The Intermediate S$_1$ Pocket of the Endometase/Matrilysin-2 Active Site Revealed by Enzyme Inhibition Kinetic Studies, Protein Sequence Analyses, and Homology Modeling*

Hyun I. Park, Yonghao Jin, Douglas R. Hurst, Cyrus A. Monroe, Seakwoo Lee, Martin A. Schwartz, and Qing-Xiang Amy Sang‡

From the Department of Chemistry and Biochemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306-4390

Human matrix metalloproteinase-26 (MMP-26/endometase/matrilysin-2) is a newly identified MMP and its structure has not been reported. The enzyme active site S$_1$' pocket in MMPs is a well defined substrate P$_1'$ amino acid residue-binding site with variable depth. To explore MMP-26 active site structure-activity, a series of new potent mercapto sulfide MMP inhibitors (MMPIs) with Leu or homophenylalanine (Homophe) side chains at the P$_1'$ site were selected. The Homophe side chain is designed to probe deep S$_1$' pocket MMPs. These inhibitors were tested against MMP-26 and several MMPs with known x-ray crystal structures to distinguish shallow, intermediate, and deep S$_1$' pocket characteristics. MMP-26 has an inhibition profile most similar to those of MMPs with intermediate S$_1$' pockets. Investigations with hydroxamate MMPIs, including those designed for deep pocket MMPs, also indicated the presence of an intermediate pocket. Protein sequence analysis and homology modeling further verified that MMP-26 has an intermediate S$_1$' pocket formed by Leu-204, His-208, and Tyr-230. Moreover, residue 233 may influence the depth of an MMP S$_1$' pocket. The residue at the equivalent position of MMP-26 residue 233 is hydrophilic in intermediate-pocket MMPs (e.g. MMP-2, -8, -9) and hydrophobic in deep-pocket MMPs (e.g. MMP-3, -12, -14). MMP-26 contains a His-233 that renders the S$_1$' pocket to an intermediate size. This study suggests that MMPIs, protein sequence analyses, and molecular modeling are useful tools to understand structure-activity relationships and provides new insight for rational inhibitor design that may distinguish MMPs with deep versus intermediate S$_1$' pockets.

Matrix metalloproteinases (MMPs, $^*$ matrixins) are believed to participate in angiogenesis, embryonic development, morphogenesis, reproduction, tissue resorption and remodeling, and tumor growth, progression, invasion, and metastasis through breakdown of the extracellular matrix, cell surface proteins, and processing growth factors, cytokines, and chemokines (1–3). Recently, human MMP-26 (endometase/matrilysin 2) was identified and its mRNA expression was detected in normal tissues of the human uterus and placenta, and in many types of malignant tumors (4–7). Characterization of the MMP-26 promoter suggests that this proteinase may be expressed in cancer cells of epithelial origin (8). MMP-26 may play an important role in human prostate and breast cancer invasion (9–10).

MMP-26 cleaves type I gelatin, $\alpha_1$-proteinase inhibitor, fibronectin, vitronectin, type IV collagen, and insulin-like growth factor binding protein-1 (4, 7, 11). Studies of MMP-26 indicate that it has substrate specificity similar to other MMPs, with the exception of a preference for Ile at the P$_3$ and P$_4'$ positions, for small residues at the P$_3$ and P$_4'$ positions, and Lys at the P$_5'$ position (11). MMP-26 also hydrolyzes several synthetic fluorogenic peptide substrates designed for stromelysin-1, gelatinases, collagenases, and tumor necrosis factor-$\alpha$ converting enzyme (4, 11). According to these peptide substrate studies, MMP-26 may be capable of cleaving a broad range of substrates, although it less catalytic efficiency than other MMPs.

