Replacement of Conserved Cysteines in Human Tissue Inhibitor of Metalloproteinases-1*

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Tissue inhibitor of metalloproteinases-1 (TIMP-1) is resistant to extremes of temperature and pH. This is thought to be due in part to the presence of six sulfhydryl bridges presumed to maintain the structural integrity of the molecule. As part of a study looking at structure-function relationships, a number of the conserved cysteine residues in TIMP-1 were targeted for replacement with serine. Single and double replacements of these conserved cysteines, as well as replacements around these cysteines, were expressed using a vaccinia virus system and analyzed for functional and structural competence. Analysis by circular dichroism indicated that these mutants maintained secondary structures similar to those of wild-type TIMP-1. Trypsin susceptibility experiments indicated that the tertiary structure of the mutants had not been drastically changed. Analysis of functional competence demonstrated that there were significant changes in some of these mutants. Assays using collagen fibrils or gelatin as substrates indicated that the double mutant C1S/C70S, but not C3S/C99S, had lost inhibitory activity against human fibroblast-type collagenase (FIB-CL) and at high concentrations only had slight activity against M, 72,000 gelatinase (M, 72,000 gelatinase). Kinetic analysis of TIMP-1 inhibition of FIB-CL cleavage of a peptide substrate indicated that mutants C1S/C70S, C3S/C99S, and CEEC → CQQC retained their ability to inhibit FIB-CL in a manner similar to wild-type TIMP-1, while mutants C1S and C70S showed little inhibitory activity. The mutants C99S and C137S could also inhibit FIB-CL cleavage of the peptide substrate. The results indicated that the degree of inhibition by the TIMP-1 mutants varied somewhat depending on the choice of substrates. Interestingly, replacing both cysteines from a disulfide bond in the wild-type molecule resulted in a more competent inhibitor than either of the single site “parent” mutations. Taken together, these experiments indicate that TIMP-1 can be rendered inactive by the loss of a single cysteine.

The tissue inhibitors of metalloproteinases (TIMPs)³ are a family of inhibitors which specifically block the proteolytic activity of the matrix metalloproteinases (MMPs). These two families of proteins are thought to play a significant role in tissue remodeling, a complex process that is necessary for a variety of normal biological functions from embryonic development and growth to wound healing. Tissue remodeling is a tightly regulated process under normal conditions that is characterized by the breakdown and synthesis of extracellular matrix (ECM) components. The degradation of the ECM can be accomplished to a large extent by the MMPs, which are able to cleave most of the components of the ECM (1).

Due to their specificity for the MMPs, the TIMPs are thought to play a major role in the regulation of the remodeling process. Treatment of cells in vitro by a number of agents including growth factors (2), cytokines (3), and phorbol esters (4) may regulate transcription of the genes that encode both the MMPs and the TIMPs. Transforming growth factor-β (5), glucocorticoid compounds (6), and concanavalin A (7) have been shown to differentially regulate expression of the various MMPs and inhibitors, indicating the complexity of the transcriptional regulation of genes involved in tissue degradation.

Upon stimulation, cells secrete the proenzyme forms of the MMPs which must be activated before ECM degradation can occur (1). This activation process serves as another step in controlling the proteolytic degradation of the ECM. The TIMPs have been shown to function both as inhibitors of active metalloproteinases and as inhibitors of the activation process itself. Pathological conditions such as rheumatoid arthritis (8–10), as well as tumor invasion and metastasis (11, 12), are thought to involve aberrant regulation of the degradative process.

Currently, cDNAs that encode four members of the TIMP family (TIMP-1, -2, -3, and -4) have been cloned (13–15, 44). TIMPs-1 and -2 have been shown to be regulated differently (14, 16, 17) and to have varying affinities for binding to the active forms of different MMPs. These inhibitors show a high degree of sequence homology at the amino acid level with the most highly conserved regions including the amino-terminal region and the 12 cysteines present in each molecule. The disulfide bonding pattern has been determined for TIMP-1 (18) (Fig. 1) and is probably similar in all TIMPs, due to the high degree of conservation of the cysteines.

