The precursor of a metalloendopeptidase from human rheumatoid synovial fibroblasts

Purification and mechanisms of activation by endopeptidases and 4-aminophenylmercuric acetate

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Two active forms (M_r 45000 and 28000) of a metalloendopeptidase that digest proteoglycans and other extracellular matrix components of connective tissues have previously been purified from rheumatoid synovial cells and characterized [Okada, Nagase & Harris (1986) *J. Biol. Chem.* **261**, 14245–14255]. To study the mechanisms of activation the precursor of this metalloendopeptidase has now been purified. The final products are homogeneous on SDS/polyacrylamide-gel electrophoresis and identified as a set of zymogens of M_r 57000 and 59000, in which the latter form is probably the product of post-translational glycosylation of the M_r 57000 zymogen, as it binds to concanavalin A. The zymogen can be activated by trypsin, chymotrypsin, plasma kallikrein, plasmin and thermolysin, but not by thrombin. Although the activated metalloendopeptidase is further degraded by trypsin, plasma kallikrein and thermolysin during a prolonged incubation, it is relatively stable against plasmin and chymotrypsin. Activation with 4-aminophenylmercuric acetate is dependent on its concentration. It requires the reaction with the zymogen, possibly through thiol groups, and the continued presence of the agent. During this treatment the zymogen undergoes a sequential processing; first it becomes active without changing its apparent molecular mass, and then it is processed to low- M_r species of M_r 46000, 45000 (HMM) and 28000 (LMM). The rate of conversion of the precursor into an initial intermediate of M_r 46000 follows first-order kinetics ($t_{1/2}$ 2.0 h with 1.5 mM 4-aminophenylmercuric acetate at 37 °C) and is independent of the initial concentration of the zymogen or the presence of up to a 676-fold molar excess of substrate, whereas the generation of HMM and LMM species is affected by these parameters. These results indicate that activation of the prometalloendopeptidase by an organomercurial compound is initiated by the molecular perturbation of the zymogen that results in conversion into the 46000- M_r intermediate by an intramolecular action; the subsequent processing of this intermediate in HMM and LMM species is a bimolecular reaction. *In vivo* it is probable that the precursor of this metalloendopeptidase is activated either by direct limited proteolysis by tissue or plasma endopeptidases, or, alternatively, by factors that cause certain conformational changes in the zymogen molecule.

INTRODUCTION

Human rheumatoid synovial fibroblasts in culture stimulated by conditioned media from macrophages synthesize and secrete at least three distinct metalloendopeptidases, which all have the potential to digest the major macromolecules of the connective-tissue matrix at neutral pH (Okada *et al.*, 1986). Previously, we have described an efficient way to separate these three enzymes, and one of them, referred to as matrix metalloendopeptidase-3 (MMP-3), has been further purified and characterized (Okada *et al.*, 1986). MMP-3 digests proteoglycans, gelatin, laminin, type IV collagen and fibronectin, and removes the *N*-terminal propeptide of type I procollagen (Okada *et al.*, 1986). Studies reporting the 3–10-fold elevation of proteoglycan-degrading

activity in osteoarthritic compared with normal cartilage (Martel-Pelletier *et al.*, 1984; Woessner *et al.*, 1986) suggest that an enzyme such as MMP-3 may play a significant role in pathological destruction of cartilage. However, like two other matrix metalloendopeptidases [collagenase and MMP-2 ('gelatinase')] from rheumatoid synovial cells (Vater *et al.*, 1978; Okada *et al.*, 1986) and from other connective tissues (Sellers *et al.*, 1978; Vaes *et al.*, 1978; Meikle *et al.*, 1980; Heath *et al.*, 1982), MMP-3 is secreted in an inactive form, such that its full activity *in vitro* cannot be detected unless the culture medium is first treated with trypsin or mercurial compounds. In our previous paper, we activated latent MMP-3 by NH_2PhHgAc and isolated two active forms with M_r values of 45000 (HMM) and 28000 (LMM) (Okada *et al.*, 1986). Since both forms had similar

Abbreviations used: MMP, matrix metalloendopeptidase; HMM, high molecular mass; LMM, low molecular mass; NH_2PhHgAc , 4-aminophenylmercuric acetate; Cm-Tf, reduced carboxymethylated transferrin.

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specific activities, specificities and related peptide maps we concluded that they were derived from the same precursor protein upon activation. The properties of MMP-3 purified from human rheumatoid synovial cells in culture are essentially identical with those described for the enzyme purified from the culture media of rabbit bone explants, named 'proteoglycanase' (Galloway *et al.*, 1983), and of rabbit synovial fibroblasts, named 'stromelysin' (Chin *et al.*, 1985). The precursor form of rabbit MMP-3 (stromelysin or proteoglycanase) has been identified as a polypeptide of M_r 51 000 by using immunochemical techniques (Chin *et al.*, 1985).

To identify the precursor of human MMP-3 and to investigate steps of its activation by a proteolytic enzyme or a mercurial compound, we have isolated the latent form of MMP-3 from human rheumatoid synovial cells. The precursor of human MMP-3 (proMMP-3) when purified, has been shown to be activated by a number of endopeptidases by limited proteolysis regardless of their substrate specificities, whereas the initial step of the activation of proMMP-3 by NH_2PhHgAc involves the conformational changes of the zymogen molecule, which is then processed to lower- M_r active species.

