Articles

Disruption of the Cysteine-75 and Zinc Ion Coordination Is Not Sufficient To Activate the Precursor of Human Matrix Metalloproteinase 3 (Stromelysin 1)†

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ABSTRACT: Matrix metalloproteinases (MMPs) are activated in vitro from their precursors (proMMPs) by multiple means such as treatment with proteinases, mercurial compounds, chaotropic agents, sodium dodecyl sulfate, HOCl, and heat. The latency of proMMPs is stabilized by intramolecular interaction of the single cysteine residue in the conserved sequence PRCG(V/N)PD of the propeptide and the zinc atom at the active site. The activation of proMMP-1 (interstitial procollagenase) by multiple treatments has been explained by the "cysteine switch" model, in which the disruption of the Cys–Zn interaction is considered to be critical for activation [Springman, E. B., Angleton, E. L., Birkedal-Hansen, H., & VanWart, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 364–368]. To further test this hypothesis we dissociated the Cys–Zn interaction by specifically modifying Cys-75 of proMMP-3 (prostromelysin 1) with iodoacetamide, (4-aminophenyl)mercuric acetate (APMA), or 5,5′-dithiobis(2-nitrobenzoate) and examined the expression of enzymic activity. The enzymic assays of the modified proMMP-3s against protein and synthetic substrates did not reveal any significant activity. The modified 57-kDa proMMP-3s were stable and did not show spontaneous activation. Activation of the modified proMMP-3s required further treatment with APMA or a proteinase and was accompanied by conversion of the proMMP-3 to a 45-kDa species. Circular dichroism studies of proMMP-3 treated with $HgCl_2$ demonstrated time-dependent conformational changes in proMMP-3 prior to the expression of proteolytic activity and processing of the zymogen to lower molecular weight species. These results indicate that the disruption of the Cys–Zn coordination alone is not sufficient to activate proMMP-3. The activation of proMMP-3 by a mercurial compound is triggered by perturbation of the conformation of the precursor molecule but not by its reaction with Cys-75 of the propeptide.

Matrix metalloproteinases (MMPs)[1] are a group of zinc metalloendopeptidases that participate in degradation of extracellular matrix components. MMPs are synthesized in a variety of cell types and secreted from the cells as inactive zymogens (proMMPs) which share structural homology with interstitial collagenase (MMP-1) consisting of three distinct domains, i.e., propeptide, catalytic, and C-terminal hemopexin/vitronectin-like domains [see Woessner (1991) and Birkedal-Hansen et al. (1993) for reviews]. The recent X-ray crystal structure of astacin, a zinc endopeptidase from the crayfish *Astacus astucus* (Bode et al., 1992) suggests that three histidines in the consensus sequence HEXXHXXGXXH also found in the members of MMPs, snake venom, and serratia metalloproteinase families (Jiang & Bond, 1992), are the three ligands of zinc. All propeptides of MMPs contain a highly conserved sequence PRCG(V/N)PD located three residues before the N-terminus of the activated mature enzymes. This sequence is critical for maintaining the latency of the proMMPs through coordination of the zinc atom at the active site with the SH group of the cysteinyl residue (Salowe et al., 1992; Holz et al., 1992), thus preventing formation of a water–zinc complex that is required for catalytic reaction (Vallee & Auld, 1990; Springman, 1990; VanWart & Birkedal-Hansen, 1990).

ProMMPs can be activated in vitro by various agents, including proteinases, SH reagents (e.g., mercurial compounds, iodoacetamide, and oxidized glutathione), sodium dodecyl sulfate (SDS), HOCl, and chaotropic agents [see Springman et al. (1990), Woessner (1991), and Birkedal-Hansen et al. (1993) for reviews] and by heat treatment (Koklitis et al., 1991). To explain the activation of proMMPs by these multiple means, VanWart and colleagues have proposed the "cysteine switch" model (Springman et al., 1990; VanWart & Birkedal-Hansen, 1990). In this model it is postulated that activation occurs when the cysteine residue is transiently dissociated from the zinc atom and reacts with SH reagents, thus preventing the reassociation of the cysteine side chain and the zinc. Disruption of the cysteine–zinc coordination can be also induced by denaturants, oxidants, or partial proteolytic removal of the propeptide, which results in expression of the proteolytic activities of MMPs. The cysteine switch mechanism is proposed to be applicable to all members of the MMP family (VanWart & Birkedal-Hansen, 1990). A series of mutants of rat proMMP-3 expressed in Cos cells, in which the residues around the Cys-75 in the sequence PRCGVPD were changed, showed a markedly increased tendency to undergo spontaneous activation accompanied by autolytic loss of propeptide (Sanchez-Lopez et al., 1998; Park et al., 1991), suggesting the critical role of this sequence in maintaining the latent proMMP-3. On the other hand, mutation of Cys-75 to Ser, His, or Asp resulted in degradation

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[1] Abbreviations: MMPs, matrix metalloproteinases; SDS, sodium dodecyl sulfate; APMA, (4-aminophenyl)mercuric acetate; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); α_2M, α_2-macroglobulin; DNP-, dinitrophenyl-; [^{3}H]Cm-Tf, reduced, [^{3}H]carboxymethylated transferrin; PAGE, polyacylamide gel electrophoresis; CD, circular dichroism.