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1 The abbreviations used are: TIMP-1, tissue inhibitor of metalloproteinases-1; rTIMP-1, recombinant TIMP-1 expressed in a recombinant vaccinia virus system; APMA, 4-aminophenylmercuric acetate; FIB-CL, human fibroblast-type collagenase; HEP2, human epidermoid carcinoma cell line; MMPs, matrix metalloproteinases; PAGE, polyacrylamide gel electrophoresis; ECM, extracellular matrix.
Cysteine Mutants of TIMP-1

TIMP-1 is resistant to extremes of temperature and pH (13). This structural stability may be due in part to the six disulfide bonds within the molecule. To investigate structure-function relationships, a number of mutants were made in which the cysteines in TIMP-1 were replaced with serines. Although there was a significant loss of inhibitory activity in some of the mutants, circular dichroism and trypsin susceptibility experiments indicated that the structural integrity of the mutant molecules had not been significantly changed. The effects of each of these mutations has been studied with regard to their ability to complex with and inhibit FIB-CL or M, 72,000 gelatinase.

MATERIALS AND METHODS

Site-directed Mutagenesis—The cDNA clone for TIMP-1 was a gift of Dr. David Carmichael (Synergen, Boulder, CO). The signal sequence was not present in the original cDNA, but was obtained from the published sequence of TIMP-1 (19). The complete signal sequence for TIMP-1 was added for export of the protein by polymerase chain reaction using a 5' oligonucleotide that contained a unique BamHI cleavage site (underlined) upstream of the start codon (double underlined) and the coding sequence for the signal peptide. The downstream oligonucleotide as a probe. The sequences of the isolated mutants were determined by digestion of each mutant with the restriction endonuclease NcoI.

I. The 270-base pair insert containing either the C1S or the C3S phenotype. Complex formation was examined using a modification of the technique of DeClerck (39) method for secondary structure analysis.

Resistance of rTIMP-1 and Mutants to Proteolysis—rTIMP-1 and TIMP-1 mutants were incubated with 10 μg/ml trypsin for 2 h at room temperature in 50 mM Tris-HCl, pH 7.5, 0.2 mM NaCl, 5 mM CaCl2, and 1 μM ZnCl2. At time points 0, 30, 60, and 120 min, an aliquot of the digest was removed and added to soybean trypsin inhibitor and phenylmethylsulfonyl fluoride (at a final concentration of 0.1 mg/ml and 1 mM respectively). After 5 min, 10 mM diithiothreitol and Laemmli reducing sample buffer were added. Trypsin study effects on fully denatured and reduced rTIMP-1, rTIMP-1 was reduced and alkylated prior to exposure to trypsin. For reduction and alkylation, rTIMP-1 was boiled in 10 mM diithiothreitol for 10 min followed by addition of 100 mM iodoacetamide. This solution was then dialyzed against 50 mM Tris-HCl, pH 7.5, 0.2 mM NaCl, 5 mM CaCl2, 1 mM ZnCl2, and 10 mM iodoacetamide for 6 h. The sample was then treated with trypsin as described above and sample dye added. Samples were resolved on 13% SDS-PAGE gels and visualized by Western analysis using polyclonal antibody 2315 (29).

Circular Dichroism—Structural analysis of wild-type and mutant TIMP-1 molecules was performed using circular dichroism. Fractions of purified TIMP-1 or TIMP-1 mutants, following reverse phase chromatography, were dried down in a SpeedVac (Savant, Farmingdale, NY) and resuspended in deionized water (dH2O) containing 0.05 mM Brij-35. Spectra were recorded with an AVIV 62DS spectropolarimeter (Aviv Associates, Lakewood, NJ) on solutions of TIMP-1 and TIMP-1 mutants in a 0.01-cm cell. The spectra were measured every 0.5 nm with a 1-s averaging per point and a 2-nm bandwidth. Five scans per sample were signal averaged and baseline corrected for any drift in the spectrum for buffer alone obtained in an identical manner. The data were analyzed using the computer program PROSEC, which is based on Yang’s (39) method for secondary structure analysis.

