Biochemical Characterization of Matrilysin. Activation Conforms to the Stepwise Mechanisms Proposed for Other Matrix Metalloproteinases†

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Received January 24, 1992; Revised Manuscript Received June 17, 1992

ABSTRACT: The latent precursor of matrilysin (EC 3.4.24.23; punctuated metalloproteinase (PUMP)) was purified from transfected mouse myeloma cell conditioned medium and was found to contain one zinc atom per molecule which was essential for catalytic activity. Promatrilysin could be activated to the same specific activity by (4-aminophenyl)mercuric acetate, trypsin, and incubation at elevated temperatures (heat activation). Active matrilysin hydrolyzed the fluorescent substrate 2,4-dinitrophenyl-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 at the Gly–Leu bond with a maximum value for k_{cat}/K_m of 1.3×10^4 M^{-1} s^{-1} at the pH optimum of 6.5 and pK_a values of 4.60 and 8.65. Activity is inhibited by the tissue inhibitor of metalloproteinases-1 in a 1:1 stoichiometric interaction. Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis in conjunction with N-terminal sequencing revealed that, as with all other matrix metalloproteinases similarly studied, promatrilysin activation was accompanied by the stepwise proteolytic removal of an M_r 9000 propeptide from the N-terminus. The intermediates generated were dependent on the mode of activation used but, in all cases studied, activation terminated with an autocatalytic cleavage at E^{77}–Y^{78} to yield the final M_r 19 000 active matrilysin. From an analysis of the stability of the various intermediates, we propose that the sequence L^{13}–K^{33} is particularly important in protecting the E^{77}–Y^{78} site from autocatalytic cleavage, thereby maintaining the latency of the proenzyme.

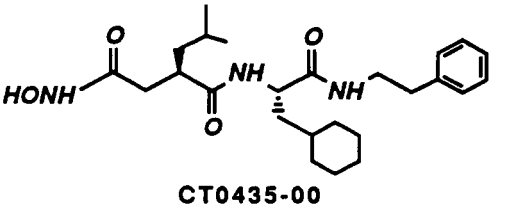
Human matrilysin (formally punctuated metalloproteinase (PUMP)[1]) was initially identified as a cDNA isolated from a mixed tumor library (Muller et al., 1988) and was characterized as a matrix metalloproteinase by expression in eukaryotic systems (Quantin et al., 1989; Murphy et al., 1991a). Although the precise physiological role of matrilysin remains unknown, its mRNA is present in biopsy material from prostate cancer patients (Pajouh et al., 1991) as well as gastric and colon carcinomas (McDonnell et al., 1991), and matrilysin has been purified from both resorbing rat uterine tissue (Woessner & Taplin, 1988) and a human tumor cell line (Miyazaki et al., 1990). It conforms precisely to the criteria for classification as a matrix metalloproteinase (Woessner, 1991), being secreted as a latent precursor (promatrilysin) activatable by organomercurials (Murphy et al., 1991a) and inhibited by the tissue inhibitor of metalloproteinases-1 (TIMP-1) (Murphy et al., 1991b). However, matrilysin is unique in that it lacks the C-terminal hemopexin-like domain possessed by the other members of this family. Currently there is a lack of detailed structural or mechanistic information concerning any member of the matrix metalloproteinase family, but the relatively low molecular weight of matrilysin and its lack of N-linked glycosylation suggest that it may be a suitable candidate for such studies.

Activation is thought to be a key point in the regulation of matrix metalloproteinase activity and has been the subject of several recent studies. For example, the activation mechanisms of human interstitial collagenase and stromelysin-1 by organomercurials and endoproteinases have been analyzed in detail by Suzuki et al. (1990) and Nagase et al. (1990) and shown to occur via a stepwise series of cleavages in an N-terminal propeptide to yield the final active forms. Recombinant stromelysin-1 has recently been shown to also be activated by incubation at 55 °C (Koklitis et al., 1991) in a process termed heat activation. In this study, we have instigated a biochemical characterization of matrilysin and investigated whether promatrilysin conforms to the same patterns of activation.

MATERIALS AND METHODS

Materials

All chromatography materials were from Pharmacia Ltd. U.K. Spectrosol grade reagents were from BDH Ltd. The substrate 2,4-dinitrophenyl(Dnp)-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 was from Novobiochem U.K. TIMP-1 was kindly supplied by Dr. S. Angal (Celltech Ltd., U.K.). CT0435-00, a competitive and specific inhibitor of matrix metalloproteinase activity, was kindly supplied by The Oncology Chemistry Department of Celltech Ltd., U.K., as a 10 mM solution in methanol. CT0435-00 has a K_i against matrilysin of 14 nM with Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 as the substrate (results not shown).



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† G.M. is supported by the Arthritis and Rheumatism Council, U.K.
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[1] Abbreviations: PUMP, punctuated metalloproteinase; TIMP-1, tissue inhibitor of metalloproteinases-1; Dnp, 2,4-dinitrophenyl; APMA, (4-aminophenyl)mercuric acetate; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

