Mechanism of Ca\(^{2+}\)-dependent Activity of Human Neutrophil Gelatinase B*

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Matrix metalloproteinases (MMP) are a family of zinc enzymes that have the ability to mount a concerted degradative attack on virtually all components of the extracellular matrix (1–4). It is widely assumed that normal physiological processes depend on careful spatial and temporal regulation of the activities of these enzymes. The uncontrolled expression of MMP has been reported to be associated with many pathological processes such as angiogenesis, rheumatoid arthritis, and tumor metastasis (5–10). These proteinases are often divided into three subgroups based on their substrate specificities: collagenases, gelatinases, and stromelysins. Gelatinase B, which specifically degrades types IV, V, and XI collagens as well as denatured collagens (gelatins), is believed to play an important role in both physiological and pathological processes. Several researchers have recently shown that cells bearing the gelatinase B CDNA are able to metastasize in nude mice, whereas inhibition of the gelatinase activity prevents metastasis (11–15), thereby providing direct evidence that this enzyme is involved in the destruction of the basement membrane associated with invasion and metastasis.

Gelatinase B activity is controlled at several levels (2, 4), including proenzyme activation. Progelatinase B isolated from various cell lines is activated by autoproteolytic processing on the NH\(_2\)-terminal propeptide and COOH-terminal hemopexin domain. Activation can be initiated in vitro by either a reaction with an organomercurial compound, such as 4-aminophenylmercuric acetate (APMA), or by limited proteolysis by other proteases such as trypsin, chymotrypsin, and stromelysin (16–18). The autoprocessing of gelatinase B in the presence of APMA occurs with a stepwise truncation of the NH\(_2\)-terminal propeptide to Met\(^{75}\), followed by a Ca\(^{2+}\)-dependent loss of COOH-terminal domain (19), apparently to Ala\(^{506}\), and generates a 70-kDa active species (16). Activation of the enzyme by trypsin, however, generates Phe\(^{88}\) as the amino terminus (19). It is noteworthy that the presence of amino acid residues 75–87, corresponding to the Met\(^{75}\)-Arg-Thr-Pro-Arg-Cys-Gly-Val-Pro-Asp-Leu-Gly-Arg sequence in the APMA-activated gelatinase (E\(_a\)), renders the enzyme activity dependent on Ca\(^{2+}\), whereas removal of this fragment by trypsin abolishes the Ca\(^{2+}\) dependence of the enzyme activity (19). This finding suggests that Ca\(^{2+}\) affects the enzyme activity by interaction with this fragment. Ca\(^{2+}\) has also been found to be indispensable for activity of other members of the MMP family (20–23). As early as 1975, Seltzer et al. (20) demonstrated that Ca\(^{2+}\) plays a dual role in collagenase catalysis. It acts as an activator (20) and a stabilizer (20, 21) of collagenase and a stabilizer of stromelysin (22, 23) at physiological temperature and pH. Recent crystallographic studies of the human collagenases (24–27) have identified at least three Ca\(^{2+}\)-binding sites. One of the

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‡ The abbreviations used are: MMP, matrix metalloproteinase; APMA, 4-aminophenylmercuric acetate; E\(_a\), APMA-activated gelatinase B; E\(_t\), trypsin-activated gelatinase B; WT, wild-type; Mca, (7-methoxycoumarin-4-yl) acetic acid; Dpa, 3-(2-fluorophenyl)-2,3-dihydroxypropionic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid.
Ca\(^{2+}\) sites is sandwiched between the surface S-shaped double loop Arg\(^{45}\) to Leu\(^{160}\) and the surface of the \(\beta\)-sheet. Because this loop structure is rather compact, it is assumed that this Ca\(^{2+}\) contributes to the stability of the collagenase structure. However, we have recently shown that Ca\(^{2+}\) is only required for the stability of the trypsin-activated gelatinase B (E\(_1\)), whereas it is involved in both stability and activity of E\(_2\) (19). These observations led us to propose that Ca\(^{2+}\) affects gelatinase B catalysis by binding to the NH\(_2\)-terminal region of E\(_1\), inducing a conformational change in the protein and rendering the enzyme active (Ref. 19; Fig. 1, A1 or A2). It has been suggested that in the catalytic domain of neutrophil collagenase, the presence of Phe\(^{79}\) at the NH\(_2\)-terminal amino acid of E\(_1\) is critical for the stability of the trypsin-activated gelatinase B (E\(_1\)), (27) because the NH\(_2\)-terminal amino acid of E\(_1\), Phe\(^{79}\), is essential for catalytic activity (Ref. 19; Fig. 1, A1 or A2). It has been suggested that in the catalytic domain of neutrophil collagenase, the presence of Phe\(^{79}\) at the NH\(_2\)-terminal amino acid of E\(_1\), Phe\(^{79}\), is essential for catalytic activity (Ref. 19; Fig. 1, A1 or A2).

