Stepwise Activation Mechanisms of the Precursor of Matrix Metalloproteinase 3 (Stromelysin) by Proteinases and (4-Aminophenyl)mercuric Acetate†

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ABSTRACT: The mechanisms of activation of the precursor of human matrix metalloproteinase 3 (proMMP-3/prostromelysin) by proteinases and (4-aminophenyl)mercuric acetate (APMA) were investigated by kinetic and sequence analyses. Incubation of proMMP-3 with neutrophil elastase, plasma kallikrein, plasmin, or chymotrypsin at 37 °C resulted in the formation of MMP-3 of M_r = 45 000 by cleaving of the His^{82}–Phe^{83} bond. Since this bond is unlikely to be cleaved by these proteinases it was postulated that an initial attack of an activator proteinase on proMMP-3 creates an intermediate form, which is then processed to a more stable form of M_r = 45 000. To test this hypothesis proMMP-3 was incubated with these serine proteinases under conditions that minimize the action of MMP-3. This led to the accumulation of major intermediates of M_r = 53 000 and two minor forms of M_r = 49 000 and 47 000. The 53 000 M_r intermediate generated by human neutrophil elastase resulted from cleavage of the Val^{35}–Arg^{36} bond, whereas plasma kallikrein cleaved the Arg^{36}–Arg^{37} and Lys^{38}–Asp^{39} bonds and chymotrypsin the Phe^{34}–Val^{35} bond, all of which are located near the middle of the propeptide. Conversion of these intermediates to the fully active 45 000 M_r form of MMP-3 resulted from a bimolecular reaction of the intermediates. A similar short-lived intermediate of M_r = 46 000 generated by APMA was a result of the intramolecular cleavage of the Glu^{68}–Val^{69} bond, and it was then converted to a stable MMP-3 of M_r = 45 000 by a intermolecular reaction of MMP-3. However, MMP-3 failed to activate proMMP-3. These results indicate the removal of the NH_2-terminal 34–38 residues by proteinases or 68 residues by APMA is the crucial step for activation of proMMP-3. This initial processing of the propeptide allows the His^{82}–Phe^{83} bond, which is hindered from proteolysis in native proMMP-3, to be correctly oriented for cleavage by activated intermediates, thereby producing stable 45 000 M_r MMP-3. This stepwise activation process allows proMMP-3 to be activated by various proteinases with distinct substrate specificities.

Matrix metalloproteinases 3 (MMP-3[1]/stromelysin) is synthesized and secreted from connective tissue cells, as are tissue collagenase (MMP-1) and MMP-2 (also called gelatinase/type IV collagenase) (Okada et al., 1986). The primary structures deduced from cDNA sequencing indicate that these enzymes are homologous to each other, but their substrate specificities are distinct (Goldberg et al., 1986; Whitham et al., 1986; Fini et al., 1987; Wilhelm et al., 1987; Collier et al., 1988; Murphy et al., 1988; Saus et al., 1988). Tissue collagenase specifically cleaves collagen types I, II, III, VII, and X (Welgus et al., 1981; Schmid et al., 1986; Seltzer et al., 1989). MMP-2 has the ability to degrade collagen types IV, V, and VII as well as gelatins (Murphy et al., 1985; Seltzer et al., 1989). MMP-3 has a broader activity on a variety of extracellular matrix macromolecules including proteoglycans, fibronectin, collagen types IV and IX, and laminin and is able to remove the NH_2-terminal propeptides from type I procollagen (Galloway et al., 1983; Chin et al., 1985; Okada et al., 1986, 1989a). More recently it has been shown that the presence of MMP-3 is essential for full activation of procollagenase and that MMP-3 is the previously described procollagenase activator (Murphy et al., 1987; Ito & Nagase, 1988; He et al., 1989), indicating that it also plays a key step in collagenolysis. The production of MMP-3 by rheumatoid synovial hypertrophic lining cells, but not normal synovial cells (Okada et al., 1989b), and the elevated level of proteoglycan-degrading activity found in osteoarthritic compared with normal cartilage (Martel-Pelletier et al., 1984; Dean et al., 1989) indicate that this enzyme may play an important role in pathological destruction of cartilage. Indeed, the synthesis of MMP-3 and other matrix metalloproteinases by connective tissue cells is elevated by various inflammatory mediators such as interleukin 1 (Mizel et al., 1981; Gowen et al., 1984; Saus et al., 1988) and tumor necrosis factor (Dayer et al., 1985), substance P (Lotz et al., 1987), several growth factors (Bauer et al., 1985; Moscatelli et al., 1986; Edwards et al., 1987), and oncogenic viruses (Matrisian et al., 1985, 1986).

