Involvement of a region near valine-69 of tissue inhibitor of metalloproteinases (TIMP)-1 in the interaction with matrix metalloproteinase 3 (stromelysin 1)

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Tissue inhibitors of metalloproteinases (TIMPs) inhibit matrix metalloproteinases (MMPs) by forming a 1:1 stoichiometric complex, but the inhibition mechanism of these inhibitors is not known. Here we have investigated the reactive site of TIMP-1 by its proteinase susceptibility before and after forming a complex with MMP-3 (stromelysin 1). When TIMP-1 was allowed to react with human neutrophil elastase, its inhibitory activity was destroyed. This resulted from cleavage of the Val⁶⁹–Cys⁷⁰ bond. However, cleavage of this bond by neutrophil elastase was prevented when TIMP-1 formed a complex with the catalytic

domain of MMP-3, and full TIMP-1 activity was restored after dissociation of the complex at pH 3.0 in the presence of EDTA. These results indicate that the region around Val⁶⁹ closely associates with an active MMP. The three-dimensional structure of the N-terminal domain of TIMP-2 elucidated by NMR studies [Williamson, Martorell, Carr, Murphy, Docherty, Freedman and Feeney (1994) *Biochemistry* 33, 11745–11759] reveals that Val⁶⁹ and Cys⁷⁰ form part of an extended ridge that also includes the N-terminal section of the inhibitor. This region is probably involved in the interaction with the catalytic domains of MMPs.

INTRODUCTION

Matrix metalloproteinases (MMPs), also called matrixins, play a central role in degradation of the extracellular matrix during a number of biological processes such as morphogenesis, embryo implantation and development, wound healing and tissue resorption [1–3]. Enhanced activity of MMPs is linked with many connective tissue diseases such as arthritis, glomerulonephritis, periodontitis, tissue ulcerations and tumour cell invasion and metastasis [2–4]. Currently, 14 members of the matrix family have been identified in humans [5–7] (see ref. [3] for a review); their activities are regulated by endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), which form tight 1:1 molar stoichiometric complexes with MMPs [3,8]. Thus the balance between matrixins and TIMP activity is critical for the catabolism of extracellular matrix.

Four TIMPs have been identified and they share about 40 % identity in sequence including 12 conserved cysteines [8,9]. Human TIMP-1 is a 29–30 kDa glycoprotein consisting of 184 amino acids, whereas human TIMP-2 is a 22 kDa unglycosylated protein with 194 amino acids [3,8]. Human TIMP-3 is comprised of 188 amino acids [10–14]. Chicken TIMP-3 isolated from Rous sarcoma virus-transfected embryonic fibroblasts is not glycosylated [15], but recombinant human and mouse TIMP-3s expressed in mouse myeloma cells are primarily glycosylated inhibitors of molecular mass 27 kDa [16]. More recently, a cDNA clone encoding TIMP-4 has been isolated from a human heart cDNA library [9]. Disulphide bond arrangements of TIMP-1 indicate that it consists of N-terminal (residues 1–124) and C-terminal (residues 127–184) domains [17]. The recombinant N-

terminal domains of TIMP-1 (N-TIMP-1) [18,19] and TIMP-2 (N-TIMP-2) [20,21] expressed in mammalian cells or in *Escherichia coli* demonstrate that MMP-inhibitory activity is located in this domain. TIMP-1 and TIMP-2 can bind tightly to the precursors of MMP-9 (gelatinase B) and MMP-2 (gelatinase A) respectively, but their interactions are through the C-terminal domains of TIMPs and the C-terminal hemopexin-like domains of gelatinases (see refs [3] and [8] for reviews). Thus the TIMP-1–proMMP-9 and the TIMP-2–proMMP-2 complexes are capable of inhibiting active MMPs through the unoccupied N-terminal domain of the inhibitor in the complex [22–24]. The solution structure of N-TIMP-2 determined by NMR spectroscopy indicated that the protein has a five-stranded antiparallel β -barrel and two short α -helices [25]. The topology of this β -barrel is similar to that of proteins of the oligonucleotide/oligosaccharide binding fold family [25]. However, it is not known which residues of TIMP are involved in complex-formation with MMPs and inhibit MMP activity.

