Accelerated Publications

Site-Directed Mutations That Alter the Inhibitory Activity of the Tissue Inhibitor of Metalloproteinases-1: Importance of the N-Terminal Region between Cysteine 3 and Cysteine 13†

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ABSTRACT: The tissue inhibitor of metalloproteinases-1 (TIMP-1) was subjected to single-site mutations within the N-terminal three loops using an oligonucleotide-directed polymerase chain reaction method. All the histidines, and a number of other residues conserved between TIMP-1 and TIMP-2, were individually modified and the mutant TIMPs expressed in mammalian cells. Purified mutant TIMPs were shown to be correctly folded by measuring the effect of guanidine hydrochloride on intrinsic fluorescence. Kinetic analyses of mutants using a quenched fluorescent peptide substrate and the metalloproteinase PUMP indicated that mutation of His7 and Gln9 caused an increase in the apparent dissociation constant, largely due to an increase in the rate of dissociation of complexes. The data indicate that the anchored sequence between Cys 3 and Cys 13 is a key region for interaction of TIMP-1 with metalloproteinases.

The matrix metalloproteinases (MMPs)[1] are a group of enzymes that have the combined ability to degrade the major components of the extracellular matrix (Docherty & Murphy, 1990; Nagase et al., 1991; Woessner, 1991). Several mechanisms exist for controlling their catabolic activity at the DNA and protein levels (Docherty et al., 1992). The tissue inhibitors of metalloproteinases (TIMPs), by virtue of their ability to inhibit all known MMPs through the formation of tight binding complexes, play an important role in regulating the activity of these enzymes.

Two members of the TIMP family (TIMP-1, 184 amino acids, and TIMP-2, 194 amino acids) are known to exist (Docherty et al., 1985; Boone et al., 1990). Human TIMP-1 and TIMP-2 display 40% identity of primary sequence within which the locations of all 12 cysteines are conserved. In TIMP-1 these cysteines form disulfide bonds, giving a protein structure of six loops and two domains (Williamson et al., 1990). The interaction between TIMP and active MMPs occurs with 1:1 stoichiometry and is noncovalent; TIMP is not modified during complex formation and retains inhibitory activity after dissociation (Cawston et al., 1983; Stricklin & Welgus, 1983; Murphy et al., 1989). Precise details of the interaction between TIMP and the MMPs are unknown, but recent findings have shown that the N-terminal three loops alone are sufficient for both the binding and inhibition of active MMPs (Murphy et al., 1991a). This study also indicated that the C-terminal three loops of TIMP-1 play a part in the binding of TIMP-1 to 95-kDa progelatinase B.

To elucidate further the mechanism of action of TIMP, we have used systematic site-directed mutagenesis to mutate individual amino acids in TIMP. Chemical modification of TIMP-1 had indicated that histidine residues are required for inhibitory activity (Williamson et al., 1992). There was also some evidence for the involvement of lysine, tryptophan, and acidic residues. We therefore initiated a program of single-site mutagenesis of TIMP-1, changing each of the histidine residues as well as Glu28 and Trp105. We also mutated residues within the motifs Val18-Lys22, Tyr38-Gly48, and Glu82-87, particularly lysine and glutamic acid, which are known to be conserved between TIMP-1 and TIMP-2 from a number of species. Mutant TIMPs were initially screened for inhibitory activity in a collagenase diffuse fibril assay. It is known that full-length active forms of MMPs bind more tightly to TIMPs than engineered short forms consisting of the catalytic domain only (Murphy et al., 1992a,b), making kinetic analyses difficult. To characterize TIMP mutants in more detail, we therefore carried out kinetic studies using PUMP as a representative catalytic domain. We now report our initial conclusions on the nature of some of the primary structural features of the TIMP molecule that govern its interaction with the catalytic domain of MMPs.

METHODS

Site-Directed Mutagenesis of Human TIMP-1. The cDNA for human TIMP-1 was cloned into pSP64 (Melton et al.,

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[1] Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; SDS, sodium dodecyl sulfate; GdnHCl, guanidine hydrochloride; PUMP, putative (punctuated) metalloproteinase or matrilysin; PCR, polymerase chain reaction; McaPLGLDpaAR, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-ArgNH_2.

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1984) between the *Hin*dIII and *Bam*HI sites of the polylinker. This was achieved by using oligonucleotide adapters to convert the 5′ *Nco*I site adjacent to the ATG start codon (nucleotides 62–67; Docherty et al., 1985) into a *Hin*dII site and the 3′ *Hin*fI site adjacent to the TIMP stop codon (nucleotides 686–690) into a *Bam*HI site. Mutagenesis was performed by the "overlap extension" method using this template, the polymerase chain termination reaction, and complementary pairs of mutagenic oligonucleotides in conjunction with oligonucleotides that prime from within vector sequences that flank the TIMP-1 sequence (Mullis & Faloona, 1987; Ho et al., 1989). The pairs of oligonucleotides that were used for mutagenesis of single amino acids to alanine, or in some cases to more structurally related residues, and in one case to make a deletion, are shown in Table I. The mutated full-length cDNAs generated as described above were digested with *Hin*dIII and *Bam*HI, purified, and ligated into *Hin*dIII- and *Bcl*I-digested pEE12 between the hCMV promoter and the SV40 early polyadenylation signal as described previously (Murphy et al., 1992a,b). The sequence of the mutated cDNA was verified using the dideoxy chain termination sequencing method (Sanger et al., 1977).