In the present report, we have extended our previous study (19) and clearly demonstrated that a salt linkage between Phe\(^{88}\) and Asp\(^{432}\) (corresponding to Phe\(^{79}\) and Asp\(^{232}\) in collagenase) exists in stabilizing the active-site conformation of gelatinase B. The side chain of Asp\(^{432}\) can also ligand to Ca\(^{2+}\). The side chain of Asp\(^{432}\) can also ligand to Ca\(^{2+}\) in the absence of the salt linkage, thereby generating a catalytically competent enzyme.

**EXPERIMENTAL PROCEDURES**

Materials—APMA, dimethyl sulfoxide, gelatin attached to 4% beaded agarose, trypsin treated with N-tosyl-L-phenylalanine chloromethyl ketone, soybean trypsin inhibitor insolubilized on 4% beaded agarose, chymotrypsin treated with N-p-tosyl-L-lysine chloromethyl ketone, disopropyl phosphorofluoridate, iminodiacetic acid chelating resin, and all buffers were from Sigma. Calcium chloride, ultradry, 99.99%, was from Alfa (Ward Hill, MA). Calcium chloride hydrate, 99.99% was from Aldrich. Transin, the rat homologue of stromelysin, was a kind gift of Dr. Lynn Matrisian (Vanderbilt University, Nashville, TN). All of the materials for site-directed mutagenesis have been described previously (28). (\[^{14}C\]Gelatin was prepared from calf skin as described previously (29). The fluorogenic peptide, (7-methoxyxycoumarin-4-y1) acetyl (Mca- Pro-Leu-Gly-Lys-Glu-Pro-Asp-Ala-Arg-NH\(_2\)) was purchased from Bachem Bioscience, Inc. (Philadelphia, PA). Buffers and (\[^{14}C\]Gelatin were rendered metal free, as described previously (19). Mutagenesis, Expression, and Purification of Recombinant Progelatinase B—Mutagenesis of the gelatinase B cDNA (pETNG; Ref. 30) was performed by polymerase chain reaction, as described previously for creating the Asp\(^{232}\) to Gly mutation (28). To substitute Glu, Asn, or Lys for Asp\(^{432}\), a degenerate mutagenic oligonucleotide, 5'-TTTCTT GACCTGTTTCATGACATCTGGCTGTCGACGACGG-3' was designed to modify the codon for Asp\(^{326}\) (GAC) to that for Glu (TAG), Asn (AAT), or Lys (AAG). The underlined sequence is the mutated codons. All mutant cDNA was sequenced to verify that the desired substitution was the only one generated by polymerase chain reaction. Plasmids encoding for wild-type (WT) and mutant enzymes were introduced into Escherichia coli strain BL21(DE3)(pLys), the cells bearing the plasmids were induced by isopropyl \(\beta\)-D-thiogalactopyranoside, and the recombinant proteins were purified as described (30). Protein concentration was determined by the Bradford dye binding techniques (a standard Bio-Rad assay), using bovine serum albumin as a standard.

Activation of Recombinant Progelatinase B—WT and each mutant enzyme (~15 \(\mu\)g/ml) were activated in 50 mM Tris-HCl, 0.1 mM NaCl, 0.5 mM ZnCl\(_2\), and 5 mM CaCl\(_2\), pH 7.5 (activation buffer) either by APMA (1.0 mM) for 16 h, by trypsin (20 \(\mu\)g/ml), for 4 h, by chymotrypsin (20 \(\mu\)g/ml), for 4 h, or by stromelysin (1 unit/ml, 1 unit of stromelysin degrades 1 \(\mu\)M fluorogenic peptide, Mca-PLGL(Dpa)AR-NH\(_2\), per min at 37°C) for 4 h at 37°C. Trypsin was removed by passage through a soybean trypsin inhibitor column (2 ml). Chymotrypsin- or stromelysin-activated enzyme was separated from chymotrypsin or stromelysin on a gelatin-agarose affinity column (1 ml) at 4°C. The column was washed thoroughly with the activation buffer to remove the activators. The activated gelatinase was eluted with the activation buffer containing 15% dimethyl sulfoxide. Ca\(^{2+}\) was removed from the activated enzymes, as described previously (19).

