2 Substrate Specificity of MMPs

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CONTENTS

INTRODUCTION
ACTIONS ON PROTEIN SUBSTRATES
PEPTIDE SUBSTRATE SPECIFICITY
CONCLUSIONS

1. INTRODUCTION

Matrix metalloproteinases (MMPs) are secreted or cell surface-bound zinc metalloendopeptidases that act on extracellular matrix (ECM) macromolecules. Thus, isolated MMPs have been tested against various components of ECM. Based on similarities in primary structure and the abilities to cleave ECM components, MMPs are grouped into collagenases, gelatinases, stromelysins, membrane-type MMP(MT-MMPs), and others which do not belong to those subgroups. Most MMPs consist of four typical domain structures: propeptide, catalytic, linker region, and a C-terminal hemopexin-like domains. The catalytic domain share structural similarity with interstitial collagenase (MMP-1). The propeptide domain has least similarities among MMPs, all but except MMP-23 *(1)* have the so-called cysteine switch sequence motif, PRCG[V/N]PD, whose cysteinyl residue ligates the catalytic zinc atom of the active site as the fourth ligand and maintain inactive proenzyme. Another conserved sequence is the zinc binding motif HEXGHXXGXXH, in which three histidines bind to Zn^{2+}. Three dimensional structures of the catalytic domains of MMPs [MMP-1 *(2–6),* MMP-3 *(7–9),* MMP-7 *(10),* MMP-8 *(11,12),* MMP-14 *(13)*] indicate that the polypeptide fold of the catalytic domains are essentially identical, although their substrate specificities are sufficiently different when peptide substrates were tested *(14).* In addition, the action of MMPs on natural protein substrates is not only dectated by the subsite requirement of the

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catalytic domains, but it is often influenced by the domains other than the catalytic domain. This chapter describes activities of MMPs on natural substrates and substrate specificity based on synthetic substrates.

2. ACTIONS ON PROTEIN SUBSTRATES

Earlier studies distinguished collagenases that degrade triple helical region of interstitial collagens *(15)* and gelatinases *(16,17)* that ready degrade denatured collagens (gelatins). A third metalloproteinase (MMP-3/stromylsin 1) was characterized for its ability to degrade various noncollagenous ECM components such as core protein of aggrecan, fibronectin, laminin, and type IV collagen *(18–20).* Recent studies, however, have revealed that the actions of those enzymes are not restricted to those substrates but they can act on a large number of native protein substrates. Currently twenty-one vertebrate MMPs have been identified, and their potential substrates are listed in Table 1.

2.1. Collagenases

Collagenases are characterized for their abilities to cleave triple-helical regions of interstitial collagnes (types I, II, and III) at a site about three-fourths away from the N-terminus. Four MMPs belong to this subgroup: MMP-1 (interstitial collagenase/collagenase 1), MMP-8 (neutrophil collagenase/collogenase 2), MMP-13 (collagenase 3), and MMP-18 (collagenase 4). They consist of propeptide, catalytic domain, hinge region, and the C-terminal hemopexin-like domain, and they are about 50–55% identical in sequence. To express collagenolytic activity, they have to retain both the catalytic and hemopexin domains (the full-length enzyme), although the catalytic domain alone has proteolytic activity against other protein substrates and synthetic substrates. Those properties have been shown for MMP-1 *(21,22),* MMP-8 *(23),* and MMP-13 *(24).* A similar principle probably applies to MMP-18. Nonetheless, it is not clear how the hemopexin domain help to cleave triple-helical collagens because the isolated hemopexin domains of MMP-8 and MMP-13 does not bind to collagen *(23,24),* although the hemopexin domain of MMP-1 was reported to bind to type I collagen *(22).* The hemopexin domain has two conserved cysteines that are disulfide bonded. Mutation of those cysteines to alanines *(25)* or reduction and alkylation destroys collagenolytic activity (K. Suzuki and H. Nagase, unpublished results). The three dimensional structures of collagenases *(2–6,11,12,26,27)* indicate that the active center has an extended substrate binding site about 5Å wide, but it is too narrow to accommodate a triple-helical structure of about 15 Å wide diameter *(28).* This suggests that the binding of collagenases to collagen must partially unwind triple helix to allow cleavage of the individual α chains. Since heat-denatured collagens (gelatins) are poorer substrates at 37°C *(29),* interaction of collagenase to interstitial collagen must induce conformational change in α chain of collagen that fits the substrate bind-

ing site of the catalytic domain, but the molecular basis of this mechanism is not known. The model of this triple helicase activity proposed by Gomis-Rüth et al. *(30)* suggests that the hemopexin domain folds over the catalytic site, sandwiching and trapping the triple-helical collagen molecule, and partially unwinding the triple helix. On the other hand, de Souza et al. *(31)* have hypothesized that the prolin-rich hinge region, which contains a repeated motif (Pro-Xaa-Yaa), might adopt a poly-proline II-like conformation. Their energy-minimized model of the complex of collagen triple helix and collagenase linker region proposes a "proline zipper-like" alignment between the linker and the two helix of the collagen, but experimental evidence for such interaction has not been provided.

Although all collagenases cleave interstitial collagens, each collagenase exhibit different preferences on collagen types. Table 2 lists the kinetic studies of three collagenases. Human MMP-1 degrade human collagens in the following preference: type III > type I >> type II. Whereas human type III collagen is most readily cleaved by MMP-1, guinea pig type III is a poorer substrate, suggesting that the action of MMP-1 on a particular collagen type may depend on species. Type II collagen is a poor substrate for MMP-1. However, MMP-13 digests type II collagen more readily than MMP-1 or MMP-8 (Table 2). Recently, Billinghurst et al. *(32)* reported that MMP-1, MMP-8, and MMP-13 initially cleave the Gly775-Leu776 bond of type II collagen then the Gly778-Gln779 bond of the C-terminal fragment. MMP-13 also digests aminotelopeptides of type I collagen and depolymenzes the crosslinked fibrillar collagen *(33)*. This action is primarily due to the catalytic domain of MMP-13 *(33,34)*. Digestion of types IV, IX, X, and XIV by MMP-13 does not require the hemopexin domain *(24)*. The best natural substrate of MMP-1 is α_2-macroglobulin ($\alpha_2 M$) with the association rate constant $k_2/K_i = 280 \times 10^4\ M^{-1}\mathrm{s}^{-1}$, which is approx 150-fold better substrate compared with human type I collagen ($k_{cat}/K_m = 1.8 \times 10^4\ M^{-1}s^{-1}$) *(35)*.

