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The interaction of purified rabbit bone collagenase with purified rabbit bone metalloproteinase inhibitor

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1. Pure rabbit bone metalloproteinase inhibitor (TIMP) bound tightly to pure rabbit bone collagenase with an apparent K_d of 1.4×10^{-10}M. 2. The molecular weight of the enzyme–inhibitor complex was found to be 54000, but no enzyme activity could be recovered from the complex after treatment with either mercurials or proteinases. The complex thus differed from latent collagenase in terms of size, susceptibility to mercurials and behaviour on concanavalin A–Sepharose. 3. The interaction of the purified components was compared with that of crude collagenase and crude inhibitor in culture medium. Mercurial treatment partially reversed the inhibition in the crude system, but not when the purified components were used. 4. The significance of the results is discussed in relation to the extracellular control of the activity of collagenase.

Connective tissues in culture can synthesize metalloproteinases that are able to degrade extracellular-matrix macromolecules (Murphy *et al.*, 1981*b*). These enzymes are usually found in a latent form and require activation before they can digest their substrates. Collagenase, the most widely studied mammalian metalloproteinase, can be activated by trypsin, mercurials and chaotropic ions (Sellers & Murphy, 1981), but the processes by which activation is achieved *in vivo* are unclear. A specific metalloproteinase inhibitor (TIMP) is found in the culture media of many tissues (Murphy *et al.*, 1977), and in the case of rabbit bone medium it was reported to combine with crude mercurial-activated collagenase to form a complex of similar size to latent collagenase (Sellers *et al.*, 1977). Reynolds *et al.* (1977) suggested that latent collagenases consist of enzyme in combination with the inhibitor, TIMP, and that both the amount of collagenase and TIMP produced by tissue cells is important in the control of the extracellular activity of collagenase and other metalloproteinases. Subsequently a number of collagenase inhibitors from the culture media of tissues from various species have been purified (Vater *et al.*, 1979; Welgus *et al.*, 1979; Cawston *et al.*, 1981*b*; Murphy *et al.*, 1981*a*; Nolan *et al.*, 1980).

Abbreviations used: APMA, 4-aminophenylmercuric acetate; TIMP, tissue inhibitor of metalloproteinases: SDS, sodium dodecyl sulphate.

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In order to determine if latent collagenase consists of active collagenase in combination with TIMP, we have purified collagenase and TIMP from rabbit tissue-culture medium (Cawston *et al.*, 1981*b*). We now report that a complex is formed between the purified collagenase and inhibitor which differs from the latent enzyme found in crude culture medium and discuss the significance of our findings in relation to the recent work of others.

Experimental

Materials

Chemicals were obtained from the following suppliers: fluorescamine from F. Hoffman–LaRoche, Basel, Switzerland; concanavalin A–Sepharose and Sephadex G-100 (superfine grade) from Pharmacia, Hounslow, Middx., U.K.; *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl) propionate from The Radiochemical Centre, Amersham, Bucks., U.K.; Ultrogel AcA 44 from LKB, Croydon, Surrey, U.K. All other chemicals and biochemicals were either commercially available analytical-grade reagents or have been previously described (Cawston & Tyler, 1979; Tyler & Cawston, 1980; Cawston *et al.*, 1980).

Methods

Enzyme assays. Collagenase activity was measured as described by Cawston & Barrett (1979). For collagenase-inhibitor assays a known amount of

collagenase (0.06 unit) was added to the incubation mixture and assayed in the presence of inhibitor activity. One unit of collagenase degrades 1 μg of collagen/min at 37°C and one unit of inhibitor is defined as that giving 50% inhibition of two units of collagenase. Gelatin-degrading activity and casein-degrading activity were assayed as described by Cawston *et al.* (1981*b*).

Protein determination and iodination. Protein was measured with fluorescamine as previously described (Cawston & Tyler, 1979) and iodinated with ^{125}I by using the Bolton and Hunter reagent, as described by Cawston *et al.* (1981*b*).

Gel filtration. Columns of either Ultrogel AcA 44 or Sephadex G-100 were equilibrated with 25 mM-sodium cacodylate, pH 7.5, containing 1 M-NaCl/10 mM-$CaCl_2$/0.05% Brij 35/0.02% NaN_3. Molecular weights were estimated by using calibrated columns as described by Sellers *et al.* (1977).

