Delineating the Molecular Basis of the Inactivity of Tissue Inhibitor of Metalloproteinase-2 against Tumor Necrosis Factor-α-converting Enzyme*

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Tumor necrosis factor-α (TNF-α)-converting enzyme (TACE, ADAM-17) is a zinc-dependent ADAM (a disintegrin and metalloproteinase) metalloproteinase (MP) of the metzincin superfamily. The enzyme regulates the shedding of a variety of cell surface-anchored molecules such as cytokines, growth factors, and receptors. The activities of the MPs are modulated by the endogenous inhibitors, the tissue inhibitor of metalloproteinases (TIMPs). Among the four mammalian TIMPs (TIMP-1 to -4), TACE is selectively inhibited by TIMP-3. The rationale for such selectivity is not fully understood. Here, we examine the molecular basis of TIMP-TACE selectivity using TIMP-2 as the scaffold. By systematically replacing the surface epitopes of TIMP-2 with those of TIMP-3 and a TIMP-1 variant V4S/TIMP-3 AB-loop/V69LT98L, we created a novel TIMP-2 mutant that exhibits inhibitory potency almost equal to that of the TIMP-3. The affinity of the mutant with TACE is 1.49 nM, a marked improvement in comparison to that of the wild-type protein (Kᵢ 883 nM). The inhibitory pattern of the mutant is typical of that of a slow, tight binding inhibitor. We identify phenylalanine 34, a residue unique to the TIMP-3 AB-loop, as a vital element in TACE association. Mutagenesis carried out on leucine 100 also upholds our previous findings that a leucine on the EF-loop is critical for TACE recognition. Replacement of the residue by other amino acids resulted in a dramatic decrease in binding affinity, although isoleucine (L100I) and methionine (L100M) are still capable of producing the slow, tight binding effect. Our findings here represent a significant advance toward designing tailor-made TIMPs for specific MP targeting.

The matrix metalloproteinases (MMPs), the ADAM (a disintegrin and metalloproteinase) and the ADAM-TS (ADAM with thrombospondin repeats) proteinases are members of the mammalian metalloproteinases (MPs) of the metzincin superfamily. The enzymes are multidomain, zinc-dependent endopeptidases with a common structural feature, they all share the same tertiary configuration, termed “metzincin” fold, at the catalytic domain. Despite the similarity in structure, the functions of MPs are enormously diverse. To name a few, matrix degradation, transmembrane protein shedding, and regulation of growth factors are just some of the many physiologic processes that are known to involve the MPs (reviewed in Refs. 1–3).

The enzymatic activities of the MPs are regulated by the endogenous inhibitors, the tissue inhibitor of metalloproteinases (TIMPs). There are four variants of mammalian TIMPs (TIMP-1 to -4), and each TIMP has its own profile of MP selectivity. For instance, whereas the majority of the soluble MMPs are well inhibited by all TIMP-1 to -4, membrane type MPFs and MMP-19 are less sensitive to TIMP-1 (4, 5). The ADAMs, on the other hand, are generally more sensitive to TIMP-3 than TIMP-1, -2, or -4 (6–10). TIMPs are small molecules of ~22 kDa in molecular mass and variably glycosylated. The molecules are composed of two recognizable domains: (i) a distinctly oligonucleotide/oligosaccharide binding-folded N-terminal domain that encompasses nearly two-thirds of the total molecule and (ii) a structurally less well defined C-terminal domain that is largely made up of β-sheets (11–13). As a natural MP inhibitor, TIMP exerts its functions by slotting its “MMP-binding ridge” into the active site cleft of the target MP in a manner similar to a substrate. The result is the formation of a high affinity 1:1 stoichiometric enzyme-inhibitor complex. This MMP-binding ridge, by its very definition, is decisive for MP association and consists largely of the first four residues at the N terminus, the AB-loop and the CD- and EF-connecting loops of the molecule. The precise molecular elements that govern the selectivity profile of a TIMP are infrequently discussed in the literature, far less studied.