As listed in Table 1, collagenases cleave a number of other ECM components and serum proteins.

2.2. Gelatinases

Gelatinase A (MMP-2) and gelatinase B (MMP-9) are in this subgroup, and both enzymes readily degrade heat-denatured collagens. These enzymes were once referred to as "type IV collagenases" because of their abilities to cleave type IV collagen, but their activity on type IV collagen is much weaker than that on type V collagen *(36,37)*. Senior et al. *(38)* reported that both gelatinases have elastolytic activity, and on a molar basis recombinant MMP-9 was 30% as active as human leukocyte elastase in solubilizing elastin and similar to that of macrophage metalloelastase (MMP-12) *(39)*. Both gelatinases possess three fibronectin type II-like repeats inserted in tandem just before the catalytic zinc

Table 1
Protein Substrates for the Matrix Metalloproteinases

Enzyme	*MMP no.*	*Substrates*
Collagenases		
Interstitial collagenase (collagenase 1)	MMP-1	Collagens I, II, III, VII, VIII[a], X, and XI[b], gelatin, Clq[c], entactin, tenascin, aggrecan, link protein[d], fibronectin[e], vitronectin[f], myelin basic protein[g], α_2-macroglobulin[h], ovostatin[h], α_1-proteinase inhibitor[i], α_1-antichymotrypsin[i], IL-1β[j], proTNF-α[k], IGFBP-3[l], casein[m], proMMP-2, proMMP-9
Neutrophil collagenase (collagenase 2)	MMP-8	Collagen I, II, and III, Clq[e′], aggrecan, α_2M[f′], ovostatin[g′], α_1PI[h′], substrate P[i′]
Collagenase 3	MMP-13	Collagen I[w′], II[w′], III[w′], IV[x′], IX[x′], X[x′] and XIV[x′], gelatin[z′], collagen telopeptides[y′], Clq[a″], fibronectin[x′], SPARC[x′], aggrecan[b″], α_2M[c″], casein[y′]
Collagenase 4 (*Xenopus*)	MMP-18	Collagen I[g″], gelatin[g″]
Gelatinases		
Gelatinase A	MMP-2	Collagen I, III, IV, V, VII and X, gelatin, fibronectin, laminin, aggrecan, link protein[d], elastin, vitronectin[f], tenascin[p], SPARC[q], decorin[r], myelin basic protein[g], α_1PI[s], α_1-antichymotrypin[s], IL-1β[j], proTNF-α[k], IGFBP-3[l], substance P[t]
Gelatinase B	MMP-9	Collagen IV, V, XI[k′], XIV[l′], elastin, aggrecan, link protein[d], decorin[r], laminin[n′], entactin, SPARC[q], myelin basic protein[m′], α_2M[n′], α_1PI[i′], IL-1β[j], proTNF-α[k], substrate P[i′], casein[o″]
Stromelysins		
Stromelysin 1	MMP-3	Collagen III[u], IV, V[u], IX, X and XI, teropeptides (collagen I and II), gelatin, aggrecan, link protein[d], elastin, fibronectin, vitronectin[f], laminin, entactin[w], tenascin, SPARC[q], decorin[r], myelin basic protein[g], α_2-macroglobulin[h], ovostatin[h], α_1-PI[s], α_1-antichymotrypsin[s], IL-1β[j], proTNF-α[k], IGFBP-3[l], substance P[x], T kininogen[y], casein[z], proMMP-1, proMMP-3[a′], proMMP-8, proMMP-9
Stromelysin 2	MMP-10	Collagen III[u], IV and V[u], gelatin, fibronectin, elastin, aggrecan, link protein[d], casein[p′], proMMP-1, proMMP-7[q″], proMMP-8, proMMP-9[q″]

MT-MMPs		
MT1-MMP	MMP-14	proMMP-2, Collagen I[d''], II[d''], and III[d''], gelatin[e''], fibronectin[d''], vitronectin[d''], laminin[d''], entactin[e''], aggrecan[f''], α_2M[f''], α_1PI[f''], proTNF-α[f''], decorin[r]
MT2-MMP	MMP-15	ProMMP-2[i''], laminin[f''], fibronectin[f''], tenascin[f''], entactin[f''], aggrecan[f''], perlecan[f''], proTNF-α[f'']
MT3-MMP	MMP-16	ProMMP-2[k'']
MT4-MMP	MMP-17	Not known
MT5-MMP	MMP-24	Gelatin[e''], proMMP-2[l'']
Others		
Matrilysin	MMP-7	Collagen IV, gelatin, aggrecan, link protein[d], elastin, fibronectin, vitronectin[f], laminin[b'], SPARC[q], enactin, docorin[r], myelin basic protein[g], tenascin, fibulin-1 and −2[h''], proTNF-α[k], casein[c'], α_1-PI[d'], proMMP-1, proMMP-2, proMMP-9
Stromelysin 3	MMP-11	Collagen IV, gelatin[q'], fibronectin, laminin, aggrecan, α_1PI[r'], α_2M[r']
Metalloelastase	MMP-12	Elastin, collagen IV[t'], gelatin[s'], fibronectin[t'], vitronectin[t'], laminin[t'], entactin[t'], aggrecan[t'], myelin basic protein[s'], α_2M[u'], α_1PI[v'], proTNF-α[s']
	MMP-19	Gelatin[m''], large tenascin C[m''], aggrecan[m'']
Enamelysin	MMP-20	Amelogenin[n'']
XMMP (*Xenopus*)	MMP-21	Not known
CMMP (Chicken)	MMP-22	Caseine[o''], gelatin[o'']
	MMP-23	Autoproteolysis of proMMP-23[p''], Mca-peptide[p'']

