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Human Skin Fibroblast Procollagenase: Mechanisms of Activation by Organomercurials and Trypsin†

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ABSTRACT: Pure human skin fibroblast procollagenase has been utilized in this study as a model system in which to examine the pathways of organomercurial and trypsin activation. Three organomercurials, *p*-(hydroxymercuri)benzoate, mersalyl, and *p*-aminophenylmercuric acetate, were able to fully activate human skin procollagenase with no accompanying loss of molecular weight. Lower molecular weight species were subsequently produced, particularly with a fourth organomercurial, phenylmercuric chloride. The activation process was dependent upon the concentration of the organomercurial compound and the time of incubation, but not on enzyme protein concentration. No evidence of a role for free sulfhydryls was found. Trypsin produced an initial cleavage product of procollagenase which was collagenolytically inactive yet underwent a concentration independent autocatalysis. Thus, procollagenase appeared to have an autocatalytic property which was enhanced by treatment with a variety of agents, all of which may function by perturbation of the zymogen conformation.

The degradation of collagen is initiated by a specific class of proteases, the collagenases. In general, these enzymes, at least in mammalian species, are found in an inactive state, and, consequently, the nature of this latency and its modulation are factors crucial to the regulation of the connective tissue matrix [for recent reviews, see Murphy & Sellers (1980) and Bauer et al. (1982)]. This latency has been variously attributed to the secretion of the enzyme as a zymogen (Vaes, 1971, 1972a,b; Harper et al., 1971; Stricklin et al., 1977), or as an enzyme–inhibitor complex (Abe & Nagai, 1972; Abe et al., 1973; Sellers et al., 1977). Much uncertainty concerning the nature of latent collagenase has been engendered by the multitude of pathways by which activation occurs such as proteolysis by trypsin (Vaes, 1972a; Harper et al., 1971; Bauer et al., 1975) or tissue proteases (Vaes, 1972a; Harper et al., 1971), incubation with chaotropic ions such as I^- or SCN^- (Abe & Nagai, 1972; Abe et al., 1973), the action of nonenzymatic tissue activators (Tyree et al., 1981), autoactivation

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(Stricklin et al., 1977), and exposure to a number of organomercurial compounds (Sellers et al., 1977). The ability of several of these agents, especially the chaotropic ions and the organomercurials, to activate a particular collagenase has been proposed as a major criterion for indicating the presence of an enzyme–inhibitor complex (Abe & Nagai, 1972; Abe et al., 1973; Sellers et al., 1977). On the other hand, several collagenases which have been shown to be activated by these same reagents (Sakamoto et al., 1981; Nagase et al., 1981) are apparently present as zymogens rather than enzyme–inhibitor complexes.

The use of highly purified latent collagenases in the study of their activation appears essential in approaching a clear understanding of this process. Human skin fibroblast collagenase has been purified to homogeneity (Stricklin et al., 1977), and its secretion in zymogen form has been demonstrated by biochemical (Stricklin et al., 1977, 1978) and in vitro labeling techniques (Valle & Bauer, 1979). Also, enzymatic properties of this protease have been thoroughly studied (Welgus et al., 1981a,b). Interestingly, a specific collagenase inhibitor was found in the fibroblast culture medium from which the proenzyme itself was purified. However, the two proteins were completely separated during purification, and, indeed, the inhibitor had essentially no affinity for the zymogen (Stricklin et al., 1977; Welgus et al., 1979). Moreover, this particular inhibitor appeared to interact with active collagenase only in the presence of collagen substrate (Welgus et al., 1979). Thus, it did not form a tight enzyme–inhibitor complex such as those disrupted by organomercurial compounds (Sellers et al., 1977; Vater et al., 1978) or chaotropic ions (Abe & Nagai, 1972; Abe et al., 1973).

As previously reported (Stricklin et al., 1977; Tyree et al., 1981), human skin fibroblast collagenase can be activated by trypsin, nonenzymatic tissue activators, an autoactivation process, and presumably by tissue proteases. In addition, this proenzyme is activated by a number of organomercurial compounds (vide infra). In this study, a detailed examination is presented of the activation of procollagenase by several organomercurial compounds and by a proteolytic zymogen activator, trypsin.

Materials and Methods

Materials. Mersalyl, *p*-(hydroxymercuri)benzoate (PHMB),[1] phenylmethanesulfonyl fluoride (PMSF), iodoacetamide, reduced glutathione, soybean trypsin inhibitor (SBTI), Trizma base, and bovine pancreas trypsin (type III) were obtained from Sigma Chemical Co. *p*-Aminophenylmercuric acetate (APMA) was obtained from Aldrich Chemical Co. Phenylmercuric chloride (PMC), β-mercaptoethanol, acrylamide, and bis(acrylamide) were purchased from Eastman Kodak. Ultrapure sodium dodecyl sulfate was purchased from BDH Biochemicals. Sephadex G-25 was obtained from Pharmacia Fine Chemicals. ^{14}C-Labeled glycine and ^{14}C-labeled PHMB were purchased from New England Nuclear. All other reagents used were of the highest grade obtainable.

Production and Purification of Human Skin Fibroblast Collagenase. Normal human skin fibroblasts (CRL-1224) were procured from the American Type Culture Collection and cultivated at 37 °C in glass roller bottles (1585 cm^2, Bellco Glass, Inc.) in 100 mL of Dulbecco's modified Eagle's medium (high glucose) + glutamine containing 0.03 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer, pH 7.6, 10% newborn calf serum, and 200 units of penicillin and 200 μg of streptomycin per mL. Medium from confluent bottles was collected as a source of collagenase, and the enzyme was purified as previously described by using a combination of ammonium sulfate precipitation, ion-exchange chromatography (carboxymethylcellulose), and gel filtration on Ultrogel AcA-44 (LKB, Inc.) (Stricklin et al., 1977). Purity was established by NaDodSO$_4$–polyacrylamide gel electrophoresis, and all collagenase preparations used were homogeneous by this criterion.

