Protein Engineering of the Tissue Inhibitor of Metalloproteinase 1 (TIMP-1) Inhibitory Domain

IN SEARCH OF SELECTIVE MATRIX METALLOPROTEINASE INHIBITORS*

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Studies of the structural basis of the interactions of tissue inhibitors of metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) may provide clues for designing MMP-specific inhibitors. In this paper we report combinations of mutations in the major MMP-binding region that enhance the specificity of N-TIMP-1. Mutants with substitutions for residues 4 and 68 were characterized and combined with previously studied Thr⁴ mutations to generate mutants with improved selectivity or binding affinity to specific MMPs. Some combinations of mutations had non-additive effects on ΔG of binding to MMPs, suggesting interactions between subsites in the reactive site. The T2L/V4S mutation generates an inhibitor that binds to MMP-2 20-fold more tightly than to MMP-3(ΔC) and over 400-fold more tightly than to MMP-1. The T2S/V4A/S68Y mutant is the strongest inhibitor for stromelysin-1 among all mutants characterized to date, with an apparent Kᵢ for MMP-3(ΔC) in the picomolar range. A third mutant, T2R/V4I, has no detectable inhibitory activity for MMP-1 but is an effective inhibitor of MMP-2 and -3. These selective TIMP variants may provide useful tools for investigation of biological roles of specific MMPs and for possible therapy of MMP-related diseases.

Degradation of the extracellular matrix is essential for normal biological processes including embryonic development and morphogenesis (1, 2), reproduction (3) and wound healing (4), and enhanced turnover is associated with diseases including arthritis (5, 6), tumor angiogenesis and metastasis (7), multiple sclerosis (8), and cardiovascular diseases (9). The matrix metalloproteinases (MMPs) are a family of more than 20 zinc-dependent proteases that catalyze extracellular matrix turnover (10). Activity andzymogen activation in MMPs are regulated by a group of endogenous proteins named the tissue inhibitors of metalloproteinases (TIMPs) (11).

TIMPs are distributed in both invertebrates and vertebrates (11–13). The mammalian TIMPs are a family of four members (TIMP-1–4) that have about 40% sequence identity and fold into two domains, each containing three disulfide bonds (11). The isolated N-terminal domains (N-TIMPs) are able to form the correct native structure that carries the inhibitory activity against the MMPs (14). Although there are four TIMPs, their inhibitory activities toward different MMPs are not particularly specific. A notable exception is that TIMP-1 is a weak inhibitor of MT-MMPs, whereas TIMP-2 and TIMP-3 are much more effective (15–17).

Reported structures of TIMPs include crystal structures of TIMP-1 in a complex with the MMP-3 catalytic domain (18), TIMP-2 in a complex with the catalytic domain of membrane type MT1-MMP (19) and in a free form (20), and the solution NMR structures of N-TIMP-1 (21) and N-TIMP-2 (22). These structures show that the N-terminal inhibitory domain consists of a 5-stranded β-barrel with three associated α-helices resembling the folds of members of the oligonucleotide/oligosaccharide binding (OB) protein family (23). The structure of the TIMP-1/MMP-3 complex reveals that about 75% of all intermolecular contacts are made by residues adjacent to the disulfide bond between Cys¹ and Cys⁷⁰, especially residues 1–5 and 66–70. These two sections of chain insert into the active site cleft of the MMP, thus blocking its accessibility to substrates (18). The N-terminal Cys¹ coordinates the catalytic zinc²⁺ through the α-amino group and the peptide carbonyl group and is crucial for the inhibitory activity of TIMPs for MMPs, as shown by the complete loss of inhibitory activity for MMPs in TIMP-2 on carboxylation of the α-amino group of the N-terminal Cys¹ (24) or mutation to append an alanine extension to the amino terminus (25).

