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The purification of tissue inhibitor of metalloproteinases-2 from its 72 kDa progelatinase complex

Demonstration of the biochemical similarities of tissue inhibitor of metalloproteinases-2 and tissue inhibitor of metalloproteinases-1

Robin V. WARD,* Rosalind M. HEMBRY, John J. REYNOLDS and Gillian MURPHY
Department of Cell and Molecular Biology, Strangeways Research Laboratory, Cambridge CB1 4RN, U.K.

Human gingival fibroblasts in culture were shown to secrete a 72 kDa progelatinase, of which a proportion in the medium was found to be complexed with tissue inhibitor of metalloproteinases-2 (TIMP-2). A purification procedure was devised to purify free enzyme and inhibitor. We also describe the purification of both 95 kDa progelatinase bound to TIMP-1 and free 95 kDa progelatinase from the medium of U937 cells. A polyclonal antiserum to TIMP-2 was prepared and it was shown that TIMP-1 and TIMP-2 are antigenically distinct. The ability to form stable complexes and the relative inhibitory activities of TIMP-1 and TIMP-2 towards 95 kDa and 72 kDa gelatinases, collagenase, stromelysins 1 and 2 and punctuated metalloproteinase were determined; only minor differences were found. Complex-formation between TIMP-2 and 72 kDa progelatinase was demonstrated not to reduce the metalloproteinase-inhibitory activity of TIMP-2, a finding that led to the characterization of high-molecular-mass TIMP activity. Competition experiments between progelatinases and active gelatinases for TIMPs indicated that the affinity of TIMPs for progelatinases is weaker than that for active gelatinases. In a study of the effects of TIMP-1 and TIMP-2 on progelatinase self-cleavage we found that both TIMP-1 and TIMP-2 inhibit the conversion of 95 kDa and 72 kDa progelatinases and prostromelysin into lower-molecular-mass forms. TIMP capable of complexing with progelatinase was shown to be no more efficient an inhibitor of gelatinase self-cleavage than TIMP not able to complex with progelatinase.

INTRODUCTION

The matrix-degrading metalloproteinases are a family of zinc-dependent endopeptidases that have the combined ability to degrade the components of connective-tissue matrices (reviewed by Docherty & Murphy, 1990). These proteinases are synthesized and secreted by connective-tissue cells and are known to be important both in normal remodelling processes (Brown *et al.*, 1989) and in the accelerated destruction occurring in many diseases, including the arthritides (Case *et al.*, 1989*a*,*b*; Okada *et al.*, 1989; Hasty *et al.*, 1990). Regulation of matrix metalloproteinases is stringent, occurring not only at the level of gene expression but extracellularly, following secretion, by the actions of activators of proenzyme forms and of inhibitors of active forms (Murphy & Docherty, 1988).

A major type of matrix metalloproteinase inhibitor that has been clearly identified is the tissue inhibitor of matrix metalloproteinases, TIMP (Docherty *et al.*, 1985). More recently two forms of TIMP have been delineated. TIMP-1, a 30 kDa glycoprotein, is synthesized by most connective-tissue cells as well as by macrophages and is found in most body fluids (reviewed by Cawston, 1986). It acts specifically against the matrix metalloproteinases, forming essentially irreversible complexes, and has no activity against other classes of metalloendopeptidases. TIMP-1 has been shown to bind to the latent proenzyme form of 95 kDa gelatinase (also called type IV collagenase; Wilhelm *et al.*, 1989), although the physiological significance of this is not known. Both immunohistochemical and hybridization studies *in situ* (Chowcat *et al.*, 1988; Brown *et al.*, 1989; Nomura *et al.*, 1989; Flenniken & Williams, 1990) and the use of cell-culture models of degradation (Mignatti *et al.*, 1986; Gavrilovic *et al.*, 1987) have indicated that TIMP-1 has a key role in the regulation of matrix metalloproteinase activity. TIMP-2 has more recently been isolated (Stetler-Stevenson *et al.*, 1989) from human melanoma cells as a 21 kDa protein bound to the proform of 72 kDa gelatinase and from bovine aortic endothelial cells as a 27.5 kDa protein (De Clerck *et al.*, 1989). The distribution of TIMP-2 is not well documented, but it has been isolated from articular-cartilage extracts (Bunning *et al.*, 1984; Murray *et al.*, 1986).

Both TIMP-1 (Docherty *et al.*, 1985) and TIMP-2 (Boone *et al.*, 1990) have been cloned from a number of species. Although their overall primary sequence identity is only about 40%, the cysteine residues that form six disulphide bonds in TIMP-1 (Williamson *et al.*, 1990) are completely conserved, as are other key residues thought to be important in their activity, suggesting that they act in a similar way.

In the present paper we describe protocols for the purification of the free forms of TIMP-2, 72 kDa progelatinase and 95 kDa progelatinase. We describe their interactions and complex-formation and compare the relative activities of TIMP-1 and TIMP-2 on the known metalloproteinases. We also assess the effect of these inhibitors on the activation of prometalloproteinases.

MATERIALS AND METHODS

Materials

Phorbol 12-myristate 13-acetate, 4-aminophenylmercuric acetate, 4-chloro-1-naphthol, concanavalin A–Sepharose, gelatin–Sepharose and CNBr-activated Sepharose 4B were purchased from Sigma Chemical Co. DEAE-Sepharose and Sephacryl S-200 were from Pharmacia. Peroxidase-conjugated anti-(sheep immunoglobulin) antibodies were from Dakopatts.

Abbreviations used: TIMP, tissue inhibitor of metalloproteinases; PUMP, punctuated metalloproteinase.
*To whom correspondence should be addressed.

Bolton & Hunter reagent was from Amersham International. Purified recombinant TIMP-1 was generously donated by Peter Koklitis and Saroj Angal, Celltech Ltd., Slough, Berks., U.K., and natural TIMP-1 was purified from cultures of human lung fibroblasts (Docherty *et al.*, 1985). The two sources of purified TIMP-1 had identical specific activities against matrix metalloproteinases. A monoclonal antibody to TIMP-1, MAC 015, was prepared and characterized as described by Cooksley *et al.* (1990). Recombinant prostromelysin-1 was prepared as described by Docherty & Murphy (1990), and procollagenase, prostromelysin-2 and proPUMP were prepared as described by Cockett *et al.* (1990), except that mouse myeloma cells were used for the expression of cDNAs.