Analysis of SDS-resistant Complexes between FIB-CL and rTIMP-1 or Mutants—Complex formation was examined using a modification of the technique of DeClerck et al. (30) as described previously (31). Native
FIB-CL was purified from conditioned medium of human gingival fibroblast cells by immunoaffinity chromatography (32). The protein concentrations were determined using a commercial protein assay kit (Bio-Rad) in which the ODmax of known quantities of bovine serum albumin was used as a standard. FIB-CL (40 μg/ml) was activated with 1 mM APMA for 90 min at 37 °C. FIB-CL (3–150 nM) was mixed with 40 μg/ml rTIMP-1 or mutants in a total volume of 100 μl and incubated at room temperature for 30 min. Following addition of diethiothreitol (final concentration 10 mM) and modified Laemmli reducing sample buffer containing only a final concentration of 0.1% SDS, the samples were loaded without boiling and resolved on two companion 12.5% SDS-PAGE gels. Complexes were visualized by Western analysis using a combination of monoclonal anti-FIB-CL antibodies (III12 and V1L3) (32).

Native Substrate Assays—Two native substrate assays were used for analysis of TIMP-1 inhibitory activity. The fibrillar collagen assay described by Birkedal-Hansen (33) was used to determine the ability of the mutants to inhibit FIB-CL. FIB-CL was activated by incubation with 1 mM APMA for 90 min at 37 °C. Ninety-six well microtiter plates containing 30 μg of reconstituted 4H-labeled type I collagen fibrils were incubated at 35 °C in the presence of APMA-activated FIB-CL (final concentration 1 μg/ml) and serial dilutions of rTIMP-1 and mutants in 50 mM Tris-HCl, pH 7.5, 0.2 mM NaCl, 5 mM CaCl2, and 1 mM ZnCl2. Collagenase activity was measured by the release of radioactivity from the gel. Addition of active TIMP-1 results in the inhibition of FIB-CL and could be determined by a decrease in the amount of radioactivity released. TIMP-1 inhibition of M, 72,000 gelatinase was analyzed by an assay modified from Lyons et al. (34), which uses Xenobind 96-well plates (Xenopore, Saddlebrook, NJ) coated with [3H]gelatin (collagen denatured by heating to 57 °C for 20 min). The Xenobind plates allow for the covalent attachment of gelatin, which can be released by cleavage of the gelatin by gelatinase. The M, 72,000 gelatinase (TIMP-free) was purified by gelatin-Sepharose chromatography in a procedure (29) modified from Hibbs et al. (35). M, 72,000 gelatinase was activated using 1 mM APMA for 30 min at 37 °C. The activated enzyme was incubated (final concentration 20 ng/ml) in the presence of varying amounts of purified TIMP-1 or mutants in 50 mM Tris-HCl, pH 7.5, 0.2 mM NaCl, 5 mM CaCl2, and 1 mM ZnCl2 at 37 °C. The inhibitory ability of the mutant TIMPs or rTIMP-1 was measured by a decrease in the amount of radioactivity released in the presence of rTIMP-1 or TIMP-1 mutants as compared with M, 72,000 gelatinase alone.

Kinetic Analysis of TIMP-1/FIB-CL Interactions—Kinetic analysis was carried out using the coumarinyl peptide derivative MCA-P-L-G-L-Dpa-A-R (36) (Bachem, King of Prussia, PA). The peptide (2.5 μM) and inhibitor (3–150 mM) were combined in 50 mM Tris-HCl, pH 7.5, 0.2 mM NaCl, 5 mM CaCl2, and 1 mM ZnCl2 at 25 °C. APMA-activated FIB-CL (3 mM) was added and emergence of florescence was monitored using a SLM AMINCO SPF-500C Spectrofluorometer (excitation wavelength 328 nm; emission wavelength 393 nm). Time course data were taken as estimates were produced for the model selection criterion. The model selection criterion, calculated by the computer program (37), is a modification of the Akaike information criterion (38) and is proportional to the goodness of fit of the model. It is the natural logarithm of the sum of the residuals, normalized for the magnitude of the data and adjusted for the number of unknown parameters in the model.

