

## Chapter 2

# Specificity of Binding with Matrix Metalloproteinases

Satya P. Gupta and Vaishali M. Patil

**Abstract** Matrix metalloproteinases (MMPs) regulate a wide range of biological functions; hence, they have invited great attention for the studies on their structures and functions, and since their overactivation leads to several diseases, the design and discovery of their potent inhibitors have become the need of the day. Since there have been so far discovered 28 different types of human MMPs, the specificity of binding of inhibitors with each different MMP needs special attention. The chapter presents the X-ray crystallographic and NMR studies on three-dimensional structures of a number of MMPs to reveal their catalytic site, subsites, specificity of binding with substrate and inhibitors, and catalytic mechanism. In addition to catalytic site, MMPs possess some subsites designated by unprimed and primed S, e.g., S1, S2, S3 and S1', S2', S3'. Among these, the S1' pocket varies the most among the different MMPs varying in both the amino acid makeup and depth of the pocket (shallow, intermediate, and deep pocket MMPs). This, along with the flexibility in the structures of MMPs, could be of great help in the design and the development of selective MMP inhibitors (MMPis). The determination of affinity of inhibitors and the cleavage position of peptide substrates is mainly based on P1'–S1' interaction (P1', the group in inhibitor or substrate binding to S1' pocket of the enzyme), and it is the main determinant for the affinity of inhibitors and the cleavage position of peptide substrates.

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## Abbreviations

ACE	Angiotensin-converting enzyme
ADAMs	A disintegrin and metalloproteinases
CPOD	Chronic obstructive pulmonary disease
ECM	Extracellular matrix protein
GPI	Glycosylphosphatidylinositol
Hpx domain	Hemopexin-like domain
MMPI	Matrix metalloproteinase inhibitor
MMPs	Matrix metalloproteinases
MT-MMPs	Membrane-type MMPs
TIMPs	Tissue inhibitors of metalloproteinases
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
ZBG	Zinc-binding group

## 2.1 Introduction

Cleavage of peptide bonds is essential for life, and the factors responsible for peptide cleavage are the enzymes metalloproteases, which are mostly zinc-dependent peptide-bond hydrolases (Neurath and Walsh 1976). Regulation of a wide range of biological functions makes structural studies of these proteins indispensable to understanding of their function and to the design of novel, highly specific therapeutic agents to modulate their activity (Lopez-Otin and Overall 2002). Most metalloproteases are members of a protease tribe, the zincins, and are divided into the gluzincin, aspzincin, and metzincin clans (Hooper 1994). Metzincins are the metallopeptidases, which are mostly multidomain proteins with ~130–260-residue globular catalytic domains and a characteristic zinc-binding consensus motif, HEXHXXGXX (H/D) and a methionine-containing Met-turn. Metzincins are subdivided into astacins, ADAMs/adamalysins/reprolysins, serralsins, matrix metalloproteinases, snapalysins, leishmanolysins, and pappalysins (Gomis-Ruth 2003).

From a pharmaceutical and medical perspective, the best known zinc-containing metalloproteinase has been angiotensin-converting enzyme (ACE), and now a new class of zinc-containing metalloproteinases, called matrix metalloproteinases (MMPs) or matrixins, has drawn the attention, since its importance as a therapeutic target has been well recognized (Wyvratt and Patchett 1985; Waller and Marshall 1993).

MMPs collectively participate in *in vitro* and *in vivo* degradation of all kinds of extracellular matrix protein (ECM) and are thus implicated in many connective tissue

remodeling processes (Woessner 1991) associated with embryonic development, pregnancy, growth, and wound healing. MMPs participate in diverse physiological processes such as shedding and release of latent growth factors, growth-factor-binding proteins, cytokines, and cell-surface receptors and inactivation of pro-MMPs, other proproteinases, proteinase, and angiogenesis inhibitors (Murphy and Gavrilovic 1999; Sternlicht and Werb 2001). Overactivation of these enzymes makes them destructive that may lead to pathologies such as arthritis, inflammation, cancer, atherosclerosis, aneurysms, nephritis, tissue ulcers, and fibrosis (Woessner 1998). The tissue inhibitors of MMPs (TIMPs) are specific inhibitors of matrixins that are involved in controlling the local activities of MMPs in tissues (Gomez et al. 1997; Brew et al. 2000).

## 2.2 Classification of MMPs

So far 28 types of human matrix metalloproteinases designated as MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-16, and so on have been recognized, which have been classified as follows.

### 2.2.1 Collagenases

Collagenases constitute the first family of MMPs, comprising of MMP-1, MMP-8, MMP-13, and MMP-18 (*Xenopus*). The key feature of these enzymes is their ability to cleave interstitial collagens I, II, and III at a specific site, three-fourths from the N terminus. Collagenases can also digest a number of other ECM and non-ECM molecules.

### 2.2.2 Gelatinases

Two MMPs, MMP-2 and MMP-9, called gelatinase A and gelatinase B, respectively, belong to this group. They readily digest the denatured collagens, gelatins. Gelatinases have been considered as promising target for cancer therapy on the basis of their massive upregulation in malignant tissues and their unique ability to degrade all components of ECM.

### 2.2.3 Stromelysins

MMP-3 and MMP-10 are called stromelysins, the former being known as stromelysin 1 and the latter as stromelysin 2. Both stromelysins 1 and 2 have

similar substrate specificity, but the former has a proteolytic efficiency higher than that of the latter. MMP-11 is also called stromelysin (stromelysin 3) but is usually grouped with other MMPs since its sequence and substrate specificity significantly differ from those of MMP-3 (Suzuki et al. 1990).

### 2.2.4 *Matrilysins*

Matrilysin 1 (MMP-7) and matrilysin 2 (MMP-26), also called endometase, are in this group. MMP-26 digests a number of ECM components, and MMP-7 processes, besides ECM components, a few surface molecules, such as pro- $\alpha$ -defensin and pro-tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Uria and Lopez-Otin 2000; Park et al. 2000).

### 2.2.5 *Membrane-Type MMPs*

Six MMPs, namely, MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25, are known as membrane-type MMPs (MT-MMPs). Out of these, the first four MMPs are type I transmembrane proteins and the last two glycosylphosphatidylinositol (GPI)-anchored proteins. They all are, however, designated as MT1- to MT6-MMP, respectively. All MT-MMPs, with the exception of MT4, are capable of activating pro-MMP-2.