Cell culture

Human gingival fibroblasts were grown from gingival explants as described previously (Heath *et al.*, 1982). Fibroblasts were grown to confluence in Dulbecco's modified Eagle's medium (Gibco) containing 10% (v/v) fetal-calf serum. For the collection of conditioned medium, cultures were washed with fresh Dulbecco's modified Eagle's medium to remove fetal-calf serum and cultured for 48 h in Dulbecco's modified Eagle's medium containing 0.2% (w/v) lactalbumin hydrolysate, 10 ng of phorbol 12-myristate 13-acetate/ml and 3% (w/v) pig mononuclear-cell cytokines partially purified by Ultrogel AcA 34 gel-filtration chromatography (Saklatvala *et al.*, 1983). The monocytic cell line, U937, was grown to about 10^6 cells/ml in RPMI medium (Gibco) containing 10% (v/v) fetal-calf serum. Before the collection of conditioned media, cultures were stimulated for 16 h by the addition of 50 ng of phorbol 12-myristate 13-acetate/ml to the medium. Cells were then pelleted and resuspended in fresh RPMI medium containing 0.2% (w/v) lactalbumin hydrolysate and 20 ng of phorbol 12-myristate 13-acetate/ml for a period of 96 h.

Purification of 72 kDa progelatinase and TIMP-2

Human gingival fibroblast conditioned medium (6.8 litres) was adjusted to 10 mM-Tris/HCl buffer, pH 7.6, containing 2 mM-phenylmethanesulphonyl fluoride and 0.02% NaN_3. This medium was concentrated to 100 ml on an Amicon Hollow Fibre Concentrator and chromatographed on a DEAE-Sepharose column (21 cm × 2.2 cm) equilibrated with 25 mM-Tris/HCl buffer, pH 7.6, containing 10 mM-$CaCl_2$ and 0.04% Brij 35 (TCB buffer). Gelatinase activity was eluted with 0.5 M-NaCl in TCB buffer. The pool of gelatinase activity was adjusted to 1 M-NaCl in TCB buffer and applied to a gelatin–Sepharose column (12 cm × 1 cm) equilibrated in TCB buffer containing 1 M-NaCl. Gelatinase activity was eluted by the addition of 10% (v/v) dimethyl sulphoxide to the equilibration buffer. Fractions containing gelatinase activity were pooled and chromatographed on a concanavalin A–Sepharose column (2 cm × 1 cm) equilibrated in TCB buffer containing 1 M-NaCl. Concanavalin A-binding proteins were eluted with 0.5 M-methyl α-D-mannoside in the equilibration buffer. Fractions containing gelatinase activity, which did not bind to concanavalin A, were pooled and chromatographed on a Sephacryl S-200 column (92 cm × 1.5 cm) in TCB buffer containing 1 M-NaCl. Fractions from this gel-filtration step were pooled into either a free 72 kDa gelatinase pool or a 72 kDa gelatinase–TIMP-2 pool as ascertained by SDS/PAGE and gelatinase assays. The 72 kDa gelatinase–TIMP-2 pool was concentrated to 3 ml (Amicon Centricon 10) and adjusted to 150 mM-glycine/HCl buffer, pH 2.8, containing 25 mM-EDTA. This pool was then incubated for 30 min at 45 °C before gel filtration on an Ultrogel AcA 44 column (90 cm × 1.5 cm) equilibrated in 50 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EDTA, 0.04% Brij 35 and 1 M-NaCl. TIMP-2-containing fractions were pooled and concentrated before storage at −70 °C.

Purification of 95 kDa progelatinase

U937-cell-conditioned medium (4.2 litres) was adjusted to 10 mM-Tris/HCl buffer, pH 7.6, containing 2 mM-phenylmethanesulphonyl fluoride and 0.02% NaN_3 before concentration to a final volume of 100 ml on an Amicon Hollow Fibre Concentrator. The concentrated conditioned medium was chromatographed on a gelatin–Sepharose column (12 cm × 1 cm) equilibrated in TCB buffer containing 1 M-NaCl. Gelatin-bound proteins were eluted by the addition of 10% (v/v) dimethyl sulphoxide to the equilibration buffer. Fractions containing gelatinase activity were pooled and chromatographed on a column (1 cm × 1 cm) of monoclonal antibody to TIMP-1 (MAC 015) immobilized on Sepharose 4B in TCB buffer containing 1 M-NaCl. Non-binding proteins (TIMP-free 95 kDa progelatinase) from this column were collected and stored at −70 °C. Bound proteins were eluted with 100 mM-glycine/HCl buffer, pH 2.5, containing 1 M-NaCl and 0.04% Brij 35 followed by rapid Tris neutralization and storage at −70 °C.

Electrophoresis, zymography and electroblotting

The purity of samples was analysed by electrophoresis on 8–12% (w/v) polyacrylamide gels (Laemmli & Favre, 1973). Proteins were detected either by staining with Coomassie Brilliant Blue or by silver staining (Merril *et al.*, 1981). Gelatin-degrading activity was assessed by electrophoresis on non-reducing SDS/10%-polyacrylamide gels (Laemmli & Favre, 1973) incorporating 0.5 mg of gelatin/ml (Heussen & Dowdle, 1980). Metalloproteinase-inhibitory activity was detected by incubating such gels for 1 h at 37 °C in a preparation of active rabbit skin gelatinase (7 units/ml) before incubation overnight in TCB buffer at 25 °C. For electroblotting the proteins were transferred from the gel to nitrocellulose at 100 mA and 30 V overnight (Towbin *et al.*, 1979). Nitrocellulose papers were blocked for 1 h at 37 °C with 10 mM-Tris/HCl buffer, pH 7.4, containing 150 mM-NaCl, 1% BSA, 0.5% casein and 0.05% Tween 20. Blocked nitrocellulose was incubated with anti-TIMP-1 serum (prepared as indicated above) at 100 μg/ml or anti-TIMP-2 serum at 200 μg/ml for 4 h at 25 °C. Nitrocellulose was re-blocked and incubated with 0.1% peroxidase-conjugated rabbit anti-(sheep immunoglobulin) antibodies for 1 h at 25 °C. Reactive bands were detected with 4-chloro-1-naphthol.

Enzyme and inhibitor assays

Collagen (collagenase)-, gelatin (gelatinases)- and casein (stromelysins and PUMP)-degrading activities were all assayed as described previously (Galloway *et al.*, 1983). One unit of enzyme degrades 1 μg of substrate/min. For the purification of TIMP-2, activity was measured by inhibition of purified 72 kDa human fibroblast gelatinase activity on gelatin. One unit of TIMP inhibits 2 units of gelatinase by 50%. For the other metalloproteinases the relevant substrate was used and 1 unit of inhibitor was defined as that inhibiting 2 units of enzyme by 50%.