[a]Sage et al. *(106)*; [b]Gadher et al. *(107)*; [c]Menzel and Smolen *(108)*; [d]Nguyen et al. *(109)*; [e]Fukai et al. *(110)*; [f]Imai et al. *(111)*; [g]Chandler et al. *(112)*; [h]Enghild et al. *(35)*; [i]Desrochers et al. *(113)*; [j]Ito et al. *(114)*; [k]Gearing et al. *(115)*; [l]Fowlkes et al. *(116)*; [m]Cawston and Taylor *(117)*; [p]Siri et al. *(118)*; [q]Sasaki et al. *(119)*; [r]Imai et al. *(120)*; [s]Mast et al. *(121)*; [t]Nakagawa and Debuchi *(122)*; [u]Nicholson et al. *(50)*; [w]Mayer et al. *(123)*; [x]Stein et al. *(124)*; [y]Sakamoto et al. *(125)*; [z]Chin et al. *(19)*; [a']Nagase et al. *(83)*; [b']Miyazaki et al. *(126)*; [c']Quantin et al. *(127)*; [d']Sires et al. *(128)*; [e']Fletcher et al. *(129)*; [f']Murphy et al. *(130)*; [g']Kudo et al. *(131)*; [h']Desrochers et al. *(132)*; [i']Diekmann and Tschesche *(133)*; [k']Hirose et al. *(134)*; [l']Sires et al. *(135)*; [m']Gijbels et al. *(136)*; [n']Morodomi et al. *(37)*; [o']Lyons et al. *(137)*; [p']Sanchez-Lopez et al. *(138)*; [q']Murphy et al. *(70)*; [r']Pei *et al. (72)*; [s']Chandler et al. *(139)*; [t']Gronski et al. *(39)*; [u']Banda and Werb *(73)*; [v']Banda et al. *(140)*; [w']Welgus et al. *(141)*; [x']Knäuper et al. *(24)*; [y']Lamaître et al. *(34)*; [z']Welgus et al. *(142)*; [a'']Eeckhout et al. *(143)*; [b'']Fosang et al. *(144)*; [c'']Nethery and O'Grady *(145)*; [d'']Ohuchi et al.*(67)*; [e'']Imai et al. *(146)*; [f'']D'Ortho et al. *(68)*; [g'']Stolow et al. *(147)*, [h'']Sasaki et al. *(148)*; [i'']Butler et al. *(61)*; [k'']Takino et al. *(62)*; [l'']Pei *(63)*; [m'']Murphy et al. *(149)*; [n'']Llano et al. *(78)*; [o'']Yang and Kurkinen *(80)*; [p'']Velsaco et al. *(82)*; [q'']Nakamura et al. *(57)*

The list is after Sang and Douglas *(105)* unless otherwise noted.

Table 2
Kinetic Parameters of Collagenases (at 25°C, pH7.5)[a]

Enzyme	*Substrate*	K_m (μM)	k_{cat} ($s^{-1} \cdot 10^3$)	k_{cat}/K_m ($M^{-1}\, s^{-1} \times 10^{-3}$)	*Ref.*
MMP-1 (human)	Type I (human)	0.8	15	18	*(150)*
	Type I (calf)	0.8	9.5	12	*(150)*
	Type I (guinea pig)	0.9	6.0	7.0	*(150)*
	Type I (rat)	0.9	5.5	6.0	*(150)*
	Type II (human)	2.1	0.28	0.13	*(150)*
	Type II (calf)	1.6	0.75	0.47	*(150)*
	Type II (rat)	1.1	1.3	1.1	*(150)*
	Type III (human)	1.4	160	69	*(150)*
	Type III (guineas pig)	0.7	5.0	7.2	*(150)*
	α2-Macroglobulin (human)	0.17	483	2800	*(35)*
	Ovostatin (chicken)	0.32	0.17	1.8	*(35)*
MMP-8 (human)	Type I (human)	0.7	1.8	2.5	*(151)*
	Type II (human)	1.1	0.65	0.59	*(151)*
	Type III (human)	1.8	0.23	0.13	*(151)*
MMP-13 (human)	Type I (human)	ND	ND	ND	
	Type II (human)	2	6.3	3.9	*(152)*
	Type III (human)	ND	ND	ND	
MMP-13 (rat)	Type I (human)	1.1	3.8	3.4	*(141)*
	Type II (human)	0.9	3.9	4.3	*(141)*
	Type III (human)	1.7	5.6	3.2	*(141)*

[a]ND: not determined

binding site. Those repeats are responsible for binding of MMP-2 and MMP-9 to gelatin *(40,41),* type I collagen *(42,43),* collagen IV and laminin *(44).* Deletion of the fibronectin-like domains from MMP-2 reduced the gelatinolytic activity and altered the digestion patterns of type IV collagen, although it did not influence the activity on a synthetic substrate *(45).* MMP-2 and MMP-9 lacking the fibronectin domains did not exhibit elastase activity *(46).* Matrilysin (MMP-7) and macrophage metalloelastase (MMP-12) lack fibronectin domain, but they possess elastolytic activities. Aimes and Quigley *(47)* reported that MMP-2 digests native type I collagen at the Gly775-Ile776/Leu776 bond and generates the ¾- and ¼-fragments characteristic of vertebrate collagenases. The k_{cat} and K_m values of MMP-2 against type I collagen were 4.4×10^{-3} sec^{-1} and 8.5 μM, similar to those of MMP-1 *(47).* Both gelatinases digests many other ECM components and soluble proteins. Cleavage of γ_2 chain of laminin 5 induces migration of normal breast epithelial cells *(48).*

2.3. Stromelysins

Three MMPs are coined stromelysins, i.e., stromelysin 1 (MMP-3), stromelysin 2 (MMP-10), and stromelysin 3 (MMP-11). The enzymic activity and amino acid sequence of stromelysin 3 are significantly diverse from those of stromelysin 1 and 2. Thus, it is appropriate to place stromelysin 3 in the uncategorized subgroup.

MMP-3 and MMP-10 in humans are 79% identical in amino acid sequence and they are about 55% identical with MMP-1. However, neither of the enzymes are able to cleave triple-helical region of type I and II collagens. A weak activity was reported on type III collagen *(49,50).* Both enzymes degrade a similar ECM component although the information of MMP-10 is limited (Table 1). In addition, the catalytic efficiency of MMP-10 is lower than that of MMP-3 *(50–52).* Unlike collagenases, the catalytic domain of MMP-3 (28 kDa) and the full-length MMP-3 (45 kDa) have indistinguishable substrate specificities against protein substrates so far tested *(20).* MMP-3 exhibits an acidic pH optimum activity around 5.5–6.0 for digestion of aggrecan and synthetic substrates, but it retains about 30–50% activity at pH 7.5 *(49,53).* MMP-10 has optimal activity against Azocoll and synthetic substrates around pH 7.5–8.0 (K. Suzuki and H. Nagase, unpublished results). Both MMP-3 and MMP-10 participate in activation of proMMP-1, proMMP-7, proMMP-8, and proMMP-9 *(54–58).* MMP-3 activates proMMP-13 *(59).*

2.4. MT-MMPs

The first member of the MT-MMP subgroup was discovered by Sato et al. *(60)* and identified as the cell surface bound activation of proMMP-2. Currently five MT-MMPs have been identified and these enzymes have a single transmembrane and cytosolic domains after the hemopexin domains. In addition to

