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Mechanisms of Activation of Tissue Procollagenase by Matrix Metalloproteinase 3 (Stromelysin)†

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ABSTRACT: The mechanism of activation of tissue procollagenase by matrix metalloproteinase 3 (MMP-3)/stromelysin was investigated by kinetic and sequence analyses. MMP-3 slowly activated procollagenase by cleavage of the Gln^{80}–Phe^{81} bond to generate a fully active collagenase of M_r = 41 000. The specific collagenolytic activity of this species was 27 000 units/mg (1 unit = 1 μg of collagen digested in 1 min at 37 °C). Treatment of procollagenase with plasmin or plasma kallikrein gave intermediates of M_r = 46 000. These intermediates underwent rapid autolytic activation, via cleaving the Thr^{64}–Leu^{65} bond, to give a collagenase species of M_r = 43 000 that exhibited only about 15% of the maximal specific activity. Similarly, (4-aminophenyl)mercuric acetate (APMA) activated procollagenase by intramolecular cleavage of the Val^{67}-Met^{68} bond to generate a collagenase species of M_r = 43 000, but with only about 25% of the maximal specific activity. Subsequent incubation of the 43 000-M_r species with MMP-3 resulted in rapid, full activation and generated the 41 000-M_r collagenase by cleaving the Gln^{80}–Phe^{81} bond. In the case of the proteinase-generated 43 000-M_r species, the action of MMP-3 was approximately 24 000 times faster than that on the native procollagenase. This indicates that the removal of a portion of the propeptide of procollagenase induces conformational changes around the Gln^{80}–Phe^{81} bond, rendering it readily susceptible to MMP-3 activation. Prolonged treatment of procollagenase with APMA in the absence of MMP-3 also generated a 41 000-M_r collagenase, but this species had only 40% of the full activity and contained Val^{82} and Leu^{83} as NH_2 termini. Thus, cleavage of the Gln^{80}–Phe^{81} bond by MMP-3 is crucial for the expression of full collagenase activity. These results suggest that the activation of procollagenase by MMP-3 is regulated by two pathways: one with direct, slow activation by MMP-3 and the other with rapid activation in conjunction with tissue and/or plasma proteinases. The latter event may explain an accelerated degradation of collagens under certain physiological and pathological conditions.

Mammalian tissue collagenases (matrix metalloproteinase 1) (E.C. 3.4.24.7) are metalloendopeptidases capable of degrading interstitial collagen types I, II, and III at specific sites to generate 3/4 and 1/4 fragments of the native molecules (Miller et al., 1976; Hofmann et al., 1978; Dixit et al., 1979). It has recently been shown that they also digest collagen types VII (Seltzer et al., 1989) and X (Schmid et al., 1986). The involvement of collagenase in pathological breakdown as well as normal remodeling of connective tissues has been proposed (Harris & Krane, 1974; Woolley & Evanson, 1980). The production of collagenase by connective tissue cells is greatly enhanced by monocyte-derived inflammatory mediators such

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as interleukin 1 (Mizel et al., 1981; Gowen et al., 1984) and tumor necrosis factor (Dayer et al., 1985), growth factors such as epidermal growth factor (Edwards et al., 1987), platelet-derived growth factor (Bauer et al., 1985), and fibroblast growth factor (Moscatelli et al., 1986), and several other substances (Harris et al., 1984; Lotz et al., 1987; Brinckerhoff et al., 1989). While the synthesis of collagenase is regulated at the transcriptional level (Gross et al., 1984; Schönthal et al., 1988; Brenner et al., 1989), the enzyme is secreted from the cells as an inactive zymogen (Stricklin et al., 1977; Nagase et al., 1981, 1983a; Wilhelm et al., 1986). Therefore, the activation process of procollagenase in the extracellular milieu is an additional key step in the regulation of collagenolysis.

It has been reported that procollagenase in crude culture medium can be activated in vitro by treating with proteinases such as trypsin, kallikrein, or plasmin or organomercurial compounds (Eeckhout & Vaes, 1977). This process has been shown to be dependent on the presence of connective tissue derived procollagenase activators (Vater et al., 1983, 1986; Treadwell et al., 1986; Ishibashi et al., 1987; Murphy et al., 1987; Ito & Nagase, 1988). The endogenous activators have been purified from the culture media of rabbit synovial fibroblasts (Vater et al., 1983), rabbit uterine cervical fibroblasts (Ishibashi et al., 1987), and bovine articular cartilage (Treadwell et al., 1986). Although the modes of procollagenase activation by these activators were shown to be variable (see Discussion), recent work by Murphy et al. (1987) and by ourselves (Ito & Nagase, 1988) demonstrated that the procollagenase activator is matrix metalloproteinase 3 (MMP-3)[1]/stromelysin. This was also substantiated by the similarities of the deduced primary structures of rabbit procollagenase activator (Fini et al., 1987) and human MMP-3 (Whitham et al., 1986; Wilhelm et al., 1987; Saus et al., 1988) from cDNA sequencing. In addition to its participation in procollagenase activation, MMP-3 is capable of degrading various connective tissue matrix macromolecules including proteoglycans, fibronectin, collagen type IV, laminin (Galloway et al., 1983; Chin et al., 1985; Okada et al., 1986; Bejarano et al., 1988), and type IX collagen (Okada et al., 1989). MMP-3 is also secreted from the connective tissue cells as an inactive zymogen, thus the activation of proMMP-3 is a rate-limiting step for collagenolysis. Purified proMMP-3 can also be activated by various proteolytic enzymes and organomercurial compounds (Okada et al., 1988; Nagase et al., 1990), but the kinetics and specificity of the action of MMP-3 on procollagenase activation are not known.

In this report we have purified procollagenase from the culture medium of human rheumatoid synovial fibroblasts and investigated the activation mechanisms of procollagenase by MMP-3. We describe here two major regulatory pathways in which MMP-3 is involved in procollagenase activation.

Experimental Procedures

Materials. Materials were obtained as follows: (4-aminophenyl)mercuric acetate (APMA), Brij 35, 5-bromo-4-chloro-3-indolyl phosphate, chymotrypsin, DFP, immunoglobulin G (human), α-methyl-D-mannoside, nitro blue tetrasolium, plasminogen (human), urokinase, transferrin (human), and alkaline phosphatase conjugated donkey anti-(sheep IgG) IgG were from Sigma Chemical Co.; YM-10 membrane and Green A Dyematrex gel were from Amicon Corp; DEAE-cellulose (DE-52) was from Whatman; bicinchoninic acid (BCA) protein assay reagents were from Pierce Chemical Co.; prestained protein standards were from Bio-Rad; reagents and solvents for the gas/liquid phase sequencer were from Applied Biosystems Inc.; nitrocellulose filter paper and poly(vinylene difluoride) Immobilon membrane (PVDF membrane) were from Millipore. Human plasma kallikrein was purified as described by Nagase and Barrett (1981).

