The Activity of the Tissue Inhibitors of Metalloproteinases Is Regulated by C-Terminal Domain Interactions: A Kinetic Analysis of the Inhibition of Gelatinase A[†]

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ABSTRACT: The cloning and expression of the full-length tissue inhibitor of metalloproteinase 2 (TIMP-2), $\Delta_{187-194}$TIMP-2, and $\Delta_{128-194}$TIMP-2 and the purification of these inhibitors and a cleaved version of TIMP-2 lacking nine C-terminal amino acids ($\Delta_{186-194}$TIMP-2) are described. The mechanism of inhibition of gelatinase A by the TIMPs was investigated by comparing the kinetics of association of TIMP-1, TIMP-2, the C-terminal deletions, and the mutants of both TIMPs which consisted of the N-terminal domain only. The full-length TIMPs inhibited gelatinase A rapidly with association constants of 3.2×10^6 M^{-1} s^{-1} for TIMP-1 and 2.1×10^7 M^{-1} s^{-1} for TIMP-2 at $I = 0.2$. The C-terminal peptide of TIMP-2 is proposed to exist as an exposed "tail" responsible for binding to progelatinase A and for increasing the rate of inhibition of active gelatinase A through electrostatic interactions with the C-terminal domain of the enzyme. The C-terminal domains of both TIMP-1 and TIMP-2 participate in low-affinity interactions with the C-terminal domain of gelatinase A which increase the rate of association by a factor of about 100 in both cases.

The matrix metalloproteinases constitute a group of zinc-dependent enzymes which includes the collagenases, gelatinases, and stromelysins. Between them, these enzymes are capable of degrading all of the proteins of the extracellular matrix and have been implicated in numerous physiological and pathological processes involving tissue turnover. Gelatinase A (EC 3.4.24.24, 72-kDa gelatinase, type IV collagenase) has been shown to be expressed by a number of tumor cell lines (Stetler-Stevenson, 1990; Liotta et al., 1991) as well as by the stromal cells surrounding tumors (Pyke et al., 1992; Poulsom et al., 1992) and consequently has been identified as a target for inhibitor therapy in a number of cancers (Docherty et al., 1992).

Gelatinase A is typical of the matrix metalloproteinase family in that it is secreted as a proenzyme, which is activated by proteolytic processing to remove an 80 amino acid propeptide from the N-terminus. The N-terminal domain contains the active site of the enzyme and a gelatin binding region that is found only in the gelatinases. The C-terminal domain is not required for catalysis but does play a role during the cell membrane-mediated activation of the proenzyme (Murphy et al., 1992b).

Matrix metalloproteinase activity is controlled at several levels (Docherty et al., 1992) including proenzyme activation and regulation by the general proteinase inhibitor α_2-macroglobulin and by the specific metalloproteinase inhibitors

TIMP-1[1] and TIMP-2. TIMP-1 is a glycoprotein of molecular mass 30 kDa whereas TIMP-2 is unglycosylated with a molecular mass of 21 kDa, and the two proteins possess approximately 40% amino acid sequence identity. The activated matrix metalloproteinases are inhibited by both TIMPs with a 1:1 stoichiometry. In addition, TIMP-2 can form a complex with the proform of gelatinase A, and TIMP-1 can bind to progelatinase B. It has been reported that the progelatinase A–TIMP-2 complex can autoactivate in the presence of organomercurials to produce enzyme that has low activity toward gelatin and can be inhibited by the further addition of free TIMP-2 (Goldberg et al., 1989; Stetler-Stevenson et al., 1989), indicating the presence of two distinct TIMP-2 binding sites on gelatinase A. The progelatinase A–TIMP-2 complex has also been shown to be an inhibitor of collagenase activity which has led to the conclusion that there are two distinct metalloproteinase binding sites on TIMP-2 (Kolkenbrock et al., 1991). The use of autoproteolytically generated fragments of gelatinase A has provided evidence that there is a TIMP-2 binding site on both the N- and C-terminal domains of gelatinase A (Howard et al., 1991). We have confirmed these observations by using a genetically engineered short form of gelatinase A consisting of the N-terminal domain only ($\Delta_{418-631}$GL), and demonstrated that TIMP-2 does not bind to the proform of the truncated enzyme. It was also shown that for both TIMPs the rate of interaction with and the affinity for gelatinase A were decreased by the removal of the enzyme C-terminal domain. This indicates the existence of an additional TIMP binding site on the C-terminal domain of active gelatinase A that is distinct from

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[1] Abbreviations: TIMP, tissue inhibitor of metalloproteinases; Mca-PLGLDpaAR, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH$_2$; APMA, (4-aminophenyl)mercuric acetate; Δ_{x-y} before a protein denotes the region that has been removed from its full-length counterpart.

the TIMP-2-specific binding site on the proenzyme (Murphy et al., 1992b).

The aim of the present work was to identify the key features of the enzyme and inhibitor domain structures involved in TIMP binding. A comparison of the sequences of the two TIMPs (Docherty et al., 1985; Boone et al., 1990) reveals the existence of a highly charged sequence (QEFLDIEDP) at the C-terminus of TIMP-2 that is not present in TIMP-1. By analogy with other proteinase–inhibitor interactions [e.g., thrombin–hirudin (Stone et al., 1989) and trypsin–bovine pancreatic trypsin inhibitor (Matthew et al., 1985)], it seemed possible that this sequence might be involved in an initial docking interaction between TIMP-2 and the enzyme which serves to align the two proteins correctly and thus reduce nonproductive complex formation. To test this hypothesis, modified forms of TIMP-2, which lack the charged C-terminal sequence, were prepared by using limited proteolysis and genetic engineering. Since we have previously demonstrated that the N-terminal domain of TIMP-1 alone is capable of inhibiting matrix metalloproteinases (Murphy et al., 1991), we also included in this study the N-terminal domains of both TIMPs ($\Delta_{127-184}$TIMP-1 and $\Delta_{128-194}$TIMP-2) in order to define more precisely the interactions of the C-terminal domains of enzyme and inhibitor. The work concentrates on a kinetic analysis of factors affecting the rate of association of the inhibitors with gelatinase A and demonstrates that the C-terminal domains of both proteins play an important role in controlling the rate of reaction with active enzyme. C-Terminal domain interactions are also responsible for the binding of TIMP-2 to progelatinase A. On the basis of these and previous results, we propose a model for the binding sites involved in the inhibition of gelatinase A.

