The 1.8-Å Crystal Structure of a Matrix Metalloproteinase 8-Barbiturate Inhibitor Complex Reveals a Previously Unobserved Mechanism for Collagenase Substrate Recognition*

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The individual zinc endopeptidases of the tissue degrading matrix metalloproteinase (MMP) family share a common catalytic architecture but are differentiated with respect to substrate specificity, localization, and activation. Variation in domain structure and more subtle structural differences control their characteristic specificity profiles for substrates from among four distinct classes (Nagase, H., and Woessner, J. F. J. (1999) J. Biol. Chem. 274, 21491–21494). Exploitation of these differences may be decisive for the design of anticancer or other drugs, which should be highly selective for their particular MMP targets. Based on the 1.8-Å crystal structure of human neutrophil collagenase (MMP-8) in complex with an active site-directed inhibitor (RO200-1770), we identify and describe new structural determinants for substrate and inhibitor recognition in addition to the primary substrate recognition sites. RO200-1770 induces a major rearrangement at a position relevant to substrate recognition near the MMP-8 active site (Ala206–Asn218). In stromelysin (MMP-3), competing stabilizing interactions at the analogous segment hinder a similar rearrangement, consistent with kinetic profiling of several MMPs. Despite the apparent dissimilarity of the inhibitors, the central 2-hydroxypyrimidine-4,6-dione (barbiturate) ring of the inhibitor RO200-1770 mimics the interactions of the hydroxamate-derived inhibitor batimatstat (Grams, F., Reinemer, P., Powers, J. C., Kleine, T., Pieper, M., Tschesche, H., Huber, R., and Bode, W. (1995) Eur. J. Biochem. 228, 830–841) for binding to MMP-8. The two additional phenyl and piperidyl ring substituents of the inhibitor bind into the S1* and S2* pockets of MMP-8, respectively. The crystal lattice contains a hydrogen bond between the Oγ group of Ser209 and Nε1 of His207 of a symmetry related molecule; this interaction suggests a model for recognition of hydroxyprolines present in physiological substrates. We also identify a collagenase-characteristic cis-peptide bond, Asn198–Tyr199, on a loop essential for collagenolytic activity. The sequence conservation pattern at this position marks this cis-peptide bond as a determinant for triple-helical collagen recognition and processing.

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We dedicate this work to Prof. H. Tschesche on the occasion of his 65th birthday.

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The matrix metalloproteinases (MMPs),¹ one of the five families that form the metzincin group of zinc proteinases (3), function to degrade the extracellular matrix during embryonic development, reproduction, and tissue remodeling (1) but are disregulated in arthritis, cancer, and other diseases. The “minimal” MMPs matrilysin and endometase (MMP-7 and MMP-28, respectively), have a Zn²⁺ and Ca²⁺ binding catalytic domain, and an N-terminal pro-domain. All other known MMPs possess additionally a hemopexin-like domain near the C terminus, and further domain insertions differentiate MMP subfamilies. Gelatinases A and B (MMP-2 and MMP-9) possess three fibronec tin type II-like repeats inserted at a loop in the catalytic domain; these form an independent folding domain adjacent to the catalytic domain. Membrane-type MMPs possess an anchoring transmembrane helix C-terminal to the hemopexin-like domain (4). Hierarchical regulation of MMP activity occurs on many levels, including gene expression control (1, 5), proteolytic activation of MMPzymogens (6), inhibition by endogenous tissue inhibitors of metalloproteinases (7), and both positive and negative proteolytic feedback loops (8, 9). Crystal structures of several MMPs have been determined for a review, see, e.g., Ref. 10, revealing overall domain structures, catalytic mechanisms, and many aspects of MMP regulation mechanisms; these include collagenase 1 (MMP-1) (11, 12) and collagenase 2 (MMP-8). Structures of the latter are represented by two forms of the catalytic domain, resulting from activation cleavage alternately at two cleavage sites, leaving either Met⁸⁰ (13, 14) or Phe⁷⁸ as the N-terminal residue (15). The latter form is “superactivated,” as Phe⁷⁸ forms a salt bridge with Asp³⁵² and thereby prevents the N-terminal sequence from transient or other interference with the active site. The result is a 3-fold increase in activity compared with activation cleavage at Met⁸⁰ (16).