Enzyme Assays. Collagenolytic activity was measured by the diffused fibril method of Cawston and Barrett (1979) using [^{14}C]acetylated type I collagen (guinea pig) at 37 °C. Gelatinolytic activity was measured by using heat-denatured [^{14}C]acetylated type I collagen (guinea pig) according to Harris and Krane (1972). One unit of collagenolytic or gelatinolytic activity was defined to be that amount of enzyme capable of degrading 1 μg of the respective substrate per minute at 37 °C. MMP-3 activity was measured using reduced, [^{3}H]carboxymethylated transferrin ([^{3}H]Cm-Tf) as described previously (Okada et al., 1986). One unit of MMP-3 was defined as the production of 1 μg of [^{3}H]Cm-Tf fragments soluble in 3.3% (w/v) trichloroacetic acid in 1 min at 37 °C. All the enzyme assays and procollagenase activation studies were carried out in 50 mM Tris-HCl buffer (pH 7.5)/0.15 M NaCl/10 mM Ca^{2+}/0.02% NaN_3/0.05% Brij 35.

Electrophoresis. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) was performed with or without reduction by the method of Bury (1981), a modification of the 2-amino-2-methylpropane-1,2-diol/glycine/HCl discontinuous system of Wyckoff et al. (1977). After electrophoresis the proteins were stained with silver nitrate (Wray et al., 1981) or Coomassie Brilliant Blue R 250.

Western Blot Analysis. Samples resolved by SDS/PAGE were electrotransferred onto nitrocellulose filters according to Burnette (1981). Nonspecific binding of IgG was blocked by 22% (w/v) fat-free milk solution. The filters were reacted with anti-(human collagenase) sheep serum at a 1:100 dilution at room temperature for 16 h. After extensive washing of the filters anti-collagenase antibodies bound to the antigens were complexed with alkaline phosphatase conjugated donkey anti-(sheep IgG) IgG at a 1:1 000 dilution for 2 h at room temperature. Then protein bands were visualized by using 165 μg/mL of 5-bromo-4-chloro-3-indolylphosphate and 330 μg/mL of nitro blue tetrazolium in 0.1 M Tris-HCl buffer (pH 9.5)/0.1 M NaCl/5 mM $MgCl_2$ (Blake et al., 1984).

Purification of ProMMP-3. ProMMP-3 was purified from the culture mediums of human rheumatoid synovial fibroblasts stimulated by rabbit macrophage conditioned medium as described by Ito and Nagase (1988). In a typical preparation, 250 mL of crude culture medium was applied to an anti-(human MMP-3) column (75 mL). The bound proMMP-3 was eluted with 6 M urea in 50 mM Tris-HCl buffer (pH 7.5)/0.15 M NaCl and immediately dialyzed against the above buffer without urea. The purified proMMP-3 was homogeneous on SDS/PAGE and identified as a typical doublet of M_r = 57 000 and 59 000. For activation of proMMP-3, the zymogen (1 mg) was incubated with 1.5 mM APMA at 37 °C for 24 h and APMA removed by a Sephadex G-10 spin column as described by Salvesen and Nagase (1989). The specific activity of MMP-3 was 860 units/mg.

Purification of Procollagenase. Procollagenase was purified from the same culture medium of rheumatoid synovial fibroblasts. The first step was the complete removal of proMMP-3 by an immunoadsorbent column as described above. The culture medium free of proMMP-3 (510 mL) was concentrated to 50 mL by a Amicon Diaflo apparatus with

[1] Abbreviations: MMP-3, matrix metalloproteinase 3; APMA, (4-aminophenyl)mercuric acetate; PVDF, poly(vinylene difluoride); SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

Table I: Purification of Procollagenase[a]

step	total protein (mg)	total act. (units)	specific act. (units/mg)	purification (-fold)	recovery (%)
concentrated medium		10,900			100
DEAE-cellulose	28.5	8,268	90	1	76
Green A Dyematrex	0.64	5,344	8,350	29	49
Sephacryl S-200	0.18	4,860	27,000	93	45

[a] See the text for the experimental detail.

a YM-10 membrane and applied to a column of DEAE-cellulose (1.5 × 22 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0)/0.15 M NaCl/10 mM $CaCl_2$/0.02% NaN_3 to remove glycosaminoglycans. The sample was then concentrated to 10 mL by a YM-10 membrane and applied a column of Green A Dyematrex gel (1.5 × 14 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5)/0.15 M NaCl/10 mM $CaCl_2$/0.02% NaN_3. The majority of the precursor of a metalloproteinase with gelatinolytic activity (proMMP-2) was eluted with 0.3 M NaCl and procollagenase with 0.5 M NaCl in 50 mM Tris-HCl buffer (pH 7.5) and 10 mM $CaCl_2$. Fractions containing procollagenase (40 mL) were mixed with Brij 35 (final, 0.05%) and concentrated to 1.5 mL with a YM-10 membrane and applied to a column of Sephacryl S-200 (1.5 × 120 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5)/0.4 M NaCl/10 mM Ca^{2+}/0.02% NaN_3/0.05% Brij 35. In some cases, a concanavalin A–Sepharose 4B column was used after the Green A Dyematrex step in order to remove the glycosylated form of procollagenase as described for proMMP-3 previously (Okada et al., 1988).

NH_2-Terminal Sequence Analysis. Automatic sequence analyses were performed with an Applied Biosystems 477A pulse liquid-phase sequencer with "on line" 120A PTH amino acid analysis. To determine the NH_2-terminal sequence of procollagenase, the sample (200 pmol) was directly applied to a polybrene-treated filter. APMA- or proteinase-treated procollagenase was separated by SDS/PAGE with 7% (w/v) total acrylamide under either reducing or nonreducing conditions and transferred from the polyacrylamide gel to the PVDF membrane according to the procedure described by Matsudaira (1987). The proteins transferred to the PVDF membrane were located by staining with Coomassie Brilliant Blue R-250 and the bands of interest were excised, placed directly onto a polybrene-treated glass filter, and sequenced. The net yields of amino acids were calculated by subtraction of the amount of PTH derivatives in each cycle from the background in the previous cycle. For double sequences gross yields were used without subtraction of the PTH derivatives from the previous cycle.

Protein Determination. The concentrations of protein was determined by BCA protein assay according to Smith et al. (1985) using crystalline bovine serum albumin as standard. The $A^{1\%}_{280nm,cm}$ values for procollagenase and MMP-3 were 12.5 and 13.9, respectively.