Expression of Wild-Type and Mutated TIMPs. pEE12, a mammalian cell expression vector, is a derivative of pEE6HCMV (Stephens & Cockett, 1989) and contains a functional glutamine synthetase cDNA from pSV2GS under the control of the SV40 early promoter (Bebbington et al., 1992). This allows selection of stable plasmid-bearing cell lines through growth in the absence of glutamine. The pEE12 plasmid containing either the wild-type or mutated TIMP cDNA was transfected into NSO mouse myeloma cells (ECACC catalog no. 85110503) by electroporation. Plasmid DNA (40 μg), which had been linearized with *Sal*I, was added to 10^7 NSO cells which had been washed and resuspended in 1.0 mL of ice-cold PBS in an electrophoretic cuvette (Bio-Rad, 165-2088). The cells were incubated on ice for 5 min and were then given two pulses of 1500 V at 3 μF with a Bio-Rad gene pulsar. After a further 5 min on ice, the cells were taken up in growth medium, diluted over several 96-well plates (50 μL/well), and incubated at 37 °C in 5% CO_2 for 24 h. Glutamine-free selection medium was added (100 μL/well), and the cells were incubated until colonies appeared (2–3 weeks). Conditioned media from selected colonies grown in mass culture were analyzed on substrate gels and in either activity (Murphy et al., 1981) or ELISA assays (Cooksley et al., 1990). The most productive cell lines were used to produce serum-free conditioned medium from which the recombinant wild-type and mutant TIMP proteins were purified.

Purification of TIMPs. Wild-type and mutant TIMPs were purified from culture media using a monoclonal antibody (MAC 015; Cooksley et al., 1990) linked to Sepharose, as described previously (Murphy et al., 1991a). The abilities of mutants to bind to this matrix and the elution conditions were checked before bulk purification. All of the mutants described in this study were bound (pH 7.5) and eluted (pH 2.8) from the matrix under the same conditions as for wild-type TIMP, with the exception of K41A-TIMP, which could be eluted at pH 4.5.

Yields of mutant inhibitors varied markedly from 30 mg/L of original culture medium for the majority, to 0.5 mg/L. In the case of ΔVal 18–Lys 22 negligible amounts of inhibitor were produced. An $A^{1\%}_{280,1cm} = 10.0$ was used for all TIMPs (calculated from total amino acid analysis and by assuming a molecular mass of 28.5 kDa; S. Angal unpublished results), but prior to kinetic studies each mutant was titrated against stromelysin-1 (see below).

Inhibitory activity of TIMP preparations against a stable preparation of rabbit collagenase was monitored using a ^{14}C-labeled collagen assay (Murphy et al., 1981).

Gel Electrophoresis. Samples of inhibitors were analyzed by SDS–polyacrylamide gel electrophoresis on 11% gels using the Laemmli system (Laemmli & Favre, 1973).

Preparation of Metalloproteinases. Recombinant prostromelysin and proPUMP were purified from the conditioned medium from transfected mammalian cell lines as described previously (Docherty & Murphy, 1990; Murphy et al., 1991b) and activated (Murphy et al., 1991b).

Intrinsic Fluorescence. Fluorescence measurements were carried out using a Perkin-Elmer LS-5B luminescence spectrometer with a thermostatically controlled cuvette holder set to 25 °C. Denaturation analyses of wild-type and mutant TIMP-1s (10 μg/mL) were carried out in 25 mM Tris-HCl and 125 mM NaCl, pH 7.5, containing guanidine hydrochloride (GdnHCl) from 0 to 7 M. Unfolding was monitored by a change in emission wavelength at maximum emission (λ_{max}). Measurements were taken from an average of five scan passes (315–365 nm) at an excitation wavelength of 280 nm. Emission scans were taken when the fluorescent intensity at 365 nm had reached a steady value (10–40 min). The excitation and emission slits were both set at 10 nm. The concentration of the GdnHCl stock solution (8 M) was checked by refractometry (Nozaki, 1972).

The data collected for each GdnHCl titration were analyzed as described by Pace (1986) and Pace et al. (1989) to obtain values for the midpoint of denaturation and the standard free energy change between the native and denatured states in the absence of denaturant ($\Delta G°_{H_2O}$). Briefly, each point in the transition region was expressed as a fraction of folded (f_f) and unfolded (f_u) molecules by extrapolating the pre- and post-transitional zones. The equilibrium constant (K) and the standard free energy change ($\Delta G°$) were calculated using the relationships:

$$K = f_u/f_f \qquad \Delta G° = -RT \ln K$$

$\Delta G°$ varies linearly with denaturant concentration and is equal to zero when $f_u/f_f = 1$ (the midpoint). This line was extrapolated to [GdnHCl] = 0 to give an estimate of $\Delta G°_{H_2O}$.

