Biochimica et Biophysica Acta, 483 (1977) 493—498

BBA Report

BBA 61323

THE DETECTION AND CHARACTERISATION OF COLLAGENASE INHIBITORS FROM RABBIT TISSUES IN CULTURE

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(Received February 28th, 1977)

Summary

As tissue cultures, rabbit bone, skin and non-gravid uterus synthesise inhibitors of collagenase (EC 3.4.24.3). An assay for the inhibitors is described and their action on collagenase from different tissue sources demonstrated.

Evidence for the involvement of the tissue inhibitors of collagenase in the latency of the enzyme in culture media is presented. Latent collagenase was activated by treatment with 4-aminophenylmercuric acetate, and then reacted with the inhibitors to form inactive complexes with properties similar to the naturally occurring latent enzyme forms. The associated changes in molecular weight are detailed, and discussed in relation to the observations of other workers concerning the extracellular control of collagenase activity.

A precise understanding of the occurrence of collagenase (EC 3.4.24.3) in active and inactive forms in the culture media of a large variety of tissues is of vital importance for further research into the mechanisms of normal and pathological collagen catabolism. The discovery of the activation of latent enzyme forms of collagenase by trypsin has been followed by attempts to identify more physiologically relevant proteolytic activities which might control the activation process [1—3]. We have recently shown that the thiol binding reagent 4-aminophenylmercuric acetate (APMA) activates latent collagenase from rabbit tissues as effectively as proteolytic enzymes [4]. With this reagent, the observed reduction of 10—20 000 in molecular weight upon conversion of latent to active collagenase is similar to that observed after trypsin activation; either treatment yields active enzyme of comparable

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molecular weight to that of naturally active collagenase [5]. We have also described [4] a collagenase inhibitor synthesised by rabbit bones in culture, that will react with either naturally active or APMA-activated enzyme to yield latent enzyme forms (which can again be activated by either trypsin or APMA) of similar molecular weight to that of the natural latent collagenase found in culture media. We now present data to show that rabbit tissues other than bone produce similar collagenase inhibitors, suggesting that this is a phenomenon common to all connective tissues, and providing supporting evidence for our proposal that latent collagenases are enzyme-inhibitor complexes [4].

Rabbit bone [4,6] and rabbit skin and non-gravid uterus [7] were cultured as described. Media were routinely assayed for (a) latent collagenase, using the reconstituted [^{14}C]collagen fibril assay [8] in the presence of 0.5 mM APMA [4], and (b) for inhibitor, using APMA-activated collagenase from skin cultures (0.035—0.060 units, sufficient to give 30—50% lysis of 100 μg collagen in 15 h at 35°C; 1 unit is defined as 1 μg collagen degraded per min) in the same assay under the conditions described below. 1 unit of inhibitor is defined as that giving 50% inhibition of 2 units of collagenase.

Active enzyme was prepared from pools of media containing latent collagenase by activation for 4 h at 35°C with 0.5 mM APMA, followed by either exhaustive dialysis against 50 mM Tris·HCl (pH 7.6), 200 mM NaCl and 20 mM $CaCl_2$, or partial purification by chromatography on Ultrogel ACA44 in a similar buffer (but 1 M in NaCl), the peak of active enzyme being pooled and dialysed. Media with inhibitory activity were either dialysed against the above buffer or chromatographed as above, and the active fractions pooled and dialysed similarly. The action of inhibitory preparations on activated enzyme was assayed either by preincubation of varying proportions of each for 1—2 h at 35°C or, more usually, by direct inclusion in the fibril assay. No significant difference in results between these two methods was observed. Inactivation of inhibitor preparations was effected by either boiling for 20 min or by incubation at 25°C for 30 min with 100 μg/ml trypsin, with subsequent inactivation of the trypsin with a final concentration of 500 μg/ml soybean trypsin inhibitor.

