Identification of the Active Site of Gelatinase B as the Structural Element Sufficient for Converting a Protein to a Metalloprotease †

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Identification of the Active Site of Gelatinase B as the Structural Element Sufficient for Converting a Protein to a Metalloprotease†

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ABSTRACT: Gelatinase B is a member of the matrix metalloproteinase family that efficiently cleaves gelatin, elastin, and types V and X collagen. To understand the contribution of the active site of the enzyme (amino acid residues 373−456) in these activities, we studied catalytic properties of a fusion protein consisting of maltose binding protein and the active site region of gelatinase B. We found that addition of the active site of gelatinase B, which corresponds to 12% of the total protein molecule, to maltose binding protein is sufficient to endow the protein with the ability to cleave the peptide substrates Mca-PLGL(Dpa)AR-NH_2 and DNP-PLGLWA-(D)-R-NH_2. The fusion protein hydrolyzed the Mca-PLGL-(Dpa)AR-NH_2 peptide with the same efficiency as that of the stromelysin, $k_{cat}/K_m \simeq 1.07 \times 10^6$ M^{-1} h^{-1}. The fusion protein, however, was not able to degrade the large substrate, gelatin. Inhibition of the activity of the protein by EDTA suggested that its activity was metal dependent. ESR analyses indicated that the fusion protein bound one molecule of Zn^{2+}. In addition, Z-Pro-Leu-Gly-hydroxamate and TIMP-1 inhibited the activity of the protein, suggesting that the structure of the active site of the fusion protein is similar to that of the other metalloproteinases. These data provide fundamental information about the structural elements required for transforming a protein to a metalloprotease.

The matrix metalloproteinases (MMP)[1] are a unique family of zinc binding endopeptidases that have the ability to mount a concerted degradative attack on virtually all components of the extracellular matrix (*1*−*3*). The uncontrolled expression of MMP reportedly leads to many physiological and pathological conditions and events including rheumatoid arthritis, cardiovascular diseases, angiogenesis, and tumor metastasis (*4*−*12*). Members of this important protease family are expressed in an inactive proenzyme form and subdivided according to their substrate specificity: the collagenases (MMPs 1, 8, and 13), the gelatinases (MMPs 2 and 9; gelatinases A and B, respectively), the stromelysins (MMPs 3, 10, and 11), and the membrane-type MMPs (MT-MMP; MMPs 14−17).

Gelatinase B is a 92 kDa glycoprotein that upon activation digests gelatin; native types IV, V, and XI collagen; and elastin (*1*, *2*, *13*−*16*). Endogenous inhibitors, α2-macroglobulins, and tissue inhibitor of metalloproteinase-1 (TIMP-1) control the proteolytic activity of gelatinase B in vivo (*17*, *18*). TIMP-1, a 28 kDa secreted glycoprotein, interacts with and inhibits almost all members of the MMP family (*19*, *20*). The MMP complexes with TIMP-1 are noncovalent and are not stable in the presence of SDS (*21*). It has been reported that progelatinase B isolated from fibroblasts copurifies with TIMP-1 (*22*), apparently due to the interaction of the inhibitor with the carboxyl-terminal domain of the proenzyme (*18*). The region(s) of the activated gelatinase B that interacts with TIMP-1 has (have) yet to be identified.

The gelatinase B molecule consists of a propeptide fragment that is lost upon activation, a catalytic domain consisting of an amino-terminal fragment, a fibronectin type II homology unit (Fib domain), and a zinc binding active site, a type V collagen-like domain, and a hemopexin-like carboxyl terminus (*13*, *22*). The catalytic domain of the other MMP family members, except MMP-2, lacks the Fib domain. According to the crystal structure of the catalytic domain of MMP-1, MMP-8, and MMP-2 (*23*−*29*), the active center of MMP contains a zinc binding motif, His-Glu-X-X-His-X-X-Gly-X-X-His, and a conserved sequence of Leu-X-X-Asp-Asp-X-X-Gly at its carboxyl terminus. Site-directed mutagenesis studies have revealed that His 400, Glu 401, Asp 432, and Asp 433 in the conserved motifs are critical for the enzymatic activity of gelatinase B (*30*, *31*). His 400

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[1] Abbreviations: MMP, matrix metalloproteinase; MT, membrane type; FC, fibroblast collagenase; APMA, *p*-aminophenylmercuric acetate; Mca, (7-methoxycoumarin-4-yl)acetyl; Dpa, 3-(2′,4′-dinitrophenyl)-L-2,3-diaminopropionyl; Gel B/AS, gelatinase B active site; TIMP, tissue inhibitor of metalloproteinase; Fib, fibronectin type II homology unit; MBP, maltose binding protein; MBP + Gel B/AS, maltose binding Gel B/AS chimeric protein.