EXPERIMENTAL

Materials

Materials were obtained as follows: NH_2PhHgAc , Brij 35, carbonic anhydrase, chymotrypsin, cysteine, diisopropyl phosphorofluoridate, EDTA, gelatin, immunoglobulin G (human), iodoacetamide, lysozyme, 2-mercaptoethanol, α -methyl D-mannoside, ovalbumin, phenylmethanesulphonyl fluoride, phosphorylase *b*, plasminogen (human), serum albumin (bovine), soya-bean trypsin inhibitor, streptokinase, thermolysin, thrombin (bovine), Tos-Lys- CH_2Cl , transferrin (human) and Tos-Phe- CH_2Cl -treated trypsin were from Sigma Chemical Co.; phosphoramidon was from Peninsula; Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) was from Pierce Chemical Co.; pressure filtration apparatus, YM-10 membrane and Green A Dyematrix gel were from Amicon Corp; DEAE-cellulose (DE-52) was from Whatman; Affi-gel 10 was from Bio-Rad Laboratories; concanavalin A-Sepharose and Sephadex G-10 were from Pharmacia Fine Chemicals; Ultrogel AcA-44 was from LKB; [^{14}C]acetic anhydride (20 mCi/mmol), Na^{125}I (16.4 mCi/mg) and [^3H]iodoacetic acid (90 mCi/mmol) were from Amersham; Dulbecco's modified Eagle's medium, antibiotics and fetal-calf serum were from Gibco. Rabbit alveolar-macrophage-conditioned medium was a gift from Dr. C. L. Mainardi, Veterans Administration Medical Center, Memphis, TN, U.S.A. Pro-Phe-Arg- CH_2Cl was a gift from Dr. C. Kettner, Brookhaven National Laboratory, Upton, NY, U.S.A. Human plasma kallikrein was prepared by the procedure of Nagase & Barrett (1981). Anti-(human collagenase) IgG-Affi-Gel 10 column was prepared as described elsewhere (Vater *et al.*, 1981). HMM and LMM forms of MMP-3 were purified as described previously (Okada *et al.*, 1986).

Cell cultures

Human synovial tissue obtained at arthroplasty from patients with definite or classical rheumatoid arthritis

was minced and dissociated from matrix enzymically according to the method of Dayer *et al.* (1976). Cells from the first-passage generation were stimulated to produce metalloendopeptidases with rabbit alveolar-macrophage-conditioned medium in serum-free medium as described previously (Okada *et al.*, 1986).

MMP-3 activity was assayed by using [^3H]Cm-Tf as a substrate according to a method devised by T. Yee, D. R. Howard, E. D. Harris, Jr. & H. Nagase (unpublished work). One unit of MMP-3 activity was defined as the production of 1 μg of Cm-Tf fragments soluble in 3.3% (w/v) trichloroacetic acid in 1 min at 37 °C.

Iodination of proMMP-3

Purified proMMP-3 (11 μg) and its concanavalin A-bound form (2 μg) were iodinated according to the method of Fraker & Speck (1978). After labelling the proteins, free I^- was removed from iodinated samples by spin columns of Sephadex G-10 equilibrated with 50 mM-Tris/HCl buffer (pH 7.5)/0.15 M-NaCl/5 mM- Ca^{2+} /0.05% Brij 35/0.02% NaN_3 as described previously (Okada *et al.*, 1986).

Gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed by the method of Barrett *et al.* (1979), a modification of the 2-amino-2-methylpropane-1,2-diol/glycine/HCl discontinuous system of Wyckoff *et al.* (1977). ^{125}I -labelled protein samples were electrophoresed on 10% polyacrylamide gels with SDS and 2-mercaptoethanol, fixed, stained and autoradiographed. Gelatin-substrate-gel electrophoresis was performed as described by Hibbs *et al.* (1985).

Purification of proMMP-3

All purification procedures were carried out at 4 °C with buffer containing 10 mM- Ca^{2+} , 0.05% Brij 35 and 0.02% NaN_3 unless otherwise stated. The activity of proMMP-3 was measured against [^3H]Cm-Tf after activation with 1.5 mM- NH_2PhHgAc [60 min at 37 °C for the column fractions; for 4 h at 37 °C for the pooled sample obtained after each purification step (see Table 1)].

Partial purification of proMMP-3. The first three steps used for the purification of proMMP-3 were essentially the same as those reported previously for the purification of MMP-3 (Okada *et al.*, 1986).

Separation of the glycosylated form of proMMP-3. The proMMP-3 pool (37.5 mol) obtained after gelatin-Affi-Gel chromatography was applied to a column of concanavalin A-Sepharose (0.7 cm $\times$ 15 cm) equilibrated with 50 mM-Tris/HCl buffer (pH 7.5)/0.4 M-NaCl. ProMMP-3 bound to concanavalin A was eluted with 1 M- α -methyl D-mannoside.

Further purification of proMMP-3. The concanavalin A-unbound proMMP-3 fraction (46.5 ml) was passed through an anti-(human collagenase) immunoadsorbent column (0.7 cm $\times$ 15 cm) equilibrated with 50 mM-Tris/HCl buffer (pH 7.5)/0.4 M-NaCl to remove contaminating procollagenase. It was then concentrated to 1.5 ml by a YM-10 membrane and applied to an Ultrogel AcA-44 column (1.5 cm $\times$ 115 cm) equilibrated with 50 mM-

Table 1. Purification of proMMP-3

See the text for experimental details. Concentration of protein was taken from the absorbance at 280 nm assuming that 1 A_{280} unit $\equiv$ 1 mg/ml.

Step	Total protein (A_{280} units)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Concentrated medium	—	9472	—	—	100
DEAE-cellulose	130	8300	64	1	88
Green A Dymatrex	4.0	4158	1040	16	44
Gelatin-Affi-Gel	3.9	4125	1058	17	44
Concanavalin A-Sepharose*	4.4	2697	613†	10	28
Anti-(human collagenase) IgG immunoadsorbent	4.1	2522	615†	10	27
Ultrogel AcA-44	1.3	2091	1608	25	22
Ultrogel AcA-44	0.9	1463	1626	25	17

* Data denote concanavalin A-unbound proMMP-3.

† Decrease in specific activity is due to the contamination of the sample with concanavalin A and other proteins from concanavalin A-Sepharose.

Tris/HCl buffer (pH 7.5)/0.4 M-NaCl. The fractions of proMMP-3 (17 ml) were pooled, concentrated and re-chromatographed on the same Ultrogel AcA-44 column. Similarly, concanavalin A-bound proMMP-3 was further purified by gel-permeation chromatography.

Fig. 1. Separation of two species of proMMP-3

Two species of proMMP-3 (M_r 57000 and 59000) were separated by a column of concanavalin A-Sepharose, and each species was further purified as described in the text. Final products were labelled with ^{125}I by the Iodogen method (Fraker & Speck, 1978) and identified by SDS/polyacrylamide-gel electrophoresis followed by autoradiography. Lane 1, concanavalin A-bound and -unbound proMMP-3; lane 2, concanavalin A-bound proMMP-3; lane 3, concanavalin A-unbound proMMP-3. Protein standards are phosphorylase *b* (M_r 94000), transferrin (M_r 77000), bovine serum albumin (M_r 68000), heavy chain of IgG (M_r 55000), ovalbumin (M_r 43000) and carbonic anhydrase (M_r 29000).

