Proactivator-dependent activation of procollagenase induced by treatment with EGTA

Carol A. VATER,* Hideaki NAGASE†‡ and Edward D. HARRIS, Jr.†§

*Department of Medicine and *Department of Biochemistry, Dartmouth–Hitchcock Medical Center, Hanover, NH 03756, U.S.A., and †Departments of Medicine and ‡Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Rutgers Medical School, New Brunswick, NJ 08903, U.S.A.

A new mechanism for activation of the proactivator of procollagenase [Vater, Nagase & Harris (1983) *J. Biol. Chem.* **258**, 9374–9382] has been found. Collagenolytic and other proteolytic enzyme activities in the medium of cultured rabbit synovial fibroblasts were found to be activated by a new mechanism: short-term incubation at 37 °C performed in the presence of EGTA followed by replacement of Ca^{2+} during enzyme assay. The crucial event in procollagenase activation is the production of a functional activator enzyme. Activation of procollagenase in the culture medium did not occur when proactivator was removed by immunoprecipitation. Proteolytic activity of proactivator was fully activated, whereas procollagenase alone could not be activated by the same sequence. EGTA treatment of the culture medium at 0 °C did not result in enzyme activation if Ca^{2+} was replaced before incubation at 37 °C. Certain other bivalent metal ions (e.g. Sn^{2+} , Cd^{2+} , Zn^{2+} and Mn^{2+}) could substitute for Ca^{2+} to stabilize the proactivator as a zymogen and therefore prevent the appearance of proteolytic activity in culture medium. Isolation of proactivator and procollagenase from EGTA-treated radiolabelled culture medium by immunoprecipitation and subsequent analyses by fluorography revealed that a time-dependent proteolysis of both molecules occurred after replacement of Ca^{2+} and incubation at 37 °C. However, comparison of enzyme activity with fluorographic analyses showed that the maximal activation of both enzymes was achieved before any detectable decrease in M_r . The results suggest that the activation of proactivator and the subsequent activation of procollagenase may be initiated by conformational changes in structure of the proactivator molecule produced by removal of stabilizing bivalent metal ions.

INTRODUCTION

Collagenolytic and/or gelatinolytic metalloenzymes have been found in medium from numerous cultured tissues and cells, including rabbit bone (Murphy *et al.*, 1981), human skin (Bauer *et al.*, 1975; Shinkai & Nagai, 1977; Seltzer *et al.*, 1981), rheumatoid synovium (Harris & Krane, 1972; Woolley *et al.*, 1975; Vater *et al.*, 1978), pig synovium (Cawston & Tyler, 1979), mouse bone (Vaes, 1972), chick bone (Sakamoto *et al.*, 1978), bovine gingiva (Birkedal-Hansen *et al.*, 1976) and rat uterus (Woessner, 1977, 1979; Halme *et al.*, 1980). In most cases these neutral metalloproteinases have been isolated in latent form, requiring activation by limited proteolysis or by organic mercurials before significant activity is detected.

Latent collagenase from rabbit synovial fibroblasts is secreted as a proenzyme (Nagase *et al.*, 1981). An activator of procollagenase, also synthesized by cultured rabbit synovial fibroblasts, is secreted in latent form (proactivator) as a doublet protein of M_r 53400 and 51900 (Vater *et al.*, 1983). Activation of rabbit synovial procollagenase (M_r 57800 and 53500) by either $\text{NH}_2\text{Ph-Hg-Ac}$ or trypsin has been shown to be mediated by the activator, which itself contained proteolytic activity against gelatin, azocasein and reduced and carboxy-

methylated bovine serum albumin, but not against collagen. Procollagenase treated with trypsin in the absence of activator is converted into an inactive lower- M_r doublet (M_r 49000 and 45000) form that could be activated subsequently by the addition of activator (Vater *et al.*, 1983). In the present paper we describe studies on the role of Ca^{2+} in the activation of proteolytic and collagenolytic activities found in culture medium from stimulated rabbit synovial fibroblasts. The Ca^{2+} requirement for activity of collagenase has been well established and is thought to be related to conformational stability of the activated enzyme (Seltzer *et al.*, 1976; Berman, 1980). Little is known, however, about the importance of Ca^{2+} in the process of activation of the latent enzymes. We describe a new mechanism for the activation of proactivator. The incubation of the zymogen at 37 °C in the absence of available Ca^{2+} followed by the addition of Ca^{2+} converts the proactivator into an active neutral proteinase and an effective activator of procollagenase.

