Characterization of the 46-kDa Intermediates of Matrix Metalloproteinase 3 (Stromelysin 1) Obtained by Site-directed Mutation of Phenylalanine 83*

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The precursor of matrix metalloproteinase 3 (MMP-3/stromelysin 1) is activated in vitro by proteinases or mercurial compounds by stepwise processes which include the initial formation of short-lived intermediates and the subsequent intermolecular cleavage of the His82–Phe83 bond to generate the fully activated mature enzyme. To study the enzymatic properties of the intermediates we have mutated either His82 or Phe83 to Arg to obtain a stable MMP-3 intermediate. The mutant proteins were expressed in Chinese hamster ovary K-1 cells using a mammalian expression system. The proMMP-3(H82R) mutant was activated by chymotrypsin, elastase, and 4-aminophenylmercuric acetate to the 45-kDa MMP-3 with similar mechanism and kinetics as the wild-type. In contrast, the activation of the proMMP-3(F83R) mutant by proteinases or 4-aminophenylmercuric acetate resulted in 46-kDa forms, which retained 13, 14, or 15 amino acids of the pro-domain depending on the activators. The proteinase-activated MMP-3(F83R) intermediates exhibited little enzymatic activity, but they were partially active after treatment with SH-reacting reagents. These molecules could bind to the tissue inhibitor of metalloproteinases-1 and a-2-macroglobulin. However, the SH group of Cys75 in the intermediates was not modified by SH-reactants, indicating that the enzymatic activity generated by SH-reactants resulted from molecular perturbation of the enzyme rather than their interaction with Cys75. When gelatin and transferrin were digested with the 46-kDa intermediates the products were different from those generated by the wild-type MMP-3, suggesting an alteration in substrate specificity. The treatment of proMMP-3 with trypsin resulted in the formation of a 45-kDa MMP-3 with an NH2-terminal Thr69, whose activity and substrate specificity were similar to those of the 46-kDa MMP-3(F83R) obtained from the proMMP-3(F83R) mutant. These observations indicate that the correct processing at the His82–Phe83 bond is critical for expression of the full activity and the specificity of MMP-3.

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Matrix metalloproteinase 3 (MMP-3), also designated stromelysin 1, is a member of the matrixin family which plays a pivotal role in the degradation and remodeling of the extracellular matrix. MMP-3 degrades a number of extracellular matrix constituents such as aggrecan core protein, fibronectin, laminin, collagen types I, II, III, IV, and VI (Okada et al., 1986; Wu et al., 1991); and the large tenasin C (Siri et al., 1995). The overproduction of MMP-3 has been implicated in connective tissue diseases such as rheumatoid arthritis and osteoarthritis (Okada et al., 1989; 1992; Harris, 1990; Gravallese et al., 1991; McCachren, 1991; Firestein et al., 1991; Walakovits et al., 1992) and tumor cell invasion and metastasis (see Stetler-Stevenson et al., 1993). The importance of MMP-3 in tissue matrix catabolism is further substantiated by its role in generating the fully active interstitial collagenase (MMP-1) (Suzuki et al., 1990) and neutrophil collagenase (MMP-8) (Knäuper et al., 1993), and its ability to activate progelatinase B (MMP-9) (Ogata et al., 1992).