Enzyme Assays—Gelatinolytic activity of activated WT and mutants was determined by incubating the enzymes (1–23 \(\mu\)g/ml) with 100 \(\mu\)mol of (\[^{14}C\]Gelatin (77,000 cpm/mg gelatin) in a reaction mixture containing 10 mM HEPES-NaOH, pH 7.5, 0.1 mM NaCl, 0.5 mM ZnCl\(_2\), and 0–1.0 mM CaCl\(_2\) in a final volume of 150 \(\mu\)l at 37°C for 5 min to 2 h, as described (31). Duplicates were performed for each assay. The effect of pH on gelatinolytic activity of the enzymes was determined using the following buffers for the indicated pH ranges: 50 mM HEPES, pH 7.35–7.80; 50 mM CHES, pH 8.25–9.24; and 50 mM CAPS, pH 9.77.

The kinetic parameters of activated WT and mutant enzymes were obtained by assaying the enzymes (0.02 \(\mu\)M) at 37°C against 0.5–20 \(\mu\)M fluorogenic peptide, Mca-PLGL(Dpa)AR-NH\(_2\), as described previously (32). The initial rates of substrate hydrolysis were determined by measuring fluorescence in real time with a multichannel 96-well reaction plate reader. The reaction mixture containing 10 mM HEPES-NaOH, pH 7.5, 0.1 mM NaCl, 0.5 mM ZnCl\(_2\), and 1 mM CaCl\(_2\) in a final volume of 150 \(\mu\)l. The assay was performed using an Aminco-Bowman Luminescence Spectrometer (SLM Instruments, Inc., Urbana, IL).
shown previously that APMA-activated gelatinase B (Ea), activity in the absence of Ca\(^{2+}\) activated gelatinase B, on the other hand, exhibited significant<br>HEPES (pH 7.5) and 10 mM Ca\(^{2+}\)NaCl and 0.5 mM ZnCl\(_2\) in the absence or presence of 1 mM Ca\(^{2+}\) for various times (0–60 min). The samples were then adjusted with 0.5 mM HEPES (pH 7.5) and 10 mM Ca\(^{2+}\) to a final concentration of 50 and 1 mM, respectively. The activities remaining were assayed using [\(^{14}\)C]gelatin or fluorogenic peptide as described above.

Table I

| Activator       | Gelatinolytic activity (units/mg protein) | \(-\text{Ca}^{2+}\) | \(+\text{Ca}^{2+}\) | \(-\text{Ca}^{2+}/+\text{Ca}^{2+}\) (%) | NH\(_2\)-terminal sequences |
|-----------------|-----------------------------------------|---------------------|---------------------|----------------------------------|--------------------------|
| Chymotrypsin    |                                         | 96.17               | 2236.33             | 4.3                              | \(\text{MRT}^{a}\)     |
| Stromelysin     |                                         | 1990.17             | 2726.83             | 73.0                             | \(\text{QTF}^{b}\)    |
| APMA            |                                         | 53.17               | 1972.17             | 2.7                              | \(\text{EGD}^{b}, \text{Glu}^{b}\) |
| Trypsin         |                                         | 1885.66             | 2418.83             | 78.0                             | \(\text{QTF}^{b}\)    |

\(^a\) One unit of enzyme degrades 1 \(\mu\)g of gelatin in 1 min at 37°C.

\(^b\) 7MRT, Met\(^{75}\)-Arg-Thr; \(8\text{FQT}\), Phe\(^{88}\)-Gln-Thr; \(8\text{QTF}\), Gln\(^{89}\)-Thr-Phe; \(92\text{EGD}\), Glu\(^{92}\)-Gly-Asp; \(8\text{QTF}\), Phe\(^{88}\)-Gln-Thr.

