Metal and pH Dependence of Heptapeptide Catalysis by Human Matrilysin[†]

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Received August 19, 1996; Revised Manuscript Received October 2, 1996[⊗]

ABSTRACT: Human matrilysin devoid of its propeptide is expressed in *Escherichia coli* and purified to homogeneity by heparin chromatography after refolding of the guanidine hydrochloride solubilized protein. Matrilysin autolytically removes its N-terminal tripeptide Met-Tyr-Ser during the refolding process. The enzyme contains 1.91 ± 0.08 zinc atoms/mol of protein and retains full activity when stored several months at 4 °C. It hydrolyzes the fluorescent substrate Dns-PLALWAR at the Ala−Leu bond with a k_{cat} of 3.1 s^{-1} and K_m of 1.8 × 10^{-5} M at pH 7.5, 37 °C, values closely similar to those for the matrilysin produced by activation of the Chinese hamster ovary and *E. coli*-expressed promatrilysin. The properties of this form of matrilysin demonstrate that the propeptide is not essential for proper folding or stability of the enzyme but likely determines the N-terminal amino acid of the mature enzyme. The pH dependence of k_{cat}/K_m for Dns-PLALWAR shows that matrilysin has a broad pH optimum (5.0−9.0) and the pK_a values obtained are 4.3 and 9.6 at 25 °C. The activity is inhibited by several metal binding agents including 1,10-phenanthroline, OP, but not by the nonchelating isomer, 1,7-phenanthroline. OP inhibits instantaneously by likely forming a transient ternary enzyme·metal·chelator complex. The zinc atom is then removed from the protein in a time-dependent manner. In agreement with the kinetic studies, dialysis in the presence of OP and $CaCl_2$ removes only the catalytic zinc atom. The monozinc enzyme can be reactivated to 90%, 56%, 27%, and 17% of the native activity by addition of zinc, manganese, nickel, and cobalt, respectively. Cadmium, on the other hand, forms an inactive Cd/Zn hybrid. The differences in the chelator accessibility properties of the two zinc sites can thus be exploited to yield metallohybrids of matrilysin.

Matrilysin is the smallest member of the matrix metalloproteinases, MMPs,[1] the class of zinc enzymes that remodel the extracellular matrix. Expression of these enzymes *in vivo* is important for a number of normal and pathological processes, including morphogenesis, differentiation, wound healing, bone and uterine resorption, tumor invasion and metastasis, and rheumatoid arthritis (Birkedal-Hansen et al., 1993; Stetler-Stevenson et al., 1993).

The alignment of cDNA-predicted amino acid sequences of the MMPs demonstrates a high degree of conservation between these enzymes. All members share two common domain structures: (1) a propeptide domain that includes a single Cys residue important for maintaining a latent form and (2) a catalytic domain containing the metal binding signature, HExxHxxGxxH, seen for a large group of zinc metalloproteases (Auld, 1992; Jiang & Bond, 1992; Bode et al., 1993; Stöcker et al., 1995). Recently, the structural analyses of the catalytic domain of fibroblast, MMP-1 (Borkakoti et al., 1994; Lovejoy et al., 1994; Spurlino et al., 1994), and neutrophil, MMP-8 (Grams et al., 1995; Bode et al., 1994; Stams et al., 1994) collagenases, stromelysin-1, MMP-3 (Becker et al., 1995; Van Doren et al., 1995; Gooley et al., 1993), and the native matrilysin, MMP-7 (Browner et al., 1995), bound with synthetic inhibitors have been reported. These structures identify the zinc and calcium binding sites and serve to predict which active-center amino acid side chains might interact with substrates and inhibitors.

Matrilysin is an important member of the MMPs. It displays limited proteolysis toward fibronectin (Quantin et al., 1989), transferrin, elastin (Abramson et al., 1995), type IV collagen (Murphy et al., 1991), prourokinase (Marcotte et al., 1992), aggrecan (Fosang et al., 1992), and entactin (Sires et al., 1993). Its substrate specificity toward small peptide substrates is also distinct from the specificities of other MMPs (Netzel-Arnett et al., 1993).

Promatrilysin has been expressed in mammalian cells (Barnett et al., 1994; Imai et al., 1995) and as GST- and ubiquitin fusion proteins in *Escherichia coli* (Soler et al., 1995; Welch et al., 1995). However, since the MMPs participate in their own activation, mutations that affect activity will slow or prevent formation of the mature enzyme. Direct production of an active matrilysin will thus facilitate the study of the catalytic mechanism of matrilysin using mutagenesis techniques. We have succeeded in producing an active form of matrilysin from *E. coli* and have performed kinetic studies on the effect of pH and metal substitution on its catalysis of a fluorescent heptapeptide substrate.

[†] This research was supported by NIH Grant GM-53265.

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[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1996.

[1] Abbreviations: CHO, Chinese hamster ovary; MMP, matrix metalloproteinase; MMP-7, matrilysin; APMA, 4-aminophenylmercuric acetate; Dns, 5-(dimethylamino)naphthalene-1-sulfonyl; OP, *o*- (1,10-) phenanthroline; MP, *m*- (1,7-) phenanthroline; HQSA, 8-hydroxyquinoline-5-sulfonic acid; DPA, dipicolinic acid or pyridine-2,6-dicarboxylic acid; Dnp, dinitrophenyl; BP, 2,2′-bipyridyl; PMSF, phenylmethanesulfonyl fluoride; Ampso, 3-[dimethyl(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TFA, trifluoroacetic acid; GuHCl, guanidine hydrochloride; GST, glutathione transferase.