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appears to provide a ligand to the bonded zinc, and Glu 401 may act as a general base catalyst. The Asp residue at position 432 apparently stabilizes the active site via interaction with calcium (*31*, *32*). The elastinolytic and type V and type XI collagenolytic activities of the enzyme, however, depend on the presence of the Fib domain (*16*, *33*, *34*). We recently demonstrated that addition of the Fib domain to fibroblast collagenase (FC) and replacement of the active site region of the enzyme (amino acids 212−254) with that of gelatinase B (amino acids 395−437) endowed the collagenase with the ability to cleave type V collagen (*34*). This addition also substantially increased the activity of FC toward gelatin and a peptide substrate (*34*). This observation led to identifying amino acid residues Leu 397, Ala 406, Asp 410, and Pro 415 in the active site of gelatinase B as being important for efficient catalysis. Leu 397 and Ala 406 are important for the general proteolytic activity of the enzyme, while Asp 410 and Pro 415 specifically enhance its ability to cleave type V collagen and gelatin, respectively. In this report, we have achieved a definitive understanding of the structural elements that are required and sufficient for proteolytic activity by studying catalytic properties of a fusion protein containing maltose binding protein (MBP) and the active site region of gelatinase B (Gel B/AS, amino acid residues 373−456). We were able, for the first time, to demonstrate that addition of Gel B/AS, which corresponds to 12% of the enzyme molecule, to MBP is sufficient to endow the protein with the ability to cleave an MMP−peptide substrate. In addition, our analysis gives insight on the inhibition of gelatinase-B by TIMP-1.

EXPERIMENTAL PROCEDURES

Materials. Reagents for LB media, Bacto-agar, Bacto-tryptone, and yeast extracts were obtained from Difco Laboratories (Detroit, MI); isopropyl β-D-thiogalactoside (IPTG) was purchased from Gold Biotech (St. Louis, MO); sequenase was from U.S. Biochemical Co. (USB). Gene Clean and Mermaid Kit were purchased from Bio 101 (La Jolla, CA). The *Escherichia coli* expression system (pMal-C), bacterial strain TB1, and amylose resins were from New England Biolabs (Beverly, MA). The fluorogenic peptide, Mca-PLGL(Dpa)AR-NH_2, and Z-Pro-Leu-Gly-hydroxamate were purchased from Bachem Bioscience, Inc. (Philadelphia, PA). $^{65}ZnCl_2$, 5 Ci/g, was from Dupont NEN (Boston, MA). pMal-F was a gift from Dr. Harry W. Jarrett III, University of Tennessee Health Science Center.

Construction of the Maltose Binding Active Site of Gelatinase B Fusion Protein (MBP + Gel B/AS). The NG1188−1440 cDNA fragment (corresponding to amino acid residues 373−456 of gelatinase B, Figure 1) was generated by polymerase chain reaction (PCR) using pNG 4.1 as a template (*13*) and 20-mer oligonucleotide primers. The 3′ oligonucleotide (5′-CAGGTCGACTCTAGACTATT-AGGTGGTGGTTGAGG-3′) was designed to introduce a stop codon at position 1440 followed by an *Xba*I restriction site at the 3′ end of the PCR product. The 5′ oligonucleotide primer (5′-ATCGAGGGTAGGCCTGCTACCACCTCGAAC-3′) complementary to nucleotide 1188 of pNG4.1 incorporated a *Stu*I restriction site. The 280 bp PCR product was digested with restriction enzymes *Stu*I and *Xba*I and cloned into pMal-C, a prokaryotic expression vector containing an *E. coli* maltose binding protein gene (Mal-E). The result was

