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Dissociation of tissue inhibitor of metalloproteinases (TIMP) from enzyme complexes yields fully active inhibitor

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Recombinant human tissue inhibitor of metalloproteinases (TIMP) forms complexes with high-M_r active recombinant stromelysin that are stable over long periods under physiological conditions. TIMP–stromelysin complexes could be dissociated in the presence of EDTA at pH 3, releasing free TIMP and destroying stromelysin activity. The dissociated TIMP was apparently unmodified, in contrast with other known protein inhibitors of metalloproteinases and many classes of serine-proteinase inhibitor, which are slowly cleaved.

INTRODUCTION

The tissue inhibitor of metalloproteinases, TIMP, occurs in most body fluids and is synthesized by many types of cell in culture, as well as *in vivo*. It is the only known mammalian inhibitor specific for connective-tissue metalloproteinases and is thought to have an important role in the regulation of matrix turnover [1]. Detailed studies of TIMP interaction with interstitial collagenase have been made. Cawston *et al.* [2] have shown that rabbit TIMP is a tight-binding inhibitor with a K_d of 1.4×10^{-10} M. Welgus *et al.* [3] carried out a kinetic analysis of human TIMP interaction with collagenase that suggested a non-competitive mechanism of inhibition and the formation of a complex with a K_i of less than 10^{-9} M. Stromelysin is a typical member of the collagenase family of enzymes, which share closely related primary structures. It exhibits a wide substrate specificity, including proteoglycan core proteins, type IV collagen and elastin [4]. As part of a further analysis of TIMP binding to metalloproteinases we have analysed its dissociation from the enzyme stromelysin.

MATERIALS AND METHODS

Materials

General chemicals were from Sigma, Bolton and Hunter reagent was from Amersham International, Ultrogel AcA44 was from IBF Biotechnic and Sephacryl S-200 was from Pharmacia. Human α_2-macroglobulin (α_2M) was the gift of Dr. U. Tisljar [5].

TIMP and stromelysin preparation

Recombinant TIMP and stromelysin were generated from C127 cells, as described previously [6,7]. TIMP was purified by using a monoclonal antibody linked to Sepharose (P. Koklitis, unpublished work; details available from P.K. on request) and pro-stromelysin was purified by using Procion Red–agarose [8]. The concentration of both purified proteins was determined by assuming $A^{1\%}_{280,\,1\,cm} = 1.0$. TIMP preparations had a specific activity of 4800 units/mg and stromelysin one of 360 units/mg (see under 'Activity assays' below). High-M_r (45000) active stromelysin ('HMW form') was generated from pro-stromelysin by treatment with trypsin (10 μg/ml) at 37 °C for 30 min, followed by the addition of 2 mM-phenylmethanesulphonyl fluoride or soya-bean trypsin inhibitor (50 μg/ml) [7]. Proteins were trace ^{125}I-labelled [9], without loss of activity [2].

Activity assays

Stromelysin activity was assessed by using a casein assay. β-Casein was ^{125}I-labelled [9] to a specific radioactivity of $(2–3) \times 10^3$ c.p.m./μg (counted on a Packard Multi Prias γ-radiation counter) and used in an assay similar to that described by Galloway *et al.* [8], except that 10 μg of casein in a final assay volume of 100 μl was employed. Stromelysin activity is expressed in units of μg of casein hydrolysed/min. TIMP activity was assessed by using collagenase [10]; 1 unit of TIMP inhibits 2 units of collagenase by 50%. TIMP protein levels were assayed by a sandwich e.l.i.s.a. technique [11]. The ability of TIMP to inhibit stromelysin was also assessed using the casein assay described above.

Gel electrophoresis

Proteins were analysed using SDS/polyacrylamide gels [12], followed by silver staining [13] or Coomassie Blue staining.

