A mutational study of the amino-terminal domain of human tissue inhibitor of metalloproteinases 1 (TIMP-1) locates an inhibitory region for matrix metalloproteinases.

Wen Huang, Qi Meng, Ko Suzuki, Hideaki Nagase, and Keith Brew

The solution structure of the inhibitory amino-terminal domain of tissue inhibitors of metalloproteinases (TIMPs) is important for understanding their inhibitory action on matrix metalloproteinases. Although the inhibitory activity of TIMPs has been attributed to the N-terminal domain (N-TIMP-1), the molecular basis of this inhibition has not been fully elucidated. In this study, we used a bacterial expression system to produce an N-terminal domain of N-TIMP-1 that is 17-fold more effective than wild-type N-TIMP-1 for MMP-1 (interstitial collagenase) and MMP-2 (gelatinase A). The mutations introduced that disrupt the Cys1–Cys70 disulfide bond and by substitution of Ala for Thr2. Most mutations that perturb the interaction with MMP-3 have parallel effects on the affinity of N-TIMP-1 for MMP-1 (interstitial collagenase) and MMP-2 (gelatinase A). However, the Thr2 mutation produces an inhibitor that is 17-fold more effective against MMP-3 than MMP-1, suggesting that it is feasible to engineer TIMP-1 variants that are more specifically targeted to selected matrix metalloproteinases. The active site identified by these studies is a structurally constrained but elongated region of TIMP that can fit the matrix metalloproteinase substrate-binding site.

The region of TIMP that is important for its inhibitory activity has also been investigated using synthetic peptides and antibodies to inhibit its interaction with interstitial collagenase, MMP-1. The results were interpreted as indicating that the second “disulfide knot” (Cys125–Cys124 and Cys127–Cys177) plays a major role in activity. However, low molecular weight peptides generally have little structure in solution and can potentially inhibit TIMP-1 action by nonspecific binding or by acting as alternative substrates for the metalloproteinase. Such mechanisms appear likely since peptides were required at concentrations in the millimolar range to exert effects as compared with nanomolar concentrations of TIMP-1. The reliability of information obtained with antibodies is also suspect since immunoglobulins are very large as compared with TIMP-1.

The solution structure of the inhibitory amino-terminal do-
main of TIMP-2 has been determined at low resolution by multidimensional NMR (19). This shows that N-TIMP-2 has the fold similar to that of the OB-fold family of oligonucleotide/oligosaccharide-binding proteins (see Fig. 1) and gives a basis for designing and interpreting the results of structure-function relationships in N-TIMP-1. With the goal of studying structure-function relationships of TIMP, we have developed a bacterial expression system for producing high yields of recombinant human N-TIMP-1 that has been characterized as having a fully native fold by CD spectroscopy and two-dimensional NMR (20). This system is convenient for designed mutagenesis studies to probe structure-function relationships and allows appropriate physical characterization of the structural state of variants. We describe here 20 mutants of N-TIMP-1 that have been expressed as inclusion bodies in *Escherichia coli*, refolded successfully *in vitro*, and characterized with respect to activity and CD spectra. Their functional properties emphasize the importance of a region that includes Met66–Cys70 and Cys1–Thr2 for TIMP inhibitory activity. Substitution of Thr2 differentially influences the activity of TIMP-1 for binding to MMP-1, MMP-2 (gelatinase A), and MMP-3 (stromelysin 1), suggesting that it may be possible to engineer TIMPs that are selective inhibitors of MMPs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ultima DNA polymerase with proofreading function was purchased from Perkin-Elmer. Wizard™ PCR Preps purification kits were purchased from Promega. Other reagents, separation media, and kits were from the same sources as described in a previous work (20). Dr. R. Werner (Department of Biochemistry and Molecular Biology, University of Miami School of Medicine) provided synthetic oligonucleotides. MMP-1 and MMP-2 were isolated aszymogens as described previously (21, 22). C-terminally truncated pro-MMP-3(–53)C was expressed in *E. coli* as inclusion bodies, from which it was extracted, folded, and purified. Pro-MMP-2 and pro-MMP-3(–53)C were activated with 4-aminophenylmercuric acetate and pro-MMP-1 with MMP-3 in the presence of 4-aminophenylmercuric acetate (21). The MMP-3 used for pro-MMP-1 activation was removed by anti-MMP-3 affinity chromatography (23).