Like other matrixins, MMP-3 is secreted from cells as an inactive zymogen (Okada et al., 1986, 1988). ProMMP-3 comprises an NH2-terminal propeptide of 82 amino acids, a catalytic domain of 165 amino acids, and a COOH-terminal heparin/vitronectin-like domain of 213 amino acids (see Wessner, 1991). Latent proMMPs can be activated in vitro by proteolytic and non-proteolytic pathways (Okada et al., 1988; Nagase et al., 1990). The “cysteine-switch” model has been proposed to explain non-proteolytic activation of proMMPs by SH-reacting reagents (e.g. mercurial compounds, iodoacetamide, N-ethylmaleimide, oxidized glutathione) and chondroitin agents (Springman et al., 1990; VanWart and Birkedal-Hansen, 1990). This model suggests that activation occurs when the cysteiny1 residue in the conserved propeptide sequence PRCG[Val/Leu]Pro-Leu is cleaved from the zinc atom at the active site and reacts with SH-reacting reagent, thereby preventing the reassociation of the cysteine-zinc complex. Studies on proMMP-3 activation with organomercurials and proteinases have indicated that the zymogen is processed in a stepwise manner (Nagase et al., 1990): treatment with APMA results in the initial formation of a 42-kDa intermediate by cleavage of the Glu68–Val69 bond; and proteinases also produce an intermediate by cleaving a peptide bond in a short segment (residues 34–39), referred to as “bait” region, located within the segment (residues 34–39), referred to as “bait” region, located...
near the middle of the pro-domain. The intermediates are then converted into the 45-kDa active form by a bimolecular reaction cleaving the His92-Phe93 bond. Stepwise activation has been also shown for interstitial collagenase (MMP-1), gelatinase B (MMP-9), and matrilysin (MMP-7) (Suzuki et al., 1990; Ogata et al., 1992; Crabbe et al., 1992), but the enzymatic properties of the transiently generated intermediates are not known. We have hypothesized that the intermediates generated during activation may bind to endogenous MMP inhibitors such as TIMP-1 and α2M, so that this unique activation system provides an additional regulatory mechanism in matrixin activities.

In this study, we aimed to generate a stable intermediate of MMP-3 by mutating residues involved in the final activation site. Such a mutant was obtained by substitution of Phe88 with Arg in proMMP-3. The intermediates generated from the proMMP-3(F88R) mutant have limited proteolytic activity but they interact with TIMP-1 and α2M. In addition, these intermediates exhibit an altered substrate specificity when compared with the fully processed wild-type MMP-3. Our studies suggest that intermolecular processing of the His92-Arg83 bond is critical for the expression of the full enzymatic activity and the specificity of MMP-3.

MATERIALS AND METHODS

Reagents—Human neutrophil elastase (HNE) was from Athens Research and Technology, Athens, GA. Chymotrypsin (bovine), transglutaminase, diisopropyl fluorophosphate (DFP), APMA, iodoacetamide, and l-methionine sulfoximine was from Sigma. 5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB) was from Calbiochem. Green A dye matrix gel and Centricon-10 concentrators were from Amicon Corp. (^14C)-Lodoacetamide was from Amersham. Tissue culture materials including glutamine-free Dulbecco’s modified Eagle’s medium, dialyzed fetal bovine serum, 25 mM Tris-HCl buffer (pH 7.5), penicillin, streptomycin, and 10 mM of neomorphic (mM) and Lipo-pectin were from Life Technologies, Inc. Chinese hamster ovary (CHO) K-1 cells were from the American Type Culture Collection. Poly(vinylidene difluoride)-Millipore Immobilon transfer membrane was from Millipore. The expression vector pEE-14 was provided from Celltech Research Ltd., Slough, United Kingdom. α-Macroglobulin (αM) was a generous gift from Dr. Janet Englel at the Dept. of Pathology, Duke University Medical Center. The synthetic substrate NFF-3(7-methoxy coumarin-4-yl)acetyl-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys-(2,4-dinitrophenyl)-NH2 was synthesized and kindly provided by Dr. G. B. Fields at the Dept. of Laboratory Medicine and Pathology, University of Minnesota. Recombinant human TIMP-1, expressed from CHO K-1 cells using the pEE-14 vector, was purified by sheph (anti-human TIMP-1) IgG coupled to Affi-Gel 10.

Expression of proMMP-3, proMMP-3(3HR2), and proMMP-3(3FR3) Mutants—Full-length human MMP-3 DNA (Saus et al., 1988) was subcloned into the expression vector pEE-14 between the human cytomegalovirus promoter and the SV40 early polyadenylation signal (Englel et al., 1990). For transfection of CHO cells, 1 mg of plasmid DNA was added to 100 μl of culture medium. After incubation at 4°C for 15 min, 2 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 10% dialyzed fetal bovine serum and 25 μM of methionine sulfoximine for 2 weeks. After colony formation, cells were harvested by treating with trypsin, split into 24-well plates, and grown for additional 2 weeks in the same medium in the presence of increasing concentration of methionine sulfoximine (100–400 μM). Expression of proMMP-3 and its mutants was confirmed by Western blot analysis.