Collagenase Assay. The routine activation of latent collagenase was accomplished proteolytically with trypsin at 25 °C for 10 min as described previously (Bauer et al., 1975). For each enzyme preparation, a range of trypsin concentrations, usually 0.1–5.0 μg of trypsin per 50 μL (1 μg) of enzyme, was used to ensure that maximal collagenase activity was obtained. After trypsin activation, at least a 4-fold molar excess of soybean trypsin inhibitor was added to inhibit further trypsin activity. The mixture was then assayed for collagenase activity at 37 °C in 0.05 M Tris-HCl, pH 7.5, containing 0.01 M $CaCl_2$ (Tris–Ca) by using reconstituted [^{14}C]glycine-labeled guinea pig skin collagen fibrils (30 000 cpm/mg of collagen) as a substrate gel (Nagai et al., 1966). Following incubation with enzyme, solubilized collagen was separated by centrifugation and counted in a liquid scintillation spectrometer (Hewlett-Packard).

Other Assays. Protein concentrations were determined spectrophotometrically by the method of Groves et al. (1968). Bovine serum albumin was used to establish a standard curve.

Free sulfhydryl group determinations were performed spectrophotometrically as described by Boyer (1954) using PHMB. Assays were performed in 0.05 M Tris-HCl, pH 7.5, containing 0.01 M $CaCl_2$ and 0.15 M NaCl. Urea (8 M), freshly deionized, was incorporated into this buffer in some determinations to denature the protein. Reduced glutathione was used as a standard.

Electrophoresis. NaDodSO$_4$–polyacrylamide gel electrophoresis was performed in 10% polyacrylamide slab gels as described by King & Laemmli (1971). Coomassie blue (1%) was used to stain the protein bands. Densitometry of these bands was performed at 600 nm by using a Zeiss PM6 spectrophotometer equipped with a gel-scanning linear-transport device.

Activation of Procollagenase by Trypsin. The mechanism of tryptic activation of proenzyme was studied by using incubations both at a lower temperature (0 °C) and at a much lower (1:100 vs. 1:1) weight ratio of trypsin to procollagenase than that used for routine assays. The action of trypsin was terminated by the addition of PMSF to a final concentration of 1 mM and/or SBTI to a final concentration of 400 μg/mL in order to permit the determination of collagenase activity.

Several types of experiments were performed. The time course of trypsin activation was studied by incubating 1 mL of procollagenase (400 μg/mL) with 4 μg of trypsin at 0 °C. At various times, 5-μL aliquots were diluted with 100 μL of Tris–Ca buffer containing 100 μg of SBTI and assayed for 30 min at 37 °C on collagen gels. Simultaneous 50-μL aliquots were mixed immediately with an equal volume of NaDodSO$_4$ sample buffer and boiled for 5 min. Other studies involved the termination of trypsin activity after 5 min. Collagenase preparations so treated were then incubated at 37 °C and

[1] Abbreviations: PHMB, *p*-(hydroxymercuri)benzoate; PMSF, phenylmethanesulfonyl fluoride; GSH, reduced glutathione; SBTI, soybean trypsin inhibitor; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; CM, carboxymethyl; APMA, *p*-aminophenylmercuric acetate; PMC, phenylmercuric chloride; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

aliquots removed as described above for both enzymic and electrophoretic analysis.

Activation of Procollagenase by Organomercurial Compounds. Activation of proenzyme by organomercurials was studied by using four of these compounds, PHMB, mersalyl, APMA, and PMC. Stock solutions (0.01 M PHMB, APMA, and PMC; 0.05 M mersalyl) were prepared just prior to use. These solutions and any dilutions thereof were made in 0.1 N NaOH. Proenzyme samples to be incubated with these solutions were buffered with 0.1 M Tris-HCl, pH 7.5, to avoid significant changes in pH upon the addition of a constant, 1:10 volume ratio of the organomercurial compound to the enzyme.

Incubation of proenzyme with these compounds was performed in two ways. First, 50-μL aliquots of procollagenase (18 μg/mL) in various concentrations of organomercurial compound were incubated in the presence of collagen gel substrate for varying times at 37 °C. Alternatively, procollagenase was preincubated with the specific organomercurial compound for various times followed by a short (30 min) assay on a collagen gel. Samples taken for electrophoretic analysis were immediately mixed with sample buffer and boiled for 5 min or, if necessary, concentrated by dialysis vs. 0.1 M acetic acid followed by lyophilization.

The effect of iodoacetamide upon the activation of proenzyme by PHMB was determined by preincubating the protein (20 μg/mL) with 1 mM iodoacetamide for 10 min at 25 °C before the addition of PHMB to a final concentration of 1.0 mM. Controls with PHMB alone and iodoacetamide alone were also performed. The ability of two sulfhydryl compounds, reduced glutathione (GSH) and β-mercaptoethanol (BME), to prevent PHMB activation of procollagenase was examined by using concentrations of these reagents up to 1.5 mM, an amount which does not significantly inhibit the collagenase itself.

[^{14}C]PHMB was incubated with proenzyme in order to assess the binding of PHMB to activated collagenase. Two milliliters of enzyme (600 μg or 0.01 μmol) was added to 0.285 mL of an 8 mM solution of labeled PHMB (18×10^6 cpm/μmol of PHMB), and the solution was incubated at 37 °C for 5 h. If a minimal content of one sulfhydryl group per mole of procollagenase is assumed, then 0.01 μmol $\times$ 18 $\times$ 10^6 cpm/μmol = 1.8×10^5 cpm should have bound. Separation of protein from free PHMB was accomplished by using a 0.9 $\times$ 150 cm siliconized glass column of Sephadex G-25 equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 0.005 M $CaCl_2$. A flow rate of 27 mL/h was maintained, 5-min fractions were collected, and 0.5-mL aliquots were assayed for radioactivity. The effluent was monitored at 230 nm.