Like other matrix metalloproteinases (Nagase et al., 1981; Murphy et al., 1985; Okada et al., 1986), MMP-3 is synthesized and secreted from cells as an inactive zymogen (Okada et al., 1988). Full activity of proMMP-3 in vitro can be attained by treatment with proteinases or organomercurial compounds such as (4-aminophenyl)mercuric acetate (APMA) (Okada et al., 1988). Our previous studies on the activation of proMMP-3 suggested that treatment of proMMP-3 with APMA induces a molecular perturbation of the zymogen that

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[1] Abbreviations: MMP, matrix metalloproteinase; APMA, (4-aminophenyl)mercuric acetate; HMW, high molecular weight; LMW, low molecular weight; HNE, human neutrophil elastase; 3,4-DCI, 3,4-dichloroisocoumarin; Cm-Tf, reduced, carboxymethylated transferrin; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

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results in its conversion to an intermediate of M_r = 46000 by an intramolecular self-catalyzed action. This is followed by a bimolecular reaction in which the intermediate converts to 45000 M_r (HMW) and eventually to 28000 M_r (LMW) species (Okada et al., 1988), both of which have similar substrate specificities and specific activities (Okada et al., 1986). None of these active forms of MMP-3 can activate proMMP-3. However, a variety of proteinases including trypsin, chymotrypsin, plasmin, plasma kallikrein, and thermolysin activate proMMP-3 regardless of their substrate specificities (Okada et al., 1988). This led us to postulate that hydrolysis of a specific region, rather than a specific peptide bond, of the zymogen may be sufficient for the expression of MMP-3 activity.

The present study was conducted to test this hypothesis by detailing the events that lead to the full activation of proMMP-3. The zymogen was allowed to be activated by enzymes with distinct specificities, human neutrophil elastase (HNE), plasmin, plasma kallikrein, or chymotrypsin, some of which are also likely pathological activators of proMMP-3 in the inflammatory site. We demonstrate here that all of these treatments generated an identical active form of MMP-3 with M_r = 45000. This process is the result of a unique stepwise reaction in which the initial intermediate forms of MMP-3 produced by the proteinases rapidly convert to a stable 45000 M_r MMP-3. A similar process was found during the activation of proMMP-3 in the presence of APMA. The key event in activation by both treatments lies on the initial removal of a portion of the NH_2-terminal propeptide from proMMP-3. The formation of the 45000 M_r MMP-3 either by proteinases or by mercurial compounds is due to bimolecular reactions of the partially processed intermediates, a step common to proMMP-3 activation by these treatments.

Experimental Procedures

Materials. HNE and human plasma kallikrein were purified according to Baugh and Travis (1976) and Nagase and Barrett (1981), respectively. Chymotrypsin (bovine), plasminogen (human), urokinase, transferrin (human), and diisopropyl fluorophosphate were from Sigma. Plasminogen was activated by treating with urokinase (10 units) at 37 °C for 60 min. A general serine proteinase inhibitor, 3,4-dichloroisocoumarin (3,4-DCI), was from Böhringer-Mannheim. Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce Chemical Co. Reagents and solvents for the gas/liquid phase sequencer were from Applied Biosystems.

Purification of the 57000 M_r ProMMP-3. The activity of proMMP-3 was assayed after activation with 1.5 mM APMA or an appropriate proteinase using reduced, [^{3}H]carboxymethylated transferrin ([^{3}H]Cm-Tf) as substrate (Okada et al., 1986). One unit of MMP-3 activity produced 1 μg of Cm-Tf fragments soluble in 3.3% (w/v) trichloroacetic acid in 1 min at 37 °C. ProMMP-3, showing a typical doublet of M_r = 57000 and 59000 in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), was purified by immunoadsorbent chromatography from the culture medium of human rheumatoid synovial fibroblasts stimulated with rabbit macrophage conditioned medium as described by Ito and Nagase (1988). The glycosylated form of proMMP-3 (M_r = 59000) was removed by passing the proMMP-3 preparation through a column of concanavalin A–Sepharose (2.5 × 15 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5)/0.4 M NaCl/10 mM Ca^{2+}/0.02% NaN_3. The concanavalin A unbound fractions were concentrated by a YM-10 membrane and applied to an Sephacryl S-200 column (1.5 × 120 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5)/0.4 M NaCl/10 mM Ca^{2+}/0.05% Brij 35/0.02% NaN_3. The final product migrated as a single band of M_r = 57000 on SDS/PAGE. The specific activity of proMMP-3 was 840 units/mg when fully activated with 1.5 mM APMA for 16 h at 37 °C.

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 proMMP-3, the sample (200 pmol) was directly applied to a polybrene-treated filter. APMA-activated MMP-3 and the intermediate forms of MMP-3 produced by proteinases were separated by SDS/PAGE with 7% (w/v) total acrylamide under reducing conditions (Bury, 1981), transferred from the polyacrylamide gel to poly(vinylidene difluoride) membrane (PVDF-Millipore Immobilon transfer membrane) according to the procedure described by Matsudaira (1987). The proteins transferred to PVDF membranes 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. Amino acid net yields were calculated by substraction 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.