S0006-2960(96)02085-5 CCC: $12.00

MATERIALS AND METHODS

Materials. A plasmid containing the entire matrilysin cDNA, pUN121, was provided by Dr. R. Breathnach (Strasbourgh, France). *E. coli* BL21(DE3) cells and the pET-11a vector were obtained from Novagen; oligonucleotide primers for amplification were from Amitof Biotech Inc; restriction and ligation enzymes were from New England Biolabs; heparin−agarose was from Sigma; Chelex-100 resin was from Bio-Rad; 2,2′-bipyridyl (BP), 1,10-phenanthroline (OP), and 8-hydroxyquinoline-5-sulfonic acid (HQSA) were from Aldrich; 1,7-phenanthroline (MP) was from G. F. Smith Chemical Co.; and pyridine-2,6-dicarboxylic acid (DPA) was from Eastern Organic Chemicals.

Construction of the pET-MMP-7 Expression Vector. Two oligomers were synthesized in order to express the portion of matrilysin that has homology to the human stromelysin-1 catalytic domain (SCD) (Ye et al., 1992). One oligomer 5′-GGAATTC*CATATG*TACTCACTATTTCCA-3′, places a *Nde*I site immediately prior to the 78th amino acid. The *Nde*I generates an additional methionine for the initiation codon. The other oligomer, 5′-CGC*GGATCC*TATTT-CTTTCTTGA-3′, is complementary to the termination codon of matrilysin. In addition a *Bam*HI site was added 3′ to the termination codon. These oligomers were used to amplify the desired region of the matrilysin cDNA using PCR under standard conditions: denaturation at 92 °C for 3 min, annealing at 55 °C for 2 min, synthesis at 72 °C for 3 min, denaturation at 92 °C for 1 min 15 s, annealing at 55 °C for 2 min, synthesis at 72 °C for 3 min; 30 cycles and extra synthesis at 72 °C for 7 min.) The PCR-amplified DNA was electophoresed on a 1% NuSieve GTG agarose gel (FMC Corp.), and the band was cut out, purified using Wizard Prep (Promega), and digested with *Nde*I and *Bam*HI. The DNA was ligated into *Nde*I/*Bam*HI-digested pET-11a (Novagen) and transformed to competent NovaBLUE *E. coli* cells. Plasmids containing the appropriate size insert (pET-MMP-7) were sequenced using the dideoxy protocol for the Sequenase system (Biochemical Corp.) for sequencing of double-stranded DNA. Clones containing approximately 500 bp of the matrilysin gene were transformed into the expression host, BL21(DE3) cells.

Expression and Purification of Matrilysin. E. coli BL21-(DE3) cells containing the pET-MMP-7 plasmid were grown overnight in 100 mL of LB medium containing 50 μg/mL ampicillin. The culture was diluted 1:100 in the same medium and cells were grown at 37 °C to an OD_{600} of 0.8−1.0 before induction by adding IPTG (0.1−0.2 mM final concentration). In order to maximize the production of matrilysin, the incubation was continued for 3−4 h after induction. The cells grown at 37 °C were resuspended in 20 mM Tris, pH 7.6, 200 mM NaCl, 10% sucrose, and 1 mM EDTA (buffer A), with 0.5 mM PMSF and lysozyme added to 0.1 mg/mL. The cells were incubated for 45 min on ice and sonicated four times (15 pulses each). The pellet obtained after centrifugation (12 000 rpm, 10 min) was washed and treated with buffer A containing 0.5% Triton X-100. The pellet was solubilized with 6 M GuHCl and 20 mM Tris-HCl, pH 7.6, and centrifuged (12 000 rpm, 20 min). The supernatant was adjusted to a protein concentration of 0.3 mg/mL using 4 M GuHCl and 20 mM Tris-HCl, pH 7.6. It was dialyzed overnight against 50 mM Tris-HCl, 10 mM $CaCl_2$, 20 μM $ZnCl_2$, pH 7.5, and 0.05% Brij 35 at 4 °C. The refolding mixture was centrifuged at 12 000 rpm for 10 min and the supernatant was applied to a heparin−agarose column previously equilibrated with 50 mM Tris-HCl and 10 mM $CaCl_2$, pH 7.5. The column was washed with the same buffer and the protein was eluted using a 1 M NaCl linear gradient. The fractions showing high activity were collected and pooled. The purity of refolded active matrilysin was examined by SDS−PAGE. The molecular weight was estimated using the molecular weight markers phosphorylase *b* (97 400), serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), trypsin inhibitor (21 500), and lysozyme (14 400).

Protein concentration during the purification was measured using Bio-Rad assays and bovine serum albumin as the protein standard. Purified enzyme concentration was determined by amino acid analysis. An ϵ_{280} of 33 000 M^{-1} cm^{-1} was determined for the purified enzyme. Subsequent protein concentrations were based on the absorbance at 280 nm.

Enzyme Assays and Determination of Kinetic Parameters. Enzyme activity was measured with the synthetic fluorescent peptide Dns-PLALWAR as a substrate. The peptide substrate was synthesized, purified, and characterized by methods previously described (Ng & Auld, 1989). Substrate concentrations were determined spectrophotometrically by the absorbance of the dansyl group at 340 nm (ϵ_{340} = 4300 M^{-1} cm^{-1}).

