Crystal Structure of the Catalytic Domain of Matrix Metalloproteinase-1 in Complex with the Inhibitory Domain of Tissue Inhibitor of Metalloproteinase-1*

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Shalini Iyer 1, Shuo Wei 1, Keith Brew 2,3, and K. Ravi Acharya 4

From the 1 Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom and 4 Department of Biomedical Science, Florida Atlantic University, Boca Raton, Florida 33431

The mammalian collagenases are a subgroup of the matrix metalloproteinases (MMPs) that are uniquely able to cleave triple helical fibrillar collagens. Collagen breakdown is an essential part of extracellular matrix turnover in key physiological processes including morphogenesis and wound healing; however, unregulated collagenolysis is linked to important diseases such as arthritis and cancer. The tissue inhibitors of metalloproteinases (TIMPs) function in controlling the activity of MMPs, including collagenases. We report here the structure of a complex of the catalytic domain of fibroblast collagenase (MMP-1) with the N-terminal inhibitory domain of human TIMP-1 (N-TIMP-1) at 2.54 Å resolution. Comparison with the previously reported structure of the TIMP-1/stromelysin-1 (MMP-3) complex shows that the mechanisms of inhibition of both MMPs are generally similar, yet there are significant differences in the protein-protein interfaces in the two complexes. Specifically, the loop between β-strands A and B of TIMP-1 makes contact with MMP-3 but not with MMP-1, and there are marked differences in the roles of individual residues in the C-D connector of TIMP-1 in binding to the two MMPs. Structural rearrangements in the bound MMPs are also strikingly different. This is the first crystallographic structure that contains the truncated N-terminal domain of a TIMP, which shows only minor differences from the corresponding region of the full-length protein. Differences in the interactions in the two TIMP-1 complexes provide a structural explanation for the results of previous mutational studies and a basis for designing new N-TIMP-1 variants with restricted specificity.

The matrix metalloproteinases (MMPs) are a family of zinc endopeptidases that share homologous catalytic domains. These enzymes catalyze the cleavage of the protein components of the extracellular matrix and play a pivotal role in connective tissue remodeling during development, wound healing, and other physiological processes. The collagenases, a subgroup of MMPs that cleave collagens type I, II, and III, include MMP-1 (fibroblast collagenase), MMP-8 (neutrophil collagenase), and MMP-13 (collagenase-3) (2–4). MMP-1 is considered a prototype for all interstitial collagenases and plays an important role in the turnover of collagen fibrils in the matrix. Much evidence indicates that collagenolysis is an important event in diverse physiologic processes such as development, tissue morphogenesis, and wound repair (5), whereas unregulated collagen breakdown has been implicated in a variety of human diseases including cancer, rheumatoid arthritis, pulmonary emphysema, and fibrotic disorders (6). Therefore, knowledge of the structural basis of collagenase action and its regulation is crucial for understanding the molecular basis of these diseases and opening new therapeutic avenues.

MMPs are initially synthesized as inactive precursors (pro-MMPs), and the levels of MMP activity in tissues are closely regulated at the levels of gene expression, pro-MMP activation, and inhibition of the active enzymes. The principal endogenous inhibitors of active MMP in tissues are four tissue inhibitors of metalloproteinases (TIMP-1 to TIMP-4). These two-domain proteins are 40–50% identical in sequence (7). Their N-terminal domains have about 120 amino acids, whereas the C-domains contain about 65 residues; in mammalian TIMPs each domain is stabilized by three disulfide cross-links (8). The N-terminal domains (designated N-TIMPs) can fold independently and carry the full inhibitory activity toward their target MMPs (9, 10).

TIMPs form tight non-covalent 1:1 inhibitory complexes with MMPs albeit with relatively low selectivity (10). An exception to this is TIMP-1, which is a weak inhibitor of some membrane-type MMPs. Crystal structures have been determined for two inhibitory MMP-TIMP complexes; that is, the catalytic

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The atomic coordinates and structure factors (code 2J0T and R2J0TSF) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 Present address: Dept. of Cell Biology, University of Virginia Health Sciences Center, P. O. Box 800732, Charlottesville, VA 22908.

2 To whom correspondence may be addressed: Dept. of Biomedical Science, FL Atlantic University, 777 Glades Rd., Boca Raton, FL 33431. Tel.: 561-297-0407; Fax: 561-297-2221; E-mail: kbrew@fau.edu.