Radioiodination of ProMMP-3. Purified proMMP-3 (10 μg/mL) was labeled with $^{125}I^-$ according to the method of Fraker and Speck (1978). After the protein was labeled, free $^{125}I^-$ was remoed from the ^{125}I-labeled sample by spin column chromatography on Sephadex G-10 equilibrated with 50 mM Tris-HCl buffer (pH 7.5)/0.15 M NaCl/5 mM Ca^{2+}/0.05% Brij 35/0.02% NaN_3 as described by Salvesen and Nagase (1989).

Protein Determination. ProMMP-3 concentrations were determined by the method of Smith et al. (1985) using bicinchoninic acid with crystalline bovine serum albumin as standard. After sodium dodecyl sulfate/polyacrylamide gel electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 unless otherwise stated. In some cases MMP-3 proteins were visualized by the immunoblotting technique using specific sheep antibody raised against human proMMP-3 as described previously (Ito & Nagase, 1988).

Results

NH_2-Terminal Sequences of ProMMP-3 and MMP-3. ProMMP-3 purified by an immunoadsorbent column showed a typical doublet of M_r = 57000 and 59000 (Figure 1). The two forms of proMMP-3 were separated by concanavalin A–Sepharose. The NH_2-terminal sequence of both forms of proMMP-3 was Tyr-Pro-Leu-Asp-Gly-Ala-Ala-Arg-Gly-Glu-Asp-Thr-Ser-Met-Asn, identical with that inferred from cDNA sequencing (Whitham et al., 1986; Wilhelm et al., 1987; Saus et al., 1988). Upon treatment with 1.5 mM APMA at 37 °C for 16 h, proMMP-3 converted to two active species of MMP-3: HMW (M_r = 45000 and 47000) and LMW (M_r = 28000 and 30000). These forms were separated by SDS/PAGE, transferred to PVDF membranes and subjected to sequence analyses. All forms revealed an identical sequence starting with Phe^{83} (see Table I), indicating that treatment of proMMP-3 with APMA results in the cleavage of the His^{82}–Phe^{83} bond, and that the conversion of HMW to LMW species occurred by the removal of an approximately 17000 M_r polypeptide from the COOH-terminal end. Our sequence analyses of each doublet of proMMP-3, HMW and LMW species, together with other biochemical evidence (Okada et al., 1988), demonstrated that the two species of proMMP-3 are single gene products of which the 59000 M_r

Table I: NH_2-Terminal Sequence Analyses of HMW, LMW, and Intermediate Forms of MMP-3 Generated by APMA or Proteinases[a,b]

	APMA									
cycle no.	HMW (47 kDa)	yield[c]	HMW (45 kDa)	yield[c]	LMW (30 kDa)	yield[c]	LMW (28 kDa)	yield[c]	HNE (45 kDa)	yield[c]
1	F	2	F	12	F	9	F	6	F	30
2	R	+	R	+	R	+	R	+	R	+
3	T	+	T	+	T	+	T	+	T	+
4	F	4	F	4	F	9	F	5	F	25
5	P	6	P	7	P	3	P	+	P	26
6	G	5	G	9	G	7	G	4	G	15
7	I	4	I	2	I	8	I	4	I	+
8	P	3	P	2	P	2	P	+	P	12
9	K	1	K	2	K	3	K	1	K	10

cycle no.	HNE (53 kDa)	yield[c]	CT (53 kDa)	yield[c]	PKK (53 kDa)	yield[d]		yield[d]	APMA (46 kDa)	yield[c]
1	R	+	V	38	R	+	D	12	V	15
2	R	+	R	+	K	25	S	+	M	9
3	K	9	R	+	D	24	G	25	R	+
4	D	8	K	10	S	+	P	16	K	5
5	S	+	D	10	G	37	V	13	P	4
6	G	12	S	+	P	25	V	20	R	+
7	P	5	G	15	V	31	K	ni	C	ni
8	V	5	P	11	V	30	K	ni	G	8
9	V	+	V	6	K	ni	I	15	V	4
10	K	2	V	+	K	ni	R	+	P	5
11	K	+	K	2	I	17	E	8	D	4
12	L	5	K	+	R	+	M	4	V	4
13	R	+	L	16	E	13	Q	7		
14	E	2	R	+	M	10	K	+		
15	M	3	E	5	Q	9	F	15		

[a] Intermediates were generated by human neutrophil elastase (HNE), chymotrypsin (CT), plasma kallikrein (PKK) and APMA. [b] +, identified but not quantified. ni, not identified. [c] Net yield, picomoles. [d] Gross yield, picomoles.