In this work, we examine the issue of MP-TIMP selectivity using TIMP-2 (ADAM-17) as the model MP. Among the ADAM proteinases uncovered, TACE is the best characterized to date. The enzyme is unique among the MPs, in function and TIMP sensitivity alike. Functionally, TACE is thought to be involved in the shedding of an array of cell surface-anchored bioactive molecules; notable examples include the growth factors pro-TNF-α and fractalkine, receptors such as Notch, TNF-α receptors I and II, as well as molecules that are associated with cell adhesion such as L-selectin (reviewed in Ref. 14). In terms of TIMP sensitivity, TACE displays a markedly different reactivity profile from those of the MMPs. Although the majority of the MMPs are sensitive to more than one species of TIMPs, TACE is exclusively inhibited by TIMP-3. TIMP-1, -2, and -4 are poor inhibitors of the enzyme (6, 15).

In our previous mutagenesis study with TIMP-1 (15), we showed that replacement of the threonine 98 (Thr98) residue by leucine (T98L) transformed the N-terminal domain of TIMP-1 (N-TIMP-1) into a fully active, slow, tight binding inhibitor
against TACE. Thr\textsuperscript{98} is situated just before the second disulfide bond on the EF-loop, and the equivalent residues in TIMP-2, -3, and -4 are, without exception, leucine. It is precisely this reason that Thr\textsuperscript{98} was chosen for mutation in the study. To become a TACE inhibitor that exhibits slow, tight binding characteristics, we further showed that the requirement for leucine at the Thr\textsuperscript{98} locus is absolute. A direct implication of the findings, however, is a conundrum: if leucine is so critical for TACE recognition, why are TIMP-2 and -4 such poor TACE inhibitors even though both are endowed with leucine at the equivalent positions (i.e. Leu\textsuperscript{100} in TIMP-2; Leu-101 in TIMP-4)? It is thus the aim of the current study to address this issue.

To this end, we generated a series of TIMP-2 variants using the N-terminal domain of the molecule (N-TIMP-2) as a scaffold. The mutants, each carrying a different mutation at the MMP-binding ridge, were subjected to in vitro assays with TACE, and the inhibitory behavior was monitored throughout the mutagenesis process. In accordance with the design and progress of the work, the results are presented in three sections. In the first section, we dissect the MMP-binding ridges of TIMP-2 and -3 and a TIMP-1 variant to delineate the amino acid composition that is essential for TACE recognition. The combined effects of “good” mutations leading to the creation of a new, potent TIMP-2 variant will be described. The second section deals specifically with the AB-loop of TIMP-3. The vital role of phenylalanine 34 (Phe\textsuperscript{34}), a residue unique to TIMP-3, will be the subject of discussion. In the last section, we devote our investigation to Leu\textsuperscript{100}, a residue that has previously been reported to be the key of TIMP-TACE recognition, and analyze the impacts of its mutation on TIMP-TACE interaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless stated otherwise, all chemicals and reagents were purchased from Sigma. Vent DNA polymerase and the restriction en-
zymes for sub-cloning were obtained from New England Biolabs (Hitchin, Hertfordshire, UK). TACE enzyme (the catalytic domain of TACE or TACE-473) used in this work was a kind gift from Dr. J. David Becherer, GlaxoSmithKline, Research Triangle Park, NC (16). The fluorescent substrate for TACE (ADAM-17) assay ([7-methoxyxycoumarin-4-yl]-acyethyl-Ser-Pro-Leu-Ala-Gln-Ala-Ala-Arg-Ser-Ser-Ser-Lys-2,4-dinitrophenyl-NH2) was synthesized by Dr. Graham Knight, Department of Biochemistry, University of Cambridge. 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 LS-50B spectrofluorometer equipped with thermostatic cuvette holders as described in our previous studies (17, 18).