RESULTS

Appearance of the NH\(_2\)-terminal Phe\(^{88}\) Correlates with the Loss of Ca\(^{2+}\) Dependence of Gelatinase B Activity—We have shown previously that APMA-activated gelatinase B (Ea) absolutely depends on Ca\(^{2+}\) for activity, but the activity of trypsin-activated enzyme (E\(_{\text{a}}\)) is Ca\(^{2+}\) independent (19). Because gelatinase B can be activated by other proteases, such as chymotrypsin and stromelysin (17), the effect of these activators on Ca\(^{2+}\)-dependent activity of gelatinase B was assessed. The activity of gelatinase B that had been activated by various activators (APMA, trypsin, chymotrypsin, and stromelysin) was determined by assaying the enzyme against [\(^{14}\)C]gelatin in the absence or presence of 1 mM Ca\(^{2+}\). As shown in Table I, chymotrypsin-activated gelatinase B, similar to Ea, had negligible activity in the absence of Ca\(^{2+}\). They were able to catalyze the degradation of gelatin substrate only in the presence of 1 mM Ca\(^{2+}\). Trypsin- or stromelysin-activated gelatinase B, on the other hand, exhibited significant activity in the absence of Ca\(^{2+}\) (78 and 73% of maximal activity, respectively). Ca\(^{2+}\)-independent activity of Ea was not due to trypsin contamination, because the activity of the enzyme was inhibited by a zinc chelating agent, 1,10-phenanthroline (19). It has been demonstrated that stromelysin requires Ca\(^{2+}\) for activity (21); thus, the possibility that Ca\(^{2+}\)-independent activity of stromelysin-activated gelatinase B resulted from the contaminated stromelysin is unprecedented. Zymographic analyses of gelatinase B activated by each activator showed that the 57.5-kDa unglycosylated progelatinase B was converted to a 41.5-kDa species by APMA (Fig. 2A, lane 2) and to a 40-kDa species by trypsin, chymotrypsin, or stromelysin (Fig. 2A, lanes 3–5). This finding agrees with the result obtained using the gelatinase B (92-kDa gelatinase) from HT 1080 human fibrosarcoma cells (17). The NH\(_2\)-terminal sequence analyses of the activated species (Table I and Fig. 2B) indicated that gelatinase B activated by APMA, trypsin, chymotrypsin, or stromelysin had Met\(^{79}\), Phe\(^{88}\), Gin\(^{88}\), or Glu\(^{92}\) or Phe\(^{88}\) as its NH\(_2\)-terminus, respectively. It is interesting to note that both the trypsin- and stromelysin-activated species that did not require Ca\(^{2+}\) for activity had the same NH\(_2\)-terminus, Phe\(^{88}\), whereas APMA and chymotrypsin-activated enzymes that depended on Ca\(^{2+}\) for activity displayed different NH\(_2\)-termini.

These observations suggest that the loss of Ca\(^{2+}\) requirement for the enzyme activity is associated with the presence of Phe\(^{88}\) at the NH\(_2\)-terminus of the activated enzyme.

Effect of pH on Ca\(^{2+}\) Dependence of Trypsin-activated Gelatinase B—X-ray crystal structure of the catalytic domain of collagenase (27) has shown that the ammonium group of the NH\(_2\)-terminal Phe\(^{79}\) makes a salt linkage with the side chain carboxylic group of Asp\(^{232}\) to stabilize the active site. Although the X-ray structure of gelatinase B has not been determined, considering the homology between members of MMP, it is reasonable to assume that the enzyme has a tertiary structure similar to collagenase. As shown above, the presence of the NH\(_2\)-terminal Phe\(^{88}\) (corresponding to Phe\(^{79}\) in collagenase) in gelatinase B correlates with the loss of Ca\(^{2+}\) dependence of the enzyme. Therefore, it is possible that the formation of a salt linkage between Phe\(^{88}\) and Asp\(^{432}\) (corresponding to Asp\(^{232}\) in collagenase) is responsible for the observed gelatinase activity in the absence of Ca\(^{2+}\). If a salt linkage that mimics the effect of Ca\(^{2+}\) on enzyme activity exists in Ea, disruption of the salt linkage at high pH should render activity of the enzyme to be Ca\(^{2+}\) dependent.

The effect of pH on gelatinolytic activity of Ea in the presence or absence of Ca\(^{2+}\) was investigated. As pH increased, Ca\(^{2+}\)
dependence of gelatinolytic activity shifted gradually from being Ca\textsuperscript{2+} independent to Ca\textsuperscript{2+} dependent (Fig. 3). When the pH of the reaction mixture shifted from 7.4 to 9.8, the gelatinolytic activity of the enzyme decreased by about 35% in the presence of 1 mM Ca\textsuperscript{2+}. However, about 95% of the activity was lost in the absence of Ca\textsuperscript{2+}. This suggests that deprotonation of a positively charged group, probably associated with a salt linkage, is responsible for the loss of enzyme activity at pH 9.8 in the absence of Ca\textsuperscript{2+}. The data also support the idea that disruption of the salt linkage at high pH can be compensated for by Ca\textsuperscript{2+} binding to the enzyme.