Results

Purification of Procollagenase. To investigate the activation mechanisms of procollagenase by MMP-3, it was essential to isolate the inactive precursor of collagenase completely free of other proteinases. We used the culture medium from human rheumatoid synovial fibroblasts stimulated with rabbit macrophage conditioned medium as a source of procollagenases and the results of purification are summarized in Table I. An initial step to remove proMMP-3 from the culture medium was essential to prevent procollagenase from autoactivation during purification. A trace amount of proMMP-3 and/or MMP-3 in the medium often resulted in partial activation of procollagenase during purification or storage. Green A Dyematrex gel chromatography after the DEAE-cellulose step was effective in separating procollagenase from proMMP-2. Procollagenase was purified about 29-fold with 64% recovery in this step. A protein peak from the final step of gel permeation chromatography on Sephacryl S-200 showed a doublet of proteins with M_r = 52000 and M_r = 56000 on SDS/PAGE, of which the 56000-M_r form was bound to concanavalin A–Sepharose and eluted with 1 M methyl α-D-mannoside (Figure 1). This confirms previous reports that the 56000-M_r species is a glycosylated form of the 52000-M_r procollagenase (Nagase et al., 1981, 1983; Wilhelm et al., 1986). Both species of procollagenase had a similar specific activity of 27000 units/mg when fully activated in the presence of MMP-3 (see below). The overall yield of procollagenase was 45%. The NH_2-terminal sequence of procollagenase was the Phe-Pro-Ala-Thr-Leu-Glu-Thr-Gln-Glu-Gln-Asp-Val-Asp-Leu-Val (see Table III). The purified procollagenase remained inactive in the buffer used for the Sephacryl S-200 step at least for 2 months at 4 °C or for 7 days at 37 °C. For investigation of molecular weight changes and the NH_2-terminal sequence analyses after activation of procollagenase, only the major unglycosylated form of procollagenase (M_r = 52000) was used to simplify the analyses.

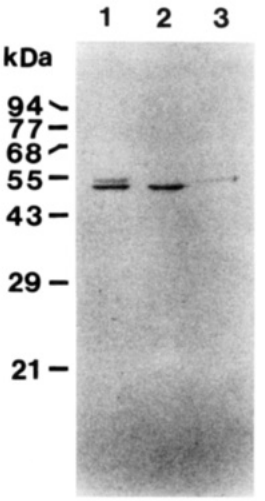

FIGURE 1: SDS/PAGE analyses of procollagenase. Lane 1, procollagenase (0.4 μg) obtained after Sephacryl S-200; lane 2, concanavalin A unbound procollagenase (0.4 μg); lane 3, concanavalin A bound procollagenase (0.1 μg). Proteins were analyzed by SDS/PAGE with 7.5% total acrylamide and stained with Coomassie Brilliant Blue R-250. Protein standards are phosphorylase *b* (94000), transferrin (77000), bovine serum albumin (68000), heavy chain of IgG (55000), ovalbumin (43000), carbonic anhydrase (29000), and soybean trypsin inhibitor (21000).

Direct Activation of Procollagenase by MMP-3. The ability of MMP-3 to activate procollagenase was first investigated. As shown in Figure 2, MMP-3 activated procollagenase and generated a fully active collagenase of M_r = 41000 in a time- and dose-dependent manner. NH_2-terminal sequence analysis of this species indicated that the Gln^{80}–Phe^{81} bond of procollagenase was cleaved by MMP-3 (see Table III). However, this activation process was very slow. Full collagenase activity

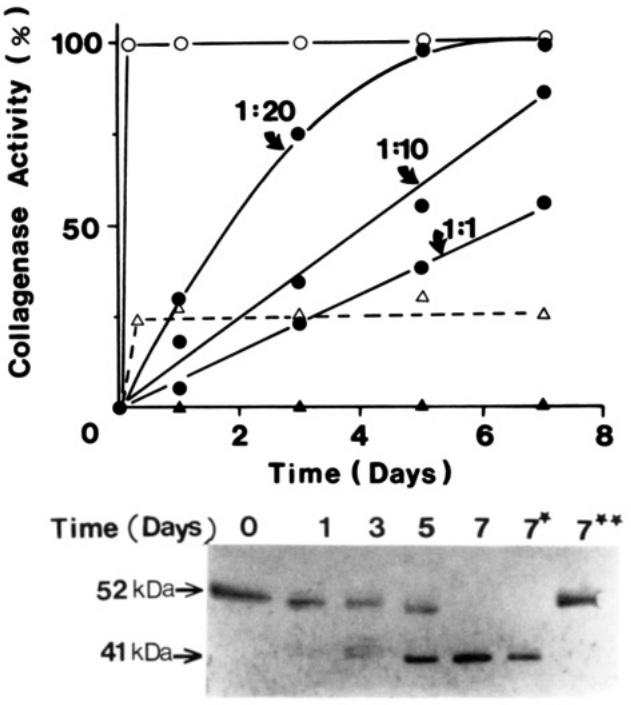

FIGURE 2: Activation of procollagenase by MMP-3. (A) A 60-μL portion of procollagenase (2 μg/mL) was mixed with MMP-3 containing both HMW and LMW species at the indicated molar ratio of procollagenase and MMP-3 (●) and incubated for various periods at 37 °C. The mixtures were then assayed for collagenolytic activity for 3 h. (○) procollagenase and MMP-3 (1:20 molar ratio) in the presence of 1 mM APMA; (△) procollagense incubated with 1 mM APMA; (▲) procollagenase alone. A portion of the mixture of procollagenase and MMP-3 at a 1:10 molar ratio was taken for analyses of M_r changes by SDS/PAGE and subsequent immunoblotting using anti-(human procollagenase) antibody. (★) Procollagenase and MMP-3 (1:20 molar ratio) in the presence of 1 mM APMA; (★★) procollagenase alone.

(27 000 units/mg) was observed only after several days of incubation with a 20-fold molar excess of MMP-3 at 37 °C. In order to accomplish procollagenase activation within 20 h at 37 °C the zymogen had to be incubated with a 200-fold molar excess of MMP-3 (data not shown).

Since a large amount of MMP-3 was required for the activation of procollagenase in a relatively short period of time we considered that the effect of MMP-3 may be on the collagen substrate rather than on procollagenase. This possibility was ruled out by the following observations. First, once collagenase is fully activated it exhibits similar specific activity regardless of the presence or the absence of MMP-3, indicating that the high specific activity is not due to the synergistic effect of MMP-3 and collagenase on the collagen substrate. Second, when procollagenase was incubated with three different inactive forms of MMP-3 [i.e., (i) apo-MMP-3 prepared by treatment of MMP-3 with 20 mM EDTA and the subsequent removal of metal ion–EDTA complex by a spin column, (ii) heat-inactivated MMP-3, and (iii) proMMP-3] at a molar ratio of 1:400 at 37 °C for 24 h, no activation of procollagenase was detected with any of these MMP-3 preparations (Table II). Thus, it is concluded that the activation of procollagenase by MMP-3 is by enzymic action of MMP-3.

Partial Activation of Procollagenase by APMA and Proteinases. It has been generally accepted that organomercurial compounds such as APMA and serine proteinases such as trypsin, plasma kallikrein, and plasmin are activators of procollagenase (Eeckhout & Vaes, 1977; Nagase et al., 1982; Stricklin et al., 1983; Grant et al., 1987). However, when purified procollagenase was treated with 1 mM APMA at 37 °C the maximal specific activity detected was about 6500 units/mg, only about 25% of that of fully activated collagenase (Figure 2). SDS/PAGE analyses of procollagenase after APMA treatment showed that the 52 000-M_r zymogen was first converted to the 43 000-M_r species, which was then gradually converted to M_r = 41 000 (Figure 3). The initial generation of the 43 000-M_r species by APMA was not affected by the concentration of procollagenase nor by the presence of substrate, suggesting that this reaction is due to an intramolecular event. However, the conversion rate of the 43 000-M_r species to M_r = 41 000 was dependent on the initial concentration of procollagenase, suggesting that it is a bimolecular reaction (data not shown). The specific activity of the 41 000-M_r form was 10 800 units/mg, about 40% of that of the fully active collagenase. Upon a longer incubation, the 41 000-M_r species was further converted to a fragment of M_r = 22 000 with a concomitant decrease of collagenolytic activity (Figure 3).