As their early nomenclature implies, collagenases I, -II, and -III (17) (MMP-1, -8, and -13, respectively) degrade mainly fibrillar collagens (18–20), although the structural origin of this specificity is not well understood (4). Disruption of MMP tissue remodeling function causes a variety of disorders, including cancer (tumor growth, invasion and metastasis), rheumatoid arthritis and osteoarthritis, and a variety of diseases involving neovascularization. The resulting clinical need has fostered an enormous interest in the development of inhibitors against MMPs. As part of these efforts, crystal structures of MMPs with a variety of synthetic inhibitors have been determined. For MMP-8, complexes reported include peptide mimet-
ics, hydroxamic acid derivatives (2, 14, 21, 22), phosphonamides and sulfodiimines (23, 24), thiadiazole (25), and malonic acid derivatives (26, 27).

Here we describe the 1.8-Å crystal structure of MMP-8 inhibited by a barbituric acid derivative. Conformational rearrangements accompanying the inhibitor binding lead to a new and highly ordered crystal packing arrangement. The high resolution structural data enables a thorough analysis of determinants of MMP selectivity toward both low molecular weight substances as well as substrate classes. A previously unreported cis-peptide bond (Asn\textsuperscript{188}—Tyr\textsuperscript{189}) could be unambiguously identified. The conservation patterns of the sequence at the cis-peptide bond position support the hypothesis that this cis-peptide plays a critical role in substrate recognition mechanisms specific to the collagenases I and II (MMP-1 and MMP-8).

**EXPERIMENTAL PROCEDURES**

**Materials**—MMP-1, MMP-3, and MMP-8 were kindly provided by Prof. G. Murphy (University of East Anglia, Norwich, United Kingdom), H. Nagase (Imperial College, London, United Kingdom), and H. Tschesche (University of Bielefeld, Bielefeld, Germany), respectively; MMP-2 and MMP-9 were obtained from Roche Molecular Biochemicals (Penzberg, Germany); MT1-MMP and MT3-MMP were provided by Prof. J. Foidart (Université de Liège, Liège, Belgium). The inhibitors RO200-1770, RO204-1924, I-COL043, RO206-0027, and RO206-0032 were synthesized as described previously (28). The fluorogenic substrate M-1855 ([Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH\textsubscript{2}]) was purchased from Bachem (Heidelberg, Germany); all other chemicals were of highest purity commercially available.

**Inhibition Assay**—All measurements were performed at 25 °C using a buffering solution of 50 mM Tris, pH 7.6, 100 mM NaCl, 10 mM CaCl\textsubscript{2}. Based on multiple measurements, all data are precise to within 5%. Depending on activity, enzymes were used at 5–50 nM concentration range with a substrate concentration of 2.5 μM. The enzyme was briefly pre-incubated with the inhibitor at a resultant Me\textsubscript{2}SO concentration of 1%. The reaction was started with the addition of the substrate M-1855. Substrate was excited at 280 nm and the substrate fluorescence was monitored at 346 nm using the FuoroMax-3 fluorometer (SPEX, Horiba Group, Grasbrunn/Munich, Germany).

**Crystallization, Data Collection, and Structure Refinement**—MMP-8 was concentrated to 8 mg/ml and then mixed with 3-fold molar excess of an aqueous solution of RO200-1770 for a final MMP-8 concentration of 6 mg/ml. 3 μl of protein-inhibitor complex was mixed with 2 μl precipitant solution containing 100 mM cacodylate pH 5.5–6.5, 10 mM CaCl\textsubscript{2}, 100 mM NaCl, and 10% polyethylene glycol 6000. The hanging drop was equilibrated by vapor diffusion at room temperature against a reservoir containing 1.0–1.5M phosphate buffer. Data were collected on a multiscan rotating anode X-ray source (SPEX, Horiba Group, Grasbrunn/Munich, Germany).

For a final resolution of 1.20 Å, the crystal was determined as I\textsuperscript{2}222 with unit cell dimensions a = 61.02 Å, b = 69.24 Å, c = 88.47 Å. The orientation and translation of the molecule within the crystallographic unit cell was determined with Patterson search techniques (30–32) using the program AMoRe (33).