Purification of Recombinant Proteins—Wild-type proMMP-3 and its mutants were purified from the conditioned media of stably transfected CHO K-1 cell line by immunoadsorption chromatography as described by Ito and Nagase (1988). In a typical preparation about 2 mg of proMMP-3 and 1–1.5 mg of proMMP-3 mutants were purified from 1 liter of the conditioned medium of a 4-day culture.

**NH2-terminal Sequence Analyses**—The activated proteins were separated by SDS-PAGE with 7.5% (w/v) total acrylamide under reducing conditions (Bury, 1981), and transferred to a poly(vinylidene difluoride)-Millipore Immobilon transfer membrane as described by Matsu-daira (1987). The proteins transferred to the poly(vinylidene difluoride) 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 by Applied Biosystem 447 A pulse liquid sequenator with “on-line” 120 A phenylthiohydantoin analysis.

Transverse Urea Gradient-Polyacrylamide Gel Electrophoreses (PAGE)—A three channel peristaltic pump was used to cast three 7.5% polyacrylamide gels simultaneously, containing a continuous 0–8 μ urea gradient in the buffer system (2-amino-2-methyl-1,3-propanediol/glucine/HCl) as described by Mast et al. (1991). After acrylamide polyacrylamide, the gel was rotated 90°, and a silver staining was allowed. Approximately 20 μg of protein in a total volume of 200–300 μl was loaded evenly across the top of the gel. Electrophoresis was performed at 10 mA (constant current) at 23 °C for 3 h. Proteins were stained with Coomassie Brilliant Blue R-250.

Protein Determination—ProMMP-3 concentration was determined by using the bicinchoninic acid (Smith et al., 1985) with bovine serum albumin as standard. The amount of activated MMP-3 was determined by titration with TIMP-1 as described by Nagase (1995).

Enzyme Assay—MMP-3 activity was measured against [3H]Cm-Tf as described by Nagase (1995). One unit of [3H]Cm-Tf activity was defined as the production of 1 μg of [3H]Cm-Tf fragments soluble in 3.3% (w/v) trichloroacetic acid in 1 min. 1 Unit of enzyme was defined as the production of 1 μg of [3H]Cm-Tf fragments soluble in 3.3% (w/v) trichloroacetic acid in 1 min.

**Kinetic Analysis**—Enzyme kinetic studies of MMP-3 and its mutants were carried out by using the trichloroacetic acid (W. B. Smith et al., 1985) with bovine serum albumin as standard. The amount of activated MMP-3 was determined by titration with TIMP-1 as described by Nagase (1995).

Characterization of Gelatin or Cm-Tf Digestion Products (Substrate Mapping)—Various forms of the wild-type MMP-3 and the mutant proMMP-3 and its mutants were incubated with either gelatin or Cm-Tf at 37 °C for the indicated periods of time. The reaction was stopped by adding 20 mM EDTA and the products were analyzed on 10% SDS-PAGE under reducing conditions.

Binding of the MMP-3 Intermediates to TIMP-1—The ability of intermediates to bind TIMP-1 was examined by performing the samples to a column of Affi-Gel 10 coupled with TIMP-1. The proteins bound to the column were eluted with 5% formic acid (1-ml fractions), neutralized with 2 mM Tris-HCl, pH 8.6, and precipitated with 3.3% (w/v) trichloroacetic acid. Precipitated proteins were solubilized in SDS-PAGE sample buffer and analyzed by Western blotting using anti-human MMP-3 sheep serum.

Alkylation of Cys25 of MMP-3 Intermediates—The 46-kDa intermediates were purified from the conditioned media of stably transfected CHO K-1 cell line by immunoadsorption chromatography as described by Ito and Nagase (1988). Various concentrations of the sub-
MMP-3, Processing and Characterization of Intermediates

Expression of Recombinant Proteins—ProMMP-3 and the mutant cDNAs were cloned into the expression vector pEEl-14, and stably transfected into CHO K-1 cells. The expression levels of recombinant proteins from the selected CHO K-1 cells were 2-3 ng/ml of the conditioned medium after a 4-day culture. The proMMP-3 mutants isolated by immunoadsorbent affinity chromatography were homogenous on SDS-PAGE (see below). Transverse urea gradient-PAGE analyses of the wild-type and two mutant proteins showed a similar unfolding pattern by urea (data not shown), indicating that the mutation did not interfere with the folding and the stability of the protein significantly. The correct folding of the mutant protein is also deduced from the similar processing and the enzymatic activity observed between the wild-type and the mutant proMMP-3 when treated with trypsin (see below).

