Residue 2 of TIMP-1 Is a Major Determinant of Affinity and Specificity for Matrix Metalloproteinases but Effects of Substitutions Do Not Correlate with Those of the Corresponding P1′ Residue of Substrate*

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The unregulated activities of matrix metalloproteinases (MMPs) are implicated in disease processes including arthritis and tumor cell invasion and metastasis. MMP activities are controlled by four homologous endogenous protein inhibitors, tissue inhibitors of metalloproteinases (TIMPs), yet different TIMPs show little specificity for individual MMPs. The large interaction interface in the TIMP-1-MMP-3 complex includes a contiguous region of TIMP-1 around the disulfide bond between Cys1 and Cys70 that inserts into the active site of MMP-3. The effects of fifteen different substitutions for threonine 2 of this region reveal that this residue makes a large contribution to the stability of complexes with MMPs and has a dominant influence on the specificity for different MMPs. The size, charge, and hydrophobicity of residue 2 are key factors in the specificity of TIMP. Threonine 2 of TIMP-1 interacts with the S1′ specificity pocket of MMP-3, which is a key to substrate specificity, but the structural requirements in TIMP-1 residue 2 for MMP binding differ greatly from those for the corresponding residue of a peptide substrate. These results demonstrate that TIMP variants with substitutions for Thr2 represent suitable starting points for generating more targeted TIMPs for investigation and for intervention in MMP-related diseases.

The matrix metalloproteinases (MMPs)1 are a family of about twenty Zn2+–dependent endopeptidases that have important roles in connective tissue turnover during physiological processes including development, morphogenesis, and wound healing (1, 2). Their activities in the extracellular matrix arestringently regulated through transcriptional control, zymogen activation, and the actions of four endogenous inhibitory proteins, tissue inhibitors of metalloproteinases (TIMPs)1 to 4 (3–7). Normal matrix homeostasis is associated with an appropriate balance between the levels of TIMPs and active MMPs, whereas an imbalance involving excess MMP activity is linked with disease processes including arthritis, tumor cell metastasis, and tissue invasion and atherosclerosis (1, 2).

Mammalian TIMPs have an N-terminal domain of about 125 amino acids and a smaller C-terminal domain of about 65 amino acids; each domain is stabilized by three disulfide bonds (8). The N-terminal domains of different TIMPs fold into a correct native structure which carries the inhibitory activity against MMPs (9–11). Although correctly folded and functional C-terminal domains have not been described, truncation experiments indicate that this region is responsible for the interactions of TIMPs with pro-MMPs (12, 13). There is little specificity in the inhibitory actions of TIMPs on metalloproteinases, with the exception of the ability of TIMP-2 and TIMP-3 to inhibit membrane-type metalloproteinases-1 and -2, whereas TIMP-1 is a poor inhibitor of these enzymes (12–14). However, the interactions of TIMPs with pro-MMPs are more specific. For example, TIMP-2 and TIMP-4 form specific complexes with pro-MMP-2 (progelatinase A), whereas TIMP-1 can bind to pro-MMP-9. In addition to their activities as MMP inhibitors and in binding to pro-MMPs, TIMPs promote the growth of various types of cells in tissue culture (15, 16) and have antiangiogenic activity (17). However, the structural basis of these activities is unknown.

Crystallographic structures have been recently reported for a complex of TIMP-1 with the catalytic domain of stromelysin-1, MMP-3ΔC (18), and a complex of TIMP-2 with the catalytic domain of membrane-type matrix metalloproteinase 1 (19). Together with a solution NMR structure of the N-terminal domain of TIMP-2, N-TIMP-2 (20, 21), these reveal that the inhibitory domain of TIMP consists of a 5-stranded β-barrel with three associated α-helices, resembling the folds of members of the OB (oligonucleotide/oligosaccharide binding) protein family (22). The TIMP-1-MMP-3 structure reveals that the principle interactions between TIMP and the metalloproteinase involve the N-terminal pentapeptide and part of the loop between β-strands C and D; other interactions are through the A-B loop and some residues in the C-terminal domain (Fig. 1A). Three quarters of all contacts are by residues adjacent to the disulfide bond between Cys1 and Cys21, specifically residues 1–5 and 66–70 (18). The N-terminal Cys1 is a key to the inhibitory strategy of TIMP because it sits on top of the catalytic Zn2+ of the metalloproteinase and coordinates the metal ion through the α-amino group and peptide carbonyl group (Fig. 1B). Similar contacts are seen in the TIMP-2-MT1-MMP complex, although there are differences in the relative orienta-