**RESULTS**

**Inhibitor Conformation**—The inhibitor RO200-1770 is a barbituric acid derivative, doubly substituted with phenyl and 4-ethanolpiperidyl rings as depicted in Fig. 1. The barbiturate ring chelates the zinc and rigidly orient the two cyclic substituents into the S1’ and S2’ substrate binding sites. Neither substituent ring system appears strained by the protein environment, although their relative orientations may be induced by protein binding. The phenyl moiety occupies the MMP-8 binding site and is perfectly planar to within the 1.8-Å resolution.

**Electron Density Calculation**—Electron density was calculated with Patterson search techniques (30–32) using the program AMoRe. Patterson search techniques allow an interpretation whereby two chair conformations related by a 180° rotation along the C\textsubscript{(N\textsuperscript{1})}-C\textsubscript{(N\textsuperscript{2})} bond might be superimposed; either conformation would allow favorable hydrophobic contacts in the S2’-site. Adopting an all-trans conformation, the alcohol group points toward the solvent.

**Relative Orientations of the Rings**—The relative orientations of the rings of the inhibitor may be described by considering the ring planes and the bonds linking the substituent rings to the C\textsubscript{6} atom of the barbiturate ring. The C\textsubscript{5}—C\textsubscript{9} bond linking the phenyl ring is nearly perpendicular to the plane of the barbiturate ring (excluding C\textsubscript{6}). This arrangement necessarily orients the plane of the phenyl ring likewise perpendicular to the barbiturate plane. The dihedral angle C\textsubscript{6}—C\textsubscript{9}—C\textsubscript{1}—C\textsubscript{2} fixes the ring orientation with an eclipsed geometry (at 1.2°, while the C\textsubscript{5}—C\textsubscript{6}—C\textsubscript{1}—C\textsubscript{2} dihedral is staggered at 60.8°). In contrast, the C\textsubscript{5}—pN\textsubscript{1} bond lies nearly in the plane of the barbiturate, extending the P2’-piperidyl ring away from the barbiturate; all dihedrals across the C\textsubscript{5}—pN\textsubscript{1} bond have staggered orientations. This results in an arrangement where all three rings are mutually perpendicular, as follows: the angle between (the normal vectors) of the barbiturate and phenyl rings is 91°, between the barbiturate and the piperidyl rings is 103°, and between the phenyl and piperidyl rings is 111°.
**Protein-Inhibitor Interaction**—The MMP-8 substrate recognition sites are shown schematically in Fig. 3A. Comparison with the binding mode of RO200-1770 as depicted in Fig. 3B highlights the inhibitor binding at the “primed” substrate recognition sites and at the Zn$^{2+}$ ion. The Zn$^{2+}$ is coordinated by atoms N$_2$ and O$_2$ of the barbiturate ring. The Zn$^{2+}$-$\text{N}_3$ coordination has a favorable distance of 2.09 Å and highly symmetric Zn$^{2+}$-$\text{N}_3$-$\text{C}_2$ and Zn$^{2+}$-$\text{N}_3$-$\text{C}_4$ angles of 119° and 117°, respectively. Positioned where the catalytic water is expected for peptidic substrates, a partial negative charge at the hydroxyl rim of the active site as defined by other MMP-8 structures (2, 14, 26). Instead, the inhibitor induces a reorientation at the Pro$^{217}$ position at an energy cost we discuss below.

The pentacoordinated Zn$^{2+}$ binding geometry resembles a highly distorted trigonal bipyramidal structure with O$_2$, N$_{CHy}(\text{His}^{197})$, and N$_{CHy}(\text{His}^{207})$ approximately in plane with the Zn$^{2+}$ ion, with N$_{CHy}(\text{His}^{201})$ and N$_3$ lying above and below the basal plane, respectively (Table I). Alternatively, the coordination can be described as a distorted square pyramid where O$_2$, N$_5$, N$_{CHy}(\text{His}^{201})$, and N$_{CHy}(\text{His}^{207})$ form the basal ligands (dihedral deviation from planarity 15°, Table I). The metal ion lies outside of the basal plane but within 0.5 Å, and the fifth ligand N$_{CHy}(\text{His}^{197})$ forms the apex of the pyramid Table I. (If considering O$_3$ to be a sixth ligand, the geometry may be described as a pentagonal pyramid with O$_4$ as basal ligand in addition to O$_2$, N$_5$, N$_{CHy}(\text{His}^{201})$, and N$_{CHy}(\text{His}^{207})$).