RESULTS

Expression and Purification of Wild-type TIMP-1 and Mutants—Wild-type and mutant TIMP-1 were expressed using a vaccinia virus system and purified from the media of infected HeLa cells. Both single and paired cysteines were mutated to serine throughout the TIMP-1 molecule (Fig. 1). A replacement mutant at Cys127 was chosen to serve as a negative control, since previous work had shown that deletion of the TIMP-1 molecule at amino acid 127 (Cys127→stop) retains most of the inhibitory capacity of wild-type TIMP-1 (40). Therefore, residues beyond amino acid 127 seem to be relatively less important for inhibitory activity. Single replacement mutants of Cys1, Cys70, Cys99, and Cys137 with serine all yielded sufficient quantities of secreted protein for purification. In addition, double replacement mutants were constructed in which both partners in a particular sulfhydryl bridge were replaced; in these proteins, the possibility of aberrant formation of sulfhydryl bridges might be avoided. The mutation of C3S resulted in insufficient levels of protein for purification. However, a double mutant of C3S and C99S was produced and secreted at wild-type levels and could be purified. Following purification, 2 μg of each mutant was analyzed by SDS-PAGE and stained with Coomassie Blue (Fig. 2).

The region surrounding the disulfide bonds of Cys13–Cys124 appears to be very important. Attempts to replace the cysteines at position 13, 124, or 127 resulted in proteins which could be made transiently inside infected cells, but for which a virus recombinant that expressed the mutant protein in culture medium could not be isolated. A truncation mutant in which Cys124 was replaced with a stop codon for translation failed to give any protein even in transient expression assays, although a similar truncation mutant at Cys127 does make functional protein (40). These results suggested the potential importance of the region between Cys124 and Cys127. Accordingly, two other mutations within this region were made: a mutation in which the CEEC residues were replaced with CQQC and a mutation in which CEEC was replaced with CDDC. Interestingly, the CQQC mutant could be expressed and purified in the same manner as wild-type, whereas the CDDC mutation failed to show any protein expression even in transient assays. The observation that most mutants in this region have severe defects that apparently preclude expression and/or secretion of protein suggests that alterations of these residues may have profound effects on protein structure, sensitivity to cellular proteinases, and/or perhaps also on function.

Trypsin Susceptibility—Because TIMP-1 is relatively resistant to cleavage by trypsin,2 trypsin sensitivity can serve as a sensitive and semiquantitative probe of the alterations in structural integrity of the mutant compared with wild-type. Trypsin sensitivity of TIMP-1 has shown that TIMP-1 was slowly cleaved to yield a major form of ~24 kDa, whereas reduced and alkylated TIMP-1 was degraded rapidly and completely, after incubation with 10 μg/ml trypsin for 2 h (Fig. 3A). Analysis of the relative trypsin susceptibility of TIMP-1 mutants is shown in Fig. 3B. The mutants were resistant to total degradation by trypsin. Although mutants C70S and C1S/C70S showed numerous breakdown products, they did not undergo the complete degradation characteristic of random-coil structures as seen with the reduced and alkylated TIMP-1. C99S and C3S/C99S showed more trypsin sensitivity than wild-type, but the molecules were not completely degraded during the course of the reaction. These results indicate that there are some minor changes in the structure of several of the mutant TIMPs, which make sites within them slightly more accessible to trypsin digestion.