0006-2960/92/0431-8500$03.00/0

Methods

Enzyme Purification. Human promatrilysin was purified from culture medium conditioned by NSO mouse myeloma cells (ECACC catalog no. 85110503) that had been transfected with a vector containing a 1.08-kb *Eco*RI fragment encoding human promatrilysin. The cDNA was kindly provided by Dr. R. Breathnach, Universite de Nantes, France (Muller et al., 1988), and the expression vector, transfection method, and culturing conditions were as previously described (Murphy et al., 1991a). Four liters of conditioned medium was diluted 1:1 with distilled water before application to an S-Sepharose Fast Flow column (5.3 $cm^2 \times 30$ cm) equilibrated with buffer A (25 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.5) containing 30 mM NaCl. Unbound protein was washed from the column with the same buffer followed by elution of the bound proteins with buffer A containing 300 mM NaCl. After centrifugation at 20000*g* for 15 min to remove a proteinaceous precipitate, the eluate was loaded onto a Blue Sepharose CL-6B column (2 $cm^2 \times 40$ cm) previously equilibrated with buffer A containing 300 mM NaCl. After the column was washed with the same buffer, bound proteins were eluted using a linear gradient of 0.3–2 M NaCl in buffer A. Pooled fractions containing promatrilysin were desalted by gel filtration with Sephadex G-25 into buffer A containing 30 mM NaCl and then concentrated by application to a Mono-S HR5/5 column previously equilibrated with the same buffer, followed by elution using a linear gradient of 30–500 mM NaCl in buffer A run at 1 mL/min. Promatrilysin eluted at approximately 250 mM NaCl, and the promatrilysin-containing fractions were pooled to give a 40 μM (1 mg/mL) solution which was stored at 4 °C.

Active matrilysin for subsequent kinetic studies was prepared by the incubation of promatrilysin at 37 °C for 2 h in the presence of 1 mM (4-aminophenyl)mercuric acetate (APMA) added from a 20 mM stock solution made up in 0.5 M NaOH titrated to pH 10. The activated matrilysin was separated from propeptide fragments and APMA by Sephadex G-25 gel filtration into buffer A containing 30 mM NaCl and stored at −20 °C. No alteration in the activity or electrophoretic mobility occurred during 6 months storage under these conditions. Amino acid analysis of active matrilysin gave a molar extinction coefficient of 30 000 M^{-1} cm^{-1} at 280 nm.

Analysis of Promatrilysin Zinc Content. To determine the zinc content of promatrilysin, precautions were taken to prevent metal contamination of samples. Milli-Q water, "Spectrosol" grade reagents and metal-free containers and pipets were used throughout. Trace levels of metal ions were removed by Chelex-100 resin (Bio-Rad) followed by addition of $CaCl_2$ and adjustment of the pH if required. Samples were rendered free of contaminating zinc by Sephadex G-25 gel filtration, digested with 6 M nitric acid at 55 °C for 18 h, and diluted to the appropriate concentrations using 1% (v/v) nitric acid. Standards were used in the range of 0.03–1 ppm in 1% (v/v) nitric acid. Suitable controls were performed to demonstrate that the presence of Tris or Ca^{2+} ions did not interfere with the results. Determinations were performed using a Shandon Southern A3300 atomic absorption spectrophotometer with an A3370 carbon rod analyser. Samples of 10 μL were analyzed in duplicate at 213.9 nm.

Matrilysin Activity Assays. The fluorescent substrate Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2, described by Stack and Gray (1989) for pig collagenase and gelatinase, was used throughout. Routine assays were performed at 37 °C using 20–50 μM substrate and 2–10 nM enzyme in an assay buffer comprised of 0.1 M Tris-HCl, 10 mM $CaCl_2$, 0.1 M NaCl, and 0.05% (v/v) Brij-35, pH 7.5. Substrate solutions were stored in 10% (v/v) DMSO, and the final DMSO concentration in the assays was 1%. At time intervals up to 2 h, 20-μL aliquots were withdrawn and added to 500 μL of 0.1 M sodium acetate buffer, pH 4.0, to stop the reaction. The increase in tryptophan fluorescence due to hydrolysis of the Gly–Leu bond was measured using a Perkin-Elmer LS5B luminescence spectrometer with microcell adaptors for the use of 300 μL of cells. Excitation was at 280 nm and emission was at 346 nm, and the assay was calibrated using a range of tryptophan solutions. The substrate concentration was determined from its absorption at 372 nm and extinction coefficient of 16 000 M^{-1} cm^{-1} (Stack & Gray, 1989). The initial rate of reaction was determined by performing a linear regression analysis on the time points.

k_{cat}/K_m Determinations. k_{cat}/K_m determinations were performed essentially as described above at a substrate concentration of 20 μM. This substrate concentration was shown to fulfill the condition of $[S] \ll K_m$ required to give progress curves that were first order in substrate, allowing direct determination of k_{cat}/K_m. Measurements were taken across the pH range 4–9 using the following buffers: sodium acetate (pH 4.0–5.6), MES (pH 5.6–6.6), Bis-Tris (pH 6.0–7.5), HEPES (pH 7.0–8.0), Tris (pH 7.5–8.5), and glycine (pH 8.0–9.0). Each buffer was at a concentration of 50 mM and contained 10 mM $CaCl_2$ and NaCl to give an ionic strength of 0.1 M. The stability of matrilysin across the pH range was tested by incubation of buffer and enzyme for the length of time and at the concentration used in the kinetic measurements followed by assay of enzyme activity at pH 7.5. Data of k_{cat}/K_m versus pH were fitted using eq 1 (where $\bar{k}_{cat}/\bar{K}_m$ is the pH-independent value and K_I and K_{II} are the apparent ionization constants for the ascending and descending limbs, respectively, of the bell-shaped curve) and the Enzfitter program (Leatherbarrow 1987; Elsevier-Biosoft, Hills Road, Cambridge, U.K.).

$$k_{cat}/K_m = \frac{\bar{k}_{cat}/\bar{K}_m}{1 + \frac{[H^+]}{K_I} + \frac{K_{II}}{[H^+]}} \quad (1)$$

Stoichiometry of the Inhibition of Matrilysin by TIMP-1. Titrations were performed using an enzyme concentration of 30–50 nM and TIMP-1 concentrations in the range of 5–100 nM. Enzyme and inhibitor were incubated for 30 min at 25 °C before addition of substrate to a final concentration of 20 μM. Assays were performed at 25 °C, and samples were taken at intervals up to 30 min and analyzed as described above.

