Different Domain Interactions Are Involved in the Binding of Tissue Inhibitors of Metalloproteinases to Stromelysin-1 and Gelatinase A†

Quang Nguyen,‡ Frances Willenbrock,§ Mark I. Cockett,⊥ Mark O'Shea,‡ Andrew J. P. Docherty,⊥ and Gillian Murphy*,‡

Department of Cell and Molecular Biology, Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K., Department of Biochemistry, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, U.K., and Department of Oncology Biology, Research Division, Celltech Limited, 216 Bath Road, Slough SL1 4EN, U.K.

Received September 22, 1993; Revised Manuscript Received December 14, 1993⊛

ABSTRACT: The matrix metalloproteinases gelatinase A and stromelysin-1 have definable N-terminal (catalytic) and C-terminal domains. In order to analyze their interactions with the N- and C-terminal domains of the tissue inhibitors of metalloproteinases TIMP-1 and -2, mutants of both the enzymes and the inhibitors were prepared in which the C-terminal domains had been deleted. Since the K_i values for TIMP inhibition of the matrix metalloproteinases are in the picomolar range, it was not possible to measure these accurately within the sensitivity of available activity assays. Rate constants for the association of the wild-type proteins were therefore determined and systematically compared with those for the deletion mutants. It was found that TIMP-1 binds more rapidly than TIMP-2 to stromelysin-1 and that the C-terminal domain of the enzyme does not affect the rate of association of enzyme and inhibitor. This is in contrast to gelatinase A, where the C-terminal domain has been shown to play an important role in increasing the rate of complex formation with the TIMPs (Willenbrock *et al.*, 1993). The TIMPs are also comprised of an N- and C-terminal domain. By deletion mutagenesis, we found that the C-terminal domain of both TIMPs contributed less to the rate of complex formation with stromelysin-1 than to that with gelatinase A. Hybrids of the N- and C-terminal domains of gelatinase A and stromelysin-1 were prepared and used to analyze further the differences in domain interactions with the TIMPs. They demonstrated that the interactions between the C-terminal domains of enzyme and inhibitor can occur irrespective of the nature of the N-terminal domain. We can conclude that the TIMPs have two major binding regions which associate in different ways with the domains of the enzymes gelatinase A and stromelysin-1. The N-terminal domains of the TIMPs bind to the enzyme catalytic domains to inhibit activity. The TIMP C-terminal domain acts to increase the association rate constant by binding to the N-terminal domain of stromelysin or the C-terminal domain of gelatinase A.

The matrix metalloproteinases (MMPs) are thought to play an important role in the physiological and pathological remodeling of connective tissues, where they are capable of hydrolyzing collagens, proteoglycans, and other matrix components. Extracellular activity of the MMPs is controlled *in vivo* by a family of inhibitors, the tissue inhibitors of MMPs, or the TIMPs.[1] To date, the primary sequences of three human TIMPs have been obtained by cDNA cloning: TIMP-1 (Docherty *et al.*, 1985), TIMP-2 (Boone *et al.*, 1990), and TIMP-3 (Apte *et al.*, 1993). Studies are now directed toward the understanding of the mechanism of action of the TIMPs. Early studies showed that they made tight-binding noncovalent complexes with active MMPs with a 1:1 stoichiometry (Cawston *et al.*, 1981, 1983; Welgus *et al.*, 1985; Okada *et al.*, 1986; Murphy *et al.*, 1987). TIMP-1 and TIMP-2 have previously been shown to have similar abilities to inhibit all the active MMPs when assessed using macromolecular substrates (Ward *et al.*, 1991). This is consistent with the fact that these inhibitors have related primary and secondary structures. The TIMPs consist of six disulfide-bonded loops, and deletion mutagenesis studies have demonstrated that two structurally distinct domains can be defined, the N-terminal domain consisting of loops 1–3 and the C-terminal domain consisting of loops 4–6 (Murphy *et al.*, 1991; Willenbrock *et al.*, 1993). The N-terminal domain can act as a functional inhibitor (Murphy *et al.*, 1991; DeClerck *et al.*, 1993) and is thought to bind at or near the enzyme active site, since competition has been observed between TIMP-1 and low-molecular-weight synthetic inhibitors that are directed at the catalytic zinc (Leliévre *et al.*, 1990). Very tight complexes are formed between the TIMPs and MMPs with K_i values of less than 1 nM. We have previously reported approximate K_i values of 2 and 30 pM, respectively, for the inhibition of gelatinase A and a mutant of gelatinase A that lacks the C-terminal domain ($\Delta_{418-631}$ gelatinase A, N-GL) by either TIMP-1 or TIMP-2 (Murphy *et al.*, 1992b). For the inhibition of stromelysin and a similarly truncated mutant, K_i values are less than 200 pM (Teahan & Stein, 1990; Murphy *et al.*, 1992a).

† Q.N. is supported by the Wellcome Trust, U.K., F.W. by the Medical Research Council, U.K., and G.M. by the Arthritis and Rheumatism Council, U.K.

* To whom correspondence should be addressed.
‡ Strangeways Research Laboratory.
§ Queen Mary and Westfield College.
⊥ Celltech Ltd.

⊛ Abstract published in *Advance ACS Abstracts,* February 1, 1994.