Collagenase and inhibitor preparations. Collagenase was prepared from rabbit bone culture medium as described by Cawston & Tyler (1979). Collagenase inhibitor was prepared as described by Cawston *et al.* (1981*b*).

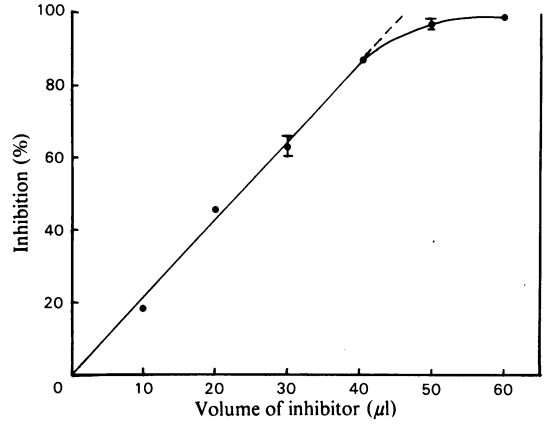

Fig. 1. *Interaction of purified rabbit bone inhibitor (TIMP) with purified rabbit bone collagenase, in the absence or presence of mercurial*

Increasing amounts of a solution of purified inhibitor (1.9 units/ml; 70 ng/ml) were added to assay tubes containing 30 μl of purified collagenase (2.93 units/ml; 160 ng/ml). The enzyme was inhibited with increasing concentrations of inhibitor in the absence of 4-aminophenylmercuric acetate; data obtained in the presence of mercurial were identical and for clarity are not drawn in the Figure.

Results

Interaction of purified collagenase and purified TIMP

Fig. 1 illustrates the inhibition of purified collagenase by increasing amounts of purified TIMP. The inhibitor binds to the enzyme stoichiometrically, 1 mol of TIMP binding to 0.93 mol of collagenase, even at low concentrations. An apparent K_d was estimated from the graph by using the method of Green & Work (1953) and found to be 1.4×10^{-10} M. This represents a minimum value, as it assumes that both enzyme and TIMP samples contain only active protein, but indicates that tight binding between the enzyme and inhibitor occurs. The collagenase was inhibited in an identical manner when the assay was performed in the presence of 0.7 mM-APMA. Other mercurials, such as mersalyl, phenylmercuric chloride and 4-chloromercuribenzoate, also did not prevent the inhibition. Additionally, preincubation of either TIMP or collagenase with these mercurials over a wide concentration range did not prevent their interaction. Alternative methods of reversing the inhibition were also attempted: no re-activation of collagenase after inhibition by TIMP was achieved with either trypsin or plasmin. These two proteinases are known to activate the latent enzyme (Cawston *et al.*, 1981*a*).

The interaction of collagenase and TIMP was studied by using gel filtration. Sufficient purified inhibitor was added to purified enzyme to give complete inhibition and the mixture chromatographed on a column of Ultrogel AcA 44. No collagenase activity could be detected in the column fractions in either the presence or absence of mercurials or proteinases, although small amounts of excess inhibitor were detected (results not shown). In view of the apparent irreversible nature of the inhibition of collagenase it was decided to investigate the complex formed by using ^{125}I-labelled collagenase. Purified collagenase was iodinated with Bolton and Hunter reagent and chromatographed on Ultrogel AcA 44. Two peaks of ^{125}I-labelled protein were eluted: the first peak at the void volume contained aggregated enzyme and the second peak eluted with an apparent molecular weight of 33 000 (results not shown). All the detectable collagenase activity was associated with this latter peak, which was then pooled. Portions of this pool were then rechromatographed on the same column in both the presence and absence of unlabelled purified inhibitor and the fractions counted for ^{125}I radioactivity in a gamma counter (Fig. 2). In the presence of inhibitor a higher-molecular-weight (54 000) complex was formed. When no inhibitor was present the enzyme eluted with an apparent molecular weight of 33 000, as before. A corresponding experiment using ^{125}I-labelled inhibitor with unlabelled enzyme gave similar results. At the first stage two peaks of ^{125}I-labelled TIMP were eluted from a gel-filtration column, which was loaded with ^{125}I-labelled purified inhibitor alone. The peak at the void volume