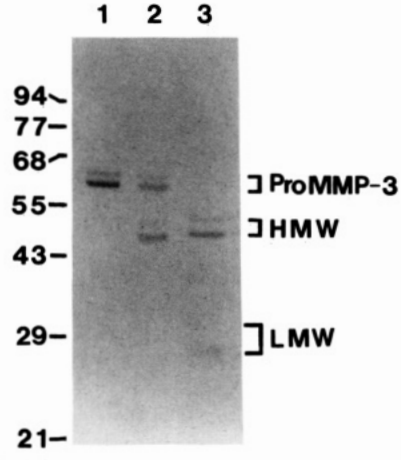

FIGURE 1: SDS/PAGE analyses of various forms of MMP-3. Lane 1, ProMMP-3 isolated after an immunoadsorbent column; lanes 2 and 3, proMMP-3 treated with 1.5 mM APMA at 37 °C for 4 and 24 h, respectively. Proteins were strained with Coomassie Brilliant Blue R-250. HMW, MMP-3 of M_r = 45000 and 47000; LMW, MMP-3 of M_r = 28000 and 30000.

species is a glycosylated form of the 57000 M_r proMMP-3. To simplify the study of the mechanisms of proMMP-3 activation only the 57000 M_r species was used.

Activation of ProMMP-3 by HNE. Incubation of proMMP-3 with HNE at 37 °C resulted in activation in a concentration- and time-dependent manner (Figure 2). SDS/PAGE analyses of the reaction products showed proMMP-3 was rapidly processed to a polypeptide of M_r = 45000, identical in size with the HMW form generated by APMA treatment (Figure 3). Only a small amount of MMP-3 was converted to M_r = 28000 after a longer incubation, but a decrease of enzymic activity was not detected even after 16 h.

NH_2-terminal sequence analysis of the 45000 M_r MMP-3 generated by HNE indicated activation by cleavage at the His^{82}–Phe^{83} bond (Table I). Since this bond is an unlikely

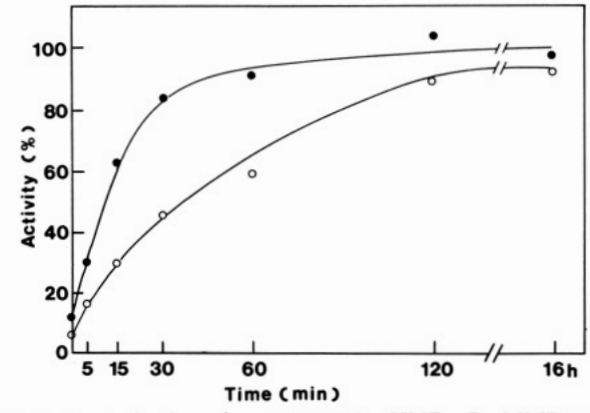

FIGURE 2: Activation of proMMP-3 by HNE. ProMMP-3 (15 μg/mL) was incubated with 2 μg/mL HNE (●) and 0.5 μg/mL HNE (○) at 37 °C for various times in 50 mM Tris-HCl buffer (pH 7.5)/0.15 M NaCl/10 mM Ca^{2+}/0.02% NaN_3/0.05% Brij 35. MMP-3 activity was measured against the substrate [^{3}H]Cm-Tf. Full activation was taken after treating proMMP-3 with 1.5 mM APMA for 20 h at 37 °C.

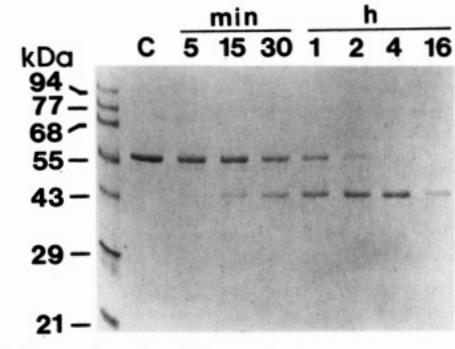

FIGURE 3: Processing of proMMP-3 by HNE at 37 °C. ProMMP-3 (70 μg/mL) was incubated with 2 μg/mL HNE at 37 °C for the indicated period, and the HNE was subsequently inactivated with 2 mM 3,4-DCI. The samples were mixed with an equal volume of SDS/PAGE sample buffer containing 20 mM EDTA, boiled under reducing conditions, and applied to SDS/PAGE of 7.5% total acrylamide.

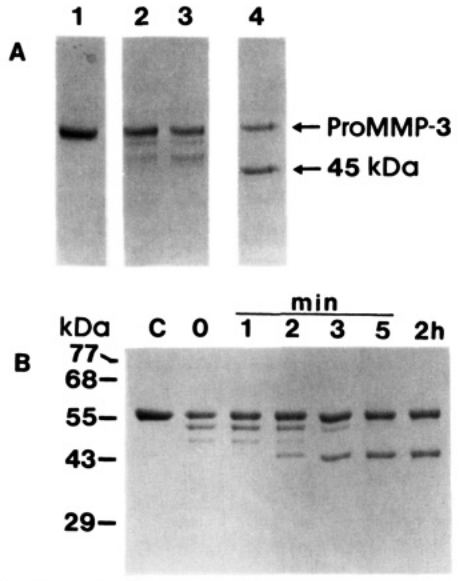

FIGURE 4: Identification of short-lived intermediates of MMP-3 produced by HNE. (A) ProMMP-3 (170 μg/mL) was reacted with 2 μg/mL HNE in the presence of 1 mM 1,10-phenanthroline at 37 °C for 30 min (lane 2) and 1 h (lane 3). Lane 1, proMMP-3 alone; lane 4, proMMP-3 treated with 2 μg/mL HNE for 1 h at 37 °C in the absence of 1,10-phenanthroline. (B) ProMMP-3 (170 μg/mL) was reacted with 2 μg/mL HNE at 0 °C for 16 h after which the activity of HNE was terminated with 2 mM 3,4-DCI (lane 0). The sample was further incubated at 37 °C for the indicated periods. Lane C, proMMP-3 alone.