[1] Abbreviations: TIMP, tissue inhibitor of metalloproteinases; $\Delta_{127-184}$ TIMP-1 (N-TIMP-1), TIMP-1 from which amino acids 127–184 have been deleted; $\Delta_{128-194}$ TIMP-2 (N-TIMP-2), TIMP-2 from which amino acids 128–194 have been deleted; $\Delta_{248-460}$ stromelysin (N-SL), prostromelysin from which amino acids 248–460 have been deleted; N-GL.C-SL, hybrid of gelatinase A residues 1–417 and stromelysin-1 residues 248–460; N-SL.C-GL, hybrid of stromelysin-1 residues 1–247 and gelatinase A residues 418–631; PCR, polymerase chain reaction; McaPLANvaDpaAR, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Ala-Norval-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2; McaPLGLDpaAR, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2.

0006-2960/94/0433-2089$04.50/0

The MMPs also have definable domains, which, in the case of gelatinase A and stromelysin-1, include an N-terminal (catalytic) domain and a noncatalytic C-terminal domain (Matrisian, 1992). Studies aimed at understanding the nature of TIMP–MMP complex formation in the case of human gelatinase A have identified two distinct TIMP-2 binding sites, one at the active site within the N-terminal domain and one in the noncatalytic C-terminal domain (Howard & Banda, 1991). These observations were supported by the work of Kleiner *et al.* (1992), who used cross-linking methods to demonstrate two binding sites for TIMP-2 on gelatinase A. Binding to one site appeared to decrease the affinity of binding of the inhibitor to the other site. Using kinetic analyses, we found that TIMP-2 bound to active gelatinase A more rapidly than TIMP-1 (Willenbrock *et al.*, 1993). This was due to the fact that TIMP-2 has an exposed charged C-terminus which promotes rapid binding to the C-terminal domain of gelatinase A. We also found that at least three sites of interaction between TIMP-2 and gelatinase A could be defined by the study of binding rates of different inhibitor and enzyme mutants. Here, we compare the binding of human TIMP-1, TIMP-2, and deletion mutants of both TIMPs to human gelatinase A and stromelysin-1. We also compare the binding of TIMPs to deletion mutants of both enzymes lacking their C-terminal domain. By measuring the second-order rate constants for binding, we reveal that the C-terminal domain of gelatinase A contributes more to the rate of inhibitor association than the corresponding domain in stromelysin-1. This is a unique feature of the gelatinase A C-terminal domain and is independent of the catalytic domain, since it can be transferred to stromelysin by exchanging the C-terminal domain between the two enzymes.

MATERIALS AND METHODS

Chemicals. All chemicals were of the highest purity available commercially. The stromelysin substrate Mca-PLANvaDpaAR, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Ala-Norval-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2, was a gift from Dr. C. G. Knight (Niedzwiecki *et al.*, 1992; Knight *et al.*, 1992), as was McaPLGLDpaAR (Knight *et al.*, 1992).

Preparation of Stromelysins and TIMPs. Recombinant human prostromelysin was purified from the medium of C127 cells expressing this enzyme (Murphy *et al.*, 1987; Koklitis *et al.*, 1991). $\Delta_{248-460}$ stromelysin (N-SL) was prepared as described by Murphy *et al.* (1992a). Prostromelysins were activated by treatment with trypsin (10 μg/mL, 37 °C, 30 min) followed by a 10-fold excess of soybean trypsin inhibitor. TIMP-1, $\Delta_{127-184}$ TIMP-1 (N-TIMP-1), TIMP-2, $\Delta_{128-194}$ TIMP-2 (N-TIMP-2), and $\Delta_{187-194}$ TIMP-2 were prepared as described previously (Murphy *et al.*, 1991; Willenbrock *et al.*, 1993).

Preparation of the N-Terminal Gelatinase A–C-Terminal Stromelysin Hybrid (N-GL.C-SL), the N-Terminal Stromelysin–C-Terminal Gelatinase A Hybrid (N-SL.C-GL), Progelatinase A, and $\Delta_{418-631}$ Progelatinase A (N-GL). The N-terminal gelatinase A–C-terminal stromelysin-1 hybrid (gelatinase A 1-417–stromelysin 248–460, N-GL.C-SL) was assembled using the polymerase chain reaction (PCR) overlap extension method of Ho *et al.* (1989), pEE12.gelatinase A (Murphy *et al.*, 1992b) was used as the template in the presence of an oligonucleotide that primes within the hCMV promoter and a second mutagenic oligonucleotide that is complementary to the sequence encoding residues 413–417 of gelatinase A and residues 248–252 of stromelysin (Figure 1) to generate

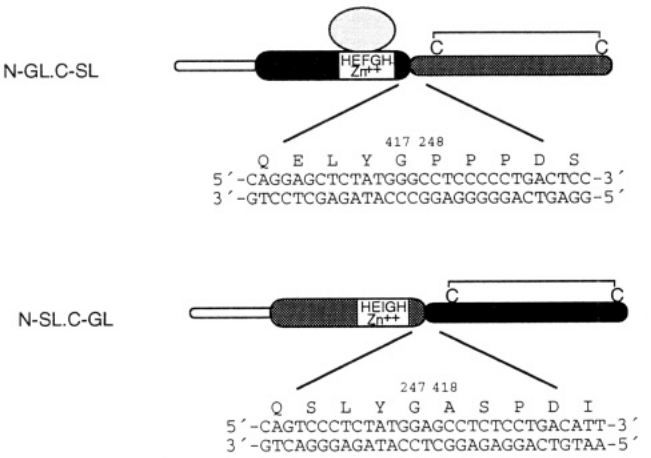

FIGURE 1: Construction of gelatinase A–stromelysin hybrids. The gelatin binding domain is shown as a lightly stippled region, mature gelatinase A sequences are shown in black, and mature stromelysin-1 sequences are shown heavily stippled; the prodomain of each enzyme is shown unshaded. The putative zinc binding region within each N-terminal region is labeled, and the two cysteine residues in each C-terminus are shown. The sequences of the oligonucleotides used to generate each hybrid molecule are shown together with the amino acids encoded. The junctions are identified by the numbered residues of the wild-type proteins (Collier *et al.*, 1988; Docherty & Murphy, 1990).