Activation Procedures. Promatrilysin (40 μM) was diluted using buffer A containing 30 mM NaCl and activated by incubation with APMA or *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (from bovine pancreas) or by incubation at elevated temperatures. APMA was added to a final concentration of 1 mM as previously described. TPCK-treated trypsin was added to a final concentration of 0.2 μM (5 μg/mL) from a stock solution of 40 μM in 1 mM HCl, 10 mM $CaCl_2$. Phenylmethane sulfonyl fluoride (PMSF) (100 mM in 1-propanol) was added to a final concentration of 1 mM to aliquots taken from TPCK-treated trypsin activations prior to their analysis.

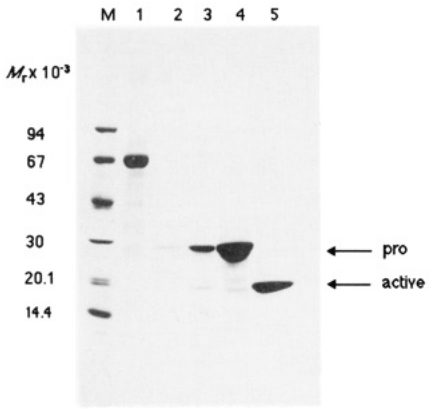

FIGURE 1: Purification of active matrilysin. Sixteen-microliter aliquots taken from each stage of the purification were analyzed by SDS–polyacrylamide gel electrophoresis as described in Materials and Methods. Lane M, marker proteins; lane 1, promatrilysin-containing conditioned medium; lane 2, pooled eluate from S-Sepharose Fast Flow; lane 3, pooled promatrilysin-containing fractions from Blue Sepharose CL-6B; lane 4, pooled promatrilysin-containing fractions from Mono-S; lane 5, APMA-activated matrilysin after Sephadex G-25 gel filtration.

Gel Electrophoresis. Samples to be analyzed by sodium dodecyl sulfate– (SDS–) polyacrylamide gel electrophoresis (Laemmli, 1970) were first inactivated by the immediate addition of EDTA to a final concentration of 30 mM before being boiled for 2 min in standard running buffer containing 0.02% (v/v) β-mercaptoethanol. Two-microgram samples of matrilysin were run on precast 3–27% polyacrylamide gels (Integrated Separation Systems, Hyde Park, MA) and stained for protein using Coomassie Blue R-250. Reduced M_r marker proteins run on all gels were phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400).

Electroblotting and N-Terminal Sequence Analysis. Matrilysin was subjected to SDS–polyacrylamide gel electrophoresis according to the method of Hunkapiller (1983). Protein was then electroblotted onto poly(vinylidene difluoride) membranes (Matsudaira, 1987) and stained using 0.1% (w/v) Ponceau S dye in 1% (v/v) aqueous acetic acid (Aebersold et al., 1987), and the relevant bands were removed. Blotted matrilysin was applied to Bioprene-treated glass fiber discs which were then loaded onto an Applied Biosystems 470A gas-phase sequencer and N-terminally sequenced by Edman degradation. The resulting phenylthiohydantoin-derivatized amino acids were analyzed with an Applied Biosystems 120A HPLC.

RESULTS

Expression and Purification of Matrilysin

The transfected NSO cells grown at a density of 5×10^6 cells/mL yielded approximately 10 μg/mL matrilysin following enzyme purification. The purification of matrilysin was confirmed by N-terminal amino acid sequencing of the major protein bands visible after analysis of the samples by SDS–polyacrylamide gel electrophoresis (Figure 1). S-Sepharose Fast Flow removed the major contaminant, albumin, but concentration of the promatrilysin-containing fraction revealed the presence of low levels of other contaminants. These were separated from promatrilysin through the use of Blue Sepharose CL-6B (Woessner & Taplin, 1988). The additional protein bands visible in the final two stages of promatrilysin purification were not present after activation by APMA and may, therefore, be either contaminating proteins which were removed by matrilysin proteolysis or autoactivation products produced by SDS-induced promatrilysin autoactivation occurring as a result of the mixing of the samples with running buffer (Van Wart & Birkedal-Hansen, 1990). N-terminal amino acid sequencing of promatrilysin gave the sequence LPLPQEAGGMSELQW which is consistent with the cDNA predicted sequence (Figure 9) and allows the first leucine shown above to be assigned as the first residue of the mature secreted proenzyme. In common with the other members of the matrix metalloproteinase family of enzymes, treatment with the organomercurial APMA resulted in a reduction in the M_r of matrilysin (Figure 1) and produced an active enzyme with the N-terminal amino acid sequence YSLFPNS.

Table I: Zinc Content of Promatrilysin[a]

promatrilysin (μg/mL)	Zn (μg/mL)	mol of Zn/ molecule of enzyme
41.5	0.088	0.91
83.8	0.190	0.97
126.0	0.300	1.02

[a] Protein concentration was determined by amino acid analysis and zinc concentration by atomic absorption spectroscopy as described in Materials and Methods.