The remaining seven MMPs, i.e., MMP-12, MMP-19 to MMP-23, and MMP-28, have not been classified, but they all play some important roles in the degradation of ECM. Whatsoever, the design and development of the inhibitors of all classes of MMPs have appeared to be of great importance, and consequently, a lot of attention has been paid to the study of structure–activity relationships (SARs) on different kinds of MMP inhibitors (MMPIs). A recent review article by Gupta (2007) on quantitative structure–activity relationships of zinc-containing enzyme inhibitors has presented a detailed picture of mechanism of interaction of MMPIs with MMPs, and a discussion of specificity of binding of these inhibitors with MMPs may be of further importance to the design and development of potent MMP inhibitors.

## 2.3 3D Structures of MMPs

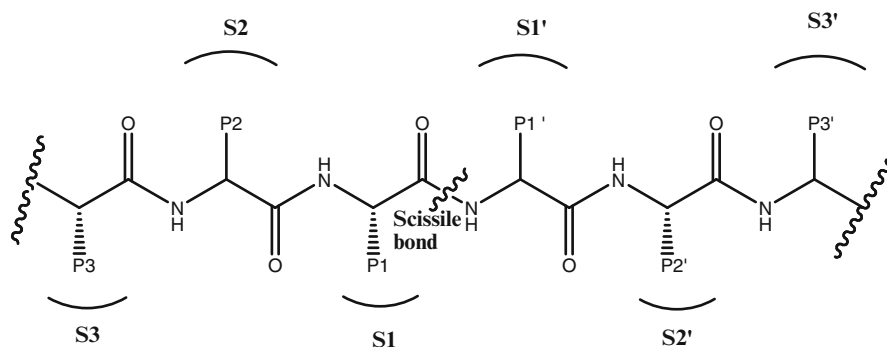
MMPs are mosaic proteins constituted by a series of inserts and domains that may include an ~20-residue secretory signal peptide, an ~80-residue propeptide, a 160–170-residue zinc- and calcium-dependent catalytic proteinase domain, a linker region, and a fourfold propeller hemopexin-like C-terminal domain. Besides, there can be many more domains, such as fibronectin type II-related domains; a cysteine-rich, a protein-rich, and an interleukin-1 receptor-like domains; and so on.

The 3D structures of a number of MMPs have been determined by X-ray crystallography and NMR methods.

From a structural point of view, most MMPs are organized into basic, well-conserved domains: an N-terminal signal sequence (“pre” domain), followed by an N-terminal propeptide, a catalytic domain, and a C-terminal Hpx (hemopexin-like) domain (Gross and Lapiere 1962; Murphy and Gavrilovic 1999; Sternlicht and Werb 2001; Ganea et al. 2007). In this, the N-terminal propeptide contains a conserved cysteine, which chelates the catalytic  $\text{Zn}^{2+}$ , keeping pro-MMP inactive. The catalytic domain contains the zinc-binding motif XEXXHXXGXXH, where three histidine residues coordinate a zinc ion. Additionally, the catalytic domain also contains a conserved methionine residue, forming a “Met-turn,” which contributes to protect the catalytic zinc (Ganea et al. 2007; Nagase et al. 2006).

The C-terminal Hpx domain is a regulatory subunit, which is present in all MMPs, but MMP-7 and MMP-26, and is supposed to control the substrate specificity of MMPs. It is separated from the catalytic domain by a variable hinge region, which also contributes to the specificity of MMPs, either by direct binding of the substrate or by influencing the orientation of hemopexin and the catalytic domain (Ganea et al. 2007; Roeb et al. 2002).

The X-ray crystallographic and NMR studies on three-dimensional structures of a number of MMPs have revealed that, although the primary structures of their domains show little homology, the polypeptide folds of their catalytic domains are almost superimposable. The catalytic domain consists of 5-stranded  $\beta$ -pleated sheets, three  $\alpha$ -helices and connecting loops, two zinc ions (structural and catalytic), and three calcium ions, which stabilize the structure. A hydrophobic “S1 pocket” (standard nomenclature is used to designate substrate residues and their corresponding binding sites on the enzyme; Fig. 2.1) is found to be present in the substrate-binding site, which contributes to MMPs substrate specificity



**Fig. 2.1** The standard nomenclature of amino acid residues of a peptide substrate and the corresponding binding sites in a protease enzyme.  $P_n$  and  $P_n'$  ( $n = 1, 2, 3, \dots$ ) refer to the residues of the substrate, and  $S_n$  and  $S_n'$  refer to the corresponding binding sites in the enzyme. Reprinted with permission from Babine and Bender (1997). Copyright 1997 American Chemical Society

(Nagase et al. 2006). In the substrate-binding pocket, there is also a “cysteine switch” in which the cysteine sulfhydryl group interacts with the catalytic zinc ion.

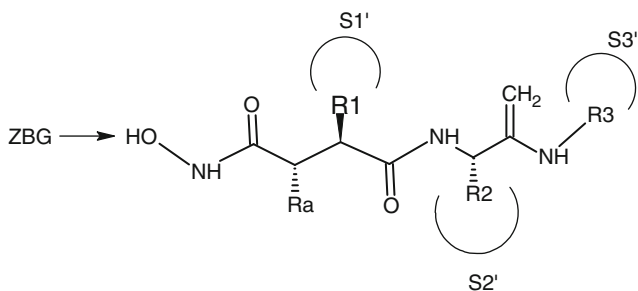
### 2.3.1 Subsites

In addition to catalytic site in MMPs, there are several other active sites in them, known as subsites (S), which interact with substrate or inhibitors. As shown in Fig. 2.1, these subsites are designated as S1, S2, S3, ... S<sub>n</sub> on the left side of the Zn<sup>2+</sup> ion and as S1', S2', S3', ... S<sub>n</sub>' on the right side of the Zn<sup>2+</sup> ion. The functional groups in the substrate or inhibitors interacting with these subsites are correspondingly designated as P1, P2, P3, ... P<sub>n</sub> and P1', P2', P3', ... P<sub>n</sub>', respectively (Babine and Bender 1997; Schechter and Berger 1967). A variety of residues present in these subsites offer substrate specificity to different MMPs (Whittaker et al. 1999).