X-ray crystal structures of MMPs illustrate that overall topology and secondary structures are conserved (12–18). The S$_1$' pocket, a hydrophobic pocket of variable depth, is a well defined substrate P$_1'$-binding site in MMPs. Three types of S$_1$' pockets can be distinguished from the available structures of MMPs (19–20). One type is a shallow pocket, as found in MMP-1 (human fibroblast collagenase; 13) and MMP-7 (matrilysin; 16), where the pockets are limited by the side chains of Arg and Tyr, respectively, crossing the pockets. Many of the structurally known MMPs possess Leu at the corresponding site, and its side chain forms the top of the pocket rather than crossing the pocket. These Leu-containing MMPs may be further classified as deep and intermediate S$_1$' pocket MMPs. A deep, tunnel-like pocket is found in MMP-3 (stromelysin-1; 12), MMP-12 (matloelastase; 17), and MMP-14 (MT1-MMP; 21), whereas MMP-2 (gelatinase A; 22), MMP-8 (human neutrophil collagenase; 15), and MMP-9 (gelatinase B; 23) possess an intermedi-
ate-sized pocket, which is neither deep nor shallow. An enzyme with a shallow pocket prefers large, aliphatic residues in the P1 position, such as Leu and Met (24–25). The remainder of the MMPs can accommodate larger amino acid derivatives, such as homophenylalanine, in the P1 position (26).

**TABLE I**

| Inhibitor | Shallow | Deep | Intermediate |
|-----------|---------|------|--------------|
|           | MMP-1   | MMP-7| MMP-3 | MMP-12 | MMP-14 | MMP-2 | MMP-8 | MMP-9 | MMP-26 |
| MAG-181   | 680     | 710  | 2500  | 1,300  | 259    | 85    | 4.1   | 44    | 81     |
| MAG-182   | 49      | 40   | 470   | 130    | 24     | 1.1   | 0.89  | 0.57  | 17     |
| YHJ-72    | >12 × 10^3 | 5,500 | 150  | 380    | 930    | 530   | 180   | 160   |
| YHJ-294   | >12 × 10^3 | 1,000 | 100  | 100    | 13     | 15    | 13    | 8.6   | 28     |
| YHJ-294-1 | 5,200   | 3,500 | >40 × 10^3 | 16,000 | 3,000  | 430   | 130   | 550   | 450    |
| YHJ-294-2 | 100     | 26   | 360   | 93     | 13     | 6.1   | 1.2   | 1.2   | 2.8    |
| YHJ-74    | 3800    | 270  | 117   | 20     | 88     | 300   | 220   | 82    |
| YHJ-75    | 2400    | 300  | 21    | 5.6    | 3.7    | 6.9   | 44    | 3.0   | 8.6    |

Abbreviations: Me, Methyl; Ph, Phenyl; Pmp, p-methoxyphenyl.

**FIG. 1.** Structures of new mercaptosulfide MMP inhibitors. The diastereomer notation in the ring system is started from the α carbon of the mercapto group.

MMP-26, composed of 261 amino acid residues and lacking a hemopexin-like domain, represents the smallest member of the MMP family. The primary structure of MMP-26 can be divided into three regions that include a signal peptide, a propeptide domain, and a catalytic domain. MMP-26 identification, ex-
Fig. 2. Structures of commercially available hydroxamate MMP inhibitors. Calbiochem 444237, 444238, and 444225 are three known inhibitors of deep S1′ pocket MMPs.

Fig. 3. The sequence alignment of eight MMPs. The alignment was determined using the Genetic Computer Group (Wisconsin Package, version 10, Madison, WI, 2002) program PILEUP with a default gap weight of 8 and a gap length weight of 2 based on the full protein sequences without propeptide regions. Italicized amino acid residues form the S1′ pocket. Underlined sequences are metal binding consensus sequences. Boldface amino acid residues may determine S1′ pocket characteristics. To align MMP-2 and MMP-9, the 183-residue insert of fibronectin type II-like modules were deleted before the alignment. The residue numbering system is based on the sequence of MMP-26 (4).