EXPERIMENTAL PROCEDURES

All chemicals were of the highest purity available commercially. The substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH$_2$ (McaPLGLDpaAR) was a gift from Dr. C. G. Knight (Knight et al., 1992).

Preparation of TIMPs. TIMP-1 was expressed from NSO myeloma cells and purified (Murphy et al., 1991). As described previously (Murphy et al., 1991), TIMP-1 was fully glycosylated by this expression system in a manner typical for a complex glycoprotein, including heterogeneous substitution (Murphy & Werb, 1985). The precise nature of the carbohydrate substitution of natural or wild-type TIMP-1 varies with cell type. However, this was found to have no effect on the inhibitory activity of TIMP-1 (data not shown). It has previously been shown that the complete absence of carbohydrate does not modify TIMP activity (Stricklin, 1986).

Human TIMP-2 cDNA-containing plaques were identified in a λgt10 mixed-tumor library kindly provided by Dr. R. Breathnach, Université de Nantes, France (Muller et al., 1988), by using a synthetic oligonucleotide probe based on the amino acid sequence reported by Stetler-Stevenson et al. (1989). As shown in Figure 1a, the 47-nucleotide-long probe contained a mixture of nucleotides at 2 positions, and the remainder of the sequence was based on preferred codon usage in humans (Grantham et al., 1981). The probe encodes amino acids 30–45 of TIMP-2 which differ significantly from the corresponding residues in TIMP-1 (Stetler-Stevenson et al., 1989). A TIMP-2 encoding cDNA was isolated from one of the plaques and subcloned into pSP65 as an *Eco*RI fragment (Melton et al., 1984). As shown schematically in Figure 1b, this DNA was used to generate an 800 bp *Eco*RI/*Stu*I
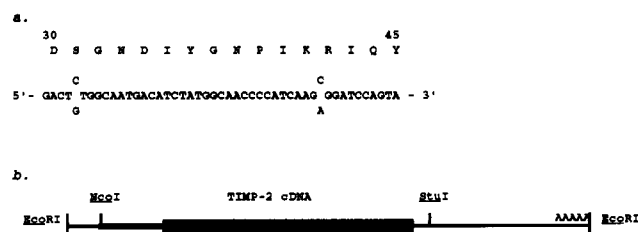

FIGURE 1: Cloning and expression of TIMP-2. (a) Oligonucleotide probe (mixed at two positions) that was used to screen the human tumor library. The amino acid numbering is taken from Stetler-Stevenson et al. (1989). (b) Schematic representation of the TIMP-2 cDNA; the coding region is shown in boldface.

fragment of the cDNA lacking the 3' untranslated region that was inserted between the *Eco*RI and *Eco*RV sites of the pSP73 vector polylinker (Promega Corp., Madison, WI). The sequence of the TIMP-2 cDNA was obtained from this construct by use of a series of overlapping oligonucleotides (Sanger et al., 1977). This revealed that the coding sequence was identical with that reported by Boone et al. (1990), except for an adenine residue substituting for a guanine reported at position 393. This gives rise to a threonine at position 21 instead of an alanine. Ala$_{21}$ is part of a well-conserved motif (VIRAK), and in six species of TIMP-1 and four species of TIMP-2, an alanine is reported at this position (Docherty et al., 1985; Horowitz et al., 1990; Freundenstein et al., 1990; Stetler-Stevenson et al., 1990; Boone et al., 1990; Pavloff et al., 1992, and unpublished results). Whether the threonine noted here is the result of a cDNA cloning artifact or an allelic variant is not clear. Preliminary kinetic analyses have revealed no differences between natural TIMP-2 and TIMP-2 expressed from the cDNA sequence. Using a 3' *Bgl*II site that is downstream from the *Eco*RV in pSP73 and a *Hin*dIII–*Nco*I adapter (5'-AGCTTTGCCAC-3', 5'-CATGGTG-GCAA-3') to place a Kozak consensus sequence (Kozak, 1987) adjacent to the ATG initiating codon, the entire coding sequence was subcloned into the mammalian expression vector pEE12 (Bebbington et al., 1992). Transfection of NSO murine myeloma cells with the resulting DNA and scaled-up culturing in serum-free medium of TIMP-2-producing cells were as previously described for TIMP-1 (Murphy et al., 1991).

Preparation of Truncated TIMPs. $\Delta_{127-184}$TIMP-1 cDNA was prepared as previously described (Murphy et al., 1991). As shown previously (Murphy et al., 1991), this mutant was largely glycosylated at both available sites in a heterogeneous manner comparable to the wild-type TIMP-1. This form was separated from the forms with single-site substitution and no carbohydrate substitution by gel filtration (Murphy et al., 1991). The preparation of cDNAs encoding $\Delta_{187-194}$TIMP-2 and $\Delta_{128-194}$TIMP-2 was achieved essentially as described by Murphy et al. (1991) by oligonucleotide-directed mutagenesis of the pEE12 TIMP-2 cDNA template and polymerase chain reaction (Mullis & Faloona, 1987). For $\Delta_{187-194}$TIMP-2, E$_{187}$ was converted to a stop codon followed by an *Eco*RI site; for $\Delta_{128-194}$TIMP-2, C$_{128}$ was converted to a stop codon followed by an *Eco*RI site. The sequences of the products were confirmed by the dideoxy chain termination method (Sanger et al., 1977). Transformation of NSO cells with the plasmid and expression were as described above and by Murphy et al. (1991).

