 Multiple Sites of the Propeptide Region of Human Stromelysin-1 Are Required for Maintaining a Latent Form of the Enzyme*

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Latency of matrix metalloproteinase 3 (MMP-3) is regulated by the interaction of a free cysteine residue (Cys-75) in the conserved amino acid sequence Pro-Arg-Cys-Gly-Val-Pro-Asp located in the COOH-terminal portion of the propeptide with a chelated zinc atom in the active site of the catalytic domain. Proteolytic activation of full-length human pro-MMP-3 involves the removal of ~35 amino acids from the NH₂-terminal portion of the propeptide, forming a 55-kDa unstable intermediate that undergoes intramolecular autocatalysis to form the 45-kDa mature active enzyme. In this study, we have evaluated the contribution of the NH₂-terminal 35 amino acids to the maintenance of latency. Full-length human pro-MMP-3 was expressed in Escherichia coli and refolded to form latent pro-MMP-3 capable of activation by chymotrypsin or aminophenylmercuric acetate. Renaturation of pro-MMP-3 expressed in bacteria with 20 or more amino acids removed from the NH₂-terminal region of the propeptide yielded only an active enzyme. COS-7 cells transiently transfected with pro-MMP-3 expression vectors containing the single amino acid substitutions Y20A, L21A, and C75S also secreted active forms of the enzyme. These data suggest that simultaneous interactions of the NH₂ and COOH-terminal regions of the propeptide are required for maintenance of the latent form of the enzyme.

Matrix metalloproteinases (MMPs) are single-chain zinc metalloenzymes sharing homologous domains consisting of an ~80-amino acid propeptide, a catalytic domain, and a carboxyterminal (hemopexin-like) region associated with substrate specificity and binding. Seven members of the MMP protein family have been characterized extensively, each containing distinct substrate degradation profiles that can be grouped into three general categories: collagenases (MMP-1 and MMP-8) cleave collagen types I, III, VII, and X; gelatinases (MMP-2 and MMP-9) degrade collagen types IV, V, and VII and gelatins; and stromelysin (MMP-3), which has been shown to degrade a wide spectrum of extracellular matrix macromolecules (including proteoglycan core protein (aggrecan); fibronecin; laminin; gelatin; type I collagen telopeptide; and collagen types III, IV, and IX) and is an activator of procollagenase (1–4). Particular

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‡ The abbreviations used are: MMPs, matrix metalloproteinases; APMA, aminophenylmercuric acetate; PCR, polymerase chain reaction; Tricine, N,2-hydroxy-l,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis.

attention has been focused on MMP-3 as an important etiopathological agent in rheumatoid and osteoarthritis due to its broad specificity of cartilage extracellular matrix substrates, elevated levels observed in arthritic compared with normal cartilage (5), and inducibility by a variety of inflammatory stimuli such as interleukin-1 (6, 7), tumor necrosis factor (8), and oncogenic viruses (9). Understanding the mechanisms by which MMP-3 and other protein family members are activated and degrade substrates in vivo will enhance pharmacological intervention of disease.

Similar to other family members, MMP-3 is secreted as a latent enzyme, orzymogen, from a variety of cell types, including synoviocytes, fibroblasts, chondrocytes, and macrophages. Latent MMP-3 can be activated by a variety of methods, including proteases, organomercurials, perturbants such as sodium dodecyl sulfate and sodium thiocyanate, disulfide agents such as oxidized glutathione, oxidants such as HOCl (1, 3, 10), and heat (11). Enzyme activation of 57-kDa pro-MMP-3 coincides with cleavage of the NH₂-terminal 82-amino acid propeptide region from the catalytic domain in a stepwise bi-molecular autocatalytic mechanism to yield a 45-kDa mature active enzyme (12). A mechanism of activation by these various reagents that is common to all members of the MMP family has been proposed in the "cysteine switch" model (10, 13, 14). In this model, coordination of the zinc molecule in the active site of the catalytic domain with a sulfhydryl-containing cysteine residue in the conserved sequence Pro-Arg-Cys-Gly/Asn-Val-Pro-Asp located at the COOH terminus of the propeptide region is required for latency. Disruption of the zinc-sulfhydryl ligation by sulfhydryl reagents or removal of the prorogen by denaturants, oxidants, and proteases allows coordination of the zinc with a water molecule, resulting in the formation of an active enzyme. Evidence to support this proposal has been obtained by mutagenesis of Cys-75 to Ser, His, or Asp in rat pro-MMP-3, in which spontaneous enzyme degradation was observed (15, 16). Similarly, expression of human pro-MMP-1 with the replacement of Cys-73 with Ser resulted in the formation of active enzyme fragments (17). Spectrometric studies have revealed that MMP-3 contains an integral zinc in the active site that is critical for catalytic activity; however, the stoichiometry of ~2 mol of zinc/mol of protein indicates the presence of a second metal-binding site (18). Detailed structural resolution of the active site of MMP-1 by x-ray crystallography has confirmed, in addition to the catalytic zinc, a second zinc ion and a calcium ion that are involved in the tertiary structure (43).