3 To whom correspondence may be addressed: Dept. of Biology and Biochemistry, Bldg. 4 South, University of Bath, Claverton Down, Bath BA2 7AY, UK. Tel.: 44-1225-386238; Fax: 44-1225-386779; E-mail: bssskra@bath.ac.uk.

4 The abbreviations used are: MMP-1, matrix metalloproteinase-1; MMP-1cd, catalytic domain of MMP-1; TIMP-1, tissue inhibitor of metalloproteinase-1; TIMP-1 NT, N-terminal domain of TIMP-1; Bis-Tris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol; r.m.s.d., root mean square deviation.
domain of MMP-3 (MMP-3cd) in complex with the full-length TIMP-1 (PDB code 1UEA (11)) and the complex of MT1-MMPcd with TIMP-2 (PDB code 1BQQ (12)). In addition, a structure has been determined for the complex of pro-MMP-2 with the full-length TIMP-2 (PDB code 1GXD (13)), which exemplifies a distinct type of non-inhibitory complex stabilized by interactions between the C-terminal domain of TIMP and the non-catalytic hemopexin domains of pro-MMP.

The previously determined structures show that the N-terminal inhibitory domains of TIMPs have the widely distributed oligonucleotide binding fold. In the inhibitory complexes this domain makes most of contacts with the protease. The core of the interaction site is a continuous ridge stabilized by a disulfide bond between Cys-1 and Cys-70, that links the N-terminal four residues (CTCV) with residues MESVC. Cys-1 interacts bidentately with the catalytic zinc through the N-terminal amino group and carbonyl group, displacing the water molecule that is needed for peptide bond hydrolysis, whereas residue 2 (Thr-2 in TIMP-1) interacts with the so-called SI specificity pocket of the MMP, a region that has a dominant role in enzyme specificity. In addition, the structures also show that the loops of the N-terminal domain of TIMP-1 between β-strands A and B and between strands E and F also facilitate interactions of the inhibitor with the protease. Mutational analyses of residues implicated in binding have shown that changes in the interaction site in TIMPs can significantly change its relative affinity for different MMPs, raising the possibility of generating TIMP variants that are selective inhibitors of disease-related MMPs that could open a novel approach to disease treatment (14, 15).

The structures of different TIMP-MMP complexes can provide information about differences in interaction interfaces that are crucial for the rational design of selective TIMP variants. For this reason we have conducted an x-ray crystallographic analysis of the complex formed between N-TIMP-1 and the catalytic domain of MMP-1 (MMP-1cd). The crystal structure provides new insights into features that influence the affinity of MMP-1 and, thus, helps provide a crucial platform for further engineering of MMP-selective TIMPs and small molecule inhibitors.

**EXPERIMENTAL PROCEDURES**

**Cloning, Protein Expression, and Protein Purification**—Human N-TIMP-1 was expressed in *Escherichia coli* (BL21 DE3) cells, partially purified, and folded as described previously (16), with the exception that the protein was extracted from inclusion bodies and initially purified by gel filtration in 6 M guanidine HCl rather than 8 M urea. After partial purification of the folded protein by cation exchange chromatography with carboxymethylcellulose, further purification was performed by medium pressure cation exchange chromatography using a Bio-Rad Biologic DuoFlow chromatography system to separate active and inactive forms of the protein (17). After dialysis against 20 mM Bis-Tris-HCl, pH 5.5, the N-TIMP-1 was applied to a Mono S HR (5/5) column (Amersham Biosciences) that had been previously equilibrated with the same buffer. Elution was performed with a linear gradient from 0 to 0.5 M NaCl over 60 min at a flow rate of 1 ml/min, and the effluent was monitored by absorbance at 280 nm. Two similarly sized slightly overlapping peaks were collected manually of which the second contained the MMP-inhibitory activity.

The vector for bacterial expression of the catalytic domain of MMP-1 (pET3a, MMP-1cd) was kindly provided by Dr. H. Nagase. Protein expression and isolation of inclusion bodies was carried out as for N-TIMP-1. Recombinant protein was extracted from inclusion bodies with 8 M urea containing 20 mM Tris HCl, pH 8.5, containing 10 mM dithiothreitol, and insoluble material was removed by centrifugation at 12,000 rpm for 30 min at 4 °C. The supernatant was applied to a column (1.5 × 10 cm) of MacroPrep 50Q anion exchange resin that had been equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 8 M urea that was subsequently washed with the same buffer at a flow rate of 1.5 ml/min. The protein was eluted with a linear gradient from 0 to 0.5 M NaCl in equilibration buffer at the same flow rate and collected in 4.5-ml fractions. Fractions containing MMP-1cd were identified by SDS-PAGE and pooled. For folding, the denatured protein was dialyzed twice (16 h each) against 50 mM Tris HCl, pH 8.0, containing 250 mM NaCl, 20 mM CaCl₂, and 50 μM ZnCl₂ at 4 °C. The solution was centrifuged at 12,000 rpm at 4 °C for 30 min to remove precipitate, and the supernatant was purified by gel filtration with a column (2.5 × 35 cm) of Superdex-75 that was equilibrated and eluted with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 20 mM CaCl₂, at a flow rate of 1.5 ml/min.