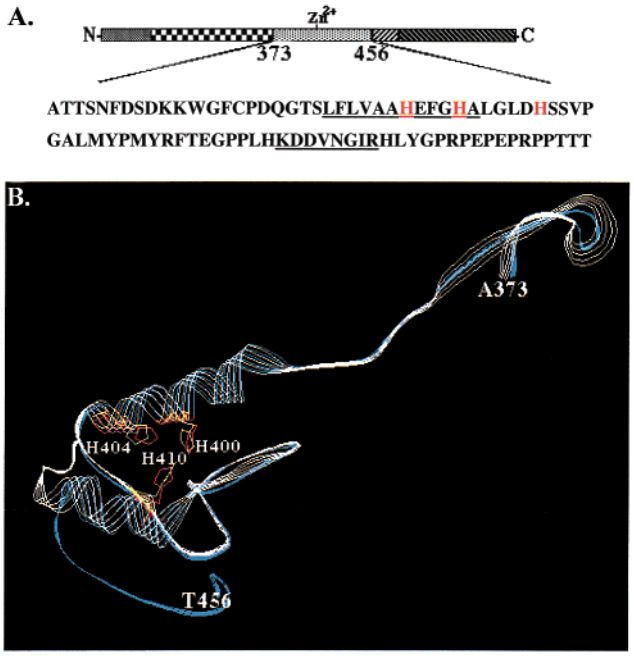

FIGURE 1: Domain structure and active site of gelatinase B. (A) The region of the protein that was amplified by PCR and fused to MBP is shown together with the amino acids encoded. The number refers to the corresponding amino acids. The conserved His residues are shown in red, and the α-helices are underlined. Bar graph (from left to right): amino-terminal domain, fibronectin type II homology unit (Fib domain), zinc binding region, collagen-like domain, and carboxyl-terminal domain of the full-length gelatinase. (B) Predicted 3-D structure of Gel B/AS (blue ribbon) superimposed on the corresponding region of MMP-2 (white ribbon). The three conserved His residues are shown in red and in yellow for Gel B/AS and MMP-2, respectively. This figure was modeled on the basis of the X-ray structure of MMP-2 (*58*).

a chimeric gene between the NG1188−1440 cDNA fragment and the Mal-E gene of the vector. The chimeric DNA (pMal + Gel B/AS) was under control of the tac promoter. The sequence of the NG1188−1440 cDNA fragment and the junction of Mal-E/Gel B/AS in the clone were confirmed by DNA sequencing. The pMal + Gel B/AS construct was used to transform *E. coli* TB1 cells. Induction of the cell with 0.4 mM IPTG resulted in expression of the maltose binding gelatinase B active site fusion protein, MBP + Gel B/AS. The MBP + Gel B/AS could be released from the cell as a soluble protein by sonication.

Isolation and Purification of Recombinant Proteins. The MBP + Gel B/AS and maltose binding protein (MBP) were purified from *E. coli* extracts using amylose affinity chromatography as recommended by the manufacturer (New England Biolabs). Briefly, the bacterial cultures were grown in superbroth containing 50 μg/mL ampicillin at 37 °C with shaking until the cultures reached an OD_{600} of 0.6−1.0; IPTG was then added to a final concentration of 0.4 mM. The cultures were incubated with shaking at 37 °C for an additional 3 h. The bacteria were pelleted by centrifugation at 4000 rpm for 10 min and resuspended in 15 mM phosphate buffer, pH 7.3, containing 30 mM NaCl, 25 mM benzamidine, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.2% Tween 20, 1 mM PMSF, and 4 mg/mL lysozyme. The cell suspension was incubated at room temperature for 15 min followed by 15 min incubation at 4 °C and then sonicated for 60 s. RNase A (1 unit/mL) and NaCl (0.5 mM) were

added, and the suspension was centrifuged at 10000 rpm for 30 min at 4 °C. The supernatant was then applied to an amylose column (2.5 cm × 18.5 cm) equilibrated with the column buffer (15 mM phosphate buffer, pH 7.3, 0.5 M NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.2% Tween 20, 1 mM PMSF). The column was washed with two column volumes of the above buffer and two column volumes of wash buffer containing 50 mM Tris, pH 7.5, 1 mM PMSF, 50 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol until the OD_{280} was near zero. The bound enzyme was then eluted with wash buffer containing 10 mM maltose. The fractions containing the recombinant proteins were pooled and dialyzed against 50 mM Tris buffer, pH 8.0, containing 100 mM NaCl and 2 mM $CaCl_2$ and stored at −20 °C.

Zinc Binding Assay. Five micrograms each of MBP + Gel B/AS, MBP, carbonic anhydrase, and ovalbumin was blotted on a nitrocellulose membrane using a slot blot apparatus. The membrane was washed with 100 mM Tris-HCl, pH 6.8, and 50 mM NaCl for 1 h (*35*) and incubated in 10 mL of the same buffer containing $^{65}ZnCl_2$ (1.0 μC/mL) for an additional 1 h at room temperature. The membrane was washed with ^{65}Zn-free buffer for 1 h at room temperature. The membrane was then dried under vacuum and exposed to X-ray film (Eastman Kodak, Rochester, NY).