Structural Analysis by Circular Dichroism—The observed functional effects of the mutants may be due to a global change in the structure of the molecule or be due to direct disruption of inhibitory function without major disruption of the secondary structure. As a more quantitative measure of the effect that the cysteine replacements may have had on the secondary structure of the TIMP-1 molecule, the CD spectra of the wild-type and mutants were analyzed. The CD spectrum of each purified mutant was measured from 180 to 260 nm and the resulting data were converted to mean molar ellipticity per residue (degree cm2/dmol) and smoothed by a program which used the Stavitsky-Golay algorithm (39). The spectra of the cysteine mutants showed relatively minor differences from the CD sig-
nal of the wild-type spectrum. The C1S mutant had a negative shoulder in the range of 225 nm consistent with an increase in α-helical structure. This same change was also seen in the C1S/C70S mutant.

These data were then analyzed by PROSEC, which uses the method of Yang and co-workers (39) to determine the percentage of secondary structure (α-helix, β-sheet, turn, and random coil) (Table I). The levels of secondary structure by this type of analysis had an error of ±5% for helices, but the error increases with the other secondary structures (37). As can be seen in Table I, the secondary structure of these molecules was remarkably similar. Further support for this analysis comes from the published solution structure of TIMP-2 by NMR, which indicates that the inhibitor is comprised primarily of β-sheets surrounding short α-helical regions (42).

Formation of Complexes between Active FIB-CL and rTIMP-1 or TIMP-1 Mutants—Binding of TIMP-1 to active FIB-CL allows complexes between these two molecules to be detected in 0.1% SDS conditions. Both C137S and CEEC3CQQC formed complexes as did wild-type (Fig. 5). On the other hand, all of the other single mutations and the double cysteine mutations failed to form complexes with FIB-CL under these conditions.

Previous work showed that after deletion of the TIMP-1 molecule at amino acid 127 (C127STOP), the molecule still retained its ability to form complexes in 0.1% SDS conditions with activated FIB-CL and had nearly wild-type activity against

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**Fig. 1. The proposed structure of TIMP-1.** The diagram shows the schematic representation of the amino acid sequence of TIMP-1, including the disulfide bonds assigned by Williamson et al. (18). Loops created by disulfide bridges are indicated (L1 through L6).
activated FIB-CL on native substrate assays (40). Since C137S is located within the region that was deleted in the C127 → stop mutant, it was expected that C137S would have activity at least comparable to C127 → stop; however, this mutant showed reduced inhibitory activity on native substrates (see below).

Analysis of TIMP-1 Mutants by Native Substrate Assays—The single cysteine replacement mutants of TIMP-1 all showed significant loss of inhibitory activity against FIB-CL (Fig. 6) and M_r 72,000 gelatinase (Fig. 7). In these experiments a fixed amount of proteinase was titrated with increasing concentrations of inhibitor. C137S, although able to form complexes, showed some loss of inhibitory activity against both FIB-CL and M_r 72,000 gelatinase in the native substrate assays. The decrease in inhibitory activity for C137S was not as dramatic as with some of the other cysteine mutants. Interestingly, the double mutant containing replacement of Cys^9^ and Cys^99^ with serine together showed only 2-fold less activity on collagen in titration experiments than wild-type yet was unable to form SDS-resistant complexes. These two mutants suggest that the failure of TIMP-1 molecules to form SDS-resistant complexes with FIB-CL does not preclude their ability to inhibit FIB-CL (and M_r 72,000 gelatinase) under less stringent assay conditions, for example, lacking the SDS. The other mutants gave results consistent with the data regarding complex formation: the mutants C1S, C70S, C1S/C70S, and C99S showed no measurable inhibitory activity on native substrates, while the CEEC → CQQC mutant showed wild-type inhibitory activity. As mentioned previously, the C3S mutant was expressed in low quantities and could not be further characterized.

Kinetic Analyses—Due to the length of time required for the assays discussed above and the necessity of using relatively large amounts of enzyme, kinetic analysis of the data on the natural substrates would have been difficult to accomplish. Therefore, the kinetic parameters of the FIB-CL/TIMP-1 interactions were examined using an assay that measured cleavage of a fluorescent peptide by activated FIB-CL rather than cleavage of native substrates. This peptide allowed for a lower concentration of enzyme and inhibitor to be used and also allowed for measurement over the entire time course of the experiment.