**Preparation of N-TIMP-1-MMP-1cd Complex**—Samples of N-TIMP-1 (3 ml; 330 nmol) and MMP-1cd (25 ml; 230 nmol) in TNC buffer (50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 10 mM CaCl₂) were mixed and incubated overnight at room temperature to ensure complex formation. The solution was concentrated to about 12 ml, and the complex was purified by gel filtration with a column of Superdex-75 (3.5 × 40 cm) equilibrated and eluted with TNC buffer at a flow rate of 1.5 ml/min. Fractions of 6 ml were collected, and protein was detected by absorbance at 280 nm. The elution profile (not shown) consisted of a major peak followed by a minor peak; SDS-PAGE of the major peak (complex) gave bands with apparent molecular masses of 14.5 and 19 kDa, corresponding to N-TIMP-1 and MMP-1cd, whereas the minor peak contained only N-TIMP-1.

**Crystallization, X-ray Diffraction Data Collection, and Processing**—Crystals for the N-TIMP-1-MMP-1cd complex were grown at 16 °C by the hanging drop vapor diffusion technique with 10% polyethylene glycol 8000, 8% ethylene glycol, and 0.1 M HEPES, pH 7.5, as the reservoir solution. A full dataset to 2.54 Å resolution was collected on station PX 9.6 of the Synchrotron Radiation Source, Daresbury (UK), at 100 K from a single crystal. Data processing was performed with the HKL package (18) in the C-centered monoclinic space group, C2. Data reduction carried out using the program TRUNCATE of the CCP4 suite (19) estimated an overall B-factor of 59.9 Å² from the Wilson plot.

**Structure Determination**—Cell content analysis suggested the possibility of 2–3 complexes in the asymmetric unit, and the native Patterson revealed a strong pseudo-translation at 1/3,0,0. The structure was solved by molecular replacement method using the program AMoRe (20). The catalytic domain...
Structure of N-TIMP-1-MMP-1cd Complex

of MMP-1 (PDB code 1CGL (21)) and the N-terminal domain of TIMP-1 (PDB code 1UEA (11)) were used as search models for the individual components of the complex. The command script for AMoRe was edited to include the NCS translation vector (0.33 0.00 0.00). Once the three MMP-1cd molecules were located and their position in the asymmetric unit defined, the N-TIMP-1 molecules were located one by one. Structures for the three MMP-1cd molecules and the three N-TIMP-1 molecules were built and ensured that there were no symmetry-related clashes using the program COOT (22).

Structure Refinement—Crystallographic refinement was carried out using the program CNS (23) at 2.54 Å resolution against 91.2% of the measured data. A test set of random reflections of 2.7% was excluded from the full data set for cross-validation purposes (24) by calculating the free R-factor ($R_{free}$) to monitor refinement trend. An initial round of refinement with the three complexes found by AMoRe (20) resulted in an $R_{cryst}$ of 37.6% and an $R_{free}$ of 43.0%. Few cycles of refinement (energy minimization, simulated annealing, and individual temperature factor (B-factor) refinement) using NCS restraints in CNS (23) and model building with reference to $2F_o - F_c$ and $F_o - F_c$ maps using the program COOT (22) progressively improved the phases. In the later stages of refinement, REFMAC (25) was used to incorporate TLS (26) thermal refinement. Each molecule within the asymmetric unit was defined as a TLS tensor group for the purpose of refinement. Refinement proceeded in 15–30 cycles of TLS refinement with atomic residual isotropic B-factors set at 45 Å$^2$ followed by 15 cycles of individual atomic restrained refinement. Water molecules with peaks greater than 3$\sigma$ in the $F_o - F_c$ maps and those within hydrogen bonding distances from appropriate atoms were incorporated into the structure. The final refined structure at 2.54 Å resolution had an $R_{cryst}$ of 24.8% and an $R_{free}$ of 27.5% (Table 1).