Organomercurials and Human Fibroblast Collagenase Inhibitor. Human skin fibroblast collagenase inhibitor was prepared as previously described (Welgus et al., 1979). Procollagenase (7 μg) was incubated with either PHMB for 1 h at 37 °C or PMC for 40 min at 37 °C. Another portion of procollagenase was fully activated with trypsin. Aliquots were then assayed alone or with a 1:1 molar ratio of inhibitor (3.5 μg). So that the influence of this inhibitor upon the actions of PMC could be assessed, a 2.12-mL solution of procollagenase (104 μg) and purified inhibitor (372 μg) was made 1.0 mM in PMC and incubated at 37 °C. Aliquots (0.5 mL) were dialyzed against 0.1 M acetic acid, lyophilized, and then redissolved in sample buffer for analysis by NaDodSO$_4$–polyacrylamide electrophoresis.

Results

The activation of human skin fibroblast collagenase by both organomercurials and trypsin was assessed by using pure

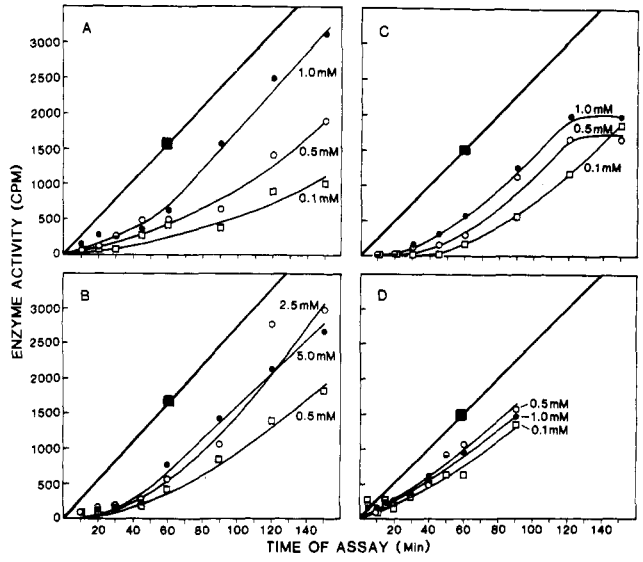

FIGURE 1: Activation of procollagenase by four organic mercurial compounds. (A) PHMB: Duplicate 50-μL aliquots of proenzyme (18 μg/mL) were incubated in the presence of collagen substrate and varying concentrations of PHMB [1.0 (●), 0.5 (○), or 0.1 mM (□)]. At the indicated times, solubilized radiolabeled collagen was determined as a measure of enzyme activity. Maximal collagenase activity (■) was determined by using trypsin to fully activate the proenzyme. All incubations were done at 37 °C. (B) Mersalyl: Incubation of procollagenase with mersalyl was performed as described in (A). Mersalyl concentrations used were 5.0 (●), 2.5 (○), and 0.5 mM (□). The maximal collagenase activity is also shown (■). (C) APMA: Incubation of procollagenase with APMA was performed as described in (A). APMA concentrations used were 1.0 (●), 0.5 (○), and 0.1 mM (□). Maximal collagenase activity (■). (D) PMC: Incubation of procollagenase with PMC was performed as described in (A). PMC concentrations used were 1.0 (●), 0.5 (○), and 0.1 mM (□). Maximal collagenase activity (■).

procollagenase preparations with low intrinsic activity (0–8%) which did not increase by autocatalysis during the incubation periods used (up to 5 h). Although this enzyme is secreted as a set of two very similar zymogens of M_r ~60 000 and 55 000 (Stricklin et al., 1977, 1978), the preparations used in this study were predominantly of the M_r 55 000 species. As will be seen, this considerably simplified the electrophoretic patterns produced upon the activation of procollagenase by these agents. Thus, we were able to approximate the more usual situation of a single proenzyme converted through a series of intermediates to a final stable form. Any major intermediates seen were therefore derived from the M_r ~ 55 000 species; those of the M_r ~60 000 species were often faint or obscured.

Organomercurial Activation of Procollagenase. The time course of activation of procollagenase by four organomercurial compounds is seen in Figure 1. The control represents collagenase which has been maximally activated by trypsin at zero time. In all four cases, a lag period was evident followed by the gradual achievement of slopes parallel to those of the trypsin-activated control (100% activity). PHMB (Figure 1A) and APMA (Figure 1C) were quite similar, whereas a somewhat higher (2–3-fold) concentration of mersalyl was required to produce a similar rate of activation (Figure 1B). Phenylmercuric chloride proved to be the most potent activator, producing a more rapid attainment of maximal activity of collagenase (~10–20 min) at a concentration 10–25 times lower than that used with the other compounds (Figure 1D). Figure 1 also demonstrates that the concentration of the organomercurial compound markedly affected both the rate of activation and the final activity achieved. Thus, as the or-

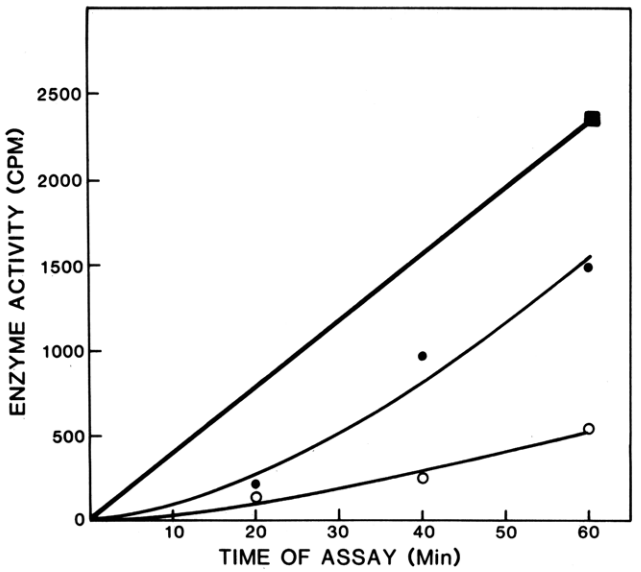

FIGURE 2: Dependence of organomercurial activation upon the continuing presence of reagent. Procollagenase (1.0 mL of 140 μg/mL) was incubated at 25 °C with 0.5 mM PMC for 20 min before gel filtration on a 0.90 × 150 cm column of Sephadex G-25. Aliquots from the protein peak were then assayed with (●) or without (○) readdition of PMC to 0.5 mM. An identical aliquot of proenzyme was gel filtered and maximally activated by trypsin (■).