ESR Analysis. X-band ESR spectra of 2.3 mM solutions of the MBP and MBP + Gel B/AS proteins in either 50 mM acetate or 50 mM Tris, pH 7.5, buffer containing 100 mM NaCl and 2 mM $CaCl_2$ were obtained on a Varian E spectrometer equipped with a TM_{110} microwave cavity using 100 kHz modulation in the absence or presence of an equimolar concentration of Mn^{2+}. The ESR signals were recorded at 298 K and on frozen solutions at 77 K. The difference spectra of a 2.3 mM solution of metal ions in the absence and presence of proteins were used to examine the state of the bound versus free metals. The data were analyzed using software written by Duling (NIEHS) (*36*).

Kinetic Analysis. The enzyme was assayed against Mca-PLGL(Dpa)AR-NH_2, DNP-PLGLWA-(D)-R-NH_2, and DNP-PQGIAGQ-(D)-R-OH peptide substrates as described (*37*−*39*). The Mca-PLGL(Dpa)AR-NH_2 peptide substrate was used in all the experiments described in this paper.

Hydrolysis of the fluorogenic peptide substrate, Mca-PLGL(Dpa)AR-NH_2, was monitored by measuring the increase in fluorescence (λ_{ex} = 328 nm, λ_{em} = 393 nm) as a function of time as previously described (*37*). All assays were performed at 25 °C in a total volume of 0.5 mL of assay buffer (50 mM Tris, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, 10 μM $ZnCl_2$) containing 1−5 μM substrate. Under this condition, the rate of substrate hydrolysis in the presence and absence of MBP was negligible. The k_{cat}/K_m value for MBP + Gel B/AS was then determined under the condition when $[S] \ll K_m$ using the $k_{cat}/K_m \cong v_0/[E]_T[S]$ equation (*37*).

Z-Pro-Leu-Gly-Hydroxamate Inhibition. The full-length recombinant gelatinase B (5 nM) and MBP + Gel B/AS (6 μM) were incubated with various amounts of the MMP's inhibitor, Z-Pro-Leu-Gly-hydroxamate (0−200 μM), in 50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$, 0.5 μM $ZnCl_2$, and 100 mM NaCl at 25 °C for 30 min. The amount of the proteolytic activity remaining as a function of the hydroxamate concentration was then determined using the Mca-PLGL(Dpa)-AR-NH_2 peptide substrate (1 μM) as described above.

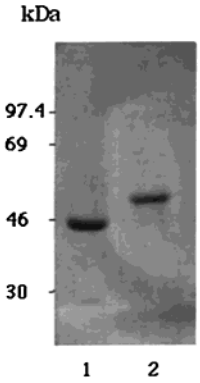

FIGURE 2: SDS−PAGE analysis of the purified recombinant proteins. Purified MBP and MBP + Gel B/AS proteins were boiled for 5 min and electrophoresed on a 10% SDS−PAGE gel under reducing conditions. Protein bands were visualized by Coomassie Blue staining. Lanes: 1, MBP; 2, MBP + Gel B/AS. Positions of molecular mass standards are shown to the left of the gel.

TIMP-1 Binding Assay. Recombinant TIMP-1 was prepared and purified as described previously (*40*). Microtitration plates were coated with 100 μL of TIMP-1 at a concentration of 300 ng/mL in coating buffer (50 mM Na_2CO_3, pH 9.6) at 4 °C overnight. The plates were washed several times with wash buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Tween 20) and blocked in blocking buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 containing 0.25% BSA) at room temperature for 1 h. Plates were washed and incubated with different concentrations of MBP, MBP + Gel B/AS, or soybean trypsin inhibitor in blocking buffer at 37 °C for 2 h. The bound proteins were then detected by anti-MBP antibody (1:1000) using the alkaline phosphatase detection method. The optical density of the color developed was measured at 405 nm using Titerscan Multiskan Plus (EF Labs, Finland). MBP and soybean trypsin inhibitor were used as an internal and an independent control, respectively.

RESULTS

Proteolytic Activity of MBP + Gel B/AS. Our previous experiments indicated that the catalytic efficiency of gelatinase B toward its substrates, gelatin, peptide substrate, and type V collagen depends on the presence of amino acid residues Leu 397, Ala 406, Asp 410, and Pro 415 in the active site of the enzyme (*34*). To assess whether the Gel B/AS containing the catalytic zinc binding ligands and the above residues has the capability to render a protein protease activity, the catalytic properties of the MBP containing this peptide fragment were examined. Gel B/AS was defined by the predicted C-terminus of the Fib domain (amino acid residue 373) and the N-terminus of the type V collagen-like domain (amino acid residue 456, Figure 1). The fusion protein MBP + Gel B/AS consisted of full-length MBP with its carboxyl terminus affixed to the Gel B/AS.