For molecular weight determination, latent enzyme was activated with APMA as above and up to 10 units of activated collagenase loaded on to a column of Ultrogel ACA44 (130 × 1.5 cm) equilibrated with 50 mM Tris·HCl (pH 7.6), containing 1 M NaCl and 20 mM $CaCl_2$. Similar amounts of dialysed, APMA-activated enzyme preparations were preincubated with sufficient inhibitory material to give 95% inhibition, previously determined by direct assay, and the latent collagenase complexes chromatographed on the same column. The single enzyme peak was detected by assaying fractions in the presence of APMA in the fibril assay. Inhibitor preparations (up to 15 units) were similarly chromatographed. Apparent molecular weights were calculated using the method and standards of Andrews [9].

Organ cultures of rabbit skin and non-gravid uterus were found to produce inhibitors of the activated form of collagenase released by the same tissues. A general pattern of first inhibitor and then latent collagenase appear-

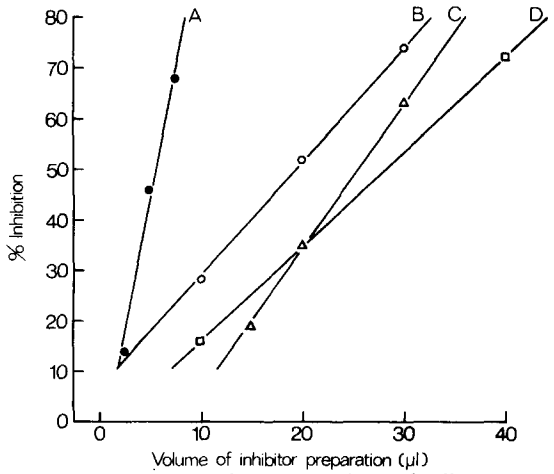

Fig. 1. Typical assays of tissue inhibitors of collagenase to determine the volume giving 50% inhibition of enzyme. A: APMA-activated collagenase from skin culture medium with partially purified uterus inhibitor. B: Partially purified APMA-activated collagenase from skin with inhibitor from skin culture medium. C: Partially purified APMA-activated collagenase from skin with inhibitor from bone culture medium. D: APMA-activated collagenase from uterus medium with partially purified uterus inhibitor.

ance in the media was observed for both tissues, similar to that previously described for rabbit bones [4,6]. The inhibitors were detected in the medium in the early days of culture but free levels declined with the appearance of latent collagenase. Unlike bone cultures [6] little naturally active collagenase was produced by skin cultures, even after 30 days, and none was ever produced by non-gravid uterus in culture [7]. Inclusion of cycloheximide (50 µg/ml) in the medium blocked production of inhibitor by bone and uterus, but only freezing and thawing was effective in preventing inhibitor production by skin cultures.

TABLE I

REACTION OF INHIBITORS FROM THE MEDIA OF RABBIT TISSUES IN CULTURE WITH ACTIVATED COLLAGENASE FROM SKIN AND UTERUS

The results show the volumes (µl) of a crude (culture medium) and a partially purified preparation of each tissue inhibitor required to give 50% inhibition of 0.06 units of APMA-activated collagenase from either crude or partially purified rabbit uterus and skin culture media. n.d. = not determined.

Inhibitor source	Enzyme source			
	Skin		Uterus	
	Culture medium	Partially purified	Culture medium	Partially purified
A. Medium from skin cultures	30	25	120	n.d.
B. Medium from uterus cultures	n.d.	0.8	10	n.d.
After partial purification	6	4	43	54
C. Medium from bone culture	11	n.d.	55	n.d.
After partial purification	34	34	148	n.d.

The tissue inhibitors were found to prevent the action of naturally active bone collagenase and the APMA-activated form of the enzyme from the three tissues. Operating within the linearity of the fibril assay for collagenase (up to 70% collagen fibril lysis; [8]), the inhibition of collagenase activity was linear with respect to inhibitor concentration over the range 15—75% inhibition (Fig. 1). Table I compares the volumes of different inhibitory preparations required to give 50% inhibition of activated collagenase (0.06 units) from each tissue source. These volumes were calculated from plots such as those in Fig. 1. Although there is little difference in inhibitor activity on either crude or partially purified collagenase preparations, there is some variation between the tissue sources; notably larger amounts of inhibitor were needed to inhibit the uterus enzyme compared with an equivalent activity of skin or bone collagenase. The exact significance of these observations is as yet unclear; it could be due to variable amounts of APMA bound to the protein of individual preparations of enzyme or may be a reflection of a real difference in inhibitor/enzyme binding capacities.

