

Mercurial activation of human PMN leucocyte type IV procollagenase (gelatinase)

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Autoproteolytic activation and processing of human polymorphonuclear leucocyte (PMNL) type IV procollagenase (gelatinase) was initiated by HgCl_2 and was investigated by kinetic analysis and N-terminal sequence determination of the reaction products. In the first instance the propeptide domain was lost by subsequent cleavage of the Asp¹⁵-Leu¹⁶, Glu⁴⁰-Met⁴¹, Leu⁵²-Leu⁵³ and Ala⁷⁴-Met⁷⁵ peptide bonds. The PRCGVDP sequence motif (residues Pro⁷⁸-Asp⁸⁴), which is conserved in all metalloproteinases and expected to be relevant for latency, remained uncleaved at the activated enzyme. The generated intermediate was further processed by three C-terminal cleavages. The Glu⁶⁶⁶-Leu⁶⁶⁷, Ala⁵⁰⁶-Glu⁵⁰⁷ and Ala³⁹⁸-Leu³⁹⁹ bonds were hydrolysed successively. From the fragmentation products we were able to conclude that three released fragment peptides contained unpaired free cysteine with the residues Cys³⁹⁷, Cys⁶⁵³, Cys⁶⁶³. Cleavage of the first C-terminal peptide bond resulted in the loss of one of the conserved Cys residues of the hemopexin-like domain, whereas the Cys residue of the PRCGVDP motif was retained at the fully active enzyme. The possibility of an entirely different activation mechanism for PMNL type IV procollagenase is discussed.

Type IV collagenase; Neutrophil; Mercurial activation

1. INTRODUCTION

Type IV collagenases (gelatinases) play key roles in matrix remodelling and basement membrane degradation and contribute to the metastatic potential of tumor cells [1]. The two type IV collagenases are unique members of the matrix metalloproteinase family with regard to their domain structure. The M_r 72,000 and the M_r 98,000 type IV collagenase share a fibronectin-like domain, which is inserted 175 residues preceding the proposed zinc binding motif. This domain is homologous to the collagen binding motif of fibronectin and responsible for gelatin binding of the proenzymes [2]. The M_r 98,000 type IV collagenase contains an additional domain with sequence similarities to the α_2 chain of type V collagen [3]. The function of this insertion behind the zinc binding motif has still to be determined.

The mode of activation of metalloproteinases in vivo is unknown. Activation in vitro was the target of intensive studies during the last ten years [3–9]. Exposure of the purified proenzymes to proteinases, organomercurials, oxygen radicals or chaotropic agents led to the generation of enzymatic activity.

Abbreviations: PMNL, polymorphonuclear leucocytes; RP-HPLC, reversed-phase high-performance liquid chromatography; TBST, Tris-buffered saline Tween; TIMP-1, tissue inhibitor of metalloproteinases-1.

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As sequence data became available different groups developed theoretical activation mechanisms to explain the experimental data [10,11]. A stable complex between the conserved Cys residue of the PRCGVDP sequence motif within the propeptide domain and the active site zinc might be the basis for latency. It is believed that all modes of activation require the removal of this Cys residue out of the coordination sphere of the zinc binding centre as the most important step. This has recently been supported by site directed mutagenesis experiments with transin, the rat homologues of human stromelysin [12,13]. Mercurial compounds as well as oxygen radicals are assumed to activate by modifying the chelating cysteine sulfhydryl group. Latent metalloproteinases are activated by human neutrophils by an oxidative process in vivo [14].

In this paper we present the first data for the individual steps in the activation process of PMNL type IV procollagenase induced by HgCl_2 and discuss a modified activation mechanism for this enzyme.

2. MATERIALS AND METHODS

2.1. Materials

Dinitrophenyl-peptide was purchased from Bachem (Bubendorf, Switzerland). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Purification of PMNL type IV procollagenase, activation and enzyme assay

PMNL type IV procollagenase was purified as recently published [15]. Proenzyme activation was achieved by incubation with HgCl_2 at 37°C. Type IV collagenase activity was determined by proteolytic

degradation of the synthetic octapeptide (dinitrophenyl-Pro-Gln-Ile-Ala-Gly-Gln-D-Arg-OH) as described by Masui [16].