The MBP + Gel B/AS and MBP were expressed in *E. coli* as soluble proteins and purified as described in Experimental Procedures. The MBP was recovered as a 45 kDa species (Figure 2, lane 1) as expected (*41*), and MBP + Gel B/AS was a 54 kDa protein (Figure 2, lane 2), consistent with the addition of the Gel B/AS fragment to the MBP molecule. The activities of these proteins toward gelatin, Mca-PLGL(Dpa)AR-NH_2, DNP-PQGIAGQ-(D)-R-OH, and

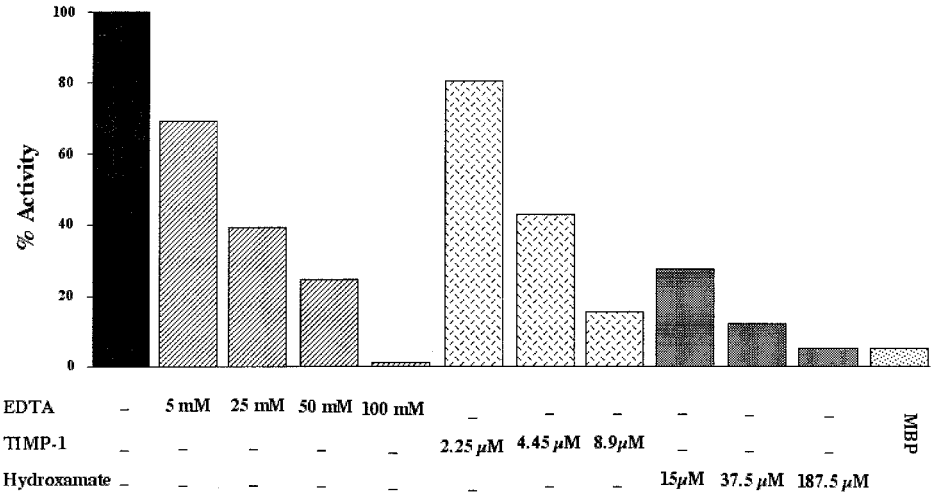

FIGURE 3: Enzymatic activity of MBP + Gel B/AS. Hydrolysis of Mca-PLGL(Dpa)AR-NH_2 peptide by MBP + Gel B/AS (6.7 μM) in the presence or absence of various inhibitors was continuously determined over a period of 10 min at 25 °C as described under Experimental Procedures. The activity of the enzyme was then expressed as micromoles of substrate degraded per minute. The activity of MBP + Gel B/AS in the absence of the inhibitor was counted as 100%.

Table 1: Catalytic Efficiency of Matrix Metalloproteinases toward Mca-PLGL(Dpa)AR-NH_2 Substrate

enzyme	k_{cat}/K_m (M^{-1} h^{-1})	reference
MBP + Gel B/AS[a,b]	1.07×10^6	this work
gelatinase B[a,c]	7.47×10^8	*30*, *34*
FC[a,c]	$(1.55-3.75) \times 10^7$	*34*, *42*
FC + Gel B/AS[a,c]	5.76×10^7	*34*
stromelysin[c]	6.5×10^6	*42*
collagenase-3[c]	4.36×10^7	*43*
MT1-MMP catalytic domain[c]	2.98×10^{12}	*44*

[a] The k_{cat}/K_m value was determined at 25 °C. [b] The concentration of protein was determined by the Bradford dye binding technique using bovine serum albumin as standard. [c] The active protein concentration was determined by active site titration using either TIMP-1 or TIMP-2.

DNP-PLGLWA-(D)-R peptide substrates were assessed using gelatinase B as a standard. MBP + Gel B/AS showed no activity toward gelatin up to 7 μM, and its activity toward the DNP-PQGIAGQ-(D)-R-OH peptide was negligible (431 M^{-1} h^{-1}). On the other hand, it was able to efficiently hydrolyze Mca-PLGL(Dpa)AR-NH_2 (Figure 3 and Table 1) and DNP-PLGLWA-(D)-R NH_2 peptides. The activity of MBP + Gel B/AS toward the Mca-PLGL(Dpa)AR-NH_2 (1×10^6 M^{-1} h^{-1}) peptide was, however, an order of magnitude higher than its activity toward the DNP-PLGLWA-(D)-R-NH_2 peptide (8.5×10^4 M^{-1} h^{-1}). The rate of substrate hydrolysis was directly dependent on the concentration of the enzyme in the reaction mixture (data not shown). The substrate specificity of the fusion protein was consistent with the peptide substrate specificity of gelatinase B. Gelatinase B hydrolyzed Mca-PLGL(Dpa)AR-NH_2 peptide 2.6 and 300 times faster than DNP-PLGLWA-(D)-R-NH_2 and DNP-PQGIAGQ-(D)-R-OH peptides, respectively (data not shown). MBP at the same concentration was not able to cleave either one of the peptide substrates, indicating that the addition of Gel B/AS to MBP was necessary and sufficient to confer proteolytic activity on the protein.