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1 The abbreviations used are: MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of metalloproteinases; N-TIMP-1, N-terminal domain of tissue inhibitor of metalloproteinases-1.
Thr², Met⁶⁶, or Val⁶⁹ had large effects on activity (24). Most significantly, whereas other substitutions that perturb N-TIMP-1 activity have approximately equal effects on binding to MMP-1, MMP-2, and MMP-3, the substitution of Ala for Thr² produces a 17-fold greater loss in binding to MMP-1 relative to MMP-3 (24). This is in accord with the crystallographic structures that indicate that Thr² of TIMP-1 and Ser² of TIMP-2 interact with the region of the metalloproteinases that correspond to the binding site for the P1' residue of peptide substrates, the residue that has a dominant role in MMP specificity (18, 19).

As part of a study of the structural basis of TIMP-1 specificity and as a step toward generating variants that are more selective as MMP inhibitors, we have characterized fifteen N-TIMP-1 mutants with substitutions for Thr². The results show that this residue has a major influence on the specificity of TIMP for different metalloproteinases but also show that there is little correlation between the effect of an amino acid at position 2 in TIMP-1 and the same residue at the P1' site of a peptide substrate on their respective activities with an MMP as inhibitor or substrate. The structural basis of these observations is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Vectors, cell lines, and enzymes for cloning, plasmid purification kits and polymerase chain reaction purification kits were from the same sources as in previous studies (11, 24). C-terminal truncated MMP-3 (MMP-3ΔC) and active forms of MMP-1 and MMP-2 were generated as described (11). Synthetic oligonucleotides were synthesized in the laboratory of Dr. R. Werner, Dept. of Biochemistry and Molecular Biology, University of Miami School of Medicine or at the University of Kansas Medical Center. The primers used for mutagenesis are listed in Table I.

**Construction and Expression of N-TIMP-1 Mutants**—Mutations were introduced by the polymerase chain reaction megaprimer method (25) with previously described modifications and using the pET3a-N-TIMP-1 expression vector as template (11, 24). The megaprimer was amplified either using the T7 promoter primer or T7 terminator primer and a mutagenic primer. The megaprimer (200 base pairs) was purified by electrophoresis in 2% low melting agarose gel, followed by the use of a Magic PCR™ purification kit. The megaprimer and the cognate T7 terminator or promoter primer were used in the second amplification, and the product was purified, digested with BamHI and NdeI, and cloned into pET3a. The sequence of each mutant was confirmed by DNA sequencing.

**Other Methods**—MMP assays were conducted using synthetic fluorogenic substrates. MMP-3 activity was assayed using (7-methoxy-coumarin-4-yl)acetyl-Arg-Pro-Val-Glu-norvalinyl-Trp-Arg-Lys-(2,4-dinitrophenyl)-NH₂ (26) and MMP-1 and MMP-2 with (7-methoxy-coumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-N(3-(2,4-dinitrophenyl)-l-2,3-diaminopropyl)-Ala-Arg-NH₂ (27) as described (24). The MMP (0.1–5

**TABLE I**

| Primer | Sequence | Orientation |
|--------|----------|-------------|
| T7 promoter | 5′CTATACGACTCACTATAGG3’ | Ceding |
| T7 terminator | 5′GTTCAGACAGGATACATAG3’ | Complementary |
| T2S | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| T2G | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| T2H | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| T2Y | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| T2L | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| T2I | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| T2M | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| T2F | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| TN | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| T1K | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| T1R | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| T1N | 5′ACGACGAGGAGATACATAG3’ | Complementary |
| T1F | 5′ACGACGAGGAGATACATAG3’ | Complementary |