Previous investigators (41) have used an integrated mathematical model designed for slow-binding inhibitors to analyze time course data of this type. This analysis is limited by the number of approximations required as well as by the concentrations of reagents necessary to make these approximations valid. The use of numeric integration allowed for the use of 1:1 molar ratios of enzymes and inhibitors. The method also permitted the testing of a greater variety of models than otherwise possible (43).

The data were fitted to the differential equations derived for several potential mechanisms of binding and inhibition by TIMP-1 (43): 1) a one-step mechanism; 2) a two-step mechanism in which a highly reversible binary complex is slowly converted to a tight complex; 3) a one-step mechanism in which the inhibited complex subsequently breaks down at a rate
proportional to the concentration of TIMP-1; 4) a two-step mechanism in which a highly reversible loose binary complex is slowly converted to a tight binding complex which, in turn, breaks down at a rate proportional to the concentration of TIMP-1 (Scheme I); 5) a single-step reversible mechanism in which the binary complex is in rapid equilibrium with the enzyme and TIMP-1; and 6) a one-step irreversible mechanism. Each data set fit best to one mechanism, whereas the goodness-of-fit parameters, model selection criterion, for the other mechanisms were more difficult to distinguish.

Data from the inhibition by wild-type TIMP-1 of FIB-CL cleavage of the peptide substrate fit best to the fourth mechanism (Scheme I) as described by Taylor et al. (43). Data from experiments of each of the double mutants, C1S/C70S and C3S/C99S, fit best to the same mechanism as the wild-type (Table II). For these two mutants, the binding parameters ($K_d$) for the first step in the two-step mechanism (Scheme I) obtained were generally within the same respective populations as those from seven independent data sets with the wild-type TIMP-1 ($6.33 \times 10^{-9}$ to $5.34 \times 10^{-8}$ M). The mutant, CEEC $\rightarrow$ CQQC, also fit clearly best to the same model as the wild-type TIMP-1 and had a $K_d$ also within the range of $6.33 \times 10^{-9}$ to

**TABLE I**

Structural analysis of TIMP-1 and mutants using circular dichroism

| Sample       | % $\alpha$-Helix | % $\beta$-Sheet | % Turn | % Random coil |
|--------------|-------------------|-----------------|--------|--------------|
| Wild type    | 6                 | 68              | 0      | 27           |
| C1S          | 7                 | 54              | 4      | 34           |
| C70S         | 5                 | 64              | 0      | 31           |
| C1S/C70S     | 7                 | 53              | 0      | 40           |
| C99S         | 5                 | 58              | 2      | 36           |
| C3S/C99S     | 3                 | 62              | 0      | 36           |
| C137S        | 7                 | 48              | 5      | 41           |
| CQQC $\rightarrow$ CEEC | 6         | 57              | 2      | 35           |

**Fig. 4. Analysis of rTIMP-1 and rTIMP-1 mutants by circular dichroism.** Samples were scanned 5 times from 260 to 185 nm and the signals averaged. The data were converted to mean molar ellipticity and are shown above. In the first panel the rTIMP-1 is shown alone and in a bold dashed line. The mutants are shown in the next panels as bold lines overlaid on the wild-type which is shown as a dashed line.