To further substantiate this endowed activity, the effect of EDTA and the Z-Pro-Leu-Gly-hydroxamate inhibitor on the proteolytic activity of MBP + Gel B/AS was examined by assaying the enzyme in the presence and absence of

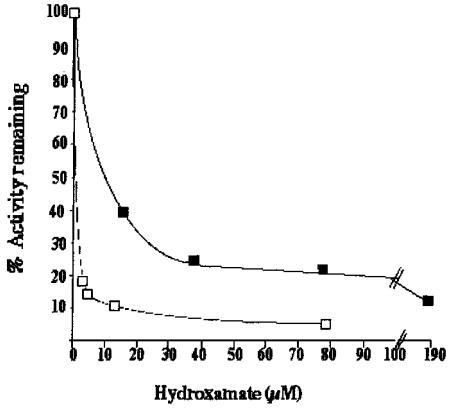

FIGURE 4: Inhibition of MBP + Gel B/AS and full-length gelatinase B by Z-Pro-Leu-Gly-hydroxamate. MBP + Gel B/AS (6.7 μM, closed squares) and recombinant gelatinase B (5 nM, open squares) were incubated with Z-Pro-Leu-Gly-hydroxamate (0−200 μM) in 50 mM Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl, 5 mM $CaCl_2$, and 0.5 μM $ZnCl_2$ at 25 °C for 30 min. The amount of the proteolytic activity remaining as a function of the inhibitor concentration was then determined using the fluorogenic peptide substrate. The activity of each enzyme in the absence of the inhibitor was counted as 100%.

various amounts of these inhibitors using Mca-PLGL(Dpa)-AR-NH_2 as a substrate. The MMP's substrate analogue, Z-Pro-Leu-Gly-hydroxamate, has been shown to inhibit the activity of all of the MMPs tested to date. As shown in Figures 3 and 4, similar to gelatinase B, Z-Pro-Leu-Gly-hydroxamate and EDTA completely inhibited the activity of MBP + Gel B/AS. The apparent K_i value of MBP + Gel B/AS for the hydroxamate inhibitor was 6.35 μM. This value was ~15 times higher than that of the full-length gelatinase B (~0.410 μM). Similarly, the activity of the enzyme was reduced by ~60% in the presence of 25 mM EDTA and completely abolished by adding 100 mM EDTA to the reaction mixture. These data indicated that the proteolytic activity of the MBP + Gel B/AS was specific, and the observed activity of MBP + Gel B/AS did not result from a minor contaminant in the protein solution. The crystal structure of the catalytic domain of the FC- and MMP-8-hydroxamate complex revealed that the inhibitor directly