**FIG. 1. Structural features of the N-TIMP-1-MMP-3 (ΔC) interaction.** A, the structure of the N-TIMP-1-MMP3 (ΔC) based on coordinates extracted from the crystallographic structure of the TIMP-1-MMP-3 complex (20). N-TIMP-1 is colored blue and MMP-3 is red. The β-strands MMP-3 are labeled s through sV and the helices are hA to hC, whereas the β-strands of N-TIMP-1 are designated sA through sF and the helices are hI to hIII. The green spheres are zinc ions, and the dark spheres calcium ions. N and C denote the N terminus of MMP-3 and C terminus of N-TIMP-1, respectively. The figure was drawn using MOLSCRIPT (36). B, a schematic representation of the N-terminal region of N-TIMP-1 indicating residues that form the part of the reactive site for MMP-3.

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nm) and a range of concentrations of N-TIMP-1 variant were preincubated in 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, 10 mM CaCl₂, and 0.02% Brij 35 at 37 °C for 1 h. An aliquot (60 μl) of substrate (15 μM) was added to 540 μl of TIMP mixture and activity determined at 37 °C by following product release by fluorescence. Inhibition data for higher affinity mutants, where the level of bound inhibitor significantly reduces the concentration of free inhibitor, were analyzed using a treatment for tight binding inhibitors (28) but with lower affinity variants (Kᵢ $\geq$ 100 nM), and data were analyzed as for a normal reversible inhibitor.

CD spectra were determined using a Jasco J-710/720 spectropolarimeter. Proteins were dissolved in 20 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl at concentrations of approximately 0.5 mg/ml, and 20 scans from 250–320 nm were collected and averaged. Automated DNA sequencing was performed with a Perkin Elmer/Applied Biosystems DNA Sequencer model 373 in the laboratory of Dr. R. Werner, University of Miami School of Medicine.

RESULTS

Expression, Folding, and Purification—All 15 substitutions at position 2 (Table I) were compatible with N-TIMP-1 expression as inclusion bodies. The proteins were extracted, partially purified under denaturing conditions by ion exchange chromatography and gel filtration, and treated to generate native protein as described. During folding, losses of some variants occurred, resulting in different yields of folded material after cation exchange chromatography with Cm-cellulose. Folded protein was not obtained from the His² variant at this step, and it appears that this substitution is incompatible with in vitro folding under the conditions used. Mutants with substitutions of Phe, Arg, Lys, Arg, Asp, Glu, and Gln, produce two protein species that are separated by cation exchange chromatography. The component eluting at lower salt concentration appears, based on its CD spectrum, to have a similar structure to the wild-type protein (11) and was characterized with respect to activity, whereas the second component had very low activity as an inhibitor of MMP-3. Variants with substitutions of Thr, Met, or Asn were isolated in higher yields (6–20 mg/liter of culture), whereas the yields of the folded forms of the other mutants were less than 3 mg/liter.

Analysis of Structural and Functional Properties of Position 2 Mutants—The near UV CD spectra of variants of N-TIMP-1 provide a sensitive guide to the presence of native tertiary structure (24), whereas the far UV CD spectrum is dominated by a trough at around 208 nm that is not characteristic of any type of secondary structure. As shown in Fig. 2, all of the mutants with substitutions for Thr² appear to have correctly folded native structures. However, the second component separated by cation exchange chromatography after folding of some variants has a perturbed tertiary structure (Fig. 2, B and D). Non-reducing SDS gel electrophoresis indicates that this component is monomeric, but its low activity and modified structure suggest that it is a highly populated metastable conformation of some residue 2 variants. Studies are in progress to further characterize the structure of this conformer.