MT1-MMP, MT2-MMP *(61),* MT3-MMP *(62),* and MT5-MMP *(63)* were also shown to activate proMMP-2. Studies by Strogin et al., *(64),* Butler et al., *(65)* and Kinoshita et al., *(66)* suggests that the cell surface activation of proMMP-2 by MT1-MMP requires the formation of a ternary complex, proMMP-2-TIMP-2-MT1-MMP on the cell surface which allows proMMP-2 to be correctly oriented and activated by free MT1-MMP. Such mechanism is considered to accumulate MMP-2 activity and locally express proteolytic activity. In addition to the ability activating proMMP-2, the soluble form of MT1-MMP was shown to digest interstitial collagen types I, II, and III into ¾- and ¼-fragments, but it is about 5–7-fold less efficient in cleaving type I collagen than MMP-1 *(67).* Several other ECM components are also digested by MT1-MMP and MT2-MMP *(68).* D'Ortho et al. *(68)* also reported that proTNF-α was processed by those two MT-MMPs. Localization of MT-MMPs restricts their actions on extracellular proteins near the cell surface.

2.5. Matrilysin

Matrilysin (MMP-7), consisting of a propeptide and a catalytic domain, is the smallest member of the family. MMP-7 digests a number of ECM components and nonmatrixin proteins, and its activity tends to be more proteolytic than that of MMP-3 *(51).* Both human and rat MMP-7s have similar activities on protein substrate but a major difference is that the human enzyme digests elastin at a reasonable rate (190 μ*g/h/nmol* of enzyme) *(51),* but little elastolytic activity is detected with the rat enzyme *(69).*

2.6. Stromelysin 3

Only weak activity was reported to digest collagne IV, gelatin, fibronectin, laminin, and agrecan with mouse stromelysin 3 (MMP-11), but human MMP-11 expressed myeloma cells is unstable *(70).* This instability of human enzyme is considered to be due Ala235 in place of the conserved Pro at this position immediately after the "Met-turn" that form a hydrophobic basis for the catalytic zinc atom in the metzincins *(71).* Pei et al. *(72)* reported that MMP-11 cleaved α_1PI synthesized by breast cancer cell line MCF-7. It also cleaves α_2*M (72).*

2.7. Metalloelastase

Metalloelastase (MMP-12) was first purified from the conditioned medium of mouse macrophage and characterized as a metal-dependent elastase *(73).* Recombinant human MMP-12 is approx 30% as active as human leukocyte elastase in solubilizing elastin *(39).* The predicted molecular mass of the human MMP-12 is 54 kDa which contains the hemopexin domain. However, the enzyme readily undergoes N-terminal and C-terminal processing to the 22 kDa form which degrades insoluble elastin. Therefore, the C-terminal hemopexin

domain does not appear to be an essential component for the elastolytic activity of MMP-12. In addition to elastin, the enzyme digests various other ECM components, α_1PI and processes proTNFα (Table 1).

2.8. Other MMPs

This group includes more recently discovered MMPs, i.e., MMP-19, MMP-20, MMP-21, MMP-22, and MMP-23. Thus, characterization of their enzymic actions on ECM components and other substrates is very limited.

MMP-19 was first identified by cDNA cloning and reported as MMP-18 by Cossins et al. *(74).* Later, it is designated MMP-19 following collagenase 4 in *Xenopus* as MMP-18. The enzyme was also recognized as autoantigen by autoantibodies found in sera of patients with rheumatoid arthritis and systemic lupus erythematosus, and it was detected on the surface of activated peripheral blood monocytes, Th1 lymphocytes, and Jurkat T lymphocytes *(75).* The recombinant enzyme hydrolyzes Mca-Pro-Leu-Ala-Nva-His-Ala-Dpa-NH_2 with $k_{cat}/K_m = 1.96 \times 10^4\ M^{-1}\ s^{-1}$ and Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 with $k_{cat}/K_m = 1.3 \times 10^3\ M^{-1}\ s^{-1}$ *(76).* It also digests gelatin, large tenascin C, and aggrecan *(149).*

MMP-20 (enamelysin) is expressed by ameloblasts and odontoblasts immediately prior to the onset of dentin mineralization and continues to be expressed throughout the secretory stage of amelogenesis *(77).* The recombinant MMP-20 digests amelogenis *(77,78),* suggesting that the enzymes poly a role in enamel and dentin formation.

MMP-21 and MMP-22 were found in *Xenopus (79)* and chicken, respectively. MMP-21 (XMMP) mRNA is not detected in the blastula stage embryo, but expressed in gastrula stage and neurula stage embryos and down-regulated in pretailbud embryo *(79).* However, the enzymic activity of MMP-21 was not reported. MMP-22 (CMMP) was found in chicken *(80).* It is structurally related to collagenases, but it has a unique cysteine in the catalytic domain which is also found in MMP-19 and XMMP (MMP-21). Recombinant MMP-22 digests gelatin and casein *(80).*

Two human MMP genes, originally designated as MMP-21 and MMP-22 *(81),* were identified in two identical genomic regions in human chromosome 1p36.3, each of which contains a MMP gene in a tail-to-tail configuration *(1).* This duplicated MMP gene, now designated as MMP-23 *(82),* encodes an MMP with unique structural motifs. It lacks a recognizable signal sequence and has a short prodomain without a typical cysteine switch sequence. It also lacks a hemopexin domain. Instead, it has cysteine-rich, proline-rich, and IL-1 receptor like regions after the catalytic domain *(1,82).* The recombinant catalytic domain of MMP-23 hydrolyzes Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, but not gelatin *(82).* TIMP-1 prevents the autolysis of the proenzyme *(82).*

3. PEPTIDE SUBSTRATE SPECIFICITY

One of the goals of peptide-based specificity studies is to gain insights into the behavior of enzymes toward their natural substrates. However, peptide sequence specificities of MMPs do not necessarily match with protein specificities. This is due to conformational constraint of a potentially susceptible sequence in proteins. In addition, short peptides do not adopt a specific secondary structure and therefore they may be more readily accommodated in the substrate-binding groove of the enzyme. For example, MMP-3 cannot activate its proenzyme by cleaving the His-Phe bond of Asp-Val-Gly-His82#Phe83-Arg-Thr-Phe (# indicate the bond cleaved) in the junction of the propeptide and the catalytic domain in its native conformation, whereas this bond is readily cleaved by MMP-3 when an N-terminal part of the propeptide is removed by a proteinase *(83).* Collagen sequence-based peptides are cleaved by most MMPs, but only a limited number of MMPs can cleave the triple-helical region of native collagens. On the other hand, although the Gly679-Leu680 in the bait region of α_2M is cleaved by MMP-1 very rapidly, the oligopeptide containing the corresponding sequence is a poor substrate of MMP-1 *(84).* Nonetheless, substrate specificity studies with peptides give us insights into the interaction of the substrate and the active site of the enzyme. The activity toward peptide is usually restricted to the catalytic domain. The truncation of the C-terminal hemopexin domain has little effect on peptide hydrolysis.