In contrast to the polar interactions of the barbiturate ring, the interactions mediated by the phenyl and piperidyl rings are predominantly hydrophobic and involve the S1’ and S2’ pockets, respectively. The most prominent interaction in the S1’ pocket is the ideally parallel planar stacking of the phenyl ring and His$^{197}$ at a distance of 3.6 Å (Fig. 4). The conserved Leu$^{160}$ contributes to ligand binding also with its side chain in the S1’ site. The phenyl ring does not by itself fill the S1’ site, but leaves space filled by a network of three ordered water molecules. The first of these (Sol$^{595}$) is probably incompletely occupied and forms hydrogen bonds with the inhibitor, with MMP-8, and with a second water molecule. The proximity of the inhibitor phenyl C$_4$ atom to Sol$^{595}$ (3.1 Å) indicates a O...H-C interaction (38). The carbonyl group of Leu$^{193}$ forms a 2.9-Å hydrogen bond with Sol$^{595}$ with, however, an unfavorable C=O$^{193}$-O$^{595}$ angle of 113°. The second water molecule, Sol$^{602}$, is positioned deeper inside the S1’ pocket at a hydrogen bonding distance of 2.7 Å from Sol$^{595}$ Sol$^{602}$ in turn is hydrogen-bonded (2.8 Å) with the third solvent molecule in the S1’ pocket, Sol$^{1502}$. Sol$^{1502}$ is in a channel bounded by Arg$^{222}$, which forms a link between the three water network in S1’ and, via Sol$^{1507}$ (2.9 Å from Sol$^{1602}$), water in the adjacent cavity. Muta-
Arg, MMP-8 has a comparatively restricted S1 site. Since most MMPs lack an equivalently stabilized pOH for solvent access. The guanidinium group atoms of the "southern" rim of S2 Leu160 at the "northern" rim. The latter residue (Leu 160) separation of Arg222 would connect the two cavities, opening a "back door" to S1 for solvent access. The guanidinium group atoms of Arg222 are fixed by hydrogen bonds to the carbonyl oxygen of Ala213 (3.3 Å) and the O of Tyr217 (3.3 Å), respectively. Since most MMPs lack an equivalently stabilized Arg, MMP-8 has a comparatively restricted S1 site.

The hydrophobic interactions of the piperidine ring are mediated by aliphatic surfaces from Pro217-Asn218-Tyr219 at the "northern" rim of S2 and by the main chain Gly158-Ile159. Leu160 at the "northern" rim. The latter residue (Leu160) separates the S1' and S2' pockets. No ordered water molecule can be detected in the vicinity of the hydroxyl group pOH, although the position of this solvent exposed ethanol group is well defined by electron density (Fig. 2) and is thus presumably hydrated by disordered water.

**Protein Conformational Changes**—Significant differences are apparent in the protein structure compared with previously determined MMP-8 structures (2, 14, 21). The catalytic Zn$^{2+}$ ion of the three reference structures occupies the same position to within 0.2 Å; it is, however, shifted from that average position by 0.6 Å in the RO200-1770 complex structure. Corresponding shifts of the Zn$^{2+}$-protein ligand positions are also apparent, with the respective N$_{e2}$ and C$_{ar}$ values measured as follows: His197 (0.4 Å, 0.3 Å), His201 (0.3 Å, 0.3 Å), and His207 (0.6 Å, 0.7 Å). Consistent with this overall shift, the side chain of the catalytic Glu198 is translated by 0.2 Å. This displacement of the catalytic Zn$^{2+}$ and its protein ligands is evidently induced by inhibitor binding, as the net effect of the optimization of barbiturate-Zn chelation geometry and the inhibitor orienting forces arising from the other inhibitor-protein interactions.

Of the two partial sequences harboring the Zn$^{2+}$ binding histidine residues, the loop Ala206-His207-Asn218 is more exposed to the solvent and anchored by fewer protein contacts than the internal helix L191-H197-EXXT191-L203. The conformation of this loop is altered by several effects associated with the binding of RO200-1770. First, the greater inherent plasticity of this loop leads to greater compensation by the Zn ligand His207 for shear stresses induced at the catalytic site. Second, the Pro217-Asn218 peptide bond is rotated by ~100°, evidently to prevent a repulsive interaction between the barbiturate C$_{ar}$=O group and the Pro217 carbonyl. Third, residues Ser209, Tyr216, Pro217, and Asn218 form crystal contacts. These effects in combination lead to a translation along the entire loop from Ala206 to Pro217, which, however, is relatively rigid, leaving most dihedral angles similar to those in the reference structures. In the "north" rim of the active site, the largest change compared with the inhibitor free MMP-8 structure is a 0.98 Å displacement and disorder of the Ile145 side chain; the electron density shows a branched but symmetric side chain interpretable as two equally populated rotamers, which "swap" C$_{ar}$ and C$_{e2}$ positions.