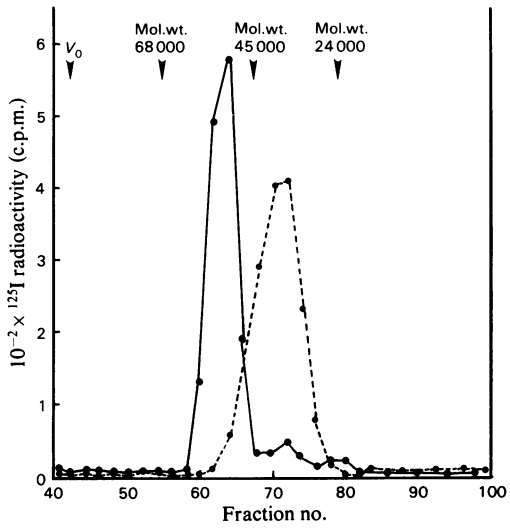

Fig. 2. *Gel filtration of ^{125}I-labelled enzyme with and without inhibitor*

A sample of ^{125}I-labelled rabbit bone collagenase was divided into two equal portions. One was mixed with 9 units of purified rabbit bone inhibitor and immediately loaded on to a column of Ultrogel AcA 44 (1.6 cm × 88 cm) equilibrated with column buffer. The column was eluted with this buffer at a flow rate of 12 ml/h. Fractions (1.7 ml) were collected and counted for ^{125}I radioactivity. The other portion was subsequently loaded on to the same column and eluted in an identical manner. The elution profile is shown for collagenase chromatographed with (——) and without (– – – –) inhibitor.

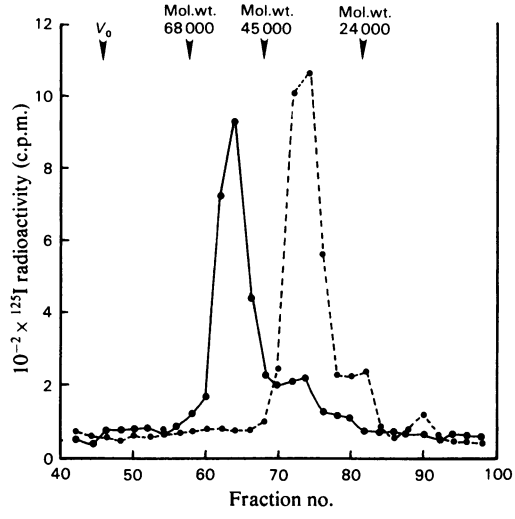

Fig. 3. *Gel filtration of ^{125}I-labelled TIMP with and without enzyme on Ultrogel AcA 44*

A sample of ^{125}I-labelled rabbit bone inhibitor was divided into two equal portions. One was mixed with 10 units of purified rabbit bone collagenase and immediately loaded on to a column of Ultrogel AcA 44 (1.6 cm × 88 cm) equilibrated with column buffer. The column was eluted with this buffer at a flow rate of 12 ml/h. Fractions (1.7 ml) were collected and counted for ^{125}I radioactivity. The other portion was subsequently loaded on to the same column and eluted in an identical manner. The elution profile is shown for inhibitor chromatographed with (——) and without (– – – –) collagenase.

corresponded to aggregated inhibitor and did not inhibit the enzyme, whereas an active inhibitor peak was eluted with a molecular weight of 34 000. This peak was pooled and rechromatographed both with and without purified unlabelled enzyme. The elution profile shows that a 54 000-mol.wt. complex was formed when ^{125}I-labelled inhibitor was combined with enzyme (Fig. 3). The complex could not be re-activated with either mercurial or proteinase treatment. However, SDS/polyacrylamide-gel electrophoresis of the complex peak showed that both enzyme and inhibitor protein were present in the complex.

Interaction of complex with concanavalin A–Sepharose

^{125}I-labelled purified collagenase was loaded on to a concanavalin A–Sepharose column in either the presence or absence of unlabelled purified inhibitor. Fig. 4 shows that the enzyme alone passed straight through the column but bound to the column when combined with inhibitor, and could then be eluted with glycoside. This experiment demonstrates that the behaviour of the complex formed between enzyme and inhibitor differs from that of the latent enzyme on this column matrix, because latent enzyme does not bind to concanavalin A–Sepharose.