MATERIALS AND METHODS

Materials

Materials were purchased from the following sources: phorbol 13-myristate 12-acetate from Consolidated Midland; L-tosylphenylalanylchloromethane-treated tryp-

Abbreviations used: CM, crude conditioned culture medium from rabbit synovial fibroblasts stimulated with either phorbol 13-myristate 12-acetate or crystals of monosodium urate monohydrate; F(ab')₂, bivalent antigen-binding fragment of IgG; [³H]Cm-Tf, reduced and [³H]carboxymethylated human transferrin; $\text{NH}_2\text{Ph-Hg-Ac}$, 4-aminophenylmercuric acetate.

§ To whom all correspondence should be addressed.

sin from Millipore Corporation; $\text{NH}_2\text{Ph-Hg-Ac}$ from Aldrich Chemical Co.; EGTA, di-isopropyl phosphorofluoridate, Brij-35, bovine serum albumin, soya-bean trypsin inhibitor and L-arginine hydrochloride from Sigma Chemical Co.; $[1\text{-}^{14}\text{C}]\text{acetic anhydride}$ (21 mCi/mmol) and $[4,5\text{-}^3\text{H}]\text{leucine}$ (108 mCi/mmol) from Amersham; M_r standard proteins and chemicals for SDS/polyacrylamide-gel electrophoresis from Bio-Rad Laboratories; pressure-filtration apparatus and UM-10, PM-10 and YM-10 membranes from Amicon Corp.; Protein A-Sepharose CL-4B from Pharmacia Fine Chemicals.

Methods

Cell culture and preparation of proactivator and procollagenase. Culture medium from rabbit synovial fibroblasts grown in monolayer cultures in the absence of serum and stimulated with phorbol 13-myristate 12-acetate (Brinckerhoff *et al.*, 1979) or crystals of monosodium urate monohydrate (McMillan *et al.*, 1981) was used as the source of latent enzymes. Culture medium was concentrated 10–20-fold by pressure filtration on Amicon UM-10, PM-10 or YM-10 membranes. Radiolabelled proactivator and procollagenase were produced by culturing cells in the presence of $[^3\text{H}]\text{leucine}$ (Nagase *et al.*, 1983). Proactivator and procollagenase were purified by immunoabsorption affinity chromatography on F(ab')_2 -agarose columns prepared from specific anti-activator or anti-collagenase IgG as described elsewhere (Vater *et al.*, 1981, 1983).

Assays for collagenolytic and proteolytic activities. ^{14}C -labelled guinea-pig skin collagen was prepared by standard methods (Glimcher *et al.*, 1964; Gisslow & McBride, 1975). Collagenolytic activity was measured with 150 μg of reconstituted radiolabelled collagen fibrils as substrate (Harris *et al.*, 1969). Unless stated otherwise, trypsin blanks (1 μg) as controls for non-specific substrate degradation were subtracted for the calculation of percentage lysis of the substrate. Non-collagenolytic proteolytic activity was measured against $[^3\text{H}]\text{Cm-Tf}$. The substrate was prepared by reaction of reduced transferrin with $[^3\text{H}]\text{acetic anhydride}$ (T. Yee, H. Nagase & E. D. Harris, Jr., unpublished work). In assays with this substrate, 80 μg of $[^3\text{H}]\text{Cm-Tf}$ (20 μl) was incubated in 50 mM-Tris/HCl buffer, pH 7.6, containing 200 mM-NaCl and 10 mM- Ca^{2+} at 37 °C with the sample to be tested in a total volume of 50 μl . Buffer blanks and trypsin (2 μg) positive controls were included to monitor reagent lysis and total degradation of substrate respectively. Assays were terminated by the addition of 250 μl of 3% (w/v) trichloroacetic acid. After centrifugation in a Beckman Microfuge B, radioactivities of portions of the supernatants were counted to determine the percentage release of radioactivity. Unless otherwise noted, final Ca^{2+} concentrations were adjusted to approx. 10 mM for all collagenase and proteinase assays. $\text{NH}_2\text{Ph-Hg-Ac}$ at 1.0–1.7 mM was added to some assays as a control for maximal activation of latent enzymic activity. One unit of collagenase and proteinase activity was defined to be that amount of enzyme capable of degrading 1 μg of substrate/min at 37 °C.