Activation of proMMP-3 (H82R) and proMMP-3(F83R) Mutants by APMA—Incubation of the proMMP-3(H82R) mutant with 1.5 mM APMA at 37 °C converted the precursor to the 45-kDa active species, and the rate of this reaction was similar to that of the wild-type proMMP-3 (Fig. 1). In contrast, the proMMP-3(F83R) mutant was rapidly converted to a stable 46-kDa intermediate form (Fig. 1B). After a prolonged incubation it was slowly converted to 45 and 28 kDa. While the APMA-activated proMMP-3(H82R) expressed a similar enzymatic activity as the wild-type, the 46-kDa MMP-3(F83R) exhibited only about 20% of the MMP-3 activity. NH₂-terminal amino acid sequence analysis of the 46- and 45-kDa MMP-3(F83R) revealed that the initial cleavage occurred at the Glu⁶⁸-Val⁶⁹ and then slowly at the Thr⁸⁵-Phe⁸⁶ bonds (Fig. 2). The inability of the proMMP-3(F83R) mutant to undergo similar processing as the wild-type precursor suggests that the Phe⁸³ residue at P₁ is a critical residue for the intermolecular processing to generate the fully active MMP-3.

Since the treatment of the proMMP-3(H82R) mutant with proteinases also processed this precursor in a similar manner and kinetics as the wild-type (data not shown), further studies were carried out with the F83R mutant.

Activation of proMMP-3(F83R) by HNE and Chymotrypsin—To assess if a stable intermediate can be obtained during proteinase activation, proMMP-3(F83R) was treated with HNE or chymotrypsin. HNE initially converted the mutant to an intermediate of 49 kDa and then to a 46-kDa species (Fig. 3B) which was stable for 48 h at 37 °C (data not shown). The proteolytic activity of the HNE-activated 46-kDa MMP-3(F83R) intermediate was less than 1% of that of the mature wild-type enzyme. NH₂-terminal sequence analysis of these two forms indicated that the Val⁵⁶-Thr⁵⁹ and the Val⁶⁹-Met⁷⁰ bonds were directly cleaved by HNE. Treatment with chymotrypsin resulted in the formation of a 51-, 46-, and 45-kDa species (Fig. 4B). The proteolytic activity of the chymotrypsin-activated 46-kDa MMP-3(F83R) was also less than 1% of that of the mature MMP-3, while the chymotrypsin-activated 45-kDa species exhibited approximately 20% activity (Figs. 3 and 4, Table I). The NH₂-terminal sequencing indicated that chymotrypsin cleaved at the Phe⁵³-Leu⁵⁴ bond, and subsequently the Leu⁶⁷-Glu⁶⁸ and the Phe⁸⁶-Pro⁸⁷ bonds, generating the 46- and 45-kDa forms, respectively (Fig. 2).

Activation of ProMMP-3 and ProMMP-3(F83R) Mutant by Trypsin—An activation time course of the wild-type proMMP-3 with 5 μg/ml trypsin at 37 °C indicated that maximal activity was reached after 5 min, and then the activity fell rapidly to the level of about 25% (Fig. 5A). SDS-PAGE analysis showed that proMMP-3 was rapidly processed from high molecular mass intermediates to 45 kDa (Fig. 5B). The treatment of proMMP-3(F83R) mutant also resulted in a 45-kDa species but with a faster kinetics than the wild-type. The specific activity generated from the mutant precursor was approximately 25% of the fully active MMP-3 (Fig. 5A). The NH₂-terminus of the trypsin activated wild-type and mutant was Thr⁸⁵, indicating that trypsin cleaved the Arg⁸⁴-Thr⁸⁵ bond in both cases.

