R., Nagase, H., and Fields, G. B. (2006) *J. Biol. Chem.* **281**, 38302–38313

30. Yu, Y.-C., Tirrell, M., and Fields, G. B. (1998)

31. Yu, Y.-C., Berndt, P., Tirrell, M., and Fields, G. B. (1996) *J. Am. Chem. Soc.* **118**, 12515–12520

32. Malkar, N. B., Lauer-Fields, J. L., Borgia, J. A., and Fields, G. B. (2002)

33. Malkar, N. B., Lauer-Fields, J. L., Juska, D., and Fields, G. B. (2003) *Biomacromolecules* **4**, 518–528

34. Lauer-Fields, J. L., Nagase, H., and Fields, G. B. (2004)

35. Suzuki, K., Kan, C. C., Hung, W., Gehring, M. R., Brew, K., and Nagase, H. (1998) *Biocatal. Biotransform.* **16**, 541–549

36. Chung, L., Dinakarpandian, D., Yoshida, N., Lauer-Fields, J. L., Fields, G. B., Visse, R., Nagase, H., and Fields, G. B. (2004) *J. Biol. Chem.* **279**, 1128–1136

37. Chalmers, M. J., Busby, S. A., Pascal, B. D., He, Y., Hendrickson, C. L., Marshall, A. G., and Griffin, P. R. (2006) *Anal. Chem.* **78**, 1005–1014

38. Bai, Y., Milne, J. S., Mayne, L., and Englander, S. W. (1993) *Proteins* **17**, 75–86

39. Iyer, S., Visse, R., Nagase, H., and Acharya, K. R. (2006) *J. Mol. Biol.* **362**, 78–88

40. Ottl, J., Gabriel, D., Murphy, G., Knäuper, V., Tominaga, Y., Nagase, H., Kröger, M., Tschesche, H., Bode, W., and Moroder, L. (2000) *Chem. Biol.* **7**, 119–129

41. Li, J., Brick, P., O’Hare, M. C., Skarzynski, T., Lloyd, L. F., Curry, V. A., Clark, I. M., Bigg, H. F., Hazleman, B. L., Cawston, T. E., and Blow, D. M. (1995) *Structure* **15**, 541–549

42. Minond, D., Lauer-Fields, J. L., Nagase, H., and Fields, G. B. (2004) *Biochemistry* **43**, 11474–11481

43. Knight, C. G., Willenbrock, F., and Murphy, G. (1992) *FEBS Lett.* **296**, 263–266

44. Rosenblum, G., Van den Steen, P. E., Cohen, S. R., Grossmann, J. G., Frenkel, J., Sertchook, R., Slack, N., Strange, R. W., Opdenakker, G., and Sagi, I. (2007) *Structure* **15**, 1227–1236

45. Bertini, I., Calderone, V., Fraga, M., Jaiswal, R., Luchinat, C., Melikian, M., Mylonas, E., and Svergun, D. I. (2008) *J. Am. Chem. Soc.* **130**, 7011–7021

46. Bertini, I., Fraga, M., Luchinat, C., Melikian, M., Mylonas, E., Sarti, N., and Svergun, D. I. (2009) *J. Biol. Chem.* **284**, 12821–12828

47. Liu, Y. H., and Konermann, L. (2008) *Biochemistry* **47**, 6342–6351

48. Watt, E. D., Shimada, H., Kovrigin, E. L., and Loria, J. P. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104**, 11981–11986

49. Benkovic, S. J., Hames, G. G., and Hammes-Schiffer, S. (2008) *Biochemistry* **47**, 3317–3321

50. Allan, J. A., Hembry, R. M., Angal, S., Reynolds, J. I., and Murphy, G. (1991) *J. Cell Sci.* **99**, 789–795

51. Hirose, T., Patterson, C., Pourmotabbed, T., Mainardi, C. L., and Hasty, K. A. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2569–2573

52. Johnson, K. A. (2008) *J. Biol. Chem.* **283**, 26297–26301

53. Lauer-Fields, J. L., Tuzinski, K., A., Shimokawa, K., Nagase, H., and Fields, G. B. (2000) *J. Biol. Chem.* **275**, 13282–13290

54. Perumal, S., Antipova, O., and Orgel, J. P. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105**, 2824–2829

55. Ross, P. D., and Subramanian, S. (1981) *Biochemistry* **20**, 3096–3102

56. Corzine, D. M., Bezmanova, I., Lee, S., Chalikian, T. V., and Kay, L. E. (2009) *J. Mol. Biol.* **386**, 391–405

57. Bhaskaran, R., Palmier, M. O., Lauer-Fields, J. L., Fields, G. B., and Van Doren, S. R. (2008) *J. Biol. Chem.* **283**, 21779–21788

58. Frederick, K. K., Marlow, M. S., Valentine, K. G., and Wand, A. J. (2007) *Nature* **448**, 325–329

59. Wu, B., Arumugam, S., Gao, G., Lee, G. I., Semenchenko, V., Huang, W., Brew, K., and Van Doren, S. R. (2000) *J. Mol. Biol.* **295**, 257–268

60. Lauer-Fields, J. L., Juska, D., and Fields, G. B. (2002) *Biopolymers* **66**, 19–32

61. Nerenberg, P. S., Salsas-Escat, R., and Stultz, C. M. (2008) *Biochemistry* **47**, 6342–6351

62. Maskos, K. (2005) *Biochemistry* **44**, 249–263

63. Jozic, D., Bourenkov, G., Lim, N. H., Visse, R., Nagase, H., Bode, W., and Maskos, K. (2005) *J. Biol. Chem.* **280**, 9578–9585