2.3. Protein determination

Protein concentrations were estimated from A_{280} assuming an absorption coefficient of $1.0 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

2.4. Kinetic analysis of HgCl_2 -induced activation of PMNL type IV procollagenase and separation of the reaction products by RP-HPLC

PMNL type IV procollagenase was activated for between 5 min to 2 h at 37°C using 1 mM HgCl_2 . The reaction mixture was directly subjected to a Bakerbond wide pore C_{18} -column ($4.9 \times 250 \text{ mm}$). The separations of the reaction products were performed at a constant flow rate of 0.8 ml/min using a linear gradient from 0–60% acetonitrile. Peptides and proteins were detected at 214 nm , collected, lyophilised and subjected to automated amino acid sequence determination using a microsequencer (Model 810, Knauer, Berlin, Germany).

2.5. SDS/PAGE

SDS/PAGE was performed according to the method of Laemmli [17]. The proteins were visualised by silver staining [18].

3. RESULTS

3.1. Autoproteolytic activation and processing of human type IV procollagenase induced by HgCl_2

Type IV procollagenase was purified from human PMN leucocytes. The N-terminus of the enzyme was found to be identical to that of the proenzyme from SV40-transformed lung fibroblasts [3,15]. As shown by SDS/PAGE (Fig. 1, lane 2), PMNL type IV procollagenase consists of three forms with different molecu-

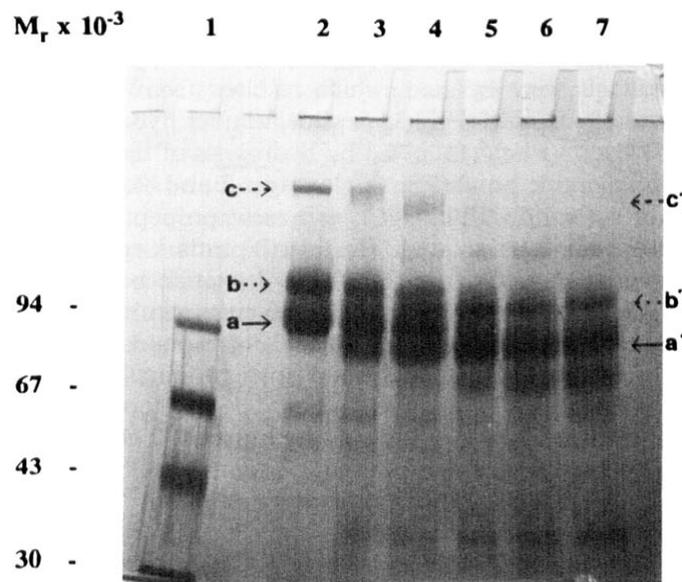


Fig. 1. Time-dependent activation of PMNL type IV procollagenase by HgCl_2 ; SDS/PAGE analysis of generated products. (Lane 1) molecular mass marker; (lane 2) starting material, PMNL type IV procollagenase; (lanes 3–7) after exposure to 1 mM HgCl_2 for 5, 15, 30, 60 and 120 min, respectively. a, latent $M_r 98,000$ form; a', first active form of a, $M_r 88,000$; b, latent $M_r 125,000$ form; b', first active form of b, $M_r 115,000$; c, latent $M_r 220,000$ form; c', first active form of c, $M_r 200,000$. The band pattern of lane 7 remains unchanged over a period of about 12 h, then further autolysis becomes visible by additional bands.

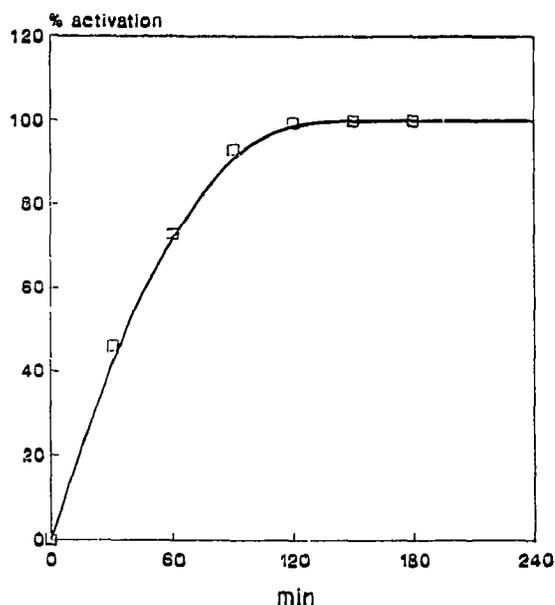


Fig. 2. Time-dependent activity of PMNL type IV collagenase during mercurial activation against DNP-octapeptide.

lar masses ($M_r 220,000$, $125,000$ and $98,000$). Under reducing conditions these forms result in $M_r 98,000$ and $M_r 28,000$ subunits [15]. The isolated latent enzyme showed no activity against the synthetic DNP-octapeptide.