The Role of the NH₂-terminal Phe⁸³ on the Substrate Specificity of MMP-3—The proteolytic activities of the APMA-activated 46-kDa MMP-3(F83R) intermediate, the trypsin-activated 45-kDa MMP-3(F83R), and the chymotrypsin-activated MMP-3(F83R) (the residues in brackets denote the NH₂-terminal amino acid) were examined for their abilities to digest type-I gelatin and Cm-Tf (Fig. 6, A and B). All three species gave similar digestion patterns against each substrate, but they were different from those of the mature MMP-3 with Phe⁸³ at the NH₂-terminus. The HNE- and chymotrypsin-activated MMP-3(F83R) intermediates in the presence of APMA gave similar digestion products to those generated by [Thr⁸⁵]MMP-3 (data not shown). The difference in digestion products was not due to the lower specific activities of these species as shown by the time course study (Fig. 6C). These results indicate that Phe⁸³ is not only essential to express the full enzymatic activity, but also influences the substrate specificity of MMP-3. A support for this was obtained by trypsin treatment of fully active Phe⁸³[MMP-3, which resulted in a time-dependent decrease in activity and changes in substrate specificity that were similar to those obtained with [Thr⁸⁵]MMP-3 (Fig. 7). This suggests that the removal of a dipeptide Phe⁸³-Arg⁸⁴ from MMP-3 results in alteration of the enzymatic activity and substrate specificity.
Kinetic Analysis of the MMP(F83R) Intermediates and the Trypsin-Activated [Thr85]MMP-3—

The changes in enzymatic activity associated with different forms of MMP-3 were examined by kinetic analysis against the synthetic substrate NFF-3. While the wild-type [Phe83]MMP-3 gave the \( K_m \) value of 28.0 ± 5.0 \( \mu \)M and the \( k_{cat} \) value of 6.3 ± 0.37 s\(^{-1} \), other forms showed about 1.8-fold increases in \( K_m \) and about 3.9-fold decrease in \( k_{cat} \) (Table II). The similar kinetic parameters for the APMA- and the trypsin-activated forms indicates that the presence of a portion of the propeptide is not the determinant factor for the reduced enzymatic activity and the changed substrate specificity of the mutated protein.

Interaction of the 46-kDa Intermediates with \( \alpha_2 \)-M and TIMP-1—The changes in enzymatic activity associated with different forms of MMP-3 were examined by kinetic analysis against the synthetic substrate NFF-3. While the wild-type [Phe83]MMP-3 gave the \( K_m \) value of 28.0 ± 5.0 \( \mu \)M and the \( k_{cat} \) value of 6.3 ± 0.37 s\(^{-1} \), other forms showed about 1.8-fold increases in \( K_m \) and about 3.9-fold decrease in \( k_{cat} \) (Table II). The similar kinetic parameters for the APMA- and the trypsin-activated forms indicates that the presence of a portion of the propeptide is not the determinant factor for the reduced enzymatic activity and the changed substrate specificity of the mutated protein.

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Reactivity of Cys\(^{75} \) of the Proteinase-generated 46-kDa MMP-3(F83R) Intermediate with SH-Reacting Reagents—The dissociation of the cysteinyl residue in the propeptide from its interaction with the zinc atom at the active center is prerequisite for proMMP activation (Springman et al., 1990; VanWart and Birkedal-Hansen, 1990; Chen et al., 1993). To test whether the lack of proteolytic activity of the proteinase-activated intermediate is due to the retention of the Cys-Zn\(^{2+} \) interaction, the 46-kDa species generated by HNE or chymotrypsin was treated

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**Fig. 2.** Sites cleaved in the propeptide of proMMP-3 and the proMMP-3(F83R) mutant after APMA or proteinases treatment. Cleavage sites identified by NH\(_2\)-terminal sequence analysis of the products generated by APMA and proteinases are shown by arrows. The His\(^{82}\)-Phe\(^{83}\) bond is cleaved by MMP-3 (Nagase et al., 1990). X indicates that the cleavage did not occur due to the substitution of Phe\(^{83}\) with Arg. The residue in brackets indicates the NH\(_2\) terminus of the individual MMP-3 species generated after a proteinase or APMA treatment. Molecular masses of individual enzyme species were estimated by SDS-PAGE. The cleavage sites in the boxed bait region are taken from Nagase et al. (1990). The conserved cysteine-switch sequence is boxed by dotted lines. CT, chymotrypsin; T, trypsin. Dotted arrows indicate sites cleaved by autolysis in the presence of APMA.

