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Threonine 98, the Pivotal Residue of Tissue Inhibitor of Metalloproteinases (TIMP)-1 in Metalloprotease Recognition*

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The mammalian matrix metalloproteinases (MMPs, I matrixins) and their close associates, proteinases of the ADAM (a disintegrin and metalloproteinase) and ADAM with thrombospondin repeats families. There are four variants of TIMPs, and each has its defined set of metalloproteinase (MP) targets. TIMP-1, in particular, is inactive against several of the membrane-type MPs (MT-MMPs), MMP-19, and the ADAM proteinase TACE (tumor necrosis factor-α-converting enzyme, ADAM-17). The molecular basis for such inactivity is unknown. Previously, we showed that TIMP-1 could be transformed into an active inhibitor against MT1-MMP by the replacement of threonine 98 residue with leucine (T98L). Here, we reveal that the T98L mutation has in fact transformed TIMP-1 into a versatile inhibitor against an array of MPs otherwise insensitive to wild-type TIMP-1; examples include TACE, MMP-19, and MT5-MMP. Using T98L as the scaffold, we created a TIMP-1 variant that is fully active against TACE. The binding affinity of the mutant (V4S/TIMP-3-AB-loop/V69L/T98L) (K_{app} 0.14 nM) surpassed that of TIMP-3 (K_{app} 0.22 nM), the only natural TIMP inhibitor of the enzyme. The requirement for leucine is absolute for the transformation in inhibitory pattern. On the other hand, the mutation has minimal impact on the MPs already well inhibited by wild-type TIMP-1, such as gelatinase-A and stromelysin-1. Not only have we unlocked the molecular basis for the inactivity of TIMP-1 against several of the MPs, but also our findings fundamentally modify the current beliefs on the molecular mechanism of TIMP-MP recognition and selectivity.

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‡ The abbreviations used are: MMP, matrix metalloproteinase; MP, metalloproteinase; ADAM, a disintegrin and metalloproteinase; MT, membrane-type; TIMP, tissue inhibitor of metalloproteinases; N-TIMP, N-terminal domain of TIMP; TACE, tumor necrosis factor-α-converting enzyme; PDB, Protein Data Bank; Mca, (7-methoxycoumarin-4-yl)-acetyl; Dnp, 2,4-dinitrophenyl; Nva, norvalyl; Dpa, N-3-(2,4-dinitrophenyl)-t-2,3-diaminopropionyl.

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about the background of the enzyme and its reactivity with some of our previous TIMP-1 and TIMP-3 mutants. The second section discusses how we extended the investigations to two MMPs also known to be insensitive to TIMP-1, namely MMP-19 and MT5-MMP, and the subsequent realization of the role of Thr-98 in MP recognition. In the final section, the impact of T98L mutation on the MMPs normally well inhibited by TIMP-1 is analyzed.

EXPERIMENTAL PROCEDURES

Materials—All kinetic assays were performed at 27 °C in fluorescence assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM CaCl₂, 0.05% Brij-35, 1% MeSO₄, 0.02% NaN₃) with a PerkinElmer Life Sciences LS-50B spectrofluorometer equipped with thermostatic cuvette holders. The enzymes and substrates for the assays of gelatinase-A (MMP-2), stromelysin-1 (MMP-3), matrixin-1 (MMP-7), collagenase-3 (MMP-13), MT5-MMP (MMP-24) (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂); MMP-19, matrixisin-2 (endometase, MMP-26) (Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH₂); and TACE (ADAM-17) (Mca-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Arg-Ser-Arg-Lys-Dnp-NH₂) have been reported in our previous papers (12, 19–21).

Site-directed Mutagenesis of N-TIMP-1—N-terminal domain TIMP-1 (N-TIMP-1) mutants were generated by PCR using Vent DNA polymerase (New England Biolabs, Huntingford, UK) as described previously (18). Mutations were incorporated into the N-terminal domain of human TIMP-1 cDNA (residue 1–126, cloned in pREST-e Escherichia coli expression vector, Invitrogen) by either forward or reverse primers, depending on the locations. All constructs have been sequenced to confirm that no unwanted mutation had been introduced during the mutagenesis process.

Protein Refolding and Assessment of Activity by Titration—N-TIMP-1 mutants were expressed as inclusion bodies in E. coli and refolded essentially as described previously (18). The activities of active N-TIMP-1 in each preparation were determined by titration against gelatinase-A and collagenase-3 as reported previously (18).