3.1. Substrate Binding Sites of MMPs

Three-dimensional structures of catalytic domains of MMPs revealed that their polypeptide folds are essentially superimposable and that all have a five stranded β-sheet, three α-helices, two zinc ions, and 1-3 calcium ions. The active site cleft is bordered by the 4th β-strand (strand IV), the 2nd α-helix, and a subsequent stretch of random coil. Based on the structure of MMP-8 catalytic domain with inhibitors Pro-Leu-Gly-NHOH that binds to the unprimed subsite (S site) of the enzyme *(11)* and 2-benzyl-3-mercaptopropanoyl-Ala-Gly-NH_2 that binds to the primed susbsite (S′ site) *(85),* Grams et al. *(85)* modeled the mode of interaction of a hexapeptide and the active site of MMP-8. In this model the peptide lies antiparallel to the edge strand IV by forming a number of hydrogen bonds. The N-terminal Pro at P_3 (the third residue on the left of the scissile bond) interacts with the hydrophobic cleft formed by side chains of His162, Ser151, and Phe164, whereas Leu at P_2 (the second residue on the right of the scissile bond) interacts with a shallow groove lined by His201, Ala206, and His207. The dominant interactions between the peptide and the enzyme are made by the phenyl side chain of the inhibitor and the large hydrophobic S_1' pocket (located to the right of the catalytic zinc) formed by side chains of His197, Val194, Tyr219, and the main chain segment of Pro217-Asn-Tyr219. The side chain of P_2' Ala points away from the enzyme and the C-terminal, P_3' Gly is located crossover segments Gly158-Leu160 and Pro217-Tyr219.

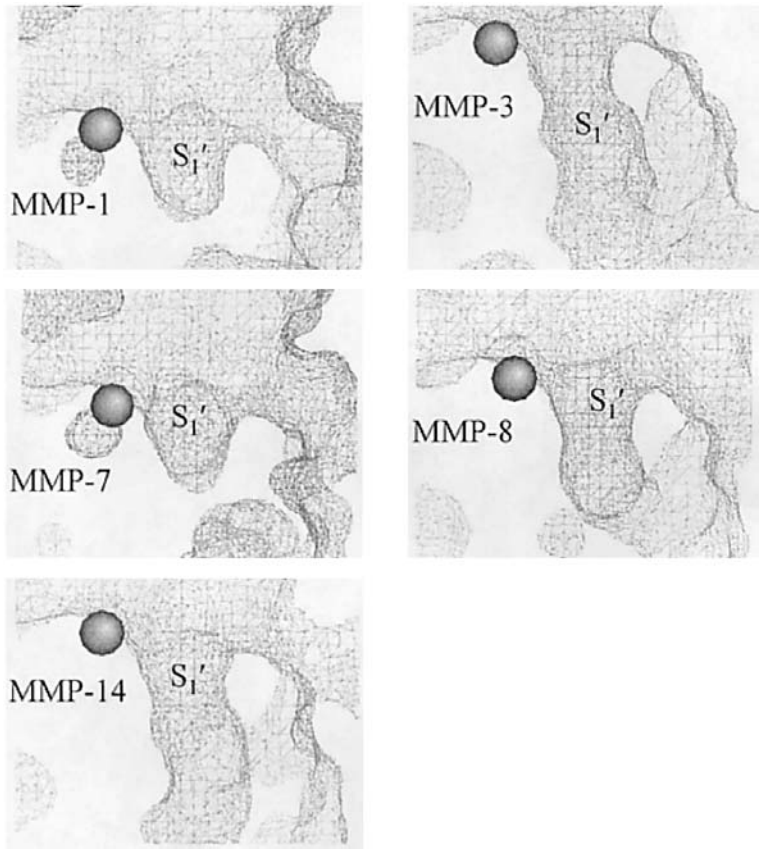


Fig. l. Comparison of the S_1' pockets. Mesh representation of the molecular surface shows S_1' pocket of MMP-1, MMP-3, MMP-7, MMP-8, and MMP-14. Images are prepared from the Brookhaven Protein Data Bank entry 1hfc (MMP-1), 1hfs (MMP-3), 1mmp (MMP-7), 1 jan (MMP-8) and 1BQQ (MMP-14) using the program GRASP v1.2 *(104).*

The most notable difference among MMP catalytic domains is the size of the S_1' specificity pocket (Fig. 1). Comparing between MMP-1 and MMP-8, the latter has the larger S_1' pocket. The main difference is due to the side chain of Arg195 in MMP-1 which delimits the S_1' pocket by projecting out toward the catalytic zinc, whereas Leu193 is found in the corresponding site in MMP-8. Leu193 enters the S_1' pocket but it orients away from the catalytic zinc. MMP-3 and MT1-MMP also have Leu at this position and form a very large, predominately hydrophobic S_1' pocket *(8,13).* MMP-7 has Tyr193 in the corresponding position, which limits the size of S_1' pocket *(10),* the enzyme prefers residues with aliphatic or aromatic side chain in the P_1' position *(86).* Mutaiton of Tyr193 to Leu in MMP-7 altered the P_1' specificity making it sim-

ilar to that of MMP-3, and reversal results were obtained with an MMP-3 (L197Y/V194A) mutant *(87)*. The S_1' pocket of MMP-2 and MMP-9 are thought to have a similar dimension as that of MMP-8 *(88)*.

Although the side chain of P_1' position is critical for substrate recognition, other subsites also influence substrate specificity of various MMPs (*see* Table 3).