Gel-filtration analysis

Proteins, some trace ^{125}I-labelled [9], were chromatographed on columns (1.6 cm × 80 cm) of Ultrogel AcA 44 or Sephacryl S-200 equilibrated with 25 mM-Tris/HCl, pH 7.5, containing 0.5 M-NaCl, 0.01 M-$CaCl_2$ and 0.05% Brij 35. Individual fractions were assessed for radioactivity (γ-radiation counter) or TIMP/stromelysin activity as described above.

N-Terminal amino acid sequencing

TIMP samples were reduced and carboxymethylated and subjected to *N*-terminal sequence analysis by automated Edman degradation on an Applied Biosystems gas-phase Sequenator (ABI 470A) with on-line (ABI 120A) analysis.

Abbreviations used: TIMP, Tissue Inhibitor of MetalloProteinases; α_2M, α_2-macroglobulin.

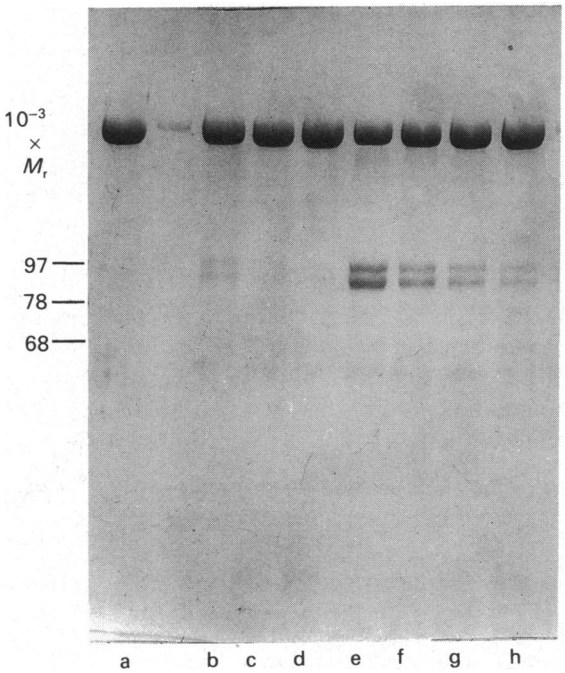

Fig. 1. Relative affinity of stromelysin for TIMP and α_2M

α_2M (10 μg) was incubated at 37 °C for 10 min (a) alone or with stromelysin (0.02 unit) pretreated with: (b) an equivalent amount of TIMP, determined by pretitration (33 ng); (c) 66 ng of TIMP; (d) 132 ng of TIMP. Negligible cleavage of α_2M occurred compared with (e) stromelysin alone (0.02 unit). If the two inhibitors were also premixed before stromelysin addition (0.02 unit), cleavage of α_2M occurred: (f) 33 ng of TIMP and 10 μg of α_2M; (g) 66 ng of TIMP and 10 μg of α_2M; (h) 132 ng of TIMP and 10 μg of α_2M. The reaction products were assessed by electrophoresis on an SDS/6.5%-(w/v)-polyacrylamide gel (reducing) and stained with Coomassie Blue. M_r values for standard proteins are indicated to the left of the gel.

RESULTS AND DISCUSSION

Inhibition of stromelysin by TIMP

TIMP was found to inhibit purified active stromelysin (HMW form) in a linear manner with respect to concentration; approx. 1 mol of TIMP bound to 1 mol of stromelysin at equivalence, in agreement with the findings of Okada *et al.* [14]. From the deviation from stoichiometric inhibition in the region of the equivalence point, an approximate apparent K_d of 3.8×10^{-10} M could be calculated [15], as described previously for collagenase [3]. This represents a minimum value, assuming both TIMP and stromelysin are in a fully active form, and indicates a tight-binding complex.

Relative affinity of stromelysin for TIMP and α_2M

Stromelysin was shown to cleave α_2M, as has been described for many other proteinases, as part of the trapping mechanism of complex-formation (Fig. 1). Stromelysin formed complexes with TIMP that were stable (hence inactive) in the presence of subsequently added α_2M (Fig. 1). However, if equivalent amounts of α_2M and TIMP were added to the enzyme together, it was found that α_2M cleavage by stromelysin still occurred. Only in the presence of a 4-fold excess of TIMP was stromelysin-produced cleavage, i.e. complex-formation, of α_2M abrogated (Fig. 1). This concurs with the observation that collagenase most rapidly binds to α_2M in the presence of TIMP, but that preformed collagenase–TIMP complexes do not permit collagenase to bind to α_2M [16].