RESULTS

Purification of proMMP-3

The results of purification of proMMP-3 from the culture medium of rheumatoid synovial cells stimulated by macrophage-conditioned medium are summarized in Table 1. Gelatin-Affi-Gel and anti-(collagenase) IgG-Affi-gel immunoadsorbent column chromatography were found to be effective steps to obtain a proMMP-2- and procollagenase-free preparation. The majority of proMMP-3 did not bind to concanavalin A-Sepharose. The final product of concanavalin A-unbound proMMP-3 exhibited a specific activity of 1626 units/ A_{280} unit against [^3H]Cm-Tf when activated with NH_2PhHgAc . It was homogeneous on SDS/polyacrylamide-gel electrophoresis with M_r 57000 (Fig. 1). The overall yield of concanavalin A-unbound proMMP-3 was 17% with 25-fold purification after the DEAE-cellulose step. About 10% of proMMP-3 was bound to concanavalin A and eluted with 1 M- α -methyl D-mannoside. The purified material had a similar specific activity of 1200 units/ A_{280} unit against [^3H]Cm-Tf and was homogeneous on SDS/polyacrylamide-gel electrophoresis with M_r 59000 (Fig. 1). When concanavalin A-unbound and -bound proMMP-3 were treated with 1.5 mM- NH_2PhHgAc , both proenzymes were processed and converted into similar low- M_r species in a parallel manner, indicating that the two forms are composed of the same polypeptide chain and that the concanavalin A-bound proenzyme with M_r 59000 represents a glycosylated form of the 57000- M_r proMMP-3. For investigation of the mechanism of proMMP-3 activation only the 57000- M_r species was used, as it was available in larger quantities.

Identification of proMMP-3 as the precursor of MMP-3

In order to establish that the purified proenzyme is indeed the precursor of the previously characterized MMP-3 we compared the substrate specificity of the purified zymogen after activation with 1.5 mM- NH_2PhHgAc with that of independently purified active MMP-3. When an equal amount of activity of the

NH₂PhHgAc-activated zymogen and the HMM or LMM active form of MMP-3 were incubated with Cm-Tf, and the digestion products were analysed by SDS/polyacrylamide-gel electrophoresis, the peptides generated by these three different enzyme preparations at various incubation times were identical (results not shown). This indicates that the purified zymogen is the precursor of MMP-3.

Stability of proMMP-3

Purified proMMP-3 was found to be stable in the buffer used for gel-permeation chromatography. Neither spontaneous activation nor loss of activity occurred during storage at 22 °C for at least 6 weeks or at 4 °C for 3 months. When the latter sample was examined by SDS/polyacrylamide-gel electrophoresis there were no changes in *M_r*.

Activation of proMMP-3 by endopeptidases

Activation by trypsin. The ability of trypsin to activate proMMP-3 was investigated first since this enzyme is commonly used to activate latent metalloendopeptidases in culture media of various connective tissues. At a low concentration of trypsin (0.1 µg/ml) proMMP-3 was gradually activated up to 82 % after a 24 h incubation, with a full activity assessed by treating the zymogen with 1.5 mM-NH₂PhHgAc for 22 h at 37 °C (see Fig. 9). At higher concentrations of trypsin (1 and 5 µg/ml), the maximally activatable level by trypsin (63–73 % of the full activity) was attained in a short time (5 min and 30 min respectively), and this was followed by a rapid decrease in activity to 20 %, but there were no further decreases in activity even after a 24 h incubation. At this stage the enzymic activity was unaltered even if the enzyme was treated with NH₂PhHgAc, suggesting that the action of trypsin on proMMP-3 was complete and produced a MMP-3 fragment that retained only 20 % of the full activity (Fig. 2).

Fig. 2. Activation of proMMP-3 by trypsin

ProMMP-3 (0.75 unit) was treated with trypsin (0.1, 1 and 5 µg/ml at final concentration) at 22 °C for 1 min–24 h. The reaction was stopped by adding a 5-fold molar excess of soya-bean trypsin inhibitor and the MMP-3 activity was assayed against [³H]Cm-Tf for 1 h at 37 °C. In the case of activation with 5 µg of trypsin/ml one set of samples was incubated with 1.5 mM-NH₂PhHgAc for 14 h at 37 °C after the termination of the trypsin reaction with soya-bean trypsin inhibitor, and then assayed for MMP-3 activity (—). Activity obtained after the incubation of proMMP-3 (0.75 unit) with 1.5 mM-NH₂PhHgAc for 24 h at 37 °C was taken as 100 % activity.

Fig. 3. Action of trypsin on proMMP-3

¹²⁵I-labelled proMMP-3 was incubated with trypsin (5 µg/ml at final concentration) for various times at 22 °C. After termination of the reaction with a 5-fold molar excess of soya-bean trypsin inhibitor and 20 mM-EDTA, the samples were applied to SDS/polyacrylamide-gel electrophoresis (10 % total acrylamide) under reducing conditions. The gel was dried and autoradiographed. Protein standards are as in Fig. 1 legend and soya-bean trypsin inhibitor (*M_r* 21 000 and lysozyme (*M_r* 14 000).

The action of trypsin on the proMMP-3 molecule was analysed by SDS/polyacrylamide-gel electrophoresis. ¹²⁵I-labelled proMMP-3 was used to facilitate the study, since the radioiodination of proMMP-3 did not cause any significant changes in the intrinsic properties of the zymogen. Fig. 3 shows that trypsin (5 µg/ml) initially converted ¹²⁵I-labelled proMMP-3 into polypeptides of *M_r* 49 000 and 43 000. These two species were gradually converted into fragments of *M_r* 32 000 and 24 000. Both bands exhibited proteolytic activity when examined by electrophoresis in a SDS/polyacrylamide gel containing gelatin in a similar manner to that shown in Fig. 8.

