The Specificity of TIMP-2 for Matrix Metalloproteinases Can Be Modified by Single Amino Acid Mutations*

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Residues 1–127 of human TIMP-2 (N-TIMP-2), comprising three of the disulfide-bonded loops of the TIMP-2 molecule, is a discrete protein domain that folds independently of the C-terminal domain. This domain has been shown to be necessary and sufficient for metalloproteinase inhibition and contains the major sites of interaction with the catalytic N-terminal domain of active matrix metalloproteinases (MMPs). Residues identified as being involved in the interaction with MMPs by NMR chemical shift perturbation studies and TIMP/MMP crystal structures have been altered by site-directed mutagenesis. We show, by measurement of association rates and apparent inhibition constants, that the specificity of these N-TIMP-2 mutants for a range of MMPs can be altered by single site mutations in either the TIMP “ridge” (Cys$^3$–Cys$^5$ and Ser$^{68}$–Ser$^{72}$) or the flexible AB loop (Ser$^{31}$–Ile$^{41}$). This work demonstrates that it is possible to engineer TIMPs with altered specificity and suggests that this form of protein engineering may be useful in the treatment of diseases such as arthritis and cancer where the selective inhibition of key MMPs is desirable.

The matrix metalloproteinases (MMPs)$^3$ are a family of zinc-dependent endopeptidases that have the combined ability to degrade the major components of the extracellular matrix. These enzymes are involved in many physiological processes (e.g. embryogenesis, growth and wound healing) and are implicated in pathologies such as arthritis, fibrosis, and tumor invasion (1, 2). TIMPs (tissue inhibitors of metalloproteinases) are a family of specific endogenous inhibitors that regulate the activity of the MMPs in vitro (3). To date, four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified (4–7) that interact with the catalytic N-terminal domain of active MMPs (16–22). The solution structure of N-TIMP-2 has been solved by $^1$H NMR (23, 24). The protein is a member of the oligosaccharide/oligonucleotide-binding protein fold family, consisting of three of the disulfide-bonded loops of TIMP-2, is a discrete protein domain that folds independently of the C-terminal domain (15). This domain is necessary and sufficient for metalloproteinase inhibition and contains the major site of interaction with the catalytic N-terminal domain of active MMPs (16–22). The solution structure of N-TIMP-2 has been preserved by $^1$H NMR (23, 24). The protein is a member of the oligosaccharide/oligonucleotide-binding protein fold family, being composed of a five-stranded anti-parallel β-sheet rolled over on itself to form a closed β-barrel with two short α-helices packed onto one face.

The aim of this study was to identify residues in human N-TIMP-2 that are responsible for specific binding to MMPs with a view to further understanding the mechanism of inhibition of MMPs by TIMPs. This was approached by a process of rational modification using site-directed mutagenesis. Initially, mutations were chosen by modelling the solution structure of N-TIMP-2 using Rasmol (Molecular Graphics). Residues such as Tyr$^{36}$, Glu$^{57}$, Lys$^{68}$, Asp$^{59}$, Glu$^{91}$, and Asp$^{93}$ appeared in the structure as prominent surface residues. Further mutagenesis was based on information emerging from several structural studies on TIMP-MMP complexes: (i) residues identified at the enzyme-inhibitor interface in the N-MMP-3/TIMP-1 crystal structure (21); (ii) residues identified by site-directed mutagenesis of TIMP-2 (25); and (iii) residues proposed to form the binding site for N-MMP-3 on N-TIMP-2 based on NMR chemical shift perturbation studies (20). All three studies mapped the region on TIMP that interacts with the MMP catalytic domain to a surface “ridge” formed by the disulfide-linked segments Cys$^3$–Cys$^5$ and Ser$^{68}$–Cys$^{72}$ (TIMP-2 numbering). We selected residues Ser$^2$, Ala$^{70}$, Val$^{71}$, and Gly$^{73}$ for modification. Mutant and nonmodified N-TIMP-2 proteins were refolded from bacterial inclusion bodies (26) and kinetically assessed for their interactions with MMP-2, -3, -7, -13, and -14.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**

Point mutations were introduced into human N-TIMP-2 (Δ128–194 TIMP-2) by the “overlap extension” method (27) using the pET23d-N-TIMP-2 construct as a template (26). The mutagenic oligonucleotides used to generate the mutations are shown in Table I and Fig. 1. The mutated DNA was digested with EcoRI and XbaI, purified, and ligated into EcoRI-XbaI-digested pET23d (Novagen). The sequence of the mu-
tated DNA was verified by dideoxy chain termination sequencing. The N-TIMP-2 DNA sequence used in this study codes for Thr at position 21. Ala is more usually found in this position, but the A21T substitution has no effect on the inhibitory activity of the N-TIMP-2 molecule (15) and therefore for the purposes of this study is referred to as the wild-type protein.