Purification of TIMP-2 and $\Delta_{186-194}$TIMP-2. Medium containing the secreted inhibitor was concentrated 4-fold using an RA2000 with an S1Y10 spiral ultrafiltration cartridge (Amicon, Boston, MA) and then diluted with distilled water to a final conductivity of 2 μS. The desalted medium was

applied at a flow rate of 4 L/h to an S-Sepharose Fast Flow column (20 cm² × 9 cm) previously equilibrated with buffer A (25 mM Tris-HCl, pH 7.5). Unbound proteins were washed from the column with buffer A before the elution of bound proteins, which included TIMP-2, using buffer A containing 0.3 M NaCl. This partially purified TIMP-2 sample was desalted into buffer A using Sephadex G-25 gel filtration and then either stored at 4 °C or further purified by elution from a second S-Sepharose Fast Flow column (2 cm² × 9 cm) using a linear gradient of 0–0.3 M NaCl in buffer A run at a flow rate of 1 mL/min. Fractions containing purified TIMP-2 eluted at 0.15 M NaCl and were pooled, and aliquots were stored at −70 °C at a concentration of 26 μM (ϵ_{280} = 39 570 M⁻¹ cm⁻¹; DeClerck et al., 1991). Partially purified TIMP-2 stored at 4 °C for 3 months became degraded such that it ran as a doublet of lower molecular mass on SDS–polyacrylamide gels. The two species were separated by using the second S-Sepharose Fast Flow step described above except that the lower band of the doublet eluted at 0.16 M NaCl whereas the upper band eluted at 0.22 M NaCl. The purified upper band was further characterized by N-terminal amino acid sequencing, which confirmed that it possessed the same N-terminus as native human TIMP-2. Its molecular mass was determined alongside that of the purified recombinant TIMP-2 by matrix-assisted laser desorption mass spectroscopy (see below).

Matrix-Assisted Laser Desorption Mass Spectrometry (MA-LDMS) of TIMP-2 and $\Delta_{186-194}$TIMP-2. The mass analyzer used was a Lasermat (Finnigan MAT Ltd., Hemel Hempstead, U.K.). Light from a pulsed nitrogen laser (337 nm) was focused onto a sample target using a single fixed-silica lens of 50-mm focal length. Crossed polarizing filters were used to adjust the power density to around 10⁶ W cm⁻². Each pulse of desorbed ions was accelerated to 20-keV energy and directed by a cylindrical extraction lens along the axis of a 0.5-m drift tube and onto a discrete dynode electron multiplier. Mass analysis was by time-of-flight. Each sample was applied (0.25 μL of a 1 mg/mL solution) to a target slide, with 0.5 μL (10 mg/mL) of sinapinic acid matrix in 70% acetonitrile. For accurate mass determination, 0.5 μL (9 pmol) of apomyoglobin was included as an internal calibrant. TIMP-2 gave an average molecular mass of 21 786 ± 7 (expected value = 21 774; 0.05% error) while that of the upper band was 20 722 ± 14. The most likely identity of the upper band is $\Delta_{186-194}$TIMP-2 (expected molecular mass = 20 687; 0.17% error), its production being as a result of cleavage of the $K_{185}–Q_{186}$ peptide bond of TIMP-2 presumably by a contaminating protease with trypsin-like specificity.

Purification of $\Delta_{187-194}$TIMP-2 and $\Delta_{128-194}$TIMP-2. The two engineered truncated forms of TIMP-2 were purified essentially as described for TIMP-2. $\Delta_{187-194}$TIMP-2 was chromatographed on S-Sepharose at pH 7.5 using the buffer system described and eluted at 0.2 M NaCl. $\Delta_{128-194}$TIMP-2 was chromatographed on S-Sepharose in 50 mM MES, pH 6.0, and was eluted with a gradient of 0.15–0.5 M NaCl.

Preparation of Metalloproteinases. Prostromelysin was expressed from C127 cells and purified according to Murphy et al. (1987) and Koklitis et al. (1991). Gelatinase A was expressed from NS0 myeloma cells and purified (Murphy et al., 1992b). The C-terminal domain of gelatinase A (Δ_1–414) was isolated during the purification of gelatinase A, its separation from the proenzyme and other autolytic breakdown products being achieved by virtue of its inability to bind to gelatin–Sepharose 4B. Confirmation of its identity was obtained by SDS–polyacrylamide gel electrophoresis and N-terminal sequencing. The C-terminal domain of collagenase

(Δ_1–269) was prepared by gel filtration of the breakdown products of organomercurial-activated collagenase stored for approximately 2 months at 4 °C. The N-terminal sequence was identified as IGPQTPKACDSK as described previously (Whitham et al., 1986).

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed according to the method of Laemmli and Favre (1973). Gels were stained for protein using Coomassie Blue R-250 or silver staining. Marker proteins run on all gels (with molecular weight values in parentheses) were phosphorylase *a* (97 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 300).

TIMP-2 Binding Studies. The ability of TIMP-2 and truncated forms to bind to progelatinase A was assessed using gelatin–Sepharose chromatography as described previously (Murphy et al., 1991). Briefly, progelatinase (1.2 μM) and TIMP (0.3 μM) were incubated together at 25 °C for 4 h. Complexes were chromatographed through mini gelatin–Sepharose columns (100 μL) and inhibitor assays carried out both on unbound material and on material remaining bound after extensive washing which was subsequently eluted with 15% DMSO. TIMP activity was assessed using the rabbit collagenase diffuse fibril assay (Murphy et al., 1991).