Properties of Matrilysin

Zinc Content. Three promatrilysin concentrations in the range 40–130 μg/mL were analyzed in duplicate for zinc content (Table I). The results obtained indicate that promatrilysin contains 1 atom of zinc per enzyme molecule. Control measurements gave a zinc concentration of less than 10 pg/mL in the buffer, a level of contamination that is negligible compared to the enzyme concentration. In studies on active matrilysin, it was found that the zinc chelator 1,10-phenanthroline was inhibitory (results not shown), indicating that the zinc atom is essential to activity. By analogy with the other metalloproteinases such as thermolysin, it is assumed that the zinc atom is involved in catalysis.

pH Dependence. N-terminal sequence analysis of the reaction products was used to confirm that matrilysin catalyzes the hydrolysis of Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 at the Gly–Leu bond. Lack of solubility of the substrate prevented the determination of all the kinetic parameters for the reaction. It was, however, demonstrated that the dependence of the initial rate of reaction on substrate concentration was linear up to a substrate concentration of 100 μM across the pH range studied. The substrate concentration of 20 μM was, therefore, considered to be low enough in comparison to the K_m to allow direct determination of k_{cat}/K_m for the reaction. The pH dependence of k_{cat}/K_m is shown in Figure 2. Lack of interference by the buffers used was demonstrated by ensuring that each pH value was determined using two different buffer systems and by establishing that matrilysin was stable under the assay conditions used in the pH range 4.4–9.0. When the data was fitted to the equation for the bell-shaped curve described in Materials and Methods, pK_a values of 4.60 and 8.65 were obtained with a maximum value for k_{cat}/K_m of 1.3×10^4 M^{-1} s^{-1} at pH 6.5.

Inhibition by TIMP-1. The inhibitor TIMP-1 was titrated against matrilysin activity to determine the stoichiometry of their interaction. The results obtained gave a straight line (correlation coefficient = 0.993) with an ordinate intercept of 44.2 nM when residual enzyme activity was plotted against TIMP-1 concentration (data not shown). The enzyme concentration in the assay as determined by amino acid analysis

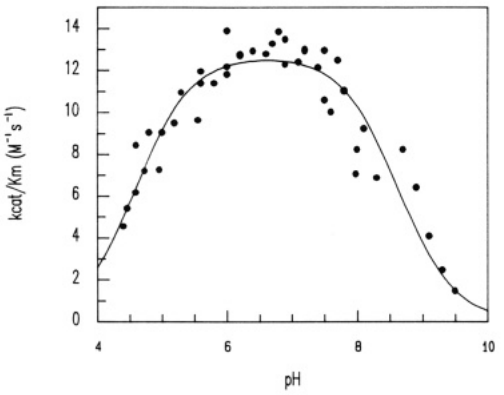

FIGURE 2: pH dependence of k_{cat}/K_m for the matrilysin catalyzed hydrolysis of Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2. Each data point is the average of duplicate determinations. The theoretical line is described by eq 1 in Materials and Methods with parameters of $pK_I = 4.60$, $pK_{II} = 8.65$, and $k_{cat}/K_m = 1.3 \times 10^4$ M^{-1} s^{-1}.

was 50 nM, giving a ratio of TIMP-1:matrilysin which results in complete inhibition of 0.88:1. The most likely stoichiometry for the interaction is therefore 1:1, a result that has been observed for other matrix metalloproteinases (Cawston et al., 1983; Murphy et al., 1989).

Activation of Promatrilysin

Multiple Modes of Promatrilysin Activation. The treatment of promatrilysin with agents or conditions which result in the activation of other members of the matrix metalloproteinase family confirmed that the organomercurial APMA, TPCK-treated trypsin, and exposure to elevated temperatures were all able to activate matrilysin to the same specific activity (Figure 3A). Maximal activity was achieved in about 30 min in the presence of 1 mM APMA and within 3 h in the presence of 0.2 μM TPCK-treated trypsin. The slow rate of activation which occurred upon incubation of promatrilysin at 37 °C (60% activation after 24 h) was increased at higher incubation temperatures; at 53 °C, maximal activity was achieved after approximately 6 h. Analysis of the activation process by SDS–polyacrylamide gel electrophoresis performed under reducing conditions indicated that concomitant with the increase in activity was a drop in relative molecular mass of the proenzyme from M_r 28 000 to M_r 19 000 (Figure 3B). The promatrilysin used in this experiment displayed some activity prior to activation but, as can be seen in Figure 3B, the starting sample contained some M_r 19 000 active form, probably the result of slow autoactivation occurring during its storage at 4 °C. Other preparations of purified promatrilysin which contained less M_r 19 000 matrilysin displayed correspondingly greater latency (results not shown). Although the final M_r 19 000 active form was not broken down further, extended incubation with trypsin or at 53 °C did lead to a slight loss of activity. Additional studies have shown that active matrilysin displays no significant loss in activity or alteration in electrophoretic mobility when stored for up to 28 days at room temperature at a concentration of 33 μM (results not shown). The N-terminal amino acid sequence of matrilysin activated by trypsin and heat was the same as that obtained after APMA activation. This indicates that the final cleavage for all the activation procedures was at E^{77}–Y^{78} which is consistent with the 9000 drop in M_r being the result of the loss of the first 77 amino acids from the N-terminus (Figure 9).

Inhibition of Promatrilysin Activation. The production of the M_r 19 000 active matrilysin could be prevented by the