Discrimination between the Effect of Ca\textsuperscript{2+} on Stability and Catalysis of Gelatinase B—It is common knowledge that the role of Ca\textsuperscript{2+} in MMPs is to stabilize the tertiary structures of the enzymes (20–23). Thus, it is reasonable to assume that the putative salt linkage plays a role similar to Ca\textsuperscript{2+} and stabilizes the conformation of E, at physiological pH in the absence of Ca\textsuperscript{2+}. This was assessed by determining the effect of pH on the stability of E\textsubscript{a} and E\textsubscript{t} in the absence or presence of Ca\textsuperscript{2+}. The enzymes were incubated for various times (0–60 min) at pH 7.5 or 9.8 at 37 °C in the presence or absence of 1 mM Ca\textsuperscript{2+} prior to assaying under normal conditions (pH 7.5, 37 °C, and 1 mM Ca\textsuperscript{2+}). Both enzymes had a similar stability at 37 °C in the presence or absence of Ca\textsuperscript{2+}, regardless of the pH of the solution (Fig. 4). They were essentially stable over 1-h preincubation at 37 °C in the presence of 1 mM Ca\textsuperscript{2+} at either pH 7.5 or pH 9.8, whereas they lost activity in the absence of Ca\textsuperscript{2+}. The loss of activity at either pH followed first-order decay kinetics, and the half-life of both E\textsubscript{a} and E\textsubscript{t} was ~22 min. At pH 7.5, in which the salt linkage should still be intact in E\textsubscript{a}, the enzyme did not show higher stability than E\textsubscript{a} in the absence of Ca\textsuperscript{2+}. This suggests that the Ca\textsuperscript{2+}-independent activity of E\textsubscript{t} does not result from the stabilization effect of the putative salt linkage, and lack of activity of E\textsubscript{a} in the absence of Ca\textsuperscript{2+} cannot be attributed to the instability of the enzyme. These data also imply that Ca\textsuperscript{2+} can act as a reversible stimulator, as well as a stabilizer, of the enzyme. This idea was further supported by assaying E\textsubscript{a} and E\textsubscript{t} at pH 7.5 or pH 9.8 with or without Ca\textsuperscript{2+} (1 mM) for only 5 min at 37 °C. Under this condition, less than 15% of total activity of the enzymes was lost in the absence of Ca\textsuperscript{2+}. E\textsubscript{a} was absolutely dependent on Ca\textsuperscript{2+} for activity, regardless of the pH of the reaction mixture, indicating that the enzyme activity resulting from addition of Ca\textsuperscript{2+} was due to the effect of Ca\textsuperscript{2+} on activity per se rather than on stability (Fig. 5). E\textsubscript{t}, on the other hand, was not active at pH 9.8 in the absence of Ca\textsuperscript{2+}. However, it regained about 60% of its activity upon the addition of 1 mM Ca\textsuperscript{2+}. The increased activity was comparable to the activity of E\textsubscript{a} stimulated by Ca\textsuperscript{2+} at pH 9.8. A decrease in pH from 9.8 to 7.5 apparently had a similar effect to that of Ca\textsuperscript{2+} on enzymatic activity of E\textsubscript{a}. When E\textsubscript{a} was assayed at pH 7.5 in the absence of Ca\textsuperscript{2+}, the enzyme displayed about 90% activity. Adding 1 mM Ca\textsuperscript{2+} increased the activity to 100%. This might be the stabilization effect of Ca\textsuperscript{2+} during the assay period. These data suggest that Ca\textsuperscript{2+} not only stabilizes gelatinase B but also stimulates the enzyme activity by reversibly binding to a distinct “activation” site. Ca\textsuperscript{2+} apparently affects catalysis by inducing a conformational change similar, if not identical, to the one generated by salt linkage in E\textsubscript{a}.
Ca\(^{2+}\) and Gelatinase B Activity

The catalytic activity of E\(_a\) (9.7 \(\mu\)g/ml) and E\(_b\) (7.7 \(\mu\)g/ml) were determined using \([\text{\textsuperscript{14}C}]\text{gelatin substrate in 10 mM HEPES (pH 7.5)} or CAPS (pH 9.8) buffer containing 0.1 mM NaCl and 0.5 \(\mu\)M Zn\(^{2+}\) in the presence or absence of 1 mM Ca\(^{2+}\) at 37 °C for 5 min. Values obtained in the presence of 1 mM Ca\(^{2+}\) at pH 7.5 were considered as 100% of activity.

**Table II**

| Mutants | Gelatinolytic activity (unit/mg protein) | \(-\text{Ca}^{2+}\) | \(-\text{Ca}^{2+}+/\text{Ca}^{2+}\) (%) |
|---------|----------------------------------------|----------------|-------------------------------|
| WT      | 1885.66                                | 2418.83        | 78.0                          |
| D432E   | 25.85                                  | 1636.0         | 1.6                           |
| D432N   | 4.84                                   | 1119.0         | 0.4                           |
| D432G   | 21.0                                   | 515.83         | 4.1                           |
| D432K   | 2.0                                    | 415.0          | 0.5                           |

*a One unit of enzyme degrades 1 \(\mu\)g of gelatin in 1 min at 37 °C.

The 57.5-kDa mutant proenzymes were processed to a 41.5-kDa species by APMA and to a 40-kDa species by trypsin, as judged by zymography (data not shown). The 57.5-kDa mutant proenzymes were processed to a 41.5-kDa species by APMA and to a 40-kDa species by trypsin, as judged by zymography (data not shown). The catalytic activity of WT and mutants were activated by APMA (1 mM) at 37 °C for 16 h. The activated enzymes were made Ca\(^{2+}\)-free as described under "Experimental Procedures." Results are average values for two assays.