Since the primary activating steps initiated by proteinases or perturbants are not involved in removal of the COOH-terminal region of the propeptide containing the zinc-ligated Cys-75 of pro-MMP-3, additional domains of the propeptide must be involved in the maintenance of latency. For example, proteolytic activation by chymotrypsin involves an initial cleavage between Phe-34 and Val-35 in the protease-sensitive "bait re-
region," forming a 53-kDa unstable intermediate, whereas activation by the organomercurial aminophenylmercuric acetate (APMA) results in the generation of a 46-kDa protein prior to a final autocatalytic step to generate the 45-kDa mature active enzyme. In addition, modification of Cys-75 with iodoacetamide does not induce activation, indicating that disruption of the cysteine switch alone is not sufficient for activation (19). Together, these data suggest that additional stabilizing domains exist in the first -35 amino acids of the propeptide region that are removed as a result of cleavage by a variety of proteinases such as plasmin and chymotrypsin (12).

In this study, we confirm by mutagenesis studies that additional amino acids located within the NH₂-terminal 35 amino acids of the proregion of pro-MMP-3 are involved in the maintenance of a latent enzyme. This observation was determined using separate experimental approaches. First, serial NH₂-terminal truncation of pro-MMP-3 expressed in bacteria allowed us to determine the minimal length of the proregion necessary to form a latent form of the enzyme. Second, expression of pro-MMP-3 in COS-7 cells containing single amino acid substitutions within the first 54 amino acids of the propeptide formed autocatalytic enzymes. These data indicate that latency to MMP-3 requires at least two separable protein interactions: the NH₂-terminal and cysteine switch regions of the propeptide. Comparison of the NH₂-terminal mutation-sensitive sequences with other MMPs reveals a similar homology, suggesting a general scheme of maintenance of latency involving this region.

MATERIALS AND METHODS

Bacterial Expression of Full-length and Truncated Forms of Pro-MMP-3—A recombinant full-length human pro-MMP-3 coding region was isolated using polymerase chain reaction (PCR) from human fibroblast cDNA (20), ligated into the T7 promoter expression vector pSRET (Invitrogen, San Diego, CA), and transformed into the BL21/DE3 (21) strain of Escherichia coli, which contains an isopropyl-β-D-thiogalactopyranoside-inducible T7 polymerase. The expressed protein contained an initiating Met codon followed by the native sequence of the latent enzyme. Construction of mutants with deletions of the NH₂-terminal residues of pro-MMP-3 were performed by PCR using a 5' oligonucleotide primer ((−)-strand) designed with an NdeI site followed by an initiating Met codon and then 18 nucleotides corresponding to peptide translation at different positions of the proregion and a 3'−primer (−)-strand) encoding the carboxyl-terminal residues and a BamHI site as previously indicated (20). The PCR products were digested with NdeI/BamHI and ligated into the NdeI/BamHI sites of the pSRET vector. DNA manipulations were performed using standard recombinant DNA methods (22).