**Enzyme Inhibition Analysis**—Utilizing the crystal structure of the MMP8-RO200-1770 complex, several follow-up compounds were synthesized and tested against the panel of metalloenzymes shown in Table II. The lead compound RO200-1770 shows broadly nonspecific micromolar inhibition, excepting onlystromelysin 1 (MMP-3) with its ~10-fold weaker binding affinity to RO200-1770. To facilitate synthesis, the piperidine of the lead compound RO200-1770 was substituted by an essentially isosteric piperazine, RO204-1924. The almost uniform decrease in binding affinity might be rationalized by higher desolvation penalties for piperazine binding. The theoretical clop values calculated for 1,4-dimethylpiperidine (1.9) and 1,4-dimethylpiperazine (0.8) support this hypothesis (39). I-COL043 and RO206-0027 represent the results of two orthogonal approaches to optimize P1'-S1' and P2'-S2' binding, respectively. For each inhibitor, an ~10-fold increase in inhibition toward MMP-8, -2, -9, and -3 was accomplished, while inhibition of MT1-MMP and MMP-1 was weakened or remained relatively unchanged. With its 4-fold weaker inhibition of MMP-1, I-COL043 showed significantly enhanced selectivity potential against the latter enzyme. The P1' and P2' optimizations of I-COL043 and RO206-0027 are combined in RO206-0032 and the inhibition values demonstrate, to a first approximation, additivity of the effects for MMP-8, -2, -9, and MT1-MMP. The improvement in its binding affinity to stromelysin (MMP-3) is less distinct, while fibroblast collagenase (MMP-1) binding averages rather than sums the effects of the precedent compounds.

**Crystal Packing Effects**—The MMP8-RO200-1770 complex did not crystallize as previously described (26), but also under the previously reported crystallization conditions formed the crystal packing arrangement described here. Thus, the inhibi-
tor induces a conformational rearrangement that leads to the new crystal packing. As discussed above, repulsion between the barbiturate C=O and Pro carbonyl groups displaces the Pro carbonyl groups displaces the Pro–Asn peptide. Its new orientation is stabilized by hydrogen bonds to the alcohol of Ser of a neighboring molecule in the crystal. This serine alcohol also forms a hydrogen bond (2.7 Å) with the N of the zinc ligand His, reminiscent of the charge relay system of serine-proteases. This interaction thus bridges Pro and His from one MMP-8 molecule with Ser of the neighboring enzyme. To create this hydrogen bond, the Ser side chain adopts a different rotamer different from earlier MMP-8 structures. A crystallographic 2-fold axis is located adjacent to Tyr and Pro. The side chain of Asn is reoriented compared with typical MMP-8 structures (where an intermolecular hydrogen to a symmetry related Thr exists) and forms a hydrogen bond with a symmetry-related Tyr. None of the crystal contacts interfere with expected peptidic binding sites. A symmetry-related Gln, however, forms a hydrophobic contact at a depression bounded by Ile and Ser, which could serve as an alternative S2/S3 binding site. There is no direct contact of the inhibitor with a symmetry-related protein molecule.

Secondary Substrate Recognition Site: A Collagenase Type I Characteristic Cis-peptide Bond—The recognition and processing of natural collagen substrates is known to involve the C-terminal hemopexin-like domain in addition to the active site (4, 40, 41). The relative domain arrangement of the catalytic and C-terminal domains, as seen for MMP-1 (11) and MMP-2 (42), shows the importance of the primed substrate recognition sites, since these are located at the interface of the two collagenase domains. Intriguingly, Asn–Tyr, located at the corridor connecting the catalytic and the C-terminal domain, adopts a cis-peptide bond (Fig. 5). Although not yet recognized, this cis-peptide bond is not unique to the present crystal form; re-inspection confirmed its presence also in the alternative crystal form (26). This cis-peptide bond is located on the solvent-exposed loop preceding the "catalytic" α-helix L191–H195EXXH201–L203. The only restraint apparent for this structural framework is a stabilizing hydrogen bond between carbonyl oxygen of Thr with the amide of Tyr. Sequence comparison of this segment with related MMPs reveals a sub-division within the MMP family. Only collagenases 1 and 2 (MMP-1 and MMP-8) lack a glycine at position 188, a feature otherwise absolutely conserved, including nonhuman species as well. We therefore predict that the Glu–Tyr peptide bond of MMP-1 also adopts a cis-conformation. As exemplified by the crystal structure of stromelysin 1 (MMP-3) (23, 43), Gly exhibits dihedral angles (φ, ψ) = (150°, 165°), which correspond to a conformation allowed only for glycine. Therefore, glycine is conserved at position 188 presumably to stabilize the local fold; conservation of a nonglycine residue (MMP-1, MMP-8) suggests a function related to the cis-peptide bond.