Experimental Procedures

Materials—The fluorescent peptide substrates for MMPs used in this study were purchased from Bachem Chemical Co. The metal salts and Brij-35 were purchased from Fisher Scientific Inc. The hydroxamate MMPIs 444237, 444238, 444225, and GM6001 were purchased from Calbiochem. All other chemicals were purchased from Sigma.

The mercaptosulfide inhibitors were prepared and characterized as previously described (27–29). cis-1-Acetylthio-2-tert-butoxycarbonylthiocyclopentane and cis-3-acetylthio-4-tert-butoxycarbonylthio-N-tert-butoxycarbonylpyrrolidine were synthesized (29) and S-alkylated with (2S)-2-bromo-4-methylpentanoic acid or (2S)-2-bromo-4-phenylbutanoic acid; the latter bromoacids were derived from L-leucine and L-homophenylalanine, respectively (27). Subsequent coupling with L-thienoHMe or L-leucine-p-methoxyanilide (27) afforded the S-Boc and N-Boc protected inhibitors as mixtures of two diastereomers. The N-Boc group was selectively removed and replaced by the other acyl groups (29). The diastereomers were separated by flash chromatography on silica gel or by reverse-phase preparative high performance liquid chromatography on a C18 column. The slower-eluting S-Boc protected diastereomer exhibited the more potent MMP inhibition in each case. Its stereochemistry was assigned by 1H NMR NOE analysis (MAG-182), x-ray crystallography (YHJ-294-2) (29), or by analogy. Finally, the S-Boc protecting groups were removed by brief treatment with 2 N HCl in acetic acid and the mercaptosulfide inhibitors were isolated by yohophilation of the reaction mixture.

| Inhibitor | MMP-26 K_i (nM) | MMP-7 K_i (nM) | MMP-12 K_i (nM) |
|-----------|-----------------|----------------|-----------------|
| GM6001    | 0.36            | 3.7            | 3.6             |
| 444237    | 1.5             | 225            | 0.20            |
| 444238    | 60              | 5.7 x 10^3     | 36              |
| 444225    | 45              | 2100           | 3.4             |

Values from Ref. 11.

Shallow S1′ Pocket MMPs

| MMP-2 | FHVAFNHGL SGGLNIQDGV KALMPFTPYSVG | 203 |
|-------|-----------------------------------|-----|
| MMP-7| FHVAFNHGL SGGLNIQDGP NAHYMPYGVNDP| 235 |

Deep S1′ Pocket MMPs

| MMP-2 | FHVAFNHGL SGGLNIQDGP KALMPFTPYSVG | 203 |
|-------|-----------------------------------|-----|
| MMP-7| FHVAFNHGL SGGLNIQDGP NAHYMPYGVNDP| 235 |
| MMP-8| FHVAFNHGL SGGLNIQDGP KALMPFTPYSVG | 235 |
| MMP-9| FHVAFNHGL SGGLNIQDGP KALMPFTPYSVG | 235 |
| MMP-10| FHVAFNHGL SGGLNIQDGP SSSYMPYGVNDP| 203 |

Intermediate S1′ Pocket MMPs

| MMP-2 | FHVAFNHGL SGGLNIQDGP KALMPFTPYSVG | 203 |
|-------|-----------------------------------|-----|
| MMP-7| FHVAFNHGL SGGLNIQDGP NAHYMPYGVNDP| 235 |
| MMP-8| FHVAFNHGL SGGLNIQDGP KALMPFTPYSVG | 235 |
| MMP-9| FHVAFNHGL SGGLNIQDGP KALMPFTPYSVG | 235 |
| MMP-10| FHVAFNHGL SGGLNIQDGP SSSYMPYGVNDP| 203 |

TABLE II

| Inhibitor | MMP-26 K_i (nM) | MMP-7 K_i (nM) | MMP-12 K_i (nM) |
|-----------|-----------------|----------------|-----------------|
| GM6001    | 0.36            | 3.7            | 3.6             |
| 444237    | 1.5             | 225            | 0.20            |
| 444238    | 60              | 5.7 x 10^3     | 36              |
| 444225    | 45              | 2100           | 3.4             |