**Construction and Expression of N-TIMP-1 Mutants**—Mutations were introduced using the polymerase chain reaction megaprimer method (24) with the pET3a-N-TIMP-1 expression vector as the template (20). The amplification to generate the megaprimer was performed in each case with the synthetic T7 promoter primer or the T7 terminator primer together with an appropriate mutagenic primer. Megaprimers were purified using the Wizard™ PCR Preps purification kit and used in a second amplification with the cognate T7-based primer. After purification by agarose gel electrophoresis, the final amplification product was digested with BamHI and Ndel, and the product was cloned into pET3a as described for N-TIMP-1. The coding sequences of all mutants were checked by DNA sequencing of the expression vector. N-TIMP-1 mutants were expressed as inclusion bodies in *Escherichia coli*, refolded successfully *in vitro*, and characterized with respect to activity and CD spectra. Their functional properties emphasize the importance of a region that includes Met66–Cys70 and Cys1–Thr2 for TIMP inhibitory activity. Substitution of Thr2 differentially influences the activity of TIMP-1 for binding to MMP-1, MMP-2 (gelatinase A), and MMP-3 (stromelysin 1), suggesting that it may be possible to engineer TIMPs that are selective inhibitors of MMPs.

Calculated values were obtained using a treatment of data for slow tight-binding inhibitors (27) or, in some cases, by a standard treatment for reversible lower affinity inhibitors.

**CD Spectroscopy**—Near- and far-UV CD spectra of N-TIMP-1 and its mutants were determined with a Jasco J-710/720 spectropolarimeter as described previously (20). Near-UV CD spectra (250–290 nm) were determined using a cell with a 1-cm path length, and far-UV spectra (200–250 nm) were determined using a cell with a 0.1-cm path length. Proteins were dissolved in 20 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl.

**RESULTS**

**Rationale for the Design of N-TIMP-1 Mutants**—Our strategy has been to probe regions of TIMP-1 that have been implicated in its activity by chemical modification studies and by analogy with other OB-fold proteins that share structural similarities to N-TIMP-1. Trace labeling studies² have indicated that groups of residues in the second disulfide loop between Cys15 and Cys70 are perturbed in reactivity toward acetic anhydride on binding to MMP-3. To investigate this region, a series of residues in this region (residues 16, 18, 22, 35, 38, 42, 45, 46, and 66) were mutated to Ala or to amino acids with similar polarity. Tyr35 and Tyr38 were chosen for replacement because, in the low resolution solution structure of TIMP-2, their equivalents are components of a surface loop that is adjacent to the binding site in other OB-fold proteins. Additional evidence that influenced our experimental design was the observation that human neutrophil elastase inactivates TIMP-1 by cleaving the Val69–Cys70 peptide bond, whereas this bond is protected and TIMP-1 activity is preserved by formation of the TIMP-MMP-3 complex. These results and the perturbed Kᵦ observed in the M66A variant prompted us to probe residues that are close to this region in covalent structure and in the solution NMR structure. This led us to construct mutants with substitutions of Val69, Cys70, Cys1, Thr2, and Pro5. The locations of the corresponding residues in N-TIMP-2 are shown in Fig. 1.

**Preparation of N-TIMP-1 Mutants**—The polymerase chain reaction method introduced some unwanted mutations adjacent to the sequence derived from the mutagenic primer, which

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² K. Suzuki, K. Brew, and H. Nagase, manuscript in preparation.

³ S. Yadav, H. Nagase, and K. Brew, unpublished observations.