To enable a more realistic comparison of the conformational stabilities of the mutant TIMP-1s, $\Delta(\Delta G°)$ values for each were calculated from an average value of m (the gradient of the $\Delta G°$ vs [GdnHCl] plot) multiplied by the difference in denaturation midpoint between the mutant and the wild type (Pace et al., 1989). This analysis avoids the large errors inherent in extrapolating $\Delta G°$ to [GdnHCl] = 0.

TIMP Concentration. The concentration of each mutant relative to that of wild-type TIMP was determined by titration against stromelysin. Each mutant (5–100 nM) was preincubated with stromelysin (50 nM) for 2 h prior to assay at an enzyme concentration of 5 nM using (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-ArgNH_2 (McaPLGLDpaAR; Knight et al., 1992). Initial velocities were plotted against the TIMP dilution and TIMP concentration determined from the intercept on the ordinate.

Kinetic Analysis of Mutants. The apparent K_i for the inhibition of PUMP by the mutants was determined using the substrate McaPLGLDpaAR (1.6 μM) in an assay buffer of 0.1 M cacodylate buffer, pH 6.5, containing 0.1 M NaCl, 10 mM $CaCl_2$, and 0.05% Brij 35. TIMP (0.1–100 nM) was incubated with PUMP (1 nM) for 2 h at room temperature

Table I: Sequence of TIMP-1 Mutagenic Oligonucleotides[a]

His_7→Ala	**His_7→Gln**
C V P P A_7 P Q T	C V P P Q_7 P Q T A
GTGTCCCACCCGCCCCACAGAC	TGTGTCCCACCCCAGCCACAGACGGCC
CACAGGGTGGGCGGGGTGTCTG	ACACAGGGTGGGGTCGGTGTCTGCCGG
Gln_9→Ala	**ΔVal$_{18}$Ile$_{19}$Arg$_{20}$Ala$_{21}$Lys$_{22}$**
P P H P A_9 T A F C	C N S D $L_{17}F_{23}$V G T P
CCCACCCCACCCAGCCACGGCCTTCTGC	GCAATTCCGACCTCTTCGTGGGGACACCAG
GGGTGGGGTGGGTCGGTGCCGGAAGACG	CGTTAAGGCTGGAGAAGCACCCCTGTGGTC
Arg_{20}→Ala	**Lys_{22}→Ala**
L V I A_{20}A K F	I R A A_{22}F V G T
CCTCGTCATCGCCGCCAAGTTCG	CATCAGGGCCGCCTTCGTGGGGAC
GGAGCAGTAGCGGCGGTTCAAGC	GTAGTCCCGGCGGAAGCACCCCTG
Glu_{28}→Asp	**Tyr_{38}→Val**
V G T P D_{28}V N Q T	L Y Q R V_{38}E I K M
GTGGGGACACCAGACGTCAACCAGACC	TTATACCAGCGTGTGGAGATCAAGATG
CACCCCTGTGGTCTGCAGTTGGTCTGG	AATATGGTCGCACACCTCTAGTTCTAC
Lys_{41}→Ala	**His_{74}→Ala/His_{77}→Ala**
R Y E I A_{41}M T K	C G Y F A_{74}R S A_{77}N R
CGTTATGAGATTGCCATGACCAAG	GCGGATACTTTGCTAGGTCTGCCAACCGC
GCAATACTCTAACGGTACTGGTTC	CGCCTATGAAACGATCCAGACGGTTGGCG
Glu_{81}→Ala/Glu_{82}→Ala	**His_{95}→Ala**
S H N R S $A_{81}A_{82}$F L I A G	D G L L A_{95}I T T C S
CCCACAACCGCAGCGCCGCCTTTCTCATTGCTGG	GGATGGACTCTTGGCCATCACTACCTGCAG
GGGTGTTGGCGTCGCGGCGGAAAGAGTAACGACC	CCTACCTGAGAACCGGTAGTGATGGACGTC
His_{95}→Gln	**Trp_{105}→Ala**
D G L L Q_{95}I T T C S	V A P A_{105}N S L
GGATGGACTCTTGCAGATCACTACCTGCAG	CGTGGCTCCAGCCAACAGCCTG
CCTACCTGAGAACGTCTAGTGATGGACGTC	GCACCGAGGTCGGTTGTCGGAC

[a] The pairs of complementary mutagenic oligonucleotides used to generate the mutations described in the text. In all cases except the deletion (shown by the prefix Δ), the modified codon is shown underlined. For example, His_7 → Ala denotes the two oligonucleotides that were used to change the histidine residue at position 7 to an alanine. The amino acids encoded by this region of DNA are shown in the single-letter code. Numbering is taken from Docherty et al. (1985).

