Unveiling the Surface Epitopes That Render Tissue Inhibitor of Metalloproteinase-1 Inactive against Membrane Type 1-Matrix Metalloproteinase*

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Membrane type 1-matrix metalloproteinase (MT1-MMP) is a zinc-dependent, membrane-associated endopeptidase of the metzincin family. The enzyme regulates extracellular matrix remodeling and is capable of cleaving a wide variety of transmembrane proteins. The enzymatic activity of MT1-MMP is regulated by endogenous inhibitors, the tissue inhibitor of metalloproteinases (TIMP). To date, four variants of mammalian TIMP have been identified. Whereas TIMP-2–4 are potent inhibitors against MT1-MMP, TIMP-1 displays negligible inhibitory activity against the enzyme. The rationale for such selectivity is hitherto unknown. Here we identify the surface epitopes that render TIMP-1 inactive against MT1-MMP. We show that TIMP-1 can be transformed into an active inhibitor against MT1-MMP by the mutation of a single residue, namely threonine 98 to leucine (T98L). The resultant mutant displayed inhibitory characteristics of a typical slow, tight binding inhibitor. The potency of the mutant could be further enhanced by the introduction of valine 4 to alanine (V4A) and proline 6 to valine (P6V) mutations. Indeed, the inhibitory profile of the triple mutant (V4A/P6V/T98L) is indistinguishable from those of other TIMPs. Our findings suggest that threonine 98 is critical in initiating MMP binding and complex stabilization. Our findings also provide a potential mechanistic explanation for MMP-TIMP selectivity.

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Materials—All chemicals and reagents were purchased from Sigma unless otherwise stated. Restriction enzymes and Vent DNA polymerase for PCRs were obtained from New England Biolabs. The fluorometric substrate for the MT1-MMP assay (QF-24: Mca-Pro-Leu-Gly-Leu-Arg-NH2) has been described in our previous papers (16, 17). Kinetic assays were performed at 27 °C in fluorescence assay buffer (10 mM CaCl2, 50 mM Tris-HCl, pH 7.5, 0.05% Brij-35, 1% Me2SO, 0.02% NaN3) with a PerkinElmer Life Sciences 50B spectrofluorometer.
Engineering TIMP-1 against MT1-MMP

RESULTS

Strategy for TIMP-1 Mutagenesis—Although members of the TIMP family are well conserved in both primary and tertiary conformation, the inability of TIMP-1 to inhibit MT1-MMP has been a long standing conundrum for protein engineers interested in the mechanism of TIMP-MMPs selectivity (20, 21). First and foremost, there is no obvious contrast between the amino acid distribution of TIMP-1 and those of TIMP-2–4 that could account for its inability to inhibit MT1-MMP (Fig. 1). Hypothetical docking of MT1-MMP and TIMP-1 models in our laboratory also failed to reveal significant interfacial clashes that could satisfactorily explain the lack of ability of TIMP-1 to form tight binary complex with MT1-MMP. The most challenging aspect, in our view, is that although the structures of TIMP-1 and -2 and MT1-MMP have been delineated by protein NMR and crystallography for a considerable length of time, all the hypotheses regarding the inactivity of TIMP-1 against MT1-MMP remained hitherto speculative.

Taken together, we propose that the lack of TIMP-1 activity against MT1-MMP is not due to simple incompatibility between the surface topology of the two molecules. Instead, we believe that TIMP-1 is not capable of inducing conformational changes in MT1-MMP, an isomerization process that needs to be overcome for tight enzyme-inhibitor (EI) complex formation (22). The reason for this, we hypothesize, is the presence of some “obstructive epitope(s)” that prevent TIMP-1 from initiating conformational changes with the enzyme. These obstructive epitope(s), in our view, must be rather subtle and are not readily identifiable by primary sequence alignment and structural examination.