DISCUSSION

Inhibitor Conformation and Its Interaction with the Protein—Although identified as potent collagenase inhibitor by an independent screening program, the barbiturate-based inhibitor family exhibits striking similarities with well characterized classes of inhibitors, namely hydroxamic and malonic acid-based compounds (2, 26). Fig. 3C illustrates that the Zn chelation geometry of the hydroxamate, exemplified by batimastat, is mirrored by the barbiturate with its N nitrogen substituting for the keto group of the hydroxamate. Additionally, the interaction of the barbiturate N-H and O with the protein backbone parallels that of batimastat (Fig. 3). A subtle difference is found at the O interaction of the barbiturate ring, since the additional amide interaction with Ala could stabilize a greater negative charge on O.

These findings present opportunities with challenges. The structural similarity of both inhibitor classes for example enables the application of knowledge of optimization criteria for one class to the other. On the other hand, the similarity might also indicate a limitation in finding specific metalloproteinase inhibitors; the presence of a similar metal chelation topology in independently identified and structurally unrelated lead compounds indicates that the Zn binding follows a rather universal recognition motif, which dominates the binding characteristics. Consequently, many if not most potent active site directed Zn protease inhibitor will exploit such a universal binding motif and are likely to exhibit a low specificity profile, at least prior to optimization.

The Barbiturate Acid Carries No Net Charge—The charge assignment of the Zn-chelating barbiturate ring aids an understanding of the binding interaction. For the crystallization experiment, the pH was adjusted to 6.0 (see "Experimental Procedures.") This information is, however, insufficient to allow for a reliable prediction of the protonation state of the
barbiturate ring. First, its $pK_a$ varies dramatically with the presence of ring substituents. Whereas the $pK_a$ of unsubstituted barbituric acid is about 4, its 5,5-diethyl substituted analog ("barbital") has a $pK_a$ of around 8 (37). Second, the surrounding protein will also strongly affect the protonation of the barbiturate.

To address this issue, we inspected each polar group of the inhibitor for possible hydrogen bonding partners. The 2.0-Å distance of the catalytic Zn$^{2+}$ to N$_3$ excludes its protonation, and the O$_2$H$_2$ hydroxyl group is necessary to avoid repulsion of the carboxylate of Glu$^{198}$. N$_3$ and O$_6$ are involved in main chain hydrogen bonds. Consequently, their protonation appears well defined as depicted in Fig. 1. O$_3$ is the only polar group without apparent attractive interactions with the protein. However, the reorientation of the Pro$^{217}$ carbonyl described above would seemingly not occur if O$_3$ is protonated as an alcohol. These arguments summarize the case for the formula depicted in Fig. 1, which carries no net charge. Tunneling of the proton H$_2$ (Fig. 1), which bridges the carboxylate group of Glu$^{198}$ however, transfers a partial negative charge to O$_2$ and by resonance also to N$_3$ (Fig. 1). (A second line of investigation using conformational correlation analysis of the 1.8-Å resolution structure presented here with barbiturate derivatives deposited in the Cambridge small molecule data base was not conclusive.)