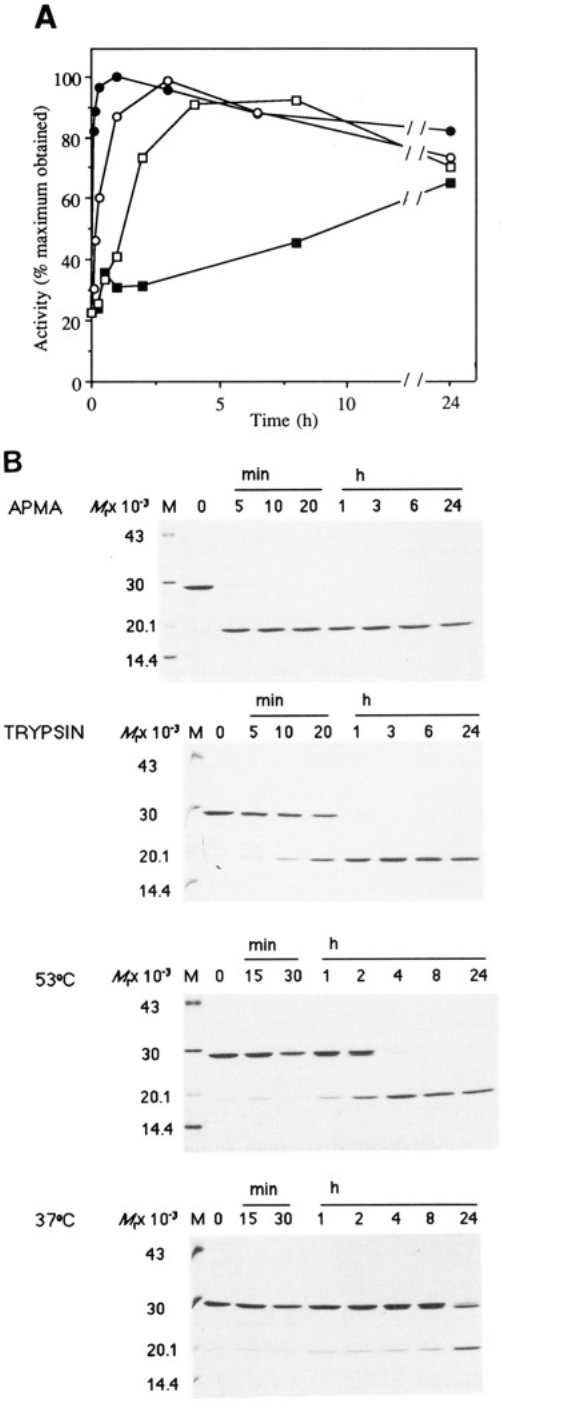

FIGURE 3: Activation of promatrilysin. (A) Aliquots taken from the incubation of 7 μM promatrilysin (●) with 1 mM APMA at 37 °C; (○) with 0.2 μM TPCK-treated trypsin at 37 °C; (■) alone at 37 °C; and (□) alone at 53 °C were immediately diluted 100-fold with assay buffer and stored at −20 °C prior to their assay in duplicate at a final enzyme concentration of 7 nM. Results are presented as a percentage of the maximum activity recorded (1 h in the presence of 1 mM APMA). (B) Samples of activating matrilysin taken from the same incubations described above and at the time points indicated were analyzed by SDS–polyacrylamide gel electrophoresis along with marker proteins (lane M) according to the procedures outlined in Materials and Methods.

presence of a synthetic inhibitor of matrix metalloproteinase activity, the peptidehydroxamate CT0435-00 (Figure 4A,B). APMA activation could not progress beyond the M_r 28 000 proform while incubation with inhibitor and trypsin produced an intermediate which migrated just above active matrilysin at M_r 20 000. This intermediate possessed the N-terminus

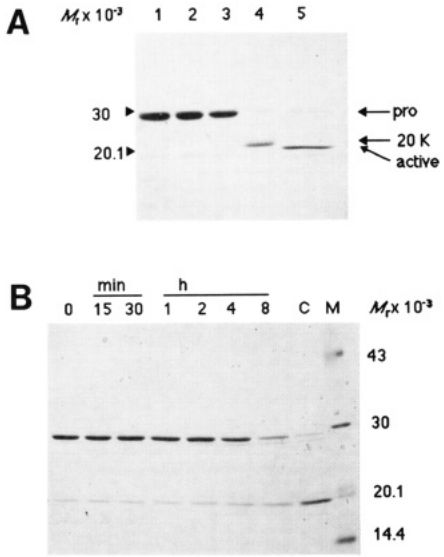

FIGURE 4: Inhibition of promatrilysin activation by CT0435-00, a synthetic inhibitor of matrix metalloproteinase activity. (A) Promatrilysin (lane 1) at a concentration of 4 μM was incubated for 1 h at 37 °C with no activators (lane 2), 1 mM APMA (lanes 3 and 5), or 0.2 μM TPCK-treated trypsin (lane 4) in either the presence (lanes 2, 3, and 4) or absence (lane 5) of 0.4 mM CT0435-00 after which samples were analyzed by SDS–polyacrylamide gel electrophoresis according to the procedures outlined in Materials and Methods. (B) Promatrilysin (7 μM) was incubated at 53 °C in the presence of 0.24 mM CT0435-00 and aliquots were removed at the indicated time points to be analyzed by SDS–polyacrylamide gel electrophoresis. A control activation (lane C) was also performed by incubation of the promatrilysin sample for 2 h at 23 °C in the presence of 1 mM APMA and 0.1% (v/v) methanol. Marker proteins (lane M) were also run.

XGVPDVAEYSLFPNS indicating that it was produced as a result of cleavage at R^{69}–C^{70} (Figure 9) which, being a typical trypsin cleavage site, demonstrates that trypsin-catalyzed cleavages in the propeptide can still occur in the presence of CT0435-00. When promatrilysin was incubated at 53 °C in the presence of CT0435-00, the accumulation of the M_r 19 000 active matrilysin was prevented (Figure 4B). These conditions did, however, result in the gradual disappearance of promatrilysin, possibly because at elevated temperatures the latent enzyme is susceptible to precipitating from solution if it is not activated.

Intermediate of APMA Activation. Promatrilysin activation by APMA at 23 °C was examined at two enzyme concentrations by following the loss of the propeptide by SDS–polyacrylamide gel electrophoresis (Figure 5A,B). Promatrilysin was transiently converted to an M_r 21 000 intermediate which was in turn cleaved to generate the final M_r 19 000 active matrilysin. The M_r 21 000 intermediate accumulated to a greater extent during the activation of the 1.3 μM sample when compared with the 40 μM sample, enabling sequencing of its N-terminus. The sequence obtained, IMQXPXXGVPDXA, and M_r of the intermediate indicate that it was produced as a result of cleavage at E^{63}–I^{64} (Figure 9).

