Pivotal Molecular Determinants of Peptidic and Collagen Triple Helicase Activities Reside in the S_{3}′ Subsite of Matrix Metalloproteinase 8 (MMP-8)

THE ROLE OF HYDROGEN BONDING POTENTIAL OF ASN^{188} AND TYR^{189} AND THE CONNECTING CIS BOND*

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The mechanism of triple helical collagen unwinding and cleavage by collagenases in the matrix metalloproteinase (MMP) family is complex and remains enigmatic. Recent reports show that triple helicase activity is initiated by the hemopexin C domain of membrane type 1-MMP, whereas catalytically inactive full-length interstitial collagenase (MMP-1) exhibits full triple helicase functionality pointing to active site determinants that are needed to complete the triple helicase mechanism. In MMP-8, the neutrophil collagenase, a conserved Gly at the S_{3}′ substrate specificity subsite is replaced by Asn^{188} that forms a highly unusual cis bond with Tyr^{189} a conserved active site residue in the collagenases. Only in MMP-1 is the S_{3}′ Gly also replaced, and there too a cis configured Glu-Tyr occurs. Thus, this high energy peptide bond coupled to the canonical Tyr may be important in the collagenolytic process. In a systematic mutagenesis investigation of the MMP-8 S_{3}′ subsite we found that introducing an S_{3}′ Gly^{188} into MMP-8 reduced collagenolytic efficiency by ~30% with a corresponding reduction in cleavage of a synthetic peptide fluorescence resonance energy transfer substrate analogue of the α2(I) collagen chain cleavage site. The substitution of Asn^{188} to Leu, a hydrophobic residue of similar size to the highly polar Asn and designed to retain the cis bond, revealed the importance of hydrogen bonding to bond substrate with both collagenolytic and peptidic activities reduced ~3-fold. In contrast, the specificity for type I collagen of the mutant Y189F dropped 3-fold without any significant alteration in general peptidase activity. Therefore, S_{3}′ and in particular the hydrogen bonding potential of Tyr^{189} is a specific molecular determinant for MMP-8 triple helicase activity. The cis bond connection to Asn^{188} juxtaposes these two side chains for closely spaced hydrogen bonding with substrate that improves collagenolytic and general catalytic efficiency that could be exploited for new collagenase-specific inhibitor drugs.

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The neutrophil collagenase matrix metalloproteinase (MMP)^{1–3} (EC 3.4.24.34) is released from the specific granules of polymorphonuclear neutrophilic leukocytes in inflammation (1–3). During wound healing neutrophils debride the focus of inflammation of bacterial and foreign matter, damaged collagen, and other tissue components (4), a process in which the activity of MMP-8 and other neutrophil proteases are well suited. In the Mmp8 knock-out mouse, a surprisingly exaggerated neutrophil accumulation and delayed clearance of inflammatory cells after application of carcinogens leads to enhanced tumorigenesis and metastasis (5). This neutrophil phenotype may result from incomplete collagen debridement with a reten-
tion of associated chemotactic signals. Clearly, the biological role of MMP-8 collagenolysis and neutrophil function in innate host defense is incompletely understood, which is a knowledge deficiency that impedes target validation in developing new antiproteolytic drugs for inflammatory diseases and cancer (6, 7).

Collagen is a triple helical macromolecule that is one of the most complex of the extracellular matrix to synthesize, fold, and assemble into fibrils (8). It is also one of the most difficult to degrade, which is reflected in the long half-lives of skin and tendon collagen in vivo (9, 10) and slow collagenolytic kinetic parameters in vitro (11, 12). In view of its structural importance the controlled physiological turnover of collagen is pivotal for normal growth and tissue repair, with abnormal breakdown or accumulation of collagen contributing to numerous pathologies including arthritis, metastasis, and fibrotic diseases (13–15). Although the collagen triple helix is proteolytically highly resistant, it was the first substrate identified for the MMP family (16). Several MMPs degrade native collagen with distinct cleavage specificities; MMP-8 has preference for type I collagen (17, 18), MMP-13 displays greatest activity against type II collagen (19), and MMP-1 preferentially cleaves type III collagen (12, 18). Membrane-type (MT) 1-MMP tethers gelatinase A (MMP-2) to the plasma membrane, and although both are individually poor collagenases, when in complex they form an important cell surface collagenolytic axis (20–23).