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of the enzyme protein rather than the generation of an active MMP-3 (Park et al., 1991). It was concluded that proMMP-3 mutated at Cys-75 became spontaneously activated and autolysed (Park et al., 1991). Expression of the proMMP-1 mutant with a replacement of Cys-73 with Ser in *Escherichia coli* resulted in primarily the 28-kDa and the 18-kDa species, of which the former contained both the propeptide and the catalytic domains and the latter contained only the catalytic domain (Windsor et al., 1991). While the 18-kDa molecule was proteolytically active and reacted with α_2-macroglobulin (α_2M), the 28-kDa species with the mutated propeptide failed to react with α_2M, indicating that this molecule was not active. The OH group of Ser-73 of the mutant is unlikely to be liganded to the zinc atom of the active site. Thus, it has not been unequivocally proven whether the disruption of the Cys–Zn interaction is sufficient to activate proMMPs.

In this report we examine the cysteine switch hypothesis using the native human proMMP-3 synthesized and secreted from rheumatoid synovial fibroblasts. The single cysteine in the propeptide of the zymogen was specifically modified by various SH reagents and the Cys-75-modified proMMP-3s were examined for their expression of enzymic activity and the stability of the molecule. Our results demonstrate that the disruption of the Cys–Zn coordination is not sufficient to activate proMMP-3.

EXPERIMENTAL PROCEDURES

Materials. Human proMMP-3 was purified from the culture medium of rheumatoid synovial fibroblasts stimulated with rabbit alveolar macrophage-conditioned medium as described previously (Ito & Nagase, 1988). (4-Aminophenyl)-mercuric acetate (APMA), chymotrypsin (bovine), iodoacetamide, and $HgCl_2$ were from Sigma Chemical Co. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was from Calbiochem. Green A Dyematrex gel was from Amicon Corp. [^{14}C]-Iodoacetamide was from Amersham. Human α_2-macroglobulin (α_2M) purified by the method of Imber and Pizzo (1981) was a gift from Dr. Jan J. Enghild, Duke University. Dinitrophenyl- (DNP-) Pro-Tyr-Ala-Tyr-Trp-Met-Arg was a gift from Dr. Harold E. VanWart, Florida State University.

Enzyme Assays. The proteolytic activity of proMMP-3 was measured before and after activation with 1 mM APMA or with chymotrypsin (10 μg/mL) using reduced, [^{3}H]-carboxymethylated transferrin ([^{3}H]Cm-Tf) as substrate in TNC buffer [50 mM Tris-HCl buffer (pH 7.5)/0.15 M NaCl/10 mM $CaCl_2$/0.05% Brij 35/0.02% NaN_3] (Okada et al., 1986). One unit of MMP-3 activity produced 1 μg of Cm-Tf fragments soluble in 3% (w/v) trichloroacetic acid in 1 min at 37 °C. The specific activity of proMMP-3 was 840 units/mg when fully activated with 1 mM APMA for 16 h at 37 °C. The enzymic activity against the synthetic peptide DNP-Pro-Try-Ala-Tyr-Trp-Met-Arg was described by Netzel-Arnett et al. (1991). The final concentration of the synthetic peptide was 1 μM, which was reacted with 20 nM modified proMMP-3 or activated MMP-3. The fluorescence of tryptophan (excitation at 280 nm with emission at 360 nm) was followed for 70 min at 23 °C with readings taken against the TNC buffer with the same concentration of the peptide as blank.

Alkylation of the Cys-75 of ProMMP-3. Purified pro-MMP-3 (1 μM) was incubated with 1 mM [^{14}C]iodoacetamide in the presence of 20 mM EDTA in TNC buffer, which contained 10 mM Ca^{2+}, at 23 °C for 15 min to alkylate Cys-75. The reaction was stopped by the addition of 5 mM cysteine, and the sample was then subjected to SDS–PAGE under reducing conditions (Bury, 1981). After proteins were stained with Coomassie Blue R-250, the gel was subjected to fluorography as described by Laskey and Mills (1977). ProMMP-3 (10 μM) was also reacted with 1 mM iodoacetamide, 1 mM APMA, or 1 mM DTNB in the presence of 20 mM EDTA in the TNC buffer at 23 °C for 15 min, and the samples were immediately dialyzed against 4 L of TNC buffer containing 50 μM $ZnCl_2$ at 4 °C for 4 h twice and then dialyzed once against TNC buffer without $ZnCl_2$. The enzymic activity of the modified proMMP-3 was measured with and without activation by 1 mM APMA or 10 μg/mL chymotrypsin.

Determination of Free SH Groups. A portion of the modified proMMP-3 (10 μM) was reacted with 1 mM DTNB at 23 °C in 0.1 M sodium phosphate buffer (pH 8) in the presence or absence of 20 mM EDTA. After 15 min the absorbance at 412 nm was read against TNC buffer with 1 mM DTNB. A molar extinction coefficient of 13 600 for 2-nitro-5-thiobenzoate was used to measure the amount of SH groups (Habeeb, 1972).

Purification of Modified ProMMP-3 by a Green A Dyematrex Gel. During the procedure to generate the Cys-75-modified proMMP-3, a small amount of the 45-kDa MMP-3 (<20%) was generated. To separate the modified proMMP-3 from MMP-3, the sample was applied to a Green A Dyematrex Gel column equilibrated with TNC buffer. The 45-kDa form of MMP-3 did not bind to Green A Dyematrex. The Green A-bound fraction containing proMMP-3 was eluted by TNC buffer with 0.5 M NaCl. This material was essentially free from the 45-kDa MMP-3.