Enzyme purification was followed by a thin-layer chromatography microassay. A total of a 10−50-μL reaction volume was used to measure the enzyme activity with 0.2 mg/mL substrate in the presence of 10 mM $CaCl_2$, 0.15 M NaCl, 10% acetonitrile, and 40 mM Hepes, pH 7.8. Aliquots, 0.5 μL, of the reaction mixture were spotted on a micropolyamide sheet (3 cm × 4 cm) (Schleicher & Schuell) and 0.3 N HCl was used for the separation of the reaction products. The dansylated substrates and products were visualized by ultraviolet light.

Assays for obtaining kinetic parameters were performed in 10 mM $CaCl_2$ and 0.15 or 0.5 M NaCl in 20 mM Hepes, pH 7.5, over the substrate concentration range 1.5×10^{-6} to 5×10^{-5} M. Initial rates corresponding to less than 10% of the reaction were obtained under steady-state conditions using a Perkin-Elmer MPF-3 spectrofluorometer equipped with a constant-temperature water bath. The product formation was monitored from the marked increase in tryptophan fluorescence at 340 nm upon bond cleavage. The relationship between fluorescence units and nanomoles of product produced was determined from the fluorescence value obtained when all the substrate was hydrolyzed. The k_{cat} and K_m values were determined from the results at 5−8 substrate concentrations and nonlinear regression analysis using the ENZFITTER program.

Stopped-flow experiments were performed on a Durrum D110 instrument interfaced to a PDP 11/34 DEC computer for studies of the pH dependence of matrilysin catalysis. The computer was programmed to analyze initial rates and progress curves in order to calculate kinetic parameters. The buffers for the pH profile were checked for inhibitory or activating effects on the reaction at 5, 20, and 50 mM concentrations, respectively, with 10 mM $CaCl_2$, 0.5 M NaCl, and 2.5% (v/v) acetonitrile at the pK_a value of the buffer at 25 °C. Acetate, Mes, Hepes, and Ampso were chosen for the pH regions 4.25−5, 5.5−6.5, 7−8.5, and 8.5−10, respectively, since they did not inhibit the reaction. The

NaCl concentration of 0.5 M ensured essentially constant ionic strength at all pH values. The reaction was carried out by a 1:1 mixing of the enzyme and substrate solutions in 10 mM $CaCl_2$, 0.5 M NaCl and 50 mM Hepes, pH 7.0. Acetonitrile [final concentration of 2.5% (v/v)] was added to the substrate to ensure solubility. The pH of the mixed solution minus enzyme was determined prior to the stopped-flow assays. The temperature was 25 ± 0.2 °C. Substrate concentrations, 1.5×10^{-6} to 3×10^{-6} M, and enzyme concentrations, 1.0×10^{-7} to 4.0×10^{-7} M, were used for obtaining k_{cat}/K_m values. Initial rates were measured in less than 20 s and k_{cat}/K_m values were calculated directly according to the Michaelis−Menten equation: $v_{init} = k_{cat}[E][S]/([S] + K_m)$, which is equal to $(k_{cat}/K_m)[E][S]$ when $[S] \ll K_m$. The stopped-flow method ensured that the enzyme was present at extreme pH conditions for only a few seconds. Each assay was performed at least 4−5 times.

Inhibition Studies. Enzyme, 7×10^{-7} M, was preincubated with metal chelators for 30 min prior to assaying in 10 mM $CaCl_2$, 0.15 M NaCl and 20 mM Hepes, pH 7.5, at room temperature. The assays were initiated by addition of substrate to give a final concentration of 1.0×10^{-4} M Dns-PLALWAR. Initial rates of hydrolysis were measured by HPLC (Waters, Nova Pak C_{18} 6 × 100 mm). Samples (50 μL) were eluted with a linear acetonitrile gradient (40−55%) in 0.1% TFA at a flow rate of 1 mL/min. Peak areas were determined at 214 nm with a Hewlett-Packard 3380 A integrator. The detailed inhibition study by OP was performed at 0.001−5 mM OP. Enzyme concentrations of 2.4×10^{-7} to 9×10^{-7} M were preincubated with OP for 1 h prior to assay in 1 mM $CaCl_2$, 0.15 M NaCl and 20 mM Hepes, pH 7.5, at 25 °C. Instantaneous inhibition was performed without preincubation with enzyme and OP. Six samples were prepared for each inhibitor concentration, and the reaction was stopped after 15, 30, 45, and 60 s, respectively, by addition of 10% glacial acetic acid. Initial rates of the reaction were determined by HPLC.

Determination of Metal Content. All the buffers and dialysis tubing, plastic, and glassware were pretreated as described previously for rendering metal-free conditions (Auld, 1988a; Riordan & Vallee, 1988). A solution of the metalloenzyme was dialyzed extensively against 5 mM $CaCl_2$ and 5 mM Hepes, pH 7.5, at 4 °C for at least 3 changes of dialysate at a volume ratio of dialysate/sample of 1000/1. After dialysis, dilutions were made in 0.2% HNO_3 and the metal concentration was determined in duplicate using a Model 4100ZL Perkin-Elmer graphite furnace Zeeman atomic absorption spectrophotometer with the 4100 application program using GEM Desktop and Windows. Metal concentrations were determined from a curve obtained with standard 2.5−20 μg/L metal samples. The metal content of the buffers was determined before and after dialysis.