SDS/polyacrylamide-gel electrophoresis. SDS/polyacrylamide-gel electrophoresis was performed by the method of Wyckoff *et al.* (1977) as described by Barrett

et al. (1979), with 7.5% or 11% polyacrylamide slab gels. Samples were prepared in buffer containing 1% (w/v) SDS and 0.5% (v/v) 2-mercaptoethanol, and heated for 3 min at 100 °C. Standard proteins for estimation of M_r were phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000) and soya-bean trypsin inhibitor (M_r 21000). Procedures for immunoprecipitation of $[^3\text{H}]\text{leucine}$ -labelled proactivator and procollagenase with specific antibodies, isolation of the immune complexes with Protein A-Sepharose and fluorography of the slab gels after electrophoresis have been described previously (Vater *et al.*, 1981; Nagase *et al.*, 1983). Immunoprecipitations were carried out in the presence of proteinase inhibitors (10 mM-EDTA and 2 mM-di-isopropyl phosphorofluoridate) at pH 8.6 in order to prevent proteolysis of the sample (Nagase *et al.*, 1983).

RESULTS

Activation of proteolytic and collagenolytic activities in CM by treatment with EGTA

Incubation of concentrated rabbit synovial fibroblast CM at 22 °C in the presence of EGTA followed by replacement of Ca^{2+} during enzyme assay resulted in the 'spontaneous' activation of both collagenase activity and neutral proteinase activity. As shown in Table 1, maximal collagenolysis was observed with the sample incubated for 10 min in the absence of Ca^{2+} that was not treated with trypsin. The collagenase activity surpassed even that of the sample activated with trypsin in the presence of Ca^{2+} . Trypsin treatment of CM in the absence of Ca^{2+} not only failed to activate procollagenase, but SDS/polyacrylamide-gel analysis of procollagenase (results not shown) revealed that enzyme proteins had been degraded under these conditions to fragments of M_r less than 10000. Analysis of the time course of activation indicated that 5 min of incubation at 37 °C in Ca^{2+} -free buffer was sufficient to activate collagenolytic activity to

Table 1. Detection of collagenase activity in CM after EGTA treatment

Samples (100 μl) of 16-fold-concentrated CM dialysed against 10 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EGTA were incubated at 22 °C for 10 min in the presence or in the absence of 2.5 mM- Ca^{2+} . Subsequently, 50 μl portions of the samples were treated with (a) trypsin (10 $\mu\text{g}/\text{ml}$, 30 min, 22 °C, followed by a 5-fold molar excess of soya-bean trypsin inhibitor) to activate residual latent enzyme or (b) a trypsin/soya-bean trypsin inhibitor (1:5) mixture to provide sham-activated controls. Collagenolytic activity was measured in the presence of approx. 10 mM- Ca^{2+} .

	Collagenase activity (units)	
	(a) With trypsin activation	(b) Without trypsin activation
EGTA-containing CM incubated with Ca^{2+}	0.72 0.75	0.15 0.28
EGTA-containing CM incubated without Ca^{2+}	0.05 0.05	0.92 0.94

Table 2. Activation of purified procollagen by EGTA treatment in the presence or in the absence of purified proactivator

Samples of purified procollagenase (proC) (20 μ l, $A_{280}/\text{ml} = 0.165$, 50 mM-Tris/HCl buffer, pH 7.6, containing 170 mM-NaCl, 1 mM- CaCl_2 and 0.05% Brij-35), purified proactivator (proA) (20 μ l, $A_{280}/\text{ml} = 0.777$, 50 mM-Tris/HCl buffer, pH 7.6, containing 170 mM-NaCl) and CM depleted of both proC and proA (CM-[proC+proA]) (30 μ l of 9-fold concentrate) and their combinations were tested for activation of collagenolytic activity. All samples were incubated for 10 min at 37 °C before assay, but only those listed under 'EGTA activation' were incubated in the presence of 3 mM-EGTA. $\text{NH}_2\text{Ph-Hg-Ac}$ activation was achieved by the inclusion of 1.7 mM- $\text{NH}_2\text{Ph-Hg-Ac}$ in the fibril assay mixture. All samples were assayed for active collagenase in the presence of 10 mM- Ca^{2+} , and the results are given as means for two determinations.