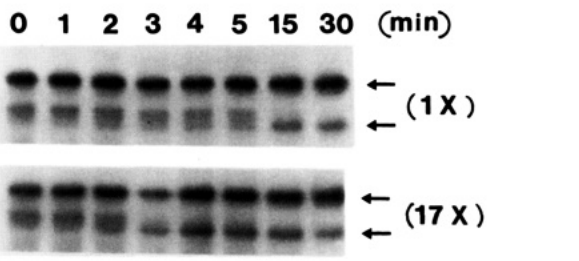

FIGURE 5: Concentration-dependent conversion of the MMP-3 intermediates to the 45 000 M_r MMP-3. ^{125}I-Labeled proMPP-3 (10 μg/mL) was reacted with HNE (10 μg/mL) at 0 °C for 4 h to generate the intermediates. They were then mixed with an equal volume of the reaction buffer (1×) or a 17-fold molar excess of the unlabeled MMP-3 intermediates (17×) generated by HNE (10 μg/mL) at 0 °C for 4 h. The HNE activity was terminated with 2 mM 3,4-DCI. Each sample was then incubated at 37 °C for the indicated times, and conversion of the intermediates to the 45 000 M_r species was examined by SDS/PAGE and autoradiography. Upper and lower arrows indicate proMMP-3 and the 45 000 M_r MMP-3, respectively.

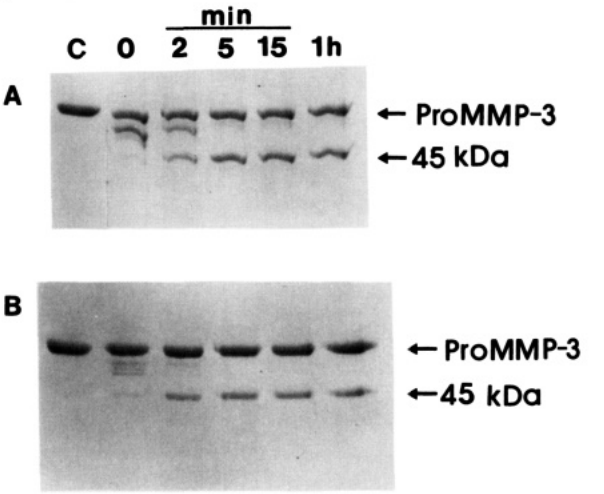

FIGURE 6: Activation processes of proMMP-3 by human plasma kallikrein and plasmin. ProMMP-3 (170 μg/mL) was reacted with (A) human plasma kallikrein (2 μg/mL) or (B) plasmin (10 μg/mL) at 0 °C for 16 h. The enzymic activity was terminated by 2 mM diisopropyl fluorophosphate and the enzyme solution was further incubated at 37 °C for the indicated periods. Lane C, proMMP-3 alone; lane 0, proMMP-3 treated with each proteinase at 0 °C for 16 h.

candidate for HNE cleavage, we postulated that HNE initially attacks proMMP-3 to produce a short-lived intermediate which then converts to a stable 45 000 M_r form following the action of the MMP-3 intermediates. To test this possibility, it is required to minimize the action of activated MMP-3. This was approached by two separate experiments: (1) treatment of proMMP-3 with HNE in the presence of 1 mM 1,10-phenanthroline and 10 mM $CaCl_2$ in which Zn^{2+} in the active site is chelated but Ca^{2+} is retained to maintain the structural integrity of proMMP-3, and (2) incubation with a large amount of HNE at 0 °C for 18 h during which the action of HNE proceeds but the MMP-3 activity is largely suppressed. In the presence of 1,10-phenanthroline, proMMP-3 was converted to M_r = 53 000, 50 000, and 47 000 by HNE (Figure 4A). In the presence of 5 mM EDTA, however, proMMP-3 was completely degraded by HNE, probably due to denaturation of the zymogen. When proMMP-3 was incubated with HNE (2 μg/mL) at 0 °C for 18 h, similar intermediates of M_r = 53 000, 49 000, and 47 000 were generated, among which the band of M_r = 53 000 was more prominent. When the reaction mixture was further incubated at 37 °C after complete inactivation of HNE by 2 mM 3,4-DCI, the intermediate forms were rapidly converted to M_r = 45 000 (Figure 4B). This conversion was observed only for partially processed intermediates but not for intact proMMP-3. This process was shown to be an intermolecular reaction of the intermediates rather than an intramolecular self-cleaving event since the rate of conversion of the intermediate forms to the 45 000 M_r form was accelerated at higher concentration (Figure 5) and decreased in the presence of Cm-Tf, a substrate for MMP-3 (data not shown). The enzymic activity detected after partial conversion of the precursor correlated with the amount of the 45 000 M_r species. Sequence analysis of the intermediate of M_r = 53 000 (Table I) revealed that the Val^{35}–Arg^{36} bond is the major initial cleavage site of proMMP-3 by HNE.