Construction and Site-directed Mutagenesis of N-TIMP-2—The N-terminal domain of human TIMP-2 cDNA (corresponding to residues Cys1 through Glu127) was amplified by PCR using Vent DNA polymerase and sub-cloned into Escherichia coli expression vector pRSET-c (Invitrogen). To simplify recombinant protein purification, a hexa-histidine tag was added to the C termini of all N-TIMP-2 constructs in this work. Mutations were introduced into the cDNAs by either forward or reverse primers, depending on the loci and the ease of sub-cloning. All constructs were sequenced to rule out any unintended mutations introduced during the mutagenesis process.

Production, Refolding, and Activity Assessment of N-TIMP-2 by Titration—N-TIMP-2 mutants were expressed as inclusion bodies in E. coli and refolded using the protocol originally developed for N-TIMP-1 (18). All mutants were amenable to in vitro refolding, and the final yields were comparable to those generated by the method developed by Williamson et al. (19) To determine the concentration of active N-TIMP-2 in each preparation, the inhibitors were titrated against a known amount of gelatinase-A (MMP-2) and/or collagenase-3 (MMP-13) enzyme and sub-cloned into pRSET-c (New England Biolabs) expression vector. The AB-loops of wild-type TIMP-2 and -3, and of TIMP-1, -2, -3, and -4. The AB-loops of TIMP-2 and -4 are known to be effective TACE inhibitors, namely wild-type TIMP-3 and a mutated form of slow, tight-binding inhibition.

**Table 1**

| Mutant                      | K<sup>app</sup> (nM) |
|-----------------------------|----------------------|
| Wild-type N-TIMP-2          | 893 ± 126            |
| First generation mutants    |                      |
| N-terminal                  |                      |
| S2T                         | 547 ± 105            |
| V6P                         | 577 ± 57             |
| V6S                         | 864 ± 97             |
| AB-loop                     |                      |
| ΔTIMP-2 AB-loop             | 12,417 ± 108         |
| TIMP-3 AB-loop transplant   | 22 ± 2.5             |
| CD-loop                     |                      |
| S69E                        | 1,145 ± 557          |
| A70S                        | 486 ± 39             |
| V71L                        | 219 ± 31             |

**RESULTS**

**Strategy for N-TIMP-2 Mutagenesis**

When the current project was first conceived, there were only two TIMP variants that were known to be effective TACE inhibitors, namely wild-type TIMP-3 and a mutated form of

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V_0 = \frac{(V_e/E_0) \times (I_e - I_a - K_{pp}^{app}) + [K_{pp}^{app} + I_e - E_0] + 4E_0K_{pp}^{app}}{E_0K_{pp}^{app}}
\]

where \( V_0 \) is the initial velocity, \( V_0 \) is the steady-state velocity, and \( k \) is the apparent first order rate constant of the enzyme and TIMP complex. \( k_{pp} \) values were calculated by linear regression of \( k \) on TIMP concentrations.

![Image](http://www.jbc.org/Downloaded_from/http://www.jbc.org/)