Hence, to approach this problem in a precise manner, we decided to confine our investigation to the N-terminal domain forms of TIMP (N-TIMP) and the catalytic domain form of MT1-MMP. Structural delineation of stromelysin-1-TIMP-1 and MT1-MMP-TIMP-2 complexes by X-ray crystallography showed that the TIMPs inhibit MMPs by inserting an “MMP-binding ridge” into the catalytic site grooves of the enzymes (8,
FIG. 2. Anatomy of N-TIMP-1. TIMPs inhibit metalloproteinases by inserting the MMP-binding ridge into the active site of the enzyme. We propose that the inactivity of TIMP-1 against MT1-MMP is most likely to be due to the presence of obstructive epitopes at the MMP-binding ridge of the molecule. Hence, we divide the ridge into five “divisions,” each composed of one or more independent epitopes. The amino acid residues chosen for mutagenesis in this work are highlighted in the ball-and-stick format.

This MMP-binding ridge, by and large, is composed of the very N terminus, the AB-loop, the CD-loop and the EF-loop of the molecule (Fig. 2). In our opinion, the obstructive epitope(s) are more likely to be located at these loci rather than the OB-core of the molecule.

Based on this hypotheses, we divided the MMP-binding ridge of TIMP-1 into five “divisions,” namely the “N terminus" division, the “Pro⁶” division, the “AB-loop” division, the “CD-loop” division, and finally the "EF-loop" division (Fig. 2). In turn, each of these divisions is subdivided into independent entities termed “epitopes.” These epitopes could either be a single or multiple amino acids, depending on the locus. Systematically, residues constituting these epitopes were swapped with the corresponding amino acids of TIMP-2—4, and the kinetic profiles of the resultant mutants were monitored throughout the mutagenesis process.

Kinetic Analysis of Wild type N-TIMP-1—4 against the Catalytic Domain of MT1-MMP—Before any mutagenesis work was carried out, we started by examining the binding affinities of wild type N-TIMP-1—4 with the catalytic domain of MT1-MMP. The binding constant (Kᵢₒₒ) and association rate (kᵢₒ) are shown in Table I. Clearly, N-TIMP-1—4 have far superior affinities than N-TIMP-1, their Kᵢₒₒ values being in the range of 0.3 to 1.5 nM. In comparison, N-TIMP-1 is at least 2 orders of magnitude higher in value (Kᵢₒₒ 178 nM). In term of association rate, N-TIMP-2 is slightly superior to N-TIMP-3 and N-TIMP-4 (kᵢₒ N-TIMP-2, 10 × 10⁻⁸ M⁻¹ s⁻¹, and N-TIMP-3 and -4, 3–6 × 10⁻⁸ M⁻¹ s⁻¹) (Table I). N-TIMP-1, on the other hand, is not capable of establishing tight binding complexes with MT1-MMP.

N-terminal Mutants—The side chains of the second and the fourth residues of TIMP (also termed P1’ and P3’ subunits) are critically important in the determination of the selectivity profile of the inhibitor. The residues dock directly into the S₁ and S₃ catalytic pockets of MMPs. The second residue of TIMP-1 and -3 is threonine and that of TIMP-2 and -4 is serine (Fig. 1).

Hence, we created only one mutant at the position of Thr² (T2S). The fourth residue of TIMP-1 is valine. The corresponding residues in TIMP-2—4 are either serine or alanine (Fig. 1). Therefore, two mutants were created at this locus, namely V4S and V4A. The results of the mutagenesis are shown in Table II. Among the three mutants of the N terminus division (T2S, V4A, and V4S), V4A exhibited substantial improvement in binding affinity with MT1-MMP (Kᵢₒₒ 66 nM versus wild type N-TIMP-1 of 178 nM), closely followed by V4S (Kᵢₒ₀ 81 nM). Nonetheless, none of the mutants was capable of potentiating tight complex formation with MT1-MMP.

Pro⁶ Mutants—Pro⁶ is located at the junction between the N terminus and the first α-helix loop of TIMP-1 (Fig. 2). TIMP-1 is unique as it is the only TIMP to have a proline at this locus. In TIMP-2—4, the corresponding residues are valine, serine, and alanine (Fig. 1). Substitution of Pro⁶ by a smaller residue might arguably release the constraints and bring about more relaxed local dynamics, although none of the crystal or NMR papers on TIMP published so far have emphasized the significance of this residue. Here we mutated Pro⁶ to valine (P6V), serine (P6S), and alanine (P6A) to mimic TIMP-2—4, respectively. Despite the 2-fold reduction in the Kᵢₒₒ value (Kᵢₒₒ 78–95 nM) for P6V and P6S mutants, there was no manifestation of tight binding inhibition. Surprisingly, replacement of Pro⁶ by alanine produced no significant changes in binding affinity with MT1-MMP.