3.2. Specificity Studies with Peptides

Initial peptide substrate studies were carried out with tadpole collagenase by Nagai and colleagues *(89,90)*. They were 6–8 amino acid residues based on collagenase cleavage site in α-chains of types I and III collagen. Weingarten and associates explored subsite preference of MMP-1 and MMP-2 *(91–94)* using a series of peptides, peptolides and peptide esters. Comprehensive sequence specificities of six MMPs were examined by Van Wart and associates *(86,95,96)* by measuring k_{cat}/K_m values of more than 45 oligopeptides covering the P_4 through P_4' subsites using the α1(I) collagen sequence Gly-Pro-Gln-Gly#Ile-Ala-Gly-Gln as the starting substrate (*see* Table 3). It is notable that the catalytic efficiency of MMPs depends on the length of the peptide substrate (Table 4). Little activity was detected with peptides less than three residues in either the P site (N-terminal side of the scissile bond) or the P′ site (the C-terminal side of the scissile bond) *(84,86,96)*. Niedzwiecki et al. *(97)* examined requirement of peptide length of the substrate for MMP-3 used more extended peptides, substance P analog, and reported that a peptide containing only three residue in the P site or two residue in the P′ site was not readily cleaved unless the N-terminal and the C-terminal ends are blocked (Table 5). Those results were somewhat different from those with collagen-based peptides but this may be due to extremely low activity of MMP-3 against Gly-Pro-Gln-Gly#Ile-Ala-Gly-Gln ($k_{cat}/K_m = 14.7\ M^{-1}\ s^{-1}$) *(14)* composed with a substance P analog ($k_{cat}/K_m = 1790\ M^{-1}\ s^{-1}$) *(97)*. These studies indicate that MMPs have an extended substrate binding site.

Comprehensive sequence specificity studies of six MMPs provided a number of important insights into differences and similarities in subsite requirement among those enzymes. As discussed earlier, the P_1' subsite plays a critical role. Little activity was detected with peptides with a charged group or proline at this position. Aliphatic side chains at P_1' position provide good substrates for all MMPs. Leu, Ile, and Met at this position are favored by all six enzymes. Aromatic groups are well tolerated by MMP-2, MMP-3, MMP-8, and MMP-9. This is due to relatively large hydrophobic S_1' pocket of these enzymes (Fig. 1). Ser is reasonably well tolerated by MMP-3, but a limited activity was detected with other MMPs.

Using dinitropheny-Arg-Pro-Leu-Ala#Leu-Trp-Arg-Ser-NH_2 peptide, Gronski et al. *(39)* examined the P_1' preference for macrophage metalloelastase

(MMP-12). Their studies indicated preference Leu>>Ala>Lys>Phe>Tyr> Trp>Arg>Ser. It is interesting that Ala-Lys and Ala-Arg bonds are cleaved by MMP-12.

A large enhancement in hydrolysis was observed with P_2' substitution. Crystal structures of MMPs with a peptide inhibitor indicate that the side chain of the P_2' position points away from the enzyme surface, but bulky aromatic side chains are much favored compared with Ala. Arg is well tolerated, but Hyp is a very poor substitution.

Substitution of the P_3' position has provided reasonably selective substrates for different MMPs. For example, Met is favored by MMP-3 and MMP-7, but the peptide with this substitution is about 10-fold less susceptible to MMP-2, MMP-8, and MMP-9. The P_4' substitution does not cause a large influence in specificity.

P_1 substitution for Gly has shown a marked influence in specificity. Ala in this position is most favored by MMP-1, MMP-3, MMP-7, and MMP-8. MMP-8 also well tolerates Glu at this position, but it only increases the rate of hydrolysis by MMP-3 about twofold. Interestingly, Pro at this position is reasonably tolerated for most cases. Bulky hydrophobic side chains are not favored by gelatinases (MMP-2 and MMP-9). Val substitution makes the peptide a poor substrate for most of the enzyme except MMP-3. For the P_2 position, however, aliphatic side chains (Leu, Met, Tyr) are favored by six MMPs. The best residue for the P_3 position for MMPs is Pro. This is also the case with substance P-based substrate for MMP-1 and MMP-3 *(97)*. When the MMP cleaved sites of naturally occurring protein substrate are aligned Pro is frequently found in P_3 position *(14)*.

3.3. Influence of Position 2 Side Chain of TIMP-1 on MMP Inhibition

The crystal structure of the complex formed between the catalytic domain of MMP-3 and TIMP-1 revealed the basic mode of interaction between MMPs and TIMPs and the inhibition mechanism of MMPs by TIMPs *(98)*. Six sequentially separate polypeptide segments of TIMP-1 interact with MMP-3 and the active site of MMP-3 is occupied by the N-terminal Cys1-Val4 and Ala65-Cys70 segments that are disulfide-bonded through Cys1 and Cys70. The α-amino group and peptide carbonyl group of Cys1 interacts with the catalytic Zn^{2+} of the metalloproteinase and the Cys1-Thr-Cys-Val4 segment bind to subsites S_1 to S_3' in a manner similar to a peptide substrate (P_1 to P_3'). The side chain of Thr2 extends into the large S_1' specificity pocket of MMP-3. Ser68 and Val69 occupy the part of S_2 and S_3 subsites, respectively, but they are arranged in a nearly opposite orientation to the P_3-P_2 segment of a peptide substrate.

Because the side chain of position 2 extends into the S_1' pocket, Meng et al. *(99)* generated fourteen variants of the N-terminal domain of TIMP-1 (N-

Table 3
Relative Substrate Specificities of Human MMPs[a,b]

Peptide sequence	*Relative rate*					
	MMP-1	*MMP-8*	*MMP-2*	*MMP-9*	*MMP-3*	*MMP-7*
Gly-Pro-Gln-Gly#Ile-Ala-Gly-Gln	100	100	100	100	100	100
Gly-Pro-Gln-Gly#Leu-Ala-Gly-Gln	130	180	88	80	110	300
Gly-Pro-Gln-Gly#Trp-Ala-Gly-Gln	<0.5	49	<5.0	<5.0	120	<5.0
Gly-Pro-Gln-Gly#Pro-Ala-Gly-Gln	<0.5	<0.5	<5.0	<5.0	<0.5	<5.0
Gly-Pro-Gln-Gly#Glu-Ala-Gly-Gln	<0.5	<0.5	<5.0	<5.0	<0.5	8.0
Gly-Pro-Gln-Gly#Tyr-Ala-Gly-Gln	50	390	50	96	150	21
Gly-Pro-Gln-Gly#Phe-Ala-Gly-Gln	20	46	55	24	140	24
Gly-Pro-Gln-Gly#Met-Ala-Gly-Gln	110	84	230	170	60	89
Gly-Pro-Gln-Gly#Val-Ala-Gly-Gln	9.1	9.0	30	25	53	17
Gly-Pro-Gln-Gly#Gln-Ala-Gly-Gln	28	10	34	20	38	<5.0
Gly-Pro-Gln-Gly#Ser-Ala-Gly-Gln	5.9	1.6	15	<5.0	45	5.5
Gly-Pro-Gln-Gly#Arg-Ala-Gly-Gln	<0.5	<0.5	<5.0	<5.0	<4.9	<5.0
Gly-Pro-Gln-Gly#Ile-Phe-Gly-Gln	360	510	380	390	130	140
Gly-Pro-Gln-Gly#Ile-Trp-Gly-Gln	840	930	310	240	280	330
Gly-Pro-Gln-Gly#Ile-Leu-Gly-Gln	430	400	400	240	280	250
Gly-Pro-Gln-Gly#Ile-Hyp-Gly-Gln	7.3	1.5	32	11	42	8.0
Gly-Pro-Gln-Gly#Ile-Arg-Gly-Gln	180	170	180	200	250	270
Gly-Pro-Gln-Gly#Ile-Glu-Gly-Gln	35	59	85	130	58	86
Gly-Pro-Gln-Gly#Ile-Ala-Val-Gln	100	57	26	49	170	170
Gly-Pro-Gln-Gly#Ile-Ala-Arg-Gln	55	34	35	45	490	220
Gly-Pro-Gln-Gly#Ile-Ala-Met-Gln	130	34	40	35	810	450
Gly-Pro-Gln-Gly#Ile-Ala-Ala-Gln	220	120	180	140	280	300
Gly-Pro-Gln-Gly#Ile-Ala-Ser-Gln	91	58	320	130	230	150
Gly-Pro-Gln-Gly#Ile-Ala-Gly-Ala	86	110	85	110	130	91