**Fig. 2** A. N-TIMP-1, -2, -3, and -4. The AB-loops of TIMP-2 and -4 are significantly longer than those of TIMP-1 and -3, as highlighted by boxes. Here, we show that the Phe<sup>34</sup> residue at the tip of the TIMP-3 AB-loop is critical for TACE association. B. The sequences of (i) the AB-loops of wild-type TIMP-2 and -3; and (ii) TIMP-2 AB-loop mutant. And (iii) TIMP-2 AB-loop mutant with 3 residues longer (Phe<sup>34FGT36</sup>) of TIMP-3. Hence, both the AB-loop mutant and the TIMP-3 AB-loop mutant mutants possess AB-loops of the same length as that of the wild-type TIMP-3. The diagram is modified from the AB-loop constructs by Williamson et al. (23).
TIMP-1, termed V4S/TIMP-3 AB-loop/V69L/T98L, originally developed in our laboratory (15) (Fig. 1A). Although the backbone of this mutant was essentially of TIMP-1 origin, the composition of its MMP-binding ridge has been extensively modified. Apart from the valine 4 to serine (V4S) mutation at the N terminus, its AB-loop is, in reality, derived from TIMP-3 (TIMP-3 AB-loop). Furthermore, the original valine 69 and threonine 98 residues on the CD- and EF-loops have also been replaced by leucine (V69L and T98L). The binding affinity of the mutant with the catalytic domain of TACE (Ki = 0.14 nM) surpasses even that of wild-type TIMP-3 (Ki = 0.22 nM). As discussed in the introduction, the key element that ensures the successful transformation of TIMP-1 into an active, slow, tight binding TACE inhibitor is the T98L mutation. The reason TIMP-2 fails to become a good TACE inhibitor despite the binding TACE inhibitor is the T98L mutation. The reason discussed in the introduction, the key element that ensures the successful transformation of TIMP-1 into an active, slow, tight binding TACE inhibitor is the T98L mutation. The reason TIMP-2 fails to become a good TACE inhibitor despite the presence of a leucine (i.e. Leu109) on the EF-loop (Fig. 1B), we surmise, is most likely to be due to the lack of ideal amino acid composition on other sections of the MMP-binding ridge, the principal candidates being the N terminus and the AB- and CD-loops.

Hence, to delineate the molecular basis of the inactivity of TIMP-2 against TACE, we modeled our N-TIMP-2 mutagenesis on two templates: the wild-type N-TIMP-3 as well as the N-TIMP-1 mutant harboring the aforementioned V4S/TIMP-3 AB-loop/V69L/T98L mutations. In terms of strategy, two criteria were followed throughout the process: (i) The amino acid composition of the N terminus and AB- and CD-loops of N-TIMP-2 was mutated systematically to mimic those of the two templates. The design of the mutants was based, however, on sequence alignment rather than the available structures of TIMPs in the Protein Data Bank. In addition, the loci chosen for mutation were restricted to the residues located within the MMP-binding ridges (ii) to study the effects of AB-loop on TACE inhibition, parts of the AB-loop of TIMP-2 were truncated or replaced. The length of the AB-loops in the resultant N-TIMP-2 mutants, however, was rendered to be exactly the same as those of the templates.

First Generation Mutants

N-terminal Mutants S2T, V6P, and V6S—Among the first six amino acids constituting the very N termini of the TIMP variants in Fig. 1A, there are two discernable point differences: residues 2 and 6 (Fig. 1B). In both TIMP-3 and the TIMP-1 variant, the second residues are, coincidently, threonine (Fig. 1A). The second amino acid of TIMP is critically important in TIMP-MP interaction, because it is the side chain of this residue that projects into the S1′ specificity pocket of the catalytic cleft, and hence determines the selectivity profile of a TIMP to a certain degree (22). We mutated serine 2 to threonine (S2T), and kinetic analysis suggested slight improvement in binding affinity with TACE (K<sub>off</sub>) S2T of 547 nM versus wild-type N-TIMP-2 of 893 nM). The second point difference is at residue 6. In TIMP-3 and the N-TIMP-1 variant V4S/TIMP-3 AB-loop/V69L/T98L, the 6th residues are serine and proline, respectively. Structurally, this amino acid is located right at the junction between the N terminus and the first α-loop (hI) of TIMP, although co-crystal structures of TIMP-MP rule out any direct interaction with the catalytic pockets of MP (MT1-MMP/ TIMP-2, PDB number 1BUV; stromelysin-1-TIMP-1, PDB number 1UEA). Hence, we generated two mutants at this point, i.e. Val-6 to proline (V6P) and Val-6 to serine (V6S) mutants in an effort to study their effects on TACE binding. Of the two, V6P has slightly better binding affinity with TACE, its K<sub>off</sub> value of 577 nM being marginally superior to that of the V6S mutant (864 nM) (Table I).