Inhibition Constant Measurement (K_{app})—With the exception of MMP-19, MPs (0.15 mM) were preincubated with increasing concentrations of N-TIMP-1 mutants (up to 800 nM, depending on the potency of the inhibitors) at room temperature. Due to the low k_{cat}/K_M rate of MMP-19 with its fluorescent substrate, significantly more MMP-19 enzyme (5 mM) was used for the inhibition constant assays. Incubation was allowed for 3 h prior to steady state (V_S measurement). Quenched fluorescent peptides were added to a final concentration of 1 μM (2 μM for MMP-19) to initiate the assays. All data were fitted into competitive tight binding equations with the computer program Grafit to obtain an inhibition constant of N-TIMP-1 mutants (up to 800 nM, depending on the potency of the inhibitors) at room temperature. Due to the low k_{cat}/K_M rate of MMP-19 with its fluorescent substrate, significantly more MMP-19 enzyme (5 mM) was used for the inhibition constant assays. Incubation was allowed for 3 h prior to steady state (V_S measurement). Quenched fluorescent peptides were added to a final concentration of 1 μM (2 μM for MMP-19) to initiate the assays. All data were fitted into competitive tight binding equations with the computer program Grafit to obtain an estimation of K_{app} values with the equation (22)

V_s = (V/V_s) × (E + K_{app}) + (K_{app} + E) \times E + (K_{app} + E)^2 + 4E^2K_{app}^{1/2}

where V_s is the rate in the absence of inhibitor, E is the total enzyme concentration, and I is the total inhibitor concentration. Association Rate Constant Measurement (k_{on})—k_{on} measurements were performed by adding N-TIMP-1 mutants (up to 400 nM) to 0.05 mM MPs (1 nM for MMP-19). The rate of inhibition was followed using a continuous fluorometric assay at 27 °C until steady state was reached. The progress curve was analyzed using the equation (22)

P = V_S + (V_S - V_S) × 1 - e^{-kt}/k

where P is the product concentration, V_S is the initial velocity, V is the steady state velocity, and k is the apparent first order rate constant of equilibrium between enzyme and TIMP complex. k_{on} values were calculated by linear regression of k on TIMP concentrations.

RESULTS

N-TIMP-1 Mutants Originally Designed against MT1-MMP Are Also Active TACE Inhibitors—TACE is unique not only because of its versatility in shedding a great variety of bioactive molecules but also because of its selective inhibition by TIMP-3 (14). TIMP-1,-2, and -4 are not capable of inhibiting the enzyme. In our previous study with MT1-MMP, we created a series of N-terminal domain TIMP-1 mutants that displayed partial to full inhibitory functions against the enzyme (18). A common feature among these N-TIMP-1 mutants is that the amino acid constituency of their N termini, AB-loops, CD-loops, and EF-loops comprises derivatives of TIMP-2, -3, and -4. At the outset of the current project, we subjected TACE to affinity measurements with three of these mutants (T98L/V4A/P6V/TIMP-2-AB-loop, T98L/V4A/P6V, and V4A/P6V/TIMP-2-AB-loop), and the results are summarized in Table I. (The composition of the TIMP-2-AB-loop mutant is explained in Fig. 1.) Interestingly, we found that N-TIMP-1 mutants originally designed against an MT1-MMP backdrop were also active TACE inhibitors. With K_{app} values between 8 and 20 nM, the binding affinity of two of the mutants (T98L/V4A/P6V/TIMP-2-AB-loop and T98L/V4A/P6V) was markedly better than the wild-type TIMP-1 (K_{app} 356 nM). In addition, their progress curves also conspicuously resembled the inhibitory pattern of a typical slow, tight binding inhibitor (Fig. 2a) (22). It was apparent that the two mutants containing T98L mutation (i.e. T98L/V4A/P6V/TIMP-2-AB-loop and T98L/V4A/P6V) had significantly better affinity than the one without (V4A/P6V/TIMP-2-AB-loop, K_{app} > 120 nM) (Table I). However, from the panel of the compounded mutants per se, we were unable to conclude whether T98L was the contributory factor for the superior potency. To clarify the ambiguity, we subject TACE to further analysis with N-TIMP-1 carrying single V4A, P6V, TIMP-2-AB-loop, and T98L mutations. As shown in Table I, T98L was indeed the source of the enhanced affinity. The affinity of the mutant with TACE (K_{app} 21 nM) was at least an order of magnitude better than that of wild-type TIMP-1. On the other hand, whereas the TIMP-2-AB-loop significantly enhanced the affinity of TIMP-1 for TACE, the individual V4A and P6V mutations had exactly the opposite effects.