Attempts at recovery of collagenase activity from complex

The finding that the interaction of the purified TIMP with purified collagenase could not be reversed by addition of mercurials differed from previous results obtained when crude fractions were used (Sellers *et al.*, 1977). Fig. 5 illustrates the reveral of inhibition by APMA (0.7 mM) when crude concentrated culture medium containing inhibitor was titrated with crude concentrated culture medium containing collagenase that had previously been activated by mercurial. The inhibition could be partially reversed by inclusion of APMA (0.7 mM) in the enzyme assay, and the inhibition was almost completely abolished by the pretreatment of the crude inhibitor with APMA (0.7 mM) for 1 h at 37°C. These results do not resemble those obtained with the purified components, where no reversal of

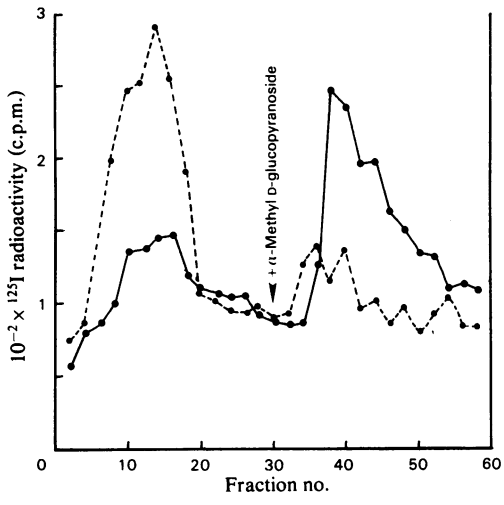

Fig. 4. *Chromatography of ^{125}I-labelled collagenase with and without inhibitor on concanavalin A–Sepharose*
A sample of ^{125}I-labelled rabbit bone collagenase was divided into two equal portions. One was mixed with 9 units of TIMP and immediately loaded on to a column of concanavalin A–Sepharose (0.9 cm × 6.5 cm) equilibrated with column buffer containing 1 mM-$MnCl_2$ and -$MgCl_2$. The sample was washed on to the column with this buffer and then the column eluted with this buffer containing 2% (w/v) α-methyl-D-glucopyranoside. The other portion was subsequently loaded on to the same column and was eluted in an identical manner. Fractions (1.0 ml) were collected and counted for ^{125}I radioactivity. The elution profile is shown for collagenase chromatographed with (——) and without (– – – –) added inhibitor.

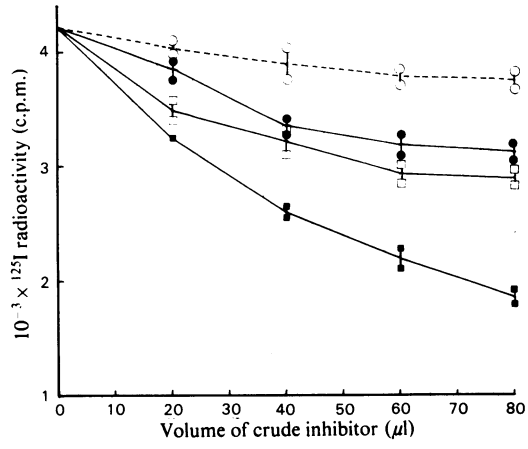

Fig. 5. *Interaction of crude inhibitor with crude active enzyme in the presence and absence of mercurial*
Increasing amounts of crude concentrated culture medium containing inhibitor (I) were added to a known amount (0.1 unit) of crude concentrated culture medium containing active collagenase (E) in either the presence or absence of APMA. ^{14}C-labelled collagen (100 μg) was added and the collagenolytic activity measured as described under 'Methods'. ■, Enzyme (E) + inhibitor (I) alone; ●, E–I complex + mercurial preincubated at 37°C for 1 h before addition to collagen; ○, I + mercurial preincubated at 37°C for 1 h before addition of E and collagen; □, E + mercurial preincubated at 37°C for 1 h before addition of I and collagen.

inhibition could be achieved, even by preincubation of purified inhibitor with APMA (see Fig. 1).