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**Fig. 3.** Activation of proMMP-3 and proMMP-3(F83R) mutant by HNE. A, proMMP-3 (□) and proMMP-3(F83R) mutant (●) (4 \( \mu \)g/ml) were treated with 10 \( \mu \)g/ml HNE at 37 °C for the indicated period of time. After inhibition of HNE by 2.5 mm DFP the MMP-3 activity was measured against \([\text{H}]\text{Cm-Tf}\). B, SDS-PAGE analyses of proMMP-3 and proMMP-3(F83R) mutant activated with HNE. ProMMP-3 (40 \( \mu \)g/ml) (lanes 1, 3, 5, and 7) and proMMP-3(F83R) (40 \( \mu \)g/ml) (lanes 2, 4, 6, and 8) were activated by HNE (4 \( \mu \)g/ml) for 30 min (lanes 3 and 4), 1 h (lanes 5 and 6), and 4 h (lanes 7 and 8). After terminating the reaction with 2.5 mm DFP and 20 mm EDTA, the samples were subjected to SDS-PAGE (7.5% acrylamide) under reducing conditions.
with \[^{14}\text{C}\]iodoacetamide in the absence and presence of 20 mM EDTA. As shown in Fig. 9, \[^{14}\text{C}\]iodoacetamide was incorporated into the 46-kDa MMP-3(F83R) intermediates only in the presence of EDTA, indicating that the SH-group of Cys\(^{75}\) in the proteinase-activated intermediates is probably bound to the zinc atom at the active site. However, the treatment of the 46-kDa intermediate with SH-reacting reagents, such as APMA, iodoacetamide, and DTNB resulted in an increase in specific activity (Tables I and II). The increased activity was not due to the modification of Cys\(^{75}\) by SH-reagents. This was concluded from the following observations. First, incubation of the proteinase-generated 46-kDa intermediates did not react with \[^{14}\text{C}\]iodoacetamide unless the reaction was carried out in the presence of EDTA (Fig. 9A). Second, even after the HNE-activated 46-kDa species were reacted with 1 mM iodoacetamide (Fig. 9B), or 1 mM APMA and 1 mM iodoacetamide (data not shown) for 2 h at 37 °C, the samples, after dialysis of these reagents, incorporated \[^{14}\text{C}\]iodoacetamide only in the presence of EDTA (Fig. 9B), indicating that the SH group of Cys\(^{75}\) was not modified by the SH reagents. The APMA-activated [Val\(^{69}\)]MMP-3 did not react with \[^{14}\text{C}\]iodoacetamide, but it incorporated \[^{14}\text{C}\]iodoacetamide in the presence of EDTA (Fig. 9B), indicating that the SH group of the [Val\(^{69}\)]MMP-3 was also not modified by APMA. These results suggest that an increase in activity of the 46-kDa intermediate by APMA or other SH reagent treatment is not due to the reaction with the SH group of Cys\(^{75}\), but most likely due to molecular perturbation of the enzyme induced by these reagents.

**DISCUSSION**

The involvement of matrixins in extracellular matrix degradation is controlled, in part, by the activation of theirzymogens and the inhibition of the activated enzymes by their endogenous inhibitors. Promatrixins are activated in vitro by several proteinases, SDS, HOCI, chaotropic agents (see Woesner, 1991), low pH (Davis and Martin, 1990), and elevated temperature (Kiskinis et al., 1991). Our previous work, demonstrating that the activation of proMMP-3 occurs in a stepwise manner (Nagase et al., 1990), led us to investigate enzymatic properties of the intermediates and their ability to interact with endogenous inhibitors, TIMP-1 and \(\alpha\)M. A stable intermediate was generated by mutating Phe\(^{83}\) to Arg, whereas the proMMP-(H82R) mutant was converted to the active 45-kDa species. These results are in agreement with the substrate specificity of MMP-3 reported by Niedzwiecki et al. (1992); i.e. the activity of MMP-3 decreased more then a 100-fold when phenylalanine at the P\(_1\) site was replaced by arginine.