RESULTS AND DISCUSSION

The structure of the N-TIMP-1-MMP-1cd complex (Fig. 1a) was determined at 2.54 Å resolution. Details of the data processing and refinement statistics are given in Table 1. The crystallographic asymmetric unit contains three copies of the complex. All the three complexes in the asymmetric unit are identical in their orientation and are translationally related to each other along the x axis by the vector 0.33, 0, 0. The MMP components of these complexes are designated A, B, and C and the cognate N-TIMP components D, E, and F, respectively. Residues 49–57 in all the three N-TIMP-1 molecules could not be positioned unambiguously because of the lack of electron density and were, therefore, modeled as glycines. The other residues modeled as glycine or alanine includes 41, 78, 91, 114, and 118 of N-TIMP-1. The disordered residues are distant from the binding interface and consequently do not affect our analysis of the interactions between the two components of the complex. The refined model includes 854 protein residues, 42 water molecules, 6 zinc ions, and 9 calcium ions. Analysis with PROCHECK (27) indicates that the $\phi$-$\psi$ conformational angles are within the limits expected for a structure at this resolution with no residues in the disallowed region of the Ramachandran plot (99.3% residues in the core region).

Overall Structure of the Complex—The inhibitory domain of TIMP-1 has a characteristic oligonucleotide binding-fold architecture consisting of a five-stranded $\beta$-sheet of Greek key topology and two or three associated $\alpha$-helices (three in N-TIMP-1). The closed $\beta$-barrel is bound by loops connecting strands B-C and strands C-D on each end. The individual N-TIMP-1 structures from the three complexes are closely similar to each other, with r.m.s.d. values of 0.7 Å. Least squares super-positioning of N-TIMP-1 (chain D) with N-TIMP-1 extracted from the published TIMP-1-MMP-3cd complex (PDB code 1UEA (11)) and the averaged NMR ensemble for free N-TIMP-1 (PDB code 1D2B (28)) yielded r.m.s. deviations of 0.93 and 1.46 Å, respectively. These alignments indicate that N-TIMP-1 in the MMP-1 complex is structurally similar to the inhibitory domain from the full-length TIMP-1 structure.

The catalytic domain of MMP-1 also exhibits the typical extended ellipsoid shape where the active-site cleft anchoring the catalytic zinc bifurcates the upper subdomain from the lower subdomain. Apart from the active-site zinc, the domain also contains a structural zinc ion and three calcium ions that are required for stability and structure. All the analyses reported here have been carried out using complex A and are replicated in the other two complexes of the asymmetric unit unless otherwise stated. The three MMP-1cd structures in the complexes are closely similar with r.m.s.d. values of 0.4 Å. Atom-by-atom r.m.s. deviations in these comparisons revealed that most of the differences lie in the loop connecting strands $sA$ and $sB$ (AB loop) and in the loop containing residues 64–73 (Fig. 1a). Similar comparisons of the catalytic domain of MMP-1 from complex A with the catalytic domain of procollagenase-1 (PDB code 1SU3 (29)), collagenase-1 (PDB code 1CGL (21)), and the averaged NMR ensemble (PDB code 2AYK (30)) resulted in r.m.s.d. values of 0.45, 0.54, and 1.30 Å, respectively. These comparisons (and others for which r.m.s.d. values are not shown) indicate that the catalytic domain in our structure is very similar to the catalytic domains of other known crystal structures of MMP-1.

The overall orientation of N-TIMP-1 relative to MMP-1cd is similar to that observed in the TIMP-1-MMP-3cd complex (PDB code 1UEA (11)) and the TIMP-2-MT1-MMPcd complex (PDB code 1BQQ (12)). The three complexes in the asymmetric unit (complexes A, B, and C) superimpose with a mean r.m.s.d. of 0.9 Å. Structural alignment of complex A with the TIMP-1-MMP-3cd complex gave an r.m.s.d. of 0.85 Å (272 C$^\alpha$ atoms), whereas alignment with the TIMP-2-MT1-MMPcd complex gave a r.m.s.d. of 1.55 Å (217 C$^\alpha$ atoms). In all these superpositions it was observed that the catalytic domains of the different complexes aligned quite well, whereas the TIMP components exhibited most of the differences.