We have previously reported that trypsin, chymotrypsin and human neutrophil elastase (HNE) cleave TIMP-1 and destroy MMP-inhibitory activity [26]. Trypsin and chymotrypsin both digest TIMP-1 into several small fragments, whereas HNE cleaves TIMP-1 into two major fragments, suggesting that a single cleavage of the inhibitor leads to its inactivation. This leads us to postulate that the site cleaved by HNE is critical for the association of TIMP-1 with MMPs for their inhibition or is important for the molecular structure. If this region is directly involved in TIMP-1 function, cleavage of TIMP-1 by HNE should be prevented when it forms a complex with an active MMP. This hypothesis was tested using 45 and 28 kDa forms of

Abbreviations used: DFP, di-isopropyl fluorophosphate; HNE, human neutrophil elastase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

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MMP-3 (stromelysin 1) as the target enzyme for TIMP-1. The results presented here indicate that Val⁶⁹ and Cys⁷⁰ are in close contact with active MMP.

EXPERIMENTAL

Materials

Human TIMP-1 was purified from the culture medium of human fetal lung fibroblasts as described previously [27]. Recombinant human TIMP-1 or human proMMP-3 was expressed in Chinese hamster ovary (CHO-K1) cells and purified as described previously [19,28]. ProMMP-3 was activated by chymotrypsin to form a 45 kDa MMP-3. The 28 kDa MMP-3 was obtained by autolysis of the 45 kDa MMP-3; both forms of MMP-3 had the same specific activity as described previously [29]. The 25 kDa MMP-3 was obtained by activating the C-terminal-domain-truncated recombinant proMMP-3 [proMMP-3(Δ C)] with 1.5 mM 4-aminophenylmercuric acetate for 16 h at 37 °C. ProMMP-3(Δ C) was purified from the inclusion body of *E. coli* transformed with the pET3a vector (Novagen) ligated with MMP-3 cDNA with replacement of a stop codon for Pro²⁵⁶ (K. Suzuki, K. Brew and H. Nagase, unpublished work; details are available from H.N. on request). HNE was from Athens Research and Technology, Inc., Athens, GA, U.S.A. Reduced S-[³H]carboxymethylated transferrin for MMP-3 assay was prepared as described previously [30].

Enzyme assays

MMP-3 activity was measured in TNC buffer (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 10 mM CaCl₂, 0.02 % NaN₃, 0.05 % Brij 35) using S-[³H]carboxymethylated transferrin as substrate [30]. The inhibitory activity of TIMP-1 was measured against a known amount of MMP-3 using [³H]carboxymethylated transferrin.

SDS/PAGE

This was performed by the method of Bury [31] under reducing or non-reducing conditions using 10 % total acrylamide. Proteins were stained with Coomassie Brilliant Blue R-250.

N-terminal sequence analysis

Native TIMP-1 or HNE-treated TIMP-1 was subjected to N-terminal sequencing either directly or after separation of the proteolytic fragments by SDS/PAGE (12.5 % acrylamide) under reducing conditions. The TIMP-1 fragments separated by SDS/PAGE were transferred to a Millipore Immobilon PVDF transfer membrane as described by Matsudaira [32]. Proteins located by staining with Coomassie Brilliant Blue R-250 were excised, placed on to a polybrene-treated glass filter and sequenced with an Applied Biosystem 447A pulse liquid sequenator with an on-line model 120A for phenylthiohydantoin analysis.