We then examined whether the effect of trypsin upon proMMP-3 is direct or whether trypsin-activated MMP-3 also participates in activation and/or inactivation of proMMP-3. To test these possibilities proMMP-3 was partially activated (10 % or 53 %) by treatment with trypsin (0.25 µg/ml for 10 min or 1 µg/ml for 5 min respectively). After the effect of trypsin had been terminated by a 5-fold molar excess of soya-bean trypsin inhibitor, the samples were further incubated at 37 °C for up to 24 h (Fig. 4). There was a slight increase in activity during the first 30 min, but no significant increase or decrease in MMP-3 activity was observed. Furthermore the partially activated samples could be activated fully by 1.5 mM-NH₂PhHgAc even after a 24 h incubation. These results indicate that trypsin activates proMMP-3 by direct proteolytic action on proMMP-3 and that the trypsin-activated MMP-3 can neither activate proMMP-3 nor inactivate proMMP-3 and MMP-3.

Activation by other endopeptidases. Several other proteolytic enzymes were tested for their ability to activate proMMP-3 (Fig. 5).

Incubation of proMMP-3 with human plasma kallikrein at a concentration of 10 µg/ml resulted in full activation in 30 min at 37 °C, but the MMP-3 activity declined to 25 % after a 24 h incubation in a manner similar to that observed with trypsin (Fig. 5). Analyses of

Fig. 4. Activation of proMMP-3 by trypsin-activated MMP-3

ProMMP-3 (0.75 unit) was activated to 10% (●) and 53% (○) of the full activity by treating with 0.25 μg of trypsin/ml for 10 min and 1 μg of trypsin/ml for 5 min at 22 °C respectively. After the addition of a 5-fold molar excess of soya-bean trypsin inhibitor, the mixtures continued to be incubated at 37 °C and a portion of the sample was removed and assayed against [^3H]Cm-Tf for 1 h at 37 °C. Effects of trypsin-inhibitor complex on proMMP-3 (Δ) activation were also examined by incubating proMMP-3 with the complex of trypsin (1 μg /ml) and soya-bean trypsin inhibitor (5 μg /ml). In order to detect residual proMMP-3 after a 24 h incubation the samples were treated with 1.5 mM- NH_2PhHgAc for 24 h at 37 °C and the activity was measured. The levels of enzymic activity after NH_2PhHgAc treatment are expressed in parentheses.

^{125}I -labelled proMMP-3 after the reaction of plasma kallikrein (10 μg /ml) showed that proMMP-3 was rapidly processed to polypeptides of M_r 46000 and 39000 (Fig. 6a). Further incubation with plasma kallikrein, however, resulted in degradation of MMP-3.

Plasmin activated proMMP-3 rather slowly: for full activation it required at least an 8 h incubation at 37 °C at a concentration of 10 μg /ml (Fig. 5). At a concentration of 1 μg /ml, only partial activation of proMMP-3 was observed even after 24 h at 37 °C. However, unlike with trypsin or plasma kallikrein, plasmin-produced active MMP-3 was stable and no decrease in enzymic activity was observed even after a 24 h incubation. SDS/polyacrylamide-gel electrophoresis showed that plasmin initially converted proMMP-3 into a polypeptide of M_r 52000, and then into the fragments of M_r 45000 and 24000 upon a longer incubation (Fig. 6b).

The time course of proMMP-3 activation by chymotrypsin showed that about 90% of the zymogen was activated at a concentration of 1 μg /ml in 2 h at 22 °C. Unlike the case with trypsin, MMP-3 activity was not diminished by the further incubation (Fig. 5). However, at a higher concentration (10 μg /ml) the activity decreased to about 60% after prolonged incubation. Fig. 6(c) shows that chymotrypsin (1 μg /ml) converted proMMP-3 initially into a polypeptide of M_r 52000 and then into fragments of M_r 50000 and 45000. Only a small portion of the 45000- M_r enzyme was converted into 25000- M_r fragment after a 24 h incubation.

Thermolysin was shown to be a potent activator of proMMP-3. Full activation was observed in 30 min at a concentration of 1 μg /ml, and in 2 h at a concentration of 0.1 μg /ml. Incubation of ^{125}I -labelled proMMP-3 with

Fig. 5. Activation of proMMP-3 by plasma kallikrein, plasmin, chymotrypsin and thermolysin

ProMMP-3 (0.75 unit) was incubated with human plasma kallikrein, human plasmin and thermolysin at 37 °C, and bovine chymotrypsin in the presence of 1 mM-Tos-Lys- CH_2Cl at 22 °C, at 10 μg /ml (●), 1 μg /ml (○) and 0.1 μg /ml (▲). The MMP-3 activity was measured against [^3H]Cm-Tf for 1 h at 37 °C after termination of the activation reaction with 2 μM -Pro-Phe-Arg- CH_2Cl (kallikrein), with 3 mM-di-isopropyl phosphorofluoridate (chymotrypsin and plasmin) or with 30 μg of phosphoramidon/ml (thermolysin). Plasmin was prepared by incubating plasminogen with streptokinase. The streptokinase itself did not activate proMMP-3 during a 24 h incubation. Activity achieved by the incubation of proMMP-3 with 1.5 mM- NH_2PhHgAc for 24 h at 37 °C was taken as 100% activity.

Fig. 6. Action of plasma kallikrein, plasmin, chymotrypsin and thermolysin on proMMP-3

^{125}I -labelled proMMP-3 was reacted with (a) human plasma kallikrein ($10\ \mu\text{l/ml}$), (b) human plasmin ($10\ \mu\text{g/ml}$) and (c) chymotrypsin ($1\ \mu\text{g/ml}$) in the presence of $1\ \text{mM-Tos-Lys-CH}_2\text{Cl}$, or (d) thermolysin ($0.1\ \mu\text{g/ml}$) at 37°C , except for chymotrypsin at 22°C . After termination of the enzymic reaction with an appropriate inhibitor, as described in Fig. 5 legend and with $20\ \text{mM-EDTA}$, the samples were subjected to SDS/polyacrylamide-gel electrophoresis (10% total acrylamide) under reducing conditions. The gels were dried and autoradiographed. Protein standards are as indicated in Fig. 3 legend.

thermolysin ($0.1\ \mu\text{g/ml}$) converted the precursor first into a fragment of $M_r\ 50\,000$, which was then processed to a $45\,000\text{-}M_r$ species that was relatively resistant to thermolysin action. After a longer incubation, however, MMP-3 was completely degraded by thermolysin (Fig. 6d).