Off the primed and unprimed subsites, the unprimed ones are more solvent exposed with less segregation between subsites. The S1 and S3 subsites are located away from the catalytic center, and the S2 subsite is located adjacent to Zn<sup>2+</sup> ion. The analogous positions in S2 subsites of MMP-2 and MMP-9, respectively, offer substrate selectivity and show the importance of unprimed subsites (Li et al. 2009).

The most efforts in the design of MMP inhibitors (MMPIs) have, however, been focused on the primed subsites. Such inhibitors are called “right-hand side” inhibitors. Three classes of compounds have been developed: (1) compounds that have amino acid residues on both sides of zinc-binding group (ZBG, the functional group in the molecule that may interact with the Zn<sup>2+</sup> ion of the enzyme), e.g., A<sub>3</sub>-A<sub>2</sub>-A<sub>1</sub>-ZBG-A<sub>1</sub>'-A<sub>2</sub>'-A<sub>3</sub>'; (2) compounds that have amino acid residues on only the right-hand side of ZBG, e.g., ZBG-A<sub>1</sub>'-A<sub>2</sub>'-A<sub>3</sub>', and are called “right-hand side” inhibitors; and (3) compounds that have amino acid residues on only the left-hand side of the ZBG, e.g., A<sub>3</sub>-A<sub>2</sub>-A<sub>1</sub>-ZBG, and are called “left-hand side” inhibitors. In all these compounds, A's and A's refer to the amino acid residues of inhibitors that can interact with S and S' subsites of the enzyme, respectively. A model of interaction of right-hand side inhibitors is shown in Fig. 2.2. The right-hand side inhibitors were reported to be potent MMPIs, while the corresponding left-hand side inhibitors were reported to possess only the modest inhibitory potency (Whittaker et al. 1999). Thus, mostly the right-hand side inhibitors were developed, although a few left-hand inhibitors were also found to be important for drug design.

The S1' pocket offers selective inhibition of MMPs due to variation in size and depth and has gotten considerable attention in the design of MMPIs. The X-ray crystallographic, NMR analysis, and homology modeling studies have classified MMPs into two broad structural classes, those with a broad S1' pocket (MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13) and those with a shallow S1' pocket (MMP-1 and MMP-7) (Lovejoy et al. 1994; Gooley et al. 1994). We may call them as class 1 and class 2 MMPs, respectively. The S2' subsite located above the opening to S1' pocket has a secondary role. However, the inhibitors with bulky hydrophobic P2' substituent exhibit selectivity for MMPs of class 1 over those of



**Fig. 2.2** A model of interaction of a right-hand side MMP inhibitor with the enzyme. R1, R2, and R3 are the substituents of the amino acid residues which can be referred to as P1', P2', and P3' substituents that may interact correspondingly with S1', S2', and S3' subsites of the enzyme. Reprinted with permission from Whittaker et al. (1999). Copyright 1999 American Chemical Society

class 2, and other MMPs have shown improved pharmacokinetic properties (Pikul et al. 2001), as the S2' cavity is hydrophobic in MMPs except MMP-1 and MMP-7, where it contains Ser and Thr residues. The S3' subsite forms the outer rim of S1' pocket entrance and has a lesser role in design of MMPIs.

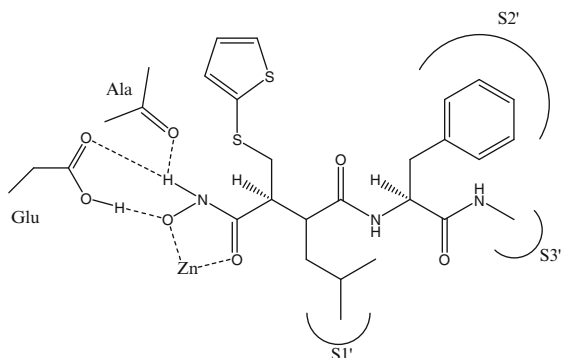
## 2.4 Specificity of Binding with MMPs

As already discussed, the catalytic  $\text{Zn}^{2+}$  ion is flanked by unprimed and primed pockets designated as S1, S2, and S3 and S1', S2', and S3', respectively. In MMPs, the main subsites for substrate recognition are the specificity pocket S1' and, to a lesser extent, S2 (Tallant et al. 2010; Overall and Kleifeld 2006). The specificity pocket S1' originates immediately to the right of catalytic  $\text{Zn}^{2+}$  ion and considerably differs in size and shape among the various MMPs. This pocket is mainly formed by the initial part of the active-site helix hB (back side), the somewhat mobile phenolic side chain of Tyr240 (right-hand flank), the main-chain atoms of underlying wall-forming segment Pro-X-Tyr (front side), the flat side of the first zinc ligand His218 imidazole (left side), and the Leu/Ile/Val235 residue of Met-turn, which together with the Leu/Tyr/Arg214 or Arg243 side chain, if present, forms its bottom or lines it toward the second exit opening at the lower molecular surface, respectively (Bode et al. 1999).

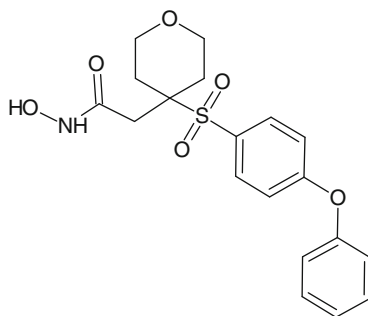
The S1' pocket varies the most among the different MMPs in both the amino acid makeup and depth of the pocket, and the MMPs can be classified based on the depth of this pocket into shallow, intermediate, and deep pocket MMPs (Jacobsen et al. 2010; Aureli et al. 2008; Park et al. 2003). The shallow S1' pocket is found in MMP-1 and MMP-7; the intermediate one in MMP-2, MMP-8, and MMP-9; and the deep one in MMP-3 and MMP-11 to MMP-14 (Park et al. 2003; Johnson et al. 2007; Gall et al. 2001). The deep S1' pocket is generally characterized as being large and an open channel.