Activation of ProMMP-3 by Other Serine Proteinases. Similar experiments were conducted with plasma kallikrein and plasmin. As shown in Figure 6, incubation of proMMP-3 with these serine proteinases at 0 °C produced intermediate forms that were subsequently converted to the 45 000 M_r species at 37 °C with similar kinetics. Again, only the intermediates were converted to the 45 000 M_r MMP-3. Sequence analysis of the intermediate (M_r = 53 000) generated by plasma kallikrein (Table I) revealed the initial cleave at the Arg^{36}–Arg^{37} and Lys^{38}–Asp^{39} bonds adjacent to the initial HNE cleavage site. This indicates that the stretch of propeptide Val^{35}-Arg-Arg-Lys-Asp^{39} is exposed on the surface of the proMMP-3 molecule and readily susceptible to proteolysis. If so, it seems reasonable to postulate that the activation of proMMP-3 by chymotrypsin (Okada et al., 1988) is likely to be triggered by the initial attack of the enzyme on the Phe^{34}–Val^{35} bond of this region. This prediction was confirmed by the NH_2-terminal analysis of the 53 000 M_r intermediate generated by chymotrypsin under conditions similar to those described for the other serine proteinases (Table I).

Processing of ProMMP-3 by APMA. We have previously demonstrated that the initial event that occurs during proMMP-3 activation by APMA treatment is the induction of conformational changes in the zymogen which, in turn,

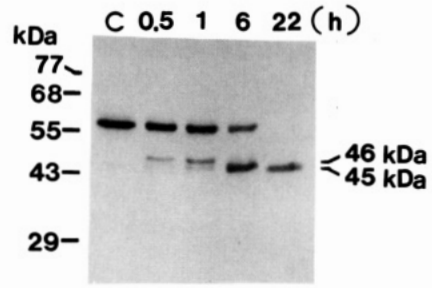

FIGURE 7: Identification of short-lived intermediates of MMP-3 produced by APMA. ProMMP-3 (7.7 μg/mL) was reacted with 1.5 mM APMA for the indicated periods and the reaction was terminated by 20 mM EDTA. The processed MMP-3 was analyzed by SDS/PAGE and subsequent immunoblotting using anti-(human MMP-3) antibody.

generates a 46 000 M_r intermediate by intramolecular self-cleavage action of the zymogen (Okada et al., 1988). Subsequently, the intermediate is converted to M_r = 45 000 (HMW) by a bimolecular reaction. The latter form was generated by the cleavage of the His^{82}–Phe^{83} bond. To identify the NH_2-terminal sequence of the initial intermediate of M_r = 46 000 generated by self-cleavage, a relatively low concentration of proMMP-3 (17 μg/mL) was reacted with 1.5 mM APMA at 37 °C for 1 h, and the reaction was terminated with 25 mM EDTA (Figure 7). The products were then concentrated and separated by SDS/PAGE. The 46 000 M_r intermediate was subjected to sequence analysis, which revealed that the initial intramolecular self-cleavage of proMMP-3 occurs at the Glu^{68}–Val^{69} bond in the presence of APMA (Table I).

Discussion

One of the common features of the activation of matrix metalloproteinases is that all of the enzymes characterized so