Similarly, only a partial collagenase activity (about 15%) was observed when procollagenase was treated with serine proteinases such as trypsin, plasmin, and plasma kallikrein. In all cases, procollagenase was converted to M_r = 43 000 (see below).

Accelerated and Full Activation of APMA-Treated Procollagenase by MMP-3. Although the activation of procollagenase by MMP-3 is very slow, when procollagenase was reacted with MMP-3 in the presence of APMA the rate of

Table II: Requirement of Enzymic Activity of MMP-3 for Procollagenase Activation[a]

	incubation time (h)	rel act. (%)
procollagenase + buffer	24	0
procollagenase + 1 mM APMA	24	25
procollagenase + MMP-3 (1:20)[b]	24	31
procollagenase + MMP-3 (1:20)[b]	72	75
procollagenase + MMP-3 (1:400)[b]	24	100
procollagenase + apo-MMP-3 (1:400)[b]	24	13
procollagenase + heat-inactivated MMP-3 (1:400)[b]	24	0
procollagenase + ProMMP-3 (1:400)[a]	24	0
procollagenase + 1 mM APMA + MMP-3 (1:20)[b]	2	100

[a] Procollagenase (2 μg/mL) and various forms of MMP-3 were mixed and incubated for indicated time at 37 °C, and the mixtures were assayed for collagenolytic activity at 37 °C for 4 h. [b] Molar ratios of procollagenase and MMP-3.

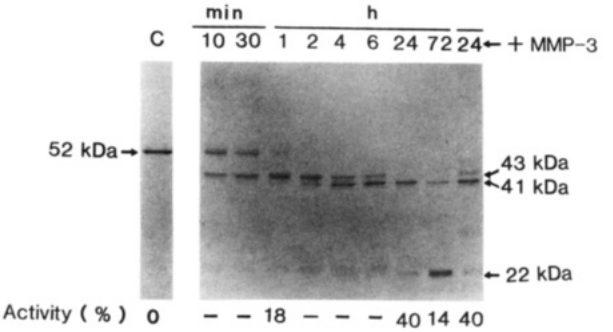

FIGURE 3: SDS/PAGE analyses of procollagenase treated with APMA. Procollagenase (70 μg/mL) was treated with 1 mM APMA at 37 °C for indicated periods. The samples were run on SDS/PAGE with 7.5% total acrylamide under nonreducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250. Procollagenase treated with 2 M excess of MMP-3 for 2 h at 37 °C after a 24-h incubation with APMA is shown in the last lane. The minor protein bands at M_r = 43 000 and M_r = 28 000 seen in the last lane are MMP-3. The percent activity of the maximal collagenase activity is shown. (–) Activity is not determined.

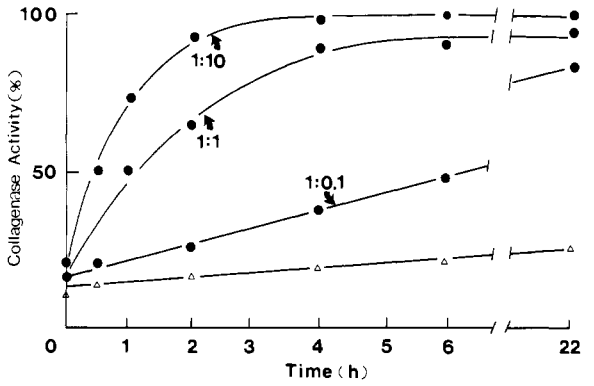

FIGURE 4: Accelerated activation of procollagenase by MMP-3 in the presence of APMA. A 60-μL portion of procollagenase (2 μg/mL) was incubated with MMP-3 at the molar ratios indicated in the presence of 1 mM APMA. Portions were removed at timed intervals and assayed for collagenase activity. (△) Procollagenase incubated with 1 mM APMA.

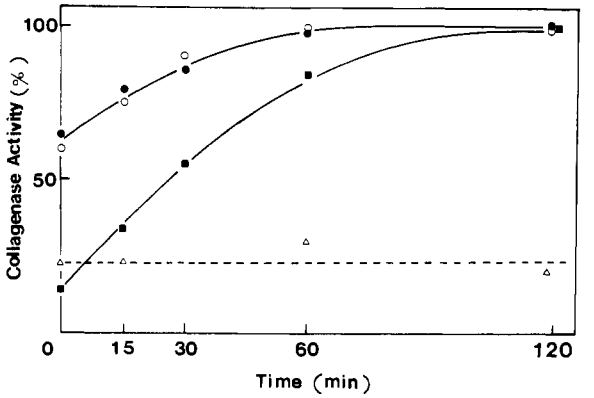

FIGURE 5: Effect of MMP-3 on the APMA-treated procollagenase (M_r = 43 000). Procollagenase (4 μg/mL) was first treated with 1 mM APMA for 2 h at 37 °C. This process converted procollagenase to a partially active form of M_r = 43 000. The sample was extensively dialyzed against the assay buffer to remove APMA at 4 °C. The sample was then reacted with MMP-3 at a molar ratio of 1:20 in the presence (●) and absence (○) of 1 mM APMA at 37 °C for the indicated time and assayed for collagenase activity. (■) Procollagenase incubated with a 20-fold molar excess of MMP-3 in the presence of 1 mM APMA; (△) procollagenase incubated with 1 mM APMA.

procollagenase activation was accelerated approximately 1200-fold compared with that of MMP-3 alone (Figure 4). We then considered whether this phenomenon is a concerted effect of APMA and MMP-3 on the procollagenase molecule or whether procollagenase treated with APMA becomes more susceptible to the MMP-3 action. To test this possibility procollagenase was first incubated with 1 mM APMA at 37 °C for 2 h to generate predominantly the 43 000-M_r species. After removal of APMA by extensive dialysis, the 43 000-M_r collagenase was incubated with MMP-3 in the presence and absence of APMA. As shown in Figure 5, the rate of activation of procollagenase was similar in both cases once procollagenase was treated with APMA, indicating the processing of procollagenase to the 43 000-M_r intermediate renders it more susceptible to full activation by MMP-3. On the other hand, once the 41 000-M_r species was generated by APMA alone after a prolonged incubation, the specific activity (40% of the full activity) did not change significantly even after treatment with MMP-3 (Figure 3).