AB-loop Mutants—The next set of mutants centered around the AB-loop of TIMP-1 (Figs. 1 and 2). MT1-MMP contains a cavity at the far “left” edge of its molecule, a result of the strategic alignment between its MT-loop and the side chains of Asp¹²⁹, Ser¹³⁰, and Phe¹³⁰ (8). Crystal structure of MT1-MMP-TIMP-2 complex (Protein Data Bank code 1BUV) shows...
that this cavity functions as a receptacle for Tyr-36, a conspicuous residue at the tip of the AB-loop. Hence, the rationale of grafting the entire AB-loops of TIMP-2–4 onto N-TIMP-1 was not to find out if any of the residues on the AB-loop of TIMP-3 and -4 could interact with the MT1-loop “receptacle” of MT1-MMP. Rather, we were interested in finding out whether the TIMP-1 AB-loop “obstructs” the molecule from initiating conformational changes with MT1-MMP. Our data indicated that TIMP-2 and TIMP-3 AB-loops were slightly beneficial in enhancing the affinity of TIMP-1 mutants against MT1-MMP (K\text{app} of 77–106 nM) (Table II and Fig. 3). Disappointingly, none of the mutants could be considered tight binding inhibitor in MT1-MMP inhibition.

**CD-loop Mutants**—The fourth group of mutants consisted of the CD-loop mutants. Only two residues were considered important at this site, namely Met-66 and Val-69. We know from the crystal structures of free and TIMP-1-bound forms of stromelysin-1 (Protein Data Bank codes 1QIA and 1UEA) that Met-66 was capable of inducing some degree of conformational change in stromelysin-1 (11). Hence, a series of mutants were created to replace Met-66 at this site. The mutations included amino acids of different characteristics, ranging from acidic to basic as well as those hydrophobic in nature (M66K, M66D, M66L, M66I, M66V, M66A, and M66G). On the other hand, given that leucine is the only variant at the Val-69 locus (Fig. 1), only one mutant was created to replace the residue (V69L). Subsequent kinetic analysis showed that the majority of the mutations only impaired the affinity against MT1-MMP (K\text{app} from 146 nM to an excess of 500 nM) (Table II).

**EF-loop Mutants**—The last mutant in the series was the Thr98 to leucine mutant (T98L) from the EF-loop division. Thr98 is situated right before the second disulfide bond (Cys3–Cys99) of TIMP-1. Interestingly, TIMP-1 is the only member of the TIMP family that has a threonine at this locus (Fig. 1). The corresponding residue in TIMP-2–4 is leucine. Indeed, replacement of threonine by leucine vastly enhanced the affinity of the resultant mutant against MT1-MMP (K\text{app} 11 nM) (Table II and Fig. 4). Indeed, the K\text{app} value is 16-fold lower than that of the wild type N-TIMP-1. The most striking effect is that the T98L mutant clearly manifested inhibitory profiles reminiscent of a slow, tight binding inhibitor (Fig. 4). Throughout the series, T98L is the first mutant exhibiting inhibitory profiles akin to those of N-TIMP-2–4.

**Thr98 Point Mutants**—So far, we have identified Thr98 to be the key obstructive residue that renders TIMP-1 inactive against MT1-MMP. It would be interesting to find out the effects of other amino acids on MT1-MMP inhibition. With the exception of cysteine, we mutated Thr98 to all the available amino acids, and the kinetic profiles of the mutants are shown in Table III. Not surprisingly, isoleucine produced the same potentiation effect as leucine, the K\text{app} value of T98I mutant (12 nM) being indistinguishable from that of T98L. The next two most potent amino acids are valine and methionine (K\text{app} T98V 989 nM and T98M 390 nM). Glutamine and tyrosine were slightly poorer, the affinity of the T98Q and T98Y mutants (90–120 nM) being marginally better than the wild type protein. Replacement of Thr98 by serine, on the other hand, produced no apparent effect on the activity of N-TIMP-1. The remaining amino acids severely impaired the affinity of N-TIMP-1 against MT1-MMP (Table III).