Kinetic Studies. Unless otherwise stated, assays were performed at 25 °C at *I* = 0.2 in a buffer consisting of 50 mM Tris, pH 7.5, containing 175 mM NaCl, 10 mM CaCl₂, and 0.05% Brij 35. Hydrolysis of the substrate McaPLGLDpaAR (0.5 μM) was followed by the increase in fluorescence (λ_{ex} 328 nm, λ_{em} 393 nm) as previously described (Knight et al., 1992) using a Perkin Elmer LS50 luminescence spectrometer.

TIMP concentration was determined by titration against human recombinant stromelysin, the concentration of which had been determined by amino acid analysis. Each TIMP (5–100 nM) was preincubated with stromelysin (50 nM) for 2 h prior to assay at an enzyme concentration of 5 nM. Initial velocities were plotted against the volume of stock TIMP solution (approximately 50 nM), and TIMP concentration was determined from the intercept on the abscissa. Titrations were performed throughout the course of the study, and TIMP activity was found to be stable when stored at −20 °C. Progelatinase A was activated in the assay buffer by incubation with 2 mM (4-aminophenyl)mercuric acetate (APMA) for 1 h at 25 °C, conditions that ensured that no proenzyme remained in the preparation and that no further breakdown products had been generated. Active gelatinase A concentration was determined by titration against TIMP-1 of known concentration (see above) and was stable for at least 1 week when stored at 4 °C.

Complexes were prepared for analysis of dissociation kinetics by incubation of 200 nM gelatinase A with 400 nM TIMP for 5 min at room temperature. Each preparation was then purified from the excess TIMP on a 0.5-mL column of gelatin–Sepharose equilibrated in the assay buffer. Complexes were eluted with 10% DMSO in assay buffer, and the concentration was determined from values of A_{280}. Complexes were diluted to the required concentration immediately before assay.

Analysis of Kinetic Data. Method 1. Initially, each TIMP was studied under conditions in which the TIMP concentration was at least 10 times greater than the gelatinase A concentration. Inhibitor concentrations used were 0.2–2.0 nM for TIMP-1, $\Delta_{186-194}$TIMP-2, and $\Delta_{187-194}$TIMP-2, 0.1–0.6 nM for TIMP-2, and 5–200 nM for $\Delta_{128-194}$TIMP-2 and $\Delta_{127-184}$TIMP-1. At the higher inhibitor concentrations, the

enzyme concentration was increased to maximize the total fluorescence change observed. Reactions were started by addition of enzyme to solutions of substrate and inhibitor in buffer. Data were collected until a steady-state velocity was attained and the progress curves analyzed by using the Enzfitter program (Leatherbarrow, 1987) and the integrated rate equation:

$$P = v_s t + (v_0 - v_s)(1 - e^{-kt})/k \qquad (1)$$

in which P is the product concentration, v_0 and v_s are the initial and steady-state velocities, respectively, for enzyme catalysis in the presence of inhibitor, and k is the apparent first-order rate constant for the establishment of equilibrium between enzyme–inhibitor complexes (Morrison & Walsh, 1988). The second-order rate constant k_{on} was provided by linear regression of k on the TIMP concentration. The linear dependence of k on the TIMP concentration provides no evidence for a model more complex than inhibition by a simple bimolecular reaction in the concentration range used for each TIMP (see Results).

Method 2. Under many conditions, the rate of inhibition was too rapid to allow the use of method 1. In these cases, it was assumed that the simple bimolecular mechanism deduced under the conditions used for method 1 is a general description of the rate-limiting step in the mechanism of each TIMP, and the following equation was used to analyze the data:

$$P = v_s t + [(v_0 - v_s)(1 - \gamma)/\lambda\gamma] \ln[(1 - \gamma e^{\lambda t})/(1 - \gamma)] \quad (2)$$

in which P, v_0, and v_s are as in method 1. The parameters γ and λ are described by the equations:

$$\gamma = E_t(1 - v_s/v_0)^2/I_t \qquad (3)$$

$$\lambda = [k_{on}K_m/(K_m + S)][(K_i' + E_t + I_t)^2 - 4E_tI_t]^{1/2} \quad (4)$$

in which E_t and I_t are the total enzyme and inhibitor concentrations, respectively, S is the substrate concentration, K_i' is the apparent K_i, and k_{on} is the association rate constant for the interaction (Williams et al., 1979).

The enzyme concentration used was 50 pM, and TIMP concentrations were in the range 100–200 pM. Since $S \ll K_m$ and $K_i' \ll E_t$ and I_t (see Results), eq 4 reduces to

$$\lambda = k_{on}[(E_t + I_t)^2 - 4E_tI_t]^{1/2} \qquad (5)$$

Values obtained for k_{on} using both methods under the same conditions were in good agreement.

RESULTS

Purification of TIMP-2 and Truncated Forms of TIMP-2. In a typical purification, approximately 2.5 mg of recombinant TIMP-2 was purified from 1 L of conditioned medium. Samples taken during the purification of recombinant human TIMP-2 and the two truncated forms were run on SDS–polyacrylamide gels (Figure 2). Like its natural counterpart, recombinant TIMP-2 migrated as a single band at a molecular mass of 24 kDa when reduced and 21 kDa under nonreducing conditions. The engineered truncated forms of TIMP-2 ($\Delta_{187-194}$ and $\Delta_{128-194}$) also migrated as single bands on electrophoresis with a molecular mass of 21 and 14 kDa, respectively (Figure 2).