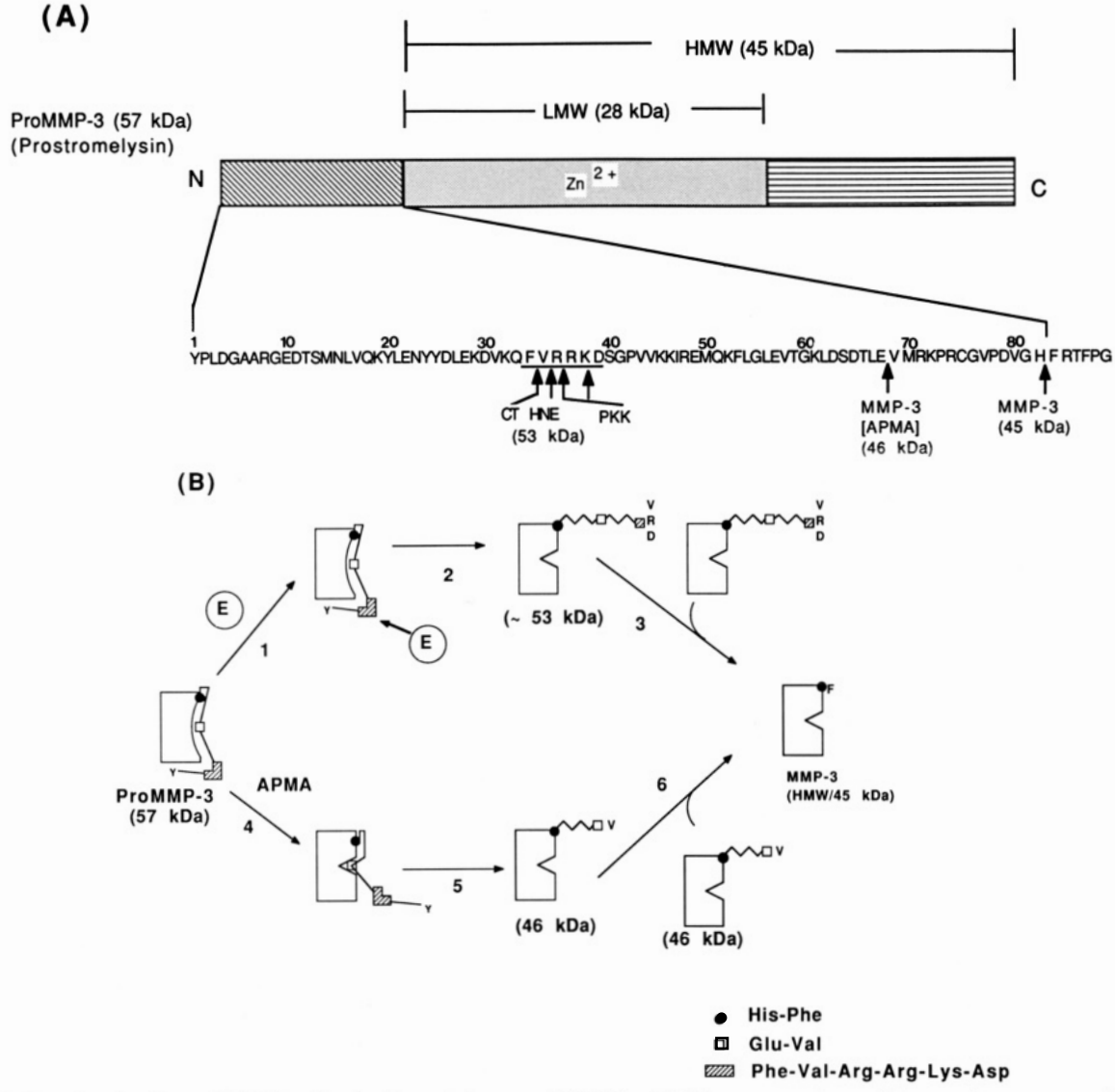

FIGURE 8: Steps involved in proMMP-3 activation by proteinases and APMA. (A) Sequence analyses of intermediates generated by human neutrophil elastase (HNE), plasma kallikrein (PKK), chymotrypsin (CT), and APMA (see Table I) revealed the cleavage sites indicated by arrows. MMP-3 cleaves the His^{82}–Phe^{83} bond. (B) Proposed steps involved in proMMP-3 activation by proteinases and APMA. The configuration of the propeptide region, indicated by a solid line, is presumed to be relatively fixed in the proenzyme. The His^{82}–Phe^{83} bond (●) is sterically hindered from proteolysis, but the region Phe-Val-Arg-Arg-Lys-Asp (34-39) (▨) is exposed and susceptible to proteolysis. Upon proteolysis in this region MMP-3 intermediates are generated (step 2), a portion of the propeptide become flexible, and the His^{82}–Phe^{83} bond becomes susceptible to the MMP-3 intermediate (step 3). This generates 45 000 M_r MMP-3 by cleavage of the His^{82}–Phe^{83} bond. The presence of APMA causes changes in conformation of the zymogen (step 4) and the Glu^{68}–Val^{69} bond (□) is cleaved by an intramolecular autocatalysis (step 5). The 46 000 M_r intermediate now has a flexible portion of the propeptide and exposes the His^{82}–Phe^{83} bond for proteolysis by MMP-3 to generate the 45 000 M_r MMP-3 (step 6). Steps 3 and 6 are a common bimolecular reaction of intermediates during proMMP-3 activation by proteinases and APMA. Amino acid residues indicated are NH_2-termini identified in various forms of MMP-3.

far are fully or partially activated from their precursors by treatment with proteinases or organomercurial compounds. This includes tissue collagenase (MMP-1) (Stricklin et al., 1983), MMP-2 (Murphy et al., 1985), MMP-3 (Okada et al., 1988), neutrophil collagenase (Mallya & Van Wart, 1989), "gelatinases" from neutrophils (Hibbs et al., 1985; Murphy et al., 1989) and macrophages (Hibbs et al., 1987), acid metalloproteinase from cartilage (Azzo & Woessner, 1986), collagen telopeptidase (Nakano & Scott, 1987), the enzyme that degrades one-quarter and three-quarter fragments of type I collagen (Overall & Sodek, 1987), and a low relative molecular mass metalloproteinase from rat uterus (Woessner & Taplin, 1988). Studies on the activation of procollagenase and proMMP-3 suggest that treatment of the zymogens with APMA initiates a molecular perturbation of the precursor zymogens that results in loss of an approximately 12000 M_r peptide from the NH_2-terminal end (Stricklin et al., 1983; Okada et al., 1988). A similar autocatalytic activation by APMA has been postulated to explain the activation of proMMP-2 by APMA (Stetler-Stevensen et al., 1989). The cleavage sites of procollagenase, proMMP-2, and proMMP-3 after APMA treatment were identified as Gln^{80}–Phe^{81} (Whitham, et al., 1986), Asn^{80}–Tyr^{81} (Stetler-Stevensen et al., 1989), and His^{82}–Phe^{83} (Whitham et al., 1986; this study) bonds located in a highly conserved region in these homologous zymogens.