Circular Dichroism Analysis. Circular dichroism (CD) spectra were obtained using a Jasco J-500A spectropolarimeter. The exact protein concentration of each sample (100 μg/mL) was determined by the bicinchoninic acid assay with crystalline bovine serum albumin as standard (Smith et al., 1985). Each sample was in TNC buffer. The CD spectra were recorded from 250 to 215 nm for proMMP-3 and the $HgCl_2$-activated proMMP-3 with a 0.5-cm or a 0.2-cm fused silica cell at 25 °C. All spectra represent the average of two runs (occasionally three runs). The sample data were obtained at 1.0-nm intervals and corrected for the solvent values. The mean residue ellipticity $[\theta]$ was calculated by the following equation and then plotted against the wavelength:

$$[\theta] = [\theta]_{obs}(MRW/10)/(cl) \quad (1)$$

where $[\theta]_{obs}$ is the observed ellipticity in cm at a particular wavelength, MRW is mean residue weight in grams per decimole, c is protein concentration in grams per cubic centimeter, and l is path length in cell in centimeters. A mean residue weight of 111 g/mol was used in the calculation.

RESULTS

Specific Modification of Cys-75 of ProMMP-3. Pro-MMP-3 has one cysteine (Cys-75) in the propeptide and two other cysteines in the C-terminal domain (Whitham et al., 1986; Saus et al., 1988). The electrophoretic mobility on SDS–polyacrylamide gels of the reduced 45-kDa MMP-3 that lacks the propeptide was less than that of the nonreduced 45-kDa MMP-3 (data not shown). This indicates that the two cysteines in the C-terminal domain are disulfide-bonded. The treatment of proMMP-3 with 1 mM DTNB did not reveal any reactive SH groups. However, when this reaction was carried out in the presence of 20 mM EDTA, 1 molecule of SH group was detected per proMMP-3 (Table I), indicating that one cysteine is cryptic by interacting with a metal ion

Table I: Determination of Free SH Groups in ProMMP-3 and the Modified ProMMP-3 in the Presence of EDTA[a]

zymogen preparation	EDTA treatment	free SH group/ proMMP-3
proMMP-3	–	0.00
	+	1.02
proMMP-3 modified with iodoacetamide	–	0.01
	+	0.03
proMMP-3 modified with APMA	–	0.07
	+	0.09
proMMP-3 modified with DTNB	–	0.00
	+	0.01

[a] ProMMP-3 (10 μM) was modified with various SH reagents in the presence of 20 mM EDTA in TNC buffer as described in Experimental Procedures. The number of free SH groups of the native and the modified proMMP-3 was determined by DTNB in the absence and presence of 20 mM EDTA.

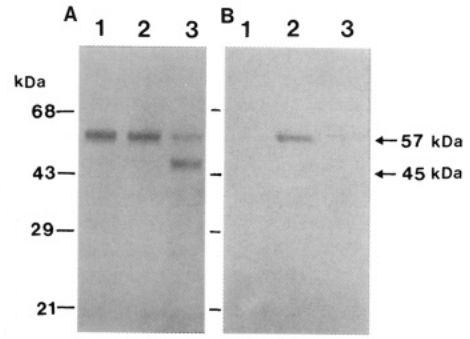

FIGURE 1: Incorporation of [^{14}C]iodoacetamide into Cys-75 of proMMP-3. Purified proMMP-3 (1 μM) was incubated with 1 mM [^{14}C]iodoacetamide and 20 mM EDTA at 23 °C for 15 min and the reaction was terminated by the addition of 5 mM cysteine. The samples were analyzed by SDS–PAGE (7.5% acrylamide) under reducing conditions and fluorography. (A) Proteins stained with Coomassie Blue R-250; (B) fluorography of the corresponding samples of (A). Lane 1, proMMP-3 (57 μg/mL) incubated with 1 mM [^{14}C]-iodoacetamide alone; lane 2, proMMP-3 treated with 1 mM [^{14}C]-iodoacetamide in the presence of 20 mM EDTA; lane 3, the sample of lane 2 was treated with chymoptrypsin (10 μg/mL) for 30 min at 37 °C.

(Salowe et al., 1992; Holz et al., 1992). Other SH reagents such as iodoacetamide and APMA also reacted with the SH group in the presence of EDTA and blocked the subsequent reaction with DTNB (Table I). To confirm the specific location of the alkylation site, [^{14}C]iodoacetamide was reacted with proMMP-3 in the presence and absence of EDTA for 15 min at 23 °C. The sample was run on SDS–PAGE and the site of [^{14}C]iodoacetamide incorporated into proMMP-3 was examined by fluorography. As shown in Figure 1, [^{14}C]-iodoacetamide reacted with proMMP-3 only in the presence of EDTA. The amount of radioactivity incorporated into the 57-kDa band was 1.02 molecules of [^{14}C]iodoacetamide/ molecule of proMMP-3. When [^{14}C]iodoacetamide-labeled proMMP-3 was treated with chymotrypsin , it was converted to an active 45-kDa MMP-3. Fluorographic analysis of this species showed that the 45-kDa MMP-3 did not contain any radioactivity. These results indicate that [^{14}C]iodoacetamide was incorporated exclusively into the propeptide. After this modification of proMMP-3 there were no detectable SH groups even in the presence of 20 mM EDTA, indicating that Cys-75 was specifically alkylated.