Gly-Pro-Gln-Gly#Ile-Ala-Gly-His	91	145	150	120	110	87
Gly-Pro-Gln-Gly#Ile-Ala-Gly-Thr	160	145	59	160	72	100
Gly-Pro-Gln-Met#Ile-Ala-Gly-Gln	200	140	22	12	110	150
Gly-Pro-Gln-Glu#Ile-Ala-Gly-Gln	28	330	15	29	190	170
Gly-Pro-Gln-Tyr#Ile-Ala-Gly-Gln	130	180	58	30	68	34
Gly-Pro-Gln-Ala#Ile-Ala-Gly-Gln	660	320	96	110	300	530
Gly-Pro-Gln-Pro#Ile-Ala-Gly-Gln	260	190	32	46	170	140
Gly-Pro-Gln-Gln#Ile-Ala-Gly-Gln	140	150	25	13	140	180
Gly-Pro-Gln-Phe#Ile-Ala-Gly-Gln	95	170	15	26	68	63
Gly-Pro-Gln-Leu#Ile-Ala-Gly-Gln	27	54	21	8.8	170	49
Gly-Pro-Gln-Val#Ile-Ala-Gly-Gln	5.5	7.9	<5.0	<5.0	32	<5.0
Gly-Pro-Gln-His#Ile-Ala-Gly-Gln	160	50	65	44	87	ND
Gly-Pro-Hyp-Gly#Ile-Ala-Gly-Gln	11	15	32	15	83	17
Gly-Pro-Asp-Gly#Ile-Ala-Gly-Gln	30	44	11	10	89	7.0
Gly-Pro-Val-Gly#Ile-Ala-Gly-Gln	32	30	130	110	160	57
Gly-Pro-Leu-Gly#Ile-Ala-Gly-Gln	150	260	330	290	190	420
Gly-Pro-Arg-Gly#Ile-Ala-Gly-Gln	17	32	160	83	96	13
Gly-Pro-Met-Gly#Ile-Ala-Gly-Gln	160	160	120	180	120	400
Gly-Pro-Tyr-Gly#Ile-Ala-Gly-Gln	200	110	200	150	230	240
Gly-Asn-Gln-Gly#Ile-Ala-Gly-Gln	17	45	60	<5.0	68	25
Gly-Ala-Gln-Gly#Ile-Ala-Gly-Gln	50	23	22	9.4	62	ND

[a]ND: Not determined.
[b]Data are from Refs. *(14,86,95,96)*.

Table 4
Effect of N- and C-terminal Truncation on the Hydrolysis of Collagen Sequence-Based Substrate by MMPs at 30°C, pH 7.5[a]

	Relative rate					
Substrate	*MMP-1*	*MMP-2*	*MMP-3*	*MMP-7*	*MMP-8*	*MMP-9*
P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'						
Gly-Pro-Gln-Gly#Ile-Ala-Gly-Gln	100	100	100	100	100	100
Ac -Pro-Gln-Gly#Ile-Ala-Gly-Gln	110	—	—	—	96	—
Pro-Gln-Gly#Ile-Ala-Gly-Gln	150	62	30	43	100	65
Gln- Gly#Ile-Ala-Gly-Gln	7.3	<5.0	26	8.3	<5.0	<5.0
Gly#Ile-Ala-Gly-Gln	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
Gly-Pro-Gln-Gly#Ile-Ala-Gly	68	60	64	99	54	93
Gly-Pro-Gln-Gly#Ile-Ala	13	<5.0	34	10	<5.0	<5.0
Gly-Pro-Gln-Gly#Ile	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0

[a]The results are after Netzel-Arnett et al. *(86,96)* and Imper and Van Wart *(84)*.

Table 5
Hydrolysis of Synthetic Peptides by MMP-3[a]

Substrate	K_{cat}/K_m $(s^{-1} M^{-1})$	*Relative activity*
P_6 P_5 P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4' P_5'		
Arg-Pro-Lys-Pro-Gln-Gln#Phe-Phe-Gly-Leu-Met-NH_2	1790 ± 140	100
Pro-Lys-Pro-Gln-Gln#Phe-Phe-Gly-Leu-Met-NH_2	800 ± 2	45
Lys-Pro-Gln-Gln#Phe-Phe-Gly-Leu-Met-NH_2	290 ± 84	16
Pro-Gln-Gln#Phe-Phe-Gly-Leu-Met-NH_2	<3	<0.1
Ac-Pro-Gln-Gln#Phe-Phe-Gly-Leu-Nle-NH_2	500 ± 200	28
Arg-Pro-Lys-Pro-Gln-Gln#Phe-Phe-Gly-Leu	1300 ± 91	73
Arg-Pro-Lys-Pro-Gln-Gln#Phe-Phe-Gly	790 ± 120	44
Arg-Pro-Lys-Pro-Gln-Gln#Phe-Phe	<3	<0.1
Arg-Pro-Lys-Pro-Gln-Gln#Phe-Phe-NH_2	1900 ± 380	106

[a]Data are from Niedzwiecki et al. *(97).*

TIMP-1) at this position 2 and examined their affinity toward MMP-1, MMP-2, and MMP-3 (Table 6). The Gly mutant was the weakest inhibitor for all three MMPs. Negatively charged side chains at position 2 are unfavorable for all three MMPs. Nonpolar side chains of increasing size increase the affinity for MMP-2 and MMP-3, except Ile and Phe, but longer aliphatic chains reduced the affinity for MMP-1. A reasonably high affinity of Arg2 mutant with MMP-2 and MMP-3 was found although it was not an effective inhibitor of MMP-1. The unfavorable interaction of MMP-1 with the Arg2 mutant may be reflected by the structural feature of the S_1' specificity pocket of MMP-1 where Arg195 and more cationic environment compared with those of MMP-2 and MMP-3.