### Protein Production

N-TIMP-2 and site-directed mutants were prepared from inclusion bodies after expression from pET23d in *Escherichia coli* BL21 (DE3) pLysS as described previously (26) with the following minor alteration. Guanidinium chloride-solubilized inclusion bodies were diluted to a final concentration of 5 mg/ml in 50 m M Tris/HCl, pH 8.75, containing 0.45 m guanidinium chloride, 0.8 m M reduced glutathione, 0.4 m M oxidized glutathione, and stirred for 2 h at room temperature. The solution was allowed to stand overnight at 4 °C and was concentrated using an Amicon RS2000 concentrator with a 3-kDa cut-off membrane. The concentrate was dialyzed into 20 m M sodium acetate, pH 5.5, and the refolded protein was purified as described (26).

TIMP-1, TIMP-2, and N-TIMP-2 were expressed from mouse myeloma cells and purified as described previously (16, 18). Matrilysin (MMP-1-7) and stromelysin-1 (MMP-3) were prepared in recombinant cell lysates from inclusion bodies using a modification of the method described by Zhang and Gray (33) for MMP-1. Refolded MMP-14 was purified using nickel-nitrilotriacetic acid-agarose and eluted with 100 m M imidazole in 20 m M sodium acetate buffer, followed by dialysis. During the dialysis step some processing of the refolded MMP-14 was observed, with a shift in molecular mass corresponding to the loss of the N-terminal His tag-vector sequences. Activation of proMMP-14 was performed by autoproteolysis at 37 °C for 15 min. Refolded active MMP-14 showed collagenolytic activity versus soluble type I collagen. The integrity of the C-terminal domain is essential for collagenolytic activity; therefore correct folding is implicated (not shown) (34). The catalytic domain of MMP-14 was produced as before (14).

### Confirmation of N-TIMP-2 Structure

### Tryptic Digests and Reverse-phase HPLC—To generate nonreduced tryptic maps, 0.2 mg/ml of wild-type N-TIMP-2 and mutants S2E and Y36G were incubated with 40 μg/ml final concentration 1-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin in 20 m M sodium acetate buffer, pH 5.5, at 37 °C for 2 h. The tryptic digests (50–100 μg) were resolved on a C18 reverse-phase column (Vydac 218TP54) at 40 °C. The column was equilibrated with solvent A (0.1% (v/v) trifluoroacetic acid in water) prior to loading and eluted with a linear gradient of 0–50% solvent B (0.1% (v/v) trifluoroacetic acid in acetonitrile) over 60 min. The flow rate was constant at 1 ml/min, and the absorbance at 220 nm was monitored.

### Kinetic Analyses—All enzyme concentrations were determined by titration using standardized preparations of TIMP-1 or TIMP-2 (18). For N-TIMP-2 mutants, titration was carried out against several enzymes to ensure that an accurate estimate of concentration was obtained. All kinetic experiments were performed in fluorimetry assay
buffer (50 mm Tris/HCl, pH 7.5, 100 mm NaCl, 10 mm CaCl$_2$, 0.05% Brij 35) using a Perkin-Elmer LS-50B spectrophotometer with a thermostatically controlled cuvette holder set to either 25 or 37 °C. Enzyme activity was assessed by measuring the rate of hydrolysis of 1 μM quenched fluorescent substrate (7-methoxycoumarin-4-yl) acetyl-Pro-Leu-Ala-Arg-NH$_2$ for fluorescent substrate cleavage: a linear, rather than curved plot of fluorescence versus time was indicative of equilibrium being reached. It was necessary to incubate MMP-13 (3–120 pm) with N-TIMP-2 overnight at 37 °C, whereas MMP-2 (10–20 pm) and MMP-14 (100 pm) were incubated overnight at room temperature.