| N-TIMP-2 mutant | K<sub>i</sub> | k<sub>i</sub> | K<sub>off</sub> |
|-----------------|-----------|----------|-----------|
| TIMP-3 AB-loop + V71L | 8.72 ± 0.71 | 5.04 ± 0.05 | 4.4 |
| TIMP-3 AB-loop + A70S | 3.40 ± 0.25 | 7.31 ± 0.33 | 2.5 |
| + V71L | | | |
| S2T + TIMP-3 AB-loop + A70S + V71L | 1.49 ± 0.31 | 5.71 ± 0.36 | 0.85 |
| S2T + V6P + TIMP-3 AB-loop + A70S + V71L | 2.29 ± 0.21 | 10.0 ± 1.7 | 2.2 |

* K<sub>off</sub> is calculated by K<sub>i</sub> × k<sub>i</sub>.
carrying an extra mutation, and measured their association profile with TACE. The results are shown in Table II. Indeed, combination of good mutations resulted in a severalfold increase in binding affinity, the best mutant being the one bearing S2T/TIMP-3 AB-loop/A70S/V71L quadruple mutations ($K_i^{app}$ of 1.49 nM). Strangely, even though V6P has been shown to be contributory toward TACE inhibition as an individual mutation, addition of this mutation to the rest only reduces the affinity of the resultant mutant. All compounded mutants display an inhibitory pattern typical of that of a slow, tight binding inhibitor (Fig. 3). The association rates ($k_{on}$) of the mutants are within the range of 5–10 $\times 10^4$ M$^{-1}$ s$^{-1}$, indistinguishable from that of the N-TIMP-1 mutant V4S/TIMP-3 AB-loop/V69L/T98L generated in our previous study ($k_{on}$ of 7.6 $\times 10^4$ M$^{-1}$ s$^{-1}$). From the calculated dissociation rate ($K_{off}$) profile, there is no doubt that the improvement in affinity is largely due to a reduction in the dissociation rate of the enzyme-inhibitor complex (Table II).

Dissecting the TIMP-3 AB-loop: Uncovering the Key Role of Phenylalanine 34 in TACE Binding

Fig. 2b illustrates the blueprint of the AB-loops of (i) wild-type TIMP-2 and TIMP-3; (ii) an N-TIMP-2 mutant with 6 residues removed from the AB-loop (i.e. the ΔTIMP-2 AB-loop mutant); and (iii) an N-TIMP-2 mutant with 6 residues removed from the AB-loop as above, in addition to a 3-amino acid replacement (i.e. the “TIMP-3 AB-loop transplant” mutant). Because the AB-loops of the ΔTIMP-2 AB-loop and TIMP-3 AB-loop transplant mutants are of the same length, it is remarkable that variation in a mere 3 amino acids could bring about a difference of over 550-fold in affinities for TACE (TIMP-3 AB-loop 22 nM versus the ΔTIMP-2 AB-loop of 12417 nM). This poses the question: which amino acid(s) within the TIMP-3 AB-loop is(are) the contributing residue(s) in TACE binding? Comparing the amino acid composition of the two constructs, the answer appeared to be straightforward: it must
either be the phenylalanine (Phe34), the glycine (Gly35), the threonine (Thr36), or, alternatively, a combination of the 3 residues (numbering in accordance with TIMP-3 sequence). Due to the lack of a side chain in glycine, Phe34 or Thr36 seemed to be the more likely suspects. Using the N-TIMP-2 mutant S2T/TIMP-3 AB-loop/A70S/V71L created in this work as a prototype, we further generated two mutants: one with Phe34 to glycine (F34G) mutation and the other with Thr36 to glycine (T36G) mutation. The results are summarized in Table III. It is clear that F34G mutation severely reduces the affinity of S2T/TIMP-3 AB-loop/A70S/V71L for TACE be diminished? To shed light on the impact of Leu100 on TIMP-TACE recognition, we created a series of "L100X" site-directed mutants, again, using S2T/TIMP-3 AB-loop/A70S/V71L as the prototype. With the exception of cysteine, we mutated Leu100 to all the existing amino acids, and the binding affinities and association rates of these mutants are shown in Table IV. Apart from isoleucine and methionine, none of the other amino acids is capable of behaving like a slow, tight binding inhibitor against TACE, the characteristics of the original S2T/TIMP-3 AB-loop/A70S/V71L prototype. The kinetic profiles of L100E, L100I, and L100M mutants are demonstrated in Fig. 4. In terms of affinity, isoleucine (L100I) and methionine (L100M) again fare best, their \( K_{i} \) being similar or slightly lower than 10 nM. Neither of them is as good an inhibitor against TACE as the original Leu100.