**Combination of Good Epitopes**—Even though T98L was much more active than wild type N-TIMP-1, the mutant was still not as potent as N-TIMP-2–3 or -4. Hence, in the second phase of this work, we combined four of the major positive epitopes in an attempt to study their effects on MT1-MMP inhibition. Two multiple mutants were made: 1) V4A/P6V/T98L triple mutant, and 2) V4A/P6V/TIMP-2 AB-loop/T98L quadruple mutant. The results of the combination are shown in Table IV. First of all, incorporation of V4A and P6V significantly improved the affinity of the T98L mutant. The affinity of the V4A/P6V/T98L triple mutant (K\text{app} 1.66 nM) was practically equal to those of N-TIMP-2 (K\text{app} 1.30 nM) and N-TIMP-3 (K\text{app} 1.38 nM). The association rate of the mutant (k\text{off} 1.48 × 10^{-5} \text{M}^{-1} \text{s}^{-1}) exceeded 10^{-5} \text{M}^{-1} \text{s}^{-1}, closely resembling N-TIMP-3 and -4 (k\text{off} 3–6 × 10^{-5} \text{M}^{-1} \text{s}^{-1}). Unexpectedly, the incorporation of the TIMP-2 AB-loop significantly impaired the affinity of V4A/P6V/T98L (K\text{app} of V4A/P6V/TIMP-2 AB-loop/T98L being 5.2 nM). The association rate, however, did not seem to be affected (Table IV).

**Effects of the C-terminal Domain on V4A/P6V/T98L Activity**—So far, we have succeeded in identifying the obstructive epitopes on the N-terminal domain of TIMP-1 that hinder the inhibitor from establishing a tight binding complex with MT1-MMP. What are the effects of the C-terminal domain on MT1-MMP association? To address this issue, we introduced V4A/
P6V/T98L mutations into full-length TIMP-1, and the results are summarized in Table V. At first sight, it appeared that the C-terminal domain might improve the affinity of TIMP-1 against MT1-MMP, because full-length wild type TIMP-1 displayed significantly better affinity than its N-terminal counterpart ($K_{iapp}$, full-length wild type TIMP-1 91 nM versus N-
Table III

\[ \text{Thr}^{98} \text{ point mutants, apparent inhibition constant (} K_{\text{app}}^{\text{on}} \text{) and association rate (} k_\text{on} \text{) with MT1-MMP} \]

With the exception of cysteine, Thr\(^{98}\) was mutated to every available amino acid to study the effects on MT1-MMP inhibition. The inhibitory profile of T98I was identical with T98L, its association rate \((k_\text{on})\) being \(1.78 \pm 0.15 \times 10^{-5} \text{ M}^{-1} \text{s}^{-1}\).

\[ K_{\text{app}}^{\text{on}} (\times 10^{-9} \text{ M}) \quad k_\text{on} (\times 10^{-5} \text{ M}^{-1} \text{s}^{-1}) \]

|       | \( K_{\text{app}}^{\text{on}} \) | \( k_\text{on} \)       |
|-------|-----------------|------------------|
| Small |      |                 |
| T98G  | 1.78 | 1.48            |
| T98A  |      | 1.45            |
| Nucleophilic |      |                 |
| T98S  |      | 1.45            |
| Hydrophobic |      |                 |
| T98L  | 1.78 | 1.48            |
| T98K  | 1.78 | 1.48            |
| Acidic |      | 1.45            |
| T98D  |      | 1.45            |
| T98E  | 0.35 | 1.45            |
| Amide |      | 1.45            |
| T98N  | 0.37 | 1.45            |
| Basic |      | 1.45            |
| T98H  |      | 1.45            |
| T98I  |      | 1.45            |
| T98V  | 0.37 | 1.45            |
| T98S  | 0.37 | 1.45            |
| T98T  | 0.37 | 1.45            |
| T98R  | 0.37 | 1.45            |