RESULTS AND DISCUSSION

Protein Expression and Purification—Previously, we had optimized the expression and refolding of N-TIMP-2 from E. coli and determined that the recombinant protein was correctly folded and able to inhibit MMPs (26). In this study, we have produced 11 mutants of N-TIMP-2, as detailed in Table I and Fig. 1, by expression in E. coli and purification of bacterial inclusion bodies using the same protocol. Purified N-TIMP-2 and mutant forms were electrophoresed on nonreducing SDS-polyacrylamide gel electrophoresis (Fig. 2). N-TIMP-2 migrated as a band of 12.5 kDa, and the mutants migrated at similar molecular masses. In some cases (Y36W and E57S/K58A/D59S) there were small differences in mobility suggesting that the mutations involved had affected slightly. Further analyses, described below, indicated that these were of minor significance. Care was taken during the purification to remove all of the misfolded forms of N-TIMP-2 that migrated just above N-TIMP-2 on nonreducing SDS-polyacrylamide gel electrophoresis (see Fig. 4 in Ref. 26). The final yield of wild-type N-TIMP-2 obtained using this scaled-up method was approximately 5 mg/liter. The yield of some of the mutants was slightly lower, e.g. Y36G, 1 mg/liter. By reverse-phase HPLC, wild-type N-TIMP-2, S2E, and Y36G were estimated to be 80% pure, and this was confirmed by active-site titration.

Structural Analysis—Refolded N-TIMP-2 has been shown previously by $^1$H NMR to be structurally indistinguishable from the protein expressed from mammalian cells (26). Furthermore, with the exceptions of S2E, A70KV71K, A70KV71K/G73F, each of the mutants showed wild-type binding kinetics to at least one of the MMPs (Table II), suggesting structural integrity. The effect of the mutations S2E and Y36G on the refolded structure of N-TIMP-2 was assessed by comparing their susceptibility to trypsin cleavage. Wild type, S2E, Y36G N-TIMP-2, and mammalian recombinant N-TIMP-2 were digested with trypsin under identical nonreducing conditions, and these digests were separated by reverse-phase HPLC. For each N-TIMP-2 the peptide map produced was reproducibly the same (data not shown), and we conclude that the tertiary structure is identical in each case and that the mutants S2E and Y36G are folded correctly.

**Table II**

| N-TIMP-2 | MMP-3 | MMP-2 | MMP-13 | MMP-14 | MMP-7 |
|----------|-------|-------|--------|--------|-------|
| **k$_{on}$**
| Mammalian | $6.01 \times 10^{-2}$ | $2.27 \times 10^{-1}$ | $1.44 \times 10^{-2}$ | $3.09 \times 10^{-2}$ | $5.10 \times 10^{-2}$ |
| Wild type, refolded | $5.38 \times 10^{-2}$ | $1.71 \times 10^{-1}$ | $9.71 \times 10^{-3}$ | $2.33 \times 10^{-2}$ | $4.43 \times 10^{-2}$ |
| S2E | $0.15 \times 10^{-1}$ | $3.00 \times 10^{-1}$ | $0.07 \times 10^{-2}$ | $0.09 \times 10^{-2}$ | $0.01 \times 10^{-2}$ |
| Y36G | $4.58 \times 10^{-2}$ | $1.11 \times 10^{-1}$ | $0.77 \times 10^{-2}$ | $0.02 \times 10^{-2}$ | $2.27 \times 10^{-4}$ |
| Y36W | $4.99 \times 10^{-2}$ | $1.80 \times 10^{-1}$ | $1.06 \times 10^{-2}$ | $0.03 \times 10^{-2}$ | $1.77 \times 10^{-4}$ |
| E57S/K58A/D59S | $3.78 \times 10^{-2}$ | $2.33 \times 10^{-1}$ | $0.85 \times 10^{-2}$ | $1.83 \times 10^{-2}$ | $3.85 \times 10^{-2}$ |
| E91S/D93S | $4.58 \times 10^{-2}$ | $2.78 \times 10^{-1}$ | $1.28 \times 10^{-2}$ | $1.56 \times 10^{-2}$ | $4.04 \times 10^{-2}$ |
| A70K | $3.46 \times 10^{-2}$ | $1.44 \times 10^{-1}$ | $0.43 \times 10^{-2}$ | $0.0003$ | $0.0029$ |
| V71K | $0.33 \times 10^{-2}$ | $1.53 \times 10^{-1}$ | $0.44 \times 10^{-2}$ | $0.0007$ | $0.0026$ |
| G73F | $5.30 \times 10^{-2}$ | $0.42 \times 10^{-1}$ | $0.45 \times 10^{-2}$ | $0.0001$ | $0.0013$ |
range of MMPs was assessed (Table II). The association rate constants for the wild-type refolded N-TIMP-2 were similar to those obtained for N-TIMP-2 expressed in mammalian cells. Association rate constants for the mutants with MMP-14 (MT1-MMP), MMP-13, MMP-3, MMP-7, and MMP-2 were expressed as a percentage of the wild-type refolded value for each MMP, because this allows the effects of the mutations described above to be clearly visualized (Fig. 3). Effects on $K_{\text{app}}$ were also measured to allow an assessment of the contribution of the mutations to the dissociation rates of the MMP complexes (Table III).