Characteristics of the Interface—N-TIMP-1 recognizes three segments of the catalytic domain of MMP-1: residues 178–186, 218–228, and 238–240 (Fig. 1c). Formation of the complex buries a total of about 1500 Å$^2$ of the solvent-accessible area of the two proteins, about 60% of the interface being contributed by MMP-1cd. In all 18 residues from N-TIMP-1 and 22 resi-
hydrogen bonds with the TIMP-1-MMP-3cd complex and 9 with the TIMP-2-MT1-MMPcd complex; 7 hydrogen bonds are conserved between all three complexes. The majority of all intermolecular contacts is made by the five N-terminal residues (Cys-1–Pro-5) and residues Met-66–Cys-70 from the connector loop (loop connecting strands sA and sD). Residues Cys-1, Thr-2, Glu-67, and Ser-68 make 62% of the van der Waals interactions and 75% of the hydrogen-bonding interactions at the interface.

Cys-1 of N-TIMP-1 coordinates the catalytic zinc via its α-amino nitrogen and the peptide carbonyl group. This interaction is crucial for the inhibitory activity of the TIMP molecule because the α-amino group of Cys-1 displaces the active-site water molecule important for peptide bond hydrolysis (Fig. 1b). The second residue of N-TIMP-1, Thr-2, extends into the S1′ specificity pocket located immediately to the right of the catalytic zinc of MMP-1. This residue forms 3 H-bonds and makes 13 van der Waals contacts with Gly-178, Ala-184, Asn-180, and Gly-186 of MMP-1cd. The connector loop of N-TIMP-1, comprising residues 66–70, occupies the active site of the MMP-1 to the left of the catalytic zinc. This loop makes many interactions with MMP-1 residues from the continuous bulge-edge strand and the active-site cleft: Ser-172, Asn-180, His-183, Ala-184, Phe-185, Gln-186, His-222, Ser-227, and His-228. Of particular interest is Glu-67 of N-TIMP-1, which makes far more extensive contacts with MMP-1 (12 contacts) than are made by the corresponding residue in the TIMP-1-MMP-3cd complex (2 contacts). Also, Pro-

FIGURE 1. a, ribbon representation of the three-dimensional structure of the N-terminal domain of TIMP-1 in complex with the catalytic domain of MMP-1. The MMP-1 component of the complex is colored pink, and the N-TIMP-1 is shown in green. There are three calcium ions and two zinc ions in the catalytic domain of the protease that are represented as spheres in gray and slate blue, respectively. The secondary structural elements of the enzyme have been annotated: helices (hA–hC) and strands (s1–s5). The secondary structural elements of N-TIMP-1 have also been annotated: helices (h1–h3) and strands (sA–sF). The cysteine residues involved in the formation of disulfide bonds in the N-TIMP-1 molecule have been numbered. The figure was made using the program Bobscript (35) and rendered using Raster3D (36). b, structure of the active site of MMP-1 from the N-TIMP-1-MMP-1cd complex superimposed onto the active site of the E200A variant of human MMP-1 (PDB code 2CLT (37)). The complex is shown in green, and the mutant enzyme is colored pink. The catalytic zinc ion is represented as a slate blue sphere. The water molecule from the mutant enzyme structure is shown in cyan. The figure was made using the program Bobscript (35) and rendered using Raster3D (36). c, molecular surfaces of the inhibitor (left panel) and the enzymes (right panel) from the N-TIMP-1-MMP-1cd complex and the TIMP-1-MMP-3cd complex. The surfaces have been color-coded according to the type of contact atoms at the interface. Non-polar atoms are shown in split pea green, uncharged polar atoms are shown in marine blue, and charged polar atoms are shown in raspberry red. N-TIMP-1 was rotated by about −90°, along the y axis, from the standard orientation shown in Fig. 1a. The enzymes have been rotated by about +90° along the y axis from the standard orientation shown in Fig. 1a. The program PyMol was used to draw the molecular surfaces (www.pymol.org).
Structure of N-TIMP-1-MMP-1cd Complex

64, Ala-65, and Met-66 form 11 van der Waals contacts in the interface with MMP-3, whereas only Met-66 makes contacts (3 contacts) with Gln-186 of MMP-1. Residues from the AB loop, 33TQ, do not interact with the enzyme in either complex A or complex C. In complex B, however, Tyr-35 of N-TIMP-1 makes two van der Waals interactions and 1 H-bond with Asn-171 of the protease. This contrasts with the multiple contacts observed in the TIMP-1-MMP-3cd complex (Table 2) and suggests that interactions involving the AB loop do not make a significant contribution to the free energy of interaction with MMP-1. There is only one water-mediated interaction observed at the interface of complex A. This water molecule, W3, bridges an interaction between Ser-68 of the inhibitor and Gln-186 of the enzyme. However, this interaction is not present in the other two complexes in the asymmetric unit.