	Collagenase activity (units)		
	EGTA activation	No activation	$\text{NH}_2\text{Ph-Hg-Ac}$ activation
ProC	0	0	0
ProA	0	0	0
CM-[proC+proA]	0	0	0
ProC+proA	0.42	0	0.37
ProC+(CM-[proC+proA])	0	0	0.02
ProC+proA+(CM-[proC+proA])	0.46	0	0.46
ProA+(CM-[proC+proA])	0	0	0

73% of the maximum achieved by $\text{NH}_2\text{Ph-Hg-Ac}$ activation.

Requirement of proactivator for procollagenase activation by EGTA treatment

We have shown previously that the activation of rabbit synovial procollagenase requires endogenous activator (Vater *et al.*, 1983). To determine whether the activation of procollagenase produced by EGTA treatment also is dependent upon the presence of proactivator and/or some other factor present in CM, we performed a recombination experiment with affinity-purified procollagenase and proactivator, and CM depleted of both zymogens (CM-[proC+proA]). As shown in Table 2, the activation of procollagenase by treatment with either EGTA or $\text{NH}_2\text{Ph-Hg-Ac}$ was dependent upon the presence of proactivator. No collagenolytic activity was observed with procollagenase alone. No change in measurable collagenase activity was observed when CM-[proC+proA] was included in the mixture during activation, indicating that no additional factor(s) in CM was required to reconstitute the EGTA effect on procollagenase activation.

We then determined whether EGTA treatment alone is sufficient for the activation of procollagenase or whether the subsequent addition of Ca^{2+} is required. This was approached by using specific anti-activator antibody. As illustrated in Fig. 1, activation of procollagenase in CM after EGTA treatment was prevented by removal of activator with anti-activator IgG before the addition of Ca^{2+} , indicating that procollagenase activation by EGTA-treated proactivator takes place after the addition of Ca^{2+} . In the absence of specific antibody, EGTA treatment produced levels of enzyme activity comparable with those generated by treatment with $\text{NH}_2\text{Ph-Hg-Ac}$ (Fig. 1).

Proteolytic activity of proactivator after EGTA treatment

When purified proactivator (in 50 mM-Tris/HCl buffer, pH 7.6, containing 0.5 M-NaCl and 0.05% Brij-35) was incubated for 10 min at 37 °C in the presence of 2 mM-EGTA, it exhibited 0.19 unit (74%) of proteolytic

Fig. 1. Inhibition of procollagenase activation by anti-activator IgG

Samples of culture medium (55 μ l, 18-fold-concentrated, dialysed into 50 mM-Tris/HCl buffer, pH 7.6, containing 200 mM-NaCl) were incubated for 10 min on ice and then for 10 min at 37 °C in the presence of either 1 mM-EGTA or 1 mM- Ca^{2+} . Either anti-activator or non-immune IgG (165 μ l, 4 mg/ml) was then added for immunoprecipitation at 37 °C for 30 min and then at 4 °C for 16 h. Di-isopropyl phosphorofluoridate (4 mM) was included to inhibit any serine proteinases possibly contaminating the IgG preparations. After centrifugation, portions of the supernatants were assayed in the presence of 10 mM- Ca^{2+} for collagenolytic activity in the absence or in the presence of $\text{NH}_2\text{Ph-Hg-Ac}$ to activate residual latent proactivator. Incubation of duplicate samples with collagen substrate was terminated at 15, 40, 65, 120 or 150 min after the start of the fibril assay. Percentage lysis was calculated after subtraction of the buffer-reagent blank rather than the trypsin blank, which produced an additional 12% lysis. Δ , (CM+EGTA)+control IgG; $\square$, (CM+EGTA)+anti-activator IgG; $\blacksquare$, (CM+ Ca^{2+})+control IgG; $\blacktriangle$, (CM+ Ca^{2+})+anti-activator IgG; $\circ$, (CM+ Ca^{2+})+control IgG, $\text{NH}_2\text{Ph-Hg-Ac}$ in assay; $\bullet$, (CM+ Ca^{2+})+anti-activator IgG, $\text{NH}_2\text{Ph-Hg-Ac}$ in assay.