Association Kinetics. Analyses of the interaction of gelatinase A with TIMP-1, TIMP-2, $\Delta_{186-194}$TIMP-2, $\Delta_{187-194}$TIMP-2, $\Delta_{127-184}$TIMP-1, and $\Delta_{128-194}$TIMP-2 were carried out using a fluorometric assay with a quenched
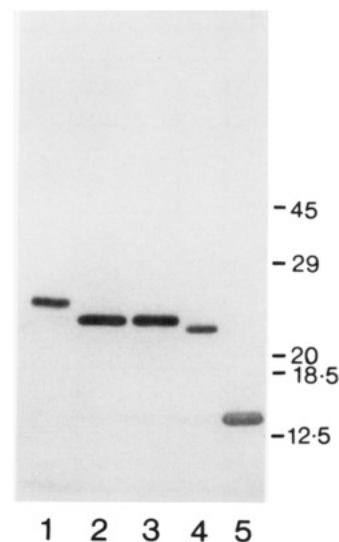

FIGURE 2: SDS–polyacrylamide gel electrophoresis of recombinant human TIMP-2 and truncated forms. Samples of the purified forms of recombinant TIMP-2 used in this study were analyzed on a 13% SDS–polyacrylamide gel under reducing conditions, followed by silver staining: wild-type TIMP-2 (lane 1); $\Delta_{187-194}$TIMP-2 (lane 2); $\Delta_{186-194}$TIMP-2 (cleavage product of wild type; lane 3); smaller cleavage product of TIMP-2 (lane 4); and $\Delta_{128-194}$TIMP-2 (lane 5). The mobilities of standard proteins (kDa) are indicated to the right of the gel.

Scheme I
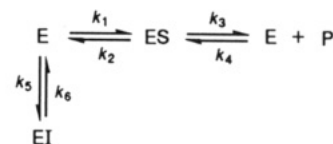
fluorescent substrate. An accurate value of K_i could not be determined for any of the TIMP molecules by using steady-state kinetics as the assay was not sufficiently sensitive to allow reliable measurements to be performed at enzyme concentrations below the K_i value. Attempts to use a substrate concentration greater than the K_m value (>8 μM; Knight et al., 1992) to compete with TIMP and thus raise the apparent K_i value were unsuccessful due to limited substrate solubility and quenching of the fluorescence at substrate concentrations greater than 10 μM. By using low reagent concentrations of 10–50 pM enzyme and 0.1–2 nM inhibitor, however, it was possible to observe the inhibition of gelatinase A by the TIMPs as curvature in the progress curves of substrate hydrolysis. The use of these low concentrations permitted the observable inhibition to be treated as "slow-binding" inhibition and analyzed by using eq 1 to provide the pseudo-first-order rate constant (k) for formation of EI. Linear regression of k on TIMP concentration then provided the value of the second-order rate constant, k_{on}. When the reaction was too fast to be monitored under pseudo-first-order conditions, eq 2–5 were used to calculate k_{on} directly. Under each set of conditions used, it was confirmed by performing equivalent assays in the absence of inhibitor that curvature was due only to inhibition by TIMP and not to enzyme instability or substrate depletion. Because it was necessary to restrict reaction conditions to [S] $\ll K_m$, it was not possible to determine whether TIMP binds only to E (i.e., competitive inhibition; Scheme I) or to both E and ES (mixed inhibition; Scheme II).

When [S] $\ll K_m$, the gradient obtained from a plot of k against [I] gives k_5 in the case of Scheme I and $k_5 + k_7[S]/K_m$ in Scheme II (Cha, 1975). In the present study, the general
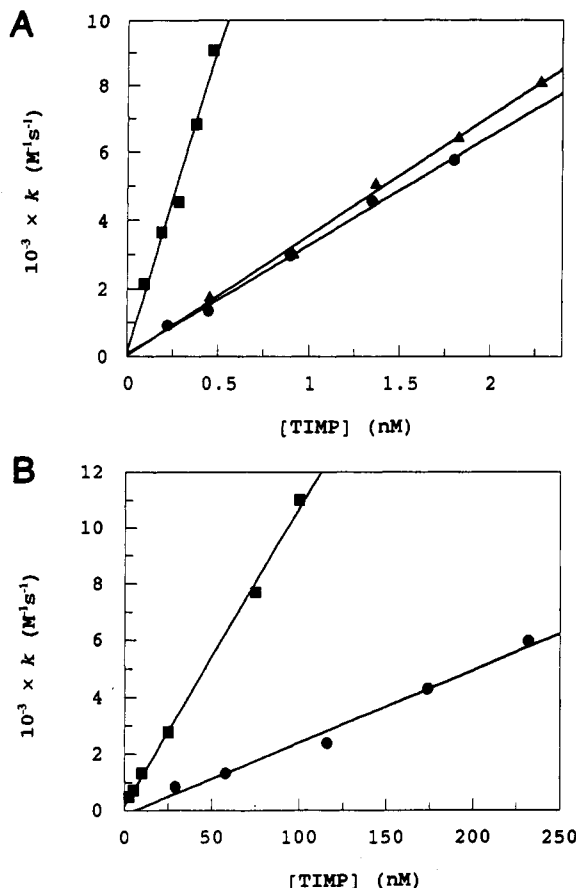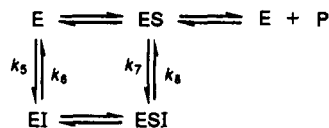
A

B

[TIMP] (nM)

FIGURE 3: Dependence of the first-order rate constant, k, for the inhibition of gelatinase A on TIMP concentration. The value of k_{on} was calculated from the gradient for (A) TIMP-1 (●), $\Delta_{186-194}$TIMP-2 (▲), and TIMP-2 (■) and (B) $\Delta_{127-184}$TIMP-1 (●) and $\Delta_{128-194}$TIMP-2 (■). Each data point is the average of triplicate determinations.

Scheme II

term k_{on} is used as the two schemes cannot be distinguished from each other.