Proteolytic Activity of the Cys-75-Modified ProMMP-3. Modification of Cys-75 was carried out by reacting proMMP-3 with 1 mM iodoacetamide, 1 mM APMA, or 1 mM DTNB in the presence of 20 mM EDTA in TNC buffer, which contains 10 mM Ca^{2+}. The samples were then immediately dialyzed against the TNC buffer containing 50 μM $ZnCl_2$.

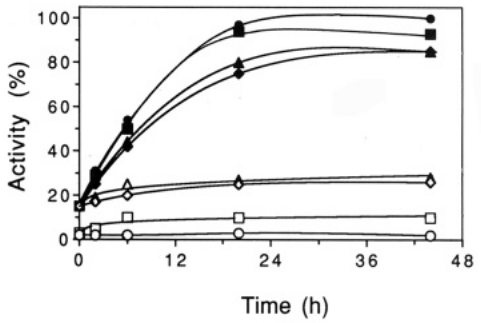

FIGURE 2: Proteolytic activity of the modified proMMP-3s before and after APMA treatment. The Cys-75 of proMMP-3 was targeted for modification of SH reagents in the presence of 20 mM EDTA as described in Experimental Procedures. The enzymic activities were measured against [^{3}H]Cm-Tf as substrate before (open symbols) and after (filled symbols) treatment with 1 mM APMA for the indicated periods of time. (○, ●) ProMMP-3; (□, ■) proMMP-3 modified with iodoacetamide; (△, ▲) proMMP-3 modified with APMA; (◇, ◆) proMMP-3 modified with DTNB.

After removal of the excess reagents by dialysis each sample was assayed for its activity before and after treatment with 1 mM APMA at 37 °C. Under these experimental conditions, the EDTA-treated proMMP-3 (apo-proMMP-3) reverted almost completely to the fully activatable proMMP-3 by replenishing Zn^{2+}. The proteolytic activity against [^{14}C]Cm-Tf showed that about 10–25% activity was detected with the modified proMMP-3s but that incubation at 37 °C up to 42 h did not produce any significant increase in activity (Figure 2). On the other hand, when the modified proMMP-3s were treated with 1 mM APMA, they were activated with kinetics similar to those of native proMMP-3 (Figure 2). Apo-proMMP-3 and apo-modified-proMMP-3 did not show any enzymic activity even after APMA treatment unless they were replenished with $ZnCl_2$ (data not shown). These results indicate that disruption of the Cys-Zn coordination did not cause much spontaneous activation of proMMP-3, but the modified proMMP-3 was still activatable by APMA. SDS–PAGE analysis of the modified proMMP-3 showed that the major component of the modified sample was the 57-kDa proenzyme, although there was about 10–20% of the 45-kDa species. Thus, it was difficult to conclude whether the 10–25% activity detected with the modified proMMP-3 was due to the low specific activity of the modified zymogen or if it was solely due to the 45-kDa active form of MMP-3. To clarify this, the reaction between the modified proMMP-3 and α_2M was examined (Figure 3). It is postulated that as long as the modified proMMP-3 is proteolytically active, even with a low specific activity, the extent of the cleavage of α_2M subunit should be similar to that of the fully active 45-kDa MMP-3, since the amount of proteolytic fragments of α_2M generated upon reaction with a proteinase depends on the number of active enzyme molecules (Barrett, 1981). However, the amount of the proteolytic fragments (94 and 80 kDa) of α_2M generated by the modified proMMP-3 was far less than the amount generated by the modified proMMP-3 activated with APMA, even after a 24-h reaction. In addition, the amount of proteolytic fragments correlated with the amount of 45-kDa species generated during the modification procedure, suggesting that the modified proMMP-3 does not possess proteolytic activity.

Separation of the Modified ProMMP-3 and the 45-kDa MMP-3. The absence of proteolytic activity of the Cys-75-modified proMMP-3 was further examined after separation of the 57-kDa proenzyme from the 45-kDa MMP-3 by Green A Dyematrex Gel. As shown in Figures 4 and 5, the isolated,