**Table III**

| Mutants | Kinetic parameters of gelatinase B mutants\(^a\) |
|---------|-----------------------------------------------|
|         | \(K_a\) (h\(^{-1}\)) | \(K_m\) (\(\mu\)M) | \(K_{cat}\) (\(\mu\)M) |
| WT      | 3654                                      | 7.60                  | 20.0                        |
| D432E   | 3255                                      | 7.45                  | 36.5                        |
| D432N   | 2523                                      | 7.0                   | 65.5                        |
| D432G   | 1122                                      | 5.6                   | 121.0                       |
| D432K   | 950                                       | 7.6                   | 194.0                       |

\(^a\) All the data are average values for two assays.

Initial rates for various concentrations of fluorogenic peptide, Mca-PLGL(Dpa)AR-NH\(_2\) (0.5–20 \(\mu\)M) of each activated species (0.02 \(\mu\)M) were measured in 10 mM HEPES (pH 7.5), 0.1 mM NaCl, 0.5 \(\mu\)M Zn\(^{2+}\) and 1 mM Ca\(^{2+}\). The kinetic constants \(K_m\) and \(K_{cat}\) were determined from Lineweaver-Burk plots.

The assay conditions are described in Fig. 6. The half-maximal stimulation by Ca\(^{2+}\) for each species (\(K_{ca}\)) was determined from Lineweaver-Burk plots.

**Discussion**

We have demonstrated previously that the removal of 13 amino acids from the NH\(_2\)-terminal of APMA-activated gelatinase B (E\(_a\)) by trypsin was accompanied by the loss of Ca\(^{2+}\)-dependence of E\(_a\) (19). Treating E\(_a\) with trypsin generated a new NH\(_2\) terminus, Ph\(_{188}\). These data led us to propose two possible mechanisms for Ca\(^{2+}\)-dependent activity of gelatinase B (Fig. 1), (a) in the absence of Ca\(^{2+}\), the 13 amino acids at the NH\(_2\)-terminus of E\(_a\) act as an autoinhibitory fragment, the inhibition of which could be released either by Ca\(^{2+}\)-binding to the enzyme (Fig. 1, A1) or by removal of this fragment from NH\(_2\)-terminus by trypsin (Fig. 1, T1), (b) the active site-conformation of E\(_a\) is not stable in the absence of Ca\(^{2+}\). A proper active-site conformation could be created either by binding of Ca\(^{2+}\) to the enzyme through interaction with NH\(_2\)- and COOH-terminal residues (Fig. 1, A2) or by a salt linkage between Ph\(_{188}\) at the NH\(_2\)-terminus and Asp\(_{432}\) at the COOH-terminal

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**Figure 5** Effects of pH and Ca\(^{2+}\) on catalytic activity of E\(_a\) and E\(_b\)

The catalytic activity of E\(_a\) (9.7 \(\mu\)g/ml) and E\(_b\) (7.7 \(\mu\)g/ml) were determined using \([\text{\textsuperscript{14}C}]\text{gelatin substrate in 10 mM HEPES (pH 7.5)} or CAPS (pH 9.8) buffer containing 0.1 mM NaCl and 0.5 \(\mu\)M Zn\(^{2+}\) in the presence or absence of 1 mM Ca\(^{2+}\) at 37 °C for 5 min. Values obtained in the presence of 1 mM Ca\(^{2+}\) at pH 7.5 were considered as 100% of activity.
region of the catalytic domain of the enzyme (Fig. 1, T2). In this study, we differentiate these proposals and provide sufficient evidence to conclude that the NH2-terminal Phe is contributes to salt linkage leading to proper active-site conformation similar, if not identical, to the Ca2+-generated conformation favorable for catalysis.

Since the activation of progelatinase B by different activators is achieved by stepwise processing of both NH2- and COOH-terminal peptides (16, 17), it is reasonable to suggest that the Ca2+-dependence of the activated species is governed by minor structural differences after processing of both ends. Although the tertiary structure of gelatinase B is unknown, according to x-ray structures of the other MMP family members (24–27), we would expect that overall structures of gelatinase activated by different activators should be identical. Therefore, the role of different NH2 termini in Ca2+-dependence of gelatinase was assessed by activating the proenzyme with trypsin, stromelysin, or chymotrypsin. Treating gelatinase B with these activators removes the putative autoinhibitory fragment (amino acid residues 75–87) do not require Ca2+ for activity. The observed differences in Ca2+-dependence of the activated enzymes do not result from differential processing of the COOH-terminal end of the enzyme, because the COOH terminus of both E2 and E1 were inferred to be identical (19). These data indicate that amino acid residues 75–87 do not constitute an autoinhibitory fragment and strongly support the idea that the presence of Phe as an NH2 terminus is responsible for the Ca2+-independent activity of trypsin- and stromelysin-activated gelatinase.