In comparison to the ~0.5-nm wide MMP-8 active site, the diameter of the collagen triple helix is 1.5 nm with the Gly-Ile and Gly-Leu scissile bonds at the helix center (24, 25). Therefore, before cleavage of the individual α-chains can occur the

1 The abbreviations used are: MMP, matrix metalloproteinase; MT-MMP, membrane type MMP; FRET, fluorescence resonance energy transfer; QF24, quenched fluorescent FRET peptide (7-methoxycoumarin-4-yl)acetetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-1,2,3-diaminopropionyl]-Ala-Arg-NH_{2}.
triple helical structure must be opened in a process termed triple helicase activity. This is difficult, as reflected by kinetic analyses (11, 12, 18), and has been as difficult to elucidate. Unlike peptides, binding and cleavage of native collagen by collagenase involves additional contacts with exosites on regions of the enzyme outside of the active site, which lowers $k_{\text{cat}}$ thus improving $k_{\text{cat}} / K_m$ (24, 26). Clark and Cawston (27) first reported that the collagenase MMP-1 hemopexin C domain alone binds collagen and is absolutely required for cleavage. The hemopexin C domains of all collagenases (28–30), including MMP-14 (22, 23) and MMP-2 (23, 31), have since been demonstrated to be critical for collagenolysis. Indeed, very recent studies have shown that the hemopexin C domain of MT1-MMP alone (23) or of an inactive MMP-1 E200A mutant (32) can initiate triple helicase activity by inducing localized conformational changes in native collagen that are separable from collagen cleavage. In MMP-2, however, a different mechanism of triple helicase occurs as reflected by a different role for the hemopexin C domain that does not bind collagen (23, 26, 33). Here the fibronectin type II modules of the MMP-2 collagen binding domain (34) that are inserted adjacent and amino-terminal to the $S_3'$ subsite are crucial and form the major exosite for collagen binding and triple helicase activity (23).

However, full triple helicase activity requires more than just interaction with exosite domains. Attempts to impart collagenolytic activity on non-collagenolytic MMPs by domain swaps using the MMP-1 hemopexin C domain have been unsuccessful (35–38). Although this may be because of domain clashes or misorientation (26) these chimera results point to the presence of other molecular determinants of collagenolytic activity. The importance of the linker or hinge region of the collagenases (35, 36, 39, 40) in the context of the hemopexin C domain (22, 23) confirmed the necessity in collagenolysis for multiple enzyme substrate contacts (26). Indeed, the chimera approach only successfully recaptured significant collagenolytic activity when the entire hemopexin C domain, linker, and catalytic subdomain up to and just beyond the $S_3'$ region of the active site of MMP-3 (stromelysin) were replaced with that of collagenase MMP-1 (41). Although the resulting collagenolytic activity is not altogether surprising because the collagen contacting interface is MMP-1-like layered on the MMP-3 hydrophobic core, it pointed to the potential importance of the $S_3'$ subsite. This too was also indicated in very recent work where the E200A MMP-1 mutant was greater than 4-fold more effective than the MMP-1 hemopexin C domain alone in inducing triple helicase activity and collagenolysis in trans by non-collagenolytic proteases (32). However, MMP-7 and trypsin in trans could not exploit the structural perturbations induced in collagen following binding by the recombinant MT1-MMP linker hemopexin C domain (23). Together, this shows that triple helicase activity is not completely recapitulated by the linker hemopexin C domain alone and revealed that important contributions are required from the active site to complete the triple helicase process. Indeed, triple helicase activity was abrogated by sterically blocking the E200A MMP-1 active site with a hydroxamate inhibitor (32).

Surprisingly, few site-directed mutagenesis studies have been performed of the catalytic domain of collagenases or, indeed, of any MMP (42). MMP scissile bond specificity is dominated by $S_1'$ interactions. Unlike most MMPs, which have deep and variably contoured $S_1'$ pockets (42), in MMP-1 it is relatively shallow (43). This accommodates the P1 residue and Leu of the $\alpha$1(1) and $\alpha$2(1) chain scissile 775–776 bonds in type I collagen (43, 44) but by excluding more bulky residues spares many other substrates. There is a considerable difference between the $S_1'$ binding pocket of MMP-1, which is significantly occluded by an Arg residue, and that of MMP-8, which is large. Hence, one key difference seen in P1 residue preference between MMP-1 and -8 is that although MMP-8 activity is enhanced nearly 4-fold by the presence of aromatic residues, MMP-1 is intolerant for such P1 residues (45). So, the $S_1'$ pocket does not appear critical for triple helicase activity per se but rather for peptide bond specificity within collagen and between collagen types (46). Therefore, it is concluded that other sites in the catalytic domain are important for triple helicase activity.