**Table 1**

Crystallographic, data processing, and refinement statistics

| Space group | Monoclinic, C2 |
|-------------|---------------|
| Unit cell dimensions (Å) | a = 158.09, b = 67.85, c = 86.24 |
| Resolution range (Å) | 23.0-2.54 |
| Total reflections measured | 191,162 |
| Unique reflections measured | 29,526 |
| Refinement statistics | |
| Rcryst (%) | 8.8 (45.1) |
| Rfree (%) | 98.4 (99.4) |
| Completeness (outermost shell) (%) | 8.3 (2.8) |
| Bond angles (°) | 0.9 |
| Bond lengths (Å) | 0.006 |
| Ions (6 zinc and 9 calcium) | 41.7 |
| Solvent molecules | 31.3 |
| Side-chain atoms | 46.3 (A), 46.1 (B), 45.0 (C) |
| Main-chain atoms | 43.8 (D), 43.9 (E), 44.0 (F) |
| All atoms (D, E, F) | 43.6 (A), 43.7 (E), 43.5 (F) |
| Ions (6 zinc and 9 calcium) | 41.7 |
| Solvent molecules | 31.3 |
| Side-chain atoms | 46.3 (A), 46.1 (B), 45.0 (C) |
| Main-chain atoms | 43.8 (D), 43.9 (E), 44.0 (F) |
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| Solvent molecules | 31.3 |
| Side-chain atoms | 46.3 (A), 46.1 (B), 45.0 (C) |
| Main-chain atoms | 43.8 (D), 43.9 (E), 44.0 (F) |
| All atoms (D, E, F) | 43.6 (A), 43.7 (E), 43.5 (F) |
| Ions (6 zinc and 9 calcium) | 41.7 |
| Solvent molecules | 31.3 |

**Table 2**

Comparison of van der Waals interactions at the binding interface of N-TIMP-1-MMP-1cd complex (present structure) and TIMP-1-MMP-3cd complex (11)

| N-TIMP-1 residue | MMP-1 interactions | MMP-3 interactions |
|------------------|--------------------|--------------------|
| Cys-1 Ala-182(2), His-183, His-218(9), Glu-219(6), His-222(2), His-228(10) | Val-163, Ala-165(2), His-166, His-201, Glu-202(2), His-203(9), His-211(9), Pro-221(14) | |
| Thr-2 Asn-180(2), Leu-181, Val-215(2), His-218, Glu-219(6), Pro-238(2), Tyr-240(2) | Ala-165, Val-198(2), His-201, Glu-204(6), Pro-221(2) | Asn-162(2), Val-163(2), Leu-222(2) |
| Cys-3 Gly-179, Ser-239(2) | Asn-162(2), Leu-164(4) | Leu-222(2) |
| Val-4 Gly-179(3), Leu-181, Tyr-211(9) | Phe-86(1) | |
| Pro-9 Ser-239(3) | Thr-85(3), Phe-86(3) | Phe-145(7), Tyr-155(10) |
| Val-29 | Tyr-155(4) | |
| Thr-33 | Phe-86(2) | |
| Leu-34 | Phe-86(4), Phe-210(5) | |
| Pro-64 | His-211(2) | |
| Ala-65 | Ala-167(2), His-205(2), His-155(9), His-166(2), Tyr-168(10) | Val-163(2) |
| Glu-67 | His-211(2), His-166(2), Tyr-168(10) | |
| Ser-68 | Val-163(2) | |
| Val-69 | His-183, Ala-184(6), His-222(2) | |
| Cys-70 Asn-180(2), His-183(4) | Pro-238(1) | |
| Thr-98 | Cys-99 Asn-180(3) | |
| Total 87 | 96 | |
the N-terminal region of the protease are not observed in the MMP-1 complex (all the comparative analyses reported here relate only to the N-terminal domain of the TIMP-1 and not the full-length TIMP-1 from the TIMP-1-MMP-3cd complex). Of the 12 hydrogen-bonding interactions between the inhibitor and the protease, 10 are equivalent between the two complexes (Table 3). Beyond these hydrogen bonds, the two complexes also share a number of van der Waals contacts. What makes the interactions more distinct in the two structures is the difference in the chemical character of the atoms participating in the interactions at the interface (Table 4). The N-TIMP-1-MMP-1cd interface is about 31% non-polar, 69% polar. This distribution is considerably more polar than that in the TIMP-1-MMP-3cd complex (which is about 42% non-polar and 58% polar) with the proportion of charged residues being higher.