Thr-98 Site-directed Mutants: Only Leucine Is Capable of Initiating Slow, Tight Binding Association—Thr-98 is situated on the EF-loop of TIMP (Fig. 3), and the residue is TIMP-1-specific. The corresponding amino acid in TIMP-2, -3, and -4 is leucine. Is leucine the only amino acid capable of transforming N-TIMP-1 into a TACE inhibitor? With the exception of cysteine, we substituted Thr-98 with all the known amino acids, and the kinetic profiles of these Thr-98 site-directed mutants are summarized in Table II. Of the 17 mutants created, only T98I, T98M, and T98V displayed clear signs of activity against TACE. With K_{app} of just over of 75 nM, isoleucine fared best, followed by methionine (K_{app} 135 nM) and valine (K_{app} > 650 nM). Judging from their progress curves, none of the mutants exhibited the inhibition characteristics of a slow, tight binding inhibitor. The rest of the amino acids were completely inactive against TACE.

The N-TIMP-1 Mutant That Surpasses N-TIMP-3 in TACE Inhibition: T98L+V4S/TIMP-3-AB-loop/V69L—As part of our

| K_{app} | μM |
|---|---|
| Wild type | 356 ± 87 |
| N-TIMP-1 | 0.22 ± 0.07 |
| N-TIMP-3 | 3.65 ± 0.33 |
| Compound mutants | |
| T98L/V4A/P6V/TIMP-2-AB-loop | 20.1 ± 6.1 |
| T98L/V4A/P6V | 8.5 ± 1.2 |
| V4A/P6V/TIMP-2-AB-loop | 124 ± 25 |
| Individual mutation | |
| V4A | 497 ± 180 |
| P6V | 754 ± 151 |
| TIMP-2-AB-loop | 57.9 ± 33 |
| T98L | 21.1 ± 4.1 |

* n/a, not able to determine.
ongoing interests in TIMP engineering, we designed previously a triple N-TIMP-1 mutant that displayed appreciable affinity against TACE (23). The mutant, termed V4S/TIMP-3-AB-loop/V69L, contains mutations at three loci: the N terminus (V4S), the AB-loop (TIMP-3-AB-loop transplant), and the CD-loop (V69L) (Fig. 3). Despite its reasonable $K_{\text{app}}$ value of 15 nM with TACE, the mutant lacks the essential quality of a slow, tight binding inhibitor. Instead, its kinetic profile with TACE was more similar to that of a fast, reversible Michaelis-Menten-type inhibitor (Fig. 2c) (23). To determine the combined effects of T98L with these mutations, we incorporated V4S, TIMP-3-AB-loop, and V69L individually and collectively into T98L to generate a group of T98L-based mutants. The effects were complementary. As shown in Table III, combination of T98L with V4S, TIMP-3-AB-loop, and V69L, be it individually or collectively, brought about a severalfold enhancement in binding affinity. Moreover, the inhibition profiles of all the mutants conformed to the kinetics of a slow, tight binding inhibitor. The $K_{\text{app}}$ of T98L/V4S/TIMP-3-AB-loop/V69L (0.14 nM), surpasses that of N-TIMP-3 ($K_{\text{app}}$ 0.22 nM), the only natural TIMP inhibitor of TACE. The $K_{\text{app}}$ and $k_{\text{on}}$ values, the corresponding dissociation rate of $1 \times 10^{-5}$ s$^{-1}$ compared with that of N-TIMP-3, $8 \times 10^{-7}$ s$^{-1}$, shows that the half-life of the mutant TIMP inhibition complex with TACE

Fig. 1. The composition of the TIMP-2-AB-loop and TIMP-3-AB-loop mutants. The AB-loop of TIMP-1 was truncated and replaced by the residues of TIMP-2 and TIMP-3. Amino acids derived from TIMP-2 and -3 are highlighted in bold for ease of identification.