ganomercurial concentration was decreased below a certain point, the lag period lengthened and the maximal slope achieved was reduced. This was readily seen for PHMB, mersalyl, and APMA (Figure 1A–C) and was also true for PMC; however, the relative potency of this compound minimized the changes (Figure 1D). Thus, a given concentration of organomercurial compound appeared to produce a characteristic level of activation which was maintained over significant periods of time. In contrast, there was no significant concentration dependence with respect to proenzyme. Using procollagenase at concentrations ranging from 18 to 400 μg/mL, maximal activation with PHMB, mersalyl, and APMA was achieved at ~60 min while PMC at all of these concentrations of proenzyme produced maximal activation at ~20 min. Furthermore, the addition of trypsin-activated collagenase to proenzyme followed by incubation with PHMB did not enhance the activation rate (not shown).

So that the need for the continuing presence of organomercurial compounds could be assessed, procollagenase was incubated with 0.5 mM PMC until 12% of maximal activity was reached (20 min at 25 °C). The protein and PMC were then separated by gel filtration on Sephadex G-25, and the enzyme was assayed with and without PMC added back to the original concentration. As seen in Figure 2, the activation of procollagenase by organomercurials displayed a requirement for the continuing presence of these compounds. Furthermore, the newly activated collagenase was unable to perpetuate the activation process.

The effects of organomercurials upon the procollagenase molecule are seen in Figure 3, and the correlation of achievement of activity with the appearance of lower molecular weight forms is quantitated in Figure 4. Conversion to lower molecular weight species ultimately occurred with all four organomercurials (Figure 3); however, this conversion was greatly delayed with respect to the attainment of activity with PHMB and mersalyl (Figure 4A,B) and significantly trailed the appearance of activity with APMA (Figure 4C). Only with PMC was conversion found to be concomitant with activity (Figure 4D). Thus, with PHMB, mersalyl, and APMA,

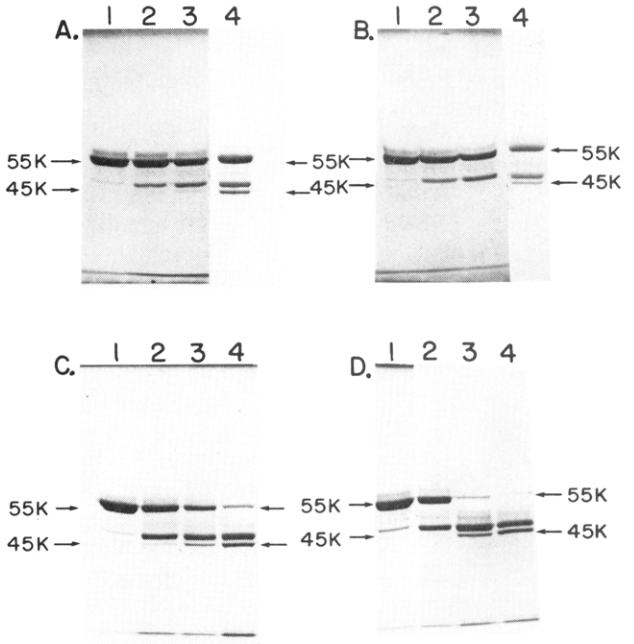

FIGURE 3: Alteration in procollagenase produced by organomercurials. The predominant procollagenase species (55K) and the position of its final active form (45K) are indicated. Only a minor amount of the 60K proenzyme was present, and intermediate products derived from this form were faint and/or obscured by those of the 55K species; thus, the major band seen just above the 45K marker represents an intermediate of the predominant 55K proenzyme. (A) PHMB: Procollagenase (180 μg/mL) was incubated with 1.0 mM PHMB at 37 °C. 18-μg aliquots were prepared for $NaDodSO_4$–polyacrylamide gel electrophoresis at 0 (lane 1), 30 (lane 2), 60 (lane 3), and 90 min (lane 4). (B) Mersalyl: Procollagenase (180 μg/mL) was incubated with 5.0 mM mersalyl at 37 °C. 18-μg aliquots were prepared for $NaDodSO_4$–polyacrylamide gel electrophoresis at 0 (lane 1), 30 (lane 2), 60 (lane 3), and 90 min (lane 4). (C) APMA: Procollagenase (180 μg/mL) was incubated with 1.0 mM APMA at 37 °C. 18-μg aliquots were prepared for $NaDodSO_4$–polyacrylamide gel electrophoresis at 0 (lane 1), 30 (lane 2), 60 (lane 3), and 90 min (lane 4). (D) PMC: Procollagenase (180 μg/mL) was incubated with 1.0 mM PMC at 37 °C. 18-μg aliquots were prepared for $NaDodSO_4$–polyacrylamide gel electrophoresis at 0 (lane 1), 5 (lane 2), 30 (lane 3), and 60 min (lane 4).