To measure the inhibitory activities of the mutants against MMP-1, MMP-2, and MMP-3, samples of N-TIMP-1 variant and protease were preincubated for 60 min at 37 °C to allow binding to reach equilibrium and remaining proteinase activity measured by the addition of 0.1 volume of fluorogenic substrate. To achieve levels of proteinase activity that can be measured precisely, MMP-1 was used at concentrations of 2–5 nM, but MMP-2 and MMP-3 could be assayed in the 0.5–1 nM range. The measurement of accurate inhibition constants (Kᵢ values) of N-TIMP-1 variants requires a range of concentrations of similar magnitude to the Kᵢ, which varies widely among the proteins studied here. As discussed previously (11), under the assay conditions used, [S] $\ll$ Kᵢ, so that the Kᵢ (apparent) determined from these analyses is insignificantly different.
from the true $K_i$. The highest errors in $K_i$ (Table II) are for some mutants with low MMP affinities because the high protein concentrations required for the accurate determination of $K_i$ were not available because of relatively low yields of the folded protein.

The Presence of a Side Chain on Residue 2 Is Crucial for Effective MMP Binding—The Gly$^2$ mutant is the weakest inhibitor for all three MMPs. Because this protein folded with reduced efficiency and was available in limited amounts, the highest concentration used in inhibition assays was 8 μM. The affinity for MMP-3 could be measured using the accessible concentration range, but only low levels of inhibition were observed with MMP-1 and MMP-2 at the highest concentration of inhibitor (31 and 7.2%, respectively) from which the provisional values in Table II were calculated. Although the $K_i$ values for Gly$^2$ with MMP-1 and MMP-2 were not determined precisely, these results show that the presence of a side chain on residue 2 is crucial for effective inhibition of all three MMPs.

The Mode of Binding of N-TIMP-1 Residue 2 Differs from the Binding of the P1’ Residue of a Substrate—Wild-type TIMPs have Thr or Ser as residue 2 and are effective inhibitors of MMP-1, -2, and -3. However, peptides with Ser and Thr at the P1’ site are poor substrates (29). This suggests that the P1’ residue of a substrate and residue 2 of N-TIMP-1 may interact with the S1’ pocket of a metalloproteinase differently. There is a very poor correlation between $-\log K_i$ for MMP-2 and $-\log K_i$ for N-TIMP-1 variant. For MMP-1 and MMP-3 the correlations are negative ($r^2$, 0.19 and 0.08, respectively), whereas the correlation for MMP-2 is weakly positive ($r^2 = 0.17$). Unfortunately, quantitative data are only available for seven amino acids in the substrate, and the comparison with $k_{cat}/K_m$ rather than $1/K_i$ is not ideal because the former relates to the affinity of an enzyme for the transition state rather than for substrate (29).

Nevertheless, there is clearly a large difference between recognition of the P1’ residue of a substrate and residue 2 of TIMP by the S1’ sites of MMPs. Possible sources of this are the different structural contexts of the P1’ residue of a substrate and residue 2 of TIMP. The binding of peptide substrates is associated with a greater loss of conformational entropy than the interaction with TIMP-1, whereas the orientation of residue 2 of TIMP in the complex is influenced by the interactions of Cys$^3$ with the active site Zn$^{2+}$. Also, the effects of substitutions for residue 2 on the conformation of the N-terminal region of TIMP could be a factor.