We then examined whether the interaction of procollagenase and MMP-3 is dependent on the forms of MMP-3 used. We previously reported that two active forms of MMP-3 with M_r = 45 000 (HMW) and M_r = 28 000 (LMW) are generated upon activation of proMMP-3 with APMA (Okada et al., 1986, 1988). Sequence analyses of the two forms indicated that LMW is a COOH-terminal truncated form of HMW (Nagase et al., 1990). We separated the two forms of MMP-3 by Sephacryl S-200 gel filtration chromatography and each component was tested for its ability to activate procollagenase. Both HMW and LMW activated procollagenase to a similar extent (data not shown), indicating that the COOH-terminal region of approximately M_r = 17 000 in MMP-3 does not play any significant role in the activation of procollagenase.

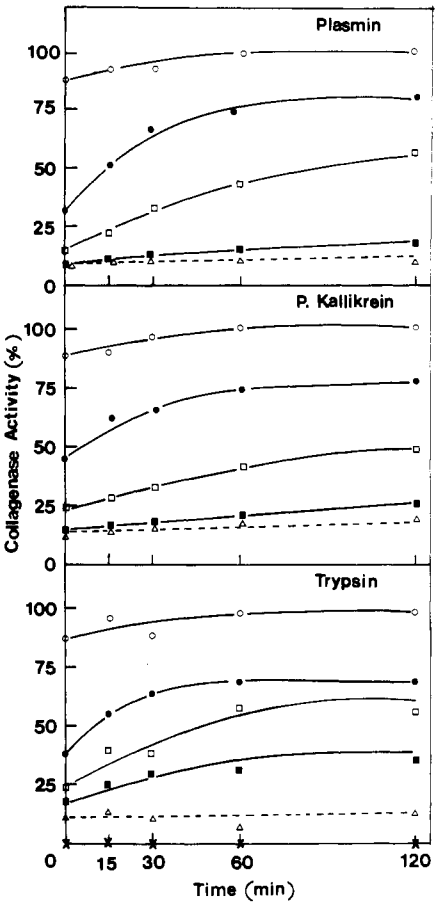

FIGURE 6: Activation of plasmin-treated, plasma kallikrein treated, or trypsin-treated procollagenase by MMP-3. Procollagenase (4 μg/mL) was treated with 10 μg/mL of plasmin, 2 μg/mL of plasma kallikrein, or 10 μg/mL of trypsin of 37 °C for 1 h. After inactivation of the serine proteinases by 2 mM DFP, the proteinase-treated procollagenase was incubated with MMP-3 at the molar ratios of 1:1 (○), 1:0.1 (●), 1:0.01 (□), and 1:0.001 (■) and without MMP-3 (△) at 37 °C for various periods and assayed for collagenase activity. (×) Procollagenase alone.

Rapid Activation of Proteinase-Treated Procollagenase by MMP-3. As presented above, trypsin, plasmin, and plasma kallikrein partially activated procollagenase and generated the 43 000 M_r species. However, when the proteinase-treated procollagenase intermediate of M_r = 43 000 was incubated with MMP-3, fully active collagenase was rapidly generated. In this case, the rate of activation was much faster than that on APMA-treated procollagenase: ony a 1/10 molar ratio of MMP-3 to the proteinase-treated procollagenase was sufficient to activate 50–70% of procollagenase within 30 min at 37 °C (Figure 6), while APMA-treated procollagenase requires approximately 20 times more MMP-3 to attain a similar rate of activation. This indicates that once procollagenase is processed to M_r = 43 000 by these proteinases, it is activated by MMP-3 about 24 000-fold faster than the intact procollagenase molecule. Proteinase-generated intermediates of M_r = 43 000 were converted to the fully active form of M_r =

Table III: NH_2-Terminal Sequence Analyses of Procollagenase and Various Collagenase Species Generated by Proteinases and APMA[a]

	procollagenase (52 kDa)		PKK (46 kDa)				APMA (43 kDa)		plasmin (43 kDa)		PKK (43 kDa)	
cycle	residue	yield[b]	residue	yield[c]	residue	yield[c]	residue	yield[b]	residue	yield[b]	residue	yield[b]
1	F	11	R	+	N	4	M	4	L	7	L	8
2	P	12	N	1	S	+	K	5	K	ni	K	3
3	A	8	S	+	G	13	Q	5	V	3	V	6
4	T	+	G	8	P	5	P	3	M	3	M	4
5	L	5	P	4	V	4	C	ni	K	ni	K	2
6	E	4	V	+	V	+	R	+	Q	3	Q	+
7	T	+	V	2	E	1	G	3	P	2	P	3
8	Q	6	E	2	K	1	V	2	R	+	R	+
9	E	2	K	1	L	4	P	2	C	ni	C	ni
10	Q	2	L	3	K	1	D	3	G	2	G	2
11	D	5	K	2	Q	ni			V	1	V	2
12	V	3	Q	ni	M	1						
13	D	3										
14	L	2										
15	V	1										

	MMP-3 (41 kDa)		APMA & MMP-3 (41 kDa)		plasmin & MMP-3 (41 kDa)		PKK & MMP-3 (41 kDa)		APMA (72 h) (41 kDa)			
cycle	residue	yield[b]	residue	yield[b]	residue	yield[b]	residue	yield[b]	residue	yield[c]	residue	yield[c]
1	F	5	F	7	F	5	F	6	V	5	L	6
2	V	6	V	6	V	4	V	4	L	6	T	+
3	L	6	L	8	L	6	L	5	T	+	E	2
4	T	+	T	+	T	+	T	+	E	3	G	9
5	E	5	E	5	E	2	E	3	G	10	N	3
6	G	4	G	6	G	4	G	5	N	5	P	4
7	N	4	N	5	N	3	N	3	P	6	R	+
8	P	3	P	4	P	3	P	1	R	+	W	ni
9	R	+	R	ni			R	ni	W	ni	E	1
10	W	ni	W	ni			W	ni	E	1	Q	1
11	E	2	E	3			E	1	Q	2	T	+
12	Q	1	Q	3			Q	1				
13			T	+								

[a] Partially active collagenase was generated by plasmin, plasma kallikrein (PKK), and APMA. Fully active collagenase was obtained by the treatment with MMP-3. +, Identified but not quantified; ni, not identified. [b] Net yield, picomoles. [c] Gross yield, picomoles.

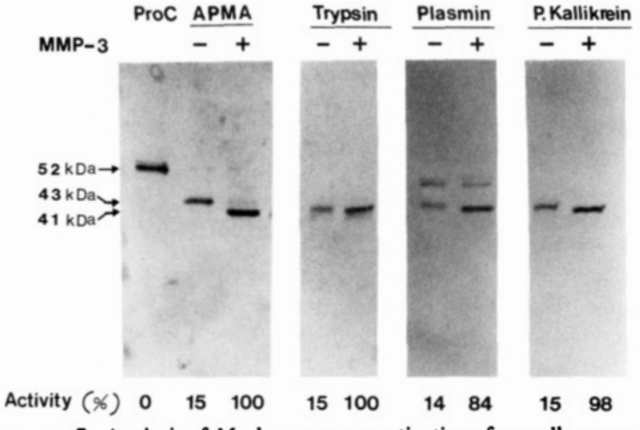

FIGURE 7: Analysis of M_r changes upon activation of procollagenase by various treatments. Procollagenase (ProC) (5 μg/mL) was treated with 1 mM APMA for 2 h at 37 °C or 10 μg/mL of trypsin, 10 μg/mL of plasmin, or 2 μg/mL of plasma kallikrein at 37 °C for 1 h (−) and then treated with MMP-3 in a molar ratio 1:1 for 2 h at 37 °C (+). Serine proteinases was inactivated by 2 mM DFP prior to MMP-3 treatment. After terminating the reaction with 20 mM EDTA, the samples were analyzed by SDS/PAGE under nonreducing conditions and the subsequent immunoblotting with anti-(human procollagenase) antibody. The percent activity indicates that of the maximal collagenase activity determined after treatment with APMA and MMP-3.