Time-dependent activation with HgCl_2 was investigated by SDS/PAGE and by determination of type IV collagenase activity as measured by proteolytic degradation of the synthetic DNP-octapeptide.

The SDS/PAGE showed that the three type IV collagenase proenzyme forms were completely transformed after 2 h into products with lower molecular masses. Determination of the activity (Fig. 2) showed that activation was complete after 2 h. The activity correlates with the formation of the products with lower molecular masses shown by SDS/PAGE. Reduction in molecular mass is therefore necessary for generation of activity. After overnight incubation with HgCl_2 at 37°C the activity decreased due to autolysis of the enzyme.

Since SDS/PAGE analysis of the activation was not very instructive, autoproteolytic processing of human PMNL type IV procollagenase ($M_r 220,000$, $M_r 125,000$, $M_r 98,000$), induced by HgCl_2 , was analysed by RP-HPLC. This allowed separation and isolation of the reaction products which could be subjected to N-terminal sequence determination. Autoproteolytic cleavage of the enzyme and further cleavage of the liberated reaction products resulted in a time-dependent generation of various peptides.

After 5 min of HgCl_2 -incubation four peptides could be isolated (Fig. 3, peptides I–IV). The N-terminal sequences of these peptides revealed that the three peptide bonds $\text{Asp}^{15}\text{-Leu}^{16}$, $\text{Glu}^{40}\text{-Met}^{41}$ and $\text{Leu}^{52}\text{-Leu}^{53}$ were

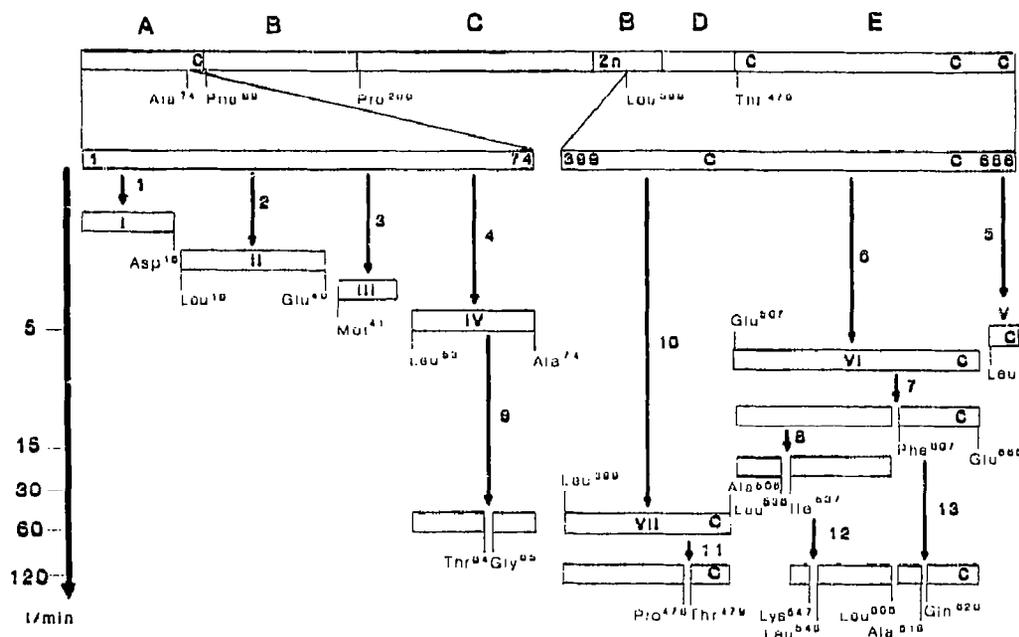


Fig. 3. Time-dependent processing of PMNL type IV procollagenase induced by HgCl_2 . (A) propeptide; (B) catalytic domain; (C) fibronectin-like domain inserted into the catalytic domain B; (D) type V collagen-like domain; (E) hemopexin-like domain; (I-IV) propeptide fragments in the order of their appearance; (V-VII) C-terminal fragments in the order of their appearance; (1-13) chronological order of formation of the reaction products.

cleaved. Sequence determinations of the enzyme isolated by RP-HPLC after 10 min activation with HgCl_2 revealed the five N-terminal residues Ala¹, Leu¹⁶, Met⁴¹, Leu⁵² and Met⁷⁵. From this we concluded that the four peptides were successively formed, without the loss of the PRCGVDP region, corresponding to a four-step propeptide truncation.