Values from Ref. 11.
Professor L. Jack Windsor (Indiana University), and Dr. C. Bruun Schiotz (OsteoPro A/S), respectively. MMP-1/human fibroblast collagenase, MMP-2/human fibroblast gelatinase, MMP-8/human neutrophil collagenase, and MMP-9/human neutrophil gelatinase were described previously (30, 31). The catalytic domain of MT1-MMP/MMP-14 was provided by Professor Harald Tschesche (Bielefeld University) (32).

MMP-26 was prepared as described previously (4, 11). Briefly, MMP-26 was expressed as inclusion bodies from a transformed BL-21 DE3 strain. After bacterial insoluble body preparation with B-Per™ reagent, the isolated insoluble protein was folded by following the procedures previously outlined (4–11). The total MMP-26 concentration was measured by UV absorption and calculated with the molar extinction coefficient ε280 = 57130 M⁻¹ cm⁻¹. The active concentration of MMP-26 was determined by titration with GM6001, a tight-binding inhibitor, as described previously (11).

**Kinetic Assays and Inhibition of Endometase—**The substrate Mca-PLGLDpaAR-NH₂ was used to measure inhibition constants (11, 33). Enzymatic assays were performed at 25 °C in 50 mM HEPES buffer at pH 7.5 in the presence of 10 mM CaCl₂, 0.2 M NaCl, and 0.01 or 0.05% Brij-35 with substrate concentrations of 1 μM. The release of product was monitored by measuring fluorescence (excitation and emission wavelengths of 328 and 393 nm, respectively) with a PerkinElmer luminescence spectrophotometer LS 50B connected to a temperature controlled water bath. All stock solutions of inhibitors were in methanol. For inhibition assays, 10 μl of inhibitor stock solution, 176 μl of assay buffer, and 10 μl of enzyme stock solution, were mixed and incubated for 30 to 60 min prior to initiation of the assay, which was accomplished by adding and mixing 4 μl of the substrate stock solution. Enzyme concentrations ranged from 0.2 to 7 nm during the assay. Apparent inhibition constant (K_i) app values were calculated by fitting the kinetic data to the Morrison equation for tight-binding inhibitors ([34, 35], where v_i and v_o are the initial (total) enzyme and inhibitor concentrations, respectively, and [I], and [E], are the initial (total) enzyme and inhibitor concentrations, respectively.

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v_i - v_o = \frac{[I] - I_{Ki} + [I]_{Ki}}{\sqrt{([I] + [I]_{Ki})^2 + 4[I]_{Ki}}} (\text{Eq. 1})
\]

**Determination of Mercaptosulfide Inhibitor Concentration—**The active inhibitor concentrations were estimated by titrating the mercapto group with 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent) as described previously (36, 37). Briefly, the reaction of 5,5'-dithiobis(2-nitrobenzoic acid) with the mercapto group produces 2-nitro-5-thiobenzoic acid. The concentration of 2-nitro-5-thiobenzoic acid is then measured by monitoring the absorbance at 412 nm. Cysteine was used to generate the standard curve with a molar extinction coefficient of 14,000 ± 500 M⁻¹ cm⁻¹, which is close to the value in the literature (37).

**Computational Protein Sequence Analyses and Homology Modeling Structure of MMP-26—**The sequence alignment of MMP catalytic domains was performed by the PILEUP program in Genetics Computer Group (GCG) software (Wisconsin Package version 10), with a default gap weight of 8 and gap length weight of 2. To align MMP-2 and -9, the alignment method (43) with the Amber force field modified to include parameters for zinc and calcium. Residues within 7 Å of the Zn(II) molecular surface. The depth of the pocket may be limited by the His-233 (light blue molecular surface). The three His residues coordinating the Zn(II) (blue sphere) are represented by tubes colored as described in A. The homology-modeled structure of MMP-26 was generated with the Swiss Model program (38–40) using the x-ray crystallographic structure of a cd-MMP-12-inhibitor complex as a template (Protein Data Bank code 1JK3) (17). The resulting MMP-26 structure was docked with YHJ-294-2 and energy-minimized as described under “Experimental Procedures.”