a 1400-bp DNA fragment. A second 780-bp fragment was generated using pEE12.stromelysin-1 (Murphy *et al.*, 1992a) as a template, with an oligonucleotide priming within the SV40 polyadenylation sequence of pEE12 and an oligonucleotide complementary to the mutagenic oligonucleotide described above (Figure 1). The two fragments were annealed and further amplified by PCR in the presence of flanking pEE12 oligonucleotide primers. The 1500-bp *Hin*dIII fragment encoding residues 1–417 of gelatinase A and residues 248–304 of stromelysin, generated by cleavage of the PCR product, was ligated into the *Hin*dIII-digested pSP65.

The N-terminal (1–247) stromelysin–C-terminal (418–631) gelatinase A hybrid (N-SL.C-GL) was generated in an analogous fashion using PCR with a pair of mutagenic oligonucleotides that are complementary to the sequence encoding residues 243–247 of stromelysin-1 and residues 418–422 of gelatinase A (Figure 1). These generated a 900-bp 5′ stromelysin-1 DNA fragment and an 830-bp 3′ gelatinase A DNA fragment. The two fragments were annealed and further amplified by PCR in the presence of the flanking pEE12 primers. The 560-bp *Hin*dIII to *Bam*HI fragment encoding residues 217–247 of stromelysin-1 and residues 418–571 of gelatinase A, generated by cleavage of the polymerase chain reaction product, was ligated into pSP65 that had been digested with *Hin*dIII and *Bam*HI.

To insert the N-GL.C-SL hybrid into pEE12, a 250-bp *Bgl*I to *Hin*dIII fragment encoding residues 393–417 of gelatinase and residues 248–304 of stromelysin-1 was purified from the vector pSP65.N-GL.C-SL and ligated in a three-way ligation to the large *Hin*dIII fragment of pEE12.stromelysin-1 containing the 3′ coding sequence of stromelysin-1 (encoding residues 305–460 in pEE12) and a 1260-bp 5′ gelatinase A *Hin*dIII to *Bgl*I fragment (encoding residues 1–392).

To insert the N-SL.C-GL hybrid into pEE12, a 280-bp *Hin*dIII to *Bgl*I fragment from pSP65N-SL.C-GL encoding residues 217–247 of stromelysin-1 and residues 418–475 of gelatinase A was ligated to *Hin*dIII, *Eco*RI-cut pEE12, and a 680-bp *Bgl*I to *Eco*RI 3′ gelatinase A fragment encoding residues 476–631. This partial reconstruction of N-SL.C-

GL in pEE12 was cut with *Hin*dIII and ligated with the 5′ stromelysin-1 810-bp *Hin*dIII fragment from pEE12.stromelysin-1.

The constructions pEE12.N-GL.C-SL and pEE12.N-SL.C-GL were shown by dideoxy chain termination sequencing to contain the correct sequences of wild-type stromelysin-1 or gelatinase A, as appropriate. These vectors were transfected into NSO myeloma cells by electroporation (Murphy *et al.*, 1991), and colonies were isolated and screened for the production of metalloproteinase activities degrading casein or gelatin. The most productive cell lines were expanded and used to produce conditioned medium from which N-GL.C-SL or N-SL.C-GL could be purified.

N-SL.C-GL-containing medium (500 mL) was purified by passage through an S-Sepharose column after equilibration of both medium and matrix with 25 mM TrisHCl, pH 7.5, containing 10 mM $CaCl_2$ and 0.02% sodium azide. The loaded column was washed with the same buffer containing 100 mM sodium chloride, and the hybrid N-SL.C-GL eluted using the same buffer containing 200 mM sodium chloride.

N-GL.C-SL medium (500 mL) was purified by binding to a gelatin Sepharose column equilibrated with 25 mM TrisHCl, pH 7.5, containing 500 mM NaCl, 10 mM $CaCl_2$, and 0.02% sodium azide and elution with the same buffer containing 10% (v/v) dimethyl sulfoxide.

Progelatinase A and $\Delta_{418-631}$ progelatinase A (N-GL) were expressed from NSO cells and purified as described previously (Murphy *et al.*, 1992b).

Activation of N-SL.C-GL hybrids was most efficiently effected with trypsin as described above for prostromelysins. Progelatinase A, N-GL, and N-GL.C-SL were activated by treatment with 2 mM (2-aminophenyl)mercuric acetate at 25 °C for 1.5 h.

Proteinase Assays. Activities of the recombinant proteinases were determined using ^{14}C-labeled β-casein or type I gelatin, as described previously (Murphy *et al.*, 1981).

Kinetic Studies. Assays were performed at 37 °C at I = 0.125 in a buffer containing 50 mM TrisHCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 1% dimethyl sulfoxide, and 0.05% Brij 35. Hydrolysis of the substrate McaPLANvaDpaAR or McaPLGLDpaAR was followed by the increase in fluorescence (λ_{ex} 328 nm, λ_{em} 393 nm) using a Perkin-Elmer LS50B fluorimeter (Knight *et al.*, 1992; Niedzwiecki *et al.*, 1992). Mutant TIMP concentrations were determined by titration against active stromelysin as described by Willenbrock *et al.* (1993). Active metalloproteinase concentrations were determined by titration against a TIMP-1 standard, the concentration of which had been determined by amino acid analysis. For this, we assumed that the preparation of TIMP-1 was 100% active and that a molar stoichiometry of binding to all the metalloproteinases and their mutants occurred.