E. coli cells transformed with pro-MMP-3 expression vectors were grown in Luria broth with 100 μg/ml ampicillin at 37 °C to a cell density of 2×10⁸ cells/ml. Protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM) at 4 °C for 16 h, following which wells were blocked with 1% bovine serum albumin in phosphate-buffered saline (pH 7.0). Plates were sequentially incubated for 1 h at each room temperature with supernatants, murine monoclonal antibody to human MMP-3 (1 μg/ml), and rabbit anti-mouse IgG alkaline phosphatase conjugate (1:2000; Jackson ImmunoResearch Laboratories, Inc.) and were developed with 1 μg/ml p-nitrophenol phosphate in 1.0 M diethanolamine buffer (pH 9.8). Absorbance values were determined at 405 nm, and MMP-3 concentrations were determined from a standard curve using recombinant human MMP-3 produced in E. coli.

Analysis of Enzymatic Activity—Enzymatic activity in supernatants was detected by an H-carboxymethylated transferrin substrate assay (28). Culture supernatants (10 μl) containing MMP-3 muteins were incubated with substrate vectors and buffer cofactors containing ANS (pH 7.5), 150 mM NaCl, 5 mM CaCl₂, and 0.02% NaN₃. Enzymatic activity was defined as 1 unit of activity equivalent to production of 1 μg/ml of soluble substrate in 3% (w/v) trichloroacetic acid in 1 min at 37 °C.

RESULTS

Refolding of Pro-MMP-3 Expressed in E. coli—Our previous studies have shown that pro-MMP-3 as a 57-kDa polypeptide expressed in E. coli strain BL21/DE3 can be isolated from insoluble inclusion body complexes, denatured, and refolded to yield a protein similar in molecular mass to the native latent proenzyme (20). Treatment of refolded pro-MMP-3 with known activators such as the organomercurial APMA or proteases such as chymotrypsin results in the formation of the same cleavage products as those obtained by treatment of the natural enzyme. Proteolytic activation of pro-MMP-3 by chymotrypsin occurs by cleavage of the proregion between Phe-34 and Val-35, forming an unstable 53-kDa intermediate that rapidly undergoes stepwise iminohemocatalytic cleavage, yielding a 45-kDa active enzyme (12). These data indicate that amino acids located within the NH₂-terminal chymotrypsin cleavage fragment, in addition to Cys-75, are essential for the maintenance of a latent enzyme. We hypothesized that construction of pro-MMP-3 muteins with serial deletion of NH₂-terminal resi-
were predicted to localize an additional stabilization domain, whereas deletions extending COOH-terminal of this residue would form spontaneous autocatalytic proenzymes. Each of the expressed mutant proteins migrated predictably on SDS-PAGE based on the truncated length of the proregion (Fig. 1B). All of the pro-MMP-3 muteins were expressed as insoluble protein complexes, similar to the full-length protein. Insoluble protein complexes of each mutein were isolated in an inactive form, renatured, and analyzed by SDS-PAGE. Renatured full-length pro-MMP-3 and pro-MMP-3 deleted to Met-14 did not change in molecular mass when compared with the denatured forms of these proteins (Fig. 1C). However, comparison of the denatured and renatured forms of pro-MMP-3 deleted to Tyr-20, Asp-36, and Glu-51 indicates that active forms of the enzyme were generated (Fig. 1C). Expression of human pro-MMP-3 with the COOH-terminal hemopexin domain removed, similar to previous studies (29), revealed that loss of the NH₂-terminal 13 amino acids did not induce autocatalytic activation. Thus, the entire latency mechanism is apparently contained within Met-14–His-82 of the proregion.