New active site structural features in MMP-8 were revealed in the crystal structure of MMP-8 in complex with the inhibitor RO200–1770 (47). A previously unreported, very rare cis peptide bond between Asn and Tyr (see Fig. 1) was found, which otherwise only occurs at the homologous bond in MMP-1 (48). The location of this cis bond is at the amino terminus of the active site $\alpha$-helix B on a loop of the $S_3'$ substrate-specificity subsite. Here, the importance of the conserved $S_3'$ Tyr in MMP-1 had been shown by mutagenesis to Thr, which reduced collagenolytic activity 5-fold (41). Moreover, in the cloning and expression of rat MMP-8 (GenBank accession AJ007288), we had previously determined that a cloning mutation to Lys of the rat homologue of human Asn reduced catalytic efficiency (49). This too pointed to the critical importance of the $S_3'$ subsite for the triple helicase activity of the collagenases. Following this initial observation we undertook a site-directed mutagenesis approach to systematically explore the molecular determinants of collagenase substrate specificity in $S_3'$ of human MMP-8.

Our results showed that hydrogen bonding to the substrate by the side chains of Asn and Tyr, which the cis bond closely positions together, is crucial for efficient catalytic and collagenolytic activities. Hence, our work identified $S_3'$ subsite interactions with collagen as being a critical component of triple helicase activity that is initiated by the linker hemopexin C domain and completed by active site interactions.

**EXPERIMENTAL PROCEDURES**

**Sequence and Three-dimensional Modeling Analyses**—The $S_3'$ substrate specificity subite of human MMP-8 was examined by three-dimensional modeling of structural data coordinates (PDB IDs 1JAN, 1JAP, 1MNC, 1KBC, 1A85, and 1A86) using Swiss-PdbViewer v3.7 (GlauxSmithKline). Residues of interest for mutagenesis were selected from the structure models and analysis of collagenolytic MMP protein sequences (see Fig. 2). Target nucleotide bases for mutagenesis were identified by sequence analysis of human neutrophil collagenase cDNA (GenBank accession AJ05556).

**Site-directed Mutagenesis**—Six site-directed mutations of the $S_3'$ subsite of MMP-8 (N188G, N188K, N188L, Y189A, Y189F, and N190L) and of the catalytic general base glutamate, E198A, were designed and modeled. Complementary oligonucleotides (Table I) were used to introduce point mutations in a double-stranded template of full-length human MMP-8 in the pGW1HG expression plasmid under regulation of a human cytomegalovirus promoter (50) using the QuickChange site-directed mutagenesis strategy (Stratagene). DNA sequence fidelity of the entire cDNAs were confirmed by automated sequencing (Applied Biosystems).

**Mammalian Cell Transfection**—Chinese hamster ovary (CHO-K1) cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (HyClone Laboratories, Inc.) and non-essential amino acids (Invitrogen). Cells were transfected with NotI-linearized pGWIHG-MMP-8 or mutant plasmids by electroporation and the transfectants selected in 25 μg/ml mycophenolic acid (Invitrogen) (50). Stable MMP-8-expressing clones were identified by Western blotting with T93-5660 or T25-88 anti-MMP-8 antibody (generously provided by Dr. H. Tschesche, Biel, DE) and cleavage of a FRET peptide designed from the collagenase-cleavage sequence in the collagen α2(1) chain (7-methoxy-coumarin-4-ylacetyl-Pro-Leu-Gly-Leu-[3-[2,4-dinitrophenyl]-l-2,3-diamino propionyl]-Ala-Arg-NH$_2$) (QF24) (51) (supplied by Dr. C. G. Knight, Cambridge, UK).