Thrombin was not an effective activator of proMMP-3.

Activation of proMMP-3 by NH_2PhHgAc

Time course of proMMP-3 activation. In contrast with the action of endopeptidases, activation of proMMP-3 by NH_2PhHgAc was gradual and concentration-dependent; about 80% activation was achieved with $1.5\ \text{mM-NH}_2\text{PhHgAc}$ after a $4\ \text{h}$ incubation at 37°C , and

Table 2. Inhibition of the NH_2PhHgAc action on proMMP-3 activation

ProMMP-3 was activated with 1.5 mM- NH_2PhHgAc for 4 h at 37 °C. All the reactions were carried out in 50 mM-Tris/HCl buffer (pH 7.6)/0.15 M-NaCl/10 mM- Ca^{2+} /0.05% Brij 35/0.02% NaN_3 .

Incubation mixture	Activation (%)
ProMMP-3 + buffer	0
ProMMP-3 + 1.5 mM- NH_2PhHgAc	100
(ProMMP-3 + 1.5 mM- NH_2PhHgAc)* + 0.5 mM-cysteine	108
(ProMMP-3 + 1.5 mM- NH_2PhHgAc)* + 1.5 mM-cysteine	116
(ProMMP-3 + 1.5 mM- NH_2PhHgAc)* + 3 mM-cysteine	91
(ProMMP-3 + 10 mM-iodoacetamide)† + 1.5 mM- NH_2PhHgAc	58
(ProMMP-3 + 20 mM-iodoacetamide)† + 1.5 mM- NH_2PhHgAc	33
ProMMP-3 + (0.5 mM-cysteine + 1.5 mM- NH_2PhHgAc)‡	42
ProMMP-3 + (1.5 mM-cysteine + 1.5 mM- NH_2PhHgAc)‡	2
ProMMP-3 + (3 mM-cysteine + 1.5 mM- NH_2PhHgAc)‡	0

* ProMMP-3 was incubated with 1.5 mM- NH_2PhHgAc at 37 °C for 4 h before the addition of cysteine.

† ProMMP-3 was treated with iodoacetamide for 20 min at 22 °C before the activation with 1.5 mM- NH_2PhHgAc .

‡ Cysteine and NH_2PhHgAc were allowed to react at the molar ratios of 1:3, 1:1 and 2:1 for 20 min at 22 °C and the mixtures were added to proMMP-3 at the final concentrations indicated.

full activation after 22 h (see Fig. 9); with 0.1 mM- NH_2PhHgAc , only about 60% of proMMP-3 was activated after 22 h at 37 °C. The effect of NH_2PhHgAc on proMMP-3 activation was blocked partially by treating the zymogen with iodoacetamide before the NH_2PhHgAc treatment and almost completely by treatment of the NH_2PhHgAc with an equimolar amount of cysteine before the incubation with proMMP-3 (Table 2). Neither iodoacetamide alone (Okada *et al.*, 1986) nor cysteine alone (Table 2) affected the enzymic activity of the activated MMP-3. These results indicate that the reaction of NH_2PhHgAc with proMMP-3, possibly through thiol groups in the proMMP-3 molecule, is essential for zymogen activation.

When ^{125}I -labelled proMMP-3 was treated with 1.5 mM- NH_2PhHgAc , the zymogen of M_r 57000 was processed initially to a polypeptide of M_r 46000, which was then converted into the 45000- M_r (HMM) species, and into the 28000 M_r (LMM) species upon longer incubation (Fig. 7).

Induction of conformational changes in proMMP-3. Since NH_2PhHgAc itself has no direct proteolytic activity it is postulated that NH_2PhHgAc treatment induces conformational changes in the proMMP-3 molecule, which, in turn, becomes a proteolytically active high- M_r form followed by processing to low- M_r species. To examine such a possibility NH_2PhHgAc -treated

Fig. 7. Conversion of proMMP-3 by NH_2PhHgAc

^{125}I -labelled proMMP-3 was incubated with 1.5 mM- NH_2PhHgAc for 0–23 h at 37 °C. Lane 1, proMMP-3 without NH_2PhHgAc treatment; lanes 2–9, proMMP-3 treated with NH_2PhHgAc for 0 min, 10 min, 30 min, 1 h, 3 h, 5 h, 8 h and 23 h respectively. The reaction was stopped with 20 mM-EDTA. The samples were subjected to SDS/polyacrylamide-gel electrophoresis (10% total acrylamide) with reduction. The gel was dried and autoradiographed. Protein standards are as indicated in Fig. 3 legend.

proMMP-3 was subjected to electrophoresis in an SDS/polyacrylamide gel containing gelatin to assess its proteolytic activity. As shown in Fig. 8, proMMP-3 became active without a change in apparent M_r after a short period of incubation with NH_2PhHgAc , but it was then converted into low- M_r forms similar to those of HMM and LMM purified previously (Okada *et al.*, 1986). In addition, the zymogram revealed an additional active band with M_r 34000. A similar observation was reported with rabbit stromelysin (Chin *et al.*, 1985).

Induction of conformational changes in proMMP-3 by NH_2PhHgAc was also supported by differential susceptibility to trypsin between the NH_2PhHgAc -treated and the untreated proMMP-3 and the decreased rate of proMMP-3 activation by NH_2PhHgAc in the presence of higher concentrations of NaCl under which the enzymic activity of already activated MMP-3 was unchanged. The latter observation indicates that the activation of proMMP-3 by NH_2PhHgAc depends on the conformational changes in the zymogen since the degree of perturbation of protein molecules is likely to be influenced by ionic strength.