Table IV

\[ \text{Apparent inhibition constant (} K_{\text{app}}^{\text{on}} \text{) and association rate (} k_\text{on} \text{) of MT1-MMP with N-TIMP-1 mutants of combined epitopes} \]

Epitopes that contribute positively towards MT1-MMP inhibition, i.e. V4A, P6V, T98L, and TIMP-2 AB-loop, were combined into two multiple mutants. The \( K_{\text{app}}^{\text{on}} \) and \( k_\text{on} \) values of the V4A/P6V/T98L mutant are indistinguishable from that of the wild-type N-TIMP-3 and -4 (see Table I). T2-AB-loop, TIMP-2 AB-loop.

\[ K_{\text{app}}^{\text{on}} (\times 10^{-9} \text{ M}) \quad k_\text{on} (\times 10^{-5} \text{ M}^{-1} \text{s}^{-1}) \]

| Combined mutants | \( K_{\text{app}}^{\text{on}} \) | \( k_\text{on} \)       |
|-------------------|-----------------|------------------|
| V4A/P6V/T98L      | 1.66 \pm 0.12  | 1.48 \pm 0.35    |
| V4A/P6V/T2-AB-loop/T98L | 5.22 \pm 0.20 | 1.45 \pm 0.14    |

Table V

\[ \text{Full-length wild-type TIMP-1 and V4A/P6V/T98L mutant, apparent inhibition constant (} K_{\text{app}}^{\text{on}} \text{) and association rate (} k_\text{on} \text{) against MT1-MMP} \]

To study the effects of the C-terminal domain of TIMP-1 on MT1-MMP inhibition, we incorporated V4A/P6V/T98L into full-length TIMP-1. Full-length wild-type TIMP-1 demonstrated better inhibition affinity than N-TIMP-1 \((K_{\text{app}}^{\text{on}} 178 \text{ nM})\). Table I). However, the C-terminal domain does not seem to exert a significant effect on the affinity and association rate of the V4A/P6V/T98L mutant. NA, not able to determine.

\[ K_{\text{app}}^{\text{on}} (\times 10^{-9} \text{ M}) \quad k_\text{on} (\times 10^{-5} \text{ M}^{-1} \text{s}^{-1}) \]

|       | \( K_{\text{app}}^{\text{on}} \) | \( k_\text{on} \)       |
|-------|-----------------|------------------|
| Wild-type TIMP-1 | 90.6 \pm 5.4  | NA               |
| Full-length V4A/P6V/T98L | 1.86 \pm 0.17 | 0.89 \pm 0.09    |

TIMP-1 of 178 nM). Subsequent comparison of the V4A/P6V/T98L mutant, however, revealed that the C-terminal domain has no beneficial effects on MT1-MMP inhibition \((K_{\text{app}}^{\text{on}} \text{ full-length V4A/P6V/T98L 1.86 nM})\). Furthermore, the association rate of the full-length mutant \((0.9 \times 10^{-5} \text{ M}^{-1} \text{s}^{-1})\) was slightly lower than the N-terminal version (Table V and Fig. 5).

**DISCUSSION**

Not only are TIMP the endogenous inhibitors of MMPs, they also modulate the enzymatic activities of the ADAM (a disintegrin and metalloproteinase) and ADAM-TS (ADAM with thrombospondin-like repeats) proteinases (reviewed in Ref. 20). Membrane-type MMPs, in general, are poorly inhibited by TIMP-1. What is the physiological significance of such inactivity and can protein engineers ever fully understand the ultimate molecular mechanism of TIMP-metalloproteinases selectivity?