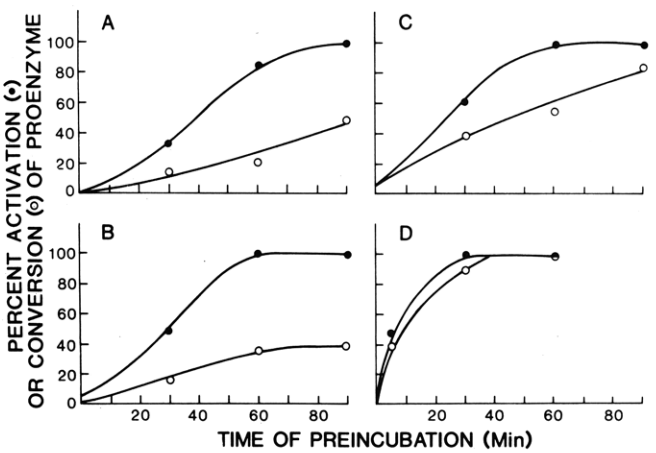

FIGURE 4: Correlation of cleavage and activation of procollagenase by organomercurials. Densitometry at 600 nm was performed on the gels seen in Figure 2 in order to quantitate the percent of enzyme present in lower molecular weight forms. Duplicate collagenase assays (1.8 μg of collagenase, 30 min at 37 °C) were performed at each time point to assess the extent of activation: (A) PHMB; (B) mersalyl; (C) APMA; (D) PMC; percent activation (●); percent conversion (○).

significant quantities of fully active collagenase could be produced which were in a form electrophoretically identical

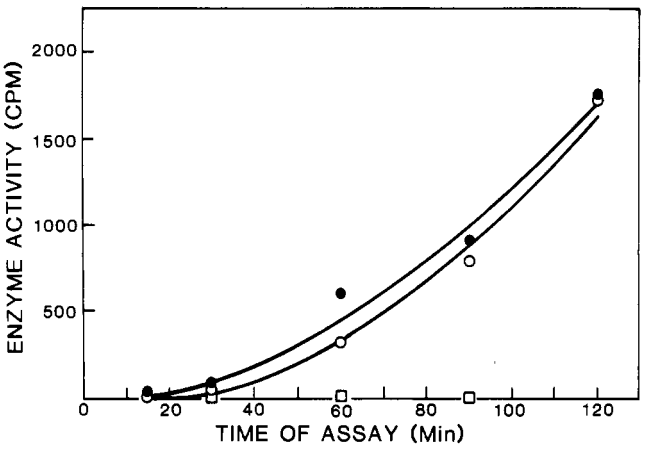

FIGURE 5: Effect of iodoacetamide upon PHMB activation of procollagenase. Procollagenase (duplicate 50-μL aliquots, 18 μg/mL) was incubated with 1.0 mM PHMB at 37 °C in the presence of collagen substrate for the indicated times (●). Identical aliquots were made 1 mM in iodoacetamide and incubated at 25 °C for 10 min before the addition of PHMB to 0.91 mM and subsequent assay (○). Incubation of procollagenase with 1 mM iodoacetamide alone produced no activation (□).

with that of the zymogen. Upon continued incubation, intermediate forms of collagenase were produced (Figure 3) which displayed electrophoretic mobilities between those of the procollagenase and the species characteristic of active collagenase produced either by trypsin activation or by purification from organ culture medium (Stricklin et al., 1977); prolonged incubation (6–12 h) was required to convert the collagenase to forms electrophoretically identical with that of fully trypsin-activated enzyme (not shown).

In view of the production of lower molecular weight forms by organomercurials, and since these compounds have not been shown to cleave peptide bonds, a series of studies were undertaken to determine the mechanism of activation and cleavage of procollagenase. These compounds have an extremely high affinity for free sulfhydryl groups; thus, the existence of an interaction of this nature was sought. The determination of free sulfhydryls in protein, performed as described by Boyer (1954) using PHMB, indicated that no such moieties were present in procollagenase, either in the native form or when 8 M urea was used to denature the protein and thus expose any "hidden" sulfhydryls. Figure 5 demonstrates that preincubation of procollagenase with iodoacetamide, a potent alkylating reagent for sulfhydryl groups, neither activated the enzyme nor interfered with its activation by PHMB, further suggesting the absence of free –SH in mediating this process. That the mercury was essential for activation, however, was indicated by the ability of sulfhydryl compounds such as GSH and BME to inhibit the activation of procollagenase by organomercurials at levels of the sulfhydryls which failed to inhibit the enzyme itself (Table I). Chelators such as EDTA or 1,10-phenanthroline also inhibited the conversion of procollagenase by PMC (not shown). Such chelating agents may inhibit this conversion through binding of the mercurial moiety itself and/or through binding of Ca^{2+} and Zn^{2+} ions crucial to the activity of this metalloprotease (Seltzer et al., 1977). The relative contribution of each of these two actions is unknown at this time. Finally, binding of the organomercurial compound to procollagenase was assessed by using ^{14}C-labeled PHMB as described under Materials and Methods. Following incubation, the protein was separated from the free PHMB by gel filtration. At a 1:1 molar binding ratio of PHMB to protein, 180 000 cpm was expected. Only

Table I: Inhibition of Organomercurial Activation of Procollagenase by Sulfhydryl Compounds

reaction mixtures	activity (cpm)	% inhibition
experiment 1[a]		
control	2118	0
0.5 mM BME	1732	18
1.0 mM BME	0	100
1.5 mM BME	0	100
experiment 2[b]		
control	1234	0
0.5 mM GSH	1135	8
1.0 mM GSH	151	88
1.5 mM GSH	0	100

[a] Procollagenase (2 μg) was preincubated with 1.0 mM PHMB alone (control) or with 1.0 mM PHMB and the indicated levels of BME. BME (2 mM) alone produces ~8% inhibition of collagenase. [b] Procollagenase (1 μg) was preincubated with 1.0 mM PHMB alone (control) or with 1.0 mM PHMB and the indicated levels of GSH. GSH (1.0 mM) alone produces no inhibition of collagenase.

Table II: Fibroblast Collagenase Inhibitor and Organomercurials[a]

activation method	activity (cpm)	% inhibition
trypsin	2476	
trypsin + inhibitor	158	94
PHMB	1845	
PHMB + inhibitor	161	87
PMC	1793	
PMC + inhibitor	40	98

[a] Procollagenase (7 μg, M_r 57 500) was activated with trypsin, PHMB, or PMC as described under Materials and Methods. Inhibitor (3.5 μg, M_r 31 000) was then added as indicated and the enzyme assayed at 37 °C for 30 min.

13 000 cpm was found in the protein pool, indicating that no significant binding occurred.