⁴ Nagase, H., Suzuki, K., Cawston, T. E., and Brew, K. (1997) *Biochem. J.*, in press.
can be attributed to the terminal transferase activity of Taq DNA polymerase. These were minimized by the use of a thermostable polymerase with proofreading activity (Ultima DNA polymerase) and by designing primers so that all substitutions at the susceptible site are silent. A few additional mutations were found outside the primer presumably because of errors made by the polymerase, which generated the double mutants C1S/F12Y, M45A/F49V, N14S/V69T, and V69A/A103V.

Wild-type and mutant forms of N-TIMP-1 were expressed in *E. coli*, solubilized, partially purified, and folded in *vitro* as described above. The final purification was carried out by cation-exchange chromatography with CM52. The yields ranged from 5 to 20 mg of purified protein/liter of bacterial culture, with the exception of the mutant Y38A, for which the yield was ~2 mg/liter of culture. Concentrations of the mutant proteins were calculated from the absorbance of solutions at 280 nm using extinction coefficients calculated from their contents of tryptophan, tyrosine, and cystine (28).

**Activity Studies**—Initially, all mutants were assayed for their activities as inhibitors of a C-terminally truncated form of MMP-3 (MMP-3(D)). The assay was developed to provide a facile means of comparing different mutants. In this assay, preincubation at 37 °C is performed for 1 h to ensure that the binding of inhibitor and metalloproteinase reaches equilibrium, but the addition of substrate results in a 10% dilution of the two proteins. Re-equilibration after this dilution will be slow and is expected to have little effect on the results. Since the substrate concentrations are very low relative to the *K_m* (1.5 μM compared with 25 μM), any correction of the apparent *K_i* determined directly from the kinetic study for competition by substrate is expected to be negligible. Table I lists the apparent *K_i* values mostly calculated using the treatment for tight-binding inhibitors (27), except with mutants for which very large reductions in affinity became apparent; the data for the latter group were analyzed as low affinity reversible inhibitors. Substitutions of Asp^{16}, Val^{18}, and Lys^{27} produced only minor changes in affinity compared with the wild type. Substitutions of the methionines at positions 42 (M42T) and 45 (M45A/F49V) and of the tyrosines at positions 35 (Y35A) and 38 (Y38F) produced significant but not major (4–6-fold) reductions in affinity, whereas substitution of Ala for Met^{66} had a larger effect (14-fold loss of binding). Mutations at positions 2 (T2A) and 38 (Y38A) reduced the affinity for MMP-3(D) by >2 orders of magnitude. Substitutions of either of the two disulfide-bonded cysteines, Cys^{1} and Cys^{70} produced a decrease of <3 orders of magnitude in the affinity for MMP-3(D). To put these mutations into perspective in relation to the proportion of molecular contacts between the inhibitor and protease affected by the substitution, the change in the Gibbs free energy change for the protein-protein interaction produced by the mutation (∆Δ*G*) was calculated for each mutation using the relationship

\[
\Delta G = -RT \ln \left( \frac{K_i(\text{wild-type})}{K_i(\text{mutant})} \right)
\]

These values are shown in Fig. 2. C70S, C1S/F12Y, T2A, and Y38A lost 3–4 kcal/mol of their free energy of interaction with MMP-3(D). The mutants with moderate to large decreases in affinity toward MMP-3(D) were selected for further investigation with MMP-1 and MMP-2 (Table II). Interestingly, whereas most mutations had parallel effects on the affinity of N-TIMP-1 for the three proteases, T2A was more selective than other mutations, showing in particular a 17-fold higher affinity for MMP-3(D) as compared with MMP-1 (Fig. 3).