During the next 10 min of HgCl_2 incubation two further peptides were isolated (Fig. 3, peptides V and VI). Their formation resulted from the hydrolysis of the Glu⁶⁶⁶-Leu⁶⁶⁷ and the Ala⁵⁰⁶-Glu⁵⁰⁷ peptide bond. The Met⁷⁵-Glu⁶⁶⁶ enzyme corresponded to the M_r 88,000 and the Met⁷⁵-Ala⁵⁰⁶ enzyme to the M_r 73,000 (Fig. 1, lane 7) active type IV collagenase forms.

The next molecular mass reduction of type IV collagenase was observed in the form of the isolation of another peptide after 60 min incubation with HgCl_2 (Fig. 3, peptide VII). This peptide was created from cleavage of the Ala³⁹⁸-Leu³⁹⁹ peptide bond, which resulted in the formation of the M_r 63,000 active type IV collagenase form. This could not be visualized by SDS/PAGE (Fig. 1). The isolation of these three peptides (V-VII) corresponded to a three-step C-terminal truncation of the enzyme.

For the M_r 125,000 proenzyme form the SDS/PAGE showed a loss of 10,000 Da, which corresponded to the formation of the M_r 88,000 enzyme form.

The dimer of the M_r 98,000 unit, which shows a molecular mass of 220,000 on SDS/PAGE, was successively cleaved into M_r 200,000 and 150,000 activation

products. The former corresponded to the M_r 88,000 monomer and the later to the M_r 73,000 monomer.

The seven primary split products of the autoproteolytic process underwent further cleavages. During the first 15 min of HgCl_2 incubation four secondary split products were isolated, which resulted from the sixth primary peptide (Fig. 3, peptide VI) via hydrolysis of the Leu⁶⁰⁶-Phe⁶⁰⁷ followed by hydrolysis of the Leu⁵³⁶-Ile⁵³⁷ peptide bond (Fig. 3, cleavages 7 and 8). After 60 min activation with HgCl_2 two new propeptide fragments could be isolated. The fourth primary split product was cleaved at the Thr⁶⁴-Gly⁶⁵ peptide bond (Fig. 3, cleavage 9). After the next 60 min incubation six further peptides occurred. They correspond to three cleavages of three C-terminal split products (Fig. 3, cleavages 11-13). The Pro⁴⁷⁶-Thr⁴⁷⁹ peptide bond of the seventh primary peptide was hydrolysed. The peptide Ile⁵³⁷-Leu⁶⁰⁶ was cleaved at the Lys⁵⁴⁷-Leu⁵⁴⁸ and the peptide Phe⁶⁰⁷-Glu⁶⁶⁶ at the Ala⁶¹⁹-Gln⁶²⁰ peptide bond.

In summary, 19 peptides were isolated during 2 h of HgCl_2 incubation. The main products of these peptides are the four propeptide split products (Fig. 3, I-IV) and the two C-terminal peptides (Fig. 3, V and VI), which are formed during the first 10 min.

3.2. Disulfide bond assignment of the hemopexin-like domain

The hemopexin-like domain of type IV collagenase contains three cysteine residues. The two cysteines,

protein fragments are generated and separated, proving that all three cysteine residues are not disulfide bridged. Such a high amount of free cysteine residues are uncommon. If the cysteine of the PRCGVPD region of type IV collagenase is assumed to be unbridged, it might interact with other disulfide bridges of the enzyme in an intramolecular disulfide exchange reaction. This intramolecular interchange might generate the free cysteines found in the isolated peptides. However, the higher molecular mass forms of type IV collagenase, which are formed from the M_r 98,000 basic subunit of gelatinase with another M_r 98,000 unit or the unidentified M_r 28,000 protein seems to indicate one or more disulfide-bridges between these molecules. This seems to support the assumption that type IV collagenase possesses more than just one free cysteine.

Some site-directed mutagenesis experiments have confirmed the cysteine switch mechanism for transin [12,13]. The activation mechanism of type IV collagenase might be homologous to those of the other metalloproteinases. The four-step propeptide truncation without the loss of the PRCGVPD region causes a conformational rearrangement around the Zn centre resulting in an active enzyme. The C-terminal split products found correspond to the loss of the C-terminal domain of stromelysin and, therefore, have no effect on the activity of this enzyme. There are also special structural features of the type IV collagenase, a unique member of the metalloproteinases with a type V collagen homologous domain and an additional cysteine residue in the hemopexin-like domain. This, taken together with our findings of a relatively early loss of the Cys⁶⁸⁶ but no loss of the Cys⁸⁰ from the PRCGVPD sequence motif, seems to indicate a mechanism of activation by intramolecular cysteine/disulfide rearrangement that is different from the cysteine-switch mechanism [10,11], but identical in the result by generating an accessible, catalytically active zinc at the enzyme's reactive site.

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