**Recombinant Protein Expression and Purification**—MMP-8-expressing clones were expanded to confluence in roller bottles and then washed with phosphate-buffered saline (138 mM NaCl, 2.7 mM KCl, 20 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.4) and incubated in 100 ml of...
serum-free CHO-S-SFM™ II medium (Invitrogen). Conditioned medium was collected every 1–2 days for up to 8 days. To remove gelatinases the medium was chromatographed over gelatin-Sepharose 4B resin (Amersham Biosciences) (10 ml) connected in tandem with a Red-Sepharose CL-6B column (Amersham Biosciences) (25 ml). The MMP-8 protein peak was eluted from Red-Sepharose with 1 M NaCl, 50 mM Tris, pH 7.4, and then loaded on a column of Zn2+/H11001-chelating Sepharose Fast Flow resin (Amersham Biosciences) (10 ml). The unbound fraction containing MMP-8 was chromatographed over lentil lectin-agarose-Sepharose 4B (Sigma-Aldrich) (2 ml) and eluted with 100 mM D-methylmannopyranoside (Sigma), 50 mM Tris, pH 7.4. Purified enzyme was exchanged into collagenase assay buffer (200 mM NaCl, 5 mM CaCl2, 0.05% Brij 35, 50 mM Tris, pH 7.4) through a PD-10 Sephadex G-25 column (Amersham Biosciences). Murine tissue inhibitor of metalloproteinases-1 was expressed in Chinese hamster ovary cells and purified (52).

Synthetic Peptide Cleavage Assays—MMP-8 mutant and wild type proteins (0.35 pmol/ml) were activated with 1 mM 4-aminophenylmercuric acetate for 2 h, 37 °C. The concentrations of activated enzymes were determined by active site titration against the tissue inhibitor of metalloproteinases-1 (53).

Rates of wild type and mutant protease cleavage of FRET peptide QF24 (10 fmol) were measured at 37 °C by continuous assay in fluorescence assay buffer (100 mM NaCl, 10 mM CaCl2, 0.05% Brij 35, 100 mM Tris-HCl, pH 7.5) in 96-well fluorimetry plates. Fluorescence was measured in a FLUOstar Optima plate reader (BMG Labtechnologies) using a 320 nm excitation and 405 nm emission filter pair. Peptide cleavage was quantitated by calibration using serial dilutions of 10 pmol of (7-methoxycoumarin-4-yl)-NH2 (Bachem). Vmax was calculated in the linear portion (500–1000 s) of the initial rate of substrate cleavage.

Collagenase Assays—Biotin-labeled porcine type I collagen (22) (100 fmol) was incubated with 0–250 fmol of MMP-8 or mutant enzymes in collagenase assay buffer for 18 h at 28 °C. The absence of collagen degradation in trypsin control assays (1:10 enzyme:substrate) confirmed the triple helical nature of the collagen during assay. Reaction products were separated by 7.5% SDS-PAGE and analyzed by in-gel detection using the Odyssey infrared imaging system (LI-COR Biosciences) at 700 nm after incubation of the gels with 2 g/ml streptavidin Alexa Fluor 680 conjugate (Molecular Probes) for 2 h. Specific activities of MMP-8 and mutants (units of collagenase activity/fmol-1 enzyme) were determined from densitometric analysis of intact collagen and the collagenase-specific 3/4-collagen A-chain cleavage products detected by the biotin-specific probe. One unit of collagenase activity degrades 2 fmol of type I collagen to 50% completion/h-1 at 28 °C.

RESULTS

Site-directed Mutagenesis of S3 Residues—The MMP-8 S3 residues Asn188, Tyr189, and Asn190 targeted by site-directed mutagenesis are located at the amino end of B helix that forms the base of the active site cleft (Fig. 2A). The cis orientation of the 188–189 peptide bond results in a closely packed, parallel, and nearly planar configuration of the Asn188 and Tyr189 side chains (Fig. 2B). These residues are shown in bold. The resulting mutant residue is identified above the mutant codon in italics.
The slightly diffuse electrophoretic migration pattern that was confirmed the identity of the purified proteins (not shown). Proteins were purified in the proenzyme form, and all displayed an apparent molecular mass of 79 kDa as analyzed by SDS-PAGE (Fig. 3). Western blotting with two anti-MMP-8 antibodies confirmed the identity of the purified proteins (not shown). The slightly diffuse electrophoretic migration pattern that is characteristic of MMP-8 is due to a high degree of glycosylation that was exploited to purify the enzymes on lentil lectin-agarose. Mutants N190L and Y189A were unstable and recalcitrant to purification. At high concentrations the wild type enzyme underwent autolytic activation resulting in the removal of the propeptide and a shift in apparent molecular mass to 59 kDa (Fig. 3, lane 1). 4-Aminophenylmercuric acetate-initiated autolytic processing to the fully activated form of the purified mutants was confirmed by electrophoretic analysis, with complete removal of the propeptide occurring by 120 min to generate 59-kDa active enzyme (not shown). The mutant E198A was not activated by 4-aminophenylmercuric acetate indicating catalytic incompetency of the enzyme.