Glu$^{57}$, Lys$^{58}$, Asp$^{59}$, Gln$^{61}$, and Asp$^{93}$ were selected for mutagenesis based on their surface location in the N-TIMP-2 1H NMR structure (23). Residues 57–59 are positioned in the loop joining strands B and C, and residues 91 and 93 are located in the loop between strands D and E (23). The mutants E57S/ K58A/D93S and E91S/D93S have association rates and apparent inhibition constants that are essentially the same as wild-type N-TIMP-2 (Tables II and III). These residues in N-TIMP-2 did not show a chemical shift change in their backbone amide signal on binding N-MMP-3 and were not found to be part of the MMP binding site in the TIMP-2/N-MMP-14 crystal structure (20, 22). The kinetic data from this study confirm that these residues are not involved in the N-TIMP-2-MMP interaction.

Tyr$^{36}$ was also selected early in the study as a prominent surface residue. The mutation of this tyrosine residue to either a glycine or a tryptophan had a dramatic effect on the specificity of N-TIMP-2 binding, according to the MMP under test. The rate of association and overall apparent inhibition constants of MMP-13, MMP-2, MMP-3, and MMP-7 were not significantly affected by Y36G or Y36W, whereas the rate of binding to MMP-14 was reduced 100-fold, and the $K_{\text{app}}$ was increased 5-fold for Y36W and 40-fold for Y36G (Tables II and III). Residues Ser$^{31}$–Lys$^{41}$ form a flexible $\beta$-hairpin structure that twists and extends away from the core $\beta$-barrel of the molecule (23, 24). Several residues within this region (including Tyr$^{36}$) were shown to undergo considerable backbone chemical shift change on complex formation with MMP-3 (20, 24) and hence are thought to be intimately involved in the binding interactions with the proteinase. However, the lack of an effect on the association rate of the mutations Y36G and Y36W with MMP-3 as well as MMP-2 and MMP-13 suggests that Tyr$^{36}$ does not make specific interactions with the enzyme that contribute to the overall binding energy. In the case of MMP-14, the slower association rate and increased inhibition constant observed for Y36G suggest that Tyr$^{36}$ does make direct and specific interactions with this MMP. This was confirmed in the recently published MMP-14 catalytic domain/TIMP-2 crystal structure (22): The long AB $\beta$-hairpin loop of TIMP-2 folds over the edge of the active site cleft and extends up into the $\beta$-sheet of MMP-14, where Tyr$^{36}$ slots into a surface gap formed by the so-called “MT loop.” The latter is unique to MMPs 14–16 (membrane-type MMPs 1–3) and is formed by an 8-residue insertion between strands II and III that results in a flexible loop. Not only do changes in Tyr$^{36}$ affect the specificity of N-TIMP-2 for MMP-14, but this is a potential site of differential inhibition between members of the TIMP family. TIMP-1 has a 7-residue deletion that significantly shortens the AB $\beta$-hairpin so that the AB loop does not extend into the pocket formed by the MT loop. This might in part explain the lack of inhibition of MMP-14 by TIMP-1 compared with TIMP-2, although TIMP-3 also has a 6-residue deletion in this region and binds MMP-14 as well as TIMP-2 (14). It is possible that other residues compensate for the small AB loop in TIMP-3, and this is under investigation. There appears to be scope for selectively improving the inhibition of MMP-14 by increasing the number or strength of interactions made by the AB loop (by mutagenesis), which are naturally suboptimal (22).