**Fig. 5. Formation of complexes between FIB-CL and rTIMP-1 or rTIMP-1 mutants.** This experiment provides a semiquantitative measure of the ability of TIMP-1 to bind to FIB-CL. Wild-type and mutant rTIMP-1 proteins were incubated for 30 min with APMA-activated FIB-CL. Samples were then electrophoresed through a 12.5% polyacrylamide gel. The gel was blotted onto nitrocellulose and stained with two monoclonal antibodies against FIB-CL as described under "Materials and Methods." The positions expected for the proenzyme (pro-FIB-CL) and activated (a-FIB-CL) forms of FIB-CL are indicated on the left side of the figure. The position of TIMP-1-FIB-CL complexes is indicated on the right side of the figure. Panel A: lane 1 latent FIB-CL; lane 2 FIB-CL activated with APMA; lane 3, activated FIB-CL + rTIMP-1; lane 4, activated FIB-CL + C1S; lane 5, activated FIB-CL + C70S; and lane 6, activated FIB-CL + C1S/C70S. Panel B: lane 1 latent FIB-CL; lane 2, FIB-CL activated with APMA; lane 3, activated FIB-CL + C99S; lane 4, activated FIB-CL + C3S/C99S; lane 5, activated FIB-CL + C137S; and lane 6, activated FIB-CL + CEEC $\rightarrow$ CQQC.
5.34 × 10⁻⁸ M. Data from experiments with at least six different concentrations of each of the two single mutants, C1S and C70S, showed poor inhibitory activity. Data sets for the mutants C99S and C137S displayed inhibitory activity, but the kinetic data for these mutants did not fit the wild-type TIMP-1 model well.

**DISCUSSION**

TIMPs-1, -2, -3, and -4 from all species sequenced to date show complete conservation of 12 cysteines, all of which are thought to be involved in disulfide bonds (18). As part of a structure-function study, the effects of cysteine replacement mutants on TIMP-1 inhibition of MMPs were investigated. Both single replacement and double replacement mutants at the positions of conserved cysteines were tested. The single cysteine mutants would be expected to have an unpaired free sulfhydryl group, while the double mutants should not. Residues between the cysteines at positions 124 and 127 were also changed because several cysteine replacement mutants in this region were not able to be expressed and/or secreted from the cells. The structural integrity of these molecules was tested by trypsin sensitivity and circular dichroism (CD). Some mutants showed a slight decrease in their resistance to trypsin cleavage compared with a completely reduced and alkylated TIMP-1 substrate and the CD analysis indicated that the loss of disulfide bonds may have loosened the structure of these mutant inhibitors, making certain regions slightly more susceptible to trypsin digestion without causing substantial changes in the secondary structure that would be detectable by CD analysis.

While structural analysis of the mutants suggested that there were no major alterations in the structure of the molecules, a range of changes in inhibitory activities was observed when these mutants were assayed for inhibitory activity against FIB-CL or Mr 72,000 gelatinase. Furthermore, the activity of these mutants against active FIB-CL did not correlate in all cases with their ability or inability to form detectable low SDS-stable complexes with this enzyme or with their resistance to trypsin digestion. The mutant C137S was able to form complexes stable under low SDS conditions, but was not able to inhibit FIB-CL at wild-type levels in the native substrate assays. However, it did inhibit at wild-type levels in the peptide assay, although by a different mechanism than wild-type. The formation of the low SDS complex is consistent with the inhibition in the presence of the peptide substrate. However, the presence of the large protein substrate seems to prevent at least partially the formation of the inhibitory complex by this mutant TIMP-1. The kinetic results with the MCA....
peptide indicate that the structure associated with the loop at Cys^{137} is important for the formation of the initial reversible complex.

The mutant C3S/C99S was able to inhibit FIB-CL with nearly wild-type activity in all the assays tried, but could not form complexes that were stable to the low SDS concentrations utilized in this electrophoresis gel assay. This mutant is clearly able to form complexes under physiological conditions, allowing it to inhibit both FIB-CL and \( M_r \) 72,000 gelatinase with near wild-type efficiency; however, these complexes apparently dissociate even in the low SDS conditions, probably due to loosening of the structure caused by the mutations. The low SDS included in the loading dye or the actual electrophoresis conditions apparently dissociate these complexes during analysis by this technique.