Zinc Replacement Studies. Assays were designed to determine the capacity of other transition metal ions to restore the activity of the apoenzyme. Apoenzyme was prepared by dialysis of matrilysin at a concentration of approximately 10 μM against three changes of 5 mM $CaCl_2$ and 20 mM Hepes, pH 7.5, in the presence of 2 mM OP at 4 °C for 3−4 h. This was followed by dialysis against two changes of 5 mM $CaCl_2$ and 20 mM Hepes, pH 7.5, without OP for an additional 3−4 h. Apoenzyme, 3.6×10^{-6} M, was preincubated with 1×10^{-3} M transition metals for 1 h for preparation of the metal-substituted matrilysin. Initial rates were measured within 5 min in 5 mM $CaCl_2$ and 20 mM Hepes, pH 7.5 containing 2.5×10^{-4} M substrate at 25 °C. HPLC was employed to determine the initial rate of each metal-reconstituted enzyme as described previously. Adventitious metals were removed from the Hepes buffer by Chelex-100.

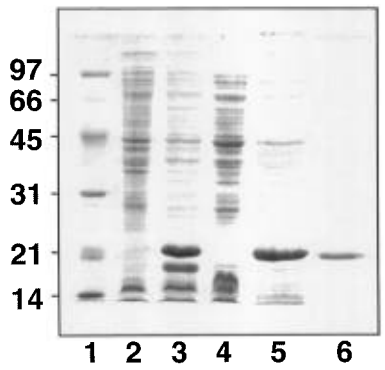

FIGURE 1: Purification steps of matrilysin. The protein samples are analyzed by SDS−PAGE on a 12% polyacrylamide Tris−glycine gel. Lane 1, low molecular weight standards; lane 2, total cell extract of uninduced BL21 (DE3) cells containing matrilysin; lane 3, total cell extract of induced BL21 (DE3) cells containing matrilysin; lane 4, soluble fraction from induced matrilysin; lane 5, insoluble fraction from induced matrilysin; lane 6, matrilysin solution after heparin column.

Amino Acid Analysis and N-Terminal Sequencing. Samples for amino acid analysis were hydrolyzed with 6 N HCl (gas phase) at 110 °C under vacuum overnight. The hydrolysate was derivatized using 6-aminoquinolyl *N*-hydroxysuccinimidyl carbamate (AccQTaq) methodology (Cohen & Michaud, 1993). N-Terminal sequences were determined using a Millgen/Biosearch ProSequencer according to the manufacturer's recommended procedures.

RESULTS

Expression of Matrilysin. An expression vector that can produce the active form of matrilysin directly was constructed using the pET-11a vector with the T7 RNA polymerase gene (Studier et al., 1990). After the induction by IPTG, the expression of the enzyme reaches its maximum within 3 h. Without IPTG addition, matrilysin is not produced (Figure 1, lane 2). The expression level of this enzyme is approximately 10% of the total cellular protein based on the purity of the SDS−PAGE analysis (Figure 1, lane 3). The variation of the concentration of IPTG does not affect the expression level.

Refolding and Purification. Most of the *E. coli* proteins remain in the soluble fraction after cell lysis (Figure 1, lane 4), while matrilysin is the predominant component in the insoluble pellet (Figure 1, lane 5) when expression is performed at 37 °C. Lowering the temperature to 30 or 25 °C fails to make the enzyme soluble. Matrilysin is solubilized using 6 M GuHCl with gentle stirring overnight. A band of 19 kDa in the SDS−polyacrylamide gel is consistent with the expected molecular weight of denatured matrilysin. The N-terminal sequence of the denatured enzyme is MYSLFPNSPKWT, in agreement with the predicted sequence plus the initiating Met (Muller et al., 1988).

Refolding is performed by dilution of the protein solution followed by dialysis against GuHCl-free buffer overnight. Keeping the protein concentration in the range 0.1−0.3 mg/

Table 1: The Amino Acid Analysis and N-Terminal Sequence of Matrilysin

amino acid	Amino Acid Analysis experimental[a]	theory[b]
Met	4.21 ± 0.76	5
Tyr	6.87 ± 0.16	8
Ser	12.88 ± 0.14	14
Leu	13.23 ± 0.03	13
Phe	7.93 ± 0.13	8
	N-Terminal Sequence	
denatured form	MYSLFPNSPKWTS	
refolded form	LFPNSPKWTS	

[a] Average values of the measurements for six different concentrations of refolded matrilysin. [b] This number is deduced from the expected sequence of matrilysin based on alignments with other MMPs.

Table 2: Kinetic Parameters for the Peptide Substrate Dns-PLALWAR

expressed form	source	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (s^{-1} M^{-1})
active form	*E. coli*[a]	2.7	2.4×10^{-5}	1.1×10^5
active form	*E. coli*[b]	0.9	1.1×10^{-5}	8.2×10^4
active form	*E. coli*[c]	3.1	1.8×10^{-5}	1.7×10^5
activated zymogen	*E. coli*[c,d]	3.3	1.3×10^{-5}	2.5×10^5
activated zymogen	CHO cells[c,d]	3.1	1.4×10^{-5}	2.2×10^5

[a] Assay conditions are 20 mM HEPES, pH 7.5, 10 mM $CaCl_2$, and 0.5 M NaCl at 25°C. [b] Assay conditions are as in *a* except 0.15 M NaCl. [c] Assay conditions are as in *a* except 0.15 M NaCl and 37 °C. [d] Soler et al. (1995).

mL minimizes the amount of protein precipitation during this step. Minor contaminants of other *E. coli* proteins are removed by heparin−agarose chromatography. Only matrilysin is bound tightly to the column. The eluted fractions containing active matrilysin are collected and pooled. The amount of enzyme produced per liter of preparation is 3−5 mg. The activity is assayed using Dns-PLALWAR as the substrate. SDS−PAGE and amino acid analysis of purified matrilysin show that the protein is homogeneous (Figure 1, lane 6). Amino acid analysis and N-terminal sequence results indicate that the active matrilysin lacks three N-terminal amino acids, Met-Tyr-Ser, that are found in its denatured form (Table 1). The refolded enzyme is stable at 4 °C for several months and possesses essentially the same kinetic properties and zinc content as the enzyme obtained from promatrilysin expressed in CHO and *E. coli* cells (Soler et al., 1995) (see below).