Organomercurials and Fibroblast Collagenase Inhibitor. Since organomercurials have been suggested to promote the dissociation of enzyme–inhibitor complexes (Sellers et al., 1977), the effects of these compounds upon human collagenase and fibroblast-derived inhibitor were examined. Human skin fibroblast collagenase inhibitor is a 31 000-dalton protein which is found in the same culture media from which the procollagenase is purified. This protein has been highly purified and displays a 1:1 stoichiometric inhibition of collagenase (Welgus et al., 1979). Table II demonstrates that both PHMB and PMC failed to affect the action of this inhibitor on pure fibroblast collagenase. In addition, for assessment of the ability of inhibitor to block the PMC-mediated cleavage of the procollagenase molecule itself, a 7-fold molar excess of inhibitor over proenzyme was incubated with 1 mM PMC at 37 °C for 90 min. Although more than sufficient inhibitor was present to fully block the collagenolytic activity, no inhibition of the cleavages within the proenzyme was seen (not shown).

Trypsin Activation of Procollagenase. The action of an enzymatic zymogen activator, trypsin, was then examined and compared to organomercurial activation. This protease, at 25 °C and at a 1:1 weight ratio, activates procollagenase within 10 min without any significant accumulation of intermediates (Stricklin et al., 1977). However, when lower weight ratios of trypsin to proenzyme (1:100) were used, and the incubation was performed at 0 °C, intermediates were now seen (Figure 6). The first and most prominent of these (Figure 6A, lane 2) was formed rapidly; however, its disappearance and the appearance of lower weight species were considerably slower processes. Figure 6B correlates these species with the attainment of collagenase activity. The first intermediate

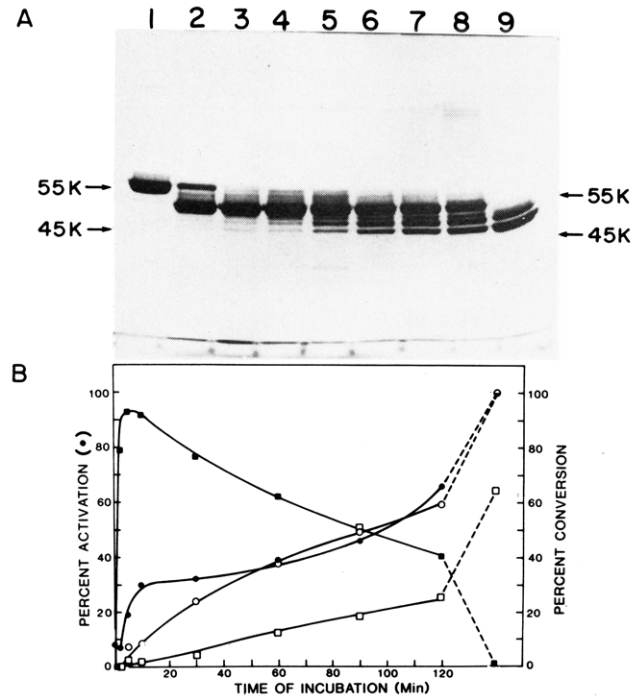

FIGURE 6: Trypsin activation of procollagenase. (A) Procollagenase (400 μg/mL, 1.0 mL) was incubated with 4 μg of trypsin at 0 °C, and 50-μL (20 μg) aliquots were taken for $NaDodSO_4$–polyacrylamide gel electrophoresis, and duplicate 5-μL (2 μg) aliquots were mixed with 100 μg of SBTI (100 μL) and assayed for collagenase activity. The 55-kdalton species of zymogen was predominant in this preparation; thus, the major intermediate bands arose from this species and obscured the corresponding intermediates derived from the minor 60-kdalton species. Complete conversion of the 55-kdalton species to its final 45-kdalton form occurred after maximal activity was obtained and is therefore not seen in this gel. Proenzyme (lane 1) for 2 min (lane 2); 5 min (lane 3); 10 min (lane 4); 30 min (lane 5); 60 min (lane 6); 90 min (lane 7); 120 min (lane 8); and 120 min at 0 °C + 10 min at 37 °C (lane 9). (B) Densitometry at 600 nm was applied to the gel seen in Figure 6A in order to quantitate the various collagenase species formed during trypsin activation and correlate their presence with enzyme activity. Percent enzyme activity (●); initial cleavage product (■); all bands below the initial product (○); final cleavage product (□).

product appeared clearly not to be active, since at 2 min of incubation 79% of the protein was in this form yet no increase in activity was detected. On the other hand, the band which corresponded to the fully trypsin-activated product appeared significantly later than the appearance of enzyme activity. However, when all of the protein bands beneath the first, inactive product were scanned and their densities summed, this quantity appeared to correlate best with activity, suggesting that all of the forms lower in molecular weight than the initial product represented active species (Figure 6B). Complete conversion of both the 60 000- and 55 000-dalton proenzymes to their respective 50 000- and 45 000-dalton forms may be seen in Figure 7.

The characteristics of the initial intermediate product (Figure 6A, lane 2) were then examined. Preparations of this species (~95–98%) could be prepared by a 5-min incubation at 0 °C of procollagenase with a 1:00 weight ratio of trypsin to enzyme. Large excesses of PMSF and SBTI were then added to block any further tryptic activity. This intermediate, upon incubation at 37 °C, slowly attained collagenase activity and converted to lower molecular weight forms (Figure 8). Like the activation mediated through organomercurials, this process showed no significant concentration dependence (Table III). In common with mercurial activation, addition of this collagenolytically inactive intermediate to procollagenase failed

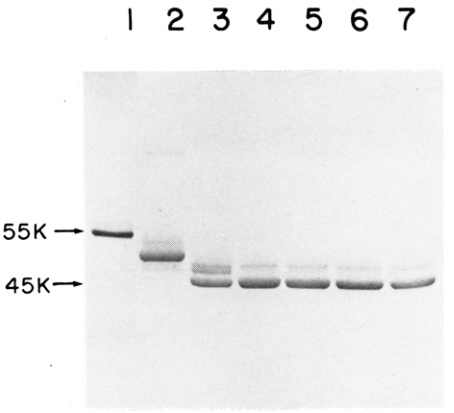

FIGURE 7: Complete conversion of procollagenase by trypsin. Procollagenase (400 μg/mL, 1.0 mL) was incubated with 4 μg of trypsin, and 50-μL (20 μg) aliquots were taken for $NaDodSO_4$–polyacrylamide gel electrophoresis. Complete conversion of both the 60K and 55K proenzymes to their respective 50K and 45K active forms may be seen. Proenzyme (lane 1) for 5 min at 0 °C (lane 2); an additional 10 min at 37 °C (lane 3); 20 min at 37 °C (lane 4); 30 min at 37 °C (lane 5); 45 min at 37 °C (lane 6); and 60 min at 37 °C (lane 7).