While the rough classification of S1' specificity pockets into shallow, intermediate, and deep ones can greatly help develop the selective MMPIs, their flexibility

**Fig. 2.3** Binding mode of batimastat showing zinc-binding mode, hydrogen bonding, and distribution of substituents into the primed pockets. Reprinted with permission from Jacobsen et al. (2010). Copyright 2010 Elsevier



can be exploited to design the compounds for targeting a given MMP which does not have the desired  $S1'$  specificity pocket for the compounds. One such example is the compound RS-104966 (**1**) with a large diphenylether backbone that can target an intermediate or deep pocket but is allowed to interact with the shallow specificity pocket in MMP-1. Of the primed subsites,  $S2'$  and  $S3'$  are relatively shallower pockets, but they also participate in bonding and can interact with the substituents of the inhibitors oriented toward them (Botos et al. 1996), as shown in Fig. 2.3.



**1, RS-104966**

### 2.4.1 Inhibitors' Specificity to $S1'$ Subsite

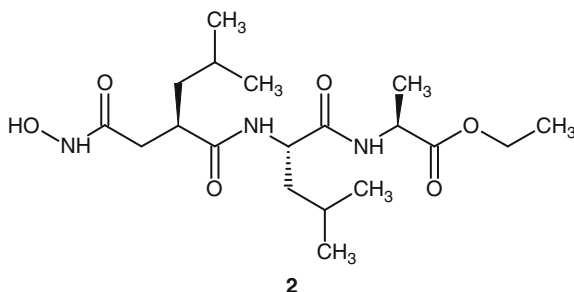
The inhibitors' specificity to MMPs depends on two important molecular features: (1) a chelating moiety which interacts with the catalytic zinc ion and (2) hydrophobic extensions protruding into the large and hydrophobic  $S1'$  pocket (Skiles et al. 2001). As the structure of  $S1'$  subsite varies between MMP family members, modifications of the  $P1'$  group can be used to introduce substrate specificity (Matter and Schudok 2004; Chen et al. 2000).

The amino acid 218 (MMP-13) and specificity loop (amino acids 244–255), surrounding the zinc-distal part of the  $S1'$  pocket, are discussed as determinants of



specificity (Lovejoy et al. 1999; Stams et al. 1994; Welch et al. 1996). Among the known zinc-chelating moieties, the hydroxamate group has been correlated with unfavorable pharmacokinetics (Babine and Bender 1997) and chronic toxicity. Thus, alternate zinc-binding groups are needed (Puerta et al. 2004; Schroeder et al. 2001; Foley et al. 2001; Jacobsen et al. 1999). Some micromolar inhibitors occupying the S1' pocket of MMP-13 without interacting with the catalytic zinc are described by Chen et al. However, optimization by introducing a zinc-binding moiety yielded a selectivity of >5,800-, 56-, and >500-fold against MMP-1, MMP-9, and TACE (Chen et al. 2000).

The S1' pocket of MMP-1 and MMP-7 is occluded by Arg214 and Tyr214, respectively, and for targeting it, smaller substituents like leucine and isoleucine residues are generally used. Ro 31-4724 (**2**) is such a potent inhibitor of MMP-1 (Borkakoti et al. 1994). There are several MMPIs with large P1' substituents as diphenylether sulfone (RS-104966, **1**) in which the group extends deep into the S1' pocket and the peptide-based inhibitor does not. It involves the induced fit mechanism where the Arg214 residue is displaced by inhibitor **1** and creates a large accommodating substrate pocket (Lovejoy et al. 1999). This induced fit mechanism fails for MMP-7 due to its shorter specificity loop creating a more rigid S1' pocket. The shallow specificity pocket of MMP-1 can accommodate a diphenylether backbone of **1** (Terp et al. 2002; Browner et al. 1995).



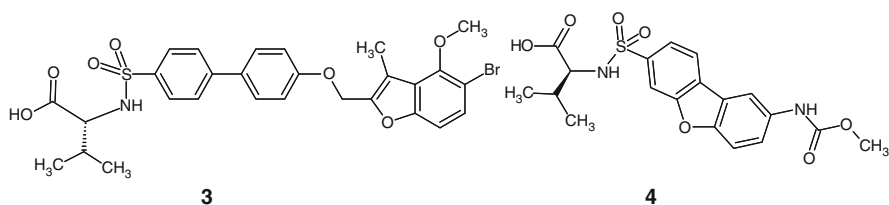
In case of MMP-2, MMP-8, and MMP-9, the intermediate pockets are present and, among this, MMP-8 belongs to collagenase family and shares similar substrate specificity as MMP-1 (Skiles et al. 2004). MMP-8 has a larger opening to the S1' pocket to accommodate the similar substrates with different pocket sizes (Aureli et al. 2008). The catalytic domains of MMP-2 and MMP-9 are highly homologous with hydrophobic S1' pockets and differ from each other in residues 425–431, which form a loop in the S1' pocket of MMP-9 (Skiles et al. 2004; Rowsell et al. 2002). The orientation of residues Thr426 and Arg424 of MMP-2 and MMP-9, respectively, causes variation in shape and size of the S1' pocket (Rowsell et al. 2002). Some MMPIs with long P1' substituent inhibit MMP-2 but fail to inhibit MMP-9 due to the occlusion by the Arg424 residue. Due to small differences in the S1' cavity, it is challenging to achieve MMPI selectivity between the gelatinases MMP-2 and MMP-9 (Tochowicz et al. 2007).

In MMP-3, MMP-11, MMP-12, MMP-13, and MMP-14, the S1' specificity pockets are characterized as large and open channels. The MMP-12 pocket is

unique as it can accommodate binding of polar groups due to the Thr215 residue within the S1' pocket, while Val or Ala residues are generally present in other MMPs. In MMP-13, the highly flexible S1' specificity loop of residues 245–253 plays major role in binding of large P1' substituents. The Leu218 residue present to the side of the pocket creates an open space (Lovejoy et al. 1999; Nar et al. 2001).