The striking features of residue 2 of N-TIMP-1 are that mutation at this site significantly alters the affinity for three different MMPs and that this side chain apparently interacts with the S_1' specificity pocket of MMPs differently from that of the P_1' residue of a peptide substrate. As shown in Table 3, MMPs favor substrate with a large aliphatic or aromatic side chains at the P_1' position. Peptides with Val, Ser, or a charged group at the P_1' position are very poor substrates. However, the best inhibitor for MMP-1 was Val2 mutant, Leu2 mutant for MMP-2 and Ser2 mutant for MMP-3. Meng et al. *(99)* reported that there was a very poor correlation between -log K_i for TIMP mutants and log (k_{cat}/K_m) for peptide substrates with the sequence Gly-Pro-Gln-Gly#X-Ala-Gly-Gln, where the same amino acid was present at the P_1' position (X) of the peptide and residue 2 of the N-TIMP-1 variant. This discrepancy is considered to be due to a greater loss of conformational entropy associated with peptide-MMP interaction a TIMP-MMP interaction. The orientation of residue 2 of TIMP-1 is probably influenced by the relatively rigid structure around the disulfide-bonded Cys1 with the catalytic Zn^{2+}.

Table 6
Inhibition Constants (K_i) of Position 2 Mutants of N-TIMP-1[a,b]

	K_i (*n*M)		
Amino Acid	*MMP-1*	*MMP-2*	*MMP-3*
Thr (wild-type)	3.0	1.1	1.9
ser	14.7	2.1	0.5
Gly	18×10^{3a}	103×10^{3a}	1380
Ala	2090	307	126
Val	1.6	4.5	3.0
Leu	93	1.0	3.2
Ile	262	5.6	20
Met	10.9	0.7	0.7
Phe	42	17	13
Asn	1970	16	44
Gln	870	12	29
Asp	8130	1250	1110
Glu	5730	433	468
Lys	1670	31	70
Arg	5010	12	28

[a] Estimated from the level of inhibition at a concentration of 8 μ*M*.
[b] The results are from Meng et al. *(99)*.

3.4. Zymogen Activiation and Substrate Specificity

Stromelysins (MMP-3 and MMP-10), MMP-2 and MMP-7 play a critical role in activation of procollagenases by cleaving the Gln80-Phe81 bond of proMMP-1 *(54)* and the Gly78-Phe79 bond of proMMP-8 *(55)*. When procollagenases are treated with trypsin or an organomercurial, 4-aminophenylmercuric acetate (APMA), those bonds are not cleaved and only partial (10–25%) collagenolytic activity is detected. This was shown to be autoprocessing of procollagenase at bonds other than the above specific sites. Treatment of proMMP-1 with APMA results in generation of [Met78]MMP-1, [Val82] MMP-1, and [Leu83]MMP-1 (residues in brackets are the N-terminus), but not [Phe81]MMP-1, since Phe81 does not fit with the S_1' specificity pocket of MMP-1. Similarly [Met80]MMP-8 and [Leu83]MMP-8 with partial collagenolytic activity are generated by treatment with a mercurial compound *(55)*. Reduced activity was also reported for MMP-3 when it retained several extra residues before the N-terminal Phe83 or when a few residues are trimmed from the N-terminus *(100)*. This was also associated with changes in substrate specificity. The reduced activity of MMP-3 is due to an increase in K_m and a decrease

in k_{cat} on a synthetic substrate *(100).* It is not known how the differently processed N-termini of MMPs influences the enzyme activity and in some cases substrate specificity, but the crystal structures of the catalytic domains of [Phe79]MMP-8 and [Met80]MMP-8 indicated the structural differences between the two forms: In [Phe79]MMP-8, the ammonium group of Phe79 forms a salt bridge with the carboxylate side chain of Asp232 in the third helix of the catalytic domain *(101),* but without the Phe79 to N-terminal hexapeptide Met80-Leu-Thr-Pro-Gly-Asn85 of MMP-8 is disordered *(11).* However, the geometry of the active site of the two forms are essentially identical. It is therefore postulated that disruption of the salt bridge may alter stabilization of the active site at the transition state or influence the substrate binding to the active site *(101)* but the experimental evidence to support these supposition is not currently available.

4. CONCLUSIONS

Substrate specificity studies of MMPs have been conducted with natural protein substrates as well as synthetic peptides. Those studies have demonstrated that many MMPs have a relatively broader substrate specificity on various ECM components and non-ECM proteins. Although degradation of interstitial collagens were originally thought to be only due to the action of the so-called collagenases, it is now known that at least six different MMPs, including gelatinase A (MMP-2) and MT1-MMP, have the collagenolytic activity. In addition, collagenases do have activities on various noncollagenous proteins. We have also learned that domains other than the catalytic domain play critical roles in expressing proteolytic activities on certain matrix components. Different propeptide processing results in active MMPs with different specific activities and substrate specificities in some cases. Although differently processed forms of MMPs are yet to be identified in the tissue, those points need to be considered in order to interpret biological and pathological implications of MMPs, since MMP activities may be altered by several fold. It should also be stressed that identification of MMP substrates in vivo is difficult. Biochemical studies using isolated macromolecules only suggest possibilities of substrates. For this purpose, antibodies that recognize specifically cleaved ECM components have been used *(32,102,103),* and those studies have been informative.

Studies with a series of synthetic substrates have provided insights into the subsite requirements of MMPs and they were useful in designing active site-directed synthetic inhibitors of MMPs. Those studies have also indicated that the susceptibility of synthetic peptides modeled after the cleavage site of a natural substrate is often different from that of the native protein. More interestingly, recent mutagenesis studies of TIMP-1 indicated that the nature of the side

chain of position 2 in TIMP-1 had a major influence on the affinity for MMPs, but it did not correlate with substrate specificity dictated by the P_1' side chain of substrates. The unique mode of interaction of TIMP with MMPs may provide the way to generate highly selective inhibitors of individual MMPs.

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