Lack of intermolecular activation. To investigate further the mechanism of activation of proMMP-3 by NH_2PhHgAc , we examined whether NH_2PhHgAc -activated MMP-3 is capable of activating the residual pro-enzyme as a positive-feedback activation. ProMMP-3 was first activated up to 33% with NH_2PhHgAc at 37 °C. This sample was then applied to a spin column to remove NH_2PhHgAc quickly from the enzyme solution, which was further incubated at 37 °C and the MMP-3 activity examined. As shown in Fig. 9, after removal of NH_2PhHgAc no further activation of proMMP-3 occurred. Re-addition of NH_2PhHgAc to the partially activated proMMP-3 resulted in full activation with very similar kinetics (Fig. 9), suggesting that MMP-3 is incapable of activating proMMP-3 and, in addition to

Fig. 8. Detection of proteolytic activity of the NH_2PhHgAc -treated proMMP-3 after SDS/polyacrylamide-gel electrophoresis

ProMMP-3 (0.8 unit) was incubated with 1.5 mM- NH_2PhHgAc for 0.5–27 h at 37 °C. The samples were mixed with reducing SDS/gel sample buffer and applied without boiling to SDS/polyacrylamide-gel electrophoresis containing 0.25 mg of gelatin/ml. After electrophoresis, the gel was washed, incubated in 50 mM-Tris/HCl buffer (pH 7.5)/5 mM- Ca^{2+} /1 μM - Zn^{2+} /1 % Triton X-100/0.02 % NaN_3 for 16 h at 37 °C and stained with Coomassie Brilliant Blue R-250.

the reaction of NH_2PhHgAc with the zymogen molecule (Table 2), the continued presence of NH_2PhHgAc is required for proMMP-3 activation. Moreover, the active HMM or LMM forms purified previously (Okada *et al.*, 1986) could not activate proMMP-3. These results are similar to those obtained with proMMP-3 partially activated by trypsin.

Kinetics of proMMP-3 conversion into HMM and LMM species by NH_2PhHgAc . Upon incubation with NH_2PhHgAc proMMP-3 undergoes a sequential proteolysis, and the conversion of proMMP-3 into the lower- M_r species appears to be correlated with the proteolytic activity of MMP-3 (Figs. 7 and 9). Inability of partially activated proMMP-3, HMM species and LMM species to activate proMMP-3 suggests that the initial proteolysis of proMMP-3 is perhaps triggered by an intramolecular rather than an intermolecular event. To test such a possibility ^{125}I -labelled proMMP-3 was incubated with 1.5 mM- NH_2PhHgAc at 37 °C in the presence of various concentrations of unlabelled proMMP-3, and the conversion of the precursor into the low- M_r species [M_r 46000, 45000 and 28000 (see Fig. 7)] was quantified by densitometric analysis of auto-

Fig. 9. Requirement of the continued presence of NH_2PhHgAc for proMMP-3 activation

ProMMP-3 (300 μl , 22.5 units) was incubated with 1.5 mM- NH_2PhHgAc for 1 h at 37 °C to achieve 33 % of the full activity. NH_2PhHgAc was removed from two-thirds of the reaction mixture (200 μl) by spin columns of Sephadex G-10 equilibrated with 50 mM-Tris/HCl buffer (pH 7.5)/0.4 M-NaCl/10 mM- CaCl_2 /0.05 % Brij 35/0.02 % NaN_3 . The NH_2PhHgAc -free sample (33 % active) was incubated at 37 °C for an additional 4 h. A half of this sample (100 μl) was again treated with 1.5 mM- NH_2PhHgAc and the further activation of proMMP-3 was monitored. The enzymic activity of activated MMP-3 was assayed against [^3H]Cm-Tf for 1 h at 37 °C. ○, Sample with NH_2PhHgAc removed.

radiographs after separating each species on SDS/polyacrylamide-gel electrophoresis. Fig. 10 shows that proMMP-3 was first converted into a polypeptide of M_r 46000, which was then processed to 45000- M_r (HMM) and 28000- M_r (LMM) species. The rate of disappearance of proMMP-3 appeared to follow first-order kinetics with $t_{1/2}$ 2.0 h, and this rate was not altered over a 100-fold range of initial concentrations of proMMP-3 (Fig. 10 inset). On the other hand, the rate of conversion of the intermediate with M_r 46000 into the HMM species was accelerated when the initial concentrations of proMMP-3 were greater. The higher rate of conversion of the HMM species into the LMM species was also observed with higher initial concentrations of the precursor. These results suggest that conversion of proMMP-3 into the 46000- M_r intermediate by NH_2PhHgAc is by an intramolecular self-catalysed action but that the subsequent processing of this form to the HMM and LMM species may occur by bimolecular reactions. This conclusion was further supported by the experiments carried out in the presence of an exogenous substrate. When ^{125}I -labelled proMMP-3 was incubated in the absence or presence of a 338-fold molar excess (1.6 mg/ml) or a 676-fold molar excess (3.2 mg/ml) of Cm-Tf, and the conversion of ^{125}I -labelled proMMP-3 into 46000- M_r , HMM and LMM forms was quantified in a similar manner, the rate of disappearance of proMMP-3 was not affected by the presence of the substrate and showed typical first-order kinetics, whereas the rate of conversion of the 46000- M_r intermediate into the HMM and LMM species was considerably decreased in the presence of the substrate and the effect was greater with a higher concentration of Cm-Tf. This indicates that

Fig. 10. Effect of proMMP-3 concentration on the appearance of an intermediate form of M_r 46000 and HMM and LMM species by NH_2PhHgAc treatment

An equal amount of ^{125}I -labelled proMMP-3 was mixed with three different concentrations of proMMP-3 and incubated with $1.5 \text{ mM-NH}_2\text{PhHgAc}$ at 37°C : (a) $0.3 \mu\text{g/ml}$; (b) $9.0 \mu\text{g/ml}$; (c) $30 \mu\text{g/ml}$. At various incubation times the reaction was stopped by adding 20 mM-EDTA , and the samples were subjected to SDS/polyacrylamide-gel electrophoresis. The gels were dried and autoradiographed. To quantify proMMP-3 (M_r 57000) (●), the 46000- M_r intermediate (▲), HMM species (M_r 45000) (○) and LMM species (M_r 28000) (■), autoradiographs were subjected to densitometric analysis. The inset in (a) shows the rate of disappearance of proMMP-3 at three different initial concentrations of proMMP-3 in the presence of $1.5 \text{ mM-NH}_2\text{PhHgAc}$ at 37°C .

conversion of the 46000- M_r species into the HMM and LMM species is a bimolecular reaction.