Molecular-weight determinations

The molecular weights of each of the components studied were carefully determined by gel filtration and are listed in Table 1. The molecular weights shown for the purified components include results obtained for three separate preparations of both inhibitor and enzyme. No differences could be detected in the molecular weights of either crude and pure inhibitor, or the crude and pure active enzyme. The molecular weight of the complex formed from the purified components, 54000, was not altered if the interaction took place in the presence of either crude inhibitor, crude enzyme or both. Equally, the molecular weight of the complex was unaltered if either pure enzyme was incubated with crude inhibitor, or crude enzyme was incubated with pure inhibitor. No decrease in molecular weight was obtained when the complex was treated with APMA (0.7 mM) either with or without crude culture

Table 1. *Molecular weights of inhibitor, collagenase, latent collagenase and complex formed from purified enzyme and inhibitor*
Molecular weights were estimated using calibrated columns of Ultrogel AcA 44 and Sephadex G-100 as described by Sellers *et al.* (1977). Results shown are the means ± S.E.M. of at least four separate determinations. Abbreviation used: nd, not determined.

	Molecular weight	
Matrix ...	Ultrogel AcA 44	Sephadex G-100
Inhibitor		
Pure	34400 ± 500	36000 ± 1400
Crude	34800 ± 700	37600 ± 800
Collagenase		
Pure	33300 ± 900	38000
Crude	32300 ± 1000	36800
Crude latent collagenase	45000 ± 1250	49300 ± 1500
Complex formed from pure components	54100 ± 700	60100 ± 700
Complex formed from pure components + AMPA	53000 ± 1300	nd

medium present. Crude latent rabbit collagenase was found to have a molecular weight of 45000 and when treated with mercurial the molecular weight of the activated enzyme was found to be 32000.

The molecular weights reported in Table 1 were also checked by gel filtration on Sephadex G-100 (superfine grade). Similar molecular-weight estimates were obtained for all components, although the results were less precise when this matrix was used.

In other experiments a complex of identical size was obtained when pure rabbit inhibitor was added to pure pig synovial collagenase (Cawston & Tyler, 1979). No complex was formed when pure inhibitor was added to crude latent rabbit collagenase.

Discussion

By using purified materials from rabbit bone culture medium we have been able to demonstrate that collagenase binds to the metalloproteinase inhibitor, TIMP, to form a tight complex which differs from latent collagenase in three respects. Firstly, the complex has a different molecular weight (54000) when compared by gel filtration with that of latent collagenase (45000). Secondly, the interaction is not prevented by mercurial treatment over a wide range of conditions, and active collagenase cannot be recovered from the complex. Thirdly, the complex binds to concanavalin A–Sepharose, a property of the inhibitor but not of the latent enzyme. We therefore conclude that the complex between active collagenase and TIMP is an entity separate from latent collagenase, although confirmation can only be obtained when methods are developed to purify latent collagenase to homogeneity for comparative purposes. The implications of these findings for understanding the control of collagenase are discussed below.

The present results extend our previous observations, but differ in some respects from those previously reported for the interaction of crude TIMP and crude collagenase (Sellers *et al.*, 1977). We have carefully repeated these earlier experiments and have confirmed that the crude inhibitor is destroyed by mercurial treatment and also that crude collagenase combined with crude inhibitor can be at least partially recovered in the presence of mercurial reagents. The difference in behaviour between the crude and the pure combinations of collagenase and TIMP and the ability of mercurials to reverse the inhibition only in the crude preparations of both components is not clearly understood. Combination of either partially purified enzyme or inhibitor (i.e. gel-filtration fractions) prevents this reversal.

We have confirmed the observation made by Sellers *et al.* (1977) that combining crude enzyme and inhibitor yields a complex of similar molecular weight to that of the latent enzyme. However, removal of the latent collagenase from the crude inhibitor preparations by concanavalin A–Sepharose chromatography, with subsequent interaction of inhibitor with active collagenase, yields no reactivatable complex. We therefore conclude that the crude inhibitor preparations used in previous studies probably contained small amounts of undetectable latent enzyme that could only be reliably assayed after removal of excess inhibitor by gel filtration.