With regard to activity against the MMPs on native substrates, the C1S, C70S, C15/C70S, and C99S showed no inhibition of FIB-CL activity and substantially decreased inhibition of \( M_r \) 72,000 gelatinase activity, even at high concentrations. The C1S and C70S single mutants are rather poor inhibitors for both the small substrate (peptide) and the large substrates (collagen and gelatin). However, the fact that the double mutant C1S/C70S behaves similar to the wild-type with the small substrate but is a poor inhibitor with the large substrates support the hypothesis above that the large substrates can interfere with the action of the inhibitor more effectively than can the small substrate. This hypothesis is consistent with our previous evidence for an initial noncompetitive inhibition step in the hydrolysis of the small substrate by TIMP-1 (43). In addition, the wild-type level of inhibition by the double mutants and the poor inhibition by either single mutant of hydrolysis of the small peptide substrate suggests that the loop produced by the disulfide bond between the two cysteines is not as important for inhibition as is the disruptive presence of an unpaired sulfhydryl group in the single mutants. Nevertheless, the cysteine loop takes on increased importance for the inhibition of hydrolysis of the large substrates.

It has been suggested in other binding studies that a sequence-based inhibitor of collagenase bearing an hydroxamate group capable of chelating the active site zinc binds the same site (probably active site) in collagenase as does TIMP-1, since this inhibitor dissociates pre-formed TIMP-collagenase complexes and prevents complex formation (45). However, dissociation of the preformed complexes required a 1000-fold molar excess of this sequence-based inhibitor to preformed TIMP-collagenase complexes to dissociate even (20–30%) of the complexes, even though the \( K_i \) for the synthetic inhibitor and for TIMP-1 were only 2–4-fold different (using either collagen or a peptide as substrate). It remains uncertain whether TIMP-1 is prevented from binding or displaced from the active site by the sequence-based inhibitor or whether the inhibitor induces a change in conformation that impairs subsequent TIMP-1 binding.

Mutation of Cys^{103} causes the TIMP-1 to be a poor inhibitor of hydrolysis of the large substrates, but a moderately good inhibitor of hydrolysis of the peptide substrate. These observations further support the hypothesis that the large substrate can retard inhibition of its own hydrolysis. The C3S/C99S double mutant retained activity in the range of wild-type against both FIB-CL and \( M_r \) 72,000 gelatinase as well as the peptide substrate. These mutants showed only minimal changes in structure, suggesting that the losses of activity were directly related to the particular mutation. The fact that the different cysteine replacements were able to maintain various levels of competence on different substrates indicates that the location of the mutation within the molecule plays an important role in the activity of TIMP-1. For example, in TIMP-2, the disulfide bond Cys^{9}/Cys^{72} is almost completely buried and abolishing it should have less effect than eliminating the disulfide bond of Cys^{9}/Cys^{72} that is located on the surface of the TIMP-2 molecule (46). This prediction is consistent with our observation that the TIMP-1 mutant C1S/C70S poorly inhibited collagenase-mediated cleavage of native substrates, while the C3S/C99S retained almost wild-type activity on these substrates. This difference was not observed in experiments with the peptide substrate.

These experiments underscore the importance of the sulfhydryl bridges to TIMP function, but not necessarily to the shape or the molecule under the conditions of the assays used; on the other hand, the sulfhydryl bridges may provide stability to the molecule under less benign conditions (for example, a low concentration of SDS). While few differences in secondary structure could be detected in many of the mutants, these molecules displayed a range of inhibitory properties, depending on the location of the mutation within the cDNA. Similarly, one mutant (C137S) was able to bind FIB-CL, but not effectively inhibit it on native substrates. Other mutants did show inhibitory activity, but were unable to form stable TIMP-1-FIB-CL complexes in low SDS; however, it is possible that complexes with these mutants are formed under the reaction conditions used to measure inhibitory activity but are not detected in the presence of low SDS utilized in this technique. This range of activities observed suggests that binding of TIMP to FIB-CL may consist of two steps: an initial binding interaction, followed by inhibition. One can alter the inhibition reaction by alteration of TIMP-1 without necessarily blocking the binding reaction. This interpretation is consistent with the analysis of the kinetic data, but awaits analysis of other mutations that may further define the domain(s) of the molecule responsible for the binding and the inhibiting reactions of TIMP-1 on FIB-CL and other MMPs.

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