**Synthetic Peptide Cleavage**—The activity of equimolar samples of wild type and mutant enzymes against the FRET collagen-sequence peptide analogue QF24 was measured by continuous fluorescence spectroscopy (Fig. 4A). Asn188 was found to be an important residue. Interestingly, reintroduction of the conserved Gly by the mutation N188G resulted in an unexpected 30% (n = 27) decrease in activity. More dramatically, the removal of the hydrogen bonding potential at position 188 by the N188L mutation reduced activity more than 3-fold (n = 7) from that of the wild type enzyme. N188K also showed a reduced specific activity for peptide cleavage by 22% (n = 13) whereas E198A was completely inactive as reported for other MMPs (54, 55) but not previously shown for MMP-8 (Fig. 4B). Lastly, Y189F displayed 88% (n = 20) of the wild type activity indicating its importance in cleavage indicating that its hydroxyl group improves catalytic efficiency by forming a hydrogen bond to the substrate. Hence, these experiments reveal that the active site S3′ specificity subsite exerts a surprisingly strong influence on peptide-substrate hydrolysis in a member of an enzyme family generally considered to be dominated by S1′ interactions. Notably, the introduction of Gly188 into MMP-8 reduced catalytic activity, which is a surprising result considering its conservation in other MMPs. Hence, in MMP-8, the hydrogen bonding potential of Tyr189, but more particularly for Asn188, improves catalytic efficiency with up to a 3-fold reduction in peptide hydrolysis following their loss.

**Effects of S3′ Mutations of MMP-8 on Native Type I Collagen Cleavage**—Having measured the general catalytic activity of the MMP-8 variants we next examined the impact of mutagenesis of S3′ residues on the specific activity for type I collagen (Fig. 5). In contrast to activities against the QF24 peptide substrate, the most significant loss of activity for type I collagen was incurred by the mutation of Tyr189 to Phe. Although this mutant retained 88% of wild type QF24 specific activity, its activity against type I collagen dropped 3-fold compared with wild type enzyme. The unstable mutant Y189A demonstrated collagenase activity in conditioned medium from the transfected Chinese hamster ovary cells, although no estimate of specific activity could be obtained (data not shown). Mutagenesis of Asn188 had a varying impact on collagenolytic activity depending on the residue introduced. The 25% decrease in collagen cleavage by N188G was similar to the decline seen in QF24 substrate activity (30%) and may therefore be attributed to a global reduction in catalytic efficiency of the enzyme rather than to a specific influence on collagenolysis. The N188L mutant showed a >2-fold reduced specific activity against type I collagen, albeit not as pronounced as its decrease in activity against QF24 (74%). Surprisingly, no change in the cleavage rate of type I collagen was demonstrated by mutation of Asn188 to Lys even though mutant competency against QF24 was reduced by 22%. P3′ in collagen is Gly that was accommodated in the engineered occlusion of the S3′ pocket, but the slightly larger Ala at P3′ in QF24, or charge repulsion from the P4′ Arg,
reduced $S_3'$-$P_3'$ complementarity and cleavage of the synthetic peptide. As expected, the E198A mutant showed no collagen cleavage activity and was therefore used as a negative control. Hence, these substitutions point to an important role for Asn188 in both the general catalytic and the more specific collagenolytic activities of MMP-8, likely through the contribution of hydrogen bonding to the enzyme-substrate interaction. Most notable though was the clear and specific role for Tyr189 in type I collagen cleavage, in particular to the importance of hydrogen bonding potential at this position.

**DISCUSSION**

Our present mutagenesis studies have characterized the mechanistic importance in collagen triple helicase activity of...
two active site residues, Asn\(^{188}\) and Tyr\(^{189}\), that are linked by a cis bond and that define the S\(_3\)/H\(_{11032}\) subsite in MMP-8. Rather than the cis bond per se it is the resulting unusual configuration of the dual H-bonding side chains of the residues, which this bond orientates more closely than is possible with a trans bond that is a crucial feature associated with triple helicase activity.