### Table II

| Amino acid | $K_i$ ± S.E. in μM | Selectivity$^a$ | MMP-1 | MMP-2 | MMP-3 |
|------------|-------------------|----------------|--------|--------|--------|
| MMP-1      | MMP-2             | MMP-3          |        |        |        |
| Thr (WT)   | 0.8 ± 0.4         | 1.1 ± 0.1      | 2.1 ± 0.3 | 0.5 ± 0.1 | 0.09   | 4.0 ± 0.1 | 3.6 | 17 |
| Ser        | 14.7 ± 1.3        | 6.1 ± 0.3      | 2.1 ± 0.3 | 0.5 ± 0.1 | 0.09   | 4.0 ± 0.1 | 3.6 | 17 |
| Gly        | (18 ± 10$^{9b}$)  | (10±10$^{9b}$) | (10±10$^{9b}$) | 1380 ± 130 | [2.9]  | [0.09]  | [43] |
| Ala$^c$    | 2090 ± 180        | 307 ± 17       | 126 ± 4 | 0.3 | 3.4 | 8.3 |
| Val        | 1.6 ± 0.2         | 4.5 ± 0.2      | 3.0 ± 0.2 | 0.02 | 48   | 15 |
| Leu        | 93 ± 14           | 1.0 ± 0.1      | 3.2 ± 0.4 | 0.02 | 48   | 15 |
| Ile        | 282 ± 55          | 5.6 ± 0.4      | 20 ± 2.7 | 0.05 | 25   | 6.7 |
| Met        | 10.9 ± 1.0        | 0.7 ± 0.1      | 0.7 ± 0.1 | 0.06 | 8.3 | 8.3 |
| Phe        | 42 ± 13           | 17 ± 2         | 13 ± 1 | 0.4 | 1.6 | 2.3 |
| Asn        | 1970 ± 400        | 16 ± 2         | 44 ± 3 | 0.02 | 63   | 23 |
| Gln        | 870 ± 220         | 12 ± 2         | 29 ± 2 | 0.02 | 36   | 12 |
| Asp        | 8130 ± 3170       | 1250 ± 140     | 1110 ± 140 | 0.15 | 3.7 | 4.2 |
| Glu        | 5730 ± 1460       | 433 ± 48       | 468 ± 28 | 0.03 | 28   | 12 |
| Lys        | 1670 ± 420        | 31 ± 5         | 70 ± 16 | 0.03 | 28   | 12 |
| Arg        | 5010 ± 1750       | 12 ± 0.5       | 28 ± 2 | 0.04 | 210 | 90 |

$^a$ This is defined for as $(K_i/M)^{1/3}/(2 \times K_i^2)$, where $x$ is the MMP under consideration and $y$ and $z$ are the cognate MMPs.

$^b$ Data estimated from the level of inhibition at a concentration of 8 μM.

$^c$ Data taken from Huang et al. (24).
most discriminating against MMP-1, being 400-fold weaker in affinity for MMP-1 as compared with MMP-2. Although the affinity for MMP-2 and MMP-3 increases with increasing side chain size for most non-polar amino acids (exceptions being Ile and Phe), in the case of MMP-1 the highest affinity is for the mutant with Val2; substitution of side chains that are either larger or smaller than valine results in weaker binding. The optimal size and shape of valine for binding to MMP-1 is supported by the fact that the wild-type protein containing threonine (isosteric with valine) at position 2 is the second most avid inhibitor of MMP-1, whereas the serine has a 5-fold lower affinity. The best inhibitors for MMP-2 and MMP-3 are the Met2 and Ser2 variants, respectively, but the affinity of Met2 for MMP-3 is insignificantly less than that of Ser2; the Leu2 mutant has the best combination of selectivity and high affinity for MMP-2. Selectivity for MMP-3 relative to MMP-2 appears to reflect a preference by MMP-3 for smaller side chains. This can be seen from the ratio of $K_i$ values for MMP-2 and MMP-3 for wild-type N-TIMP-1 and Ser2, as well as for the Gly and Ala mutants (Table II).

### DISCUSSION

The present results suggest that protein engineering of TIMP is a viable approach for generating more specific protein inhibitors of MMPs for studies of the biological roles of different MMPs and to facilitate the development of therapeutic agents for diseases linked with excess activity of specific MMPs. The development of specifically targeted high affinity variants will require substitutions at multiple sites but the inhibitory properties of the Met66 and Val99 mutants and full-length TIMP-1 and MMP-3 for wild-type N-TIMP-1 and Ser2, as well as for the Gly and Ala mutants (Table II).

**Fig. 3.** Log-log plots of $K_i$ values for pairs of MMPs. **Solid circles** are data for TIMP-1, N-TIMP-1, and N-TIMP-1 and mutants with substitutions at sites other than Thr2 (24); **open squares** represent data for N-TIMP-1 mutants with substitutions for Thr2.
cations that affect TIMP specificity.

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