**S1' and S2' Interaction, and Enzyme Inhibition Profiles—** Compared with MMP-8, human fibroblast collagenase (MMP-1) has a more restricted S1' site with its Arg instead of Leu at position 193. Its guanidinium group approximately occupies the three S1' solvent sites of MMP-8, namely SoI$^{595}$, SoI$^{592}$ and SoI$^{592}$. Conversely, three solvent molecules are found near Thr$^{222}$ in MMP-1, where in MMP-8 the guanidinium group of Arg$^{222}$ is found. It appears, therefore, possible to enlarge the MMP-1 S1 site to an MMP-8 size by swapping its Arg side chain and solvent molecules. Although such a swapped conformation has been confirmed (12) for MMP-1, the rehydration is likely to create a considerable kinetic barrier. Consequently, MMP-1 is expected to bind large P1' residues with a $k_{cat}$ kinetic rate considerably lower than for MMP-8. The S1' site of TACE appears rather too large to properly accommodate the large P1' residue of ICOL 043 and RO206-0032 (Table II). A unique feature of the TACE active site (44) is the occurrence of Ala at the equivalent position of the strictly conserved Tyr$^{219}$ (MMP-8 numbering) of MMPs. This renders the TACE S1' site both larger and less hydrophobic than in MMPs by almost completely removing the barrier to the S3' site. Consequently, the hydrophobic P1' residue of ICOL 043 and RO206-0032 is not optimally anchored in the TACE S1' pocket. Further, incomplete dehydration of the voluminous site is likely to disrupt the solvent structure within the TACE S1' site (44) without the energy compensation of a good fit.

Considering MMP-3, the southern rim of the active site, and in particular Pro$^{221}$ (Pro$^{217}$ in MMP-8), is rigidified by His$^{224}$ (Ala$^{220}$ in MMP-8), which hydrogen-bonds via its N$_3$ and N$_2$ atoms to the backbone carbonyl groups of Leu$^{222}$ and Thr$^{215}$ (Asn$^{218}$ and Pro$^{211}$ in MMP-8). The MMP-3 active site is thus incompatible with binding the barbiturate ring, as reflected by the overall lower binding constants. His$^{224}$ is unique to MMP-3. The observed progressive increase of binding affinity with enolization of around 8 (37). Second, the $pK_a$ of the O$_4$ ketone and retention of the position of the chelating group N$_3$C$_2$H$_2$ will allow the new five-membered ring to relax to a chemically reasonable geometry. With appropriate restraints for the relaxation, the result will still possess favorable hydrogen bonding interactions between the HN$_3$C$_2$=O$_4$ segment and the protein. To compensate for concomitant displacements of the phenyl ring, it would be necessary to add a spacer atom to restore unstrained occupancies of both the S1' and S2' pockets.

Additional optimization approaches are suggested by kinetic analyses of earlier x-ray structures of MMPs with peptidic, hydroxamic, and malonic acid-based inhibitors (2, 26, 27, 45), whereby significant improvement in binding affinity is achieved by better filling the respective binding pockets (see Ref. 46 for a comprehensive review of these approaches). Expansion of the P1' residue with an appropriate heterocyclic ring should supply both the necessary flexibility to optimally fill the curved S1' pocket and the hydrophilicity to adequately replace the binding sites of the three water molecules found in the S1' pocket. In summary, the major contribution to specificity can be attributed to the P1'-S1' interaction, while both substituents similarly contribute to MMP-8 binding (Table II).

**Protein Conformational Changes—** As described under “Results,” the most striking structural changes induced by inhibitor binding occur near the catalytic Zn$^{2+}$ with a major contribution from repulsive interactions between the C$_1$=O$_4$ ketone and the Pro$^{217}$ carbonyl group. For the collagenases, considerable flexibility near the active site environment appears physiologically necessary for triple helical peptide processing (47). The flexibility observed in the present structure suggests that the loop Ala$^{206}$—Asn$^{218}$ will provide much of the flexibility necessary for collagen substrate recognition (48), along with the additional plasticity seen at the catalytic Zn$^{2+}$ and its ligating residues. The generally weaker inhibition constants indicate that stromelysin 1 (MMP-3) does not possess the necessary plasticity in this segment for barbiturate binding.