Mammalian Expression of Pro-MMP-3 with Single Amino Acid Substitutions—NH₂-terminal deletions of pro-MMP-3 were analyzed using E. coli-derived proteins; however, additional studies in a mammalian expression system would prove a more accurate assessment of native structure than studies of proteins refolded from insoluble complexes. To confirm the identification of stability sequences in the refolded bacterial muteins, pro-MMP-3 containing single amino acid substitutions was characterized by mammalian expression analysis. As indicated from the bacterial refolding studies, we identified a potential stability peptide from Met-14 to Tyr-20 in the propeptide. This peptide contains charged and hydrogen-bonding residues that may potentially interact directly with the catalytic fragment to stabilize the enzyme. Comparison of the homologous region in human fibroblast collagenase (MMP-1) revealed a striking identical heptapeptide with the sequence Leu-Val-Gln-Lys-Tyr-Leu-Glu located between Asp-13 and Lys-21. The conservation of this sequence between MMP-1 and MMP-3 further suggests that it may be involved in latency. To test this hypothesis, we used PCR-directed mutagenesis of MMP-3 template cDNA to change Val-17, Gln-18, Lys-19, Tyr-20, Leu-21, and Glu-22 to alanine. Alanine was chosen as the substitution based on the truncated length of the proregion (Fig. 1C) and of the C75S mutation to the 45-kDa and smaller fragments was consistent with other studies (15-17) in which replacement of the unique cysteine residue with Ser in rat pro-MMP-3 and human pro-MMP-1 resulted in expression of an autocatalytic enzyme. In our studies, complete conversion of the C75S mutation to the 45-kDa and smaller fragments was dependent on the presence of zinc. Without the addition of  

\[ \text{Zn}^{2+} \]
Structural Requirements for Latency of Human Stromelysin-1

Enzymatic activity of supernatants from COS-7 cells transiently transfected with MMP-3 containing single amino acid substitutions was assayed using H-carboxymethylated transferrin as the substrate (28). Specific activity represents units of enzyme activity/milligram of MMP-3 protein.

<table>
<thead>
<tr>
<th>Pro-MMP-3 mutein</th>
<th>Specific activity units activity/mg enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>ND*</td>
</tr>
<tr>
<td>C75S</td>
<td>388</td>
</tr>
<tr>
<td>V17A</td>
<td>4.6</td>
</tr>
<tr>
<td>Q18A</td>
<td>22.7</td>
</tr>
<tr>
<td>L19A</td>
<td>7.7</td>
</tr>
<tr>
<td>Y20A</td>
<td>606</td>
</tr>
<tr>
<td>L21A</td>
<td>765</td>
</tr>
<tr>
<td>E22A</td>
<td>31.5</td>
</tr>
</tbody>
</table>

* ND, not detectable.

pressed compared with wild-type proenzyme. The addition of APMA to these "latent" muteins generated polypeptides comparable to those formed by the natural enzyme, thus indicating that no structural abnormalities forming inactive enzymes are introduced (Fig. 2C). Thus, the generation of protein fragments of the correct size for active enzyme in culture medium from cells transfected with C75S, Y20A, and L21A is likely to be the result of pro-MMP-3 autocatalysis and not fragmentation due to incorrect protein folding.

Enzymatic Activity of Autocatalytic Pro-MMP-3 Muteins—To test whether the autocatalytic muteins contained enzymatic activity, culture supernatants were tested for the ability to digest H-carboxymethylated transferrin. As shown in Table I, the specific enzymatic activity of pro-MMP-3 muteins expressed in COS-7 cells coincided with the formation of enzyme fragments as previously detected by Western blot analysis. Wild-type pro-MMP-3 possessed low levels of enzymatic activity, as did muteins V17A, Q18A, L19A, and E22A. Greater levels of enzymatic activity were observed with muteins C75A, Y20A, and L21A. The difference in specific activity of C75S compared with Y20A and L21A has not been resolved, but may be due to differences in activation rates.

DISCUSSION

Stabilization of latent pro-MMPs has been proposed to involve the interaction of the free cysteine residue in the conserved sequence Pro-Arg-Cys-Gly-Val/Asn-Pro-Asp in the propeptide with a zinc atom that is coordinated to 3 histidine residues in the consensus sequence His-Glu-X-X-His-X-Gly-X-X-His located in the catalytic domain (13, 43). Consistent with this model, our data indicate that transient transfection of COS-7 cells with human pro-MMP-3 containing a C75S substitution led to secretion of protein fragments consistent in size with the active forms of the enzyme. However, without the addition of exogenous zinc to culture supernatants, a large percentage of the C75S mutein was expressed as full-length protein. This observation complements data from previous studies in which mutation of Cys-75 to Ser, His, or Asp in rat pro-MMP-3 resulted in the degradation of enzyme protein rather than the generation of an active MMP-3 (16). Thus, the spontaneous processing of pro-MMP-3 containing Cys-75 substitutions is due to the zinc-dependent autocatalytic activity of the enzyme.