Stromelysin was generally used at a concentration of 0.3–0.5 nM using the substrate McaPLANvaDpaAR and gelatinase A at a concentration of 10–100 pM using either McaPLANvaDpaAR or McaPLGLDpaAR. Substrates were used in the concentration range 0.5–1.5 μM. In each case, $[S] \ll K_m$ ($K_m > 8$ μM; Knight *et al.*, 1992; Q. Nguyen, unpublished results), giving progress curves that were first-order in substrate and allowing direct determination of k_{cat}/K_m.

Inhibition by TIMP. Reaction conditions were chosen so that in each case the TIMP concentration was greater than 10 times the enzyme concentration. Reactions were started by the 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 were analyzed by using the Enzfitter program (Leatherbarrow, 1987) and the equation:

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

in which P is the product concentration, v_o 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 and inhibitor complexes (Morrison and Walsh, 1988).

In each case, the dependence of k on TIMP concentration was determined across as high a concentration range as was practically possible. The linear relationships observed in each case provided no evidence for a model more complex than inhibition by a simple bimolecular reaction in the concentration range used (see Results). Thus, the second-order rate constant (k_{on}) was provided by linear regression of k on TIMP concentration.

Table 1: Comparison of the Proteolytic Activities of the Hybrids Stromelysin (1-247)–Gelatinase A (418-631), N-SL.C-GL, and Gelatinase A (1-417)–Stromelysin (248-460), N-GL.C-SL, with Their Wild-Type Counterparts[a]

	ratio casein/gelatin-degrading activity	k_{cat}/K_m (M^{-1} s^{-1})
gelatinase A	0.28	871496 ± 6370
N-GL.C-SL	0.26	662753 ± 13100
stromelysin-1	42.9	58766 ± 1160
N-SL.C-GL	28.9	61533 ± 3164

[a] The ability of gelatinase A, stromelysin-1, and their hybrids to degrade ^{14}C-labeled casein and type I gelatin at 37 °C was compared. The k_{cat}/K_m values were determined at 37 °C using the quenched fluorescent peptide substrate McaPLANvaDpaAR, as described in the Materials and Methods, and are the mean of four to eight determinations.

RESULTS

Characterization of Hybrid Enzymes. In order to analyze the role of the C-terminal domains of gelatinase A and stromelysin-1 in interactions with the TIMPs, we prepared forms of these enzymes lacking this domain (Murphy *et al.*, 1992a,b) and hybrid enzymes consisting of the N-terminal catalytic domain of one enzyme fused to the C-terminal domain of the other (Figure 1). This is a particularly valuable approach since the C-terminal domains of all the metalloproteinases show a good degree of similarity (>50%).

The N-terminal (residues 1–247) stromelysin–C terminal (residues 418–631) gelatinase A hybrid, N-SL-C-GL, was expressed from myeloma cells and purified using S-Sepharose. It migrated during SDS polyacrylamide gel electrophoresis (reducing conditions) as a single band with a molecular mass of 57 kDa (Figure 2B). The hybrid was apparently unglycosylated, which would be predicted from our previous observation that the potential glycosylation site of the stromelysin catalytic domain was not utilized (Murphy *et al.*, 1992a). The hybrid could be activated by incubation with (4-aminophenyl)mercuric acetate or trypsin with similar kinetics to wild-type stromelysin-1 by loss of the 10-kDa propeptide (Figure 2B). Optimal activation, as assessed by β-casein or McaPLANvaDpaAR assays, was obtained using trypsin at a concentration of 10 μg/mL (data not shown), which was therefore used routinely. The hybrid N-SL.C-GL degraded casein efficiently and was a poor degrader of gelatin, with a similar ratio of activities against these substrates as that of stromelysin-1 (Table 1). The N-terminal (residues