The stable intermediates generated from proMMP-3(F83R) could bind TIMP-1 and \(\alpha\)M, but this occurred only in the presence of SH-reacting reagents. Since the activity of these intermediates against the synthetic substrate NFF-3 was very
low, their binding constants with TIMP-1 could not be determined. Nonetheless, our observations are in good agreement with those by Ward et al. (1991) reporting that TIMP-1 was able to bind to MMP-3 intermediates during activation by APMA. TIMP-2 also interferes with the proMMP-1 processing during activation (DeClerck et al., 1991).

Little activity was detected with the proteinase-activated MMP-3 (F83R) intermediates, whereas the APMA-activated intermediate expressed about 15–20% of the full MMP-3 activity against [3H]Cm-Tf and the synthetic substrate. The lack of activity with the former is likely to be due to the retention of the Cys75-Zn2+ interaction since Cys75 did not react with [14C]-iodoacetamide unless the sample was treated with EDTA. This contrasts with our previous studies with the wild-type proMMP-3 whose intermediates underwent autoprocessing, indicating that those intermediates have proteolytic activity (Nagase et al., 1990). This discrepancy may be explained by the different length of the remaining propeptide. The molecular mass of the major intermediate generated from the wild-type proMMP-3 by proteinases is 53 kDa, whereas that of the stable intermediate form the proMMP-3 (F83R) is 46 kDa. It is, therefore, speculated that the longer propeptide moiety of the 53-

FIG. 6. Substrate specificity of the wild-type MMP-3, [Thr85]MMP-3, [Pro87]MMP-3, and the APMA-activated 46-kDa [Val69]MMP-3.

Type-1 gelatin (30 µg) and Cm-Tf (30 µg) were incubated with various forms of MMP-3 (0.4 µg) at 37°C. The reaction was terminated by the addition of 20 mM EDTA, and the products were analyzed by SDS-PAGE (10% acrylamide). Lane 1, substrate without enzyme as control; lane 2, wild-type MMP-3; lane 3, [Thr85]MMP-3; lane 4, [Pro87]MMP-3; lane 5, APMA-activated 46-kDa [Val69]MMP-3. Type-1 gelatin (30 µg) was digested with an equal amount (0.4 µg) of MMP-3 (lanes 2, 4, and 6) and of the trypsin-activated [Thr85]MMP-3 (lanes 3, 5, and 7) at 37°C for the indicated period of time. The reactions were terminated by the addition of 20 mM EDTA and the digestion products were analyzed by SDS-PAGE.

FIG. 7. Treatment of MMP-3 with trypsin. A, MMP-3 was incubated in the presence of trypsin (5 µg/ml) at 23°C for the indicated time periods. After inactivation of trypsin with 2.5 mM DFP, the enzymatic activity of MMP-3 was measured against [3H]Cm-Tf or the synthetic substrate. The lack of activity with the former is likely to be due to the retention of the Cys75-Zn2+ interaction since Cys75 did not react with [14C]-iodoacetamide unless the sample was treated with EDTA. This contrasts with our previous studies with the wild-type proMMP-3 whose intermediates underwent autoprocessing, indicating that those intermediates have proteolytic activity (Nagase et al., 1990). This discrepancy may be explained by the different length of the remaining propeptide. The molecular mass of the major intermediate generated from the wild-type proMMP-3 by proteinases is 53 kDa, whereas that of the stable intermediate form the proMMP-3 (F83R) is 46 kDa. It is, therefore, speculated that the longer propeptide moiety of the 53-