activity against [^3H]Cm-Tf compared with 0.25 unit (100%) after activation with trypsin and 0.07 unit (27%) found in the unactivated preparation. More proteinase activity in purified proactivator was always revealed with activation by $\text{NH}_2\text{Ph-Hg-Ac}$ or trypsin than with EGTA treatment, possibly owing to the instability of the EGTA-treated purified activator at 37 °C. Notwithstanding, these experiments did indicate that proteolytic activity in activator could be revealed by EGTA treatment, whereas collagenase activity in procollagenase could not be detected by such treatment unless proactivator was present (Table 2).

Studies of M_r change associated with EGTA-induced activation of proactivator and procollagenase

To determine whether the EGTA-induced activation of proactivator and procollagenase was associated with decreases in M_r of the proenzyme species, [^3H]leucine-labelled and unlabelled samples were treated with EGTA for 15 min at 37 °C, and then incubated with Ca^{2+} for various times at 37 °C. Unlabelled samples were assayed for enzymic activities during a 90 min assay, and the labelled samples were immunoprecipitated to determine the changes in M_r by fluorography after SDS/polyacrylamide-gel electrophoresis.

Maximally EGTA-activated activities of collagenase and activator were seen when Ca^{2+} was added at time zero of the enzyme assays, when both enzymes were completely in high- M_r precursor form (Fig. 2). Progressive proteolysis of the EGTA-treated doublet proteins of both enzymes resulted from longer incubation at 37 °C in the presence of Ca^{2+} , but was associated with little change (decrease less than 10%) in specific activity. This suggests that the apparent decrease in M_r of proactivator and procollagenase protein observed upon prolonged incubation is not a prerequisite for activation of enzyme activity, but, rather, is a reflection of proteolysis by a catalytically active precursor form.

Fig. 2. Conversion into lower- M_r forms of proactivator and procollagenase

[^3H]Leucine-labelled CM was treated with 3 mM-EGTA for 15 min at 37 °C. Ca^{2+} (final concn. 15 mM) was then added, and aliquot samples were incubated at 37 °C for 0, 10, 20, 30 or 60 min (*60 min incubation in the presence of 1 mM- $\text{NH}_2\text{Ph-Hg-Ac}$). Samples were then immunoprecipitated with either (a) anti-collagenase serum or (b) anti-activator serum and analysed by fluorography.

Fig. 3. Effect of Ca^{2+} on protection against proactivator activation by stabilization of zymogen conformation

CM (40-fold concentrated) was dialysed at 4 °C into 50 mM-Tris/HCl buffer, pH 7.8, containing 500 mM-NaCl and 1 mM-EGTA. Portions were incubated at 37 °C for 10 min either after or before the replacement of an equal volume of various concentrations of Ca^{2+} (the final concentration of EGTA was 0.5 mM). ●, CM incubated sequentially in: (1) 1 mM-EGTA at 0 °C for 10 min; (2) 1 mM-EGTA at 37 °C for 10 min; (3) various concentrations of Ca^{2+} at 0 °C for 10 min. Δ, CM incubated sequentially in: (1) 1 mM-EGTA at 0 °C for 10 min; (2) various concentrations of Ca^{2+} at 0 °C for 10 min; (3) the same various concentrations of Ca^{2+} at 37 °C for 10 min. Following the preincubation steps, samples were assayed in duplicate against 80 μg of [^3H]Cm-Tf in the presence of 10 mM- Ca^{2+} .