Dependence on TIMP Concentration. Progress curves for the inhibition of gelatinase A by the TIMPs were obtained initially at 25 °C, pH 7.5, and $I = 0.2$ at a range of TIMP concentrations. In each case, the initial velocity of the enzyme-catalyzed reaction was independent of the inhibitor concentration, and values of k showed a linear dependence on the TIMP concentration (Figure 3). Both observations provide no evidence for a mechanism involving significant concentrations of intermediate states. This permits kinetic analysis in terms of an assumed bimolecular collision model. The slopes of the plots of k against TIMP concentration gave k_{on} values of $(3.2 \pm 0.2) \times 10^6$ M^{-1} s^{-1} for TIMP-1, $(2.6 \pm 0.17) \times 10^4$ M^{-1} s^{-1} for $\Delta_{127-184}$TIMP-1, $(2.1 \pm 0.23) \times 10^7$ M^{-1} s^{-1} for TIMP-2, $(1.1 \pm 0.12) \times 10^5$ M^{-1} s^{-1} for $\Delta_{128-194}$TIMP-2, and $(3.5 \pm 0.1) \times 10^6$ M^{-1} s^{-1} for Δ_{186^-194}TIMP-2.

Influence of Ionic Strength. The effect of ionic strength on k_{on} was studied at three NaCl concentrations for each inhibitor. The results obtained are shown in Table I and demonstrate that TIMP-2 shows a marked response to changes in salt concentration with a 3.7-fold increase in the rate of reaction on decreasing the ionic strength from 0.5 to 0.1.

Table I: Effect of Ionic Strength on the k_{on} for Inhibition of Gelatinase A[a]

	$10^{-6} \times k_{on}$ (M^{-1} s^{-1})		
	$I = 0.1$	$I = 0.25$	$I = 0.5$
TIMP-1	3.4 ± 0.15	3.15 ± 0.16	2.85 ± 0.08
$\Delta_{127-184}$TIMP-1	0.026 ± 0.002	0.028 ± 0.001	ND
TIMP-2	29.4 ± 2.7	15.3 ± 0.9	7.88 ± 0.65
$\Delta_{128-194}$TIMP-2	0.157 ± 0.008	0.125 ± 0.003	ND
$\Delta_{186-194}$TIMP-2	4.18 ± 0.15	3.45 ± 0.08	2.48 ± 0.05
$\Delta_{187-194}$TIMP-2	4.19 ± 0.22	2.98 ± 0.03	ND

[a] Each value is the average of four determinations. Gelatinase A concentration was 50 pM throughout, and data for TIMP-2 were obtained using 100 pM TIMP-2 and eq 2 and 5. All other data were calculated using eq 1.

Table II: Effect of the C-Terminal Domain of Gelatinase A (Δ_{1-414}GL) on the Inhibition of Gelatinase A by TIMP-2[a]

[Δ_{1-414}GL] (nM)	$10^{-6} \times k_{on}$ (M^{-1} s^{-1})	v_s (nM/s)	K_d (pM)
0	24.4	0.04	
0.25	16.0	0.17	37
0.5	10.8	0.22	58
2.0	4.0	0.46	54

[a] Data were analyzed using eq 2 and 5 with [gelatinase A] = 50 pM and [TIMP-2] = 100 pM. The K_d was calculated from the steady-state velocities using eq 6 and 7 as described in the text.

TIMP-1 binding is almost unaffected by ionic strength, and $\Delta_{186-194}$TIMP-2 has an intermediate response with a 1.7-fold increase in activity over the same concentration range. The other truncated forms of TIMP-2 behave in a similar fashion to $\Delta_{186-194}$TIMP-2. Higher NaCl concentrations interfered with the gelatinase activity assay and therefore could not be tested. Problems were encountered also when different salts were used. In some cases, this was due to limited solubility in the assay buffer, and in others, the gelatinase activity was affected.

Competition Experiments. The contributions of the C-terminal domains of gelatinase A (Δ_{1-414}GL) and collagenase (Δ_{1-269}CL) to the inhibitory activity of TIMP were investigated in the above assay system by measuring the rate of association of TIMP and the steady-state rate of substrate hydrolysis. The results for the effect of Δ_{1-414}GL on TIMP-2 activity are given in Table II. Neither TIMP-1 nor $\Delta_{186-194}$TIMP-2 activities were modified by the C-terminal domain of either enzyme, and TIMP-2 was unaffected by Δ_{1-269}CL. In the case of TIMP-2, the decrease in the final steady-state velocity and the rate of inhibition indicates an effective decrease in the inhibitor concentration, presumably by the formation of a complex between TIMP-2 and Δ_{1-414}GL which either cannot inhibit gelatinase A or does so very slowly so that this inhibitory activity is not detected during the assay. The data were analyzed by using eq 6 and 7 (Stone et al., 1987) to obtain

$$K_d = (F_t - FI)(I_t - EI - FI)/FI \qquad (6)$$

$$FI = I - E_t(1 - v_s/v_0) + K_i(1 - v_0/v_s) \qquad (7)$$

an estimate for K_d, the dissociation constant for the binding of TIMP-2 to the C-terminal domain of gelatinase A. In eq 6 and 7, I_t, E_t, and F_t are the total concentrations of TIMP-2, gelatinase A, and Δ_{1-414}GL, respectively, and EI and FI are the concentrations of the TIMP-2 complexes with gelatinase A and Δ_{1-414}GL, respectively. The dissociation constant calculated for the gelatinase A–TIMP-2 complex (K_i) from the steady-state data is 2.4 pM, which is in good agreement

with the previously reported value (Murphy et al., 1992b). The values of K_d given in Table II were calculated using a K_i of 2.4 pM and give an average value of 50 pM.