RESULTS

TIMP-1 purified from the conditioned medium of human skin fibroblasts inhibited both 45 and 25 kDa MMP-3 by forming a 1:1 stoichiometric complex (results not shown). When TIMP-1 was treated with HNE the individual inhibitory activity against MMP-3 was lost in a time- and dose-dependent manner (Figure 1). SDS/PAGE analysis of HNE-treated TIMP-1 under reducing conditions indicated that TIMP-1 was initially cleaved into 16 and 20 kDa fragments, which were then degraded into smaller

Figure 1 Inactivation of TIMP-1 by HNE

Human TIMP-1 (20 µg/ml) was incubated with 10 (●) or 20 (○) µg/ml HNE for the indicated period of time at 37 °C. After inactivation of HNE with 2 mM DFP, residual inhibitory activity of TIMP-1 was measured against MMP-3.

peptides on further incubation as indicated by the gradual loss of the two protein bands as well as the TIMP-1 band (Figure 2). The slower degradation of TIMP-1 by HNE demonstrated by SDS/PAGE compared with the results in Figure 1 was due to the use of a 16.8-fold higher concentration of TIMP-1. In contrast, when TIMP-1 formed a complex with an equimolar amount of the 45 kDa MMP-3 (Figure 2, top, lanes 4–6) or the 25 kDa catalytic domain of MMP-3 (Figure 2, top, lanes 7–9), it was protected from proteolysis by HNE. A small number of TIMP-1 fragments appeared after HNE treatment. This may be due to either a small excess of free TIMP-1 or dissociation of a small amount of TIMP-1 during the reaction. When free TIMP-1 and the TIMP-1–MMP-3 complex that had been treated with HNE were analysed by SDS/PAGE without reduction, all TIMP-1 molecules exhibited the same electrophoretic mobility. This suggests that HNE cleaves a bond within a disulphide loop. On the other hand, when the TIMP-1–MMP-3 complexes were subjected to trypsin digestion, TIMP-1 was degraded into smaller peptides at a rate similar to that of free TIMP-1 (Figure 3). These results suggest that the site at which TIMP-1 is cleaved by HNE is in close contact with the catalytic domain of MMP-3, since the 25 kDa form of MMP-3 protected TIMP-1 from HNE as effectively as the 45 kDa MMP-3.

To confirm further that TIMP-1 bound to MMP-3 is protected from HNE, we examined the activity of TIMP-1 that had been retrieved after HNE treatment. Both free TIMP-1 and the TIMP-1–MMP-3 complex were first treated with 20 µg/ml HNE for 1 h at 37 °C. After inactivation of HNE with 2 mM diisopropyl fluorophosphate (DFP), the samples were treated with 25 mM EDTA at pH 3.0 for 30 min to dissociate the TIMP-1–MMP-3 complex and irreversibly inactivate MMP-3 [33]. After neutralization by dialysis against TNC buffer, the inhibitory activity of TIMP-1 in the samples was determined. As shown in Table 1, the TIMP-1 component of the complex with MMP-3 was completely protected from inactivation by HNE whereas TIMP-1 without MMP-3 lost most of its inhibitory activity.

To identify the HNE cleavage site, both 16 and 20 kDa fragments were transferred to Millipore Immobilon PVDF membranes and subjected to N-terminal sequence analysis. The 20 kDa fragments gave the sequence: Xaa-Gly (45.7)-Tyr (45.7)-Phe (43.9)-His (31.1) (numbers in parentheses are in pmol). The N-terminal sequence of the 16 kDa fragment was Xaa-Thr (16.4)-Xaa-Val (16.5)-Pro (25.3), identical with the N-terminal sequence of uncleaved TIMP-1 (Xaa is a residue that was not identified by sequencing, but corresponds to a disulphide-bonded cysteine in TIMP-1). Direct N-terminal analysis of TIMP-1 treated with HNE also gave the same two sequences. These results indicate