The intermolecular crystal contact Ser$^{209}$—His$^{207}$ might provide an unexpected opportunity for synthetic drug design. In particular, this interaction offers the welcome possibility to deviate from peptide-like binding patterns at a highly ordered position in the active site. Intriguingly, since hydroxyprolines and other hydroxylated amino acids are present in the physiological substrates of the extracellular matrix, we speculate that this crystal contact may mimic a hydroxylated substrate interaction. An alternative possibility that also would exploit the common His-Ser motif would be an interaction with His$^{201}$ N$_{3\text{I}}$

**Type I Collagen Recognition Exosite—** Independent investigations by others on rat MMP-8 have shown the 188-loop to be required for collagenase activity. The single site-directed mutation to N209K, corresponding to N188K in human MMP-8, disrupts collagenolytic activity. In addition, hybrid molecule studies involving stromelysin 1 (N-terminal) and collagenase 1 (C-terminal) underscore the importance of this loop for collagenolytic activity. The segment R$^{184}$WTNNFREY$^{189}$ of collagenase 1 is critical for triple-helicase activity (49). In addition to collagenase 1, 2, and 3, the two gelatinases MMP-2 and MMP-9

*pK*$_a$ *C. M. Overall, personal communication.*
have tyrosine at position 189, both of which are preceded by a large insertion of three fibronectin II domains (Fig. 6). These domains also are known to be critical for substrate recognition (4, 48, 50). Therefore, the 188 exosite serves as a collagen substrate recognition site in both collagenases (MMP-1, -8, and -13) and gelatinases (MMP-2 and -9). This proposed substrate recognition site is the position of the cis peptide bond described here for MMP-8 and predicted for MMP-1. As such, it distinguishes collagenases 1 and 2 (MMP-1 and -8) from the other MMPs known to cleave collagen that have a glycine at this position, including MMP-13 (17), MMP-14 (51), and MMP-18 (52). Thus, collagenase 3 (MMP-13) (12, 53), MMP-14 (54), and presumably MMP-18 have a different backbone conformation in this loop segment. This structural relationship is reflected by the biochemical properties of the respective enzymes. MMP-13 is distinct from MMP-1 and -8, as it preferentially hydrolyzes type II collagen, whereas the enzyme was 5 or 6 times less efficient at cleaving type I or III collagen (17). Similarly, MMP-14 is 5–7 times less efficient at hydrolyzing type I collagen than MMP-1, whereas its gelatinolytic activity is 8 times higher than that of MMP-1 (51).

To further investigate the role of the 189 exosite for macromolecular substrate recognition, we docked a collagen triple helix to a full-length collagenase (MMP-1). In addition to optimizing overall contact areas of the substrate-enzyme complex, we were guided by the following localized interactions: (a) the contact of the collagen helix with the primary substrate recognition sites, including the catalytic Zn$^{2+}$; (b) the contact of the collagen helix with the 189 exosite; and (c) the interaction of the collagen hydroxyproline with His$^{267}$. We used published data for modeling the structures of the isolated components (11, 55, 56). The most reliable and powerful conclusion from these modeling studies is the orientation of the extended collagen peptide relative to the enzyme. Earlier models postulated that the triple helix makes major contacts with the first "blade" of the propeller-like hemopexin domain (48). We conclude, however, that the triple helix will not lie in the MMP active site oriented along the shortest route to the hemopexin-like domain, which would bring it into contact with its first blade. Instead, we propose that the substrate runs through the 188 exosite, leading to major contacts to blade 2 of the C-terminal collagenase domain, consistent with the chimera mutant studies by Nagase and co-workers (49). The extended contact of the collagen substrate with the catalytic domain is consistent with a collagenolytic activity of the catalytic domain alone, as described for MMP-1 (57). On the other hand, the conservation of the substrate exosite within MMP-1 and MMP-8 would suggest that the catalytic domain of MMP-8 should also exhibit a collagenolytic activity that however has not been observed (48). A second important contribution of these modeling studies is that at least one of the collagen strands must be bent or arched by a position, including MMP-13 (17), MMP-14 (51), and MMP-18 (52). Thus, collagenase 3 (MMP-13) (12, 53), MMP-14 (54), and presumably MMP-18 have a different backbone conformation in this loop segment. This structural relationship is reflected by the biochemical properties of the respective enzymes. MMP-13 is distinct from MMP-1 and -8, as it preferentially hydrolyzes type II collagen, whereas the enzyme was 5 or 6 times less efficient at cleaving type I or III collagen (17). Similarly, MMP-14 is 5–7 times less efficient at hydrolyzing type I collagen than MMP-1, whereas its gelatinolytic activity is 8 times higher than that of MMP-1 (51).
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José R. Domínguez-Solís, Gloria Gutiérrez-Alcalá, José M. Vega, Luis C. Romero, and Cecilia Gotor

José M. Vega was inadvertently omitted from the list of authors. The correct list is shown above.

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