**CD Spectroscopy**—The mutants that displayed major changes in *K_i* were further characterized by UV CD spectroscopy to see if the loss of activity was a direct result of the sequence change or the indirect consequence of a global change in secondary or tertiary structure in N-TIMP-1. The only feature of the far-UV CD spectrum of N-TIMP-1 is a trough with a minimum at 208 nm, which is not characteristic of any known type of secondary structure. Consequently, changes in the CD spectrum in this wavelength range do not provide useful information about the structural effects of mutations. In contrast, N-TIMP-1 has a complex near-UV CD spectrum with multiple peaks that reflect the fixed environments of aromatic side chains and disulfide bonds in the native structure. The spectrum of this region in N-TIMP-1 is therefore a preferable indicator of changes in tertiary structure introduced by mutations and can help to identify mutants with partially folded conformations (29). The near-UV CD spectra of mutants that show large changes in functional properties are shown in Fig. 4. Although the mutations affected the magnitude of the largest peak centered at 290 nm, the multiple peaks and inflections between 255 and 285 nm were preserved in most of the mutants, including M66A/C70F, which had the lowest activity of all of the mutants constructed in this study. In contrast, the T2A mutant showed some signs of being structurally perturbed, whereas Y38A and the C1S/F12A double mutant lost much of the detail in their CD spectra. It seems likely that some of the loss of activity in these variants results from a loss of fixed tertiary structure. Tyr^{28}, in particular, is an improbable candidate as a component of the interaction site because substitution with phenylalanine has little effect on activity (Table I).

### Table I

| Mutation(s) | *K_0* (nM) | Mutation(s) | *K_0* (nM) |
|-------------|------------|-------------|------------|
| None        | 1.5        | C15/F12Y    | 2020 ± 230 |
| T2A         | 126 ± 4    | M45L        | 8.8 ± 0.8  |
| F5A         | 1.2 ± 0.1  | M45A/F49G   | 5.5 ± 0.6  |
| D16N        | 2.6 ± 0.7  | M66A        | 27 ± 4     |
| V18A        | 3.2 ± 0.1  | M66A/C70I   | 12500 ± 1250 |
| K22A        | 0.7 ± 0.0  | V69I        | 7.0 ± 0.6  |
| K22R        | 1.6 ± 0.2  | V69T        | 14 ± 2     |
| K22E        | 3.7 ± 0.2  | V69A/A103V  | 29 ± 2     |
| Y35A        | 6.4 ± 0.4  | C70S        | 3690 ± 110 |
| Y38F        | 4.4 ± 0.4  |             |            |
DISCUSSION

In the absence of a three-dimensional structure for the complex of a TIMP with a MMP, site-directed mutagenesis provides an effective approach for investigating the roles of individual residues in the inhibitory action of TIMP-1. Mutation of a series of sites between Cys13 and Met45 produced relatively small reductions in affinity for MMP-3. As discussed above, the Y38A variant, which has a greatly reduced affinity for MMP-3(ΔC), also has a disturbed tertiary structure that may account for the reduction in activity. The fact that substitution with Phe at this site has little effect on inhibitory activity supports this hypothesis. In the solution structure of TIMP-2, the side chain of the residue corresponding to Tyr38 of TIMP-1 is partially buried (Fig. 1). Substitution of a large hydrophobic side chain with a small methyl group (Y38A) will introduce a cavity in the interior of the protein and destabilize the structure. The low yield of this protein obtained after folding is consistent with the effect of the mutation on folding and stability.

Substitution of Ala for Met66 close to the C-terminal section of the same disulfide loop produced a 14-fold increase in $K_i$. Evidence for the location of the interaction site in TIMP-1 derived from a "footprinting" experiment correlates well with this observation. TIMP-1 loses its inhibitory function upon incubation with human neutrophil elastase as a result of cleavage of the peptide bond between Val69 and Cys70. Cleavage of this peptide bond by neutrophil elastase and the loss of TIMP activity are prevented when TIMP-1 is preincubated with MMP-3. Although this does not prove that Val69 and Cys70 are in direct contact with MMP-3, it does suggest that they are sufficiently close for MMP-3 to block access of the peptide bond by MMP-3. In the three-dimensional structure of TIMP-2, two sections of polypeptide chain, from Met66 to Cys70 and from Cys1 to Pro5, are connected by a Cys1–Cys70 disulfide bridge and form a continuous surface ridge (19). Because the disulfide