Dissociation of stromelysin–TIMP complexes

Approximately equimolar amounts of HMW-form active stromelysin–TIMP complexes, or TIMP, or stromelysin alone, were treated with EDTA at various pH values at 4 or 37 °C before activity assay. Table 1 shows that stromelysin is sensitive to EDTA at all pH values and temperatures, although it is quite stable at pH 3 in the presence of Ca^{2+}. TIMP is stable, even at pH 3 and 37 °C. TIMP activity was apparently released from stromelysin–TIMP complexes by treatment with EDTA at 37 °C, a pH of 3 being the most efficient. TIMP could be released from complexes at 4 °C in the presence of EDTA at pH 3. 1,10-Phenanthroline was also an efficient chelator in this system, but EGTA was not (results not shown). This method may be useful for the assay of complexed TIMP in culture fluids etc. TIMP can also be quantitatively released from complexes with collagenase and gelatinase for assay of activity or e.l.i.s.a. (G. Murphy, unpublished work).

Table 1. Stability of stromelysin, TIMP and their complex

Equimolar amounts of stromelysin and TIMP alone and complexed were treated with 10 mM-EDTA at different temperatures and pH for 30 min as detailed above. Samples were subsequently adjusted to neutral pH (Tris) and 10 mM-$CaCl_2$ before assay for TIMP and/or stromelysin activity (see the Materials and methods section). Results are expressed as a percentage of the activity of untreated stromelysin and TIMP respectively.

Treatment		Recovery of activity (%)			
				Stromelysin–TIMP complex	
Temp. (°C)	pH	Stromelysin	TIMP	Stromelysin	TIMP
4	7.5	8	100	0	0
	4.5	10	—	0	24
	3.0	0	97	0	95
37	7.5	4	102	0	72
	4.5	—	—	0	79
	3.0	0	98	0	100
No treatment		100	100	0	0