Our previous mutagenesis studies of N-TIMP-1 (26, 27), together with work with N-TIMP-2 by others (28), suggest that the affinity and specificity of TIMP for MMPs can be modified by site-directed mutagenesis. A major determinant of the affinity of N-TIMP-1 for different MMPs is the residue at position 2 in the sequence (threonine 2 in the wild-type protein) which interacts with the S₁ pocket of MMPs, a key to MMP substrate specificity (27). Based on this, it is reasonable to hypothesize that other residues that make contact with MMPs also contribute to the binding affinity and specificity of N-TIMP-1 and that selective variants can be generated by combining suitable mutations at these sites. In the present study, we show that N-TIMP-1 mutants with substitutions at positions 4 and 68 showed changes in affinity and specificity for MMPs. Combinations of mutations in these positions with those in position 2 led to the discovery of N-TIMP-1 variants with higher binding affinity and specificity for individual MMPs.
Materials—Vectors and cell lines used for expression of N-TIMP-1 and variants were from the same sources as in previous studies (26, 27). Plasmid and protein expression were purchased from Qiagen. Mutagenic PCR products were exchanged from pET-3a vector using the NcoI and BamHI restriction sites. All constructs were confirmed by DNA sequencing using T7 promoter primer. The N-Timp-1 mutants were expressed in E. coli. Further purification of N-TIMP-1 and Mutants—N-TIMP-1 and mutant proteins purified by cation exchange chromatography with CM-52 were dialyzed overnight against 15 volumes of 20 mM Tris-HCl, pH 5.5 (5.0 φl) for T2S/V4A and applied to a cation exchange Mono S HR 5/5 column previously equilibrated with the same buffer and connected to a Biologic Duowave medium pressure chromatography system. The protein was eluted with a linear salt gradient of 0.5 M NaCl over 60 min at a flow rate of 1 ml/min. The activity of different fractions was estimated by the fluorescence assay method using MMP-3 (for the inhibitors with MMP-1 and MMP-3 complex) and the Biologic DuoFlow medium medium pressure chromatography system. 

Substitutions for Val<sup>68</sup> of N-TIMP-1—In the crystal structure of the N-TIMP-1/MMP-3 complex, the side chain of Val<sup>68</sup> interacts with the S9′ site of MMP-3, forming contacts at the edge of the interaction interface (Fig. 1, and Ref. 18). Four mutants with substitutions at position 4 of N-TIMP-1 were constructed and expressed in E. coli. As compared with the Thr<sup>68</sup> mutations, which generally have strong effects on MMP binding (27), these mutations produce more moderate changes (Table I). Mutations of Val<sup>68</sup> into isoleucine, lysine, and serine cause significant increases in the <i>K<sub>i</sub></i> values for MMP-1 while having only a small effect on the affinity for MMP-2. 

RESULTS

Substitutions for Val<sup>68</sup> of N-TIMP-1—In the crystal structure of the N-TIMP-1/MMP-3 complex, the side chain of Val<sup>68</sup> interacts with the S9′ site of MMP-3, forming contacts at the edge of the interaction interface (Fig. 1, and Ref. 18). Four mutants with substitutions at position 4 of N-TIMP-1 were constructed and expressed in E. coli. As compared with the Thr<sup>68</sup> mutations, which generally have strong effects on MMP binding (27), these mutations produce more moderate changes (Table I). Mutations of Val<sup>68</sup> into isoleucine, lysine, and serine cause significant increases in the <i>K<sub>i</sub></i> values for MMP-1 while having only a small effect on the affinity for MMP-2. 

Substitutions for Ser<sup>86</sup> of N-TIMP-1—The C-D loop also form part of the core of the TIMP/MMP contact site (Fig. 1 and Ref. 18), and previous studies have shown that substitutions for Met<sup>66</sup> or Val<sup>69</sup> affect TIMP activity (26). Here we mutated the Ser<sup>86</sup> to Ala, Glu, Arg, and Tyr. These mutations have large effects on MMP binding (Table I). Three of the four mutants have improved selectivity for MMP-2 relative to the other two MMPs, whereas the fourth mutant, S68Y, inhibits MMP-3 much more strongly than MMP-1 and -2. 