Almost all synthetic inhibitors that could bind with MMPs were found to contain a chelating group, such as hydroxamic acid, a carboxylate, or a thiol group, to interact with the  $Zn^{2+}$  ion, and a peptidic or peptidomimetic moiety mimicking peptide substrate binding to the substrate recognition site. In the majority of synthetic inhibitors studied so far, this peptidic moiety has been found to interact in an extended manner with the primed right-hand subsites with an L-configured P1' side chain substituent perfectly arranged to extend into the hydrophobic bottleneck of S1' pocket (Grams et al. 1995a, b).

This rough classification of S1' specificity pockets according to the shape and size and the flexibility can aid in the development of selective MMPIs. The selectivity over MMP-2 was achieved by considering the steric limitations of the shorter S1' loop. It was considered in the designing of a series of biphenyl sulfonamide carboxylate MMPIs for the treatment of osteoarthritis. Compound **3**, a potent inhibitor of MMP-13, is reasonably selective over MMP-2, MMP-3, and MMP-8 and highly selective over MMP-1, MMP-7, MMP-9, and MMP-14. During development of the carboxylic acid scaffold for the treatment of COPD, MMP-12 has been suggested as a variable target. The MMP-13 selectivity was improved by using a fused ring system, and restriction of rotation of the biphenyl group favors binding in the less flexible MMP-12 S1' pocket. Inhibitor **4** has shown 60-fold selectivity for MMP-12 over MMP-13 and also over MMP-1, MMP-3, MMP-7, MMP-9, and MMP-14 (Mitchell et al. 1996).

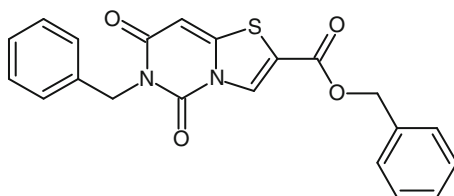


In case of hydroxamic acids, the steric bulk (sulfonamide group) was added as it favors the large opening of the S1' pocket in MMP-3 and thus selectivity over MMP-1, MMP-2, MMP-9, and MMP-14. Specific inhibitors for MMP-13 have been designed with elongated backbones to probe deep into the S1' pocket, and the rigid backbones show improved targeting of MMP-12. Also opening of the S1' pocket is more important for the development of MMP-3 selective inhibitors (Whitlock et al. 2007).

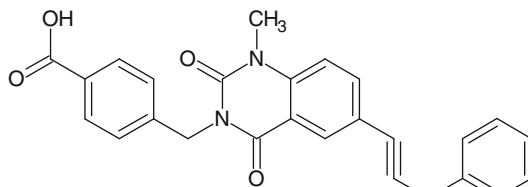
In case of phosphinate inhibitors, targeting MMP-11 vs. MMP-2, MMP-8, MMP-9, MMP-13, and MMP-14, the selectivity is due to the P1' interactions with the protein at the entrance of the S1' site and other protein–inhibitor interactions. The selectivity can be further improved by targeting MMPIs toward

the entrance of the S1' pocket rather than deep in the pocket (Matziari et al. 2004). More recently, pyrimidine-2,4,6-trione, spiropyrrolidine barbiturate, and aryl oxazoline derivatives of diphenylether have been optimized to inhibit MMP-13 (Kim et al. 2005; Blagg et al. 2005; Reiter et al. 2006; Freeman-Cook et al. 2007).

Most of the non-zinc-binding MMPIs (which do not bind to the catalytic Zn<sup>2+</sup> ion) show remarkable MMP-13 selectivity and bind deep within the S1' pocket to induce a specific protein conformation (Johnson et al. 2007; Li et al. 2008). These are long molecules linked with ring structures and are generally hydrophobic; carbonyl oxygen atoms and NH groups are involved in hydrogen bonding interactions with the S1' pocket. The Gly227 is rotated to a main-chain conformation, which opens the S1' pocket to accommodate the inhibitor. The crystal structure of inhibitor **5** shows binding in the deep S1' site where there is no overlap with the natural substrate-binding space (Li et al. 2008; Morales et al. 2004; Dublanchet et al. 2005).

**5**

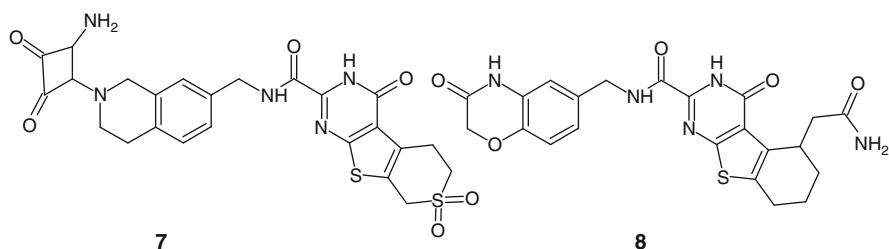
Among the various non-zinc-binding MMPIs, **6** shows remarkable selectivity for MMP-13 due to shorter S1' specificity pocket, which is not able to accommodate the especially long P1' substituent of **6** but displays substantial flexibility. Rotation of certain glycine residues opens the S1' pocket to an exosite and extends the pocket length for accommodating long inhibitors. The major reason behind the selectivity of **5** and **6** is due to their ability to induce a unique conformation in the S1' specificity loop of MMP-13 that is not accessible in other MMPs (Johnson et al. 2007; Engel et al. 2005). The use of non-zinc-binding MMPIs is based on intrinsic flexibility of the S1' specificity loop and hence most useful for the development of selective MMP-13 inhibitors.

**6**

The mode of binding of several synthetic inhibitors in the active site of MMPs has been determined by X-ray crystallography and NMR spectroscopy. A zinc-binding group (ZBG) is found to be present in the inhibitors which chelate the catalytic zinc ion bound to a substrate-like fragment designed to fit the S' primary specificity subsite and adjacent subsites. The most effective zinc-binding group, the

hydroxamate moiety, shows lack of selectivity, possesses poor pharmacokinetic properties, and may cause toxicity. To overcome the nonselective toxicity, novel MMP inhibitors, which do not bind with catalytic zinc ion, have been designed. These include micromolar inhibitors of MMP-13, highly selective pyrimidine dicarboxamides characterized by the absence of interactions with the catalytic zinc ion. Some of these inhibitors are located halfway down the S' subsite, and the potent ones extend deeply into the pocket without removing the floor formed by the F252 side chain (Johnson et al. 2007; Li et al. 2008; Engel et al. 2005).