A number of collagenase inhibitors have been purified from the culture media of tendon (Vater *et al.*, 1979), human skin fibroblasts (Welgus *et al.*, 1979), rabbit bone (Cawston *et al.*, 1981*b*) and human amniotic fluid (Murphy *et al.*, 1981*a*), and some have been partially purified (Nolan *et al.*, 1980; Sakamoto *et al.*, 1981; Pettigrew *et al.*, 1981; Simpson & Mailman, 1981). Most of these inhibitors have properties, in terms of size, heat-stability and susceptibility to trypsin and mercurials, similar to those of the material that we named 'TIMP' (Cawston *et al.*, 1981*b*), but there appears to be some differences in the way in which they interact with collagenase. In some studies no reversal of the inhibition was found with either trypsin or APMA, as in the present study (Nolan *et al.*, 1980; Murphy *et al.*, 1981*b*; Sakamoto *et al.*, 1981). Interestingly, previous studies have not described the formation of a tight-binding complex of enzyme and inhibitor, although Welgus *et al.* (1979) stated that the human skin fibroblast inhibitor bound to human skin collagenase, but only in the presence of collagen. We found a high-affinity complex was formed by collagenase and TIMP, of mol.wt. 54000, and furthermore we have found (Galloway *et al.*, 1983) that another purified metalloproteinase, proteoglycanase, also forms tight complexes with TIMP-like inhibitors from a number of sources. These complexes of TIMP and proteoglycanase are not dissociable by treatment with either trypsin or mercurials under the conditions required to activate latent proteoglycanase in the culture medium.

It is noteworthy that high-molecular-weight aggregates were found to be present when both pure TIMP and pure collagenase preparations were subjected to gel filtration. These aggregates contained no biological activity and could be dissociated with SDS. After SDS/polyacrylamide-gel electrophoresis both monomer TIMP and collagenase could be detected. Sakamoto *et al.* (1981) described high-molecular-weight forms of a collagenase inhibitor containing biological activity, whereas our results suggest that both enzyme and inhibitor become inactive after aggregation.

The present results support the idea that the control of collagenase activity is complex. Modulation of collagen degradation can take place by

control of the synthesis and secretion of collagenase, by the control of the factors responsible for the activation of collagenase, by the control of the amount of inhibitor synthesized and secreted, and by the control of its destruction in the extracellular fluid. TIMP may represent the final 'fail-safe' mechanism to control the extracellular activity of the enzyme. Recent evidence shows that collagenase is first secreted from connective-tissue cells in an inactive latent form (Nagase *et al.*, 1981). This precursor form can be activated by proteinases such as trypsin and kallikrein, with a decrease in apparent molecular weight. Although a number of mammalian proteinases have been shown to activate latent forms of collagenase in biochemical experiments (Werb *et al.*, 1977; Nagase *et al.*, 1982), knowledge as to how activation occurs *in vivo* is scanty and difficult to interpret. Both kallikrein and plasminogen activator (together with plasminogen) would be potential activators in inflamed tissues, yet much normal tissue remodelling must take place under very different conditions. It is known that activation of collagenase can take place with no decrease in molecular weight and by mechanisms other than those involving proteinases (Cawston *et al.*, 1981*a*; Stricklin *et al.*, 1977). It has not been established which mechanism constitutes the principal biological activation pathway.

A number of other important issues are raised by our results. Culture medium of connective tissues is likely to contain large amounts of masked collagenase bound to inhibitor which cannot, as yet, be detected because no way is known to activate the complex. This suggests that immunological assays of collagenase levels in resorbing tissues in culture should be set up to ensure total enzyme protein can be measured. Thus when various connective-tissue cells are treated with conditioned medium from mononuclear-cell culture (see Dayer *et al.*, 1977), and the increase in collagenase levels taken as a measure of the inflammatory potential of the stimulating factor, it is possible that the collagenase detectable (using conventional assays) does not truly reflect the total collagenase present. It is also possible that complexes may be trapped in certain tissues and could interfere with the binding of active metalloproteinases to their substrates. These points can now be investigated, since potent antisera to collagenase and inhibitor have been produced by several laboratories. Antisera to collagenase should also be useful in the purification of latent collagenase.

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