Intermediates of Trypsin Activation. The relationship between the intermediates formed during TPCK-treated trypsin activation of promatrilysin was investigated by lowering the incubation temperature to 4 °C, thereby reducing the rate of activation (Figure 6A). The first event observed was the generation of a transient M_r 25 000 intermediate with an N-terminal sequence, NAXXLXAXXXXMXXF, that showed that it was the result of cleavage at the typical trypsin site, K^{33}–N^{34} (Figure 9). Alongside the subsequent gradual production of the M_r 19 000 active matrilysin, other transient

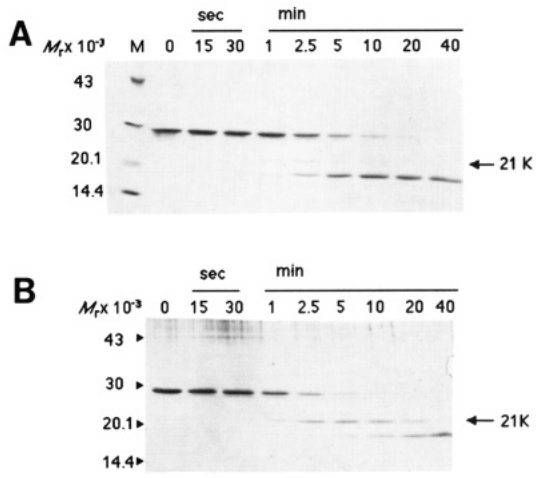

FIGURE 5: Effect of promatrilysin concentration on its rate of activation by APMA. Promatrilysin at a concentration of (A) 40.0 μM and (B) 1.3 μM was incubated at 23 °C in the presence of 1 mM APMA. Aliquots were removed at the time points indicated and analyzed by SDS–polyacrylamide gel electrophoresis according to the procedures outlined in Materials and Methods. Marker proteins (lane M) were also run.

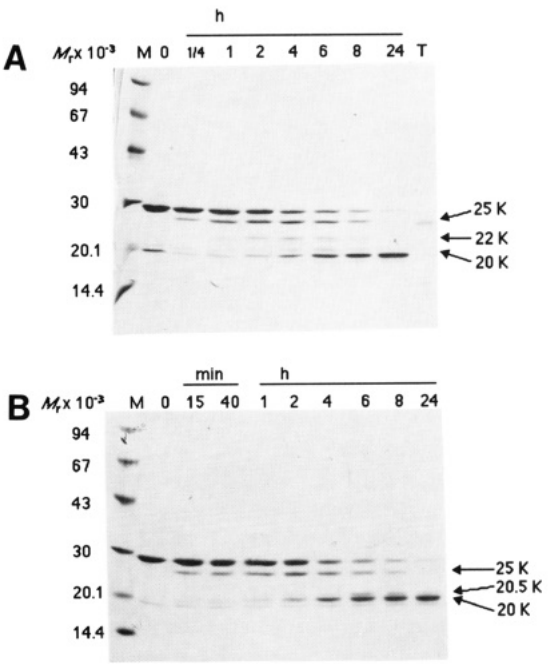

FIGURE 6: Matrilysin activation intermediates generated by incubation with TPCK-treated trypsin. Promatrilysin (6 μM) was incubated at 4 °C with 0.2 μM TPCK-treated trypsin in either (A) the absence or (B) the presence of 0.4 mM CT0435-00. At the time points indicated, aliquots were removed and analyzed by SDS–polyacrylamide gel electrophoresis according to the procedures outlined in Materials and Methods. A 2-μg sample of trypsin (gel A; lane T) was run in addition to the marker proteins (lane M).

intermediates migrating at M_r 22 000 and 20 000 were also observed. The M_r 25 000 intermediate appeared and disappeared at the same rate when trypsin activation was performed in the presence of an inhibitor of matrix metalloproteinase activity, CT0435-00 (Figure 6B), but in this instance the final product was the previously described M_r 20 000 intermediate. Although an additional transient intermediate of M_r 20 500 was observed during activation in the presence of the inhibitor, the M_r 22 000 intermediate was notably absent.

The susceptibility of the trypsin-generated intermediates to autocatalysis was examined by briefly treating promatrilysin at 4 °C with trypsin before inactivating the trypsin and

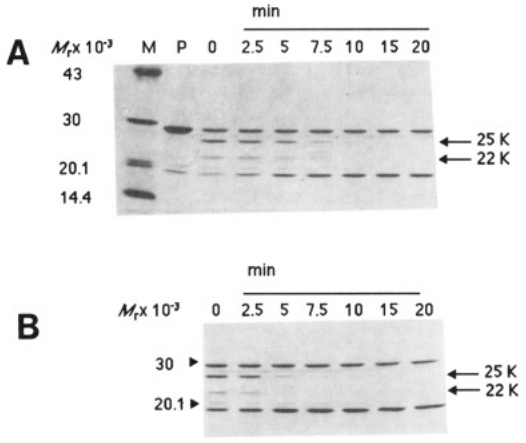

FIGURE 7: Stability of the TPCK-treated trypsin-generated matrilysin activation intermediates. Promatrilysin (6 μM; lane P) was incubated at 4 °C for 2 h with 0.2 μM TPCK-treated trypsin to generate the activation intermediates. At this point (0 h), PMSF was added to 1 mM and the sample incubated at 37 °C in either (A) the absence or (B) the presence of 2 μM activated matrilysin (generated by the incubation of a 13.4 μM promatrilysin solution at 53 °C for 5 h). The dilution caused by the addition of heat-activated matrilysin was equalized in the other sample by the addition of Tris-HCl buffer. At the time points indicated, aliquots were removed and analyzed by SDS–polyacrylamide gel electrophoresis according to the procedures outlined in Materials and Methods except that no extra PMSF was added. Marker proteins (lane M) were also run.