The significant amide chemical shift changes in N-TIMP-2 on binding N-MMP-3 were located on one face of the molecule (20, 24). A major feature of this site is a ridge of extended polypeptide chain, which is highly exposed on the surface of N-TIMP-2, composed of segments Ser$^{68}$–Cys$^{72}$ and Cys$^{1}$–Ser$^{4}$, linked by the Cys$^{1}$–Cys$^{72}$ disulfide bond (20, 22, 24). The region surrounding Val$^{69}$ in TIMP-1 is also protected from proteolysis by MMP-3 binding (40), and Huang et al. (25) showed that mutagenesis of Thr$^{4}$ to Ala in TIMP-1 reduced the binding affinity for MMP-3 by 2 orders of magnitude. The crystal structures reveal that this region forms a “wedge” that occupies the active site of the MMP in the complex. Residues of this wedge insert between two antiparallel strands of the enzyme and interact with residues at the bottom of the cleft on either side of the catalytic zinc. We mutated Ser$^{2}$, Ala$^{70}$, Val$^{71}$, and Gly$^{73}$,
Modifying TIMP-2-MMP Interactions

TABLE III

| N-TIMP-2 | MMP-2 | MMP-13 | MMP-14 | MMP-7 |
|----------|-------|--------|--------|-------|
| Wild type | 40 ± 16 | 4 ± 1 | 3 ± 1 | 2 ± 0.5 |
| S2K      | 29 ± 15 | 5 ± 5 | 0.5 ± 0.5 | 8 ± 2 |
| S2E      | 18109 ± 5886 | 1522 ± 178 | 1090 ± 550 | 8 ± 2 |
| Y36G     | 70 ± 9 | 6 ± 2 | 124 ± 72 | 3 ± 1 |
| Y36W     | 46 ± 14 | 5 ± 3 | 18 ± 6 | 2 ± 0.5 |
| E57S/K58A/D59S | 49 ± 7 | 7 ± 0.5 | 2 ± 1 | 2 ± 0.5 |
| E91S/D93S | 36 ± 4 | 7 ± 3 | 3 ± 1 | 3 ± 0.5 |
| A70K     | 3676 ± 1210 | 365 ± 101 | 82 ± 4 | 450 ± 8 |
| V71K     | 1433 ± 87 | 349 ± 100 | 266 ± 70 | 898 ± 12 |
| G73F     | 1889 ± 337 | 34 ± 10 | 22 ± 7 | 96 ± 33 |

For mutants where the $K_{\text{app}}$ appeared to be significantly different from the wild-type value, the $K_{\text{app}}$ was measured an average of three times, and the standard error is shown.

Nevertheless, it is clear that ability of the side chain to form a hydrogen bond with the catalytic glutamate is not the major factor determining specificity and neither is the larger side chain always more easily accommodated by the bigger $S_1'$ pocket. The results confirm that position 2 of N-TIMP-2 is a key residue for MMP binding and suggest that the variable nature of the MMP $S_1'$ pocket will enable binding specificity to be altered further.