Stabilization of proactivator by Ca^{2+} and other bivalent cations against activation

At this point we had established that (1) EGTA treatment activates latent neutral metalloproteinase activity in CM by a temperature-dependent process, (2) EGTA-induced activation of procollagenase is prevented by removal of proactivator by specific immunoprecipitation, and (3) the proteolytic activity of purified proactivator can be activated by the Ca^{2+} -depletion/replacement sequence. These data indicate that the direct activation effect of metal-ion chelation is on proactivator, but not on procollagenase. The activation of proactivator by EGTA could be prevented if Ca^{2+} was added before the 37 °C incubation, but the active form of the enzyme could not be reversed to a latent form once Ca^{2+} was

added after the 37 °C incubation. These observations suggest that the function of Ca^{2+} is to stabilize proactivator against spontaneous activation by molecular perturbation. Such a role of Ca^{2+} in proactivator was further investigated by the following experiments.

Concentrated CM dialysed extensively at 4 °C against 50 mM-Tris/HCl buffer, pH 7.6, containing 0.5 M-NaCl and 1 mM-EGTA (containing less than 2% activated enzyme) was placed either on ice or at 37 °C for 10 min, and then mixed with various concentrations of Ca^{2+} at 0 °C. Near-maximal proteolytic activity was observed in all samples incubated at 37 °C with EGTA (Fig. 3). On the other hand, when Ca^{2+} was replenished to samples in EGTA without warming at 37 °C, Ca^{2+} protected the zymogen from activation in a dose-dependent manner (Fig. 3).

Similar experiments were carried out to test the ability of other bivalent metal ions to protect the zymogen activation, with care being taken to account for inhibition of active proteinase activity by an excess of any particular ion. Chloride, sulphate, hydroxide or acetate salts of Ca^{2+} , Mn^{2+} , Cd^{2+} , Cu^{2+} , Zn^{2+} , Sn^{2+} , Ba^{2+} , Mg^{2+} , Fe^{2+} , Hg^{2+} and Co^{2+} were dissolved (suspended in the case of insoluble or poorly soluble salts of Sn^{2+} and Ba^{2+}) at 10 mM in 1 M-Tris/HCl buffer, pH 7.6, and tested at a final concentration of 5 mM for stabilization of latent proteolytic activity in the presence of 0.5 mM-EGTA and for inhibition of active proteinase. Several bivalent metal ions in addition to Ca^{2+} were capable of preventing autoactivation of the proenzyme. Among metal ions affording greater than 40% protection, the order of stabilization potential was as follows: Sn^{2+} (82%), Ca^{2+} (62%), Cd^{2+} (48%), Zn^{2+} (45%) and Mn^{2+} (41%).

DISCUSSION

Previously we reported that purified rabbit synovial fibroblast procollagenase is not activated directly by trypsin or organic mercurials, but that its activation is dependent upon the activator molecule found in a proenzyme form in the same culture medium (Vater *et al.*, 1983). Proactivator is activated directly by trypsin or $\text{NH}_2\text{-Ph-Hg-Ac}$ (Vater *et al.*, 1983). In the present paper we have described a new mechanism for activation of proactivator: treatment of proactivator with EGTA for 5 min at 37 °C followed by the addition of an excess amount of Ca^{2+} . This results in the 'spontaneous' activation of proactivator to a form capable of activating procollagenase as well as expressing its proteolytic activity. This activation process is temperature-dependent; it does not occur without warming the proenzyme molecule in a Ca^{2+} -free solution.

The first step in the sequence of activation of proactivator by this treatment may be perturbation of the zymogen molecule in a Ca^{2+} -free solution at 37 °C, which is sufficient to induce the molecule to an active state. Indeed, maximal activities of collagenase and activator were observed in samples containing only high- M_r precursor forms of these enzymes (Fig. 2). Subsequent incubation with Ca^{2+} induced proteolysis of the molecules that was not accompanied by changes in specific activity, indicating that the precursor forms were sufficiently active. No proteolytic activity or ability to activate procollagenase was demonstrated until Ca^{2+} was replaced. That conformational changes in proactivator, as well as procollagenase, occur when Ca^{2+} is chelated by

EGTA is supported by the observation that digestion products of both molecules produced by trypsin in the presence of EGTA are different from those produced by trypsin in the presence of Ca^{2+} (results not shown). Similar observations have been reported for other proteins, such as troponin C (Grabarek *et al.*, 1981), human coagulation Factors IX and X (Chuang *et al.*, 1974; Karpati *et al.*, 1982) and calmodulin (Walsh & Stevens, 1977; Drabikowski *et al.*, 1977), all of which become more susceptible to proteolytic digestion in the absence of Ca^{2+} , presumably owing to the exposure of peptide bonds inaccessible in a conformational state stabilized by Ca^{2+} .