Figure 2 Protection of TIMP-1 from HNE by complex-formation with MMP-3

Top, Free TIMP-1 (336 $\mu\text{g/ml}$) was allowed to react with 20 $\mu\text{g/ml}$ HNE in TNC buffer for 2 h (lane 3) at 37 $^{\circ}\text{C}$. To form TIMP-1–MMP-3 complexes, TIMP-1 (672 $\mu\text{g/ml}$) was mixed with an equal volume of the 45 kDa MMP-3 (1008 $\mu\text{g/ml}$) or the 25 kDa catalytic domain of MMP-3(ΔC) (560 $\mu\text{g/ml}$) in TNC buffer for 18 h at 23 $^{\circ}\text{C}$. The complexes were then treated with 20 $\mu\text{g/ml}$ HNE for 2 h (lanes 5 and 8) or 4 h (lanes 6 and 9) at 37 $^{\circ}\text{C}$. After reaction HNE was inactivated with 2 mM DFP, and the samples were subjected to SDS/PAGE with reduction. Lanes 1, 4 and 7; samples without HNE treatment. Lane 10, MMP-3(ΔC); lane 11, MMP-3(ΔC) treated with 20 $\mu\text{g/ml}$ HNE for 4 h at 37 $^{\circ}\text{C}$. Bottom, Free TIMP-1 (lanes 1 and 2), the TIMP-1–MMP-3 (45 kDa) complex (lanes 3 and 4) and the TIMP-1–MMP-3(ΔC) complex (lanes 5 and 6) were treated with HNE for 2 h at 37 $^{\circ}\text{C}$ as above, and the samples were run on SDS/PAGE without reduction. Lanes 1, 3 and 5, the samples without HNE treatment; lanes 2, 4 and 6, the samples treated with HNE.

that HNE specifically cleaved the Val⁶⁹–Cys⁷⁰ bond of TIMP-1 and inactivated the inhibitor.

DISCUSSION

TIMPs are important in the regulation of extracellular matrix because of their unique abilities to inhibit matrixins [1–3,8]. TIMPs bind to MMPs non-covalently, forming very tight complexes with K_i values in the range 2–1000 pM [8]. TIMP is not cleaved by the target enzyme, and TIMP activity can be completely recovered by dissociating the complex by treatment with EDTA at acidic pH [33]. Several studies aimed at identifying the functional site(s) in TIMP-1 have been reported. Williamson et al. [34] proposed, on the basis of chemical-modification studies, that His⁹⁵ of the N-terminal domain was essential for the inhibition of MMPs, but O'Shea et al. [35] reported that the mutation of His⁹⁵ to Glu or Ala did not alter the inhibitory activity. The latter investigators, on the other hand, reported that mutations of His⁷ and Gln⁹ to Ala increased K_i values about 2–6-fold against MMP-7 (matrilysin), but these are relatively minor changes. Therefore these residues do not contribute

in a major way to the interaction between TIMP-1 and matrixin. Bodden et al. [36] examined a series of peptides corresponding to the fragments of TIMP-1 for their abilities to compete with TIMP-1 for inhibition of collagenase (MMP-1), and proposed that the region surrounding the second 'disulphide knot' (Cys¹³–Cys¹²⁴ and Cys¹²⁷–Cys¹⁷⁴) located at the junction of the N-terminal and the C-terminal domains plays a major role in this activity. However, their studies also indicated that a peptide corresponding to residues 165–184 in the C-terminal domain was as effective as the peptides corresponding to the second disulphide knot [36], yet the C-terminal domain of TIMP-1 does not bind to the active site of matrixins [18–21]. On the other hand, Hanglow et al. [37] reported that a peptide corresponding to residues 70–97 inhibited MMP-3 with an IC_{50} value of 47 μM , but the nature of the interaction of this peptide and MMP-3 is not known. Thus there are a number of discrepancies among the proposed reactive sites of TIMP-1 with matrixins from those studies. In this study we have examined the sites of interaction of TIMP-1 and MMP-3 using the proteinase susceptibility of free TIMP-1 and its protection from proteolysis by forming a complex with a target enzyme. Trypsin, chymotrypsin and HNE have been shown to

Figure 3 Degradation of TIMP-1 and the TIMP-1-MMP-3 complex by trypsin

Free TIMP-1 (lanes 1–3), the TIMP-1-MMP-3 (45 kDa) complex (lanes 4–6) and the TIMP-1-MMP-3 (28 kDa form autoprocessed from the 45 kDa MMP-3) complex (lanes 7–9) were treated with trypsin (a final concentration of 20 $\mu\text{g/ml}$) for 1 h (lanes 2, 5 and 8) or 2 h (lanes 3, 6 and 9) and 37 °C. After inactivation of trypsin with 2 mM DFP, the samples were run on SDS/PAGE (10% acrylamide) under the reducing conditions. Lanes 10 and 11, MMP-3 (28 kDa) without and with trypsin (20 $\mu\text{g/ml}$) treatment at 37 °C for 2 h respectively.