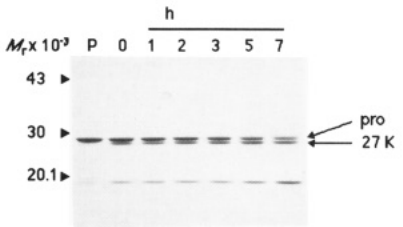

FIGURE 8: Stability of the matrilysin activation intermediate generated by incubation at 53 °C. Promatrilysin (14 μM; lane P) was incubated at 53 °C for 1 h at which point (0 h) the incubation temperature was adjusted to 37 °C. At the time points indicated, aliquots were removed and analyzed by SDS–polyacrylamide gel electrophoresis according to the procedures outlined in Materials and Methods.

raising the temperature of the incubation to 37 °C (Figure 7). In contrast to the remaining promatrilysin, the M_r 25 000 and 22 000 intermediates were found to be unstable, their reduction in relative molecular mass to the M_r 19 000 active form occurring within 10 min of PMSF addition (Figure 7A). Their rate of disappearance could be increased if fully (heat-)activated M_r 19 000 form was added along with the PMSF (Figure 7B). The final samples possessed only a fraction of the activity of similar samples that had no PMSF added. Full activity was achieved if they were subjected to a further 1-h incubation at 37 °C with 1 mM APMA (results not shown).

Heat-Activation Intermediate. An intermediate, migrating at M_r 27 000, was observed by the SDS–polyacrylamide gel electrophoresis analysis of promatrilysin samples taken during heat activation (Figures 3B and 8). Its N-terminal sequence was XQWEXAXXYLXXFXL, indicating that it was produced as a result of cleavage at E^{12}–L^{13} in the propeptide (Figure 9). The relative stability of the M_r 27 000 intermediate, generated by a brief incubation of promatrilysin at 53 °C, was examined by its incubation at 37 °C (Figure 8). Unlike the trypsin-generated intermediates, this heat-activation intermediate was as resistant to cleavage to the M_r 19 000 form as was full-length promatrilysin.

DISCUSSION

Matrilysin was expressed and purified as a latent precursor (promatrilysin) which could be activated by treatments previously used in the activation of interstitial collagenase and stromelysin. This fact, along with the demonstration that matrilysin possesses a single zinc atom essential to catalysis and its inhibition by TIMP-1 in a 1:1 stoichiometric interaction, provides further support for this enzyme's classification as a member of the matrix metalloproteinase family of enzymes.

Using the substrate Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2, matrilysin has a K_m of >100 μM with a k_{cat}/K_m value of 1.3×10^4 M^{-1} s^{-1} at the pH optimum of 6.5. These kinetic parameters contrast with those for pig collagenase and gelatinase, which have K_m values of 7.1 and 2.6 μM and k_{cat}/K_m values of 1.5×10^3 and 1.2×10^5 M^{-1} s^{-1}, respectively, using the same substrate at pH 7.7 and 37 °C (Stack & Gray, 1989). The pH optimum for k_{cat}/K_m observed here is in general agreement with pH profiles reported for other matrix metalloproteinases, although much of the previous work has been performed on protein substrates and is, therefore, complicated by the effects of pH on the substrate itself. Free enzyme pK_a values for pig synovial collagenase and gelatinase of 7.0 and 9.5 and 5.9 and 10.0, respectively, have been obtained using the same substrate as that in the present work (Stack & Gray, 1990).

Matrilysin, together with other members of the matrix metalloproteinase family, shares some amino acid sequence homology with thermolysin around the thermolysin residue E^{143} (Whitham et al., 1986; Vallee & Auld, 1990). This residue is thought to be involved in catalysis by a general base mechanism, promoting the attack of water on the scissile bond (Hangauer et al., 1984). The first ionization constant of thermolysin at 4.6 is therefore assigned to residue E^{143}, while the second ionization constant is thought to be due either to the ionization of Zn-OH_2 or to the deprotonation of H^{231}, which may play a role in stabilizing the tetrahedral intermediate. For matrilysin, we speculate that the pK_a value of 4.60 reported here could be assigned to the ionization of residue E^{198} which, by homology, would correspond to E^{143} of thermolysin. An equivalent of the thermolysin residue H^{231} in matrilysin cannot be readily identified (Vallee & Auld, 1990) so the assignment of the second pK_a of 8.65 and confirmation of the catalytic mechanism must await further structural and kinetic studies.

Promatrilysin activation by APMA, trypsin, and heat occurred alongside the stepwise loss through proteolysis of an M_r 9000 propeptide from the N-terminus which ended with cleavage at E^{77}–Y^{78}. This final cleavage did not occur in the presence of a synthetic inhibitor of matrix metalloproteinase activity, suggesting that it was the result of autocatalysis. If the conditions or reagents which induced activation were removed prior to full activation, the residual promatrilysin proved resistant to autocatalysis. It is concluded, therefore, that possession of the M_r 9000 N-terminal propeptide in its native conformation not only prevents the expression of enzyme activity but also protects the E^{77}–Y^{78} site from cleavage.

It is likely that the incubation of promatrilysin at elevated temperatures or with APMA caused conformational perturbations sufficient to disrupt these propeptide functions. Other denaturing conditions have been found to activate the matrix metalloproteinases: M_r 92 000 progelatinase can be activated by incubation at low pH (Davis & Martin, 1990) and interstitial procollagenase by treatment with SDS (Birkedal-Hansen & Taylor, 1982). As the loss of the propeptide was caused by autocatalytic cleavages, it is probable that promatrilysin activation briefly preceded propeptide removal.