A similar mechanism of Ca^{2+} -dependent activation of procaspain has been described by Pontremoli *et al.* (1984). This proenzyme purified from rat liver in the presence of EGTA is activated by 0.1–1.0 mM- Ca^{2+} in the absence of substrate or by 1–5 μM - Ca^{2+} in the presence of substrate. In contrast with our results, however, it is not clear whether the activation of procaspain in the presence of Ca^{2+} is dependent upon an initial change in enzyme conformation induced by prior removal of bivalent cations from the proenzyme by treatment with EGTA.

In a search to understand what factor(s) are ultimately responsible for activating the collagenolytic process *in vivo*, all potential mechanisms for the activation of any component of that system are worthy of investigation. Many activation strategies for collagenases have been tested, including treatment with proteinases (Birkedal-Hansen *et al.*, 1976; Werb *et al.*, 1977; Eeckhout & Vaes, 1977; Nagase *et al.*, 1982), chaotropic agents (Nagai *et al.*, 1975; Ohyama & Hashimoto, 1977; Shinkai & Nagai, 1977; Sakamoto *et al.*, 1978), mercurial compounds (Werb & Burleigh, 1974; Sellers & Reynolds, 1977; Cawston *et al.*, 1981) and detergents (Birkedal-Hansen & Taylor, 1982). The method of zymogen activation described here has allowed us to appreciate the intra- and inter-molecular interactions of two of the principal molecules involved in collagen degradation. Our results indicate that, in addition to being essential for the expression of activity of both activator and collagenase, Ca^{2+} functions to stabilize the conformation of the proactivator zymogen and thereby to prevent proactivator from autoactivation at 37 °C.

This work was supported by National Institutes of Health Grants AM 14780 and AM 20641, and by a Clinical and Research Center Grant from the Arthritis Foundation. We thank Francine Mittleman for her assistance in the preparation of the manuscript.

REFERENCES

- Barrett, A. J., Brown, M. A. & Sayers, C. A. (1979) *Biochem. J.* **181**, 401–418
- Bauer, E. A., Stricklin, G. P., Jeffrey, J. J. & Eizen, A. Z. (1975) *Biochem. Biophys. Res. Commun.* **64**, 232–240
- Berman, M. B. (1980) in *Collagenase in Normal and Pathological Connective Tissues* (Woolley, D. E. & Evanson, J. M., eds.), pp. 141–174, John Wiley and Sons, Chichester
- Birkedal-Hansen, H. & Taylor, R. E. (1982) *Biochem. Biophys. Res. Commun.* **107**, 1173–1178
- Birkedal-Hansen, H., Cobb, C. M., Taylor, R. E. & Fullmer, H. M. (1976) *J. Biol. Chem.* **251**, 3162–3168
- Brinckerhoff, C. E., McMillan, R. M., Fahey, J. V. & Harris, E. D., Jr. (1979) *Arthritis Rheum.* **22**, 1109–1116