Fig. 2. The evolution of N-TIMP-1 against TACE: the association profiles of (a) T98L+V4A/P6V, (b) T98L, (c) V4S/TIMP-3-AB-loop/V69L, and (d) T98L+V4S/TIMP-3-AB-loop/V69L mutants. The N-TIMP-1 mutant T98L+V4A/P6V was originally designed for MT1-MMP inhibition. It was subsequently shown to be also an active TACE inhibitor. On the contrary, V4S/TIMP-3-AB-loop/V69L was an N-TIMP-1 mutant tailor-made against TACE. The kinetic characteristics of the mutant were similar to a fast competitive, Michaelis-Menten-type inhibitor. Incorporation of T98L into V4S/TIMP-3-AB-loop/V69L transformed the mutant into a slow, tight binding inhibitor. The affinity of the final mutant, T98L+V4S/TIMP-3-AB-loop/V69L ($K_{\text{app}}$ 0.14 nM), surpasses that of N-TIMP-3 ($K_{\text{app}}$ 0.22 nM), the only natural TIMP inhibitor of TACE. The gray lines denote the trajectory of the slow, tight binding equation used for calculating the association rate, as explained under “Experimental Procedures.” The hydrolysis curve of TACE in the absence of inhibitor is also included in each panel to show that no significant substrate depletion occurred during the assays. (The arrow indicates the time point when inhibitor was added to the enzyme.)
is considerably longer than that of N-TIMP-3. In other words, the mutant could be the ideal archetype for the development of future clinical TACE inhibitors.

**MMP-19 and MT5-MMP: Two Metalloproteinases Also Inhibited by T98L** — So far, we have shown that T98L is the key for the successful transformation of TIMP-1 into fully active inhibitors against two of the most prominent cell surface-associated sheddases, MT1-MMP and TACE. What about other MPs not customarily inhibited by TIMP-1? MMP-19 and MT5-MMP are known to be relatively TIMP-1-insensitive (12, 24). More attractively, the enzymes are reasonably stable at room temperature, and the assays for their activity have been worked out previously (12, 24). Table IV shows that, although none of the T98L-based mutants were originally designed for MMP-19 and MT5-MMP inhibition, they did display improved activity against the enzymes (Fig. 4, a and b). In the case of MMP-19, the \( K_{\text{app}} \) of the best mutant T98L/V4A/P6V and T98L/TIMP-3-AB-loop/V69L (1.27 nM) approached that of N-TIMP-2 (0.66 nM). In terms of association rate, there was practically no difference between the mutant and those of N-TIMP-2, -3, or -4 (\( k_{\text{on}} \); 2–5 \( \times \) \( 10^{-5} \) M\(^{-1}\)s\(^{-1}\)). Examination with Thr-98 site-directed mutants again showed that residues with the best affinity were those of aliphatic and hydrophobic character, i.e., valine, leucine, and isoleucine (Table II). Interestingly, although T98I and T98V were superior to T98L (\( K_{\text{app}} \) of 12 nM for T98I and for T98V versus \( K_{\text{app}} \) of 18 nM for T98L), they failed to display the inhibition profile of a slow, tight binding inhibitor (Fig. 4c). MT5-MMP, on the contrary, had better affinity with the T98L/V4A/P6V and T98L/TIMP-3-AB-loop mutants (\( K_{\text{app}} \) of 5–6 nM) (Table IV). Although the affinity of the mutants is significantly better that of wild-type N-TIMP-1 (~65 nM), they are still not as good as those of N-TIMP-3 and -4 (0.2–0.5 nM). Intriguingly, despite the rather similar \( K_{\text{app}} \) reactivity of MMP-19 and MT5-MMP with regard to T98L mutation (Table IV), the association curves of the enzymes with T98L mutants differ significantly. An example is shown in Fig. 4d; although the T98L-bearing mutant T98L+V69L (\( K_{\text{app}} \) 7 nM) displays obvious signs of slow, tight binding association with MT5-MMP, its association curves do not strictly echo the trajectory of a slow, tight binding inhibitor (22). On the contrary, the association profiles of T98L mutants with MMP-19 are typical of that of a slow, tight binding inhibitor. Indeed, much more work is needed to delineate the fundamental kinetics of N-TIMP-1 mutants/MT5-MMP interactions in the future.

