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CHICK BONE COLLAGENASE INHIBITOR AND LATENCY OF COLLAGENASE

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SUMMARY: Collagenase and collagenase inhibitor were isolated from the culture fluid of embryonic chick bone. The inhibitor, separated as a high molecular weight aggregate (160,000-200,000 daltons) during gel filtration in 1M NaCl, dissociated in 6M urea to species of approx 25,000 daltons. The inhibition of collagenase activity by the addition of inhibitor was not reversed by the addition of trypsin or p-aminophenylmercuric acetate. However, isolated inhibitor alone was inactivated by treatment with either trypsin or p-aminophenylmercuric acetate. The results suggest that the latent form of chick bone collagenase is a proenzyme which converts into an active form without a detectable change in molecular weight and that this occurs after the inactivation of collagenase inhibitor.

Various tissues synthesize collagenase in a latent form which can be activated by tissue culture medium (1), trypsin (2), NaSCN (3), NaI (4) or by a thiol-blocking reagent such as p-aminophenylmercuric acetate (5). It is not clear from these data, however, whether the latent form of collagenase is a precursor zymogen which is enzymetically activated by limited proteolysis (1,2) or whether it is a non-covalently bound complex of active enzyme and inhibitor(s) (4,5). Indeed both types of latent collagenase may exist. Rabbit connective tissues in culture synthesize latent collagenase which has been shown to be a complex between active enzyme and tissue inhibitor. The enzyme activity could be restored (6). We have previously demonstrated latent collagenase in embryonic chick bone cultures and have reported results which also suggest the presence of a collagenase inhibitor (7). Recently, Welgus et al. (8) and Vater et al. (9) purified specific collagenase inhibitors and demonstrated the irreversible inhibition of collagenase by these inhibitors.

In this communication we report that when collagenase and inhibitor are individually isolated from the tissue culture media and then reacted, the

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Abbreviation: p-APMA, p-aminophenylmercuric acetate

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resulting inhibition cannot only be reactivated by treatment of the complex with trypsin or p-APMA. On the other hand, treatment of the isolated inhibitor with either trypsin or p-APMA prior to its reaction with collagenase prevents the inhibition of enzyme activity by the inhibitor.

MATERIALS AND METHODS

Preparation of Enzyme and Inhibitor: The cartilage-free portions of 14-day embryonic chick tibiae were cultured as described (7). In some instances the cultures were fed daily and continued for eight days. Following culture, the medium was frozen at -20°C prior to analysis. The pooled culture medium was concentrated approximately 5-fold on PM-10 membrane (Amicon Corp., Lexington, MA). Saturated $(NH_4)_2SO_4$ solution was added to the concentrated sample and the fraction, which precipitated out between 0-60% saturation, was collected. The precipitate was dissolved in 50 mM Tris-HCl, pH 7.6, and 5 mM Ca Cl_2 (Tris buffer) containing either 1M NaCl or 6M urea.

Activation of Latent Collagenase and Collagenase Assay: Culture media, ammonium sulfate fractions, and chromatographic fractions were activated with either p-APMA (5) (Aldrich Chemical Co., Milwaukee, WI) or trypsin (7). Collagenase activity was assayed as described (7). Inhibitor activity was assayed by adding inhibitor preparations to a known amount of active collagenase.

Column Chromatography: The ammonium sulfate fraction samples were chromatographed on a Sephadex G-200 gel filtration column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with Tris buffer containing 1M NaCl. They were also chromatographed on a Sephadex G-200 gel filtration column equilibrated with Tris buffer containing 6M urea. The columns were calibrated using phosphorylase b (188,000), bovine serum albumin (68,000), ovalbumin (44,500), carbonic anhydrase (29,000) and cytochrome c (12,500) as the standards (all from Sigma Chemical Co., St. Louis, MO).

Source of Other Collagenases and Enzymes: Mouse bone collagenase was prepared by heparin-Sepharose 4B gel affinity chromatography as described (10). Rat bone and human gingival collagenase were obtained from tissue culture medium (10). Bacterial collagenase from Clostridium histolyticum and trypsin-TPCK were purchased from Worthington Biochemical Co., Freehold, NJ.