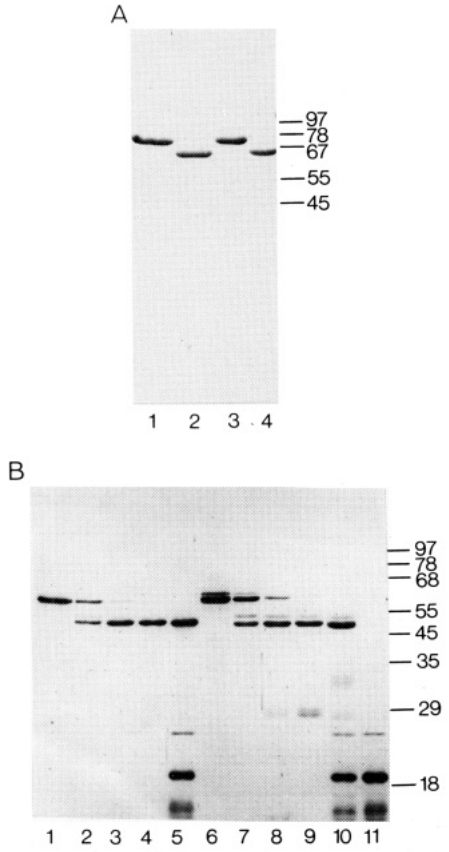

FIGURE 2: Comparison of the activation of gelatinase A–stromelysin-1 hybrids with their wild-type counterparts. (A) Progelatinase A was incubated alone (lane 1) or in the presence of 2 mM (4-aminophenyl)-mercuric acetate (lane 2) at 25 °C for 1 h. N-terminal gelatinase–C-terminal stromelysin, N-GL.C-SL, was incubated under the same conditions: alone (lane 3) and with (4-aminophenyl)mercuric acetate (lane 4). The products of the incubations were analyzed on an 11% polyacrylamide gel with SDS under reducing conditions and stained with Coomassie Blue. (B) N-terminal stromelysin–C-terminal gelatinase, N-SL.C-GL, was incubated at 37 °C alone for 2 h (lane 1) or with 2 mM (4-aminophenyl)mercuric acetate for 1 h (lane 2), 2 h (lane 3), and 4 h (lane 4). Treatment with trypsin (5 μg/mL) at 37 °C for 30 min followed by the addition of a 10-fold excess of soybean trypsin inhibitor elicited a similar fall in molecular mass (lane 5). Stromelysin-1 behaved similarly upon incubation under the same conditions: alone (lane 6), with (4-aminophenyl)mercuric acetate for 1 h (lane 7), 2 h (lane 8), and 4 h (lane 9), or with trypsin (lane 10). Trypsin and soybean trypsin inhibitor are shown in lane 11. The incubations were analyzed on an 11% polyacrylamide gel with SDS under reducing conditions and stained with silver. The mobilities of standard molecular mass (kDa) proteins are indicated to the right of each gel.

1–417) gelatinase A–C terminal (residues 248–460) stromelysin-1 hybrid, N-GL.C-SL, could be purified on gelatin Sepharose, since it retains the fibronectin-like collagen binding domain (Figure 1). It migrated during SDS polyacrylamide gel electrophoresis (reducing conditions) as a single band of molecular mass 72 kDa (Figure 2A). In some preparations, a minor higher molecular mass band was detectable, which may be due to glycosylation of the C-terminal domain of stromelysin-1. The hybrid could be activated by incubation with (4-aminophenyl)mercuric acetate with a similar time course to full-length gelatinase A and involving the apparent loss of the propeptide piece (Figure 2A). As in the case of wild-type gelatinase A, trypsin treatment did not cause the same shift in molecular mass (Figure 2A) nor activate the hybrid (data not shown). Optimal activation was obtained by 1–2-h treatment with the organomercurial (2 mM), as determined by gelatin or McaPLANvaDpaAR assays, and this treatment was used routinely. The hybrid N-GL.C-SL degraded gelatin efficiently and casein poorly, with a similar ratio of activities as that of wild-type gelatinase A (Table 1). The specificities of stromelysin-1 and gelatinase A therefore both appear to result from the N-terminal catalytic domains of these enzymes.

Inhibition Kinetics. Analyses of the inhibition of the activated purified recombinant MMPs by TIMP-1 and TIMP-2 and their truncated forms were carried out using a continuous fluorimetric assay with the appropriate internally quenched fluorescent substrate (Neidzwiecki *et al.*, 1992; Knight *et al.*, 1992). K_i values for the inhibition of wild-type and mutant enzymes by the wild-type TIMPs are all less than 200 pM. In order to obtain accurate values, it is necessary to use enzyme and inhibitor concentrations which are less than the K_i. Under these conditions, long incubations (>24 h) are needed to reach a steady-state velocity, during which time enzyme activity is lost due to instability at such low concentrations. In addition, the activity assays are not sufficiently sensitive to allow reliable measurements to be performed at enzyme concentrations below the K_i value. Attempts to determine the dissociation rate constants were also prevented by the limitations in assay sensitivity. However, half-lives for the dissociation of complexes of each enzyme and inhibitor of several hours were observed, confirming that all of the interactions are tight-binding.

Our analysis was limited, therefore, to following the association of enzyme and inhibitor by using low reagent concentrations (10–50 pM gelatinase A or 0.3–0.5 nM stromelysin). Under these conditions, inhibition was observed as curvature in the progress curve of substrate hydrolysis which could be analyzed using eq 1. For each reaction, it was confirmed in the absence of inhibitor that the curvature was due to inhibition by TIMP only and not enzyme instability or substrate depletion. Analysis of the progress curves provided the pseudo-first-order rate constant (k) for the formation of EI. The dependence of k on TIMP concentration was studied for each reaction. TIMP concentrations used were 20–400 nM for reactions with k_{on} values of 10^4 M^{-1} s^{-1}, 2–50 nM for k_{on} values of 10^5 M^{-1} s^{-1}, and 1–10 nM for k_{on} values of 10^6 M^{-1} s^{-1}. The reaction of gelatinase A with TIMP-2 was studied at a TIMP concentration of 100 pM and an enzyme concentration of 10 pM. The interaction of N-SL.C-GL with full-length TIMP-1 or TIMP-2 was too rapid to be followed under pseudo-first-order conditions, and lower limits for the values of k_{on} only were obtained. In each case, the initial rate of the enzyme-catalyzed reaction was independent of the inhibitor concentration and values of k showed a linear dependence on TIMP concentration (data not shown). Both observations provide no evidence for a mechanism involving significant concentration of intermediate states. Linear regression of k on TIMP concentration provided the value of the second-order rate constant k_{on}. As discussed previously (Willenbrock *et al.*, 1993), the second-order rate constant k_{on} determined in the present work could contain a term for the interaction of TIMP with the enzyme–substrate complex as well as with the free enzyme.