Determination of protein concentration

Protein concentrations were estimated from A_{280} measurements. For TIMP-1 the $A^{1\%}_{1\,cm} = 10$ (determined by total amino acid analysis and A_{280} measurement; S. Angal, unpublished work). Because of the similarity in structure and sequence of TIMP-2 to TIMP-1 the same absorption coefficient was used for TIMP-2.

Preparation of an antiserum to TIMP-2

Purified human fibroblast TIMP-2 was used as an antigen to raise an antiserum in a sheep. A 102 μg sample of TIMP-2 was emulsified in an equal volume of complete Freund's adjuvant and injected intramuscularly on day 0, and a further 50 μg was injected on day 29. Blood was taken from the sheep 7, 11 and 14 days after the second injection. Immunoglobulins were prepared from the serum of the 14-day bleed by triple precipitations with 0.67 vol. of 4 M-$(NH_4)_2SO_4$ pH 8.0, at room temperature. The anti-TIMP-2 serum was further purified by adsorption on immobilized TIMP-2 followed by elution according to the method of Talian *et al.* (1983). The polyclonal antibody to TIMP-1 used in this study was prepared and purified by the method of Hembry *et al.* (1985).

Inhibitor-complex studies

Purified human fibroblast TIMP-2 and human recombinant TIMP-1 were radiolabelled with ^{125}I by using the method of Bolton & Hunter (1973), and repurified by gel filtration on Sephacryl S-200. ^{125}I-labelled TIMP-1 and TIMP-2 were analysed by reverse zymography for activity and by conventional SDS/PAGE under reducing conditions followed by autoradiography. Iodination by mild acylation has previously been shown not to modify TIMP specific activity (Murphy *et al.*, 1989*a*). Gel-filtration experiments were conducted with approx. 200 ng of TIMP (8×10^4 d.p.m.). TIMP–enzyme complexes were formed with a molar excess of enzyme incubated for 30 min at 25 °C in 50 mM-Tris/HCl buffer, pH 7.5, containing 1.0 M-NaCl, 10 mM-$CaCl_2$ and 0.04% Brij 35 (final volume 1 ml) before gel filtration on a Sephacryl S-200 column (92 cm × 1.5 cm) in a buffer of identical composition. Radioactivity was assessed by counting whole fractions in a Packard Multi Prias γ-radiation counter (70% efficiency). Molecular masses were determined from the gel-filtration column by calibration with proteins of known molecular mass. These were yeast alcohol dehydrogenase (150 kDa), BSA (68 kDa), soya-bean trypsin inhibitor (22 kDa) and cytochrome *c* (12.5 kDa).

Activation studies

Proenzymes were incubated with 2 mM-4-aminophenylmercuric acetate in 10 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-$CaCl_2$ and 0.04% Brij 35 at either 25 °C or 35 °C for periods up to 24 h before analysis by SDS/PAGE. To assess the effects of purified natural TIMP-1 and TIMP-2, proenzymes were incubated with either 2 mol of inhibitor/mol of enzyme or a mixture of 1 mol of each inhibitor/mol of enzyme for 30 min at 25 °C before the addition of 4-aminophenylmercuric acetate. Metalloproteinase self-cleavage was also studied under conditions in which 2 mol of prometalloproteinase/mol of inhibitor was present.

RESULTS

Purification of TIMP-2 and 72 kDa gelatinase

Human gingival fibroblasts cultured in the presence of partially purified mononuclear-cell cytokines secrete two gelatinolytic activities, of molecular masses 95 kDa and 72 kDa respectively on reducing SDS/PAGE, and two metalloproteinase inhibitors (Fig. 1 and Table 1). Most of the inhibitory activity is due to the 30 kDa glycoprotein TIMP-1 that has been previously described (Murphy *et al.*, 1981; Docherty *et al.*, 1985). This inhibitor is largely in the free form, but a proportion is bound to 95 kDa progelatinase. A second 23 kDa inhibitor is produced in smaller amounts and is bound to a proportion of the 72 kDa progelatinase in the culture medium. This inhibitor, which had the

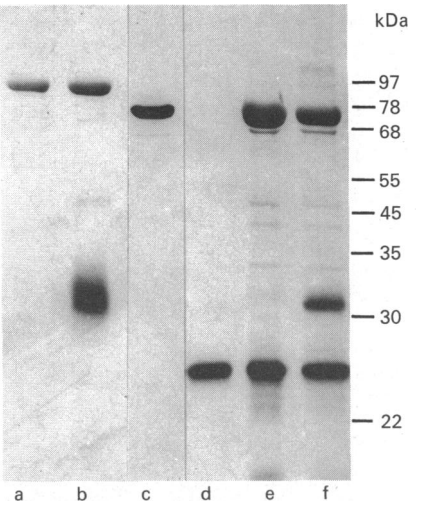

Fig. 1. Analysis of the purification of 95 kDa and 72 kDa progelatinases and TIMP-2

Samples from the purification of progelatinases and TIMP-2 were loaded on to an SDS/10%-polyacrylamide gel under reducing conditions. Materials from the purification of 95 kDa progelatinase from U937-cell-conditioned medium were (lane a) 0.8 μg of purified 95 kDa progelatinase and (lane b) 2.0 μg of gelatin–Sepharose-binding pool. Materials from the purification of 72 kDa progelatinase and TIMP-2 from human gingival fibroblasts were (lane c) 0.4 μg of purified 72 kDa progelatinase, (lane d) 0.3 μg of purified TIMP-2, (lane e) non-binding pool from concanavalin A–Sepharose and (lane f) gelatin–Sepharose-binding pool. Positions of molecular-mass standards are indicated.

Table 1. Purification of 72 kDa human fibroblast gelatinase and TIMP-2

The protocol for the gelatinase is shown in part (*a*) and that for the subsequent purification of TIMP-2 is shown in part (*b*). For experimental details see the text.