TABLE II

Comparison of the inhibition constants ($K_i$) of N-TIMP-1 and selected variants for MMP-1, MMP-2, and MMP-3(ΔC)

| TIMP-1 variant | $K_i$ (nM) | $K_i$ (nM) | $K_i$ (nM) | $K_i$ (nM) |
|---------------|-----------|-----------|-----------|-----------|
|               | MMP-1     | MMP-2     | MMP-3     | MMP-1/MMP-3 |
| N-TIMP-1      | 1.5 ± 0.4 | 1.1 ± 0.1 | 1.9 ± 0.1 | 0.8 ± 0.2 |
| C1S/F12Y     | 1800 ± 370| 1450 ± 120| 2020 ± 230| 0.9 ± 0.2 |
| T2A          | 2090 ± 180| 308 ± 17  | 126 ± 4   | 17 ± 1.5  |
| M66A         | 24 ± 1    | 21 ± 1    | 27 ± 4    | 0.9 ± 0.1 |
| V69T         | 5.7 ± 0.8 | 2.0 ± 0.3 | 7.0 ± 0.6 | 0.8 ± 0.1 |
| V69T         | 13.2 ± 0.5| 8.6 ± 1.3 | 14.1 ± 2.0| 0.9 ± 0.1 |
| V69A/A103V   | 49 ± 4    | 22 ± 2    | 29 ± 2    | 1.7 ± 0.2 |
| C70S         | 2160 ± 240| 2010 ± 250| 3690 ± 110| 0.6 ± 0.1 |

FIG. 3. Comparison of the inhibitory activity of the T2A mutant of N-TIMP-1 against MMP-1 and MMP-3. $V_i/V_0$ is the ratio of the activity in the presence of inhibitor to the activity in the absence of inhibitor, determined as described under “Results.”

FIG. 4. Near-UV CD spectra of N-TIMP-1 and variants that have perturbed functional properties. Spectra were determined as described under “Experimental Procedures.” WT denotes wild-type (unmutated) N-TIMP-1. deg, degrees.
bond arrangements are conserved in all known TIMPs, a similar structure must also be present in TIMP-1. The mutational and protection results discussed above suggest that this ridge forms part of the binding site. The sequence of this region is well conserved in different TIMPs; besides the conserved disulfide-bonded cysteines (Cys¹ and Cys⁷⁰ of TIMP-1), the only amino acids found at the position corresponding to Val⁶⁹ of TIMP-1 are Val and Leu, and only Thr and Ser are found at position 2. Proline is conserved as residue 5 throughout the TIMP family. To probe the roles of these conserved residues, the effects of substitutions of Cys¹, Thr², Pro⁵, Val⁶⁹, and Cys⁷⁰ were investigated. Replacement of either cysteine, which prevents the formation of the disulfide bond between these residues, produces a loss of affinity of >3 orders of magnitude. The near-UV spectra of the disulfide bond mutants are qualitatively different from but similar in magnitude to those of the wild-type protein. The change in CD spectrum may be partly attributable to the loss of the disulfide bond since disulfides contribute to the spectrum in this wavelength range (29), but some localized change in structure may also be introduced by the mutation. However, the change in CD spectrum is small compared with that of the misfolded Y38A mutant, suggesting that the disulfide bond between Cys¹ and Cys⁷⁰ is not essential for the overall fold of N-TIMP-1; mutants lacking this bond probably have structures that are slightly distorted versions of the wild-type structure. Treatment with Ellman’s reagent indicates that no free thiol groups are present in the folded disulfide bond mutants (data not shown), so the remaining cysteinyl residue is present as a mixed disulfide with mercaptoethanol or has become oxidized during the purification process. Various substitutions of Val⁶⁹ produce moderate changes in affinity, but replacing the totally conserved Pro⁵ with Ala had little effect. A striking functional change arose from substitution of Ala for Thr², which reduced the affinity for MMP-3 by 2 orders of magnitude. Since this residue is conserved as Thr or Ser in different TIMPs, it is possible that the hydroxyl group is important for the formation of complexes between N-TIMP-1 and MMP-3(DΔC). The CD spectrum suggests that substitution with Ala perturbs the tertiary structure. However, the effects of this substitution on TIMP-1 specificity (see below) support the view that residue 2 plays a key role in TIMP action.