Kinetic Studies. Matrilysin has a k_{cat} of 3.1 s^{-1} and a K_m of 18 μM for catalysis of Dns-PLALWAR at pH 7.5 and 37 °C. These kinetic parameters are very close to those obtained for CHO- and *E. coli*-expressed matrilysin (Table 2). Identification of reaction products by HPLC and amino acid analysis shows that the enzyme cleaves exclusively at the A−L bond.

The pH dependence of matrilysin catalysis of Dns-PLALWAR hydrolysis was examined at a substrate concentration well below K_m where the velocity of the reaction is proportional to k_{cat}/K_m. The high pK_a buffers, Gly and Ches, are not used because they inhibit the reaction at pH 9 when the concentration of buffer is increased from 5 to 50 mM (data not shown). Concentrations > 10^{-3} M of $ZnCl_2$ inhibit the activity at pH 7, while 5−100 mM $CaCl_2$ does not strongly affect the reaction rate on a short time basis (< 3 min) at low and high pHs (data not shown). $ZnCl_2$ is added

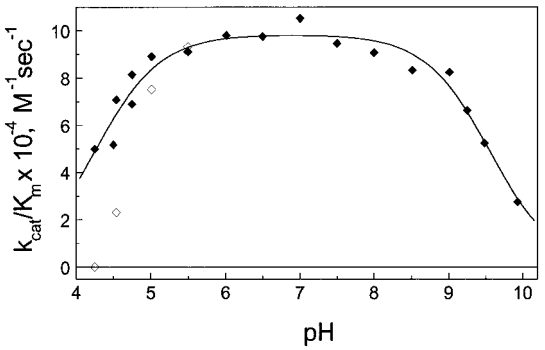

FIGURE 2: pH effect on matrilysin activity. The assays below pH 5.5 are performed in the presence of zinc (◆) and in the absence of zinc (◇). The line is theoretical, calculated using eq 1 and pK_{a1} = 4.26, pK_{a2} = 9.55, and a pH-independent k_{cat}/K_m value of 9.8 × 10^4 M^{-1} s^{-1}.

Table 3: Inhibition of Enzymatic Activity by Metal Binding Agents[a]

inhibitors	concentration (mM)	residual activity (%)
no inhibitor		100
OP	0.1	46
OP	1.0	7
BP	2.0	25
MP	0.1	100
MP	1.0	95
HQSA	1.0	40
EDTA	1.0	88
DPA	2.0	91
PMSF	1	100

[a] See Materials and Methods for conditions of assay.

to concentrations of 1×10^{-3} M and 5×10^{-5} M at pH 4.25 and 4.5−5, respectively in order to ensure maximal activity. The k_{cat}/K_m value of matrilysin is increased about 35% by increasing the concentration of NaCl or Na_2SO_4 from 0.15 to 0.5 M (Table 2).

The pH−k_{cat}/K_m profile from pH 4 to 10 is a broad bell-shaped curve (Figure 2). The two pK_a values obtained by fitting the data to eq 1 suggest a single pathway to product formation. The pK_a values are 4.3 and 9.6 and the pH-independent k_{cat}/K_m value is 9.8×10^4 M^{-1} s^{-1}.

$$k_{cat}/K_{m(observed)} = k_{cat}/K_{m(pH-independent)}/(1 + [H^+]/K_{a1} + K_{a2}/[H^+]) \quad (1)$$

where pK_{a1} and pK_{a2} control activity at low and high pH values, respectively.

Inhibition Studies. A number of structurally different metal binding agents inhibit matrilysin's relative, astacin, in a concentration- and time-dependent manner (Stöcker et al., 1988). Several of them were tested for their effect on matrilysin catalysis (Table 3). The enzyme is preincubated with all the chelators for 30 min. OP strongly inhibits enzymatic activity, while MP, a nonchelating isomer of OP, is not an inhibitor. The inhibition by OP is fully reversible upon addition of zinc (data not shown). HQSA and BP also inhibit the enzyme at millimolar concentrations. In marked contrast EDTA (1 mM) and DPA (2 mM) exhibit only 12% and 9% inhibition. The weak inhibition by EDTA and DPA is likely due to the presence of $CaCl_2$ in the assay coupled with the strong Ca-binding properties of these chelators (Auld, 1995). PMSF, 1 mM, an inhibitor of serine proteases, has no effect on enzymatic activity.

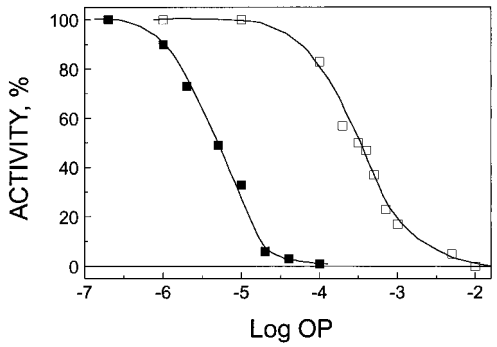

FIGURE 3: Inhibition of matrilysin as a function of the OP concentration. Assays in the presence of OP are performed after 1 h preincubation of enzyme with inhibitor (■) or without preincubation (□).