### N-TIMP-2 mutant

| Kᵢ | kₐn | \( K_{i} \) × 10⁻⁴ M⁻¹ s⁻¹ |
|----|-----|-----------------|
| S2T/TIMP-3 AB-loop/A70S/V71L prototype | 1.49 ± 0.31 | 5.71 ± 0.36 | 0.85 |
| F34G | 21.60 ± 2.88 | 0.94 ± 0.04 | 2.0 |
| T36G | 1.52 ± 0.13 | 15.8 ± 0.5 | 2.4 |

\( K_{i} \) is calculated by \( k_{a} \times k_{c} \).

### Leu100 Mutants:

Mutational analysis for total TIMP conversion and selectivity.

**DISCUSSION**

One of the most intractable problems with which the enzymologists have to contend is the reconciliation of the common three-dimensional architecture of the mammalian metzincins with the complexity of their functions (24). The enzymes, be they of MMP, ADAM, or ADAM-TS origin, display strikingly similar tertiary configuration at the catalytic domains and yet, carry out vastly diverse processes of substrate turnover. Likewise, the same conundrum applies to their natural inhibitors, the TIMPs. Although there are only four TIMPs, collectively, they regulate the enzymatic activities of most, if not all of the MMP and ADAM proteases identified to date. So far, only the structures of TIMP-1 and -2 have been delineated, but there is every reason to believe that TIMP-3 and -4 also possess the same configuration. Hence, the fundamental questions confronting the TIMP engineer are: how does a TIMP recognize and select its MP targets, and is it possible to create one or more tailor-made TIMP(s) for specific MP targeting? There have been many reviews written on the subject of TIMP engineering, but few have given much consideration to the most fundamental issue, the exact molecular elements that govern the recognition and selectivity profile of a TIMP species (25-27). It is against this background that our current series of TIMP-engineering projects is configured and initiated.

The first account of “total conversion” of TIMP, from a non-active to a fully active one insofar as the specificity is concerned, was achieved with TIMP-1 and MT1-MMP (MMP-14) in our laboratory (20). This was shortly followed by a second example, the conversion of TIMP-1 to a full-fledged TACE inhibitor (15). In both instances, the key to success was in the mutation of Thr36 to leucine (T36L). The mutation, in effect, altered the inhibitory mode of TIMP-1 with a range of MPs otherwise insensitive to the inhibitor, i.e. MT1-MMP, TACE, and MMP-19. Interestingly, the transformation was realized in both cases with virtually no understanding of the mechanism.