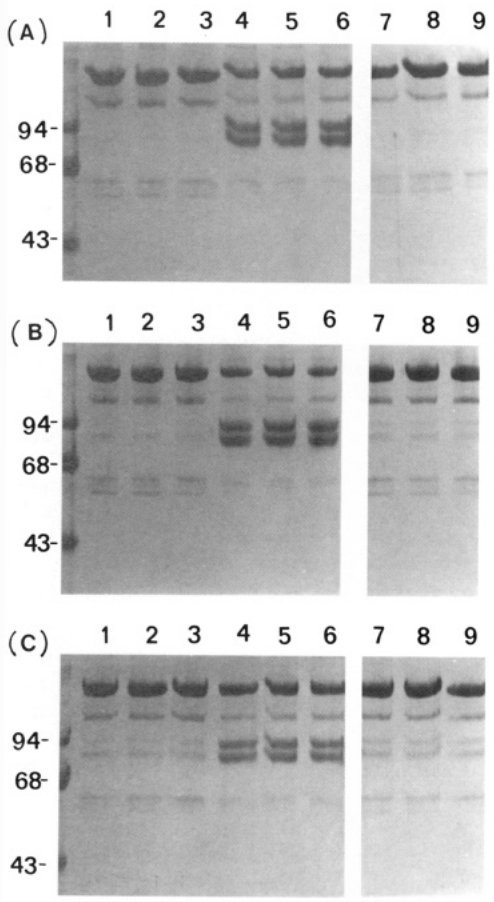

FIGURE 3: Interaction of the modified proMMP-3s with α_2M. The modified proMMP-3s were reacted with α_2M in the molar ratio of 1:8 at 37 °C for various periods of time. The reaction was stopped by adding 20 mM EDTA, and the samples were analyzed by SDS–PAGE (7.5% acrylamide) under reducing conditions. α_2M reacted with (A) proMMP-3, (B) proMMP-3 modified with iodoacetamide, and (C) proMMP-3 modified with APMA. Lanes 1–3, the modified proMMP-3s were incubated with α_2M for 2, 6, and 24 h, respectively. Lanes 4–6, same as lanes 1–3 but the proMMPs were activated with 1 mM APMA at 37 °C for 18 h. Lanes 7–9, same as lanes 1–3 but the samples were incubated at 37 °C for 18 h before reaction with α_2M. Protein bands of 94 and 80 kDa are proteolytic fragments generated from the α_2M subunit (180 kDa) after the reaction with MMP-3.

modified proMMP-3s were essentially free from the 45-kDa MMP-3 and had little activity against [^{14}C]Cm-Tf. However, these molecules were readily converted to the 45-kDa MMP-3 by APMA treatment (Figure 5). Absence of proteolytic activity was also confirmed by the lack of action on α_2M even after incubation for 24 h at 37 °C unless the modified proMMP-3s were treated with APMA (data not shown).

Lack of Enzymic Activity of the Cys-75-Modified Pro-MMP-3 against DNP-Pro-Tyr-Ala-Tyr-Trp-Met-Arg. The above results suggest that the Cys-75-modified proMMP-3 lacks proteolytic activity, but it is possible that this is due to the nature of substrates used (Cm-Tf or α_2M) since the 82-amino acid propeptide is still attached to the enzyme (Figure 5) and therefore large protein substrates may have restricted access to the active site of the enzyme. To test this possibility we examined the enzymic activity of the modified proMMP-3 using a heptapeptide substrate, DNP-Pro-Tyr-Ala-Tyr-Trp-Met-Arg. As shown in Table II, the peptidolytic activity of the modified proMMP-3 was negligible. These results further provide evidence that the modification of Cys-75 by iodoacetamide, APMA, or DTNB does not express the enzymic activity of proMMP-3.

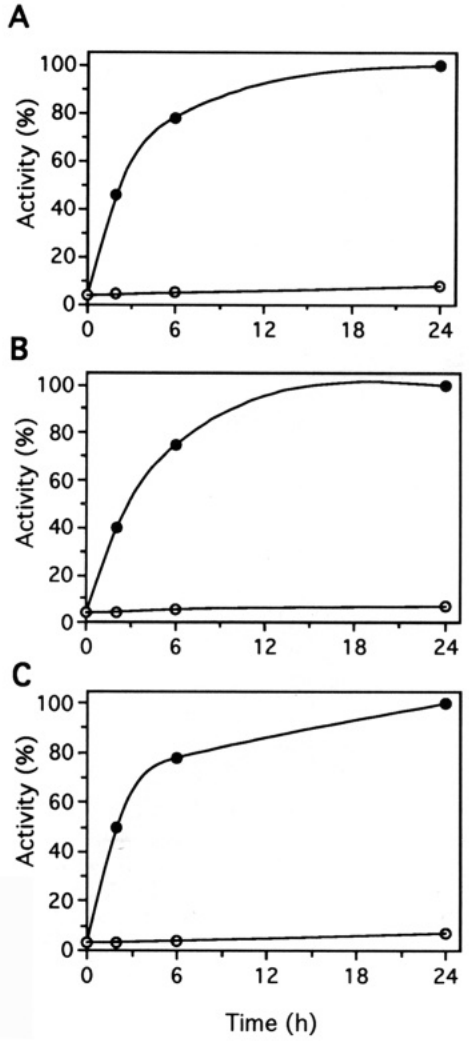

FIGURE 4: Activation of the purified Cys-75-modified proMMP-3s by APMA. The Cys-75-modified proMMP-3s were purified by a Green A Dyematrex Gel column and their enzymic activities were measured against [^{3}H]Cm-Tf before (○) and after (●) treatment with 1 mM APMA at 37 °C for the indicated periods of time. (A) ProMMP-3 modified with iodoacetamide; (B) proMMP-3 modified with APMA; (C) proMMP-3 modified with DTNB.

Table II: Enzymatic Activity of the Cys-75-Modified ProMMP-3s against DNP-Pro-Tyr-Ala-Tyr-Trp-Met-Arg[a]

enzyme	activity: pmol min^{-1} mg^{-1}	activity: %
MMP-3	4600	100
proMMP-3	8	0.2
proMMP-3 treated with EDTA	23	0.5
proMMP-3 modified with iodoacetamide	55	1.2
proMMP-3 modified with APMA	46	1.0
proMMP-3 modified with DTNB	69	1.5

[a] Cys-75 of proMMP-3 was modified by various reagents as described in Experimental Procedures and further purified by a column of Green A Dyematrex Gel. The synthetic substrate at a final concentration of 1 μM was reacted with 20 nM proMMP-3 or MMP-3 for 70 min at 23 °C, and the increase in fluorescence of tryptophan was measured with excitation at 280 nm and emission at 360 nm.