**The Impact of T98L on MMPs Inhibited Well by Wild-type N-TIMP-1: Gelatinase-A, Stromelysin-1, Collagenase-3, Matriphilin-1, and Matrilysin-2** — In an attempt to explore the impact of the T98L mutation on MPs as a whole, in particular the MMPs normally well inhibited by TIMP-1, we extended our study to include gelatinase-A, stromelysin-1, collagenase-3, matrilysin-1, and matrilysin-2. The results are summarized in Table V. In the case of matrilysin-1 and -2, a different profile of inhibition was observed with T98L and T98L+V4A/P6V mutants. T98L was a better inhibitor than T98L+V4A/P6V against matrilysin-1, yet the opposite was true for matrilysin-2. Overall, the T98L mutation had a negative impact on the inhibition of MMPs normally well inhibited by TIMP-1.
and the CD- and AB-loops (Fig. 3, inset). These residues have long been considered central to TIMP selectivity because of their direct contact with the S1', S3', and S2 pockets of the MP catalytic site, and to a lesser extent, the “MT-loop receptacle” unique to the membrane-type MMPs (Fig. 3) (25). Thr-98 is located on the EF-loop, the part of the molecule that does not come into direct association with any of the afore-mentioned MP pockets. For this reason, the residue has been ignored until our investigation with MT1-MMP began in earnest (18).

Here, we show that Thr-98 is the pivotal residue that renders TIMP-1 inactive against MT1-MMP, TACE, MMP-19, and MT5-MMP. The exact molecular rationale, however, is not clear. In our view, it is unlikely that the findings in this work could be explained satisfactorily by homology modeling or the available TIMP/MMP co-crystal structures. To date, there are only two published structures of TIMP-MMP complexes in the Protein Data Bank (PDB), namely, stromelysin-1-TIMP-1 (PDB code 1UEA) and MT1-MMP-TIMP-2 (PDB code 1BUV) complexes. Stromelysin-1-TIMP-1 co-crystal structure reveals that Thr-98 is located right before the catalytic zinc, −3 Å from the closest MMP residues, His-211 and Pro-221 (Fig. 5a). Arguably, replacement of Thr-98 by leucine might enhance the area of surface contact between TIMP-1 and the MPs, and hence the improvement in binding affinity. However, examination of MT1-MMP-TIMP-2 complex shows that Leu-100, the Thr-98 equivalent of TIMP-2, is also positioned 3−4 Å from the closest MMP residues, His-249 and Pro-259 (Fig. 5b). Furthermore, both TIMP-2 and -4 have leucine at the Thr-98-equivalent position, and yet, none of them is capable of inhibiting TACE. For these reasons, we believe that physical intimacy is unlikely to be the true answer for the versatility of the T98L mutant.

Throughout this project, we have been intrigued by the absolute requirement for leucine for such transformation. Neither isoleucine nor any amino acid could mimic the function of the residue. In addition, if T98L has such a great effect on TACE, MMP-19, MT5-MMP, and MT1-MMP, why should its impact on gelatinase-A, stromelysin-1, and collagenase-3 be so mild or even negligible? Do MPs have different modes of interactions with TIMPs at the Thr-98 site so that one class of MPs is affected by the mutation while the others are not? The hypothesis is complicated by the fact that the closest MMP residues to Thr-98 (or Leu-100 in TIMP-2) in the available TIMP/MMP structures are, in fact, highly conserved among the MPs studied. Could the answer lie in the surrounding amino acids? Sequence alignment of the zinc-binding regions (Fig. 5c) reveals no obvious trend that would allow any specific conclusion to be drawn.

It has taken a long time to realize the pivotal role of Thr-98 in MP recognition. In retrospect, this could be due to our overdependence on the TIMP/MMP co-crystal structures for the interpretation of TIMP activity. While it is relatively straightforward to postulate the action of TIMP using representations of the “ground state,” i.e. the crystal structures of TIMP-MMP complexes, we preclude the “transition state” of the molecules in solution. That is, although thermodynamics and molecular movement are important factors in protein-protein interactions, these factors have so far been overlooked in our consideration of TIMP selectivity. Studies by protein NMR do indicate that TIMP-1/stromelysin-1 association is an induced fit affair that requires subtle structural readjustment within both the enzyme and the inhibitor (26). In particular, the EF-loop where Thr-98 resides has been noted to demonstrate a high degree of mobility. What we do not know at present is the relationship between the mobility of the loop and