Part (*a*)

Purification stage	Total gelatinase activity (units)	Specific gelatinase activity (units/mg)	Purification (fold)
Concentrated conditioned medium	61000	84	–
DEAE-Sepharose fraction	221000	398	4.7
Gelatin–Sepharose fraction	80000	11600	138
* Concanavalin A–Sepharose fraction (see part *b*)	72000	16000	190
Sephacryl S-200 fraction (free gelatinase pool)	61000	22700	270
Sephacryl S-200 fraction (TIMP–gelatinase pool)	12000	8800	104

Part (*b*)

Purification stage	Total TIMP-2 inhibitory activity (units)	TIMP-2 specific inhibitory activity (units/mg)
* Con-A–Sepharose fraction (non-binding pool)	14000	3250
Ultrogel AcA 44 fraction	7900	31800

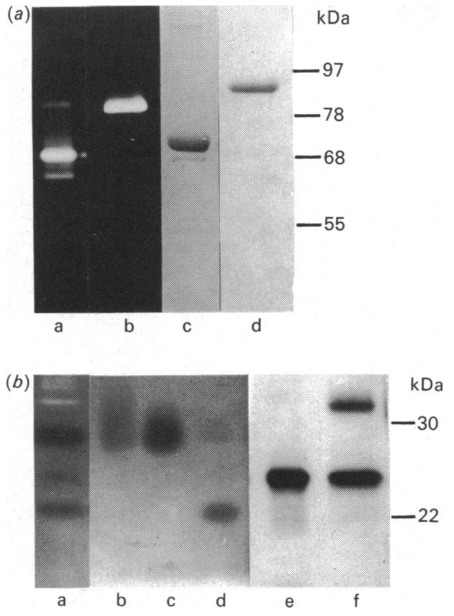

Fig. 2. Detection of (*a*) 95 kDa and 72 kDa gelatinolytic activity and (*b*) TIMP-1 and TIMP-2 inhibitory activity

(*a*) Detection of 95 kDa and 72 kDa gelatinolytic activity. Samples from the gelatin–Sepharose-binding pool of (lane a) human-gingival-fibroblast-conditioned medium and (lane b) U937-cell-conditioned media were electrophoresed under non-reducing conditions on an SDS/10%-polyacrylamide gel containing gelatin. For comparison the protein staining pattern was assessed under reducing conditions on a silver-stained SDS/10%-polyacrylamide gel for each of these two pools (lanes c and d respectively). Positions of molecular-mass standards are indicated. Under non-reducing conditions the 95 kDa and 72 kDa gelatinases run respectively at the slightly lower molecular masses of 92 kDa and 66 kDa. (*b*) Detection of TIMP-1 and TIMP-2 inhibitory activity. Metalloproteinase-inhibitory activities were detected in crude conditioned media and purified samples analysed by inhibitor gel zymography under non-reducing conditions (see the Materials and methods section). The samples were (lane a) crude human-gingival-fibroblast-conditioned medium, (lane b) crude-U937-cell-conditioned medium, (lane c) 4 ng of purified natural human TIMP-1 and (lane d) 2 ng of purified TIMP-2. For comparison the silver-staining protein pattern was assessed under reducing conditions for (lane e) 0.4 μg of purified TIMP-2 and (lane f) gelatin–Sepharose-binding pool of human-gingival-fibroblast-conditioned medium. Positions of molecular-mass standards are indicated.

characteristics of TIMP-2 (see below), was purified from conditioned culture medium by using the protocol described in the Materials and methods section (Table 1, part *b*). The progelatinase activities, of which the 95 kDa form was the smaller proportion, were separated from the other metalloproteinases and free TIMP by using DEAE-Sepharose followed by gelatin–Sepharose affinity chromatography. TIMP-1 and TIMP-2 bound to progelatinases also bound to this matrix, and the eluate was shown to contain both proteinases and both inhibitors (Figs. 2*a* and 2*b*). The 95 kDa progelatinase with TIMP-1 bound to it was separated from the free 72 kDa enzyme and its complex with TIMP-2 by using concanavalin A–Sepharose chromatography (95 kDa gelatinase and TIMP-1 are mannose-containing glycoproteins whereas 72 kDa gelatinase and TIMP-2 are unglycosylated). Free 72 kDa progelatinase (approx. 75% of total gelatinase) was separated from the inhibitor-complexed form (approx. 25% of total gelatinase) on Sephacryl S-200. It was shown to be free of TIMP-2 by substrate gel analysis. The inhibitor was subsequently dissociated from the progelatinase by treatment at pH 2.8 in the presence of the chelating agent EDTA, followed by chromatography on Sephacryl S-200 in the presence of EDTA (Fig. 1). The progelatinase was found to be partially activated and partially denatured by this treatment and was discarded. By means of this procedure the TIMP-2 was purified to a specific activity of 31800 units/mg with respect to gelatinase inhibition. It had a molecular mass of 23 kDa by SDS/PAGE under reducing conditions.

Purification of 95 kDa progelatinase

The 95 kDa progelatinase was purified from U937-cell-conditioned medium as described in the Materials and methods section. A short purification procedure for 95 kDa progelatinase was possible because of the presence of only trace amounts of 72 kDa progelatinase (Figs. 1 and 2*a*). Analysis of the gelatin–Sepharose-binding pool identified a 30 kDa protein bound to the 95 kDa progelatinase (Fig. 1). The use of a monoclonal anti-TIMP-1–Sepharose column both identified the 30 kDa protein as TIMP-1 and facilitated the separation of TIMP-1 complexed to 95 kDa progelatinase (approx. 65% of total progelatinase) from free progelatinase. The separation of TIMP-1-bound progelatinase from free progelatinase brought about a significant increase in the specific activity of the final pool of free progelatinase (Table 2). The final preparation of 95 kDa progelatinase was shown to be free from TIMP-1 by e.l.i.s.a., which has been described in detail elsewhere (Cooksley *et al.*, 1990), and by substrate gel analysis, which can detect 0.5 ng of TIMP-1.

Inhibitor properties

Sequence. The metalloproteinase inhibitor dissociated from 72 kDa progelatinase (0.46 nmol) was subjected to *N*-terminal amino acid sequencing (11.4% yield) after reduction and pyridylethylation (Amons, 1987) and found to have the sequence CXCXPVHPQQAF (where X is an undetermined residue). Consequently it seems very probable that the inhibitor is TIMP-2, which has been previously described bound to progelatinase in tumour-cell-conditioned media and its sequence determined (Stetler-Stevenson *et al.*, 1989). For comparison, the *N*-terminal sequence of TIMP-1 was shown to be CTCVPPHPQTAF (Docherty *et al.*, 1985).

Metalloproteinase inhibition. The putative TIMP-2 was compared with TIMP-1 with respect to ability to inhibit active 72 kDa gelatinase, collagenase and stromelysins in the standard assays for these enzymes. Table 3 shows that TIMP-1 is more active against 72 kDa gelatinase and that TIMP-2 is more active against 95 kDa gelatinase. Detailed analysis of TIMP-2 inhibition of 72 kDa gelatinase showed that an enzyme/inhibitor molar ratio of approx. 1:1 is found on extrapolation to 100% inhibition (Fig. 3) and that the deviation from 100% inhibition may be

Table 2. Purification of 95 kDa progelatinase

For experimental details see the text.