The results of the determination of rates for the MMPs and TIMPs are summarized in Table 2. Inhibition of full-length wild-type enzymes by wild-type TIMPs is rapid with second-order rate constants in the range of 10^5–10^7 M^{-1} s^{-1}. The relative rates of inhibition vary for each enzyme; in the case of stromelysin, TIMP-1 is a more rapid inhibitor than TIMP-2. Removal of the C-terminal domain of TIMP-1 reduces the rate constant with stromelysin by less than 2-fold, whereas

Table 2: Rate Constants for the Interaction of TIMP-1 and -2 and Their N-Terminal Domains with Wild-Type and Mutant Forms of Gelatinase A and Stromelysin[a]

	k_{on} (M^{-1} s^{-1}) × 10^{-6}			
	TIMP-1	$\Delta_{127-184}$ TIMP-1 (N-TIMP-1)	TIMP-2	$\Delta_{128-194}$ TIMP-2 (N-TIMP-2)
stromelysin	1.9	1.17	0.30	0.05
$\Delta_{248-460}$ stromelysin (N-SL)	1.9	1.2	0.47	0.05
gelatinase A	4.7	0.17	38	0.36
$\Delta_{418-631}$ gelatinase A (N-GL)	0.014	0.14	0.30	0.30
stromelysin$_{1-247}$–gelatinase$_{418-631}$ (N-SL.C-GL)	>10	1.58	>10	0.045
gelatinase$_{1-417}$–stromelysin$_{248-460}$ (N-GL.C-SL)	0.14	0.15	0.83	0.32

[a] The data are the mean of more than four determinations at 37 °C.

removal of the TIMP-2 C-terminal domain results in a 6-fold decrease in the association rate constant. Removal of the stromelysin C-terminal domain had little effect upon these patterns of interactions. The deletion of the nine amino acid charged "tail" from the C-terminal domain of TIMP-2 ($\Delta_{187-194}$ TIMP-2) only modified the k_{on} to 0.21 × 10^6 M^{-1} s^{-1}, and variation of ionic strength did not alter the value of k_{on} significantly (data not shown).

As previously reported, inhibition of gelatinase A is characterized by a higher rate constant for TIMP-2 than for TIMP-1. Removal of the C-terminal domain of TIMP results in a dramatic decrease in the rate of inhibition: 50-fold for TIMP-1 and 100-fold for TIMP-2. Removal of the enzyme C-terminal domain also affects the rate constants markedly, with a 300-fold decrease in the rate of inhibition by TIMP-1 and a 100-fold decrease in the case of TIMP-2. Rate constants for inhibition by the truncated TIMPs, N-TIMP-1, and N-TIMP-2, are unaffected by the gelatinase A C-terminal domain.

The hybrid enzymes were also inhibited rapidly by wild-type TIMPs. The hybrid consisting of the N-terminal domain of stromelysin and the C-terminal domain of gelatinase A (N-SL.C-GL) was inhibited at similar rates by TIMP-1 and TIMP-2 with k_{on} values considerably larger than for wild-type stromelysin. Limitations of the sensitivity of the stromelysin assay using the substrate McaPLANvaDpaAR prevented the use of enzyme concentrations lower than 0.3 nM which would permit accurate determination of the latter rate constant. The effect of removal of the TIMP C-terminal domain is to decrease the rate constants by approximately 7-fold for TIMP-1 and over 200-fold for TIMP-2. The rate of inhibition of the other hybrid, N-GL.C-SL, is relatively unaffected by the C-terminal of either TIMP, which was comparable to the behavior of wild-type stromelysin.

DISCUSSION

We previously reported evidence that the N-terminal domain of TIMP-1 is responsible for the inhibition of the matrix metalloproteinases, largely by interactions with the enzyme catalytic domain (Murphy *et al.*, 1991). In the case of gelatinase A, further interactions between the C-terminal domains of either TIMP-1 or TIMP-2 with the enzyme C-terminal domain play an important role in increasing the rate of formation of the inhibitory complex (Murphy *et al.*, 1992b; Willenbrock *et al.*, 1993). In order to assess whether this interaction between the C-terminal domains of MMPs and TIMPs is a general phenomenon, we have now extended the study to a comparative analysis of the inhibition of stromelysin and gelatinase A. We have determined the contribution of the C-terminal domains of enzyme or inhibitor to the rate of inhibition by comparing the full-length proteins with their genetically engineered counterparts that lack the C-terminal domain. The interaction of TIMP with each enzyme was studied by determining the rate constant for the formation of the inhibitory complex. Such an analysis does not provide information on the features of each molecule that are important in determining the stability of the complex. It does however allow us to draw some conclusions about the nature of the initial interaction. In order to do so, it is essential that the changes we have made, exchanging domains between enzymes or expressing individual catalytic domains, have not resulted in structural changes to the domains themselves. We have previously reported that the activation and catalytic activity of the N-terminal domain of both stromelysin and gelatinase A are not altered by the removal of the C-terminal domain (Murphy *et al.*, 1992a,b). In the present work, we have also demonstrated that the properties of the N-terminal domains are not altered in the hybrid enzymes. We are confident, therefore, that differences observed between the full length, truncated, and hybrid enzymes with respect to TIMP binding are not due to conformational changes in the mutant enzymes. The conformation of the truncated form of TIMP-1 has been investigated in some detail by chemical modification studies and susceptibility to trypsin. The inhibitory activity of both forms of TIMP-1 are affected to a similar extent by modification with diethyl pyrocarbonate (Williamson *et al.*, 1993b). Trypsin digestion not only releases the same peptides from the sequence common to both forms of TIMP but also releases them in the same order (Williamson *et al.*, 1993a). Therefore, it has been concluded that the structure of the N-terminal domain of TIMP-1 is not affected by the C-terminal domain. In view of the homology between TIMP-1 and TIMP-2, it is reasonable to assume that the conformation of the TIMP-2 mutant is also correct.