Dissociation Kinetics. The purity of each TIMP–gelatinase A complex was tested by addition at a final concentration of 250 pM to an activity assay containing 50 pM enzyme. In each case, no alteration in gelatinase activity was detected, indicating that there was no free enzyme or TIMP in the preparation. Dissociation curves were followed overnight for each TIMP complex at a complex concentration of approximately 0.2 pM. These conditions proved to be at the limit of the sensitivity of the assay, however, and there was too great a variation between duplicates to allow analysis of the factors affecting dissociation. In each case, it was demonstrated that the interaction is reversible, and rate constants were obtained for dissociation of the TIMPs in the range of $(6–24) \times 10^{-6}$ s^{-1}, equivalent to half-lives of between 8 and 30 h. These correspond to K_i values for the full-length TIMPs on the order of 10^{-12} M, which agrees with previously reported estimates of 2 pM (Murphy et al., 1992b) and the result obtained from the competition experiments.

TIMP-2 Binding Studies. Assays were performed on mixtures of progelatinase with TIMP-2, $\Delta_{128–194}$TIMP-2, and $\Delta_{186–194}$TIMP-2, or inhibitors alone before and after passage through gelatin–Sepharose (Murphy et al., 1991, 1992b). The inhibitors alone did not bind to the matrix. In the presence of progelatinase A, 100% of the full-length TIMP-2 was retained on the column and 0% of the truncated forms. TIMP-2 is therefore the only inhibitor that binds to the proform of gelatinase A under these conditions.

DISCUSSION

We have prepared recombinant forms of human TIMP-1 and TIMP-2 and deletion mutants that lack key structural features, namely, the three disulfide-bonded loops comprising the complete C-terminal domain ($\Delta_{127–184}$ and $\Delta_{128–194}$, respectively) and, in the case of TIMP-2, the charged C-terminal peptide "tail" of this domain ($\Delta_{187–194}$). We also isolated a cleaved form of TIMP-2 with nine amino acids missing from the C-terminus ($\Delta_{186–194}$). The ability of these inhibitors to interact with gelatinase A was assessed by kinetic methods. Complexes of gelatinase A with each inhibitor could be isolated and dissociated with half-lives of several hours, reflecting a very tight binding interaction. The linear dependence of the apparent first-order rate constant, k, on TIMP concentration over the range of concentrations used provides no evidence for an association process more complex than a bimolecular collision. Most of the values of k_{on} shown in Table II (2.5×10^6 to 2.9×10^7 M^{-1} s^{-1}) are within the range typically found for the rate constant of enzyme–substrate complex formation (10^6–10^8 M^{-1} s^{-1}; Hammes, 1982). Even the low values of k_{on} for the reactions of gelatinase A with $\Delta_{127–184}$TIMP-1 (2.6×10^4 M^{-1} s^{-1}) and with $\Delta_{128–194}$TIMP-2 (1.1×10^5 M^{-1} s^{-1}) are not unprecedented. For example, whereas k_{on} for the reaction of aspartate aminotransferase with aspartate is $>10^7$ M^{-1} s^{-1} (Fasella & Hammes, 1967), the value for the reaction with the analogue substrate α-methylaspartate is as low as 1.2×10^4 M^{-1} s^{-1} (Hammes & Haslam, 1968).

The C-terminal nine amino acid peptide of TIMP-2 appears to make an important contribution to the rate of reaction because when this is removed the rate constant decreases approximately 4-fold to resemble that of TIMP-1. The marked decrease in the rate of inhibition by TIMP-2 with increasing ionic strength confirms the importance of ionic interactions
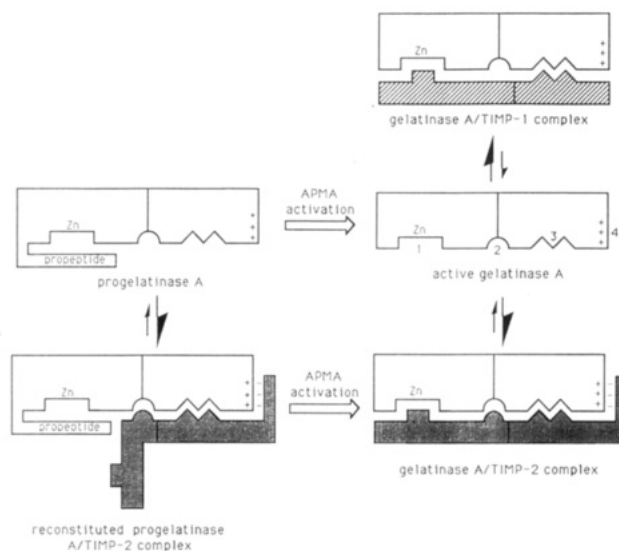

FIGURE 4: Schematic representation of the TIMP-1 and TIMP-2 binding sites on gelatinase A. Vertical lines divide the structures into domains, with the N-terminal domain on the left and the C-terminal domain on the right in each case. The binding regions 1–4 are described under Discussion.

to its mechanism. The effect is considerably decreased with the forms of TIMP-2 that lack the C-terminal peptide and is insignificant in the case of TIMP-1. Thus, the C-terminus of TIMP-2 is primarily, although not completely, responsible for the ionic interactions with gelatinase A. The salt effect seen with the TIMP-2 lacking the C-terminal peptide is observed also with $\Delta_{128–194}$TIMP-2 lacking the complete C-terminal domain. We conclude that another charged region present in the N-terminal domain of TIMP-2, but not in that of TIMP-1, plays a role in the association of TIMP-2 with gelatinase A.

On the basis of these observations, we propose a working model of the various points of interaction between gelatinase A and TIMP-1 and TIMP-2 which is depicted in Figure 4. The numbered binding sites in the active enzyme represent the following features:

(1) The active site of gelatinase A interacts with the N-terminal domain of the inhibitor. Evidence for the involvement of the N-terminal domain of TIMP in this role comes from our previously reported observation that all matrix metalloproteinases, including matrilysin which lacks a C-terminal domain, are inhibited by the N-terminal domain of either TIMP-1 or TIMP-2 (Murphy et al., 1991, and unpublished results). The interaction does not appear to depend primarily on a single specific amino acid residue of the TIMP molecule (O'Shea et al., 1992), and TIMP hydrolysis is not catalyzed by the enzyme (Murphy et al., 1989). At present, it is not clear whether the TIMPs interact with the catalytic residues directly or inhibit enzyme activity by sterically preventing substrate binding. There is some evidence for the former mechanism since competition has been observed between TIMP-1 and active-site-directed hydroxamate inhibitors for binding to both collagenase and stromelysin (S. Tickle, Celltech, personal communication; Lelièvre et al., 1990).