Purification stage	Total gelatinase activity (units)	Specific gelatinase activity (units/mg)
Concentrated conditioned medium	6000	3.8
Gelatin–Sepharose fraction	2388	850
Anti-TIMP-1–Sepharose fraction	5285	7550

Table 3. Comparison of the inhibitory activities of TIMP-1 and TIMP-2 against matrix metalloproteinases

Metalloproteinase	Inhibitory activity (units/nmol) TIMP-1	TIMP-2
95 kDa gelatinase	20.6	36
72 kDa gelatinase	1142	742
Interstitial collagenase	30	12.6
Stromelysin-1	40	12.4
Stromelysin-2	2.7	1.5
PUMP-1	16	18

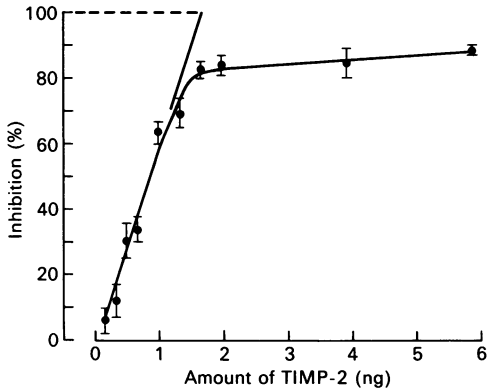

Fig. 3. Inhibition of active 72 kDa gelatinase by TIMP-2

A 4 ng portion of 72 kDa gelatinase pre-activated with 2 mM-4-aminophenylmercuric acetate (for 2 h at 25 °C) was incubated for 30 min at 25 °C with increasing amounts of purified TIMP-2. Activity remaining was assessed by using the ^{14}C-labelled gelatin assay.

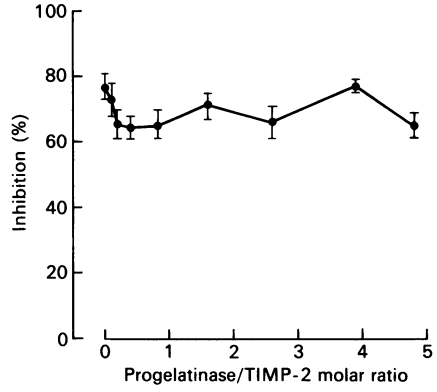

Fig. 4. TIMP-2 activity in the presence of progelatinase

A 3 ng portion of purified TIMP-2 was incubated with increasing amounts of purified 72 kDa progelatinases up to a 4.8-fold molar excess for 30 min at 25 °C. The inhibitory activity of TIMP-2 preincubated in this way was determined by measuring the percentage inhibition of a sample of active 72 kDa gelatinase by using the ^{14}C-labelled gelatin assay. Progelatinase alone (up to a 5-fold molar excess) was shown to have no gelatinolytic activity.

used to calculate an approx. K_d for the complex of 1×10^{-11} M (Green & Work, 1953). The specific activity of TIMP-2 was lower than that of TIMP-1 for collagenase inhibition as well as that of stromelysin-1 and stromelysin-2. Both inhibitors were equally active against PUMP. During the purification of TIMP-2 it was found that TIMP inhibitory activity could be measured in samples that contained an excess of progelatinase (Table 1). Fig. 4 demonstrates that the preincubation of purified TIMP-2 with amounts up to a 4.8-fold molar excess of pure progelatinase did not reduce the inhibitory activity of TIMP-2 against active 72 kDa gelatinase. Similar experiments in which the inhibitory activity of TIMP-2 against active collagenase was measured have demonstrated that the inhibition of collagenase by TIMP-2 could not be blocked by preincubation of TIMP-2 in the presence of a molar excess of progelatinase.

TIMP–metalloproteinase complexes

The use of ^{125}I-labelled TIMPs and gel filtration on Sephacryl S-200 enabled their binding to the proenzyme and active forms of metalloproteinases to be investigated. After iodination TIMP-1 was found to elute from gel filtration on Sephacryl S-200 as a single peak of radioactivity of molecular mass 28 kDa and TIMP-2 as a single peak of 22 kDa. Using this method we have confirmed that both TIMP-1 and TIMP-2 can form a stable enzyme–inhibitor complex with the active forms of 95 kDa and 72 kDa gelatinase, collagenase and stromelysin but not PUMP (Table 4). The proenzyme-binding capacity of TIMP-2 was found to be restricted to 72 kDa gelatinase, generating a proenzyme–inhibitor complex of 77 kDa by gel filtration. A TIMP-1–95 kDa progelatinase complex of 115 kDa was formed, but TIMP-1 would not complex with 72 kDa progelatinase, nor with procollagenase or prostromelysin. Studies with the active form of 72 kDa gelatinase have demonstrated that TIMP-2 could not displace TIMP-1 pre-bound to the active gelatinase when equimolar amounts of TIMP-1 and TIMP-2 were used and that TIMP-1 could not displace TIMP-2 prebound to the active gelatinase.

Two series of experiments were carried out to look at the relative affinities of TIMP-1 and TIMP-2 for active and progelatinases. Fig. 5 demonstrates that TIMP-1 preferentially complexes with 72 kDa active gelatinase rather than 95 kDa progelatinase. Incubation of ^{125}I-labelled TIMP-1 with a molar excess of 95 kDa progelatinase (final concentration 10 nM) for 30 min at 35 °C followed by gel filtration led to the detection of a 115 kDa peak of ^{125}I-TIMP-1 corresponding to a progelatinase–TIMP-1 complex as well as a peak of free TIMP-1. When the sample of 95 kDa progelatinase and TIMP-1 was incubated before gel filtration for a further 15 min at 35 °C with a 0.5 molar excess of active 72 kDa gelatinase (relative to 95 kDa gelatinase) then a single peak of ^{125}I-TIMP-1 was detected at 77 kDa corresponding to a complex of 72 kDa active gelatinase–TIMP-1. In a similar series of experiments in which ^{125}I-labelled TIMP-2 was first preincubated with 72 kDa progelatinase and

Table 4. Molecular masses of metalloproteinase complexes with either TIMP-1 or TIMP-2 (gel filtration on Sephacryl S-200)