Differential effects on the inhibition of MMPs were observed by the mutations A70K, V71K, and G73F. The rate of association with MMP-14, MMP-3, and MMP-13 were affected equally by all three mutations but to different degrees, approximately 0.01, 0.05, and 45% of the wild-type value, respectively. Only G73F association was slower for MMP-2, and V71K association was significantly slower with MMP-3. When we analyzed the overall apparent inhibition constant, there were again differential effects, but they did not correspond exactly to the $k_{\text{on}}$ data. This indicates that the mutations also affect the rate of dissociation of the TIMP-MMP complex. In the case of $K_{\text{app}}$, each of these three ridge mutants resulted in an increased value for all of the MMPs studied, although to different extents: for MMP-14 and MMP-13, A70K and V71K had large effects on $K_{\text{app}}$, increasing it 60–90-fold, whereas the $K_{\text{app}}$ for G73F was increased only 10-fold. Although G73F showed a large increase in backbone amide chemical shift change, it appears less prominent on the surface of the ridge in N-TIMP-2 (see Fig. 5 in Ref. 24) but may be important in the flexibility of the loop between strands C and D. This mutation had a large effect on the $K_{\text{app}}$ for MMP-2 (50-fold increase) comparable with A70K and V71K. Thus these residues are very important in the interaction with MMPs. This region is highly conserved in the TIMP family (9), and on the basis of mutational and enzyme protection data, Huang et al. (25) demonstrated that these residues form an essential part of the MMP binding site in TIMP-1. In TIMP-2 this region undergoes substantial conformational change on binding to allow the backbone and side chain interactions seen at the $P_2$ and $P_3$ sites in the N-TIMP-2-MMP-3 complex (24).

Mutating more than one of these residues in mutants A70K/V71K and A70K/V71K/G73F reduced binding to the MMPs so dramatically that kinetic analysis was not possible because of the quantities of protein required to measure inhibition (data not shown). The Ser$^{93}$-Val$^{77}$ segment bulges into the MMP and interacts with the $S_2$ and $S_3$ subsites. In TIMP-2, Ala$^{70}$ contacts the benzyl group of MMP-14 Phe$^{304}$ and is also hydrogen bonded to Cys$^{31}$ of the TIMP, and the isopropyl group of Val$^{77}$ binds into a hydrophobic groove (21, 22). Hence, these results confirm structural predictions that these residues make critical contacts with the MMPs such that changing a single residue can increase the $K_{\text{app}}$ by 2 orders of magnitude. It is also clear that as for Ser$^{93}$, these residues make different contributions to the interaction depending on the enzyme involved.

FIG. 4. Ribbon diagram of N-TIMP-2 showing the side chains of those residues changed by site-directed mutagenesis in ball and stick representation. The N-TIMP-2 structure shown is that determined for the free molecule by heteronuclear NMR (24). Disulfide bonds are shown in yellow. This figure was produced using MOLMOL (42).
Conclusions—The aims of this study were 2-fold: (i) to determine which residues are involved in the interaction with MMPs using structural information as it became available and (ii) to investigate the possibility that the specificity of TIMP could be altered with a view to designing selective TIMPs for therapeutic use. First, we have shown that the charged residues Glu57, Lys58, Asp59, Glu91, and Asp93 do not participate in the interaction with MMPs. We have also confirmed that residues of the pronounced ridge, Ser2, Ala70, Val71, and Gly73, are crucial for the interaction of all the MMPs and that the AB β-hairpin loop, in particular Tyr36, is involved in some cases. Second, we have already achieved our aim in designing a selective TIMP by altering Tyr36, with Y36G and Y36W showing significantly compromised inhibition of MMP-14. It is clear that mutation of a single residue in N-TIMP-2 can have profound effects on inhibition constants and that the MMPs are affected differently by mutation of a particular residue. This is clearly seen when viewing Fig. 3 from left to right where the profile of the effect by mutation of a particular residue. This is clearly seen when viewing Fig. 3 from left to right where the profile of the effect of each mutation is different for each MMP. Hence it is possible to engineer a more selective N-TIMP-2 by changing these residues, and there is further potential for altering the specificity of binding by making further substitutions with different amino acids at these sites and by using combinations of these mutations.

Although synthetic inhibitors of MMPs have been developed and are currently in clinical trials, it appears that a number of TIMPs with altered specificity, and it is anticipated that these may be useful in gene therapies for diseases such as arthritis and cancer where the selective inhibition of key MMPs is compromised inhibition of MMP-14. It is clear that mutation of a single residue in N-TIMP-2 can have profound effects on inhibition constants and that the MMPs are affected differently by mutation of a particular residue. This is clearly seen when viewing Fig. 3 from left to right where the profile of the effect of each mutation is different for each MMP. Hence it is possible to engineer a more selective N-TIMP-2 by changing these residues, and there is further potential for altering the specificity of binding by making further substitutions with different amino acids at these sites and by using combinations of these mutations.

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