**DISCUSSION**

Much of our understanding of TIMP-MP interactions is derived from the structures of TIMP-MMP complexes and the extensive kinetic analysis carried out on TIMP mutants. Despite the effort over so many years, the precise factors that enable a TIMP to recognize (or alternatively, reject) a particular MP remain elusive. Consequently, attempts to formulate a general paradigm from structural information that would allow prediction be made about the selectivity profile of a particular TIMP variant have not been successful. The issue of “TIMP-MP recognition” is a subject of great interest to protein engineers as the question is fundamental to the success of TIMP development as a potential therapeutic agent against MP-related diseases. Until very recently, TIMP mutagenesis has been confined to the second residue (P1'), the fourth residue (P3'),

| Residue | K<sup>app</sup> (nM) | k<sub>on</sub> (×10<sup>−5</sup> M<sup>−1</sup> s<sup>−1</sup>) |
|---------|---------------------|-------------------|
| Thr-98  | 76 ± 9              | 12.3 ± 1.8        |
| Thr-98  | 135 ± 25            | 34.1 ± 10.2       |
| Thr-98  | 660 ± 54            | 12.1 ± 3.3        |
| Thr-98  | >800                | >100              |
| Thr-98  | >800                | >100              |
| Thr-98  | >800                | >100              |
| Thr-98  | >800                | >100              |

**Table III**

Combining T98L with V4S, TIMP-3-AB-loop, and V68L mutations from previous study against TACE

| Previous mutant | K<sup>app</sup> (nM) | k<sub>on</sub> (×10<sup>−5</sup> M<sup>−1</sup> s<sup>−1</sup>) |
|-----------------|---------------------|-------------------|
| V4S/TIMP-3-AB-loop/V68L | 15 ± 3 | n/a* |
| T98L + mutations from previous study | T98L + V4S | 7.08 ± 0.73 | 0.34 ± 0.03 |
| T98L + TIMP-3-AB-loop | 2.38 ± 0.52 | 0.58 ± 0.06 |
| T98L + V68L | 5.69 ± 1.38 | 0.26 ± 0.02 |
| T98L + V4S/V68L | 4.37 ± 1.34 | 0.24 ± 0.02 |
| T98L + V4S/TIMP-3-AB-loop/V68L | 0.14 ± 0.06 | 0.76 ± 0.07 |

* n/a, not able to determine.

**Table II**

Inhibition constant (K<sup>app</sup>) of Thr-98 site-directed mutants with TACE and MMP-19

| Residue | TACE | MMP-19 |
|---------|------|--------|
| Hydrophobic |       |        |
| T98I     | 76 ± 9 | 12.3 ± 1.8 |
| T98M     | 135 ± 25 | 34.1 ± 10.2 |
| T98V     | 660 ± 54 | 12.1 ± 3.3 |
| T98F     | >800 | >100 |
| T98P     | >800 | >100 |

| Small     |       |        |
| T98G     | >800 | >100 |
| T98A     | >800 | >100 |

| Nucleophilic |       |        |
| T98S     | >800 | >100 |

| Aromatic  |       |        |
| T98Y     | >800 | >100 |
| T98W     | >800 | >100 |

| Acidic    |       |        |
| T98D     | >800 | >100 |
| T98E     | >800 | >100 |

| Amide     |       |        |
| T98N     | >800 | >100 |
| T98Q     | >800 | >100 |

| Basic     |       |        |
| T98H     | >800 | >100 |
| T98K     | >800 | >100 |
| T98R     | >800 | >100 |

in-2 (Table V). None of the mutations seemed to have any impact on the association rate, again indicating that they have targeted enzyme-inhibitor dissociation. On the other hand, mutants carrying T98L mutation are generally poorer inhibitors than wild-type N-TIMP-1 with gelatinase-A and collagenase-3. In contrast to both the scenarios above, the T98L mutation had a negligible effect on stromelysin-1 (Table V).
### TABLE IV