Metalloproteinase	Molecular mass of complex (kDa) TIMP-1	TIMP-2
None	28	22
Pro 72 kDa gelatinase	No complex	77
Active 72 kDa gelatinase	85	61
Pro 95 kDa gelatinase	115	No complex
Active 95 kDa gelatinase	108	107
Procollagenase	No complex	No complex
Active collagenase	80	53
Prostromelysin-1	No complex	No complex
Active stromelysin-1	76	86
Active PUMP-1	No complex	No complex

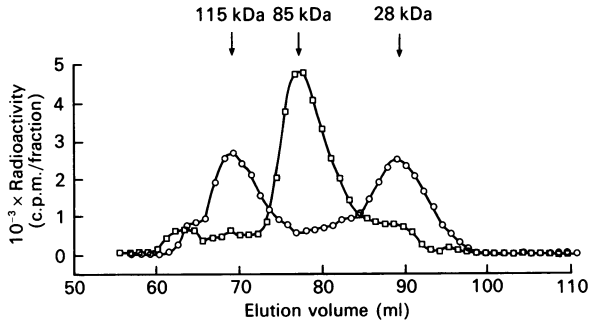

Fig. 5. Transfer of TIMP-1 from 95 kDa progelatinase to 72 kDa active gelatinase

A 68 ng portion of ^{125}I-labelled TIMP-1 was incubated for 30 min at 35 °C with a 1 molar excess of 95 kDa progelatinase (final concentration of TIMP 12 nM) and gel-filtered on Sephacryl S-200. ^{125}I-TIMP was detected in the eluted fractions as shown (○). A second sample prepared in the same way was incubated for a further 15 min with 1.5 mol of 72 kDa active gelatinase/mol of 95 kDa progelatinase before gel filtration. ^{125}I-labelled TIMP was detected as shown (□). The molecular mass of each peak is indicated.

then an equimolar amount of 72 kDa active gelatinase a shift in the ^{125}I-TIMP-2 peak from 77 kDa to 61 kDa could be detected, indicating the transfer of TIMP-2 from the proenzyme form to the active form of 72 kDa gelatinase (results not shown).

Detection of high-molecular-mass TIMPs

Gel filtration of crude conditioned medium from human gingival fibroblasts followed by assays for gelatinase-inhibitory activity showed a peak of TIMP activity of molecular mass about 25 kDa with a minor peak of 80 kDa. In a similar experiment in which the gelatin–Sepharose-binding pool of U937-cell-conditioned medium was subjected to gel filtration, gelatinase-inhibitory activity was eluted at a point corresponding to 110 kDa. This TIMP activity was identified as TIMP-1 by e.l.i.s.a. High-molecular-mass TIMPs have been detected by immunoblotting samples of crude conditioned media from the culture of U937 cells and human gingival fibroblasts as well as at stages during the purification of 72 kDa progelatinase. Immunoblotting of samples of the gelatin–Sepharose-binding pool obtained during the purification of 72 kDa progelatinase with the polyclonal antibody to TIMP-2 demonstrated the presence of TIMP-2 at 66 kDa under non-reducing conditions (Fig. 6). Under reducing conditions this TIMP-2 migrated to a position corresponding to 23 kDa (Fig. 6). Use of the polyclonal antibody to TIMP-1 identified TIMP-1 at 97 kDa and 68 kDa in the same samples. Under reducing conditions this TIMP-1 migrated to a position corresponding to 28 kDa. Fig. 6 also demonstrates that the polyclonal antibody to TIMP-2 failed to recognize TIMP-1 and that the antibody to TIMP-1 also failed to recognize TIMP-2.

Effects of TIMP-1 and TIMP-2 on prometalloproteinase self-cleavage

Incubation of 72 kDa progelatinase with 2 mM-4-aminophenylmercuric acetate for 2 h at 25 °C in the absence of TIMP led to the generation of a 66 kDa active form of specific activity 20000 units/mg. Activation at temperatures higher than 25 °C led to the generation of lower-molecular-mass products with decreased specific activity (results not shown). The 95 kDa progelatinase was most fully activated by incubation with 2 mM-4-aminophenylmercuric acetate for 1.5 h at 35 °C; the resultant 76 kDa active form had a specific activity of 7500 units/mg. In a study

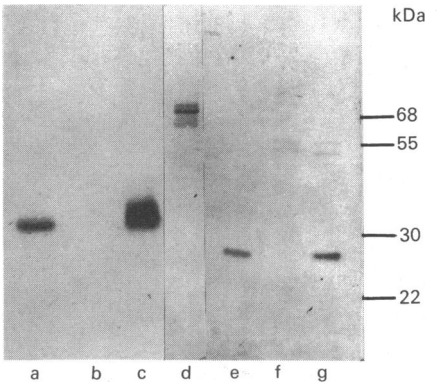

Fig. 6. Immunoblotting of TIMP-1 and TIMP-2

Samples from the purification of TIMP-2 were electrophoresed under reducing and non-reducing (lane d only) conditions on SDS/10%-polyacrylamide gels, electroblotted on to nitrocellulose and probed with antiserum to TIMP-1 (lanes a, b and c) and antiserum to TIMP-2 (lanes d, e, f and g). The samples were (lane a) gelatin–Sepharose-binding pool from the purification of 72 kDa progelatinase, (lane b) 0.25 μg of purified TIMP-2, (lane c) 0.25 μg of purified TIMP-1, (lane d) gelatin–Sepharose-binding pool from purification of 72 kDa progelatinase (non-reducing), (lane e) gelatin–Sepharose-binding pool from purification of 72 kDa progelatinase, (lane f) 0.25 μg of purified TIMP-1 and (lane g) 0.25 μg of purified TIMP-2. Positions of molecular-mass standards are indicated.