CD Spectra of ProMMP-3 Treated with $HgCl_2$. The cysteine switch hypothesis proposes that mercurial compounds initiate the activation of proMMPs by reacting with the conserved Cys in the propeptides (Springman et al., 1990). However, our results demonstrating the lack of enzymic activity after chemical modification of Cys-75 in proMMP-3,

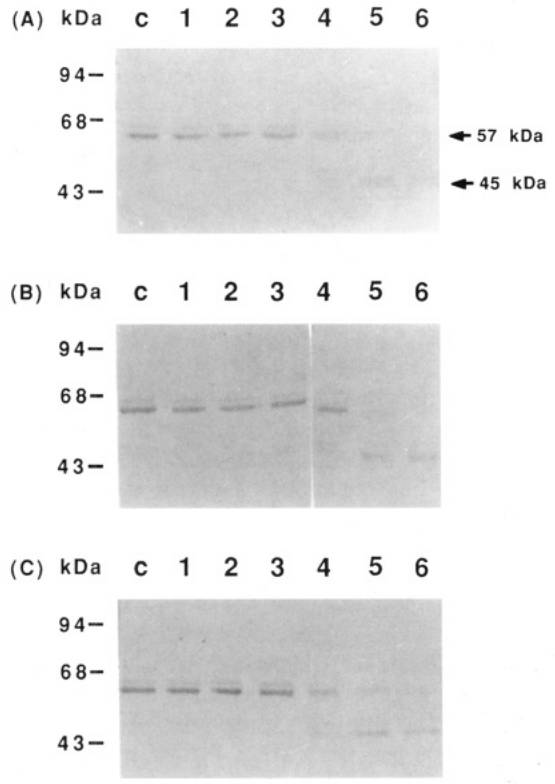

FIGURE 5: SDS–PAGE analysis of the modified proMMP-3s. ProMMP-3 modified with (A) iodoacetamide, (B) APMA, or (C) DTNB was further purified by the Green A Dyematrex Gel and incubated with or without 1 mM APMA at 37 °C. The samples were subjected to SDS–PAGE (7.5% acrylamide). Lane C, the modified proMMP-3s without incubation. Lanes 1–3, the modified proMMP-3s incubated for 2, 6, and 24 h at 37 °C, respectively. Lanes 4–6, same as lanes 1–3 but the samples were treated with 1 mM APMA at 37 °C.

even with APMA, indicate that the initial step of proMMP-3 activation by a mercurial compound is due to the molecular perturbation of the zymogen rather than the result of interaction with Cys-75. To investigate this further we examined the changes of CD spectra of proMMP-3 during activation with $HgCl_2$. The studies with $HgCl_2$ treatment at 37 °C showed that the changes in CD spectra correlated with the generation of the 45-kDa MMP-3 as well as the expression of proteolytic activity (Figure 6). The removal of the propeptide would make interpretation of CD spectral changes ambiguous. Thus, there was no clear evidence that $HgCl_2$ triggers conformational changes in proMMP-3 prior to processing of the zymogen. On the other hand, when the effect of $HgCl_2$ on proMMP-3 was examined at 25 °C, there were time-dependent progressive changes in CD spectra without any concomitant processing of proMMP-3 or significant expression of the proteolytic activity (Figure 7). This indicates that $HgCl_2$ initially perturbs the conformation of proMMP-3 prior to processing the propeptide. In addition, there was no notable difference in CD spectra between the alkylated proMMP-3 and proMMP-3, indicating that the alkylation of Cys-75 did not cause any significant changes in the secondary structure of proMMP-3. Addition of $HgCl_2$ to this sample showed similar but slightly faster changes in the CD spectrum (data not shown). After a 44-h incubation, however, the 57-kDa proMMP-3 was converted to a 54-kDa species which exhibited about 10% of the proteolytic activity. These results indicate that activation of proMMP-3 by a mercurial compound is initiated by conformational changes in the zymogen but not due to its binding to Cys-75.