before 10-fold dilution into the substrate solution. The reaction was followed at 37 °C until a steady-state rate (v_s) could be measured (usually 1–1.5 h) and the apparent K_i (K_i') obtained using the Enzfitter program (Leatherbarrow, 1987) and the equation

$$v_s = (v_0/2E_t)([(K_i' + I_t - E_t)^2 + 4K_i'E_t]^{1/2} - (K_i' + I_t - E_t))$$

(Morrison & Walsh, 1988), where v_0 is the rate in the absence of inhibitor, E_t is the total enzyme concentration, and I_t is the total inhibitor concentration.

The rates of formation and dissociation of the PUMP–TIMP complexes were studied under similar conditions. The rate of inhibition of PUMP was determined using a TIMP concentration of 5 nM, and the reaction was started by addition of 0.1 nM enzyme. To obtain dissociation rates, complexes were initially formed by incubating enzyme and inhibitor at an equimolar concentration of 50 nM. They were then diluted to 0.1 nM in an assay buffer containing 1.6 μM substrate, and recovery of enzyme activity was followed for 4 h. All curves were analyzed using the Enzfitter program and the equation $P = v_s t + (v_0 - v_s)(1 - e^{-kt})/k$ (Morrison & Walsh, 1988), where P is the product concentration at time t, to obtain the apparent first-order rate constant, k. Values for $t_{1/2}$ for the inhibition were calculated using the relationship $t_{1/2} = 0.693/k$.

RESULTS

Preparation of Mutant TIMPs. Mutagenesis of single conserved residues of TIMP-1 was effected by an oligonucleotide-directed polymerase chain reaction method. In most cases residues were mutated to Ala, but more conservative modifications were made in a few cases (Table I). In the case of Glu 81 and Glu 82 both residues were modified together to Ala (TIMP-2 has only one Glu at the corresponding position) and His 74 and His 77 were modified together to Ala, since these are not conserved residues. We attempted to express one mutant deleting the totally conserved motif Val18-IleArgAlaLys22, but this resulted in yields of TIMP-1 that were too low for further analysis. Individual mutant TIMPs were purified from NSO myeloma cell culture medium by binding to a monoclonal anti-TIMP IgG Sepharose column (Murphy et al., 1991a). Mutant inhibitors were screened for their ability to inhibit collagenase in a diffuse fibril assay. Of all the mutants listed in Table I many showed a similar specific activity to wild-type TIMP-1 (95–105%), with the exception of His7Ala (15%), His7Gln (22%), His7Glu (41%), and

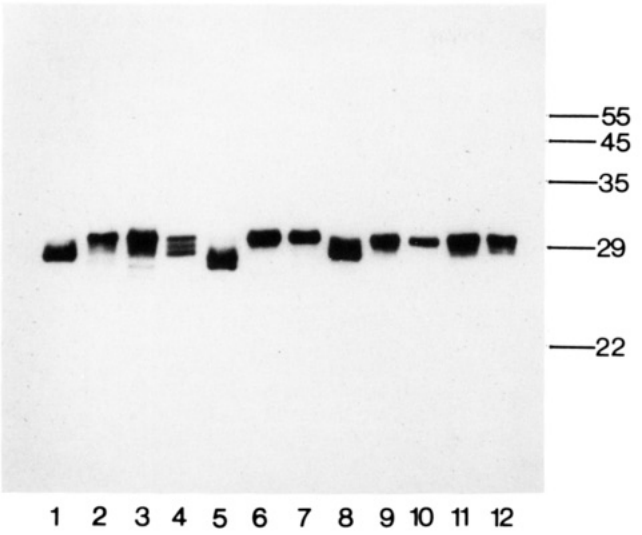

FIGURE 1: Polyacrylamide gel electrophoresis of purified mutant forms of TIMP-1. The different forms of TIMP-1 were purified using a monoclonal antibody to wild-type TIMP-1 linked to Sepharose, are described under Methods. Approximately 500 ng of each inhibitor was electrophoresed on an 11% polyacrylamide gel under reducing conditions, and the gel was silver stained: wild-type TIMP-1 (lane 1), His7Ala (lane 2), Gln9Ala (lane 3), Arg20Ala (lane 4), Lys22Ala (lane 5), Glu28Asp (lane 6), Tyr38Val (lane 7), Lys41Ala (lane 8), His71Ala/His77Ala (lane 9), Glu81Ala/Glu82Ala (lane 10), His95Gln (lane 11), and Trp105Ala (lane 12). The mobilities of standard proteins (in kDa) are indicated to the right of the gel.

Gln9Ala (19%). Analysis of the purified mutants by SDS–polyacrylamide gel electrophoresis showed that they migrated with mobilities that differed by 1–2 kDa (Figure 1). This was thought to be due to varying glycosylation patterns, since changes of charge and hydrophobicity may modify the rate of Golgi trafficking and posttranslational modification. We have also observed considerable heterogeneity in the glycosylation of wild-type TIMP using this expression system, but this has not been further investigated.