(2) There are ionic interactions between the N-terminal domain of TIMP-2 and gelatinase A. These have been demonstrated in the present work by the effect of salt concentration on the rate of inhibition and do not appear to be important in the mechanism of TIMP-1. The binding site

is depicted as involving both domains of the enzyme to emphasize that its location on the enzyme is unknown.

(3) There are low-affinity interactions between the enzyme and inhibitor C-terminal domains which increase the rate of inhibition approximately 100-fold. For simplicity, these interactions are drawn as being common to both TIMPs. Since they have a similar effect on the rate of reaction, this simplification may be valid. Evidence for the location of the binding sites on the C-terminal domain of both TIMPs comes from the difference in the rate of inhibition of gelatinase A by the N-terminal domains of both TIMPs compared to their full-length counterparts. Preliminary results indicate that full-length TIMP-2 and the TIMP-2 N-terminal domain inhibit the truncated form of gelatinase A ($\Delta_{418-631}$) equally rapidly ($k_{on} \approx 10^5$ M^{-1} s^{-1}; F. Willenbrock, unpublished observations). Thus, the binding sites on the C-terminal domain of the TIMPs are shown interacting with binding sites on the C-terminal domain of the enzyme rather than with the N-terminal domain.

(4) The C-terminal peptide of TIMP-2 interacts with the C-terminal domain of gelatinase A to increase the rate of inhibition. The electrostatic nature of this interaction has been demonstrated by studying the effect of salt concentration on the rate of inhibition. The experiments in which the C-terminal domain of gelatinase A (Δ_{1-414}GL) competes with the full-length enzyme to associate with TIMP-2 but not $\Delta_{186-194}$TIMP-2 or TIMP-1 demonstrate that there is a binding site for the C-terminal peptide of TIMP-2 on the C-terminal domain of gelatinase A. The results do not disprove the existence of other binding sites for either TIMP on the C-terminal domains of gelatinase A or collagenase, because lower affinity sites would only be detected by competition with higher C-terminal domain concentrations. Indeed, we have previously shown that for both enzymes C-terminal domain interactions with either TIMP decrease the K_i for the inhibition (Murphy et al., 1992a,b). We have reported a K_i value for the inhibition of $\Delta_{418-631}$GL by TIMP-2 of 33 pM compared with a value of ≤ 2 pM for the full-length enzyme (Murphy et al., 1992b). If the effects of the two domains are additive, then the overall K_i for the inhibition of gelatinase A by TIMP-2 would be equal to the product of the dissociation constants of the individual binding regions. The K_d of 50 pM for the interaction between the C-terminal domain and TIMP-2 reported here is therefore considerably lower than expected. Either additional interactions occur with sites on the Δ_{1-414}GL that are masked in the full-length enzyme, thus giving an artificially low dissociation constant, or the C-terminal tail of TIMP-2 is not involved in the final complex, its role being to initially bring the two molecules together.

The ability of the TIMPs to bind to progelatinase A was also tested, and the results demonstrate that the C-terminal peptide is essential for the interaction of TIMP-2 with progelatinase A. It is not known whether the other C-terminal domain binding sites interact, and they are depicted as doing so in the proenzyme complex in Figure 4 for simplicity only. It can be postulated that the binding site for the C-terminal peptide of TIMP-2 is the same on the C-terminal domain of both progelatinase A and active gelatinase A. Since gelatinase A is the only matrix metalloproteinase that binds TIMP-2 when in its proform, this binding site is probably unique to gelatinase A, although the location of this site is not obvious from inspection of the matrix metalloproteinase amino acid sequences.

We note that purified progelatinase A–TIMP-2 complexes appear to be bound very much more tightly than the reconstituted complexes described here. The purified native complexes can be dissociated only by severe treatments that denature the enzyme (Goldberg et al., 1989; Stetler-Stevenson et al., 1989; Ward et al., 1991). Reconstituted complexes, however, are not stable during electrophoresis, and free TIMP-2 dissociates readily from such complexes to inhibit exogenously added enzyme (Ward et al., 1991; R. Ward and G. Murphy, unpublished results). These observations suggest the possibility of an additional TIMP-2 binding site that is not usually exposed on the mature, free enzyme. Such a complex involving this site may be formed intracellularly, for example, during the posttranslational folding of progelatinase A in the presence of TIMP-2. The native complex could therefore have a very different conformation than the reconstituted complex depicted in Figure 4.

In summary, we have used deletion mutants of TIMP-1 and TIMP-2 to assess the significance of the C-terminal domains and motifs therein in the mechanism of interaction with gelatinase A. Our data indicate that TIMP-2 binds more rapidly to gelatinase A than TIMP-1 and that this is largely due to the charged C-terminal sequence of nine amino acids. This tail is also responsible for the specific property of TIMP-2 binding to progelatinase A. Preliminary studies of gelatinase A–TIMP-1 interactions using a macromolecular substrate had indicated that TIMP-1 lacking the entire C-terminal domain was a less efficient inhibitor than the full-length molecule. We have now used more accurate peptide assays to show that the C-terminal domain of both TIMPs is involved in gelatinase A binding interactions and that their removal decreases the rate and stability of binding. We have tentatively identified three to four binding sites between gelatinase A and TIMP-2. Further analyses of gelatinase A–TIMP binding, involving more specific mutations of all the interacting domains, coupled with kinetic analyses, should allow us to build up a model of the number and nature of all the binding sites involved.

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