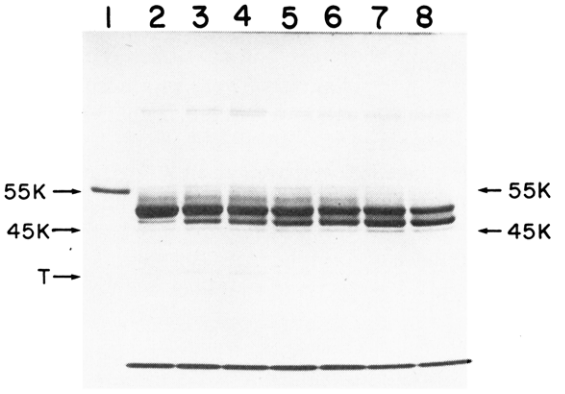

FIGURE 8: Autocatalysis of the trypsin-derived initial product of procollagenase. Procollagenase (1 mL; 400 μg/mL) was incubated with 4 μg of trypsin for 5 min at 0 °C. PMSF (1 mM) and SBTI (200 μg) were added to inhibit further tryptic activity. The mixture was then incubated at 37 °C, and aliquots were withdrawn for electrophoresis (20 μg) and a short, 30-min, assay (2 μg, duplicate samples). A trypsin titration was performed to establish maximal activity. As described for Figure 6, the 55-kdalton zymogen species was predominant, and a full conversion of this species to its 45-kdalton form is not presented. The added SBTI may be seen at the gel front, and the location of the added trypsin (T) is indicated: (lane 1) proenzyme, 8% active; (lane 2) initial product, zero time, 8% active; (lane 3) 1 h, 11% active; (lane 4) 2 h, 7% active; (lane 5) 3 h, 10% active; (lane 6) 4 h, 18% active; (lane 7) 5 h, 22% active; (lane 8) 6 h, 32% active.

Table III: Autocatalysis of the Trypsin-Derived Initial Intermediate of Procollagenase Concentration Dependence[a]

protein concn (μg/mL)	% of initial intermediate remaining at			
	0 h	1 h	3 h	5 h
831	94	62	44	32
415	94	58	43	33
207	94	58	38	37
103	94	63	40	35

[a] Procollagenase (831 μg/mL; 0.5 mL) was incubated with 4 μg of trypsin for 5 min at 0 °C. The reaction was stopped by the addition of SBTI (200 μg) and PMSF (1 mM), and the indicated dilutions were made in buffers containing the same concentrations of PMSF and SBTI. Aliquots were then incubated at 37 °C for 1, 3, and 5 h before being subjected to $NaDodSO_4$–polyacrylamide electrophoresis. Quantitation of the products was performed by densitometry of the stained protein bands.

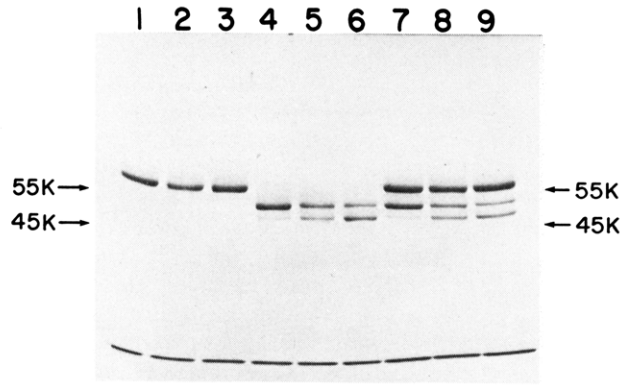

FIGURE 9: Mixture of procollagenase with the trypsin-derived initial product. Procollagenase (1 mL, 400 μg/mL) was incubated at 37 °C in the presence of PMSF (1 mM) and SBTI (200 μg) for 0 (lane 1), 2 (lane 2), and 4 h (lane 3). The trypsin-derived initial product was obtained by preincubating proenzyme (1 mL, 400 μg/mL) with 4 μg of trypsin for 5 min at 0 °C before the addition of PMSF (1 mM) and SBTI (200 μg). This initial product was then incubated alone for 0 (lane 4), 2 (lane 5), and 4 h (lane 6) or with an equal volume of proenzyme (final concentration 400 μg/mL) for 0 (lane 7), 2 (lane 8), and 4 h (lane 9). At the indicated times, 20- (lanes 1–6) or 40-μg (lanes 7–9) aliquots were taken and subjected to $NaDodSO_4$ electrophoresis. Duplicate aliquots were also taken for the assay of collagenase activity. By 4 h, the procollagenase alone (1 μg, lane 3) produced 67 cpm/h. The initial product (1 μg, lane 6) produced 503 cpm/h, and the mixture (2 μg, lane 9) yielded 565 cpm/h. Thus, the procollagenase was neither cleaved nor activated by the trypsin-derived initial product. As described for Figure 6, the 55-kdalton zymogen species was predominant, and a full conversion to its 45-kdalton form is not presented.

to produce either cleavage or activation of the proenzyme (Figure 9). This finding, as well as the concentration independence of this reaction, strongly suggested that it occurred in an intramolecular fashion. Additionally, this process cannot be attributed to residual tryptic activity since the initial cleavage of the intact proenzyme by trypsin was by far the most favored reaction (Figure 6).