The binding surface on the TIMP-1 molecule in the two complexes with the different MMPs is characteristically different in relation to the role of the AB loop. Similarly, the chemical characters of the atoms involved from the MMP counterparts in the two complexes also differ. This suggests that although the overall docking of the MMPs in the two TIMP-1 complexes analyzed so far is similar, the specific interactions at the interface are quite distinct and the versatility of TIMP-1 to inhibit both MMP-1 and MMP-3 reflects its ability to interact with both conserved features of the active sites and features unique to each of the two MMPs.

**Correlation of Structural Data with Results from Mutagenesis Experiments**—Twelve residues of N-TIMP-1 surrounding the Cys-1-Cys-70 disulfide bond make contacts with the catalytic domains of MMP-1 and MMP-3 in their respective complexes. This region is structurally constrained by the Cys-1-Cys-70 and Cys-3-Cys-99 disulfides, which reduce the flexibility of the reactive site and appear to be important for the high affinity binding of TIMP-1 to MMPs by lowering the conformational entropy loss arising from enhanced rigidity in the interaction site on complex formation. The effects of mutating six of these contact residues (Thr-2, Val-4, Met-66, Ser-68, Val-69, Cys-70) on the affinity for various MMPs has been previously investigated. Although multiple substitutions are required to produce N-TIMP-1 variants with high selectivity for MMPs (9, 14, 15, 33), substitutions for Thr-2 of N-TIMP-1 show that this residue has a dominant influence on the free energy of binding selectivity for MMPs. Consequently, this residue, which is located centrally in the MMP binding ridge and interacts with the S1’ specificity pocket of MMPs, has been designated as a hot spot in the interaction interface (14, 15).

To understand the effects of Thr-2 substitutions, we modeled selected mutants of N-TIMP-1 in the present N-TIMP-1-MMP-1cd complex and the previously reported TIMP-1-MMP-3cd complex. Each complex was subjected to energy minimization using CNS (23) to ensure that the mutated structure was devoid of steric clashes. Analyses of the effects of some mutations on N-TIMP-1-MMPcd contacts are summarized in Table 5.

The N-TIMP-1 mutant with a T2G substitution, which effectively eliminates the side chain, is greatly reduced in affinity for most MMPs, including MMP-1 and MMP-3, indicating the importance of interactions with the MMP-1 S1’ pocket. In the model containing this mutant in a complex with MMP-1 there are 33 fewer van der Waals contacts and 11 fewer H-bonding interactions at the interface. Residue 2 alone has 9 fewer interactions as compared with the complex containing wild-type

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**TABLE 3**

Comparison of hydrogen-bonding interactions at the binding interface of N-TIMP-1-MMP-1cd complex (present structure) and TIMP-1-MMP-3cd complex (11).

| Residue | Distance (Å) |
|---------|--------------|
| Cys-1 N | 2.89         |
| Cys-1 O | 2.86         |
| Cys-1 O | 3.19         |
| Thr-2 N | 2.93         |
| Thr-2 O | 2.81         |
| Thr-2 Oy| 2.63         |
| Cys-3 N | 2.89         |
| Cys-3 O | 3.00         |
| Val-4 N | 3.09         |
| Glu-67 Oe1 | 2.91    |
| Ser-68 Oy| 2.73         |
| Ser-68 O | 3.32         |
| Total   | 12           |

**TABLE 4**

Comparison of the chemical character of contact residues/atoms at the interface.

| Chemical character | N-TIMP-1-MMP-1cd | N-TIMP-1-MMP-3cd | N-TIMP-1-MMP-3cd<sup>a</sup> |
|-------------------|------------------|------------------|-----------------------------|
| Residues          | Atoms            | Residues         | Atoms                       | Residues                   |
| Non-polar (%)     | 31.2             | 41.7             | 47.7                        | 35                          |
| Polar (%)         | 68.8             | 58.3             | 52.3                        | 65                          |

<sup>a</sup> Values listed for the N-TIMP-1-MMP-3cd complex in the last column are taken from Otwinowski and Minor (18). The other calculations are our analysis based on the present structure and the crystal structure of N-TIMP-1-MMP-3cd (11).
N-TIMP-1. Interactions of other N-TIMP-1 residues, particularly Pro-5, Met-66, and Cys-99 at the periphery of the interaction site, increase in the model, but these are expected to make weaker contributions to the free energy of binding. In the case of the complex of the T2G mutant with MMP-3, fewer interactions from residue 2 are lost (9, as opposed to 10), and the higher affinity for MMP-3 relative to MMP-1 for the T2G mutant suggests that the interactions of the side chain of residue 2 are less important for binding MMP-3 than MMP-1.