The maintenance of latency of MMP-3 is not solely regulated by the cysteine switch mechanism. Proteinase activators of pro-MMP-3 have been demonstrated to remove ~35 amino acids from the NH₂ terminus, suggesting that additional stabilization motifs are located in this region (12). In addition, chem-

![Western blot analysis using anti-human MMP-3 PAGE under reducing conditions, and transferred to nitrocellulose for](image-url)
metrical modification of Cys-75 in human pro-MMP-3 with iodoacetamide does not induce activation of the enzyme, indicating that disruption of the zinc-Cys interaction alone is not sufficient to activate the enzyme (19). This indicates that latency is tightly regulated by the synergistic interaction of the COOH-terminal propeptide containing Cys-75 with a second NH$_2$-terminal propeptide region, whereby disruption of either peptide interaction leads to enzyme activation. We hypothesized that localization of stabilization motifs contained within the first 35 amino acids could be identified by serial deletion of the NH$_2$ terminus of the propeptide and assessing the ability of the truncated proteins expressed in E. coli to undergo autocatalysis following protein renaturation. Deletion of the NH$_2$-terminal 14 amino acids had no effect on the formation of a latent enzyme, indicating that these residues have a minimal role in the stabilization mechanism. However, all truncations of 20 amino acids or more had the ability to form protein fragments consistent with the active form of MMP-3, suggesting that a minimal stabilization motif lies between Met-14 and Tyr-20. Consistent with this observation, activation of the NH$_2$-terminal 14 amino acids had no effect on the formation of active enzyme consistent with active forms of MMP-3. The substitutions Y20A and L21A resulted in expression of protein fragments in culture supernatant that was the NH$_2$-terminal residue, and the presence of at least some of the 21 residues from Leu-13 to Lys-33 was critical in maintaining the propeptide in a conformation preventing autocatalysis to the mature active enzyme.

To further test whether amino acids were involved in the stabilization of a latent enzyme, pro-MMP-3 mutants with single alanine substitutions were transiently expressed in COS-7 cells, and cell culture supernatants were assayed for active forms of MMP-3. The substitutions Y20A and L21A resulted in expression of protein fragments in culture supernatants consistent with active forms of MMP-3. Mutation of Val-17, Gln-18, Lys-19, and Glu-22 to alanine had no effect on the formation of a latent enzyme. These data indicate that within the peptide Val-17–Glu-22, only Tyr-20 and Leu-21 contribute to the stabilization of the proenzyme. Expression of the Y20A mutation in culture medium without the addition of exogenous zinc generated protein fragments more rapidly than expression of the C75S mutation (Fig. 2B), indicating that disruption of protein interactions involving the NH$_2$-terminal region of the propeptide leads to faster enzyme activation than disruption of the zinc-Cys coordination. In support of these findings, previous studies (12) have indicated that faster activation rates are achieved by cleaving the NH$_2$- and COOH-terminal propeptide domains by proteases such as chymotrypsin than by APMA, which acts by interfering with the zinc-Cys interaction, but not the NH$_2$-terminal stabilization motifs. Alternatively, NH$_2$-terminal propeptide modifications induce modified substrate specificities in the catalytic domain, leading to faster autocatalysis rates.