- Cawston, T. E. & Tyler, J. A. (1979) *Biochem. J.* **183**, 647–656
- Cawston, T. E., Mercer, E. & Tyler, J. A. (1981) *Biochim. Biophys. Acta* **657**, 73–83
- Chuang, T. F., Sargeant, R. B. & Hougie, C. (1974) *Br. J. Haematol.* **27**, 281–287
- Drabikowski, W., Kuznicki, J. & Grabarek, Z. (1977) *Biochim. Biophys. Acta* **485**, 124–133
- Eeckhout, Y. & Vaes, G. (1977) *Biochem. J.* **166**, 21–31
- Gisslow, M. T. & McBride, B. C. (1975) *Anal. Biochem.* **68**, 70–78
- Glimcher, M. J., Francois, C. J., Richards, L. & Krane, S. M. (1964) *Biochim. Biophys. Acta* **93**, 585–602
- Grabarek, Z., Drabikowski, W., Vinokurov, L. & Lu, R. C. (1981) *Biochim. Biophys. Acta* **671**, 227–223
- Halme, J., Tyree, B. & Jeffrey, J. J. (1980) *Arch. Biochem. Biophys.* **199**, 51–61
- Harris, E. D., Jr. & Krane, S. M. (1972) *Biochim. Biophys. Acta* **258**, 566–576
- Harris, E. D., Jr., Dibona, D. R. & Krane, S. M. (1969) *J. Clin. Invest.* **48**, 2104–2113
- Karpati, J., Varadi, K. & Elodi, S. (1982) *Hoppe-Seyler's Z. Physiol. Chem.* **365**, 521–525
- McMillan, R. M., Vater, C. A., Hasselbacher, P., Hahn, J. & Harris, E. D., Jr. (1981) *J. Pharm. Pharmacol.* **33**, 382–384
- Murphy, G., Cawston, T. E., Galloway, W. A., Barnes, M. J., Bunning, R. A. D., Mercer, E., Reynolds, J. J. & Burgeson, R. E. (1981) *Biochem. J.* **199**, 807–811
- Nagai, Y., Hori, H., Kawamoto, T. & Komiya, M. (1975) in *Dynamics of Connective Tissue Macromolecules* (Burleigh, P. M. C. & Poole, A. R., eds.), pp. 171–187, Elsevier/North-Holland, Amsterdam
- Nagase, H., Jackson, R. C., Brinckerhoff, C. E., Vater, C. A. & Harris, E. D., Jr. (1981) *J. Biol. Chem.* **256**, 11951–11954
- Nagase, H., Cawston, T. E., DeSilva, M. & Barrett, A. J. (1982) *Biochim. Biophys. Acta* **702**, 133–142
- Nagase, H., Brinckerhoff, C. E., Vater, C. A. & Harris, E. D., Jr. (1983) *Biochem. J.* **214**, 281–288
- Ohyama, H. & Hashimoto, K. (1977) *J. Biochem. (Tokyo)* **82**, 175–183
- Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., Michetti, M. & Horecker, B. L. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 53–56
- Sakamoto, S., Sakamoto, M., Matsumoto, A., Goldhaber, P. & Glimcher, M. J. (1978) *FEBS Lett.* **88**, 53–58
- Sellers, A. & Reynolds, J. J. (1977) *Biochem. J.* **167**, 353–360
- Seltzer, J. L., Welgus, H. G., Jeffrey, J. J. & Eisen, A. Z. (1976) *Arch. Biochem. Biophys.* **173**, 355–361
- Seltzer, J. L., Adams, S. A., Grant, G. A. & Eizen, A. Z. (1981) *J. Biol. Chem.* **256**, 4662–4668
- Shinkai, H. & Nagai, Y. (1977) *J. Biochem. (Tokyo)* **81**, 1261–1268
- Vaes, G. (1972) *Biochem. J.* **126**, 275–289
- Vater, C. A., Mainardi, C. L. & Harris, E. D., Jr. (1978) *J. Clin. Invest.* **62**, 987–992
- Vater, C. A., Hahn, J. L. & Harris, E. D., Jr. (1981) *Collagen Relat. Res.* **1**, 527–542
- Vater, C. A., Nagase, H. & Harris, E. D., Jr. (1983) *J. Biol. Chem.* **258**, 9374–9382
- Walsh, M. & Stevens, F. C. (1977) *J. Biol. Chem.* **252**, 7440–7443
- Werb, Z. & Burleigh, M. C. (1974) *Biochem. J.* **137**, 373–385
- Werb, Z., Mainardi, C. L., Vater, C. A. & Harris, E. D., Jr. (1977) *N. Engl. J. Med.* **296**, 1017–1023
- Woessner, J. F., Jr. (1977) *Biochem. J.* **161**, 535–542
- Woessner, J. F., Jr. (1979) *Biochim. Biophys. Acta* **571**, 313–320
- Woolley, D. E., Glanville, R. W., Crossley, M. J. & Evanson, J. M. (1975) *Eur. J. Biochem.* **54**, 611–622
- Wyckoff, M., Rodbard, D. & Chrambach, A. (1977) *Anal. Biochem.* **78**, 459–482

Received 3 January 1986/17 March 1986; accepted 8 April 1986