Combined Mutations of Thr<sup>68</sup>, Val<sup>4</sup>, and Ser<sup>86</sup>—Based on the mutagenesis studies of Thr<sup>68</sup>, Val<sup>4</sup>, and Ser<sup>86</sup> (26), we constructed double and triple mutants combining mutations of the more selective single-site mutations. Characterization of the mutant shows that the residues do not always contribute independently to the affinities of the TIMP/MMP complex. 

The T2L mutation was combined with V4S to generate a N-TIMP-1 variant that is more selective for MMP-2. As predicted, with MMP-2 the resulting double mutant inhibits 20- fold more strongly than with MMP-3 (33) and about 470-fold more strongly than with MMP-1. Introduction of a third mutation, S68A, produced a mutant binding very weakly to MMP-1 while retaining a good activity for MMP-2. However, this inhibitor is much more effective with MMP-3 than is predicted
based on additive effects on the free energy of binding (Table II). This triple mutant, T2L/V4A/S68A, is less selective against MMP-3(ΔC) than the double mutant T2L/V4S.

The double mutant, T2S/V4A, has an increased inhibitory activity for MMP-3. Binding with MMP-2 is unchanged, whereas that with MMP-1 is weaker than the wild-type protein. Interestingly, the triple mutant T2S/V4A/S68Y has the highest inhibitory activity for MMP-3(ΔC) among all mutants characterized so far, with a $K_i$ of 50 μM. Unusually, it also has a greatly improved affinity for MMP-2, whereas binding to MMP-1 was reduced over 36-fold.

The T2R/V4I mutant was purified as a ~95% active form using a Mono S column at pH 6.0 instead of pH 5.5, which is used for the wild-type protein. We could not detect any inhibition of MMP-1 by this mutant even at a concentration of 10 μM, yet it retains good activity as an inhibitor of MMP-2 and MMP-3(ΔC).

**DISCUSSION**

The TIMPs are important regulators of extracellular matrix metabolism (11), principally through their primary activities as endogenous inhibitors of MMPs. Imbalances between the levels of TIMPs and active MMPs are linked to disease processes such as tumor metastasis, arthritis, and atherosclerosis (31, 32). Low molecular weight MMP inhibitors have been developed and extensively studied, and a few have been used in clinical trials but with little success (33). Nonspecific inhibition of housekeeping MMP functions has been proposed as the major drawback of these inhibitors (34). We proposed previously that N-TIMP-1 can be engineered to produce more selective MMP inhibitors that could be applied in gene therapy of MMP-related diseases (35, 36), and residue 2 of TIMP-1 was found to be a major determinant of affinity and specificity for MMPs (27). In this paper we report the effects of mutagenesis of two other positions with Thr2 mutations generated N-TIMP-1 variants with increased selectivity and/or affinity for specific MMPs. The side chain of Val4 occupies a site similar to the substrate P3′-subsite in currently known TIMP/MMP complexes (18, 19). In the complex of TIMP-1 with MMP-3, the side chain of Val4 sits in a shallow groove at the margin of the interaction site close to the side chains of Gly161, Asn162, Leu164, and Tyr223 of the protease (15). These residues are >4 Å from Val4, and the insignificant effect of truncating the side chain through the Ala substitution on the affinity for all three MMPs suggests that interactions between the side chain of Val4 and the protease contributes little to the free energy of binding. The Val4 side chain projects away from the protease surface in the TIMP-1/MMP-3 complex (18), and the more extended Ile side chain can be accommodated in MMP-2 and -3 without perturbing the protein-protein interaction, but in MMP-1 it produces a 14-fold
loss in affinity. The basis for this is uncertain, because the structure of complexes of TIMP-1 with MMP-1 and -2 have not been determined, but this result suggests that the S3' site of MMP-1 does not readily accommodate a larger side chain; it is also affected more than the other proteases by the Lys substitution (Table I). Differences between the MMPs in the residues that form the P3' site do not readily account for these effects; residues Leu164 and Tyr233 of MMP-3 are conserved in MMP-1 and -2, whereas residues corresponding to Gly161, Asn162 are Gly-Gly in MMP-1 and Asp-Gly in MMP-2, so changes in side chain size are not responsible for the reduced steric tolerance in this subsite in MMP-1. The binding of TIMP-1 to MMP-2 is least affected by substitutions for Val1', suggesting that there is greater separation between MMP-2 and N-TIMP-1 in this part of the interaction site.