The molecular determinants required for the catalysis of native triple helical collagen are distinct from those required for individual peptide chain cleavage, but the precise mechanism of collagen catalysis is unknown. The factors contributing to the ability of MMPs to hydrolyze collagen have been comprehensively reviewed both from the perspective of the enzyme (26) and that of the substrate (14). Three key stages of the process have been identified: collagen binding, unwinding of the triple helix, and hydrolysis of the individual \(\alpha\)-chains of the triple helix (24, 26). The very recent studies of Tam et al. (23) have revealed that localized denaturation of type I collagen, with circular dichroism spectral changes, is induced by the linker hemopexin C domain but not the hemopexin C domain of MT1-MMP alone. However, the helix was not completely opened up because non-collagenolytic proteases could not access and cleave the \(\alpha\)-chains in trans (23). Chung et al. (32) further demonstrated the necessity for an additional binding interaction at the active site in the triple helicase mechanism. Their report showed that full triple helicase activity occurs with full-length inactive MMP-1 E200A 4-fold more efficiently than with the MMP-1 hemopexin C domain alone. Moreover, hydroxamate inhibitors blocked the triple helicase activity of the inactive MMP-1 mutant (32). Therefore, interaction with the catalytic domain was also required for triple helicase activity. Indeed, cleavage of native type I collagen by the MMP-8 catalytic domain alone has also been recorded at 37 °C (56), and studies using the triple helical peptide model substrates have demonstrated binding and cleavage by truncated collagenases lacking the hemopexin C domain (57). Hence, collagenase triple helicase activity is distinct and separable from proteolytic hydrolysis; the linker hemopexin C domain interactions induce structural perturbations in collagen and so initiate triple heli-
case activity, and active site interactions with the collagen are necessary to complete the triple helicase mechanism. Our present report characterizes the pivotal importance of S₃’ in the active site for full MMP-8 triple helicase activity.

The Y189F mutation demonstrated a specific function for hydrogen bonding to collagen in MMP-8 collagenolytic activity. Compared with wild type MMP-8, the Phe substitution reduced collagen degradation by ~3-fold while only reducing synthetic peptide substrate cleavage activity ~10%. Therefore, it is likely that in Tyr¹⁸⁹ the phenolic hydroxyl forms a hydrogen bond with the collagen substrate that is critical for triple helicase activity but not collagen α-chain cleavage. No mutation of this residue in any collagenase had been reported until our study was in progress. In MMP-1, mutation of Tyr¹⁸⁹ to Thr resulted in a more than 75% decrease in collagenolytic activity (41), a result consistent with our data. Tyr¹⁸⁹ is strictly conserved through the MMP collagenases (MMP-1, -8, -13) and is also present in the collagen-degrading gelatinase (MMP-2), but interestingly, the Tyr does not occur in the membrane-type MMPs. We used structural alignments from the three-dimensional structure of MT1-MMP (58) to override the ClustalW sequence alignment to reveal that the MT1-MMP structural homologue of the Tyr is Asn¹²¹ rather than Leu¹⁰⁸ as predicted by the program (Fig. 2B). Hence, the polarity and hydrogen bonding potential to substrate is retained at this position in MT1-MMP supporting the importance of hydrogen bonding at position 189 in MMP-8 as a key collagenolytic specificity determinant.

Structural studies have revealed the rare occurrence of the cis bond connected to the collagenase-specific Tyr at the start of the active site helix between positions 188 and 189 in MMP-8 (see Fig. 1) (47) and 190 and 191 in MMP-1 (59). Here, Asn¹⁸⁸ in MMP-8 and Glu¹⁹⁰ in MMP-1 replace an otherwise conserved Gly found in all other MMPs exhibiting a trans bond (see Fig. 2B). Even MMP-2 and MMP-9 have the conserved Gly immediately after the insertion point of the three fibronectin type II modules in the collagen binding domain (34). Glycine is the only structural fit for a trans bond that turns the loop between strand V of the catalytic domain and the active site helix (see Fig. 1). Because of these structural constraints the side chain of non-Gly residues at position 188 can only be oriented in a planar manner toward the interior of the active site. The resulting distortion of the protein backbone generates a cis bond. Because of closer contacts between neighboring α-carbons and adjacent side chains, cis peptide bonds are less energetically favorable by ~20 kcal/mol than those in the trans conformation. Because cis bonds may act as an energy reservoir and play a role in enzymatic function (60) such an energy source in S₃’ is an attractive idea to explain the energy requirement of the collagenolytic process (26) or at least the catalytic domain component of triple helicase activity.