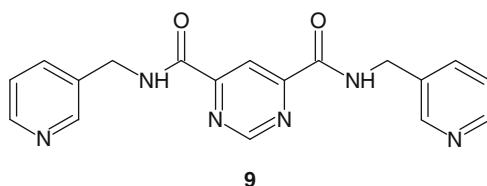
Pochetti et al. reported the binding mode of first two non-zinc-chelating inhibitors **7** and **8** of human neutrophil collagenase and their selectivity toward MMP-8 S1' pocket and other MMPs, and concluded that their high selectivity profile is due to the conformational change induced in S1' loop.



The two inhibitors have similar shape and size except the squaramide moiety bound to one terminal end of **7** and the carbamoylmethyl substituent present at the asymmetric carbon atom at the opposite end of **8**. The monoclinic and orthorhombic crystal forms were found after cocrystallization of **7** and **8** with MMP-8. The central scaffold of the two inhibitors showed similar mode of binding into the primary specificity pocket S1' and similar interactions with the enzyme in all complexes. The similar interactions are the  $\pi$ -stacking interactions of the aromatic cloud of the H197 imidazole with that of the isoquinolone of **7** and the benzoxazinone of (*S*)-**8** and the H-bondings of L214CO and A220NH with the NH and CO moieties, respectively, of the central carboxamide group. The different binding interactions include formation of bifurcated H-bond with one carbonyl of the squaramide ring of **7** and the benzoxazinone ring of (*S*)-**8**. The P217CO present at the entrance of S1' pocket is H-bond acceptor from the amino group of the squaramide ring or from that of the benzoxazinone ring. The S228NH present at the end of the S1' loop engages both sulfonyl oxygens of **7** through a bifurcated H-bond. The terminal carbamoyl group of (*S*)-**8** forms a complex H-bonding with R222CO and 227OH (Pochetti et al. 2009).

Some micromolar inhibitors occupying the S1' pocket of MMP-13 require introduction of zinc-binding moiety for improved selectivity against MMP-1, MMP-9, and TACE (Jacobsen et al. 2010). Engel et al. reported binding of pyrimidine dicarboxamide **9** in the S1' pocket, protruding into the additional S1' side pocket, which is unique to MMP-13. It exhibits a bent conformation. The central scaffold and the distal pyridyl group of **9** form intricate interactions with the specificity loop of MMP-13. The specificity loop of MMP-13 shows significant

flexibility when it is in contact with inhibitors, and it is critical for binding. The particular conformation of specificity loop defines the shape and size of the S1' pocket and the exact geometry of polar interactions between them. The role of specificity loop conformation for MMP selectivity of **9** was investigated by superimposition of MMP-13 with 11 MMPs. It was observed that conformational restrictions appear obvious for those MMPs with specificity loops shorter than that of MMP-13, but for those with similar or longer specificity loops, based on the loops' sequence. The nature of residues in position 218 and 248 and the sequence conformation of the specificity loop are the structural determinants for the high MMP selectivity. Also inhibitors with low nanomolar affinity have shown improved hydrophobic interactions (Jacobsen et al. 2010).



### 2.4.2 Mechanism of Binding at S1' Subsite

Novel MMPiS were designed which do not bind to the catalytic zinc ion and overcome the nonselective toxicity. The micromolar inhibitors of MMP-13 occupying the S1' subsite without interacting with the catalytic zinc ion were reported by Chen et al. (2000). The pyrimidine dicarboxamide inhibitors of MMP-13 (Engel et al. 2005), non-zinc-chelating selective inhibitors of MMP-12 (Morales et al. 2004), and some selective inhibitors of MMP-13 reported by Johnson et al. (2007) and Li et al. (2008) were found to be located halfway down the S1' subsite and the most potent to bind deeply into the pocket by removing the floor formed by the F252 side chain (Johnson et al. 2007; Li et al. 2008; Engel et al. 2005).

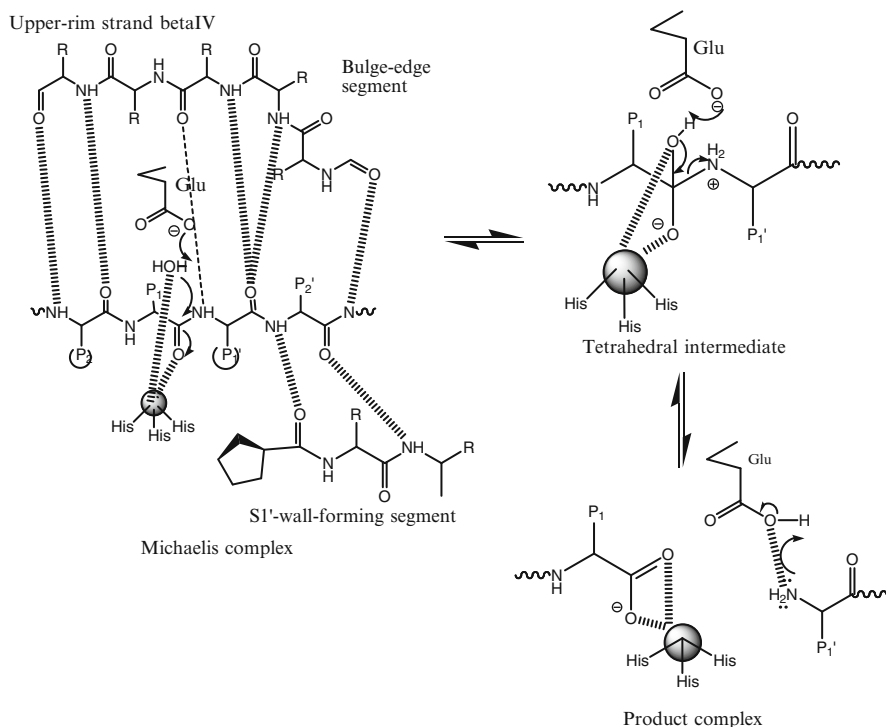
The study of crystal forms of the uninhibited MMP-8 and the inhibitor-bound complexes reveals the structural changes of the S1' specificity loop. The Zn-coordinated H207 and two consecutive turns remain practically unaltered, and the large conformational changes are on segment 219–229 and sequence R222–N226, and this leads to change in the position of Y227 side chain in both complexes. But on examining the other MMP-8 inhibitors on the PDB, the position of Y227 was found to be the same in the uninhibited and inhibited MMP-8, forming the floor of the S1' pocket, and conformation of S1' loop to remain same as the inhibitors were not long enough to approach the side chain of Y227. But in case of phosphate inhibitors, the rearrangement of the S1' loop in the region R222–Y227 was observed and the extra-binding region to become available. The MMP-8 inhibition

takes place through an induced fit mechanism operating on the loop surrounding the S1' subsite and defines the shape and size of the pocket. Thus, the Y227 residue plays the role of "selective gatekeeper" by rendering accessible the extra-binding region of the S1' specificity subsite if the interaction with the catalytic zinc ion has not to take place.