**RESULTS**

**Inhibition of MMPs with Mercaptosulfide MMPIs—**An inhibitor set consisting of eight mercaptosulfide inhibitors was chosen to evaluate the S1’ pocket of MMP-26 (Fig. 1). These inhibitors contain P1’ and P2’ residues and have a mercapto and a sulfide group as a possible bidentate metal-binding moiety. The inhibitors contain a Leu side chain (MAG-181 and -182 and YHJ-294-1 and -2) or a Homophe side chain (YHJ-72-73, -74, and -75) at the P1’ site. These inhibitors were tested against MMPs with known pocket characteristics (MMP-1-3, -7-9, -12, and -14). The inhibition potency of this class of inhibitors for the MMPs is significantly enhanced with a β-H configuration at the five-membered ring containing the mercapto and sulfide groups. The inhibitors with a Leu side chain are more potent against the shallow pocket MMPs, MMP-1/human fibroblast collagenase, and MMP-7/matrilysin than those with a Homophe side chain. Inhibitors with a Homophe side chain (YHJ-72, -73, 74, and -75) were more potent against the known deep-pocket MMPs such as MMP-3, -12, and -14 than those...
with Leu side chain. The inhibitors with the Leu side chain at the P1 site (MAG-182 and YHJ-294-2) inhibit MMP-7 (40 and 26 nM, respectively) and MMP-12 (130 and 93 nM, respectively) without significant differences in $K_{\text{app}}$ values. However, the presence of Homophe at the P1 site dramatically distinguishes MMP-12 from MMP-7. YHJ-73 efficiently inhibits MMP-12 (13 nM), however, the potency is decreased to 1 M against MMP-7. This trend is also displayed by YHJ-75, which has a high $K_{\text{app}}$ value against MMP-7 (300 nM) but retains potency against MMP-12 (5.6 nM). This dramatic change of potency because of changes in the P1 residue (H1032) of the inhibitors is consistently observed with the remaining shallow- and deep-pocket MMPs.

MMPs with an intermediate pocket can also accommodate the Homophe at the P1 residue. However, the difference in inhibitor potency observed with Leu or Homophe at the P1 residue is not as remarkable as that in the shallow- and deep-pocket MMPs. Inhibitors containing Leu at the P1 site (MAG-182 and YHJ-294-2) are only slightly more potent against MMP-2 and MMP-9 than inhibitors with Homophe (YHJ-73 and -75). These Homophe inhibitors are still potent against MMP-8 with $K_{\text{app}}$ values in the low nanomolar range. In general, these results indicate that mercaptosulfide inhibitors are suitable for characterizing the S1 pocket of MMPs.

Characteristics of the S1 Pocket of MMP-26 as Probed by Mercaptosulfide MMPiS—Inhibition constants for the inhibitors in Fig. 1 were measured with MMP-26 (Table I). YHJ-294-2 is the most potent inhibitor of MMP-26 among the mercaptosulfide inhibitors tested, with a $K_{\text{app}}$ value of 2.8 nM. MMP-26 also favors the $\beta$-$H$ configuration at the cyclopentyl or pyrrolidine ring moiety in the inhibitor. Addition of the urea-substituted pyrrolidine ring in place of the cyclopentyl ring (YHJ-294-1 and -2; YHJ-74 and -75) enhances the stereoselectivity for the $\beta$-$H$ configuration. Importantly, MMP-26 prefers Leu over Homophe at the S1 site, similar to the intermediate pocket MMPs, MMP-2, -8, and -9.