Each reaction studied in the present work has been analyzed for the existence of a rate-limiting step in the reaction pathway due to a conformational change in either of the reagents or the enzyme–inhibitor complex by determining the dependence of the apparent first-order rate constant k on TIMP concentration. The linear dependence observed provides no evidence for an association process more complex than a bimolecular collision. As discussed previously (Willenbrock *et al.*, 1993), although many of the rate constants are lower than would be expected from a diffusion-limited encounter, similar values have been reported for other enzyme interactions (see Hammes, 1982 and references cited therein). In our interpretation of the kinetic data, we therefore assume the simplest model for the interaction of a diffusion-limited bimolecular collision. This model does not preclude the possibility that conformational changes occur after formation of the inhibitory complex to give a final complex in which different interactions are important.

In contrast to our observations on TIMP interactions with gelatinase A, the association rate constant for the TIMP–stromelysin interaction is greater for TIMP-1 than for TIMP-2. In addition, the rate of inhibition of stromelysin is affected to a much lesser extent by the C-terminal domain of either TIMP than is that of gelatinase A, and ionic interactions do not appear to be important in the association of the TIMPs with stromelysin. Removal of the C-terminal domain of stromelysin also does not result in any significant changes in the association rate constants, although a small increase in the k_{on} for inhibition by TIMP-2 is observed. This differs from the results obtained with gelatinase A, in which the C-terminal domain of the enzyme is responsible for a (100–300)-fold increase in the rate of inhibition by TIMP, via interactions with the TIMP C-terminal domain. Thus, the C-terminal domains of the two enzymes appear to be functionally very different with respect to TIMP binding.

The replacement of the C-terminal domain of stromelysin with that of gelatinase A in the N-SL.C-GL mutant results in a dramatic increase in k_{on} values for both full-length TIMPs relative to those for stromelysin, whereas k_{on} values for the truncated TIMPs are unchanged. The results obtained with the wild-type enzymes demonstrated that the TIMP C-terminal domain interacts with both the N-terminal domain of stromelysin and the C-terminal domain of gelatinase A. If the two domains (N-SL and C-GL) share a common binding site on the C-terminal domain of TIMP, then (a) binding of the C-GL domain of the hybrid enzyme would result in incorrect alignment of the enzyme and inhibitor N-terminal domains and (b) there would be competition between the domains of the hybrid enzyme for binding to TIMP. Both effects would result in a lower k_{on} value for the inhibition of N-SL.C-GL than for the inhibition of stromelysin. The observed result of an increase in k_{on} leads us to conclude that the C-terminal domain of TIMP must have a separate binding site for each enzyme. The results also suggest that either there is a great deal of flexibility in the enzyme and/or inhibitor which allows both sets of domains to bind efficiently or the domain shuffling has not altered the distance between the binding sites on the two enzyme domains.

The results obtained with the C-GL.N-SL hybrid differ somewhat. As for N-SL.C-GL, the reactions with the truncated TIMPs are characterized by the same rate constant as the appropriate enzyme N-terminal domain alone, confirming that the N-terminal domain of the TIMPs do not interact with the C-terminal domain of stromelysin. However, the full-length TIMPs have higher k_{on} values than expected. In the case of the full-length TIMP-1, it is the result with truncated gelatinase A that seems to be anomalous, in that the rate constant is 10 times lower than that for N-TIMP-1. It is possible that the presence of the gelatinase C-terminal domain masks a site on the enzyme catalytic domain which interacts with the TIMP-1 C-terminal domain and is unfavorable for TIMP-1 inhibition. It is interesting to note that this site is also apparently masked by the presence of the stromelysin C-terminal domain in the hybrid. There is also an increase in k_{on} for the inhibition of N-GL.C-SL by TIMP-2 when compared to its interaction with N-TIMP-2 and the k_{on} for the N-GL-TIMP-2 interaction. This suggests that there is an interaction between the C-terminal domains of stromelysin and TIMP-2, although it plays a minor role in the association of the enzyme and inhibitor. For wild-type stromelysin, this interaction actually decreases k_{on} slightly, indicating that this low affinity interaction aligns the TIMP-2 inhibitory site incorrectly for the stromelysin–TIMP-2 interaction but correctly for the inhibition of N-GL.C-SL.

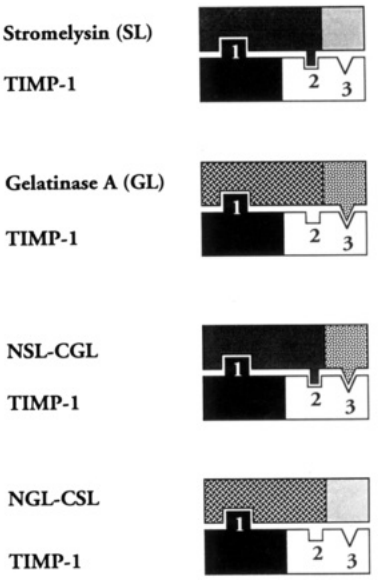

FIGURE 3: Schematic representation of the binding sites involved in formation of inhibitory complexes of TIMP-1 with stromelysin, gelatinase A, and their hybrids, N-SL.C-GL and N-GL.C-SL. The enzymes are represented as N-terminal (left side) and C-terminal domains divided by a vertical line and with different shading. TIMP-1 is denoted as consisting of two major regions made up of the N-terminal and the C-terminal three disulfide-bonded loops. The N-terminal domain contains the inhibitory site and binds the N-terminal catalytic domain of both stromelysin and gelatinase A (and their hybrids) to form a tight inactive enzyme–inhibitor complex (site 1). The C-terminal domain of TIMP-1 also binds to the enzymes to increase the rate of inhibition of each enzyme to differing extents. There are at least two discrete binding sites within this domain, each of which interacts with different enzymes. These are depicted for stromelysin (site 2) and gelatinase A (site 3).