Table 4: Time Dependence of pI_{50} and n for the Inhibition of Matrilysin by 1,10-Phenanthroline[a]

	no preincubation		1 h preincubation	
inhibitor	pI_{50}	n	pI_{50}	n
1,10-phenanthroline	3.5	1.1	5.3	1.7

[a] See Materials and Methods for conditions of assay.

Table 5: Metal Content of Matrilysin

	metal content[a] (mol/mol of protein)		
proteins	zinc	cadmium	n
matrilysin[b]	2.22 ± 0.11		3
matrilysin	1.91 ± 0.08		4
OP-treated matrilysin	1.16 ± 0.02		2
Cd-substituted matrilysin	1.18 ± 0.03	1.16 ± 0.07	3

[a] Values are expressed as the average ± standard deviation for n determinations. [b] From CHO cells; Soler et al. (1994).

The most potent inhibitor of matrilysin catalysis, OP, is used to further examine the mode of chelator inhibition. The dependence of activity on the OP concentration is measured without and with a 1 h preincubation of enzyme and OP (Figure 3). Analysis of the data is accomplished by using

$$\log (V_c/V_i - 1) = -\log K_i + n \log [I] \qquad (2)$$

where V_c and V_i are the velocities in the absence and presence of inhibitor (I) (Auld, 1988b). The resulting intercept with the log [I] axis yields the inhibitor concentration required for 50% inactivation, and the value of the slope gives n, the order of the inhibitor in the reaction. The value of n was 1.1 with no preincubation of enzyme and inhibitor, indicating inhibition by a single inhibitor molecule (Table 4). After 1 h of preincubation with OP, this value increases to 1.7, indicating that the mode of inhibition is now likely removal of the catalytic zinc (Auld, 1995). Correspondingly, the value of pI_{50} increases from 3.5 to 5.3.

Metal Replacement Studies. The zinc content of purified enzyme is 1.91 ± 0.08 (Table 5). The inhibition study by OP in the presence of $CaCl_2$ implies that the catalytic zinc atom is removed by OP. Consistent with the results of OP inhibition, dialysis of matrilysin against OP in the presence of 5 mM $CaCl_2$ yields an enzyme with 1.16 ± 0.02 zinc/mol of protein. The addition of cadmium ions to a solution of the apoenzyme yields an enzyme that contains 1 zinc and 1 cadmium/mol of protein (Table 5).

The activity of the apoenzyme toward the Dns-PLALWAR substrate is reduced to <10% but is restored to ~90% of the original level by addition of Zn^{2+}. The residual activity of the apoenzyme is likely accounted for by the trace amount of zinc in the assay since the enzyme concentration is 2.4×10^{-7} M. Mn^{2+}, Ni^{2+}, and Co^{2+} at 0.07 mM in assay restores partial activity, while Cd^{2+} and Cu^{2+} are inactive (Table 6).

Table 6: Effect of Divalent Transition Metals on the Activity of Matrilysin[a]

metal	relative activity
Zn^{2+} (native)	100
Zn^{2+} (reconstituted)	90
Mn^{2+}	56
Ni^2	27
Co^{2+}	17
Cd^{2+}	ND[b]
Cu^{2+}	ND

[a] Apoenzyme (3.6 μM) is preincubated with 1 mM metals for 1 h. Activity is determined as described under Materials and Methods. [b] ND, not detectable.

DISCUSSION

We have constructed a vector that can produce matrilysin in *E. coli* without its N-terminal propeptide, thereby excluding the need for *in vitro* activation. The deletion of the propeptide generates the active matrilysin of M_r 19 000 that is equal to the size of matrilysin activated by APMA, stromelysin-1, or autodigestion (Crabbe et al., 1992; Imai et al., 1995; Quantin et al., 1989). The enzyme is stable and contains the same zinc content as the enzymes prepared from their zymogen precursors (Table 5; Soler et al., 1994; 1995). However, the N-terminal residue of active matrilysin produced in this study has a leucine (80th position of proMMP-7) as the NH_2-terminal residue (Table 1). This observation differs from that reported for the *in vitro* activation of promatrilysin, which leads to a N-terminal tyrosine (Crabbe et al., 1992, 78th position of proMMP-7). The properties of this form of matrilysin demonstrate that the propeptide is not essential for proper folding or stability of the enzyme but likely determines the N-terminal amino acid of the mature enzyme.

The physiological mechanism of MMP activation is unknown. ProMMPs are activated *in vitro* by several different classes of reagents: mercurial compounds, chaotropic agents, oxidants, disulfide compounds, alkylating agents, and various proteinases such as trypsin and plasmin (Springman et al., 1990; Van Wart & Birkedal-Hansen, 1990). In all of these enzymes there is a highly conserved Cys residue in the propeptide domain of each enzyme (Table 7). The *cysteine switch* or *velcro* hypothesis has been advanced to explain the latency and activation of the proMMPs (Springman et al., 1990; Van Wart & Birkedal-Hansen, 1990; Vallee & Auld, 1990). The latency of the proMMPs is thus due to the displacement of the activated water bound to the catalytic zinc by the cysteine in the conserved propeptide PR*C*GxPD. All means of activation share the property that the activators cause a conformational change in the molecule that dissociates the Cys residue from the zinc atom to generate a functional active site. The activated MMPs are then capable of autoproteolysis, removing the amino-terminal prodomain, including the PR*C*GxPD sequence, and thus are converted to an active form. The importance of the conserved sequence in maintaining the latency is supported by site-directed mutagenesis and struc-