The side chain of Ser68 of TIMP-1 interacts with the S2 subsite of MMP-3 in the crystal structure of their complex and is in contact with Ala167 of the metzincin (18). It is also near (<4.5 Å distance) to His166, Tyr168, Ala169, and His205. Mutation of Ser68 to Ala, Glu, and Arg reduces the affinity of N-TIMP-1 for all three MMPs, but the effect is less for MMP-2 than for MMP-1 and MMP-3. The Tyr mutant has a major effect on inhibitor specificity, producing a 150-fold loss in affinity for MMP-1, a 7-fold reduction in affinity for MMP-2, but essentially no change in binding to MMP-3. The side chain of Ser68 of TIMP-1 projects toward Ala169 of MMP-3 in their complex (18), and it appears that the Tyr side chain can be accommodated without major perturbation of the interaction interface. Ala is conserved at this site in MMP-2, but in MMP-1 it is replaced by Gln. A steric conflict between the Gln side chain and Tyr68 in the N-TIMP-1 mutant is a possible explanation of the effects of this mutation on inhibitor selectivity.

Substitutions for residues 2, 4, and 68 with similar selectivity were combined in an attempt to engineer N-TIMP-1 variants with higher selectivity and/or affinity for specific MMPs. In some cases, the effects of these mutations are essentially additive (Table II), indicating that interactions with the S1', S3', and S2 subsites of MMPs contribute independently to the stability of the TIMP/MMP complex. However, some mutants containing combinations of mutations have much lower K_i values for particular MMPs than predicted based on the assumption that individual substitutions have additive effects on the ΔG of binding. For example, the T2L/V4S/S68A mutant has a >100-fold higher affinity for MMP-3(ΔC) than expected, yet its K_i values for MMP-1 and MMP-2 are in good agreement with predictions. Similarly, the T2S/V4A/S68Y is a 60-fold better inhibitor of MMP-1 and also a 68-fold better inhibitor of MMP-2 than predicted based on additivity. These discrepancies greatly exceed the compounded errors of the single-site mutations and suggest interactive effects between the sites in these triple mutants. Because of the complexity of protein-protein interactions, there are many possible explanations for this, such as changes in relative orientation of TIMP and protease, structural changes introduced by the mutations, and changes in dynamics (11). Structural studies are in progress to address this question.

Several combined mutants have interesting and potentially useful properties. T2L/V4S is selective for MMP-2, providing a possible tool for gene therapy of gelatinase-related diseases. Another mutant, T2S/V4A/S69Y, is the best inhibitor for stromelysin-1 among all N-TIMP-1 mutants. The third mutant, T2R/V4I, has no detectable activity for MMP-1 while retaining good inhibition for MMP-2 and -3. This mutant is of special interest, because the failure of many general MMP inhibitors in clinical trials was the result of muscular-skeletal disorders that are thought to be caused by nonspecific inhibition of MMP-1 (38). Our mutational studies have demonstrated the feasibility of generating selective MMP inhibitors by engineering TIMP. Using high throughput screening methods, it should be possible to identify TIMP variants that are specific inhibitors of individual MMPs for application in future clinical trials.
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