Comparison of the propeptide region amino acid sequence Leu-16–Glu-22 of human pro-MMP-3 reveals a striking 7-amino acid stretch of identity to the sequence Leu-Val-Gln-Lys-Tyr-Leu-Glu of the same region in human MMP-1. The Tyr-Leu sequence of this region is strictly conserved among all members of the MMP protein family (Table II). The exception to this homology is the prosegment of MMP-11, which is truncated with a deletion of the Tyr-Leu motif and is proline-rich, which may allow unique protein folding to form a latent enzyme (41). The presence of a consensus Tyr-Leu peptide among other MMP family members suggests that these residues have a central role in the latency mechanism and interact with a highly conserved hydrophobic region of the proenzyme. This implicates the highly conserved amino acids of the cysteine switch region with the Tyr-Leu peptide. Either residues flanking the Tyr-Leu peptide, although primarily acidic or basic, do not contribute directly to the latency mechanism, or their contribution was not revealed in our studies. Our experience in reactivating pro-MMP-3 from insoluble protein complexes derived from E. coli indicates that the latent form of the enzyme is reconstituted (20). Thus, the propeptide region of reactivated pro-MMP-3 assumes a native conformation prior to the formation of a functional catalytic site and is a stabilizer of the enzyme. The synthetic COOH-terminal propeptide peptide PRCGVPNV has been shown to act as a weak inhibitor of rat MMP-3 (15); however, inhibition by this peptide is likely greater when presented as part of the entire propeptide region. Together, these data support a role of the Tyr-Leu peptide in orienting the COOH-terminal autoinhibitor domain in close proximity to the active site of the enzyme.

In conclusion, we have determined that in the propeptide of MMP-3, Tyr-20 and Leu-21, in addition to Cys-75, contribute to the maintenance of the latent form of the enzyme. Our survey does not preclude, however, that other amino acids in the propeptide may stabilize latency. Crystallographic analysis of the propeptide region of pro-MMP-3 should resolve the key protein interactions contributing to latency that may extend to all MMP family members. The recent x-ray crystal structure of adamalysin II (42), a zinc endopeptidase derived from rattlesnake venom, and neutrophil collagenase (43, 44) indicate that the NH$_2$-terminal portion of the polypeptide is located on the surface. The surface location of the propeptide region of mammalian MMPs would support evidence that enzyme activation is possible by a wide variety of agents acting on an exposed portion of the proenzyme. Further analysis of peptide sequences adjacent to Tyr-20 revealed that the consensus tyrosine phosphorylation site Lys-X-X-Glu-X-X-Tyr (45) is contained within the sequence TLYLENY. It is also noteworthy that an identical tyrosine spatial configuration exists within the kinase domain of the insulin receptor (Tyr-1158, Tyr-1162, and Tyr-1163) in which phosphorylation is required for kinase activity (46, 47). It is speculated that phosphorylation of Tyr-20, Tyr-24, or Tyr-25 in the NH$_2$-terminal propeptide region could interfere with hydrogen bonding, leading to autocatalytic activation or improper protein folding, resulting in novel substrate specificities. Phosphorylation of a variety of intracellular substrates has been well documented in cells stimulated with growth factors or cytokines (48, 49); however, they are unlikely substrates for phosphorylation since they are secreted from cells. The extent to which MMPs are activated in vivo by mechanisms that do not directly disrupt the zinc-cysteine interaction of the cysteine switch mechanism is uncertain. Further characterization of the intramolecular protein-protein in-

**TABLE II**

Comparison of peptide sequences in the NH$_2$-terminal propeptide domain of different human MMPs

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sequence Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human stromelysin (MMP-3)</td>
<td>&quot;LVQKYLE&quot;</td>
</tr>
<tr>
<td>Human stromelysin-2 (MMP-10)</td>
<td>LAVQYLE</td>
</tr>
<tr>
<td>Human fibroblast collagenase (MMP-1)</td>
<td>LVQKYLE</td>
</tr>
<tr>
<td>Human neutrophil collagenase (MMP-8)</td>
<td>TVQVQYE</td>
</tr>
<tr>
<td>Human 72-kDa type IV collagenase (MMP-2)</td>
<td>LAVQYLN</td>
</tr>
<tr>
<td>Human 92-kDa type IV collagenase (MMP-9)</td>
<td>LAVQYLN</td>
</tr>
<tr>
<td>Human matrilysin (MMP-7)</td>
<td>QAQVLK</td>
</tr>
<tr>
<td>Human macrophage elastase</td>
<td>FGQVYK</td>
</tr>
</tbody>
</table>
teractions that lead to the active forms of MMPs may provide important insights into in vivo enzyme activation.

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References