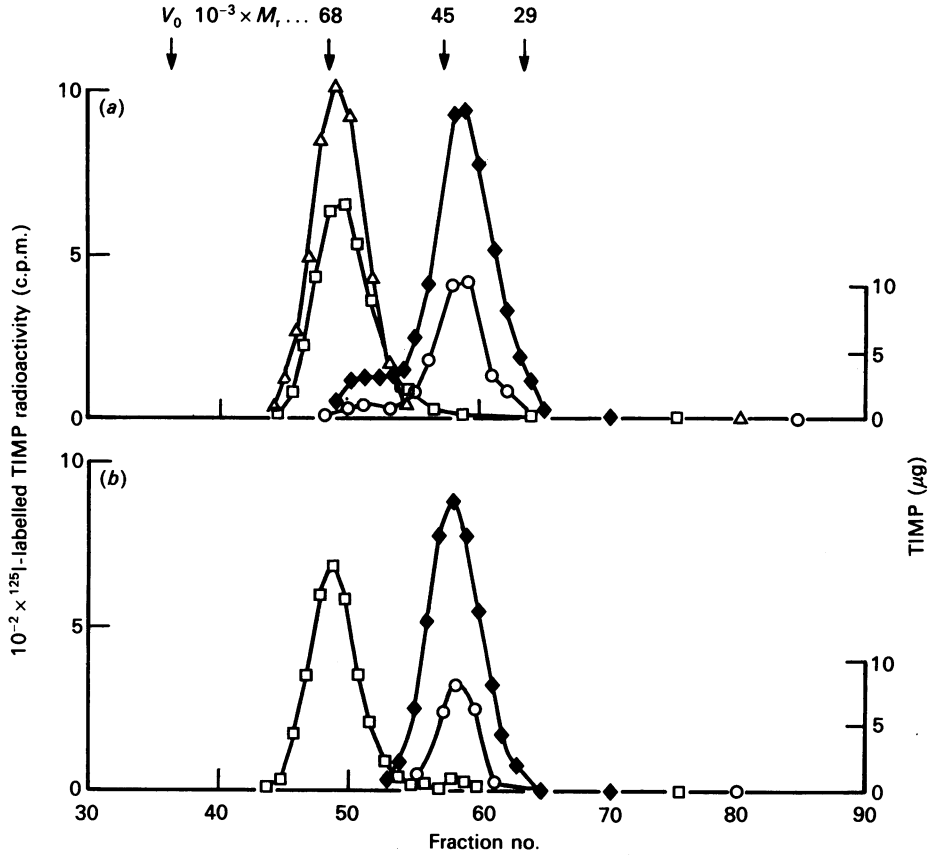

Fig. 2. Gel-filtration analysis of stromelysin–TIMP complexes

(*a*) TIMP (90 μg, containing 3×10^4 c.p.m. of ^{125}I-labelled TIMP) complexed with active stromelysin and chromatographed on Sephacryl S-200 was eluted as a single peak of ^{125}I-complex (see also Fig. 3). The pooled peak fractions were incubated at 37 °C for 24 h, and a portion (2 ml) shown to be eluted in an unchanged position on re-chromatography (△) as assessed by the distribution of ^{125}I. A further 2 ml of complex was dissociated with 20 mM-EDTA (pH 3)/glycine at 37 °C for 30 min before rechromatography: ◆; most of the radioactivity was eluted at the position of free TIMP and contained TIMP protein (○), as detected by e.l.i.s.a., as well as TIMP inhibitory activity (not shown). The peak fractions of dissociated ^{125}I-TIMP were re-complexed with active stromelysin and rechromatographed (□), being eluted only at the position of the complex. (*b*) Similar amounts of TIMP alone were similarly treated to estimate recoveries: ◆; ^{125}I-TIMP distribution on chromatography coincided with TIMP protein: ○. Complexing of peak fractions with active stromelysin and rechromatography gave a complete shift in the ^{125}I peak to the elution position of the complex: □. The elution positions of standard proteins are indicated by their M_r values.

Gel-filtration analysis of stromelysin–TIMP interaction

The interaction of active HMW-form stromelysin and TIMP was studied by gel-filtration chromatography using trace ^{125}I-labelled proteins alone or with unlabelled proteins added. Stromelysin (gel-filtration M_r 46600; $n = 2$) formed a complex with an equivalent amount of TIMP ((M_r 34000 ± 150; $n = 12$; Fig. 2) to yield a single species of M_r 61900 ± 149; $n = 11$; Fig. 2). Identical values were obtained by using labelled enzyme or inhibitor. Fractions corresponding to the complex contained negligible TIMP or stromelysin activity. No complex formed between latent pro-stromelysin and TIMP, nor between 1,10-phenanthroline-treated active stromelysin and TIMP, as described previously [8]. A stromelysin substrate, casein, or 5 mM-$ZnCl_2$ (inhibitory to enzyme activity), did not interfere with complex-formation, as assessed by gel-filtration analysis. The stromelysin–TIMP complex isolated in this way was stable to storage at 4 °C for more than 90 days or exposure to 37 °C for 40 h or 50 °C for 15 h, with complete recovery of intact complex on re-chromatography. Studies using trace amounts of proteins gave as little as 0.5 ng of complexed TIMP/ml (6×10^{-11} M) in a single elution peak, which indicates the tight-binding nature of the TIMP–HMW-form stromelysin complex.