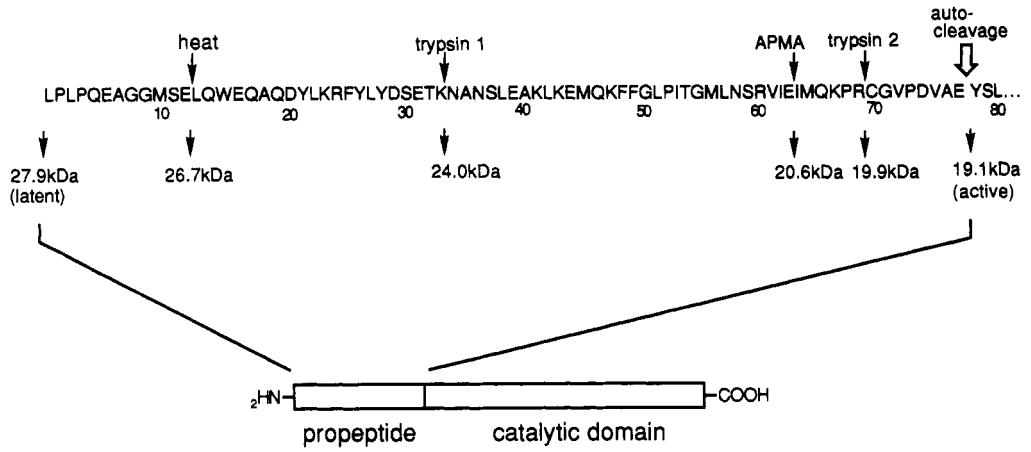

FIGURE 9: Sites of proteolytic cleavage in the promatrilysin propeptide which occur during activation. The N-terminal amino acid sequence of promatrilysin given above is that predicted by the cDNA sequence obtained by Muller et al. (1988). The points of cleavage were deduced from the N-terminal amino acid sequences of the various products of APMA, TPCK-treated trypsin, or heat activations. The molecular masses given above are as predicted from the cDNA sequence and should be compared with the estimates obtained by SDS–polyacrylamide gel electrophoresis.

Figure 8 shows that the removal of the denaturing conditions (incubation at 53 °C) resulted in the cessation of activation, suggesting that the conformational changes which generate active promatrilysin are reversible. It is the subsequent autocatalytic cleavage allowed by the concomitant exposure of the E^{77}–Y^{78} site which renders the activation process irreversible.

The relative stability of the M_r 27 000 heat-activation intermediate demonstrated that the loss of the first 12 amino acids did not affect the ability of the propeptide to protect the E^{77}–Y^{78} site from autocatalytic cleavage. At 37 °C, it was apparent that the slow disappearance of promatrilysin coincided with an increase in concentration of both the M_r 27 000 intermediate and the M_r 19 000 active matrilysin, suggesting that at elevated temperatures promatrilysin could be cleaved at either E^{12}–L^{13} or at E^{77}–Y^{78}. As the M_r 27 000 intermediate can still be cleaved at E^{77}–Y^{78}, the net result of heat activation is the production of M_r 19 000 active matrilysin.

The physiological activators of the matrix metalloproteinases are believed to be other endoproteinases. Although the pancreatic trypsin used in this study is unlikely to be the in vivo activator of matrilysin, its role might be taken by plasmin which shares the same cleavage specificity. Trypsin disrupts propeptide function by removing the first 33 amino acids by cleavage at K^{33}–N^{34}, generating an M_r 25 000 intermediate which, unlike promatrilysin, is unable to resist autocatalytic cleavage to the M_r 19 000 form. As the relatively stable heat activation intermediate had lost only the first 12 amino acids, it is concluded that the presence of at least some of the 21 amino acids, L^{13}–K^{33}, is critical in maintaining the propeptide in a conformation which prevents E^{77}–Y^{78} cleavage. The K^{33}–N^{34} site is in the so-called "bait-region" where cleavage occurs as the initial event in the endoproteinase activation of both human prostromelysin (Nagase et al., 1990) and human interstitial procollagenase (Suzuki et al., 1990), highlighting the similarity of promatrilysin activation with that of other matrix metalloproteinases. Incubation in the presence of the synthetic inhibitor demonstrated that subsequent trypsin cleavages, for example at R^{69}–C^{70}, can occur, though it is not known at which point an active matrilysin intermediate able to perform the autocatalytic cleavages is produced. Autocatalysis must occur during trypsin activation, to produce not only the final M_r 19 000 active form but also the M_r 22 000 intermediate, a point illustrated by the absence of these two forms when the matrix metalloproteinase inhibitor was included in the incubation.

The precise mechanism by which enzyme activity is precluded by the intact propeptide remains unknown. It may be that in its native conformation the propeptide blocks the access of the substrate to the active site. It has also been proposed that C^{70} in the conserved sequence PRCGV/NPD, found in the propeptide of all matrix metalloproteinases, coordinates with the active-site Zn^{2+}, thereby preventing activity (Springman et al., 1990; Van Wart & Birkedal-Hansen, 1990).

In conclusion, the relative structural simplicity and stability of matrilysin lead us to propose that this enzyme, acting as a representative member of the human matrix metalloproteinase family, is an ideal candidate for detailed crystallographic studies.

ACKNOWLEDGMENT

We are grateful to Mark Cockett for supplies of promatrilysin containing conditioned medium and to Chris Sutton for obtaining the N-terminal amino acid sequence for promatrilysin. We thank Julia Hubbard and Neil Ward at the University of Surrey for assistance with the zinc analysis.

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