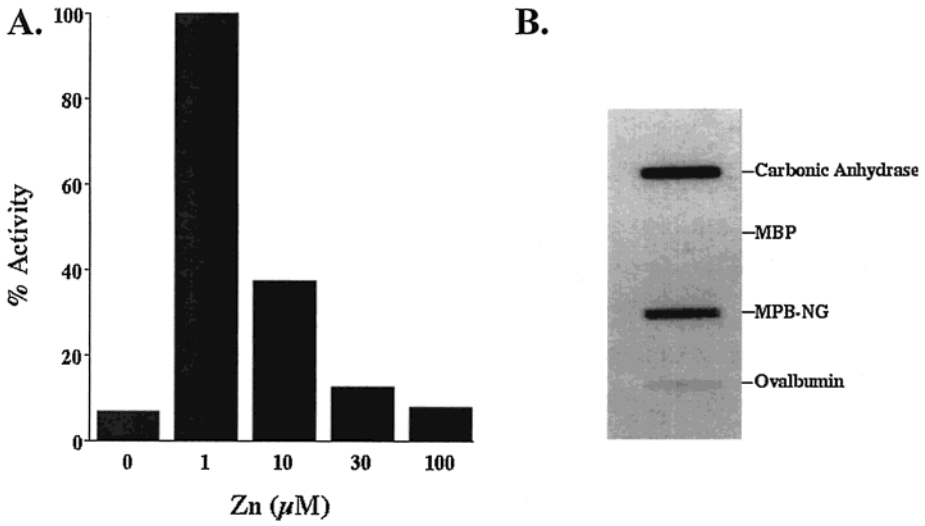

FIGURE 5: (A) Effect of the Zn^{2+} concentration on enzymatic activity of MBP + Gel B/AS. Hydrolysis of Mca-PLGL(Dpa)AR-NH_2 peptide by MBP + Gel B/AS (6.7 μM) in the presence of various concentration of Zn^{2+} was continuously determined as stated in the legend to Figure 3. **(**B) Binding of MBP + Gel B/AS to zinc as determined by ^{65}Zn blot. Five micrograms of MBP + Gel B/AS, MBP, carbonic anhydrase, and ovalbumin was blotted on a nitrocellulose membrane. The membrane was incubated in Tris buffer (100 mM, pH 6.8) containing NaCl (50 mM) and $^{65}ZnCl_2$ (1.0 °C/mL) for 1 h at room temperature. The membrane was then washed, dried under vacuum, and exposed to X-ray film. MBP was used as an internal control; carbonic anhydrase and ovalbumin were a positive and a negative control, respectively.

interacts with the catalytic Zn^{2+} (*28*, *45*). These data together suggested that the catalytic activity of the MBP + Gel B/AS is dependent on the presence of metal ions in the solution. This was confirmed by assaying the enzyme in the presence of various concentrations of $ZnCl_2$. The observed activity of the fusion protein, similar to all the MMPs, was dependent on the concentration of the Zn^{2+} in the reaction mixture (Figure 5A). The enzyme was fully active in the presence of 1.0 μM Zn^{2+}. Zn^{2+} at high concentration, however, inhibited the activity of the enzyme, consistent with the notion that MBP + Gel B/AS is a Zn^{2+} metalloenzyme.

Metal Binding Properties of MBP + Gel B/AS. To investigate the role of metal ions in MBP + Gel B/AS, the ability of the enzyme to bind Zn^{2+} was examined by ^{65}Zn blot and ESR techniques. MBP + Gel B/AS, MBP, carbonic anhydrase, and ovalbumin were treated with $^{65}Zn^{2+}$ under nondenaturing conditions at pH 6.8. Under these conditions Zn^{2+} binds only to zinc metalloproteins (Figure 5; *35*, *46*). As shown in Figure 5B, $^{65}Zn^{2+}$ was able to bind MBP + Gel B/AS and the positive control, carbonic anhydrase, whereas MBP that lacks the gelatinase B active site sequence failed to bind Zn^{2+}. Ovalbumin, on the other hand, showed a very weak interaction with Zn^{2+}; the reason is unknown. These data demonstrated that MBP + Gel B/AS is a metalloprotein and suggested that, in addition to providing the MBP with proteolytic activity, the inclusion of the Gel B/AS in MBP conferred metal dependency upon the protein.

The interaction between MBP + Gel B/AS and $Zn^{2+}$was further substantiated, and the number of metal binding sites in the fusion protein was determined by the ESR technique and use of the paramagnetic metal ion Mn^{2+} (Figure 6). This technique is based on the principle that the ESR spectra show changes in the magnitude of the Mn^{2+} hyperfine interaction when Mn^{2+} binds to a ligand. A reduction in the hyperfine coupling constant, *A* (^{55}Mn), is expected when the unpaired electron density on the metal is reduced by an increase in the covalency of the metal−ligand bond (*47*). As demonstrated in Figure 6A, the typical ESR signal from free Mn^{2+} is a sextet with splitting that comes from the hyperfine coupling between the unpaired electrons and the ^{55}Mn nucleus ($I = {}^5/_2$). Upon addition of an equal molar concentration of MBP + Gel B/AS to the metal solution, the