An analysis of the effects of side-chain chemistry on binding to different MMPs for 15 different substitutions for residue 2 of N-TIMP-1 shows that hydrophobicity favors binding to MMP-1 relative to MMP-3, whereas increased size is unfavorable for MMP-1 but favorable for MMP-3. Side-chain charge, particularly positive charge, disfavors binding to MMP-1 relative to other MMPs including MMP-3. The Arg-2 mutant shows the greatest selectivity against MMP-1 relative to MMP-2 and MMP-3 (14). This is consistent with the structure of the S1’ pocket of MMP-1. Modeled complexes for this mutant show 2 additional hydrogen-bonding interactions and 27 more van der Waals contacts in the MMP-3 complex as compared with the MMP-1 complex. Because the S1’ pocket of MMP-1 is narrower and more shallow than in other MMPs (34), it is difficult to accommodate the larger arginine side chain without structural rearrangement. Furthermore, the presence of Arg-214 in MMP-1 S1’ pocket in place of leucine at the equivalent site in MMP-3 would be expected to result in charge repulsion between the Arg-2 of N-TIMP-1 and Arg-214 of MMP-1. The modeled N-TIMP-1(T2R):MMP-1cd complex shows a rearrangement in which the side chain of Arg-214 projects away from the S1’ pocket instead of inwards. The rearrangement of surrounding residues is reflected in an r.m.s.d. of ∼1.3 Å between the wild-type and modeled complex containing the N-TIMP-1 mutant.

Substitutions of negatively charged residues for Thr-2 are unfavorable for binding to MMPs. MMP-1, however, is intolerant to both negative and positive charges at this site (15). To attempt to understand this, we have compared the interactions at the interface in models of the isosteric pairs, T2D/T2E and T2D/T2Q, in complexes with MMP-1 and MMP-3. This reveals that there are one-third fewer total van der Waals for all of these mutants in the MMP-1 complex as compared with the wild-type N-TIMP-1 complex. Similarly, the slightly larger number of van der Waals contacts for the T2N/T2Q mutants relative to the T2D/T2E mutants in the N-TIMP-1-MMP-3cd complex correlates well with the results of activity measurements (14). Substitution of different nonpolar residues at position 2 also affects the binding of N-TIMP-1 to MMPs. The preference for the smaller size and non-polarity of the side chain at position 2 for MMP-1 binding is reflected in the selectivity of the T2V mutation for MMP-1. Modeling indicates that valine at this position results in 7 more van der Waals contacts in the MMP-1 complex compared with the wild-type protein but 25 fewer in the MMP-3 complex, explaining the preference for MMP-1 relative to MMP-3.

The structures of TIMP-MMP complexes provide a basis for designing TIMP variants with increased selectivity for different MMPs. Such variants are potentially useful for investigating the roles of different MMPs in biological and pathological processes and may provide a novel route for therapy in diseases associated with excess MMP activities. The present study shows that although TIMP-1 has a similar affinity for MMP-1 and MMP-3, there are striking differences in the chemistry and structures of the interaction sites. This suggests that the elucidation of the structures of more MMP-TIMP complexes would be worthwhile.

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Structure of N-TIMP-1-MMP-1cd Complex

| Mutants | N-TIMP-1-MMP-1cd | TIMP-1-MMP-3cd |
|---------|-----------------|----------------|
|         | VWC | HB | Ki  |    | VWC | HB | Ki  |    |
| Wild type | 87  | 12 | 0.4 |    | 96  | 12 | 0.2 |    |
| T2R      | 54  | 2  | ~2000 |    | 81  | 4  | 11 |    |
| T2V      | 94  | 11 | 0.6 |    | 71  | 3  | 1.2 |    |
| T2G      | 54  | 1  | 7000 |    | 77  | 3  | 560 |    |
| T2Q      | 57  | 2  | 350 |    | 73  | 2  | 12 |    |
| T2E      | 48  | 1  | 2290 |    | 62  | 3  | 187 |    |
| T2K      | 57  | 3  | 668 |    | 83  | 4  | 28 |    |

T2N  3  57  668
T2Q  57  2  350
T2E  48  1  2290
T2K  57  3  668

a Values from Wei et al. (15).

Estimated from values reported previously (14) by correcting for fraction of active N-TIMP-1 (17).
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