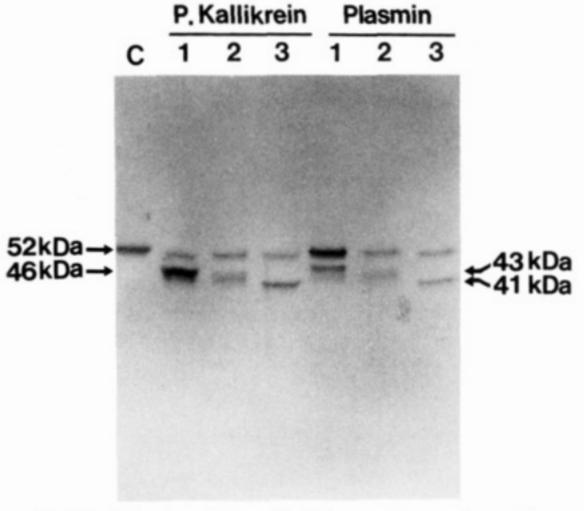

FIGURE 8: Identification of short-lived intermediates of procollagenase produced by plasma kallikrein and plasmin. Procollagenase (65 μg/mL) was incubated with plasma kallikrein (2 μg/mL) or plasmin (10 μg/mL) at 0 °C for 16 h, and the reactions were terminated with 2 mM DFP and 20 mM EDTA. A portion of the processed procollagenase was analyzed by SDS/PAGE and the subsequent immunoblotting with anti-(human procollagenase) antibody. Lane C, procollagenase; lane 1, procollagenase treated with a serine proteinase at 0 °C; lane 2, procollagenase treated with a serine proteinase at 37 °C for 1 h; lane 3, procollagenase treated with a serine proteinase at 37 °C for 1 h and then with MMP-3 at a molar ratio of 1:1 for 2 h at 37 °C after inactivation of the serine proteinase with 2 mM DFP.

41 000 upon MMP-3 treatment (Figure 7).

Sequence Analyses. To understand the precise processing events that occur during procollagenase activation, various forms of collagenase generated were separated by SDS/PAGE and their NH_2 termini were analyzed (Table III). The cleavage sites identified during procollagenase activation are shown in Figure 9A, and the steps involved in forming various intermediate forms of collagenase together with their relative