HMW-form stromelysin–^{125}I-TIMP complexes prepared by gel filtration could be dissociated by treatment with 20 mM-EDTA/150 mM-glycine, pH 3 at 37 °C, and rechromatography, ^{125}I-TIMP being eluted in a peak corresponding to the position of free TIMP (Fig. 2; M_r 34500 ± 470; $n = 5$). The putative free TIMP peak could be recomplexed with excess fresh active HMW-form stromelysin, 81 % of the radiolabel being re-eluted at an M_r equivalent to that of the complex (Fig. 2). By using identical chromatographic conditions, but in the absence of stromelysin, an equivalent amount of ^{125}I-TIMP exposed to pH 3 and rechromatographed was also able to form a stromelysin complex, with 70 % of the radioactivity being eluted at a position similar to that of the intact complex

Sequence analysis of dissociated TIMP

TIMP–stromelysin complexes isolated by gel filtreation and kept at 4 °C for 4 days or at 37 °C for 24 h were

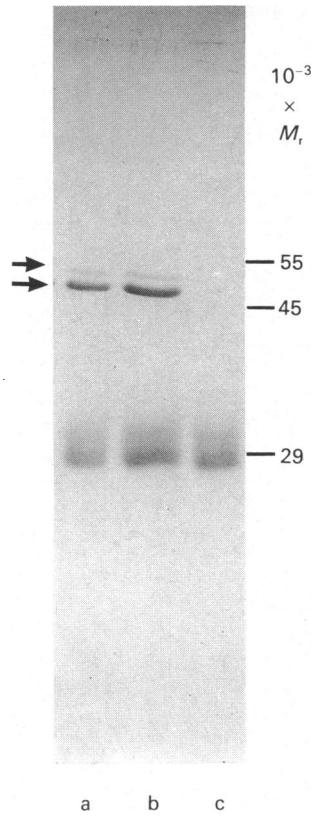

Fig. 3. SDS/polyacrylamide-gel analysis of the fate of TIMP in stromelysin complexes

Stromelysin–TIMP complex eluted from Sephacryl S-200 (Fig. 2) was (a) untreated and (b) incubated at 37 °C for 40 h. In comparison with TIMP alone (c), no detectable modification of TIMP can be observed. Proteins were compared on an SDS/12.5% polyacrylamide gel and silver-stained. The position of 57000- and 60000-M_r (glycosylated) forms of stromelysin are arrowed. Standard-protein migration positions are indicated by M_r values.

treated with EDTA at pH 3, as described above, before chromatography. TIMP was released with 54% recovery of activity and formed a stable complex with further active stromelysin (Fig. 2). Uncomplexed TIMP subjected to the same treatment gave 56% recovery of activity; these recoveries were also confirmed by e.l.i.s.a. Stromelysin–TIMP complexes dissociate on SDS/polyacrylamide-gel electrophoresis, and no evidence of an M_r change in TIMP can be detected even when complexes had been incubated for 40 h at 37 °C (Fig. 3). *N*-Terminal amino acid sequence analysis on TIMP dissociated from such stromelysin complexes gave only the sequence Cys-Thr-Cys-Val-Pro-Pro-His-Pro-Gln-Thr; this is identical with the *N*-terminal sequence previously described for intact TIMP [6]. The lack of any other significant sequence indicates that no cleavage of TIMP has occurred within the complex.

It has been shown biochemically that many protein inhibitors of serine proteinases and some inhibitors of metalloproteinases bind tightly as substrates and are cleaved slowly at a peptide bond in the reactive site [17,18]. Potato (*Solanum tuberosum*) carboxypeptidase inhibitor loses the *C*-terminal glycine residue on complexing with carboxypeptidase A, although the cleaved inhibitor is a new substrate which is bound in a non-productive fashion [19]. This rebinding of cleaved inhibitor appears to be due in part to the importance of secondary contacts between inhibitor and enzyme in the complex. The *Streptomyces* metalloproteinase inhibitor has been shown to be slowly cleaved at Cys^{64}–Val^{65} by the endopeptidase thermolysin over 52 h at 37 °C [20], but further studies of the cleaved inhibitor have not yet been described. Our preliminary observations indicate that TIMP is not modified during complex-formation. TIMP dissociated from stromelysin complexes even after long storage periods or exposure to high temperatures has the same mobility on SDS/polyacrylamide-gel electrophoresis and gel-filtration chromatography, forms stable complexes with added stromelysin and contains no detectable new *N*-terminal amino acid sequences.

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