The possibility that Phe is involved in the formation of a salt linkage was suggested by the x-ray structure of the catalytic domain of neutrophil collagenase (27). In this enzyme, the NH2 terminus Phe (corresponding to Phe in gelatinase B) apparently forms a salt linkage with highly conserved residue Asp232 (corresponding to Asp in gelatinase B). That the presence of a salt linkage is responsible for the activity of E2 in the absence of Ca2+ is supported by the observation that the enzymatic activity of E2 shifted from Ca2+-independent to Ca2+-dependent as the pH of the reaction mixture increased from 7.5 to 9.8. The enzyme lost about 95% of its activity at pH 9.5 in the absence of Ca2+. The loss of gelatinolytic activity of Ca2+-free E2 at high pH could result from ionization of catalytically essential amino acid residues that are involved in catalysis or those that are responsible for structural integrity of the enzyme. The ionization of active-site groups directly involved in catalysis does not completely account for the loss of activity of E2 in the absence of Ca2+ at pH 9.8, because high pH would have impaired the enzyme activity whether Ca2+ was present or not. A salt linkage in the protein, on the other hand, can play a role in stabilizing the enzyme structure and/or maintaining a proper active-site conformation and is sensitive to the pH of the solution (33). For example, the salt linkage formed between the α-ammonium group of Ile146 and the side chain of Asp194 in chymotrypsin (34) controls the active-site conformation of the enzyme. When the α-ammonium group is deprotonated at high pH, chymotrypsin is reversibly inactivated, displaying a pK of 8.8. A pK of –8.8 reflecting the ionization state of the α-ammonium group of Phe148 in E2 could be extracted from Fig. 3. The pK of free ammonium group of Phe is about 7.8, and it rises to about 10 when it is involved in a salt linkage (35). The observed pK suggests that the superposition of both conformations causes this residue to titrate with a pK of 8.8. These data suggest that a salt linkage involving Phe, similar to the one in α-chymotrypsin (34), is present in E2 and is able to substitute for Ca2+-in maintaining the proper active-site conformation of the enzyme favorable for catalysis. The putative salt linkage in E1 did not contribute to enzyme stability because the rate of inactivation of E1 at pH 7.5 was similar to that at pH 9.8 in the absence of Ca2+. Furthermore, the activity of E1 at different pH was determined for only 5 min. Under this condition, E1 was essentially stable. Therefore, the loss of activity of Ca2+-free E1 at high pH is apparently due to irreversible disruption of the active-site conformation rather than from irreversible denaturation of the protein.

The involvement of Asp in the salt linkage mimicking the effect of Ca2+ on enzymatic activity is supported by the observation that replacing Asp with Gly, Glu, or Lys, amino acids that conceivably could destroy the salt linkage, resulted in enzyme species the activities which were dependent on Ca2+. Even adding one more methylene group to the carboxylate side chain of amino acid 432 (D432E) resulted in an enzyme that required Ca2+ for activity. The requirement of both correct processing of the NH2 terminus and of exact length of the side chain at position 432 for Ca2+-independent activity also suggests the existence of a salt linkage between the two sites in the gelatinase molecule.

We and others (19–23) have demonstrated that Ca2+ acts as both an activator of MMPs and a stabilizer to prevent the enzymes from denaturation. In this study, we have shown that the function of Ca2+ in catalytic activity of gelatinase B could be effectively performed by the salt linkage formed between the NH2 and COOH termini of the catalytic domain. This result raised a possibility that Ca2+ forms an intramolecular bridge between the NH2 and COOH termini of the enzyme similar to the salt linkage to maintain a proper active-site conformation. Interestingly, a similar Ca2+-binding site has been identified in protein C (36). It was demonstrated that binding of Ca2+ to a high affinity site in protein C involving Glu and Glu resulted in a conformational change that is required for activation by thrombin-thrombomodulin complex, the activator of
protein C. Substituting Glu80 with Lys created a salt linkage between Glu80 and Lys80 and led to a conformational change similar to that induced by Ca2+. Our study has strongly suggested that Asp432 in the COOH-terminal helix functions as one of the Ca2+-binding ligands. The direct evidence for the participation of this residue in Ca2+ binding was obtained from the observation that affinity of gelatinase for Ca2+ was significantly affected by the side chain of the introduced amino acid residue at position 432. Replacing Asp432 with either the isosteric amide group (D432N) or removing the side chain as in D432G decreased the affinity of the enzyme for Ca2+ by factors 3 and 6, respectively, whereas the conserved substitution (D432E) had little effect on its affinity for Ca2+. Introducing a positively charged side chain (D432K), however, decreased the Ca2+ affinity by a factor of 10. This finding indicates that both the size and charge of the side chain of Asp432 is important for maintaining a proper geometry of the Ca2+-binding site and that the negatively charged carboxylate group of Asp432 apparently contributes to Ca2+ coordination. The involvement of Asp432 and Ca2+ binding in the catalysis of gelatinase B has been further suggested by the kinetic study of the WT and mutant enzymes. All of the substitution mutants had Km values for the synthetic substrate similar to the WT, but their kcat values were decreased significantly. The decreased Ca2+ affinities are apparently responsible for the observed decrease in kcat values of the mutant enzymes. The order of decrease in kcat values for the mutants is WT ≥ D432E > D432N > D432G > D432K, which correlates well with the order of decrease in Ca2+ binding affinity of the mutant enzymes. The lack of effect of Asp432 replacement on Km indicates that the active site geometry of the mutant enzymes are still intact. These observations strongly suggest that binding of Ca2+ to Asp432 triggers a minor conformational change at the active site and leads to proper orientation of the catalytically essential groups without significantly affecting the geometry of the entire active site.