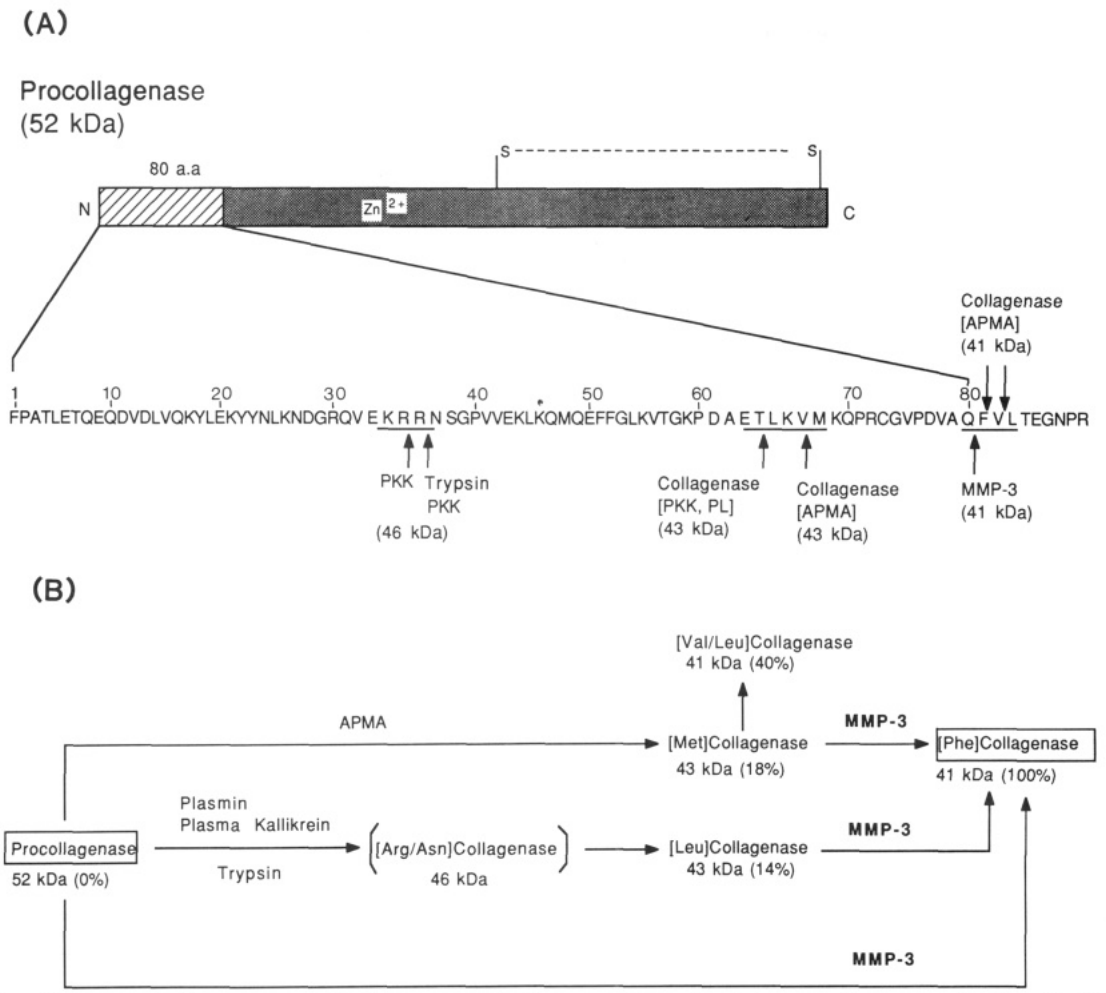

FIGURE 9: Procollagenase activation processes. (A) Cleavage sites in the propeptide region during procollagenase activation. The NH_2-terminal sequences of various collagenase species generated by plasma kallikrein (PKK), plasmin (PL), trypsin, APMA, and MMP-3 were determined and aligned according to the amino acid sequence deduced from cDNA sequencing (Goldberg et al., 1986; Whitman et al., 1986). The trypsin cleavage site is taken from Grant et al. (1987). Collagenase actions are dependent on the presence of the reagents indicated in the brackets. The molecular masses of various intermediates generated during the procollagenase activation processes are shown in the parentheses. (B) Activation pathways of procollagenase. Various forms of collagenase generated during in vitro activation are shown. Procollagenase is activated by MMP-3 directly but this process is slow. Proteinases and APMA partially activate procollagenase by removing portions of the propeptide region. Full activation is achieved by MMP-3 resulting in complete removal of 80 amino acid residues from the NH_2 terminus. In the case of proteinase treatment, 46 000-M_r intermediates are first formed, which are then processed to M_r = 43 000. The action of MMP-3 on partially activated collagenase is rapid. Amino acid residues in brackets indicate the NH_2 terminus of each collagenase species. [Leu]Collagenase of M_r = 43 000 was identified only with plasma kallikrein or plasmin treated procollagenase. In the absence of MMP-3, APMA-treated collagenase of M_r = 43 000 is converted to a 41 000-M_r species by autolysis at the Phe^{81}–Val^{82} and the Val^{82}–Leu^{83} bonds. Collagenolytic activity of each species is shown in parentheses.

collagenolytic activities are summarized in Figure 9B. The NH_2 terminus of fully activated collagenase of M_r = 41 000 generated by the direct action of MMP-3 was Phe^{81}. During APMA treatment the Val^{67}–Met^{68} bond of procollagenase was initially cleaved to generate a partially active 43 000-M_r intermediate. Full activation of this intermediate was accomplished by MMP-3 rapidly converting it to the 41 000-M_r collagenase with Phe^{81} as its NH_2 terminus. On the other hand, a longer treatment of procollagenase with APMA generated the partially active M_r = 41 000 species with the NH_2 terminus of Val^{82} and Leu^{83}. The NH_2 terminus of the partially active 43 000-M_r species generated by plasma kallikrein and plasmin was Leu^{65}. Since the Thr^{64}–Leu^{65} bond is unlikely to be cleaved by these serine proteinases, we postulated that these enzymes initially cleave peptide bonds closer to the NH_2 terminus in the propeptide to produce initial intermediates which, in turn, cleave the Thr^{64}–Leu^{65} bond. To confirm this, procollagenase was incubated with plasma kallikrein (2 μg/mL) or plasmin (10 μg/mL) at 0 °C for 16 h to minimize the proteolytic action of collagenase intermediates. SDS/PAGE analyses of these products showed intermediates with M_r = 46 000 (Figure 8). Sequence analysis of the intermediate generated by plasma kallikrein demonstrated that the Arg^{35}–Arg^{36} and Arg^{36}–Asn^{37} bonds were initially attacked by this enzyme. The results agree with the specificity of plasma kallikrein. It is therefore concluded that the formation of the 43 000-M_r species by serine proteinases is a two-step process (Figure 9B). The generation of fully active collagenase requires cleavage of the Gln^{80}–Phe^{81} bond by MMP-3, and this reaction is greatly accelerated when a portion of propeptide is removed from procollagenase by proteinases of APMA (Figure 9B).

Discussion

Human tissue procollagenase was purified from the culture medium of rheumatoid synovial fibroblasts and used for the studies on its activation mechanisms. When fully activated under appropriate conditions, collagenase of M_r = 41 000 was generated with a maximum specific activity of approximately 27 000 units/mg. This process requires enzymically active

MMP-3. When procollagenase was treated with APMA or a serine proteinase alone, collagenase of M_r = 43 000 was generated. The specific activity of this species was only about 15–25% of the maximal activity. Partially activated procollagenase was also capable of cleaving native collagen molecules into characteristic 3/4 and 1/4 fragments. The specific activity of human tissue collagenase reported here was comparable with those of 27 200 units/mg for rabbit synovial collagenase estimated by titration with ovostatin (Nagase et al., 1983b), 28 000 units/mg for rabbit bone collagenase (Murphy et al., 1982), and 53 000 units/mg for pig synovial collagenase (Cawston et al., 1979). Since these collagenase preparations were activated with APMA in the crude culture medium or at an earlier step of purification, it is most likely that MMP-3 participated in procollagenase activation. On the other hand, specific activities of purified procollagenase used for activation studies by others range from 381 to 4 600 units/mg (Stricklin et al., 1983; Vater et al., 1983; Ishibashi et al., 1987). Autoactivation that often occurs during purification of prolonged storage of procollagenase has been one of the hindrances in obtaining an absolutely latent preparation of procollagenase (Stricklin et al., 1977; Cawston et al., 1981). This phenomenon may be attributed to contamination by proMMP-3 and/or MMP-3. When procollagenase is free of other matrix metalloproteinases, the zymogen is stable to spontaneous activation.

The presence of an endogenous procollagenase activator was proposed in the early studies of Vaes (1972), which demonstrated that latent collagenase in the culture medium of mouse bone explants was gradually activated after brief treatment of the medium with trypsin. This was further substantiated by observations with other proteinases (Eeckhout & Vaes, 1977). Currently the activator molecules have been purified from several sources (Vater et al., 1983, 1986; Ishibashi et al., 1987; Treadwell et al., 1986) and they were identified as MMP-3/stromelysin (Murphy et al., 1987; Fini et al., 1987; Ito & Nagase, 1988). Nonetheless, the modes of procollagenase activation by these activators were reported to be different. While the ability of MMP-3 to directly activate procollagenase was reported by Vater et al. (1983) and Ishibashi et al. (1987), some investigators failed to see such activation of procollagenase by MMP-3 (Chin et al, 1985; Treadwell et al., 1986; Wilhelm et al., 1987). Furthermore, Ishibashi et al. (1987) reported that APMA or trypsin activated their rabbit cervical procollagenase but the activation of rabbit synovial procollagenase by these treatments was not observed (Vater et al., 1983). In the latter case, MMP-3 was required for procollagenase activation. The studies by Murphy et al. (1987) using recombinant human procollagenase and MMP-3 showed that procollagenase treated with APMA or trypsin exhibited only partial collagenolytic activity with a fall in M_r of 10 000. Full activation of collagenase required coincubation of proMMP-3 with procollagenase during the APMA or trypsin activation and was associated with an additional decrease in M_r of 2000.

These various results may be explained by our studies of the activation mechanisms of procollagenase. Our results show that MMP-3 is capable of activating procollagenase directly but this reaction is extremely slow. It requires about 5 days to activate procollagenase fully at 37 °C even at a 20-fold molar excess of MMP-3 over procollagenase. It is notable, however, that the ratio of procollagenase and proMMP-3 secreted from the human rheumatoid synovial fibroblasts stimulated with macrophage-conditioned medium is about 1:20. Such slow activation may be one of the factors that led some investigators to conclude that MMP-3 failed to activate procollagenase (Wilhelm et al., 1987; Chin et al., 1985; Treadwell et al., 1986). Nonetheless, the activation is time- and dose-dependent and insensitive to inactive MMP-3, indicating that it is an enzymic process. The identification of Phe^{81} as the NH_2 terminal of fully active collagenases of M_r = 41 000 generated by MMP-3 indicates that removal of the NH_2-terminal residues 1–80 from procollagenase is crucial for expressing maximal collagenolytic activity. However, the rate of cleavage of the Gln^{80}–Phe^{81} bond by MMP-3 was greatly accelerated when procollagenase was partially activated by a proteinase or APMA.