There are indications that different TIMPs vary quantitatively in inhibitory activity toward different MMPs (11, 30). We were therefore interested to determine if mutations that affect the affinity of N-TIMP-1 for MMP-3(DΔC) have similar effects on its interactions with other metalloproteinases. Table II shows that most substitutions have approximately parallel effects on the affinity for MMP-1, MMP-2, and MMP-3 even when the Kᵢ values are increased by >3 orders of magnitude, with the exceptions of T2A and, to a much lesser extent, V69A/A103V. The former has a particularly striking 17-fold discriminatory inhibitory action on MMP-3 over MMP-1 (see Fig. 3) as well as a 7-fold higher affinity for MMP-2 relative to MMP-1. The structural basis of this selectivity is currently unclear; a simple explanation could be that the interaction with MMP-1 involves a hydrogen bond between the Thr² hydroxyl group and the enzyme that is less important in binding to MMP-2 and MMP-3. However, this result lends support to the proposal that a region that includes residues 66–70 and residues 1 and 2, linked by the Cys¹–Cys⁷⁰ disulfide bond (TIMP-1 numbering), forms part of the inhibition site in TIMP. Bodden et al. (31) have previously suggested that this disulfide bond is particularly important for structure and/or activity in TIMP. Figs. 1 and 5 show that the disulfide bond links the N-terminal region and residues 66–70 to form a distinct ridge on the surface of the TIMP-2 solution structure.

Analyses by Jones and Thornton (32) show that, in high affinity heterologous protein-protein interactions that are comparable in nature and strength to the TIMP-MMP interaction, a large surface area of each component is buried on complex formation (785 ± 75 Å² for other protease-inhibitor systems). The interaction site defined by our current results has a surface area of <200 Å², smaller than expected for an interaction with a nanomolar dissociation constant. Although the extended ridge defined by our studies represents a suitable structure for fitting the MMP substrate-binding groove and appears to be a major component of the interaction site, additional residues in N-TIMP-1, adjacent to this region, are likely to contribute to the interaction with MMPs. A contribution to binding by a larger part of the N terminus is consistent with the previously published observation (16) that substitutions of His⁷ and Gln⁹ in TIMP-1 produce moderate 2–6-fold reductions in the Kᵢ for MMP-7 (matrilysin). Our results also indicate that similar modest reductions in affinity are produced by substitutions of Tyr⁴⁴, Tyr⁴⁸, Met⁴⁵, and Met⁴⁶.

Amino-terminal disulfide-bonded cysteines are uncommon in proteins. The bond between Cys¹ and Cys⁷⁰ appears to be functionally significant as a linker between the two key sections of the reactive site. Additional residues N-terminal to Cys¹ could potentially disrupt the substrate mimetic character of this region. Cys¹ and Cys⁷⁰ together with their associated disulfide links will also constrain the flexibility of the N-terminal region of TIMP. Structural rigidity in the reactive site may
be important for the inhibitory strategy possibly for arresting the catalytic action of MMPs and preventing proteolysis of the inhibitor by its target. The properties of the T2A mutant provide an initial indication that residue 2 of TIMP is a key to specificity and that TIMP-1 variants that are specifically targeted to individual MMPs can be potentially constructed. Such proteins could be used therapeutically against pathological processes (such as tumor cell metastasis) that are associated with the activity of specific MMPs. Mutational studies are in progress to further investigate this possibility.

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