Table 7: Comparison of Sequences Surrounding the Propeptide Cysteine of MMPs[a]

enzyme	sequence
human fibroblast collagenase (MMP-1)	PRCGVPDVAQ↓F↓V↓LTE
rabbit fibroblast collagenase (MMP-1)	PRCGVPDVAQ FVLTP
human neutrophil collagenase (MMP-8)	PRCGVPDSGG↓F↓M↓LTP
human fibroblast stromelysin (MMP-3)	PRCGVPDVGH↓FRTFP
human stromelysin 2 (MMP-10)	PRCGVPDVGH FSSFP
rabbit stromelysin (MMP-3)	PRCGVPDVGH FSTFP
human matrilysin (MMP-7)	PRCGVPDVAE↓YS⇓LFP
rat matrilysin (MMP-7)	PRCGVPDVAE↓FSLMP
human 72-kDa gelatinase (MMP-2)	PRCGNPDVAN↓YNFFP
human 92-kDa gelatinase (MMP-9)	PRCGVPDLGR↓FQTFE

[a] Amino acid sequences were acquired from the Brookhaven Protein Data Bank. [b] ↓, *in vitro* cleavage site; ⇓, cleavage site in our study.

tural studies (Sanchez-Lopez et al., 1988; Park et al., 1991; Freimark et al., 1994; Becker et al., 1995).

The product of activation depends on the means of activation used. Activation of human fibroblast collagenase (MMP-1) by proteinases, mercurials, or oxidants produces a variety of enzyme having either Leu-65, Met-72, Phe-81, Val-82, or Leu-83 as the NH_2-terminal residue (Suzuki et al., 1990). The Leu-65 form is produced by plasmin or plasma kallikrein treatment and it is easily converted to other forms which have high collagenolytic activity. The Phe-81 form, only produced by stromelysin (MMP-3), shows maximum collagenolytic activity. The Val-82 and Leu-83 forms are autolytically produced after incubation with APMA or trypsin.

The results of a recent study of the expression of the human stromelysin catalytic domain (SCD) in *E. coli* (Ye et al., 1992) may give insight into the present results for matrilysin. They constructed the SCD gene with three extra amino acids (Met-Ala-Ser) at the N-terminus in order to gain a restriction site, but these residues are removed during the purification of the refolded SCD. Our construct, which is made by sequence homology to SCD, has the expected N-terminal sequence (MYSLFPNS) in the GuHCl-denatured enzyme. However, the refolded enzyme has lost the Met-Tyr-Ser sequence. In both cases the fourth residue in the sequence of the expressed protein (Phe for stromelysin and Leu for matrilysin) is a good candidate for the P_1' site of a substrate for the respective enzyme (Netzel-Arnett et al., 1993; Sang et al., 1995). The cleavage specificity studies against protein and peptide substrates have also revealed that the best residue in the P_1' subsite is a Leu for matrilysin (Sires et al., 1993; Netzel-Arnett et al., 1993; Smith et al., 1995). The N-termini of high- and low-activity matrilysin mutants are Leu and Met, respectively, which is also consistent with autolytic cleavage (Cha & Auld, unpublished).

The k_{cat}/K_m value of 1.7×10^5 M^{-1} s^{-1} at pH 7.5, 37 °C, obtained for the matrilysin-catalyzed hydrolysis of the peptide substrate Dns-PLALWAR is essentially the same as that of the enzyme produced by the activation of promatrilysin expressed in CHO cells and *E. coli* (Table 2). The peptide substrate employed herein has a k_{cat}/K_m value as good or better than those of previously reported peptide substrates. This kinetic value is approximately 10 times higher than the value observed at pH 6.5, 37 °C, using the substrate Dnp-PLGLWAR (Crabbe et al., 1992) and closely similar to that found for Dnp-RPLALWRS, 1.9×10^5 M^{-1} s^{-1} (Welch et al., 1995).

o-Phenanthroline, OP, was chosen to study the mechanism of chelator inhibition of matrilysin because it is known to have a high binding constant for zinc and a weak one for calcium (Auld, 1995). The data presented here give some insight into the mechanism of metal removal. Chelator inhibition of a metalloenzyme can arise from formation of a transient ternary chelator·metal·enzyme complex or from metal removal by an S_N1 type of mechanism (Auld, 1988b). One criterion that distinguishes between them is the stoichiometry of the reaction as given by *n*. When *n* is near 1, it is usually due to a reversible and instantaneous ternary chelator·metal·enzyme complex formation, whereas a value which is greater than 1 implies that the chelator is removing the metal from the protein or that there is more than one chelator binding site on the protein (Auld, 1988b). In the case of matrilysin the value of *n* is 1.1 when there is no preincubation of enzyme and OP, suggesting the cause of the inhibition is due to formation of a ternary complex. After 1 h of preincubation with OP, the value increases to 1.7. The values of pI_{50} and *n* found here for the inhibition of matrilysin by OP are very close to that found for OP inhibition of MMP-1 catalysis (Springman et al., 1995). Thus, inhibition is likely a two-step process with the first step characterized by formation of an enzyme·inhibitor complex, followed by release of Zn(OP) and formation of $Zn(OP)_2$ and $Zn(OP)_3$ complexes.

The results of the OP inhibition study suggest the way to exchange metals at the catalytic zinc site is to first dialyze the enzyme in the presence of OP and Ca^{2+} to remove the catalytic zinc. Under these conditions the second Zn binding site is not replaced (Tables 5 and 6). This is reasonable since this zinc site has the properties of a structural zinc site (Vallee & Auld, 1990) in that it is bound to four protein ligands (Becker et al., 1995; Lovejoy et al., 1994; Browner et al., 1995). The monozinc enzyme can be reactivated to 90%, 56%, 27%, and 17% of the native activity by addition of zinc, manganese, nickel, and cobalt, respectively. Cadmium, on the other hand, forms an inactive Cd/Zn hybrid. These results are in marked contrast to those obtained for the catalytic domain of stromelysin, where manganese and nickel are incapable of restoring activity (Salowe et al., 1992). The differences observed for these two enzymes may be intrinsic to them or related to the different oligopeptides used for measuring activity.