Unfortunately, these instances of serendipity are the exception rather than the rule. Here, we present the third example of total TIMP conversion. In contrast to their forebears, the N-TIMP-2 mutants in this work are mostly tailor-made, using epitopes known or suspected to be beneficial for TACE inhibition, a result of the experience and knowledge accumulated from an earlier mutagenesis study on TACE and TIMP-1 (15).
By and large, our findings on N-TIMP-2 in this work are in agreement with our previous observation with N-TIMP-1. For example, the preferences of the S1 specificity pocket for threonine (S2T) and the S2 pocket for leucine (V71L) have both been noted before (15). This aside, mutagenesis analysis on Leu100 also resulted in a conclusion that conforms to our findings with N-TIMP-1; that is, for the best TACE inhibition, the occupant on the Leu100 locus (or its equivalent in TIMP-1, Thr98) should ideally be leucine. Subsequent to leucine, the amino acids of the best affinity are those of aliphatic and hydrophobic nature such as isoleucine and methionine. The combination of the good mutations, in general, additively improves the affinity of the compounded mutants toward the target enzyme, and the improvement is due to the reduction in the dissociation rate \( (K_{\text{off}}) \) of the enzyme-inhibitor complex.

There is an exception to this custom, however. Not all of the individually identified good epitopes are mutually complementary upon combination. For instance, whereas the V6P mutation has been shown to improve the affinity of N-TIMP-2 with TACE, incorporation of this epitope with the rest (i.e. S2T/TIMP-3 AB-loop/A70S/V71L) only reduces the affinity of the resultant mutant. The reason is unknown, because there are no structural insights that could explain the observation to our satisfaction. The true answer, we believe, lies in the molecular dynamics that govern the association process of the TIMP and its MP target.

For the first time, the molecular basis of the selective inhibition of TACE by TIMP-3 is revealed. Here, we showed that Phe34 is the decisive element that sets the TIMP-3 AB-loop apart from those of TIMP-1, -2, and -4 when it comes to TACE selection. The only comparable example so far is the dissection carried out on the TIMP-2 AB-loop by Williamson et al. (23). As shown in his figure 4, the contrast in the association profiles \( (k_{\text{on}}) \) of the S2T/TIMP-3 AB-loop/A70S/V71L prototype bearing mutations at the Leu100 site: L100E, L100I, and L100M. Replacement of Leu100 by other amino acids significantly reduced the affinity of the S2T/TIMP-3 AB-loop/A70S/V71L mutant with TACE. Among the eighteen Leu100 mutants created, only L100I (isoleucine) and L100M (methionine) are capable of maintaining the inhibitory profiles of a slow, tight binding inhibitor. Arrows indicate the time points when inhibitors were added to the enzyme.
report, mutation of the tyrosine residue to glycine (Y36G) reduced the affinity of N-TIMP-2 for MT1-MMP by nearly a 100-fold (K_i of 1.2 nM for wild-type N-TIMP-2 versus 124 nM for Y36G). Notwithstanding the significance of the findings, the outcomes are not wholly unexpected given that the co-crystal structure of MT1-MMP/TIMP-2 (PDB 1BUV) indicated clear inter-molecular interaction between the side chain of Tyr^36 and the MT-loop receptor at the far left edge of the MT1-MMP molecule. This type of AB-loop-MP interaction has been, until now, considered to be restricted only to TIP-2 or -4 due to the fact that the AB-loops of TIP-2 and -4 are much longer than those of TIP-1 and -3. In TIP-3, the AB-loop is short and unlikely to reach the far edge of TACE, as illustrated by our models in Fig. 5. Moreover, there is no apparent deep pocket on the far edge of TACE that could serve as a potential receptor for the side chain of Phe^34. Restricted by the lack of clear structural evidence, we are more inclined to postulate that Phe^34 interacts with TACE by forming hydrophobic bonds with a patch of shallow invagination, rather hydrophobic in nature, at the left (non-primed) side of the catalytic zinc. The exact spot, however, will always remain speculative until the advent of a TACE-TIMP co-crystal structure.

Our findings here answer two queries that surround TIP-TACE selectivity. First and foremost, we demonstrate the viability of engineering tailor-made, MP-specific designer TIP using the skeleton of another TIP species as the scaffold. The ability of a TIP to inhibit MP lies at the molecular rationale of the effectiveness of TIP-3 against such a variety of ADAM and ADAM-TS proteinases. It is our hope that the findings in this report will ultimately lead to the development of MP-specific TIP of therapeutic value.

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