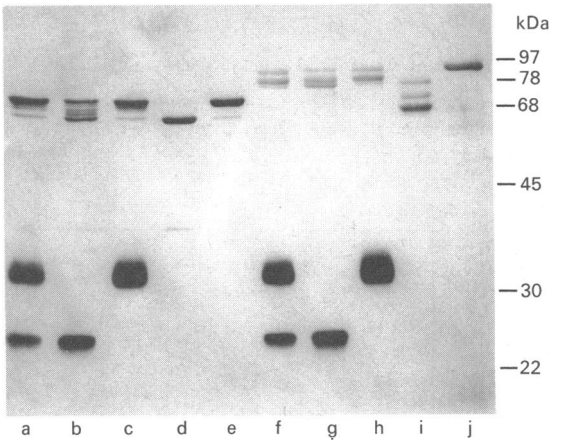

Fig. 7. Self-cleavage of progelatinases in the presence of TIMP-1 and TIMP-2

To assess the effects of TIMP-1 and TIMP-2 on the self-cleavage of 72 kDa progelatinase 0.45 μg samples of progelatinase were incubated with 2 mM-4-aminophenylmercuric acetate for 4 h at 25 °C after preincubation of progelatinase for 30 min at 25 °C with the following amounts of TIMP per mol of progelatinase: lane a, 1 mol each of TIMP-1 and of TIMP-2; lane b, 2 mol of TIMP-2; lane c, 2 mol of TIMP-1; lane d, no TIMP; lane e, no 4-aminophenylmercuric acetate. To assess the effects of TIMP-1 and TIMP-2 on the self-cleavage of 95 kDa progelatinase, 0.45 μg samples of 95 kDa progelatinase were incubated with 2 mM-4-aminophenylmercuric acetate for 4 h at 35 °C after preincubation with the following amounts of TIMP per mol of progelatinase: lane f, 1 mol each of TIMP-1 and of TIMP-2; lane g, 2 mol of TIMP-2; lane h, 2 mol of TIMP-1; lane i, no TIMP; lane j, no 4-aminophenylmercuric acetate. Samples were electrophoresed on an SDS/10%-polyacrylamide gel under reducing conditions and proteins were detected by silver staining. Positions of molecular-mass standards are indicated.

of the effects of TIMPs on the conversion of progelatinases into these lower-molecular-mass products it was found that both TIMP-1 and TIMP-2 (when present as a 1 molar excess) could

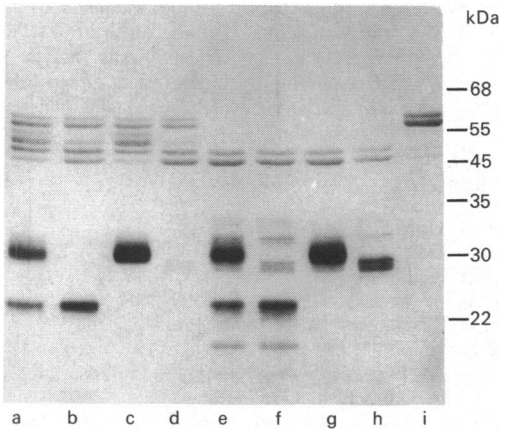

Fig. 8. Self-cleavage of prostromelysin in the presence of TIMP-1 and TIMP-2

To assess the effects of TIMP-1 and TIMP-2 on the activation of human recombinant prostromelysin-1, 0.35 μg samples of prostromelysin were incubated with 2 mM-4-aminophenylmercuric acetate for 2 h at 35 °C after preincubation of prostromelysin for 30 min at 25 °C with the following amounts of TIMP per mol of prostromelysin: lane a, 0.5 mol each of TIMP-1 and TIMP-2; lane b, 0.5 mol of TIMP-2; lane c, 0.5 mol of TIMP-1; lane d, no TIMP. Further stromelysin samples were incubated for 18 h at 35 °C after preincubation of prostromelysin for 30 min at 25 °C with the following (per mol): lane e, 0.5 mol each of TIMP-1 and TIMP-2; lane f, 0.5 mol of TIMP-2; lane g, 0.5 mol of TIMP-1; lane h, no TIMP; lane i, no 4-aminophenylmercuric acetate. Samples were electrophoresed on an SDS/10%-polyacrylamide gel under reducing conditions and proteins were detected by silver staining. Positions of molecular-mass standards are indicated.

significantly inhibit the conversion of 72 kDa progelatinase into 66 kDa gelatinase and of 95 kDa progelatinase into 76 kDa progelatinase (Fig. 7). TIMP-1 appeared to be a more effective inhibitor of the rate of appearance of the 66 kDa form of 72 kDa progelatinase and of the 76 kDa form of 95 kDa progelatinase. Preincubation of either TIMP-1 or TIMP-2 with either of the progelatinases has shown that the ability of a TIMP to bind to the proenzyme form of a gelatinase does not make it a more effective inhibitor of self-cleavage than a TIMP not able to bind to the proenzyme. The combination of TIMP-1 and TIMP-2 was found to be no more effective at inhibiting the 4-aminophenylmercuric acetate-induced conversion of progelatinases into lower-molecular-mass forms than TIMP-1 alone. TIMP-1 and TIMP-2 were found to prevent the complete conversion of 72 kDa progelatinase into 66 kDa gelatinase and of 95 kDa progelatinase into 76 kDa gelatinase after incubation with 2 mM-4-aminophenylmercuric acetate for 18 h. SDS/PAGE analysis of such incubations showed that the appearance of lower-molecular-mass forms was little different to that seen at 2 h time points. In a similar study with recombinant human prostromelysin both TIMP-1 and TIMP-2 were found to inhibit the 4-aminophenylmercuric acetate-induced conversion of the 60–57 kDa doublet of prostromelysin to 46–50 kDa stromelysin (which in the absence of TIMP is active). As in the study with progelatinases, TIMP-1 was the more potent inhibitor of this decrease in molecular mass. Preincubation of prostromelysin with a 1 molar excess of TIMP-1 and/or TIMP-2 was shown to prevent full conversion of the 60–57 kDa proenzyme form into the 46–50 kDa form even after prolonged incubation for 18 h at 35 °C. However, in experiments in which prostromelysin was present as a 1 molar excess relative to TIMP-1 or TIMP-2, full conversion into the 46–50 kDa form was observed after 18 h at 35 °C (Fig. 8).

DISCUSSION

The existence of complexes between 95 kDa and 72 kDa progelatinases and TIMP-1 and TIMP-2 respectively has been described in a number of normal and tumorigenic cell-culture systems (De Clerck *et al.*, 1989; Stetler-Stevenson *et al.*, 1989; Wilhelm *et al.*, 1989; Goldberg *et al.*, 1989), but the biochemical characterization of TIMP-2 has been especially limited by the lack of gelatinases in the uncomplexed form. The present paper describes the purification of 72 kDa progelatinase free of TIMP-2 and of TIMP-2 itself from the conditioned medium of cytokine-stimulated human gingival fibroblasts. We have found that gingival fibroblasts produce an excess of 72 kDa enzyme over inhibitor, thereby allowing separation of the uncomplexed progelatinase from the complex. We were also able to dissociate active TIMP-2 from its progelatinase complex by a method previously devised to separate TIMP-1 from complexes with active metalloproteinase (Murphy *et al.*, 1989*b*). In the case of phorbol ester-stimulated U937 cells, the conditioned medium contained 95 kDa gelatinase in free and TIMP-1-bound forms, allowing us to purify TIMP-1-free 95 kDa progelatinase. Enzyme complexed with TIMP-1 was removed on a monoclonal antibody affinity column to yield free enzyme.