DISCUSSION

We have purified the precursor of MMP-3 secreted from human rheumatoid synovial cells and identified it

as a doublet of single polypeptide zymogens of M_r 57000 and 59000. Since the 59000- M_r species, which accounts for approx. 10% of proMMP-3, bound to concanavalin A it is likely a product of post-translational glycosylation of the 57000- M_r species. Similar microheterogeneity resulting from post-translational glycosylation has been reported for procollagenase (Nagase *et al.*, 1981, 1983; Wilhelm *et al.*, 1986), a metalloendopeptidase also found in higher amounts in cultures to which macrophage-conditioned medium was added (Okada *et al.*, 1986). This suggests that these metalloendopeptidases have similar molecular structures and share a common secretory pathway. Indeed, the primary structure of human prostromelysin, presumed to be identical with that of proMMP-3, deduced from the cDNA sequencing by Whitham *et al.* (1986) has indicated that it is 55% homologous to the amino acid sequence of human procollagenase (Goldberg *et al.*, 1986; Whitham *et al.*, 1986) and that one of the two potential glycosylation sites of proMMP-3 is located at the corresponding homologous sequence of procollagenase (Whitham *et al.*, 1986).

Among six endopeptidases tested (trypsin, plasma kallikrein, thrombin, plasmin, chymotrypsin and thermolysin) for their ability to activate proMMP-3, all except thrombin were capable of activating the zymogen to some degree. Trypsin, commonly used to activate procollagenase, was not one of the better activators of proMMP-3. At high trypsin concentrations, or with prolonged incubation, activated MMP-3 was rapidly degraded to a 24000- M_r species that retained only 20% of the potential MMP-3 activity. Plasmin also produced a 24000- M_r species upon prolonged incubation. Our time-course activation study has suggested that this fragment is fully active. The primary structure of proMMP-3 deduced from cDNA indicates that the Zn^{2+} -binding site is located approximately in the middle of the 57000- M_r polypeptide (Whitham *et al.*, 1986). Thus MMP-3 with M_r 24000 must result from the removal of both N-terminal and C-terminal fragments from the precursor molecule. Therefore the discrepancy in the enzymic activity between the two similar- M_r species generated by trypsin and plasmin may be due to the production of two different proteolytic fragments. Further investigation must be conducted to resolve this point, however. Nevertheless, plasmin, being capable of activating proMMP-3 and having limited degradative effects on MMP-3, may be a good candidate for an activator of proMMP-3 *in vivo*. This may be particularly relevant to the joints in pathological conditions where the concentrations of plasminogen and plasminogen activators are elevated (Berger, 1977; Werb *et al.*, 1977; Mochan *et al.*, 1986) and plasminogen specifically binds to the connective-tissue matrix (Knudsen *et al.*, 1986). The observation that the HMM and LMM species were incapable of activating proMMP-3 suggests that the activation of proMMP-3 by endopeptidases is the result of the direct action of these enzymes on the proMMP-3, possibly by removing a peptide of M_r approx. 12000 from the precursor zymogen. Since the activation of proMMP-3 was observed with a variety of endopeptidase, regardless of their substrate specificities, the activation seems to require the hydrolysis of a specific region rather than a specific peptide bond in the zymogen molecule.

We postulate that the initial event that occurs in the

proMMP-3 molecule upon NH_2PhHgAc treatment is the induction of conformational changes in the zymogen molecule, which, in turn, becomes a proteolytically 'active' form with M_r 57000 that self-catalyses to lower- M_r active species. The primary structure of human proMMP-3 (prostromelysin) revealed only three cysteine residues (Whitham *et al.*, 1986; Saus *et al.*, 1988). Thus, upon treatment with NH_2PhHgAc it is likely that NH_2PhHgAc reacts with at least one of the three thiol groups. The requirement of this reaction for proMMP-3 activation was suggested by the observations that iodoacetamide-treated proMMP-3 was not fully activated by NH_2PhHgAc and that NH_2PhHgAc that had reacted with cysteine was not capable of activating proMMP-3. However, NH_2PhHgAc binding alone cannot activate proMMP-3, since the removal of NH_2PhHgAc from the proenzyme solution after a 1 h reaction at 37 °C prevented further activation of proMMP-3. Activation of proMMP-3 requires the continued presence of NH_2PhHgAc , and the rate of its activation is dependent on the NH_2PhHgAc concentration. Nevertheless, NH_2PhHgAc is not required for enzymic activity once the proenzyme is activated (Okada *et al.*, 1986). This effect of an organomercurial compound is similar to the one demonstrated for the activation of human skin procollagenase (Stricklin *et al.*, 1983), although the reaction of organomercurial compounds with a free thiol group of procollagenase has not been established (Stricklin *et al.*, 1983).

Kinetic analysis of the proMMP-3 conversion into low- M_r species by NH_2PhHgAc indicated that the conversion of the 57000- M_r proenzyme into the intermediate of M_r 46000 was by an intramolecular self-catalysed process, but that further processing to the HMM and LMM species was dependent upon a bimolecular reaction. This was inferred from the observations that the rate of disappearance of proMMP-3 obeyed first-order kinetics and was not affected by the initial concentration of the proenzyme or by the presence of a 676-fold molar excess of substrate. Conversely, the rate of further proteolysis of the 46000- M_r species to the HMM and LMM species was affected by these parameters. The examples of proenzyme activation by intramolecular processes have been described for proacrosin (Kennedy & Polakoski, 1981), pepsinogen (Bustin & Conway-Jacobs, 1971) and procalpain II (Coolican *et al.*, 1986; DeMartino *et al.*, 1986). In addition, inactivation of calpain II in the presence of Ca^{2+} has also been shown to be via an intramolecular process (Mellgren *et al.*, 1982).