Kinetic profiles of T98L mutants with MMP-19 and MT5-MMP

|                | MMP-19 | MT5-MMP |
|----------------|--------|---------|
|                | $K_{i,app}$ | $k_{on}$ | $K_{i,app}$ | $k_{on}$ |
| Wild type      |        | nM     | s$^{-1}$   |        | s$^{-1}$ |
| N-TIMP-1       | 52 ± 20 | n/a    | 64.5 ± 1.9 | n/a    |
| N-TIMP-2       | 0.66 ± 0.31 | 4.62 ± 0.64 | 3.60 ± 0.20 | 127 ± 50 |
| N-TIMP-3       | 0.53 ± 0.32 | 2.80 ± 0.25 | 0.25 ± 0.05 | 525 ± 50 |
| N-TIMP-4       | 0.20 ± 0.10 | 3.06 ± 0.20 | 0.53 ± 0.13 | 16 ± 9.0 |
| T98L-based mutants |  |  |
| T98L           | 18.8 ± 3.2 | 0.82 ± 0.09 | 16.75 ± 1.00 | NA$^b$ |
| T98L + V4A/P6V | 6.95 ± 1.68 | 0.80 ± 0.03 | 6.06 ± 0.08 | NA |
| T98L + V4S     | 4.46 ± 0.60 | 2.68 ± 0.10 | 14.51 ± 1.50 | NA |
| T98L + TIMP-3-AB-loop | 5.08 ± 0.60 | 1.79 ± 0.15 | 4.90 ± 0.05 | NA |
| T98L + V69L    | 5.36 ± 0.45 | 2.44 ± 0.12 | 6.60 ± 0.31 | NA |
| T98L + V4S/V69L| 3.84 ± 0.67 | 2.18 ± 0.30 | 23.44 ± 2.04 | NA |
| T98L + V4S/TIMP-3-AB-loop/V69L | 1.27 ± 0.36 | 2.15 ± 0.12 | 15.30 ± 2.20 | NA |

$^a$ n/a, not able to determine.
$^b$ NA, not available, as the progress curves do not fit the trajectory of the equation for $k_{on}$.

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**Fig. 4.** The association profiles of (a) T98L, (b) T98L + V4A/P6V, (c) T98I with MMP-19, and (d) T98L + V69L with MT5-MMP. a and b, T98L mutation transformed N-TIMP-1 into a slow, tight binding inhibitor against MMP-19. c, despite the fact that T98I ($K_{i,app}$ 12 nM) had better affinity than T98L ($K_{i,app}$ 19 nM), the mutant was not showing the inhibition profile of a slow, tight binding inhibitor. Only T98L was capable of such transformation. d, the different association profiles of N-TIMP-1 mutants with MT5-MMP, highlighting the details within 4000 s of inhibitor addition. The association curves do not strictly fit the trajectory of a slow, tight binding inhibitor as delineated by Morrison and Walsh (22). (The arrow indicates the time point when inhibitor was added to the enzyme.)
The ability of a TIMP to induce conformation changes in a particular MP. The present study addresses two important issues of TIMP engineering. Firstly, our findings with Thr-98 have allowed us to break down the functional barrier between TIMP-1 and the other TIMPs and to create a new breed of TIMP-1 variants that are fully active against MPs otherwise unyielding to the inhibitor. Secondly, our results contradict the traditional dogma that the hotspots of TIMP recognition reside at the N terminus and the AB- and CD-loops. Instead, our mutagenesis work with TACE relegates these loci to auxiliary status. We hope the findings here will lead to a fundamental rethinking of the way we approach the subject of TIMP-MP recognition.

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Table V

|        | Matrilysin-1 | Matrilysin-2 | Gelatinase-A | Collagenase-3 | Stromelysin-1 |
|--------|--------------|--------------|--------------|---------------|---------------|
| $K_{app}$ |              |              |              |               |               |
| N-TIMP-1 | 1.27 ± 0.2 | 0.54 ± 0.06 | 0.25 ± 0.05 | 0.081 ± 0.012 | 5.31 ± 0.91 |
| T98L    | 0.15 ± 0.06 | 0.63 ± 0.10 | 0.29 ± 0.07 | 0.13 ± 0.01   | 4.60 ± 0.43  |
| T98L + V4A/P6V | 0.50 ± 0.02 | 0.08 ± 0.02 | 0.58 ± 0.13 | 0.21 ± 0.04   | 3.03 ± 1.22  |

$K_{app}$ in nM, $k_{on}$ in $10^{-6} M^{-1} s^{-1}$.
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