Characterization of MMP-26 S1 Pocket Using Commercial Hydroxamate MMPiS—The S1 site of MMP-26 was further investigated with commercially available inhibitors (Fig. 2). MMP-7/matriplysin was selected as a representative member of the shallow S1 pocket MMPs and MMP-12/metalloelastase as one of the deep S1 pocket MMPs for comparison purposes. The $K_{\text{app}}$ values of the inhibitors with MMP-7, MMP-12, and MMP-26 are summarized in Table II. GM6001 is a broad-spectrum and potent inhibitor of MMPs ($K_{\text{app}} = 0.4$ nM for MMP-1, 0.5 nM for MMP-2, 27 nM for MMP-3, 0.1 nM for MMP-8, and 0.2 nM for MMP-9) (46). It is also the most potent synthetic MMP-26 inhibitor tested, with a $K_{\text{app}}$ value of 0.36 nM. It contains a Leu residue at the P1 site, and inhibits MMP-7 (3.7 nM) and MMP-12 (3.6 nM) with similar $K_{\text{app}}$ values as observed in the mercaptosulfide inhibitors with a Leu side chain at the P1 site. The potent inhibitor 444237 of deep S1 pocket MMPs and its less potent stereoisomer 444238 were designed for human MMP-8 (IC$_{50}$ = 4 nM and 1 $\mu$M, respectively; 45). Inhibitor 444225 was designed to be a potent deep S1 pocket inhibitor of MMP-3 ($K_{\text{app}} = 130$ nM; 47). The 4-methoxybenzenesulfonyl group of these inhibitors binds at the deep S1 pocket according to the crystallographic structure (45) and the structure-activity relationship of several derivatives (47). They inhibit MMP-7 and MMP-12 with at least 100-fold lower $K_{\text{app}}$ values for MMP-12 than MMP-7. These deep S1 pocket inhibitors effectively inhibited MMP-26 with at least 90-fold lower $K_{\text{app}}$ values than those of MMP-7, but were more potent against MMP-12. These results are consistent with MMP-26 having an intermediate S1 pocket.

Fig. 5. The x-ray crystallographic structure MMP-8 (Protein Data Bank number 1BZS) (45) and homology modeled MMP-26 structure are shown after superimposition of zinc (black sphere) and histidine N ligands with MacroModel version 7.2. The proteins are represented by a flat ribbon (MMP26) or by a line ribbon (MMP-8). Arg-233 and His-233 from MMP-8 and -26, respectively, may limit the depth of the S1 pocket and are represented by gray and black sticks.
Sequence Alignment and Homology Modeling Structure of MMP-26—The folding topology and patterns of all MMP catalytic domains are quite similar (19). Thus, homology modeling and protein sequence alignment may be useful tools to predict key residues involved in forming the $S_1^\prime$ pocket of MMP-26. Protein sequence alignment in Fig. 3 reveals a plausible explanation for residues participating in the formation of the $S_1^\prime$ pocket of MMP-26. According to the alignment, Leu-204, His-208, and Tyr-230 may be key residues in forming the $S_1^\prime$ pocket of MMP-26. To evaluate the prediction from the alignment, a homology modeled structure of the MMP-26 catalytic domain was constructed using the Swiss Model program (38–40) and the crystal structure of the MMP-12-inhibitor complex (Protein Data Bank number 1JK3) (17) as a template. The mercapto-sulfide inhibitors were docked into the modeled MMP-26 structure using MacroModel version 7.2. The docked structures were further energy minimized as described under “Experimental Procedures.” The overall MMP-26 structure complexed with YHJ-294-2 is shown in Fig. 4A. Consistent with other MMP family members (19), the non-primed (left) side of the MMP-26 active site is relatively flat. The primed (right) side extends deeper into the surface and the well defined $S_1^\prime$ pocket is clearly visible. The pocket that is formed by Leu-204, His-208, and Tyr-230 is illustrated in Fig. 4B. Interestingly, the depth of the pocket may be limited by His-233, consistent with the intermediate size prediction.