RESULTS

Analyses of aliquot samples of tissue culture media each day over an eight-day culture period, demonstrated that collagenase and collagenase inhibitor were synthesized daily. The maximum release of latent collagenase and collagenase inhibitor was observed during the first or second day of incubation. If the explants were first devitalized by freeze-thawing, neither collagenase nor inhibition was detected in the tissue culture medium. This demonstrated that both components were actively synthesized by the explant during the period of tissue culture. Our preliminary findings that the tissue culture medium contained both latent collagenase and collagenase

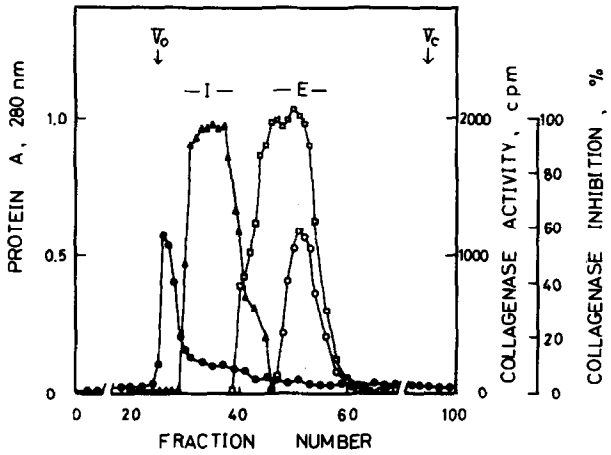

Fig. 1: Elution profile of chick bone collagenase inhibitor and collagenase from a column (1.6 x 90 cm) of Sephadex G-200 gel, equilibrated with 1M NaCl in Tris buffer. The ammonium sulfate fraction (approx 100-fold concentrated culture medium, 2.5 ml) was applied to the column and fractions (2.3 ml) were collected at 7.5 ml/h. Fractions were desalted by passing them through PD-10 columns (Pharmacia Fine Chemicals, Piscataway, NJ) prior to analysis. Desalted fractions (200 µl aliquots) were assayed for their ability to inhibit active mouse bone collagenase (0.3 units). The results are expressed as percentage inhibition of the stated amount of enzyme. Desalted fractions (25 µl aliquots) were also assayed for collagenase activity after activation with p-APMA (total enzyme) or without activation (active enzyme). (●—●): distribution of the protein measured in the absorbance at 280 nm. (Δ—Δ): distribution of collagenase inhibitor activity. □—□: distribution of total collagenase. (o—o): distribution of active collagenase.

inhibitor and that the two components could be separated by gel filtration led to the following experiments.

Gel filtration of the tissue culture medium in 1M NaCl revealed that fractions corresponding to a mol. wt. of 160,000-200,000 inhibited collagenase, whereas latent collagenase (approx 60% of total activity) and active collagenase (approx 40% of total activity) were detected in the fractions corresponding to mol. wts. of approx 60,000 and 50,000, respectively (Fig. 1). The recovery of inhibitor from the column was approx 65%. The recovery of total collagenase activity (latent plus active) from the column was approx 80%. The ratio of active enzyme over latent enzyme, however, progressively increased during the subsequent procedures (i.e., concentration, storage at $4^{\circ}C$, and rechromatography). The fractions corresponding to the enzyme were concentrated and further chromatographed either on the same column or on a 1.6 x 5 cm column of heparin-Sepharose 4B (10). A good deal of the latent enzyme was spontaneously converted to the active enzyme during further chromatography. Collagenase inhibition was also separated from the enzyme(s) by gel filtration in 6M urea. The molecular weight of the inhibitor appeared to be approx

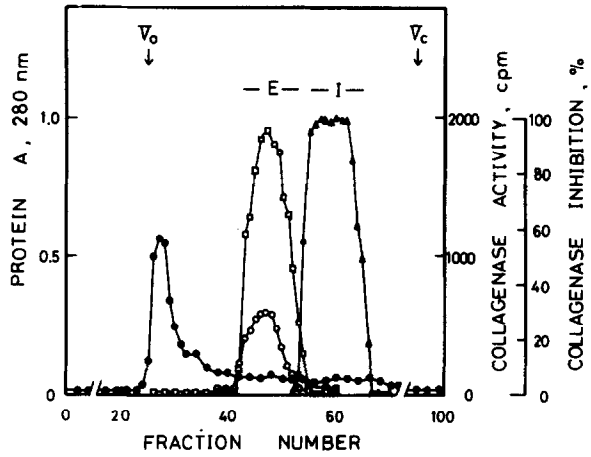

Fig. 2: Elution profile of chick bone collagenase inhibitor and collagenase from a column (1.6 x 90 cm) of Sephadex G-200 gel equilibrated with 6M urea in Tris buffer. The ammonium sulfate fraction (2.5 ml) was gel-filtered and fractions were assayed for inhibitor and enzyme activity as described in Fig. 1. (●——●): distribution of the protein measured in the absorbance at 280 nm. (Δ——Δ): distribution of collagenase inhibitor activity. (□——□): distribution of total collagenase. (o——o): distribution of active collagenase.