The pH dependence of k_{cat}/K_m for matrilysin observed herein is similar to that observed for the mouse myeloma enzyme (Crabbe et al., 1992) in terms of the activity showing a wide bell-shaped neutral pH optimum, with pK_a values in the present (and former) study of 4.3 (4.6) and 9.6 (8.7). The enzyme activity is greatly decreased in the absence of added zinc below pH 5.5 (Figure 2). This is likely due to competition between hydrogen ions and the zinc for the protein metal binding ligands, as has been observed for carboxypeptidase A (Auld & Vallee, 1970). The incubation of the enzyme at such a low pH also results in progressive loss in activity. The metal stoichiometry of matrilysin at various pHs using the electrospray ionization mass spectrometry technique also demonstrates that the free enzyme is highly sensitive to pH changes on the acid side of neutrality (Feng et al., 1995). These results indicate that the acidic pK_a value of enzyme will be significantly affected in the absence of excess zinc (Figure 2). The difference in the alkaline pK_a values might be due to the differences of the

buffers used. Glycine, used in the mouse myeloma study, inhibits activity in the alkaline pH region. The present buffers are also made metal-free. Zinc or other metals often inhibit zinc proteases in the alkaline pH region (Auld, 1995). Thus both the presence of glycine or adventitious metal ions may have decreased enzymatic activity below pH 9 in the previous study.

A specific state of ionization of at least two acid−base groups is essential for catalysis in matrilysin. MMPs are postulated to follow a similar reaction mechanism as thermolysin based on the overall structural similarity (Bode et al., 1993; Hodgkin et al., 1994; Becker et al., 1995; Browner et al., 1995). Superimposition of the active-site residues of MMPs and thermolysin reveals both their similarities and differences. The zinc ion and a Glu-198 are located at the same position as zinc and Glu-143 in thermolysin, while two residues (Tyr-157 and His-231) that are proposed to play a stabilizing role in the transition state of the tetrahedral intermediate in the thermolysin mechanism are not found. The pH−activity studies of thermolysin indicate catalysis involves two ionic groups with pK_a values of 5.0−5.6 and 7.5−8.25 (Kunugi et al., 1982; Riechmann & Kasche, 1986; Holmquist & Vallee, 1976). The lower pK_a is ascribed to the Glu-143 interacting with zinc-bound water, that is suggested to act as a proton-accepting group for one proton from the zinc-coordinated water molecule in hydrolysis (Matthews, 1988). Therefore, the acidic pK_a of 4.3 in matrilysin may reflect the ionization of the conserved Glu-198 that corresponds to Glu-143 in thermolysin. Substitution of the analogous Glu in fibroblast collagenase and gelatinase A with an Asp causes a 3−10-fold decrease in catalysis of peptide substrates (Crabbe et al., 1994; Windsor et al., 1994) and the Ala and Gln mutants reduced activity by about 10^4 in a zymogram gelatin assay (Crabbe et al., 1994). These results, while consistent with Glu-198 having a role in catalysis, are orders of magnitude less than what is observed for the mutations of the catalytic Ser and His residues in serine proteases (Carter & Wells, 1988).

The search for the corresponding group for the second pK_a value is more intriguing. A chemical modification study on His residues in thermolysin indicates the pK_a inducing the modification process is near 8 (Burstein et al., 1974), close to the kinetic alkaline pK_a value. Thus His-231 was ascribed as a proton donor in catalysis (Matthews, 1988). There is no corresponding residue to His-231 in matrilysin (Browner et al., 1995) and the alkaline pK_a value of matrilysin is over 8 (Figure 2). The most likely group responsible for this pK_a is a zinc-bound water or a tyrosine residue in the active site. Recent X-ray absorption fine structure studies of carboxypeptidase A have implicited the ionization of the zinc-bound water in the alkaline pK_a of 9.0 in this zinc protease (Zhang & Auld, 1993; 1995). Therefore, the alkaline pK_a value in matrilysin could be ascribed to the ionization of a zinc-bound water molecule. On the other hand, the pK_a of 9.6 could represent ionization of a tyrosine. A tyrosine following the Met turn in the astacin superfamily is a long-distance fifth ligand to the zinc and moves to a hydrogen-bonding position in a phosphinyl inhibitor complex (Grams et al., 1996). Tyr-219 is conserved in all MMPs (Sang & Douglas, 1996). It is the fourth residue from the methonine in the Met turn and is located in the S_1' substrate binding pocket (Browner et al., 1995). Upon ionization of the hydroxyl group of the Tyr, the pocket will become more hydrophilic, which could result in weaker binding of the peptide substrate at the S_1' position during the catalysis. Tyr-216 is conserved in a number of the subfamilies of the MMPs, including matrilysin (Sang & Douglas, 1996), but no role in catalysis has been suggested for it. Kinetic and XAFS studies on the native and mutant enzymes should be helpful in evaluating the role of Glu-198, Tyr-216, Tyr-219, and the zinc-bound water in catalysis.

ACKNOWLEDGMENT

We thank Dr. R. Breathnach for the gift of the matrilysin cDNA and Dr. Daniel J. Strydom for his assistance with the amino acid analysis and N-terminal protein sequencing.

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BI962085F