Pochetti et al. (2009) further investigated the role of the S1' specificity loop for MMP-8 by aligning the amino acid sequence of this loop with that of other MMPs. The MMP-8 differs from the other members for two residues R222 and Y227 forming the floor, and the phenylalanine residue replaces the tyrosine residue in the other MMP members. The key position in the loop of these two residues offers interesting hints for designing new and more selective inhibitors of MMP-8 (Pochetti et al. 2009).

## 2.5 Catalytic Mechanism

The catalytic mechanism involves the zinc ion, the signature glutamic acid, and zinc-bound water molecule, and the substrate binds in a groove extending across the face of a protein. Effective inhibitors show extensive van der Waals contacts within the largely hydrophobic interior of S1' and have strong electrostatic interactions with zinc ion (Kester and Matthews 1977). In the active-site cleft, the "bulge-edge segment" is of great importance for substrate and inhibitor binding, and a hydrophobic S1'-substrate-binding site or specificity pocket shows large variations in all the MMPs. In specificity loop, the initial segment Pro217-Asn-Tyr219 in MMP-8 is termed "S1'-wall-forming segment." The catalytic mechanism involves nucleophilic attack of a polarized catalytic solvent molecule so that the substrate must bind to form a Michaelis complex. The binding occurs in an extended conformation through S1'-wall-forming segment and the bulge-edge segment on the primed side and upper-rim strand  $\beta$ IV on the non-primed side. The carbonyl group coordinates the catalytic zinc ion and further ligands the three protein histidines and a solvent molecule in a pentameric fashion (Bertini et al. 2006). The specificity pocket accommodates the scissile NH bonds to a main-chain carbonyl of the upper-rim strand and the hydrophobic P1'-chain. A hydroxide formed by glutamate base attacks the scissile carbonyl carbon following the tetrahedral mechanism (Solomon et al. 2007). Therefore, the planar carbonyl group becomes negatively charged tetrahedral-reaction intermediate centered on an  $sp^3$ -hybridized *gem*-diolate group (Fig. 2.4). The latter interacts with the zinc ion, and the glutamate acts as a general acid catalyst (March 1985). The *gem*-diolate proton transfer to the secondary ammonium results in two products containing new carboxylate and  $\alpha$ -ammonium groups and remains in the form of a double-product complex (Bertini et al. 2006). The product release step is a rate-limiting step of the reaction (Hangauer et al. 1984). An incoming solvent molecule binds with the zinc ion and separates the new C terminus from the general acid/base glutamate carboxylate group and facilitates the release of the nonprimed-side product half, and the primed-side half remains



**Fig. 2.4** Catalytic mechanism of MMPs: the figure represents the cleavage mechanism proposed for MMPs. The catalytic zinc ion is shown by *sphere*, and the binding with it and the H-bonds by *dashed lines*. The three histidine ligands are represented by *sticks*. Reprinted with permission from Tallant et al. (2010). Copyright 2011 Elsevier

bound to the enzyme. Repulsion between the zinc ion and the new N terminus leads to a subtle rearrangement of the P1' side chain within the specificity pocket. Thus, the main-chain interactions of product and enzyme are weakened, leading to product release (Tallant et al. 2010).

The determination of affinity of inhibitors and the cleavage position of peptide substrates is mainly based on P1'–S1' interaction (the L-configured P1'-like side chain extends into the hydrophobic bottleneck of the S1' pocket) (Grams et al. 1995a, b). The size of the S1' pocket differs considerably among the MMPs depending on the length and character of harbored residue 214 in the active-site helix hB. The side chains of Arg214 (MMP-1) and Tyr214 (MMP-7) extend into the S1' opening with a size and shape in agreement with peptide cleavage studies on model peptides (Netzel-Arnett et al. 1991, 1993; Niedzwiecki et al. 1992). The recent MMP-1 structures allow binding of synthetic inhibitors with larger P1' side chains, as the Arg214 side chain can swing out of its normal site. In MMP-3 and MMP-14, the smaller Leu214 residues do not bar the internal S1' "pore," while the S1' pocket of MMP-8 is of medium size and is closed at the bottom by extending Arg243 side chain into the S1' space.

## 2.6 TIMP–MMP Inhibition Mechanism

The endogenous tissue inhibitors of metalloproteinases (TIMPs) precisely regulate the degenerative potential of the MMPs, and the MMP–TIMP balance results in serious disorders (Nagase et al. 1997; Yong et al. 1998; Beckett and Whittaker 1998). Therefore, the MMP structures and their TIMP complexes are attractive targets for rational inhibitor design (Beckett and Whittaker 1998; Bottomley et al. 1998). The wedge-shaped TIMPs bind into the entire length of the active-site cleft of their cognate MMPs through N-terminal segment (Cys1–Pro5), the sC-connector loop (residues Ala168 to Cys172, center), and the connecting disulfide bridge. The Cys1–Pro5 residues bind to the active-site cleft in a substrate- or product-like manner, and the sC-connector loop interacts in a somewhat substrate-inverse manner (Fernandez-Catalan et al. 1998; Gomis-Ruth et al. 1997). The Cys1 located directly above the catalytic zinc coordinates it together with the three imidazole rings from the cognate MMP. The Thr/Ser side chain of the second TIMP residue extends into the S1' pocket, and the Ser2 of TIMP2 is bonded with the catalytic Glu219. The remaining Cys3, Val/Ser4, and Pro5 side chains contact subsites S2', S3', and S4' similar to P2', P3', and P4' side chains of the substrate. In the MMP–TIMP complexes of “non-inhibited” MMP-1 catalytic domain and serralyisins, a “substrate-like” interaction is observed in which the opened-out-N-terminal segment of one molecule inserts into the S1 to S3' subsites (Lovejoy et al. 1994).