NH_2-terminal sequence analyses of partially activated collagenase showed that only a portion of the propeptide region was removed during these treatments. The APMA-treated or proteinase-treated collagenase of M_r = 43 000 contains 13 or 16 extra amino acid residues preceding the Phe^{81} residue, respectively. Although He et al. (1989) suggested that COOH-terminal truncation of approximately 15 amino acid residues by MMP-3 was associated with full activation of collagenase, our results indicate that the difference in M_r of approximately 2000 between the partially active and fully active collagenase is due to NH_2-terminal truncation. Cleavage of the Val^{67}–Met^{68} bond by APMA treatment is probably due to an intramolecular autolytic cleavage since the conversion rate of procollagenase to the 43 000-M_r species was not affected by the initial concentration of procollagenase and the presence of substrates. The self-cleavage reaction of procollagenase by APMA was originally proposed for human skin procollagenase by Stricklin et al. (1983). A similar self-catalyzed intramolecular processing as an initial activation step upon APMA treatment was also shown for proMMP-3 (Okada et al., 1988). However, the site of APMA-induced autolytic cleavage in proMMP-3 is at the Glu^{68}–Val^{69} bond (Nagase et al., 1990), one residue prior to the equivalent Val–Met bond. This may reflect differences in the specificity of the two enzymes and/or in the conformational arrangement of the propeptide region in the active site of each enzyme. Upon longer incubation of procollagenase with APMA, the 43 000-M_r collagenase underwent further conversion to M_r = 41 000 caused by intermolecular hydrolysis of the Phe^{81}–Val^{82} and Val^{82}–Leu^{83} bonds. The Gln^{80}–Phe^{81} bond was not cleaved in this process. The specific activity of these 41 000-M_r species was about 40% of that of the fully active 41 000-M_r collagenase with Phe^{81} at the NH_2 terminus (Figure 9B). These results indicate that Phe^{81} at the NH_2 terminus plays a significant role in expressing the full activity of tissue collagenase.

The identification of Leu^{65} as an NH_2 terminus of the plasmin or plasma kallikrein treated procollagenase was unexpected. We showed that cleavage of the Thr^{64}–Leu^{65} bond occurs by the partially activated collagenase produced by the initial proteolysis of the zymogen at the Arg^{35}–Arg^{36} and Arg^{36}–Asn^{37} bonds by plasma kallikrein. Grant et al. (1987) reported the initial cleavage site of procollagenase by trypsin was at the Arg^{36}–Asn^{37} bond. Since the sequence of this region, Glu^{33}-Lys-Arg-Arg-Asn^{37} is highly charged, it is likely to be exposed to the surface of the molecule and thus susceptible to proteolysis. A similar stepwise activation process was seen with the zymogen of MMP-3 (Nagase et al., 1990). Treatment of proMMP-3 with human leukocyte elastase, plasma kallikrein, plasmin, and chymotrypsin initially generates intermediates of M_r = 52 000 by the proteolytic attack of these serine proteinases within the corresponding region Phe^{34}-Val-Arg-Arg-Lys-Asp^{39}. The sites cleaved by these proteinases agree with their substrate specificities (Nagase et

al., 1990). Activation of procollagenase was not observed with neutrophil elastase or chymotrypsin, nor did such treatment result in an enhancement of susceptibility of procollagenase to MMP-3 activation. This may be explained by the lack of residues that meet the specificities of these enzymes in this region.

The sequential processing events by intramolecular and intermolecular reactions appear to be a common feature to both procollagenase and proMMP-3 whose primary structures are highly conserved (Goldberg et al., 1986; Fini et al., 1977; Whitham et al., 1987; Wilhelm et al., 1977; Saus et al., 1988). The different rates of activation by MMP-3 of procollagenase and differentially processed collagenase intermediates may be due to variation in the length of the propeptide and the conformation around the Gln^{80}–Phe^{81} bond. Slow cleavage of the Gln^{80}–Phe^{81} bond in procollagenase suggests that this bond is not readily accessible to MMP-3 in the native procollagenase. The recent studies by Springman et al. (1990) suggested that the inactive zymogen form of procollagenase is maintained by binding of Cys^{73} in the propeptide to the active-site zinc atom at its fourth coordination site. Thus, the displacement of Cys^{73} from the zinc atom is considered to be a key event for expressing the catalytic function of collagenase. Our studies demonstrated that the removal of a portion of the propeptide from the NH_2 terminus is sufficient for partial activation of procollagenase perhaps by destabilization of the Cys^{73}–zinc complex, resulting in the subsequent exposure of the Gln^{80}–Phe^{81} bond for specific proteolysis by MMP-3. This suggests that the NH_2-terminal segment of the propeptide also participates in retaining a stable conformation of the zymogen. The different rates of activation between proteinase-treated procollagenase and APMA-treated procollagenase molecules may be due to an additional positive charge of the extra tripeptide of Leu^{65}-Lys-Val^{67} in the former, which may further influence the local conformation near the Gln^{80}–Phe^{81} bond. Interestingly, the processes required for proMMP-3 activation are analogous (Nagase et al., 1990). The active form of MMP-3 with M_r = 45 000 is generaged by cleavage of the His^{82}–Phe^{83} bond, which aligns with the Gln^{80}–Phe^{81} bond in procollagenase, but this bond is not accessible to MMP-3 in the native zymogen. Once a portion of propeptide is removed by proteinase or APMA treatment (at least 34 amino acid residues from the NH_2 terminus), the His^{82}–Phe^{83} bond is readily attacked by MMP-3. Clearly, the conformation around these peptide bonds appears to govern their susceptibility to MMP-3 and the eventual activation of these two zymogens.

The differences in the rate of procollagenase activation and the state of active forms emphasize that degradation of collagen in vivo may be regulated in a very precise manner. As summarized in Figure 9B, the availability of MMP-3 and other proteinases is clearly a rate-limiting factor for collagenolysis. Relatively slow turnover of connective tissue matrix macromolecules may result from the slow, direct activation of procollagenase by MMP-3, whose activation can be achieved by a large number of proteinases with different specificities (Okada et al., 1988; Nagase et al., 1990). On the other hand, under certain pathological conditions, especially those accompanied by inflammation, an accelerated breakdown of collagen is likely to be attributed not only to enhanced production of both procollagenase and proMMP-3 by inflammatory mediators but also to the accelerated activation of procollagenase by MMP-3 in conjunction with certain plasma and tissue proteinases. Plasma kallikrein and plasmin are excellent candidates to govern such stepwise reactions (Eeckhout & Vaes, 1977; Werb et al., 1977; Nagase et al., 1982). Both enzymes are also capable of activating proMMP-3 to MMP-3 (Okada et al., 1988; Nagase et al., 1990).

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