Contradicting previous perceptions, our study shows that Thr\(^{98}\) is the pivotal obstructive epitope that renders TIMP-1 inactive against MT1-MMP. Thr\(^{98}\) is TIMP-1-specific, and other TIMPs have leucine at the equivalent position. How does leucine potentiate the binding of TIMP-1 to MT1-MMP? Re-examination of the available TIMP/MMP structures failed to provide a satisfactory answer to the question. In the stromelysin-1 (MMP-3)-TIMP-1 complex (Protein Data Bank code 1UEA), Thr\(^{98}\) is situated right before His\(^{211}\) (HEXXHXXGXXH\(^{311}\)) of the enzyme, the last of the three conserved histidines that forms the catalytic zinc-binding ligands. Distance-wise, the two MMP-3 residues closest to Thr\(^{98}\) are His\(^{211}\) and Pro\(^{221}\), the amino acids being ~4 Å from the side chain of Thr\(^{98}\) (Fig. 6). This aside, Thr\(^{98}\) does not seem to be in close contact with any particular hydrophobic residue on the surface of stromelysin-1. The TIMP-2 equivalent of Thr\(^{98}\) is Leu\(^{100}\) (Fig. 1). The crystal structure of the MT1-MMP/TIMP-2 complex (Protein Data Bank code 1BVP) again shows that Leu\(^{100}\) is located before the third conserved histidine (His\(^{249}\)) of the zinc-binding motif (HEXXHXXGXXH\(^{49}\)) (Fig. 6). The two MT1-MMP residues closest to Leu\(^{100}\) are His\(^{249}\) and Pro\(^{259}\), almost a spitting image of the setting found in TIMP-1-MMP-3 mentioned above (Fig. 6). Hence, could this “His\(^{249}$/Pro\(^{259}\) pair” in MT1-MMP be a deciding factor in its rejection of TIMP-1? As part of our modeling simulation, we replaced the TIMP-2 molecule in MT1-MMP/TIMP-2 (Protein Data Bank code 1BVP) complex with TIMP-1 bearing a Thr\(^{98}\) to leucine mutation. Our study suggests that substitution of Thr\(^{98}\) by leucine does not enhance the interfacial contact between TIMP-1 and MT1-MMP enzyme (not shown).

Thr\(^{98}\) is not the only residue we considered that is unique to TIMP-1. As mentioned earlier, Pro\(^{6}\) was also featured prominently on our list of mutagenesis study. Substitution of the residue by valine or serine improved the binding affinities against MT1-MMP significantly. Yet again, examination of the stromelysin-1/TIMP-1 structure suggests that the residue is not directly involved in MMP association.

Why should leucine and isoleucine be the best residues? Table III demonstrates that the activity of N-TIMP-1 is critical dependent on the biophysical characteristics of the amino acids occupying the Thr\(^{98}\) position. The best amino acids are...
those similar in nature to leucine, namely isoleucine, valine, and methionine. Too bulky and hydrophobic (tryptophan and phenylalanine) or minute (glycine and alanine) a side chain resulted in complete abrogation of activity. Strangely, even though phenylalanine is poorly tolerated, tyrosine is beneficial, notwithstanding its similar size with phenylalanine. In general, acidic (aspartate and glutamate) or basic residues (lysine and arginine) are poorly tolerated.

Throughout this work, we were intrigued by the fact that some of the individual positive epitopes are not always mutually complementary as hoped. Incorporation of V4A and P6V mutations into T98L, for example, enhanced the affinity of N-TIMP-1 against MT1-MMP to a level essentially equal to those of N-TIMP-2 and P6V mutations into T98L, for example, enhanced the affinity of N-TIMP-2 against MT1-MMP. Given its relative insignificance in comparison to the N terminus, AB-loop, and CD-loop regions, Thr98 has never been emphasized in any literature on TIMP engineering.

The true answer, we believe, lies in the molecular dynamics of MMP-3. Crystallographic studies of MMP-3 complex, the closest MMP residues to Thr98 are His211 and Pro221 (highlighted by yellow and orange space fill, respectively). His211 being the third conserved histidine of the zinc-binding motif (HEXXHXGXHH). The distance between Thr98 and His211/Pro221 is ~4 Å. Similar setting is observed in TIMP-2/MT1-MMP complex, the closest residue to Leu100 being His426 (the third conserved histidine of the zinc-binding motif of MT1-MMP) and Pro275. This apart, there is no intimate contact between Thr98 (or Leu100) with the surface residues of MMP. Given its relative insignificance in comparison to the N terminus, AB-loop, and CD-loop regions, Thr98 has never been emphasized in any literature on TIMP engineering.

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