DISCUSSION

The inhibition characteristics of MMP-26 with mercapto-sulfide inhibitors (Table I) and hydroxamate inhibitors (Table II) indicate that MMP-26 does not have a shallow $S_1^\prime$ pocket. According to the protein sequence alignment in Fig. 3 and the crystallographic structures of MMP-7 (16) and MMP-1 (13), Leu-204 in MMP-26 is substituted for Tyr and Arg at the equivalent position in MMP-7 and MMP-1, respectively. The side chains of Tyr and Arg terminate the $S_1^\prime$ pockets in these shallow-pocket MMPs. In the structure of MMP-26 (Fig. 4B), the side chain of Leu-204 forms the top wall of the $S_1^\prime$ pocket as found in most MMPs. Thus, MMP-26 appears to satisfy the requirement for a deep-pocket MMP. However, the inhibition profile of MMP-26 indicates a difference in the $S_1^\prime$ pocket of MMP-26 from those of other deep-pocket MMPs. The inhibitors with Homophe at the $S_1^\prime$ site (YHJ-73 and -75) do not show better potency than those with Leu (MAG-182 and YHJ-294-2). For the deep-pocket MMPs, the inhibition constants are consistently lower for the Homophe inhibitors than Leu inhibitors. The inhibition profile of MMP-26 with mercaptop-sulfide inhibitors is more similar to intermediate-pocket MMPs (MMP-2, -8, and -9) than deep-pocket MMPs (MMP-3, -12, and -14). These results suggest that MMP-26 may possess an intermediate pocket similar to those of MMP-2, MMP-8, and MMP-9.

A structural comparison of MMP-26 with MMP-8 further supports the similarity between the $S_1^\prime$ pockets of these two enzymes. The overlapping structures of MMP-8 (Protein Data Bank number 1BZS) (45) and MMP-26 at the $S_1^\prime$ pocket are displayed in Fig. 5. In MMP-8, it is known that the depth of the $S_1^\prime$ pocket is restricted by the Arg-233 side chain projecting toward the catalytic Zn(II) (14). In MMP-26, His-233 is present in place of Arg-233, which may restrict the depth of the pocket in a similar fashion, rendering the $S_1^\prime$ pocket to an intermediate size.

Based on the findings provided in this study and x-ray crystallographic structures of MMPs, the residue at the position
equivalent to His-233 of MMP-26 may play a key role in the determination of a deep or intermediate S₁´ pocket. The sequence analyses (Fig. 3) showed that the residue at position 233 is hydrophobic in MMPs with deep S₁´ pockets and hydrophilic in MMPs with intermediate pockets. The loop containing residue 233 may have a different orientation depending on the hydrophobicity of the side chain. The superimposed x-ray crystallographic structures of MMP-8 (Protein Data Bank number 1BSZ) (45) and MMP-3 (Protein Data Bank number 1CIZ) (48) in Fig. 6 reveals this type of structural difference between an intermediate-pocket MMP (MMP-8) and a deep-pocket MMP (MMP-3). These investigations suggest that it is possible to predict the S₁´ pocket properties by sequence analyses of the key residues at the Leu-204 and His-233 equivalent positions in other MMPs.

MMPs can be divided into three groups based on the characteristics of their S₁´ pockets: shallow-, intermediate-, and deep-pocket MMPs (Fig. 7). Enzyme inhibition kinetic studies using MMPs in combination with protein sequence analysis and homology modeling reveal that MMP-26 has an intermediate S₁´ pocket. Our data may provide important mechanistic and structural information to design MMP-26-specific inhibitors. As the need for innovations and new strategies for MMP inhibition in cancer and inflammation is increasing (49, 50), this study may shed light on the molecular mechanisms by which highly selective and specific inhibitors targeting an individual MMP or subgroups of MMPs may be rationally designed and developed.

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