TABLE II

| Enzyme species      | $K_m$ (µM) | $k_{cat}$ (s⁻¹) | $k_{cat}K_m$ (µM s⁻¹) |
|---------------------|------------|----------------|----------------------|
| MMP-3               | 28.0 ± 5.0 | 6.3 ± 0.37     | 267,585 ± 10,780     |
| [Glu68]MMP-3        | 50.0 ± 10.0| 0.5 ± 0.03     | 9,895 ± 157          |
| +DTNB               | 45.0 ± 6.0 | 1.6 ± 0.006    | 34,482 ± 837         |
| +APMA               | 53.0 ± 10.0| 1.6 ± 0.08     | 30,563 ± 625         |
| [Val69]MMP-3        | 45.0 ± 5.0 | 1.8 ± 0.20     | 41,011 ± 1,116       |
| [Met70]MMP-3        | 45.0 ± 6.0 | 0.4 ± 0.02     | 8,235 ± 107          |
| +DTNB               | 50.0 ± 10.0| 1.7 ± 0.32     | 35,548 ± 985         |
| +APMA               | 50.0 ± 10.0| 1.6 ± 0.35     | 32,436 ± 857         |
| +iodoacetamidea     | 50.0       | 1.6 ± 0.02     | 36,048 ± 1,091       |

*One measurement.*
used HNE partially converted proMMP-3 (F83R) to the 46-kDa form. This intermediate was not modified by APMA (Fig. 9A), suggesting that the removal of a small stretch of the propeptide by cleaving the Arg84-Thr85 bond generates an 46-kDa intermediate. This form of MMP-9 exhibited enzymatic activity only in the presence of APMA. No activity could be detected after removal of APMA by dialysis, suggesting that APMA does not covalently modify the cysteinyl residue in the propeptide of proMMP-9.

The 46-kDa MMP-3 (F83R) intermediates and the [Thr85]MMP-3 showed similar specific activity on [1H]Cm-Tf and NFF-3, which was considerably lower than that of the mature wild-type MMP-3. Kinetic parameters on NFF-3 and the cleavage patterns of Cm-Tf and gelatin indicate that these enzyme species have similarly altered substrate specificity. Thus, the reduced enzymatic activity associated with the intermediates is not simply due to the presence of a part of the propeptide at the NH2-terminal end of the enzyme. Although the altered activity and specificity cannot be readily explained, our observations are analogous to those for interstitial collagenase (MMP-1) and neutrophil collagenase (MMP-8). Both collagenases express the full collagenolytic activities when they possess Phe81 and Phe79 at their NH2 termini, respectively, but [Val82]MMP-1 and [Me80]MMP-8 have only 40 to 20% of the full activities (Suzuki et al., 1990, 1995; Knäuper et al., 1993). Recently resolved crystal structures of the catalytic domains of [Phe79]MMP-8 and [Me80]MMP-8 provides some insights into the different activities between the two forms of MMP-8. In [Phe79]MMP-8 the ammonium group of the NH2-terminal Phe79 forms a salt linkage with the side chain carboxylate of Asp232, but in the case of [Me80]MMP-8 the NH2-terminal hexapeptide was disordered (Bode et al., 1994; Reinemer et al., 1994). It is postulated that a small structural and rotational difference may result in different stabilization of the active site or of the transition state, or that the mobile NH2-terminal peptide may interfere with the substrate (Reinemer et al., 1994). The changes in both kcat and Km with the 46-kDa intermediate, and the 45-kDa [Thr85]MMP-3 suggest that both the enzymatic efficiency and the interaction with a substrate are altered without a correct positioning of the NH2-terminal Phe83.

In summary, our studies have shown that intermolecular processing of the His82-Phe83 bond is critical for the expression of full enzymatic activity and specificity of MMP-3. The importance of the correct NH2 terminus may be related to other members of the matrixin family. All matrixins identified to date have either Phe or Tyr at this position. The activation of proMMP-1 by human and rat mast cell chymases generates [Thr84]MMP-1 and [Val82]MMP-1, respectively (Saarinen et al., 1994; Suzuki et al., 1995), and reduced collagenolytic activity was detected (Suzuki et al., 1995). In the case of MMP-3, not only the reduction in activity, but also the changes in substrate specificity occur when the His82-Phe83 bond is not correctly processed or the Phe83 is removed from the NH2 terminus of the mature enzyme. Although it is not known whether altered...
processing of proMMP-3 occurs in vivo, a number of peptide bonds near the activation site can become a target of various proteases. Such proteases may arise not only from resident connective tissue cells but also inflammatory cells, plasma, and opportunistic microorganisms under certain pathological conditions.

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Characterization of the 46-kDa Intermediates of Matrix Metalloproteinase 3 (Stromelysin 1) Obtained by Site-directed Mutation of Phenylalanine 83
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