Conformational Stability Studies. Intrinsic fluorescence characteristics of the mutant TIMP species were studied in order to analyze their conformations and stabilities to denaturation; this technique offered the sensitivity to provide significant information from the limited quantities of mutant proteins available. The mutant proteins selected for detailed study were His7Ala and Gln9Ala, which showed significantly different functional properties from those of the wild type (see below), and a selection of other mutations in which the sites of substitution are distributed through the N-terminal domain of the inhibitor (Lys41Ala, Glu81Ala/Glu82Ala, His95Ala, and Trp105Ala). In the absence of a three-dimensional structure of the inhibitor, it was not possible to predict which mutations might have significant effects on folding and stability; the fluorescence studies were undertaken to test for the possibility of such major changes.

Figure 2 shows the intrinsic fluorescence spectrum for wild-type TIMP-1 in 0 and 6 M guanidine hydrochloride (GdnHCl). The λ_{max} of the peak is red shifted from 335 to 349 nm, which is consistent with denaturation of the protein and the subsequent exposure of buried tryptophan residues to a more aqueous environment. The fluorescence intensity of the folded molecule is less than that of the denatured molecule, suggesting that the three tryptophan residues are highly quenched in the native structure. The progression of denaturation over a GdnHCl titration is usually followed by measuring fluorescent intensity at the point of maximal difference between the folded and denatured spectra. For

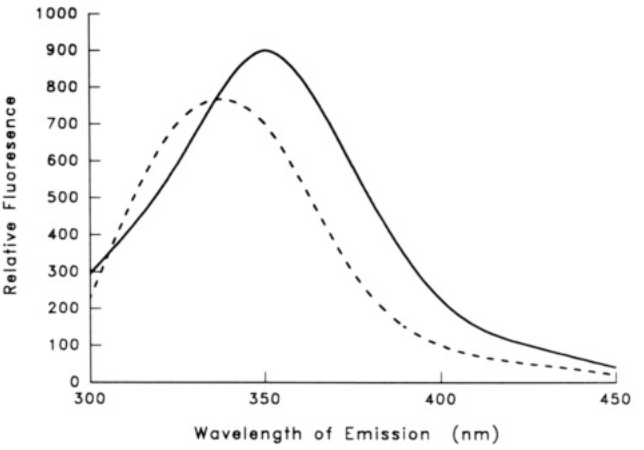

FIGURE 2: Fluorescence emission spectra of TIMP-1. Fluorescence emission spectra of wild-type TIMP-1 in 0 M (- - -) and 6 M (—) GdnHCl (λ_{ex} = 280 nm). An equal quantity of TIMP was used for each measurement (10 μg/mL).

TIMP-1 this difference is comparatively small due to the quenched nature of the folded species; consequently, we decided to monitor denaturation by the shift in wavelength at maximal emission (λ_{max}; Lindsay & Pain, 1990).

GdnHCl titration curves for wild-type TIMP-1 and the selected mutants are shown in Figure 3a,b. In each case the curve displays three distinct zones; a linear pre- and post-transition region separated by a region of rapid λ_{max} change, the unfolding transition. In all cases the unfolding event appears to be highly cooperative and occurs between 2.0 and 5.0 M GdnHCl, and the λ_{max} of the unfolded protein is always close to 350 nm. The mutant proteins show limited difference from the wild type in the λ_{max} of the native state, the behavior in the pretransition region, and the midpoint of denaturation. The greatest variation between the mutants is found in the slopes of the pretransition region. The shapes of some of the denaturation curves are very similar, most notably Lys41Ala and the wild type. Each GdnHCl titration curve was analyzed to obtain values for the midpoint of denaturation and the extrapolated conformational stability of the protein in the absence of denaturant (Table II). An example of the analysis for wild-type TIMP is shown in Figure 3c. The midpoint of denaturation ranged from 3.06 M GdnHCl for Trp105Ala to 3.88 M GdnHCl for Gln9Ala, while values for $\Delta G°_{H_2O}$ varied from 3.7 to 6.6 kcal/mol. The mutants with the lowest midpoint of denaturation also had the lowest values for $\Delta G°_{H_2O}$ and vice versa. Two of the mutants (His7Ala and Gln9Ala) appear to have similar stability to or slightly greater stability than the wild-type molecule, while the other mutants (Lys41Ala, Glu81Ala/Glu82Ala, His95Gln, and Trp105Ala) are all slightly less stable.

$\Delta(\Delta G°)$ values were calculated directly from the data in the transition region (Table II). By this analysis mutants His7Ala, Gln9Ala, and Lys41Ala were found not to differ significantly in conformational stability from the wild-type, while mutants Glu81Ala/Glu82Ala, His95Gln, and Trp105Ala were slightly less stable. This analysis gave the same trend in stabilities as was seen for the $\Delta G°_{H_2O}$ results calculated by extrapolation for each mutant separately. Due to the absence of good structural information on the wild type, it is not possible to interpret the magnitude of the small changes in stability.