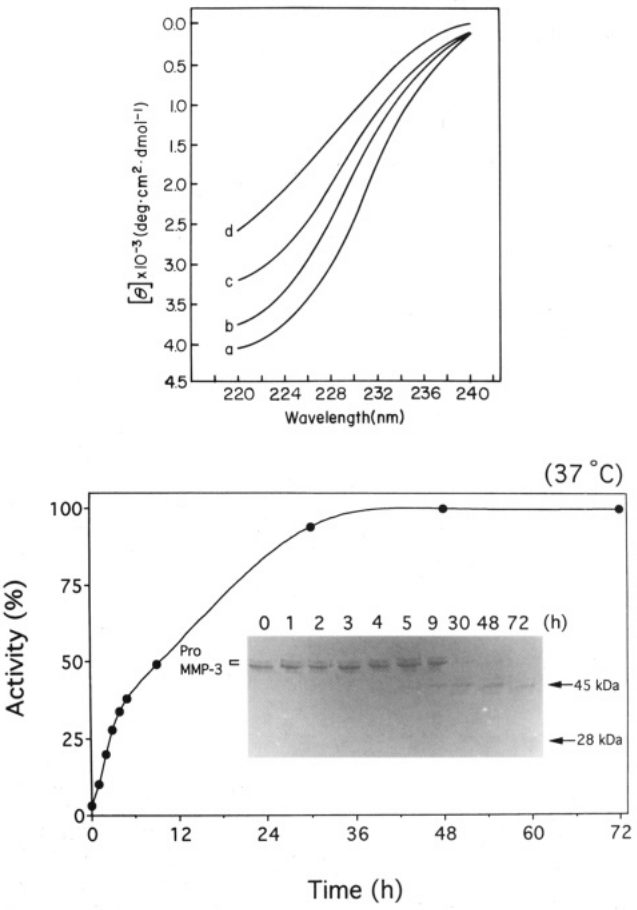

FIGURE 6: CD spectra of proMMP-3 upon activation with $HgCl_2$ at 37 °C. (Top) ProMMP-3 (100 μg/mL) was incubated with 100 μM $HgCl_2$ at 37 °C and CD spectra were obtained as described in Experimental Procedures at (a) 0, (b) 6, (c) 30, and (d) 74 h. (Bottom) The portions of the proMMP-3 sample from the top panel were removed after incubation for the indicated periods of time and subjected to enzyme assay, using [^{3}H]Cm-Tf, and to SDS–PAGE analysis (inset).

DISCUSSION

The maintenance of latency of proMMPs through the interaction of the cysteine in the propeptide and the zinc atom of the active site has been demonstrated by spectroscopic evidence of sulfur ligation to Co^{2+} in the cobalt-substituted proMMP-3 as well as in the cobalt-substituted active MMP-3 complexed with a sulfur-containing synthetic inhibitor (Salowe et al., 1992) and by extended X-ray absorption fine structure (EXAFS) spectroscopy (Holz et al., 1992). This interaction is likely to be facilitated by the conserved sequence of PRCG-(V/N)PD (73–79; the numbering is after proMMP-3) in the propeptide since a single mutation of Pro73 to Leu, Pro78 to Val or Asn, or Asp79 to Tyr (Sanchez-Lopez et al., 1988) increased the tendency to undergo spontaneous activation of rat proMMP-3. Similar observations were also made by Park et al. (1991) on other mutants involving this region. However, the mutation of Arg-74 to Lys, Gln, or Leu or of Cys-75 to Ser, His, or Asp resulted not only in low levels of expression in Cos-7 cells but also in degradation of the protein (Park et al., 1991). The failure of proMMP-3 or active MMP-3 to be expressed in Cos-7 cells by mutating Cys-75 to other residues suggests that Cys-75 may play a critical role in the folding of the proMMP-3 molecule during synthesis. Thus, the specific role of the Cys–Zn interaction in proMMP activation has not been clarified.

To test whether the disruption of the Cys–Zn interaction results in spontaneous activation of proMMPs, we employed

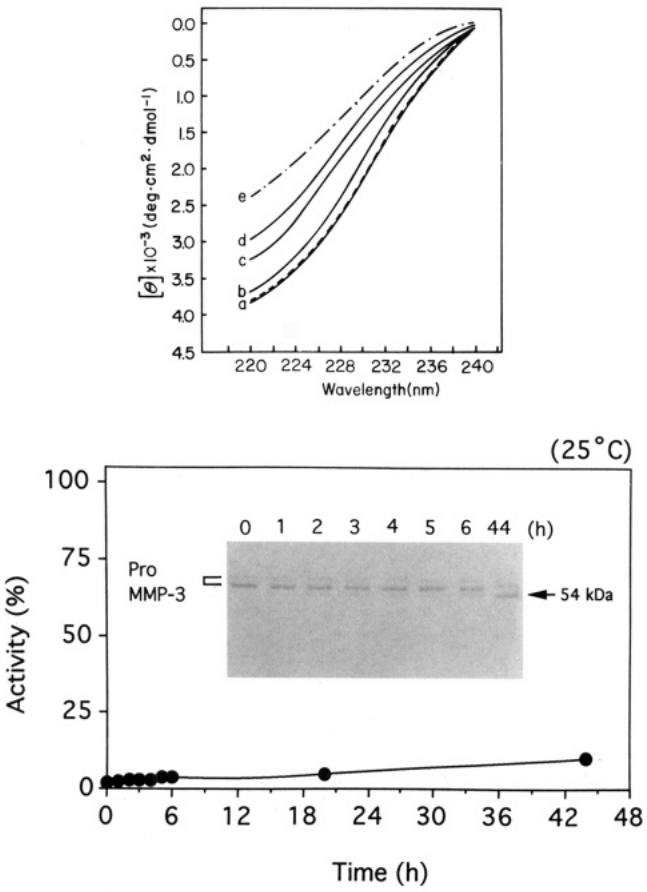

FIGURE 7: CD spectra of proMMP-3 upon activation with $HgCl_2$ at 25 °C. (Top) ProMMP-3 (100 μg/mL) was incubated with 100 μM $HgCl_2$ at 25 °C and the CD spectra (—) were determined at (a) 0, (b) 2, (c) 6, and (d) 44 h. CD spectra of the 45-kDa proMMP-3 (---) and the alkylated proMMP-3 (- - -) are shown. (Bottom) Portions of the proMMP-3 sample from the top panel were removed after incubation at indicated periods of time and subjected to enzyme assay, using [^{3}H]Cm-Tf, and to SDS–PAGE analysis (inset).