The highly conserved sequence Lys431-Asp432-Asp-Val435 in gelatinase B containing Asp432 has striking parallels with the Ca2+-binding site (Lys-Asp-Asp-Ile-Asn) in coagulation factors IX and X (37–40), further supporting the idea that this sequence in gelatinase B is one of the Ca2+-binding site. On the basis of the x-ray crystal structure of the catalytic domain of neutrophil collagenase, this conserved sequence constitutes the basement of the active site of the enzyme (27); however, no Ca2+ was detected at this particular region of the enzyme. This is not a surprising observation. Leibodi and Stec (41) have also reported that the “catalytic” metal ion in yeast enolase is not observed by x-ray diffraction. The concentration of Ca2+ and the pH of an enzyme solution from which the crystals were grown are known to affect the number of Ca2+-binding sites seen in the protein. Pantoliano et al. (42) found that occupancy of a weak Ca2+-binding site in bacterial serine protease subtilisin is a function of the Ca2+ concentration for a series of x-ray structures obtained for crystals grown with increasing amounts of CaCl2 in the range of 0–40 mM at pH 9.0. When crystals were grown in the presence of 10 mM CaCl2 and at pH 5.0, the two low affinity regulatory sites of trypsinogen (Kd ≈ 10−5 M at neutral pH) were devoid of Ca2+ ion, whereas the higher affinity structural sites (Kd ≈ 10−7 M at neutral pH) were occupied by Ca2+ in the x-ray structure of the enzyme (43). Furthermore, it has been shown that the Ca2+ binding affinity is significantly decreased by lowering the pH in stromelysin (44). The observed discrepancy between our data and the x-ray crystal structure of collagenase could be due to the fact that the reported x-ray structures of MMPs were obtained under different crystallization conditions. Lovejoy et al. (24) identified one Ca2+ site in the truncated fibroblast collagenase when they crystallized the enzyme at pH 6.5 in the absence of added Ca2+. The same group (25) found three Ca2+ sites in this enzyme only by increasing the pH to 7–9 and adding 1 mM Ca2+) to the enzyme solution. Under the conditions of pH 6.0 and 5 mM Ca2+, Bode and co-workers (26, 27) identified two Ca2+ sites in the truncated neutrophil collagenase. These Ca2+-binding sites are thought to have a high affinity for Ca2+ and be involved in stabilization of the enzyme. The site involving Asp432, however, may have a low affinity for Ca2+; thus, it is likely that Ca2+ is not able to bind to this site under the crystallization conditions used, or Ca2+ binds to this region of the molecule only during catalysis.

In summary, our data provide sufficient evidence to conclude that Ca2+ plays dual roles in gelatinase B. It is involved in catalysis by bridging NH2- and COOH-terminal regions of the catalytic domain and in structural stability by binding to several sites on the enzyme. The role of Ca2+ in catalysis can be substituted by the salt linkage between the ammonium group of Phe80 and the carboxylate side chain of Asp432. This interaction renders the enzyme independent of Ca2+ for activity. Because all MMP family members require Ca2+ for activity and contain these conserved amino acids, we anticipate that the proposed mechanism for gelatinase B will apply to other MMP members as well. For neutrophil collagenase we have demonstrated previously that the COOH-terminal region containing the conserved sequence Asp332-Asp-X-Asp is necessary for general catalytic activity of the enzyme (45). Replacing Asp332 with Gly has also been demonstrated to reduce the catalytic activity of the APMA-activated collagenase. Whether the presence of a salt linkage in stromelysin-activated collagenase substrates for Ca2+ in activity remains to be determined.

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