Kinetic Studies. To determine the concentration of active TIMP, preparations of mutant inhibitors were initially subjected to active site titration against stromelysin in the quenched fluorescent substrate assay under conditions of [E] $\gg K_i$. Active stromelysin concentration was determined by

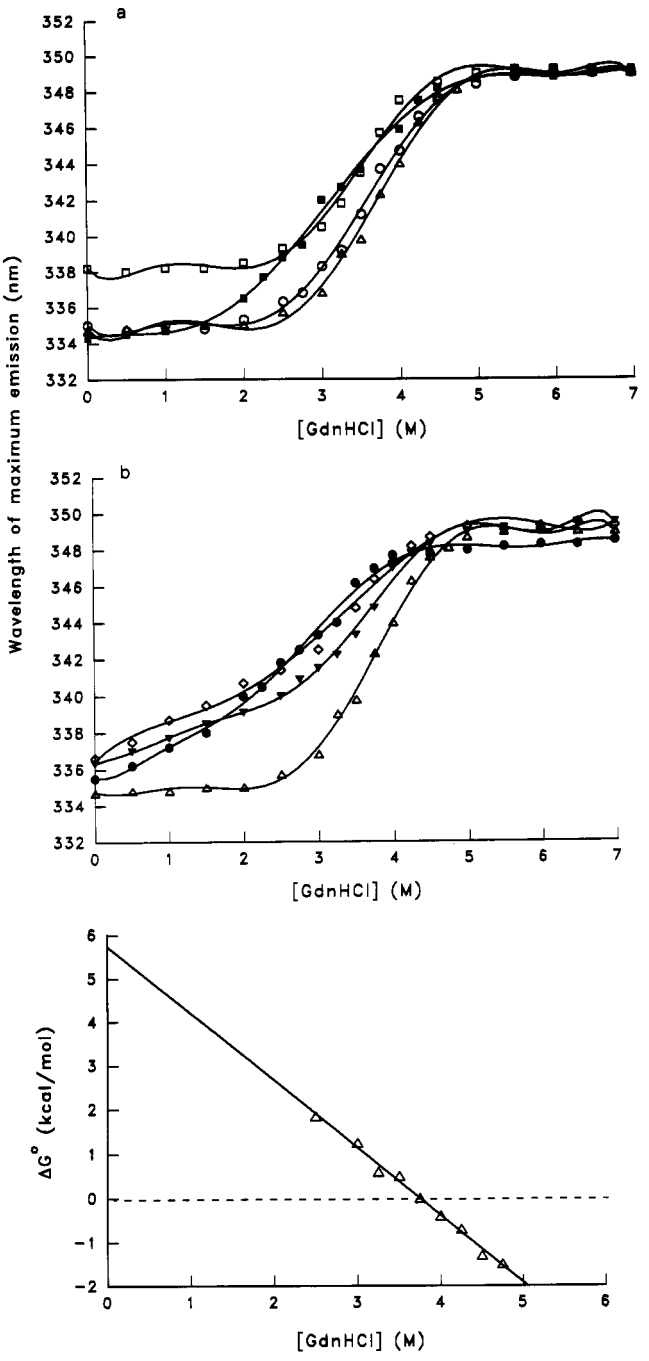

FIGURE 3: Denaturation analysis of wild-type and mutant TIMP-1s. (a, b) Effect of GdnHCl concentration on λ_{max} of fluorescence emission (λ_{ex} = 280 nm). Symbols: △, wild-type; ○, Lys41Ala; ■, His95Gln; □, Glu81Ala/Glu82Ala; ◇, Gln9Ala; ▼, His7Ala; ●, Trp105Ala. (c) $\Delta G°$ as a function of GdnHCl concentration for wild-type TIMP-1. Values for the midpoint of denaturation were taken from the x-intercept at $\Delta G° = 0$; estimates for $\Delta G°_{H_2O}$ were obtained by linear extrapolation of the line to [GdnHCl] = 0. These values and m, the gradient of the line, are given in Table II for the wild-type and mutant TIMP-1s.

titration against wild-type TIMP-1, which was considered to be fully active. In agreement with the initial collagenase inhibitory assays, the His7 mutants and Gln9Ala were found to contain a lower number of active molecules than wild-type TIMP-1 (Table III). To allow more accurate analyses to be made, kinetic studies on the mutant forms of TIMP were carried out using the quenched fluorescent peptide (Table IV) and the short metalloproteinase PUMP. Each mutant that was produced inhibited PUMP, but in some cases the affinity was modified by the mutation. The most notable