the natural human proMMP-3 secreted from rheumatoid synovial cells (Okada et al., 1988). The treatment of proMMP-3 with 20 mM EDTA in TNC buffer which contains 10 mM Ca^{2+} allowed Cys-75 to be modified by iodoacetamide, APMA, or DTNB. The specific modification of Cys-75 was shown by the observation that [^{14}C]iodoacetamide was incorporated into the propeptide and that once one molecule of [^{14}C]iodoacetamide was bound to proMMP-3, the SH group was no longer detectable even in the presence of 20 mM EDTA. This modification was not possible, however, when proMMP-3 was treated with 2 mM 1,10-phenanthroline, in good agreement with the results reported for proMMP-1 (Springman et al., 1990) and proMMP-9 (progelatinase B) (Lyons et al., 1991). Unavailability of a SH group for alkylation in the presence of 1,10-phenanthroline also agrees with the recent studies by Salowe et al. (1992), who found that the zinc in the active site of the mature MMP-3 was removed by dialysis against 1,10-phenanthroline but not the zinc in proMMP-3.

When Cys-75 was modified with iodoacetamide, APMA or DTNB, a small amount of the 45-kDa MMP-3 (10–20%) was generated, but the majority of the modified proMMP-3 was found to be the precursor form of molecular mass 57 kDa. The interaction of Cys-75 with Zn is considered to be disrupted in the modified proMMP-3, particularly with proMMP-3 containing bulky (aminophenyl)mercuric and dithionitrophenyl groups attached to Cys-75. However, all these proenzymes were essentially inactive against protein substrates and an octapeptide substrate, and an increase in enzymic activity was not observed even after incubation at 37 °C at least for 43 h. Inability to activate proMMPs by the disruption of the Cys–Zn interaction is also supported by the failure of the 28-kDa species of MMP-1 generated as a result of mutation of Cys-75 to Ser to react with α_2M (Windsor et al., 1991). Our result with APMA modification was unexpected since the mode of proMMP activation by a mercurial compound has been considered to be due to its interaction with the conserved residue in the propeptide (Springman et al., 1990; Bläser et al., 1992). The activation of the modified proMMP-3 required the continuous presence of 1 mM APMA (Figure 6) or treatment with a proteinase (Figure 1), as we reported previously for the native proMMP-3 (Okada et al., 1988). These observations suggest that the initial event in which a mercurial compound triggers the activation of proMMP-3 is not binding to the Cys-75, but rather it involves perturbation of the zymogen conformation by a mercurial compound. This was supported by initial changes of CD spectra of proMMP-3 incubated with 100 μM $HgCl_2$ at 25 °C, under which conditions little processing of the precursor and expression of the enzymic activity were observed. We postulate that the molecular perturbation induced by APMA thus destabilizes the Cys–Zn interaction, which then allows Cys-75 to readily react with the APMA and permanently dissociate the Cys–Zn complex. The failure to detect spontaneous activation of the Cys-75-modified proMMP-3 further suggests that the propeptide tightly interacts with the mature enzyme and stabilizes the proenzyme conformation. Indeed, the alkylation of Cys-75 with iodoacetamide did not cause any significant changes in the secondary structure of proMMP-3 (Figure 7). However, the ability of a number of proteinases to activate proMMP-3 by removing about 35 amino acids from the N-terminal (Nagase et al., 1990) indicates that this segment is involved in stabilization of the proenzyme. The removal of the N-terminal portion of propeptide causes the disruption of the Cys–Zn interaction, which renders the intermediates to be further processed by a bimolecular reaction involving either the intermediate or active MMP-3 (Nagase et al., 1991). These processes were also found for proMMP-1 (Grant et al., 1987; Suzuki et al., 1990), proMMP-7 (matrilysin) (Crabbe et al., 1992), and proMMP-9 (Ogata et al., 1992; Goldberg et al., 1992).

In conclusion, the disruption of Cys–Zn interaction is essential for activation of proMMPs as the association of H_2O is required for catalytic reaction, but our studies demonstrate that this process by itself is not sufficient to activate the secreted proMMP-3. The activation of proMMP-3 by a mercurial compound is triggered by perturbation of the zymogen, perhaps by the initial binding to a site(s) other than Cys-75 in the zymogen. On the other hand, proMMP-1 and proMMP-8 (neutrophil collagenase) were shown to be activated by HOCl (Weiss et al., 1985; Springman et al., 1990; Michaelis et al., 1992), and at least partially by a number of SH reagents such as DTNB, *N*-ethylmaleimide, and oxidized glutathione in the case of proMMP-1 (Springman et al., 1990). Activation of these two proMMPs by these treatments is thought to be due to the disruption of Cys–Zn interaction (Springman et al., 1990). Thus, it is quite possible that some other proMMPs may be activated by a similar mechanism depending on the stability of the interaction between the propeptide and the enzyme molecule. Such differences may give some important insights into which proMMPs may be activatable in vivo by a nonproteolytic pathway such as oxidation. The cysteine switch mechanism for other MMPs remains to be investigated in full.

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