Despite insufficient material for crystallographic studies, it is predicted that the N188G mutation may disrupt the 188–189 cis bond by relieving strain and steric clashes in the enzyme structure that was supported by our three-dimensional modeling analyses. The ~25% decrease in catalytic activity of the N188G mutant is similar for both type I collagen and the collagen α-chain peptide substrate analogue. So rather than having a specific collagenolytic role, our data indicates that it is the nature of the residues at 188 and 189 and not the high energy cis bond that is key. Hence, the cis bond is a structural consequence of this specificity requirement rather than being of primary importance in triple helicase activity. Indeed, the other collagenolytic MMPs do not have a S₃’ cis bond adding support to our conclusion. Beyond the scope of our present studies, structural studies are needed to absolutely confirm disruption of the cis bond in the Gly variant and its preservation in the other mutants generated.

The importance of hydrogen bonding potential to substrate at position 188 was supported by the mutant N188L. The side chain of the Leu substitution is of similar size to that of the Asn and three-dimensional modeling shows a preservation of the cis bond. But as evidenced by a similar 60–70% reduction in cleavage of collagen and peptide substrates, the mutation N188L shows that the introduction of energetically unfavorable changes in side chain polarity at position 188 to a residue that cannot form hydrogen bonds perturbs important stabilizing interactions with bound substrate at the S₃’ subsite. The N188K mutant further revealed the importance S₃’-P₃’ complementarity for efficient catalytic activity of MMP-8. Modeling indicated that the cis bond is also retained by this substitution and that placement of a large positively charged side chain reduces the size of the S₃’ pocket. This side chain change leads to a 22% decrease in peptide hydrolysis but not collagen cleavage indicating that the smaller pocket led to clashes with the P₃’ Ala in the simple peptide substrate but not the smaller P₃’ Gly in collagen. Potentially, charge repulsion between the MMP-8 Lys¹⁸⁸ and the peptide P₃’ Arg may also have contributed to this result. These data are consistent with substitutions of P₃’ Gly with the larger Arg or Met residue in a synthetic peptide substrate modeling the α2(I) collagen chain, which decreased activity 3-fold (45), supporting the importance of topological fit between the S₃’ subsite and P₃’ for catalysis.

Between Tyr¹⁸⁸ and the active site Zn²⁺ lies Asn¹⁹⁰. Asn is a helix capper that forms hydrogen bonds with free amides when at the end of α-helices leading to the increased structural stability of the helix. The side chain of Asn¹⁹⁰ hydrogen bonds to Val¹⁹⁴ in helix B supplementing a backbone hydrogen bond to Leu¹⁹³ and to Tyr²¹⁸. Loss of the α-helical hydrogen bond by substitution with a less preferred Leu is a significant destabilizing mutation in this molecule that was apparent from the inability to purify and characterize the N188L mutant due to precipitation problems. Hence, internal hydrogen bonding by the S₃’ Asn¹⁹⁰ is an important structural requirement in MMP-8.

Our findings reveal that in the context of the overall fold of the MMP-8 catalytic domain, the S₃’ residues and associated cis bond are both required for efficient triple helicase activity. The polar nature of the S₃’ residues, the closely packed side chains and potential to form hydrogen bonds with substrate are critical for defining and improving collagenolytic activity of MMP-8. The dual hydrogen bonds formed with substrate at S₃’ are positioned this way by the cis bond and may anchor collagen to the active site to complete the triple helicase process. Because collagen binding to the hemopexin C domain and linker initiates localized distortion of the triple helix, we suggest that the simultaneous fulfilling of the binding propensity of a third collagen binding site at S₃’ only occurs dynamically by further structural changes in the collagen, changes that now fully open up the triple helix to allow engagement by the active site of an α-chain for scission. Although resolving the molecular determinants of the collagenase-collagen interaction in the S₃’ subsite of the MMP-8 active site casts important clues in the understanding of triple helicase activity, a clear answer to this elusive collagenolytic mechanism is likely only to be forthcoming by determination of the three-dimensional structure of the collagen triple helix bound to full-length enzyme. Nonetheless, the recent advances in this area point to S₃’ and exosites in the cleft formed by the linker hemopexin C domain and the catalytic domain that may be targeted by next generation substrate-specific collagenase exosite inhibitor drugs (6).

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