Table II: Thermodynamic Analysis of the GdnHCl Denaturation Curves for Wild-Type and Mutant TIMP-1s

form of TIMP	midpoint (M GdnHCl)	$\Delta G°_{H_2O}$ (kcal/mol)	m^a (kcal/mol vs M)	$\Delta(\Delta G°)^b$
wild type	3.75	5.7 ± 0.13	−1.53	
His7Ala	3.77	6.4 ± 0.32	−1.71	0.03
Gln9Ala	3.88	6.6 ± 0.09	−1.70	0.19
Lys41Ala	3.58	4.8 ± 0.09	−1.34	−0.25
Glu81Ala/Glu82Ala	3.43	5.4 ± 0.31	−1.57	−0.47
His95Gln	3.08	3.7 ± 0.12	−1.19	−0.98
Trp105Ala	3.06	3.8 ± 0.07	−1.25	−1.01

[a] m – (gradient of slope for ΔG vs [GdnHCl] plot) (average = 1.47). [b] $\Delta(\Delta G°)$ values were calculated by multiplying the average of m with the difference in the midpoint between the mutant and wild-type TIMP-1.

Table III: Comparison of TIMP-1 Mutant Protein Concentrations and Active Molecule Concentrations[a]

	μM	
form of TIMP	A_{280}	active site titration
wild type	10.5	10.1
His7Ala	6.1	2.96
	6.3	2.53
	7.5	2.27
His7Gln	5.3	2.11
His7Glu	5.6	2.76
Gln9Ala	4.2	0.81
Arg20Ala	12.9	13.4
Lys22Ala	8.2	9.9
Glu28Asp	11.2	11.3
Tyr38Val	3.3	3.8
Lys41Ala	11.0	11.3
Glu81Ala/Glu82Ala	6.7	5.9
His95Gln	4.7	5.0
Trp105Ala	4.6	4.19

[a] TIMP concentrations were calculated from an $A^{1\%}_{280,1cm}$ of 10.0 and an M_r of 28 500, except for Trp105Ala, which was assigned a value of 6.7 (two out of three Trp residues present), and by active site titration with stromelysin as described under Methods.

Table IV: Kinetic Analysis of TIMP-1 Mutants[a]

		$t_{1/2}$ (min)	
form of TIMP	app K_i (nM)	association	dissociation
wild type	0.37	7.9	40.2
His7Ala	2.25	5.5	15.6
His7Gln	1.00	nd	nd
His7Glu	0.70	nd	nd
Gln9Ala	1.20	5.6	23.0
Arg20Ala	0.58	9.1	44.2
Lys22Ala	0.38	8.8	39.0
Glu28Asp	0.39	8.0	50.2
Tyr38Val	0.45	9.6	35.3
Lys41Ala	0.38	7.6	34.8
Glu81Ala/Glu82Ala	0.37	9.3	43.9
His95Gln	0.53	7.2	31.7
Trp105Ala	0.65	9.9	15.2

[a] The ability of active site-titrated wild-type TIMP and mutants to inhibit PUMP was analyzed using McaPLGLDpaAR as described under Methods. nd = not determined.

increases in the apparent K_i for the inhibition of PUMP were observed for His 7 mutants and Gln9Ala. Smaller changes were also detected when mutations involved residues Arg 20, Tyr 38, His 95, and Trp 105. When the rate of formation and dissociation of the enzyme–inhibitor complexes are compared, it can be seen that the differences in apparent K_i are due mainly to an increase in the rate of dissociation of the complexes (Table IV). Of the three His 7 mutants, modification to

alanine increased the apparent K_i the most markedly. Glutamine or glutamate substitutions were less effective.

DISCUSSION

The effect of single-site mutations within the N-terminal domain of TIMP-1 has been assessed in terms of conformational stability and the kinetics of interaction with representative matrix metalloproteinases. The mutant inhibitors were expressed in a mammalian cell system to allow correct folding of this complex disulfide-bonded protein to occur. No attempt was made to interfere with the disulfide bonds, nor with proline residues which may also be essential for the correct conformation of the inhibitor. Most mutants were highly expressed, possibly due to the growth factor properties of TIMP (Gasson et al., 1985; Hayakawa et al., 1992; G. Murphy and M. Cockett, unpublished observations). Poor protein yields (2–5% of wild type) were obtained in the case of His7Ala, His7Gln, Gln9Ala, and Glu81Ala/Glu82Ala.

Analyses of the conformational stability of the mutant inhibitors relative to wild-type TIMP-1 were carried out by monitoring the effect of GdnHCl on the λ_{max} of intrinsic fluorescence of each protein. The results suggested that all the mutants analyzed possessed significant secondary and tertiary structure and displayed a cooperative unfolding transition. They are, therefore, folded into a stable conformation similar to that of the native molecule. None of the mutants exist as a disordered misfolded structure. Indeed, it is unlikely that such molecules would be secreted by the cells but would be degraded intracellularly immediately after synthesis. This may, in part, explain the low level of expression of some mutants. We found that mutants with deletions of several residues from the molecule such as ΔVal 18–Lys 22 were generally hardly secreted (M. O'Shea and G. Murphy, unpublished results), the exception being Δ 127–184 TIMP described previously (Murphy et al., 1991a).