The P1'–S1' interaction is the main determinant for the affinity of inhibitors and the cleavage position of peptide substrates.

## 2.7 Conclusion

In conclusion, the chapter presents the X-ray crystallographic and NMR studies on three-dimensional structures of a number of MMPs to reveal their catalytic site, subsites, specificity of binding with substrate and inhibitors, and catalytic mechanism. According to these studies, the catalytic domain of MMPs consists of 5-stranded  $\beta$ -pleated sheets, three  $\alpha$ -helices and connecting loops, two zinc ions (structural and catalytic), and three calcium ions, which stabilize the structure. A hydrophobic “S1 pocket” is found to be present in the substrate-binding site, which contributes to MMPs substrate specificity. In addition to catalytic site in MMPs, there are several other active sites in them, known as subsites (S), which interact with substrate or inhibitors. These subsites are designated as S1, S2, S3, ...  $S_n$  on the left side of the  $Zn^{2+}$  ion and as S1', S2', S3', ...  $S_n'$  on the right side of the  $Zn^{2+}$  ion. A variety of residues present in these subsites offer substrate specificity to different MMPs.

The most efforts in the design of MMP inhibitors (MMPIs) have been focused on the primed subsites. Such inhibitors are called “right-hand side” inhibitors. These inhibitors have amino acid residues on only the right-hand side of ZBG to interact



with only S' subsites. The right-hand side inhibitors were reported to be potent MMPiS, while the corresponding left-hand side inhibitors were reported to possess only the modest inhibitory potency. Thus, mostly the right-hand side inhibitors were developed, although a few left-hand inhibitors were also found to be important for drug design.

The S1' pocket offers selective inhibition of MMPs due to variation in size and depth and has drawn considerable attention in the design of MMPiS. The X-ray crystallographic, NMR analysis, and homology modeling studies have classified MMPs into two broad structural classes, those with a broad S1' pocket (MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13) and those with a shallow S1' pocket (MMP-1 and MMP-7). We may call them as class 1 and class 2 MMPs, respectively. The S2' subsite located above the opening to S1' pocket has a secondary role. However, the inhibitors with bulky hydrophobic P2' substituent exhibit selectivity for MMPs of class 1 over those of class 2, and other MMPs have shown improved pharmacokinetic properties, as the S2' cavity is hydrophobic in MMPs except MMP-1 and MMP-7, where it contains Ser and Thr residues. The S3' subsite forms the outer rim of S1' pocket entrance and has a lesser role in design of MMPiS.

In MMPs, the main subsites for substrate recognition are the specificity pocket S1' and, to a lesser extent, S2. The specificity pocket S1' originates immediately to the right of catalytic Zn<sup>2+</sup> ion and considerably differs in size and shape among the various MMPs. The S1' pocket varies the most among the different MMPs in both the amino acid makeup and depth of the pocket, and the MMPs can be classified based on the depth of this pocket into shallow, intermediate, and deep pocket MMPs; the deep S1' pocket is generally characterized as being large and an open channel. While the rough classification of S1' specificity pockets into shallow, intermediate, and deep ones can greatly help develop the selective MMPiS, their flexibility can be exploited to design the compounds for targeting a given MMP which does not have the desired S1' specificity pocket for the compounds.

The inhibitors' specificity to MMPs depends on two important molecular features: (1) a chelating moiety which interacts with the catalytic zinc ion and (2) hydrophobic extensions protruding into the large and hydrophobic S1' pocket. As the structure of S1' subsite varies between MMP family members, modifications of the P' group can be used to introduce substrate specificity. Among the known zinc-chelating moieties, the hydroxamate group has been correlated with unfavorable pharmacokinetics and chronic toxicity. Thus, alternate zinc-binding groups are needed.

The rough classification of S1' specificity pockets according to the shape and size and the flexibility can aid in the development of selective MMPiS. Most of the non-zinc-binding MMPiS (which do not bind to the catalytic Zn<sup>2+</sup> ion) show remarkable MMP-13 selectivity and bind deep within the S1' pocket to induce a specific protein conformation.

The mode of binding of several synthetic inhibitors in the active site of MMPs has been determined by X-ray crystallography and NMR spectroscopy. A zinc-binding group (ZBG) is found to be present in the inhibitors which chelate the catalytic zinc ion bound to a substrate-like fragment designed to fit the S' primary

specificity subsite and adjacent subsites. The most effective zinc-binding group, the hydroxamate moiety, shows lack of selectivity, possesses poor pharmacokinetic properties, and may cause toxicity. To overcome the nonselective toxicity, novel MMP inhibitors, which do not bind with catalytic zinc ion, have been designed. Effective inhibitors show extensive van der Waals contacts within the largely hydrophobic interior of S1' and have strong electrostatic interactions with zinc ion. The size of the S1' pocket differs considerably among the MMPs depending on the length and character of harbored residue 214 in the active-site helix hB. The P1'–S1' interaction is the main determinant for the affinity of inhibitors and the cleavage position of peptide substrates.

The endogenous tissue inhibitors of metalloproteinases (TIMPs) precisely regulate the degenerative potential of the MMPs, and the MMP–TIMP balance results in serious disorders. Therefore, the MMP structures and their TIMP complexes are attractive targets for rational inhibitor design. In the MMP–TIMP complexes of “non-inhibited” MMP-1 catalytic domain and serralysins, a “substrate-like” interaction is observed in which the opened-out-N-terminal segment of one molecule inserts into the S1 to S3' subsites.

The catalytic mechanism of substrate–MMP interaction involves nucleophilic attack of a polarized catalytic solvent molecule so that the substrate must bind to form a Michaelis complex. The binding occurs in an extended conformation through S1'-wall-forming segment and the bulge-edge segment on the primed side and upper-rim strand  $\beta$ IV on the non-primed side. The carbonyl group coordinates the catalytic zinc ion and further ligands the three protein histidines and a solvent molecule in a pentameric fashion.

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