In summary, the N-terminal domain of TIMP binds to the enzyme catalytic domain to inhibit activity (site 1 in Figure 3). This reaction can be quite slow in many cases with second-order rate constants ranging from 5×10^4 to 1.2×10^6 M^{-1} s^{-1} for the interactions reported in the present work. The presence of the C-terminal domain of TIMP increases the rate constant for the association by binding to the N-terminal domain of stromelysin (site 2 in Figure 3) or the C-terminal domain of gelatinase A (site 3 in Figure 3). The binding sites for the two enzymes on the TIMP C-terminal domain are separate entities. The mechanism by which the binding of the TIMP C-terminal domain increases the rate of reaction need be no more complicated than increasing the probability of an interaction between the two N-terminal domains.

ACKNOWLEDGMENT

We thank Mary Harrison for cell culture, Jim Turner for oligonucleotide synthesis, and Richard Harrison and Graham Knight for the generous gifts of substrates.

REFERENCES

Apte, S. S., Mattei, M.-G., & Olsen, B. R. (1993) *Genomics*, in press.

Boone, T. C., Johnson, M. J., De Clerck, Y. A., & Langley, K. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2800–2804.

Cawston, T. E., Galloway, W. A., Mercer, E., Murphy, G., & Reynolds, J. J. (1981) *Biochem. J. 195*, 159–165.

Cawston, T. E., Murphy, G., Mercer, E., Galloway, W. A., Hazleman, B. L., & Reynolds, J. J. (1983) *Biochem. J. 211*, 313–318.

Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C., Bauer, E. A., & Goldberg, G. I. (1988) *J. Biol. Chem. 263*, 6579–6587.

DeClerck, Y. A., Yean, T.-S., Lee, Y., Tokich, J. M., & Langley, K. E. (1993) *Biochem. J. 289*, 65–69.
Docherty, A. J. P., & Murphy, G. (1990) *Ann. Rheum. Dis. 49*, 469–479.
Docherty, A. J. P., Lyons, A., Smith, B. J., Wright, E. M., Stephens, P. E., Harris, T. J. R., Murphy, G., & Reynolds, J. J. (1985) *Nature 318*, 66–69.
Hammes, G. G. (1982) in *Enzyme Catalysis and Regulation*, pp 99–102, Academic Press, New York.
Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51–59.
Howard, E. W., & Banda, M. (1991) *J. Biol. Chem. 266*, 17972–17977.
Kleiner, D. E., Unsworth, E. J., Krutzsch, H. G., & Stetler-Stevenson, W. G. (1992) *Biochemistry 31*, 1665–1672.
Knight, C. G., Willenbrock, F., & Murphy, G. (1992) *FEBS Lett. 296*, 263–266.
Koklitis, P. A., Murphy, G., Sutton, C., & Angal, S. (1991) *Biochem. J. 276*, 217–221.
Leatherbarrow, R. J. (1987) Enzfitter Software, Elsevier Biosoft, Cambridge, U.K.
Lelievre, Y., Bouboutou, R., Boizian, J., Faucher, D., Archard, D., & Cartwright, T. (1990) *Matrix 10*, 292–299.
Matrisian, L. M. (1992) *Bioessays 14*, 455–463.
Morrison, J. P., & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201–301.
Murphy, G., Cawston, T. E., Galloway, W. A., Barnes, M. J., Bunning, R. A. D., Mercer, E., Reynolds, J. J., & Burgeson, R. E. (1981) *Biochem. J. 199*, 807–811.
Murphy, G., Houbrechts, A., Cockett, M. I., Williamson, R. A., O'Shea, M., & Docherty, A. J. P. (1991) *Biochemistry 30*, 8097–8102.
Murphy, G., Allan, J. A., Willenbrock, F., Cockett, M. I., O'Connell, J. P., & Docherty, A. J. P. (1992a) *J. Biol. Chem. 267*, 9612–9618.
Murphy, G., Willenbrock, F., Ward, R. V., Cockett, M. I., Eaton, D., & Docherty, A. J. P. (1992b) *Biochem. J. 283*, 637–641.
Niedzwiecki, L., Teahan, J., Harrison, R. K., & Stein, R. L. (1992) *Biochemistry 31*, 12618–12623.
Okada, Y., Nagase, H., & Harris, E. D. (1986) *J. Biol. Chem. 261*, 14245–14255.
O'Shea, M., Willenbrock, F., Williamson, R. A., Cockett, M. I., Freedman, R. B., Reynolds, J. J., Docherty, A. J. P., & Murphy, G. (1992) *Biochemistry 31*, 10146–10152.
Teahan, J. A., & Stein, R. L. (1990) *FASEB J. 4*, A1977 (Abstr. 1652).
Ward, R. V., Hembry, R. M., Reynolds, J. J., & Murphy, G. (1991) *Biochem. J. 278*, 179–187.
Welgus, H. G., Jeffrey, J. J., Eisen, A. Z., Roswit, W. T., & Stricklin, G. P. (1985) *Collagen Relat. Res. 5*, 167–179.
Willenbrock, F., Crabbe, T., Slocombe, P. M., Sutton, C. W., Docherty, A. J. P., Cockett, M. I., O'Shea, M., Brocklehurst, K., Phillips, J. R., & Murphy, G. (1993) *Biochemistry 32*, 4330–4337.
Williamson, R. A., Smith, B. J., Angal, S., Murphy, G., & Freedman, R. B. (1993a) *Biochim. Biophys. Acta 1164*, 8–16.
Williamson, R. A., Smith, B. J., Angal, S., & Freedman, R. B. (1993b) *Biochim. Biophys. Acta 1203*, 147–154.