The inhibitory activity of the mutant TIMPs was compared to that of the wild type. The collagenase-based assay initially confirmed that each mutant was active. To perform a more detailed study of the mutants, it was necessary to know the concentration of active TIMP in each preparation, and this was determined by titration against stromelysin. Stromelysin was used as it is very stable and can readily be converted to a fully activated form. Titrations were performed at an enzyme concentration that was 100-fold higher than the reported K_i (<0.2 nM; Murphy et al., 1992b) for the interaction of wild-type TIMP-1 with stromelysin, thus ensuring that all the inhibitor is involved in complex formation. A greatly reduced affinity of a mutant for stromelysin, which would be detected by nonlinear titration of enzyme activity, was not observed for any of the mutants. Limitations in the assay sensitivity and the high affinity of TIMP for stromelysin, collagenase, and gelatinase restricted accurate kinetic analyses to the inhibition of PUMP. PUMP differs from the other MMPs in that it lacks a C-terminal domain, and this study therefore examines the interaction of active site-titrated forms of TIMP-1 with the catalytic domain alone. Justification for the use of PUMP as a representative of the MMP family in this study is as follows: (i) the fact that TIMP has a lower affinity for and binds more slowly to PUMP than the full-length enzymes is consistent with previous observations on the inhibition of genetically constructed catalytic domains of collagenase and gelatinase (Murphy et al., 1992a,b) and therefore appears to be a property of the matrix metalloproteinase catalytic domain rather than being unique to PUMP; (ii) it has previously been demonstrated that the N-terminal three loops of TIMP-1 inhibit PUMP as well as full-length MMPs, indicating that this region within which all the mutations were performed interacts predominantly with the catalytic domain of the enzymes; and (iii) any marked decrease in affinity of the mutants toward full-length enzymes would have been observed in the collagenase assays or by an inability to titrate inhibitory activity with stromelysin. Significant increases in apparent K_i values were observed for His7 mutants and Gln9Ala and Trp105Ala. Problems were encountered in studying the dissociation rates of the TIMP–PUMP complexes as association was not sufficiently tight to allow isolation of the complexes from free enzyme or inhibitor. However, the values obtained are consistent with the apparent K_i values and the half-times for the association of the complexes in indicating that the differences observed in the His7Ala, Gln9Ala, and Trp105Ala mutants lie in the increased rate of dissociation of the complexes.

These data are in contrast with data generated by studies of TIMP-1 inactivation by chemical modification with diethyl pyrocarbonate, which implicated His 95 as a potentially important residue for TIMP-1 activity (Williamson et al., 1992). In the present mutagenesis studies, conversion of His 95 to Gln (or Ala, data not shown) had no effect on the kinetics of TIMP inhibition of collagenase or PUMP. Thus it is clear that His 95 is not required as a functional group for TIMP activity. The chemical modification studies found no evidence for modification by diethyl pyrocarbonate at His 7, the mutagenesis of which has clear-cut effects on activity in the present study. This apparent inconsistency is being explored by further chemical modification studies on mutant TIMPs to clarify the mode of inactivation by diethyl pyrocarbonate. Initial results suggest that the effect of diethyl pyrocarbonate on TIMP-1 activity may not be a direct result of histidine modification.

The changes observed in TIMP activity as a result of a single amino acid change are small, and each mutant is still an efficient metalloproteinase inhibitor. We consider that the mutations of the highly conserved residues that we chose would have detected any single amino acid that is essential for the interaction of TIMP with the enzyme. Therefore, it is unlikely that TIMP resembles the serine protinase inhibitors which bind tightly at the catalytic site and are often cleaved in a manner similar to that of the substrate, allowing definition of a major active site residue in the inhibitor. We have previously shown that TIMP–MMP interactions are fully reversible, with no alteration in TIMP structure (Murphy et al., 1989). Thus, TIMP appears to belong to the class of inhibitors which includes the cysteine proteinase inhibitors and hirudin (Bode & Huber, 1992). Such inhibitors bind mainly to specific surface patches on the enzyme, forming a large number of interactions and preventing substrate binding through steric hindrance.

It was interesting to note that substitution of His 7 with Ala, a smaller residue of increased hydrophobicity, had the greatest effect on TIMP affinity for PUMP. Gln, of similar size and hydrophobicity but of reduced polarity, was more efficient, and Glu, which has a similar size and degree of polarity, was the most efficient substitute for His 7. From the results presented here, we propose that the proline-rich, structurally restricted region of TIMP between Cys 3 and Cys 13, and containing residues His7 and Gln9, plays an important role in the inhibitory mechanism of TIMP. Confirmation of such a role awaits the results of more detailed kinetic studies as well as an X-ray crystallographic analysis of enzyme–inhibitor complexes.

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