The inhibitory activities of TIMP-1 and TIMP-2 against all the purified active matrix metalloproteinases were compared and found to be similar, although TIMP-1 was more active than TIMP-2 in most instances. With the exception of PUMP, stable complexes of each active metalloproteinase with both TIMP-1 and TIMP-2 at approx. 1 nM could be demonstrated by gel filtration. From the inhibition curve of 72 kDa gelatinase by TIMP-2, and extrapolation to 100% inhibition, it has been calculated that TIMP-2 fully inhibits active 72 kDa gelatinase by the formation of a 1:1 stoichiometric complex. This has been previously shown for the complex of TIMP-1 and active collagenase (Cawston *et al.*, 1981) and for that of TIMP-1 and stromelysin (Murphy *et al.*, 1989*a*). From the calculated K_d of 1×10^{-11} M the TIMP-2–active 72 kDa gelatinase complex is a tight-binding complex, as has been shown for the TIMP-1–active collagenase complex (Cawston *et al.*, 1983). As previously demonstrated by Wilhelm *et al.* (1989) and Goldberg *et al.* (1989), we have confirmed that TIMP-1 was able to bind to 95 kDa progelatinase and that TIMP-2 was able to bind to 72 kDa progelatinase, using purified materials at concentrations of 1 nM. Although we have not specifically demonstrated a 1:1 molar stoichiometry for the binding of TIMP-2 to 72 kDa progelatinase, the yield of TIMP-2 isolated from progelatinase–TIMP-2 complexes strongly suggests that this is the case.

Assays of TIMP-2 inhibitory activity in the presence of 72 kDa progelatinase showed that the association of TIMP-2 with the proenzyme did not modify its ability to bind to and inhibit added active gelatinase or collagenase. Gel-filtration studies with purified materials showed that TIMP-1 and TIMP-2 transferred from progelatinase complexes to active gelatinase. A multiple complex of progelatinase–TIMP–active gelatinase was not demonstrable. We deduce that the interaction between TIMP-1 and TIMP-2 with progelatinases is relatively weak and does not interfere with the formation of tight-binding complexes between either TIMP-1 or TIMP-2 and active forms of metalloproteinases. It seems likely that active gelatinase–TIMP complexes and progelatinase–TIMP complexes are formed by different mechanisms (R. Ward & G. Murphy, unpublished work). The role of TIMP binding to progelatinase is not clear but may be a method of sequestering the inhibitor in tissues. Our data suggest that TIMP bound in this way could readily dissociate from the weaker progelatinase–TIMP complex to the higher-affinity binding sites of active metalloproteinases. More detailed kinetic

analyses of TIMP–metalloproteinase interactions await the availability of suitable low-molecular-mass substrates.

The stability of gelatinase–TIMP complexes to gel filtration and the ability of progelatinase–TIMP complexes to retain inhibitory activity can result in the detection of what are apparently high-molecular-mass inhibitors. Such inhibitors have previously been reported (Morris, 1989; Cawston *et al.*, 1990), and these may well be due to the presence of progelatinase–TIMP complexes. One of the major differences between the gelatinases and the collagenases and stromelysins is the insertion of a domain similar to the collagen-binding region in fibronectin (Collier *et al.*, 1988; Wilhelm *et al.*, 1989). This region is thought to be responsible for the ability of the gelatinases and fibronectin to bind specifically to gelatin–Sepharose. It seemed possible that this region might also be responsible for the ability of progelatinases to bind TIMPs. However, in initial studies we found that neither TIMP-1 nor TIMP-2 bound to fibronectin; furthermore gelatin did not interfere with TIMP-2 complexing with 72 kDa gelatinase.

Both TIMP-1 and TIMP-2 have been cloned from a number of species (Docherty *et al.*, 1985; Carmichael *et al.*, 1986; Boone *et al.*, 1990; Stetler-Stevenson *et al.*, 1990) and shown to have an overall primary sequence identity of about 40%. The 12 cysteine residues are completely conserved, as are other key residues, and it can be reasonably assumed that the disulphide bond assignments defined for TIMP-1 by Williamson *et al.* (1990) will hold for TIMP-2, yielding a similar structure with a similar mechanism of action with respect to the complexing of active metalloproteinases. Despite the predicted similarity in the structure of TIMP-1 and TIMP-2, the polyclonal antibody raised to TIMP-2 during the course of this study did not recognize natural human TIMP-1, which may in part be due to the non-glycosylated state of TIMP-2, in contrast with TIMP-1.

Our studies on the self-cleavage of prometalloproteinases in the presence of TIMPs have shown that TIMP-1 and TIMP-2 are effective inhibitors of such self-cleavage. TIMP–progelatinase complexes are no more resistant to organomercurial-induced self-cleavage than is progelatinase in the presence of unbound TIMP. For example, 4-aminophenylmercuric acetate can initiate the generation of lower-molecular-mass forms of 72 kDa progelatinase more readily after preincubation of progelatinase with TIMP-2 rather than with TIMP-1. Under conditions where there is an excess of TIMP, metalloproteinase self-cleavage was almost totally arrested after an initial period during which low-molecular-mass products were generated. This suggests that, although TIMPs may bind these early cleavage products, they are not able to inhibit their production. Under conditions where there is an excess of prometalloproteinase over TIMP full conversion into lower-molecular-mass forms was observed, although at a lower rate than in the absence of TIMP. This suggests that both TIMP-1 and TIMP-2 form reversible complexes with the intermediates generated during the conversion of proenzyme into active enzyme. In the presence of excess enzyme, TIMP complexes readily with active enzyme, enabling full conversion of the remaining intermediates into lower-molecular-mass active forms. The gelatinase self-cleavage studies reported here are all based on the use of 4-aminophenylmercuric acetate. How gelatinase is activated *in vivo* is not yet known: it is likely that any further understanding of TIMP–progelatinase complexes and the activation of gelatinase complexed in this way will benefit from a greater knowledge of physiological activation.

In summary, these studies demonstrate that TIMP-1 and TIMP-2 are essentially similar inhibitors in biochemical terms. Further analysis of their tissue distribution and relative levels of expression *in vivo* will be necessary to establish the significance of the existence of two forms of matrix metalloproteinase inhibitor.

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