25,000 in 6M urea (Fig 2), indicating dissociation of a non-covalently bound aggregate in urea. The yield of the inhibitor was approx 1.4-fold higher by gel filtration in 6M urea than in 1M NaCl. Most importantly, when the latent and active forms of collagenase were molecularly filtered on Sephadex G-200 gel in 6M urea, they eluted in an identical position corresponding to a mol. wt. of approx 50,000. The recovery of total enzyme activity was approx 65%.

Table 1 shows that isolated enzyme inhibitor equally inhibited the *in vitro* activated collagenase (expt. A) and the spontaneously activated collagenase (expt. B). Table 2 demonstrates that the activity of purified collagenase was blocked even in the presence of p-APMA or trypsin (expts. 1-4). When the enzyme and inhibitor were preincubated before the addition of p-APMA or trypsin, inhibition was slightly greater (expts. 5-7).

Most importantly, when the enzyme inhibitor was preincubated with either p-APMA or trypsin, a procedure utilized to "activate" latent collagenases in crude enzyme preparations, the inhibitor was found to be inactivated (expts. 8-10, Table 2). These results clearly demonstrate that the reactions of the active enzyme with the inhibitor is very rapid and neither trypsin nor p-APMA are capable of reversing the inhibition of the active enzyme by the inhibitor *per se*.

The isolated inhibitor was found to be stable at pH 3.2 at 22°C for 2 h, at pH 7.6 at 56°C for 30 min. It did not lose activity when dialyzed

Table 1: The inhibition of activated and spontaneously activated chick bone collagenase by enzyme inhibitor.

Experiment	Collagenase Preparation[a]	Activation Procedure[b]	Inhibitor Preparation	Activity cpm	Activity %
A	Latent enzyme	Untreated	-	0	0
		Trypsin	-	1650	100[c]
		Trypsin	1M NaCl	594	36
		Trypsin	6M Urea	347	21
		p-APMA	-	1698	103
		p-APMA	1M NaCl	560	34
		p-APMA	6M Urea	365	22
B	Spontaneously activated enzyme	-	-	1590	100
		-	1M NaCl	730	46
		-	6M Urea	512	32

[a]Latent chick bone collagenase partially purified by ammonium sulfate fractionation (0.3 units per assay after trypsin activation) and spontaneously activated enzyme (0.3 units per assay) were used.

[b]Collagenase inhibitor preparations isolated from either 1M NaCl-Sephadex G-200 gel filtration or 6M urea-Sephadex G-200 gel filtration were concentrated approx 5-fold and dialyzed against Tris buffer. One hundred µl of sample solution was used in each assay.

[c]Activity obtained by trypsin activation was arbitrarily set at 100% activity.

against water and lyophilized. Chick bone collagenase inhibitor equally inhibited the same amounts of chick bone and mouse bone collagenase, both of which were similarly prepared by heparin-Sepharose gel affinity chromatography. The inhibitor similarly inhibited rat bone and human gingival collagenase but did not inhibit bacterial collagenase.

DISCUSSION

Our previous gel filtration study of active and latent chick bone collagenase (7) indicated an apparent difference in molecular weight between the latent and active enzymes of about 11,000. The present study showed a similar difference during gel filtration in 1M NaCl (Fig. 1), which was eliminated, however, when the fractions were chromatographed in the dissociative solvent, 6M urea. Moreover, when the mixture of the latent and active enzymes separated by molecular sieving in 6M urea was now refiltered in 1M NaCl, both the latent and active enzymes co-eluted in the identical position (results not shown). These results suggest that the apparent difference in molecular weight between latent and active enzyme may be ascribed to the interaction between inhibitor and latent enzyme during gel filtration under non-dissociative conditions. Although the tissue culture medium used for

Table 2: Inhibition of chick bone collagenase by chick bone collagenase inhibitor, and inactivation of the inhibitor by p-APMA or trypsin[a]