Discussion

An understanding of the latent nature of collagenases as well as the mechanisms whereby these enzymes are activated is of crucial importance in defining the role of these proteases in connective tissue metabolism. The number of seemingly disparate ways which lead to the activation of latent collagenase has resulted in significant confusion concerning the nature of inactive collagenase (Vaes, 1971, 1972a,b; Harper et al., 1971; Abe & Nagai, 1972; Abe et al., 1973; Sellers et al., 1977). The zymogen nature of human skin fibroblast collagenase has been well established (Stricklin et al., 1977, 1978; Valle & Bauer, 1979). Thus, the activation of this procollagenase by organomercurials makes this study applicable to the broader question of collagenase latency. First, the availability of human skin fibroblast collagenase in pure form permits its study in the absence of any contaminating proteins such as proteases or inhibitors which might alter or obscure the true nature of the activating agent being studied. Second, the human skin proenzyme is activated by many of the same reagents which have been reported to activate other collagenases and appears to be a good model system for collagenase activation. In addition to its activation by trypsin, tissue proteases, an autoactivation process, and nonenzymatic tissue activators (Stricklin et al., 1977; Tyree et al., 1981), this enzyme is also activated by chaotropic ions (unpublished experiments) and organomercurials. The actions of these last two agents, often suggested to be indicative of the presence of an enzyme–inhibitor complex (Abe & Nagai, 1972; Abe et al., 1973; Sellers et al., 1977), are in this case mediated through the modification of a zymogen molecule. This situation also appears to be true with rabbit synovial collagenase (Nagase et al., 1981) and chick bone collagenase (Sakamoto et al., 1981).

In the present study, human skin collagenase appears to be activated by a variety of pathways which are conformational and/or proteolytic in nature. In the case of organomercurials, both mechanisms appear to be at work. Three of the four organomercurials were found to produce quantities of fully active collagenase in the absence of concomitant molecular weight changes. However, continued incubation with these three or with a fourth (PMC) organomercurial did produce lower molecular weight species. Since organomercurials have no intrinsic ability to cleave peptide bonds, any cleavage products seen during the course of activation could only have arisen from the action of the collagenase itself. This cleavage was concentration independent with respect to enzyme which suggested that it occurred in an intramolecular fashion. Suggestive of the intramolecular nature of this cleavage was the finding that trypsin-activated enzyme did not enhance the organomercurial-mediated activation of procollagenase. Also, the presence (Figure 1) or absence of collagen substrate (Figure 4) did not appear to significantly affect the time course of activation. This cleavage, at least with PHMB, mersalyl, and APMA, was not essential for the assumption of maximal activity. This finding was very reminiscent of the autoactivation of human skin collagenase, a phenomenon which yields full activity without any detectable change in molecular weight (Stricklin et al., 1977).

The complete activation of procollagenase by organomercurials was dependent upon the continuing presence of relatively high concentrations of these agents. Although an unaltered mercurial moiety was required, no evidence was obtained which demonstrated a reaction of this group with a free sulfhydryl within the procollagenase. Thus, no free sulfhydryls were detectable in either native or denatured procollagenase, and no inhibition of activation was seen upon preincubation of proenzyme with iodoacetamide. In addition, no significant binding of [^{14}C]PHMB to enzyme protein occurred. It is of interest that the more hydrophobic organomercurials (PMC and APMA) were more effective with regard to activation and conversion than the relatively hydrophilic compounds (PHMB and mersalyl). We can only speculate that this hydrophobicity allows for more penetration and disruption of the inner domains of the zymogen molecule.

Studies of the interactions between the homologous collagenase inhibitor (Welgus et al., 1979) and organomercurial compounds revealed that the ability of this inhibitor to block collagenolysis was not altered. This particular inhibitor does not form a tight complex with the proenzyme or even the active collagenase except in the presence of collagen substrate (Welgus et al., 1979); thus, it appears to be fundamentally different from those inhibitors reported to form tight complexes with collagenase which may be disrupted by these reagents (Sellers et al., 1977; Vater et al., 1978). The inability of the fibroblast inhibitor, even in great excess, to block the PMC-induced cleavages in procollagenase was an interesting finding and may be a result of the low affinity of this inhibitor for collagenase in the absence of collagen substrate as well as the intramolecular nature of the cleavages.

The study of trypsin activation also indicated that collagenase has the potential to cleave itself, again in an intramolecular fashion. Here, it is of interest that the collagenolytic activity of this enzyme and its autocatalytic potential are

dissociable; i.e., an intermediate is rapidly formed which is inactive toward collagen yet which slowly cleaves itself to form collagenolytically active species.

Although the collagenase zymogen may be trypsin activated in classical fashion, other agents capable of activating this protease, specifically nonenzymatic tissue activators (Tyree et al., 1981), chaotropic ions, and the organomercurials, have no intrinsic protein cleavage properties. Even the action of trypsin may be limited to yield a collagenase species which though collagenolytically inactive has the potential to undergo autocatalysis. What all these agents appear to have in common is the ability to disrupt the conformation of the procollagenase molecule, i.e., by interaction with small molecules (chaotropic ions, organomercurials), macromolecules (nonenzymatic tissue activators), or proteases (trypsin). Thus, all of these agents may act by enhancing the autoactivation potential resident in the zymogen. It is also of note that, like autoactivation, cleavage of the zymogen is not a prerequisite for the appearance of collagenase activity. However, quite stable collagenase species $\sim$10 000 daltons less than their respective zymogen precursors are found as an end product of many of these activation processes. Indeed, these are the only products found when whole skin is cultured in serum-free media (Stricklin et al., 1977). These findings suggest that both conformational and/or proteolytic processes are important in the activation of procollagenase and in the production of its in vivo form.

Acknowledgments

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Registry No. *p*-(Hydroxymercuri)benzoate, 1126-48-3; mersalyl, 492-18-2; *p*-aminophenylmercuric acetate, 6283-24-5; procollagenase, 39287-99-5; phenylmercuric chloride, 100-56-1; trypsin, 9002-07-7.

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