Our earlier studies on proMMP-3 activation by proteinases indicated that the activation of proMMP-3 by proteinases is a result of the removal of NH_2-terminal propeptide of approximately 12000 M_r by direct proteolysis (Okada et al., 1988). However, in the present study we demonstrated that generation of the 45000 M_r MMP-3 by HNE treatment resulted from hydrolysis of the His^{82}–Phe^{83} bond. Direct hydrolysis of this bond by HNE is unlikely as it has little selectivity for histidine in its primary specificity pocket (Bode et al., 1989). Instead, the cleavage of the His^{82}–Phe^{83} bond is more likely to result from the action of MMP-3 since this bond is also cleaved upon APMA activation of proMMP-3. Indeed, under the conditions where the activity of MMP-3 was largely suppressed (incubation with a larger amount of HNE at 0 °C or in the presence of 1,10-phenanthroline), HNE cleaved proMMP-3 at the Val^{35}–Arg^{36} and at two additional bonds (unidentified) to generate intermediate forms of M_r = 53000, 49000, and 47000. Intermediates with similar relative molecular mass were generated by plasma kallikrein, plasmin, and chymotrypsin. Plasma kallikrein cleaved the Arg^{36}–Arg^{37} and Lys^{38}–Asp^{39} bonds and chymotrypsin the Phe^{34}–Val^{35} bond in the same region of the propeptide. When these intermediates were incubated at 37 °C following the inactivation of the serine proteinases, all of them were rapidly converted to the 45000 M_r species with Phe^{83} as its NH_2 terminus. This process was shown to be due to a bimolecular reaction of the intermediates of MMP-3 since their conversion rate to the stable 45000 M_r MMP-3 was accelerated at higher concentration and decreased in the presence of the substrate. However, it was not possible to demonstrate whether the 53000 M_r intermediate possesses proteolytic activity against the [^{3}H]Cm-Tf substrate as it rapidly converted to M_r = 45000 during the assay at 37 °C.

Figure 8 illustrates the stepwise activation schemes of proMMP-3 after treatment with a proteinase or APMA. When the proenzyme is treated with an organomercurial compound, the Glu^{68}–Val^{69} bond become accessible to the active site of the enzyme, resulting in the release of peptide residues 1–68 by an intramolecular autocatalysis. Once this NH_2-terminal region is removed, the His^{82}–Phe^{83} bond becomes readily susceptible to the intermediates or to MMP-3, thereby allowing the bimolecular processing. We postulate that the His^{82}–Phe^{83} bond is not accessible to proteolysis in the native proMMP-3 molecule. This is supported by our previous observation that MMP-3 cannot directly activate proMMP-3 (Okada et al., 1988) and by the results presented in Figures 4–6. The exposure of the His^{82}–Phe^{83} bond may also be attained by removing the portion of the propeptide region by proteolysis. All of the serine proteinases tested attacked bonds within the stretch of the propeptide Phe^{34}-Val-Arg-Arg-Lys-Asp^{39}, according to their known specificities. This highly charged region is likely to be exposed and readily accessible to proteinases with different specificities. The removal of the NH_2-terminal 34–38 residues from the precursor by proteinases results in changes in the local conformation of the propeptide, thus allowing the His^{82}–Phe^{83} bond to be correctly oriented for specific proteolysis by MMP-3 intermediates. The cleavage of the His^{82}–Phe^{83} bond is a common pathway for proMMP-3 activation regardless of the initial triggers for activation, i.e., by proteinases or organomercurial compounds. This two-step activation process of proMMP-3 contrasts to the activation of serine proteinase zymogens where propeptide removal occurs from a single proteolytic event, usually following a Lys or Arg residue, by activators with trypsin-like specificity (Neurath, 1984). The promiscuity of the region encompassing residues 34–39 in the propeptide allows the activation of proMMP-3 to be triggered by a number of proteinases with different specificities. Thus, proteolytic enzymes from plasma, tissues, and inflammatory cells are likely to participate in proMMP-3 activation under certain physiological and pathological conditions.

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Registry No. Pro-MMP, 99332-75-9; APMA, 6283-24-5; elastase, 9004-06-2; kallikrein, 9001-01-8; plasmin, 9001-90-5; chymotrypsin, 9004-07-3.

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