Table 1 Recovery of TIMP-1 activity after HNE treatment

TIMP-1 (25 $\mu\text{g/ml}$) was mixed with an equal volume of either TNC buffer or the 45 kDa MMP-3 (39.2 $\mu\text{g/ml}$) for 4 h at 23 °C. The mixtures were then allowed to react with HNE (20 $\mu\text{g/ml}$) for 1 h at 37 °C. After inactivation of HNE with 2 mM DFP, the samples were treated with 25 mM EDTA in 0.1 M citrate, pH 3.0, for 30 min at 23 °C. The samples were then dialysed against TNC buffer, and the TIMP-1 activity was determined against MMP-3 activity.

Sample	Treatment	Recovered TIMP-1 activity (%)
TIMP-1	Without HNE	100
TIMP-1-MMP-3 complex	Without HNE	85
TIMP-1	With HNE	5
TIMP-1-MMP-3 complex	With HNE	93

Figure 4 The HNE cleavage site of TIMP-1 superimposed with the solution structure of N-TIMP-2

Val⁶⁹ and Cys⁷⁰ are illustrated in the solution structure of N-TIMP-2 determined by Williamson et al. [25]. The arrow indicates the bond cleaved by HNE. The co-ordinates of N-TIMP-2 were kindly provided by Dr. Richard A. Williamson of the University of Kent.

cleave TIMP-1 readily, but the former two enzymes cleave TIMP-1 at multiple sites. In contrast, HNE initially hydrolyses the inhibitor at a single site, the Val⁶⁹-Cys⁷⁰ bond. This cleavage of TIMP-1 inactivates the inhibitor. Similarly, TIMP-1 that is bound to proMMP-9 (progelatinase B) through their C-terminal domains is also inactivated by HNE without significant destruction of proMMP-9, as the N-terminal domain of TIMP-1 is exposed in this complex [38]. From these observations we have postulated that the integrity of the HNE-susceptible region is important for the interaction with matrixins. Strong evidence that the region around the Val⁶⁹-Cys⁷⁰ bond is close to the site involved in MMP binding is provided by the fact that this bond was protected from proteolysis by HNE when TIMP-1 forms a complex with the catalytic domain of MMP-3. This notion is also supported by mutants with replaced Val⁶⁹ that show greatly reduced TIMP-1 reactivity against MMP-3(ΔC) (W. Huang, Q. Meng, H. Nagase and K. Brew, unpublished work).

Val⁶⁹ and Cys⁷⁰ are covalently joined to the N-terminus of the protein through the Cys⁷⁰-Cys¹ disulphide bond. Together these residues and the N-terminal part of the polypeptide chain form a continuous surface-exposed ridge in the solution NMR structure of TIMP-2 (Figure 4). This region appears to be a good candidate for being at least part of the interaction site for MMPs. Functionally active TIMPs require that the part of the chain before and including Val⁶⁹ must be covalently attached to the N-terminus, since cleavage of the Val⁶⁹-Cys⁷⁰ bond by proteolysis eliminates inhibitory activity. Disruption of the Cys¹-Cys⁷⁰ disulphide bond by mutagenesis essentially eliminates the inhibitory activity of TIMP-1 ([39]; W. Huang, Q. Meng, H. Nagase and K. Brew, unpublished work). MMPs have a relatively elongated substrate-binding site which can be occupied by a peptide chain in an extended conformation that forms multiple hydrogen bonds between its peptide groups and residues in the binding site in a manner similar to an antiparallel β -structure [40,41]. The surface ridge in TIMP-1 could conceivably fit into this substrate-binding groove. The structure of this region in TIMPs is constrained by the presence of two disulphide bonds, Cys¹-Cys⁷⁰ and Cys³-Cys⁹⁹ (residue numbers are in TIMP-1), together with a conserved Pro at position 5. Whether this

apparent rigidity is functionally important in blocking the catalytic action of MMPs is being investigated by a series of mutagenesis studies.

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