Either heat treatment or incubation with trypsin abolished the activities of the skin and uterus inhibitors. Inclusion of 0.5 mM APMA in assays antagonised the action of bone, skin and uterus inhibitors on collagenase activity (Table II), although to varying extents. In each case gel chromatography of inhibitory media gave a single species of molecular weight approximately 30 000 (Table III). Incubation of a variety of combinations of activated enzyme (molecular weight approximately 25 000—29 000) and inhibitor, either from crude medium or after partial purification, was found to produce latent enzyme forms of higher molecular weight of approximately 42 000 (Table III).

On the basis of the data presented in this paper and in the previous communications [4,6] we propose that the latent collagenases of all rabbit tissues are composed of either very similar or identical enzyme-inhibitor complexes. Whether the inhibitors themselves are either identical or tissue specific can only be ascertained by further studies on purified materials. The apparent molecular weights of collagenase after activation of skin and uterus latent enzymes, and of the enzyme/inhibitor combinations, agree well with the values for naturally occurring active and latent forms respectively. It is possible that the inhibitors as detected are in a dimeric (or polymeric) form and that only a single monomeric unit combines with each molecule of collagenase; such a

TABLE II

INHIBITION BY APMA (0.5 mM) OF THE ACTIVITY OF TISSUE INHIBITORS ON ACTIVATED COLLAGENASE FROM DIFFERENT SOURCES

The results are expressed as the percentage inhibition of 0.06 units of collagenase. n.d. = not determined.

Inhibitor source		Enzyme source		
		Skin	Uterus	Bone
Skin	—APMA	50	50	n.d.
	+APMA	5	0	n.d.
Uterus	—APMA	50	50	n.d.
	+APMA	35—50	0	n.d.
Bone	—APMA	50	50	50
	+APMA	35	0	5

monomeric unit would then be comparable in molecular weight to that of a bovine cartilage inhibitor of human collagenase [10] and an inhibitor of collagenase from human synovial cell cultures (Harris, E.D., unpublished). Collagenase inhibitors are apparently produced by a wide variety of tissues in several different species. We have also found inhibitors of molecular weight approximately 30 000 in the culture media of rat and mouse bones and an inhibitor from human skin fibroblasts has been described [12].

The precise function of the tissue inhibitors in the extracellular control of collagenase activity remains to be elucidated. The most likely roles are either to inhibit the enzyme, post-function, or to form complexes with enzyme, for which a proteolytic activation step is required. However, we have no evidence for the presence of activators or proactivators in the culture media from either bone, skin or non-gravid uterus, as suggested by Vaes and Eeckhout [1] and Woessner [11]. Treatment of media with either 2 mM phenylmethane sulphonyl fluoride or up to 2.5 mg/ml soybean trypsin inhibitor does not alter the activation of latent collagenase by APMA, eliminating any role for serine proteinases in our systems. Also the addition of 4 mg/ml bovine serum albumin, which might be expected to modify the activity of any proteinases on latent collagenase, did not change the rate of APMA activation and did not cause any lag phase. The presence of proteinases from either serum [3] or invading cells in pathological conditions however, could be important in activating collagenase in situations where it would otherwise be latent.

TABLE III

APPARENT MOLECULAR WEIGHTS OF LATENT AND ACTIVATED FORMS OF COLLAGENASE, INHIBITORS, AND ACTIVATED ENZYME-INHIBITOR COMPLEXES FROM RABBIT TISSUES AS DETERMINED BY ACA44 ULTROGEL FILTRATION

A	Source		
	Skin	Uterus	Bone
Inhibitor	27 500	28 500	29 500
Latent enzyme	42 000	39 000	39 000
APMA-activated enzyme	25 000	25 500	29 000
B			
APMA-activated uterus enzyme combined with uterus inhibitor	42 000		
APMA-activated skin enzyme combined with uterus inhibitor	42 000		
APMA-activated skin enzyme combined with skin inhibitor	39 000		

Acknowledgements

We thank Mrs Wendy Beard and Mr Gary Dew for their expert technical assistance. This work was supported by funds from the Medical Research Council and Smith, Kline and French Foundation. E.C. is a Junior Beit Fellow.

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