Experiment		Activity cpm	Activity %
1	Collagenase alone	1085	100
2	Collagenase + inhibitor	305	28
3	Collagenase + inhibitor + p-APMA	293	27
4	Collagenase + inhibitor + trypsin	325	30
5	Collagenase + inhibitor, preincubated then added to the assay	225	21
6	Collagenase + inhibitor, preincubated then added to the assay with p-APMA	240	22
7	Collagenase + inhibitor, preincubated then added to the assay with trypsin	228	21
8	Inhibitor + p-APMA, preincubated then added to the assay with collagenase	1074	99
9	Inhibitor + p-APMA, preincubated, dialyzed then added to the assay with collagenase	944	87
10	Inhibitor + trypsin, preincubated, SBTI[b] added, then added to the assay with collagenase	1075	91

[a]Active chick bone collagenase prepared by heparin-Sepharose affinity chromatography (specific activity approx 2,100 units/mg of protein, 0.3 units per assay) and collagenase inhibitor purified by 6M urea-Sephadex gel filtration (3 µg per assay) were used in all experiments. p-APMA (final concentration 1 mM) or trypsin-TPCK (1.0 µg per assay) was added to appropriate experiments. Preincubation of the mixture of enzyme and inhibitor was carried out at 37°C for 10 min. Preincubation of inhibitor with either p-APMA or trypsin was carried out at ambient temperature (22°C) for 10 min; the procedure was essentially the same as for activation of latent collagenase in crude enzyme preparations.

[b]SBTI: soybean trypsin inhibitor.

the gel filtration showed no active collagenase activity, the emergence of the active enzyme during gel filtration of the tissue culture medium and its progressive conversion during subsequent procedures may be due to an autocatalytic conversion (11), activation mediated by other tissue components (12,13) or a combination of these mechanisms.

The results of the present experiments on the failure of trypsin or p-APMA to reactivate the collagenase after its reaction with inhibitor, which are similar to those reported by Welgus et al. (8) and Vater et al. (9), plus the additional data that the action of the inhibitor can be prevented by its prior treatment with trypsin or p-APMA, make it quite clear that an enzyme-inhibitor complex, originally presumed to be responsible for the latency of the enzyme (7) does not completely describe the chick bone enzyme system. In addition to the data noted above, we have also found that gel filtration of a mixture of isolated inhibitor and active enzyme under the conditions described in the present study results in complete loss of

enzyme activity; neither latent nor active enzyme can be recovered (results not shown).

There are clearly a number of different schemas which one might formulate to explain the data. These include the proposal that chick bone collagenase is synthesized as a proenzyme which only interacts weakly if at all with the major inhibitor isolated in this study. This would explain why latent enzyme (in the form of a proenzyme) and inhibitor were so easily separated from each other in 1M NaCl during gel filtration. The result is consistent with those of the study by Welgus et al. (8). Tissue activators which are also released into the tissue culture system (13) activate the proenzyme to an active enzyme with little or no loss in molecular weight (14), and it is this active enzyme which is inhibited by the inhibitor isolated in this study (and possibly others) and thus keeps control and regulates local collagenolytic activity. The action of trypsin and p-APMA in activating latent chick bone collagenase may be then visualized as the result of inactivation of inhibitor(s) in the crude tissue culture medium and/or enhancement of the activation of the proenzyme.

In trying to formulate any schema to explain these data and those in the literature, the characteristics of the inhibitor are of great interest. We have therefore investigated some of the characteristics of isolated chick bone collagenase inhibitor. The inhibitor is susceptible to trypsin. Preincubation of the inhibitor with trypsin results in a dose-dependent inactivation of the inhibitor (data not shown). The inactivation of the inhibitor by thiol-blocking agent (p-APMA) suggests that the inhibitor molecule contains a sulfhydryl group which is essential for its inhibitory activity. The removal of p-APMA by dialysis from the preincubation mixture resulted in slightly less inactivation of inhibitor (Table 2) indicating that inactivated inhibitor partially recovers its activity after dialysis. The inhibitor is different from α_2-macroglobulin (15) and β_1-anticollagenase (16) in its molecular weight and acid stability, but similar to other collagenase inhibitors isolated from fibroblasts (8), tendon (9), and smooth muscle cells (17). The inhibitor specifically inhibits tissue collagenases; however, there appeared to be no species specificity between the inhibitor and various tissue collagenases. In addition to chick bone collagenase, the chick bone collagenase inhibitor inhibited mouse bone, rat bone, and human gingival collagenase at a similar rate. The results are consistent with other recent studies (8,9). The control of the local activity of tissue collagenase synthesized by chick bone tissue may depend in part on the activity of collagenase

inhibitor which is also synthesized in this tissue. Whether the same or different bone cells produce both the enzyme and inhibitor remains to be investigated.

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