The complete amino acid sequence of proMMP-3 (Whitham *et al.*, 1986; Saus *et al.*, 1988) has also revealed that human proMMP-3 is equivalent to the previously described major protein product (M_r 53000) with unknown function called 'transin' from rat fibroblasts either treated with epidermal growth factor or transformed by oncogenic viruses (Matrisian *et al.*, 1985, 1986). This suggests that MMP-3 may play a significant role in tumour invasion and contribute to the metastatic potential of cells, especially since it is capable of degrading basement-membrane components (Galloway *et al.*, 1983; Okada *et al.*, 1986). However, one of the crucial steps before proMMP-3 can participate in such an activity involves the activation of the zymogen. Our studies suggest that under certain pathological conditions proteolytic enzymes derived from plasma, tissues or

inflammatory cells may activate it directly. In certain biological processes such as embryonic development, plasminogen activator/plasmin may participate in the activation of proMMP-3. On the other hand, the ability of NH_2PhHgAc to activate proMMP-3 in a non-enzymic fashion suggests the possibility that the initial step in proMMP-3 activation may occur simply by changes in the molecular conformation in response to a certain microenvironment of the zymogen within the tissues.

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REFERENCES

- Barrett, A. J., Brown, M. A. & Sayers, C. A. (1979) *Biochem. J.* **181**, 401–418
- Berger, H., Jr. (1977) *Arthritis Rheum.* **20**, 1198–1205
- Bustin, M. & Conway-Jacobs, A. (1971) *J. Biol. Chem.* **246**, 615–620
- Chin, J. R., Murphy, G. & Werb, Z. (1985) *J. Biol. Chem.* **260**, 12367–12376
- Coolican, S. A., Haieck, J. & Hathaway, D. R. (1986) *J. Biol. Chem.* **261**, 4170–4176
- Dayer, J.-M., Krane, S. M., Russell, R. G. G. & Robinson, D. R. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 945–949
- DeMartino, G. N., Huff, C. A. & Croall, D. E. (1986) *J. Biol. Chem.* **261**, 12047–12052
- Fraker, P. J. & Speck, J. C., Jr. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849–857
- Galloway, W. A., Murphy, G., Sandy, J. D., Gavrilovic, J., Cawston, T. E. & Reynolds, J. J. (1983) *Biochem. J.* **209**, 741–752
- Goldberg, G. I., Wilhelm, S. M., Kronberger, A., Bauer, E. A., Grant, G. A. & Eisen, A. Z. (1986) *J. Biol. Chem.* **261**, 6600–6605
- Heath, J. K., Gowen, M., Meikle, M. C. & Reynolds, J. J. (1982) *J. Periodontal Res.* **17**, 183–190
- Hibbs, M. S., Hasty, K. A., Seyer, J. M., Kang, A. H. & Mainardi, C. L. (1985) *J. Biol. Chem.* **260**, 2493–2500
- Kennedy, W. P. & Polakoski, K. L. (1981) *Biochemistry* **20**, 2240–2245
- Knudsen, B. S., Silverstein, R. L., Leung, L. L. K., Harpel, P. C. & Nachman, R. L. (1986) *J. Biol. Chem.* **261**, 10765–10771
- Martel-Pelletier, J., Pelletier, J.-P., Cloutier, J.-M., Howell, D. S., Ghandur-Mnaymneh, L. & Woessner, J. F., Jr. (1984) *Arthritis Rheum.* **27**, 305–312
- Matrisian, L. M., Glaichenhaus, N., Gesnel, M.-C. & Breathnach, R. (1985) *EMBO J.* **4**, 1435–1440
- Matrisian, L. M., Leroy, P., Ruhlmann, C., Gesnel, M.-C. & Breathnach, R. (1986) *Mol. Cell. Biol.* **6**, 1679–1686
- Meikle, M. C., Sellers, A. & Reynolds, J. J. (1980) *Calcif. Tissue Int.* **30**, 77–82
- Mellgren, R. L., Repetti, A., Muck, T. C. & Easley, J. (1982) *J. Biol. Chem.* **257**, 7203–7209
- Mochan, E., Uhl, J. & Newton, R. (1986) *J. Rheumatol.* **13**, 15–19
- Nagase, H. & Barrett, A. J. (1981) *Biochem. J.* **193**, 187–192
- Nagase, H., Jackson, R. C., Brinckerhoff, C. E., Vater, C. A. & Harris, E. D., Jr. (1981) *J. Biol. Chem.* **256**, 11951–11954
- Nagase, H., Brinckerhoff, C. E., Vater, C. A. & Harris, E. D., Jr. (1983) *Biochem. J.* **214**, 281–288

- Okada, Y., Nagase, H. & Harris, E. D., Jr. (1986) *J. Biol. Chem.* **261**, 14245–14255
- Saus, J., Quinones, S., Otani, Y., Nagase, H., Harris, E. D., Jr. & Kurkinen, M. (1988) *J. Biol. Chem.* **263**, 6742–6745
- Sellers, A., Reynolds, J. J. & Meikle, M. C. (1978) *Biochem. J.* **171**, 493–496
- Stricklin, G. P., Jeffrey, J. J., Roswit, W. T. & Eisen, A. Z. (1983) *Biochemistry* **22**, 61–68
- Vaes, G., Eeckhout, Y., Lenaers-Claeys, G., François-Gillet, C. & Druetz, J.-E. (1978) *Biochem. J.* **172**, 261–274
- Vater, C. A., Mainardi, C. L. & Harris, E. D., Jr. (1978) *J. Clin. Invest.* **62**, 987–992
- Vater, C. A., Hahn, J. L. & Harris, E. D., Jr. (1981) *Collagen Relat. Res.* **1**, 527–542
- Werb, Z., Mainardi, C. L., Vater, C. A. & Harris, E. D., Jr. (1977) *N. Engl. J. Med.* **296**, 1017–1023
- Whitham, S. E., Murphy, G., Angel, P., Rahmsdorf, H.-J., Smith, B. J., Lyons, A., Harris, T. J. R., Reynolds, J. J., Herrlich, P. & Docherty, A. J. P. (1986) *Biochem. J.* **240**, 913–916
- Wilhelm, S. M., Eisen, A. Z., Teter, M., Clark, S. D., Kronberger, A. & Goldberg, G. I. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3756–3760
- Woessner, J. F., Jr., Azzo, W. & Dean, D. D. (1986) *J. Cell. Biochem.* **10A** (Suppl.), 255
- Wyckoff, M., Rodbard, D. & Chrambach, A. (1977) *Anal. Biochem.* **78**, 459–482

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