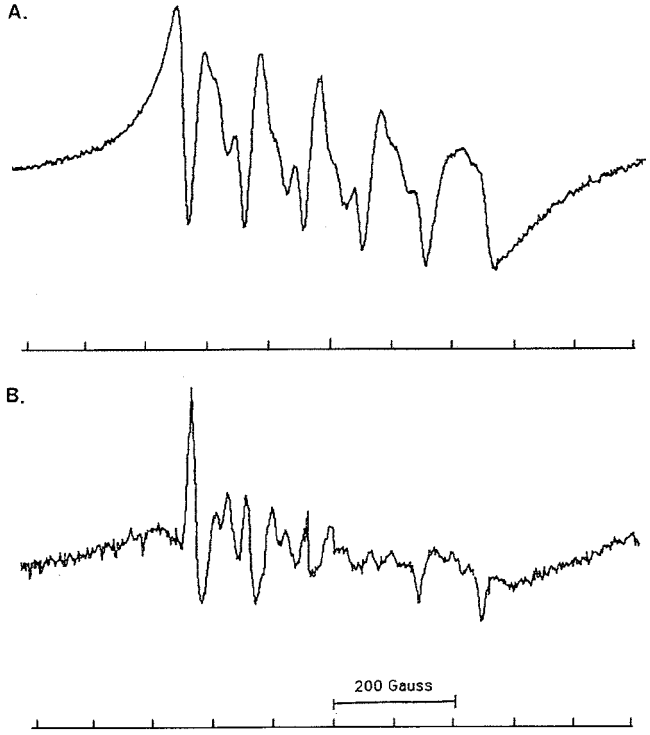

FIGURE 6: ESR spectrum of Mn^{2+} in the absence and presence of MBP + Gel B/AS. (A) First derivative X-band ESR spectrum obtained at 77 K showing the Mn^{2+} signal for free Mn (1.0 mM) in Tris buffer (50 mM, pH 7.5). Spectra represent the central fine structure transition ($Ms = {}^1/_2 \leftrightarrow Ms = {}^{-1}/_2$). (B) First derivative X-band ESR spectrum obtained at 77 K showing the Mn-MBP + Gel B/AS signals remaining after computer-subtracting out the signal for free Mn. The frozen sample contained 1.0 mM MBP + Gel B/AS in Tris buffer (50 mM, pH 7.5) containing 0.95 mM $MnCl_2$.

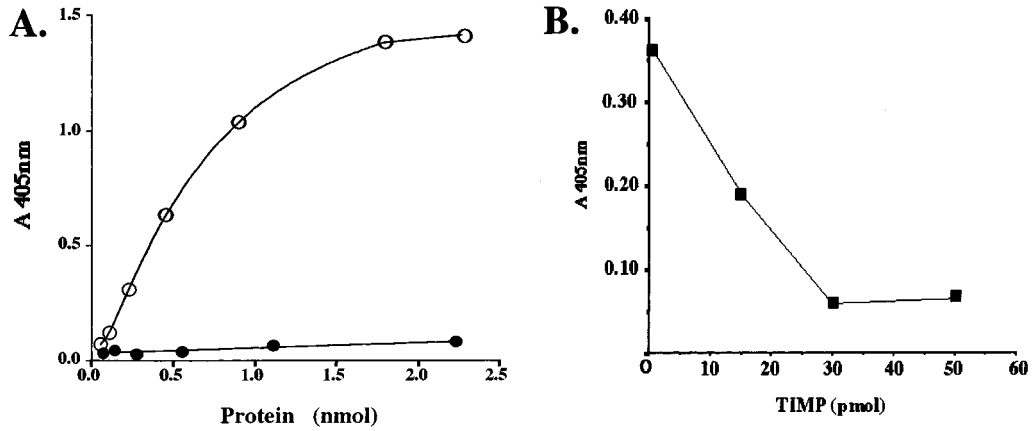

FIGURE 7: (A) Binding of MBG + Gel B/AS (○) and MBP (●) to TIMP-1 as determined by sandwich ELISA. Various concentrations of either MBP + Gel B/AS (○) or MBP (●) were added to wells coated with TIMP-1 as described under Experimental Procedures. The plates were washed and incubated with anti-MBP antibody (100 μL, 1:1000 dilution) at 25 °C for 1 h. The bound proteins were detected with alkaline phosphatase labeled Goat anti-rabbit IgG (100 μL, 1:1000) at 25 °C using *p*-nitrophenyl phosphate as substrate. The optical density of the developed color was measured at 405 nm. (B) TIMP-MBP + Gel B/AS competitive ELISA. MBP + Gel B/AS (18.5 pmol) was incubated with various concentrations of TIMP-1 (0−50 pmol) at 37 °C for 1 h. The samples were then placed on a TIMP-1 coated microtiter plate, and the amount of MPB + Gel B/AS bound to the immobilized TIMP-1 as a function of TIMP-1 concentration in the reaction mixture was determined as above.

magnitude of the hyperfine coupling constant, A (^{55}Mn), was reduced by about 6.5 gauss, which demonstrated the formation of the Mn^{2+}−MBP + Gel B/AS complex (Figure 6B). Further addition of an equal molar concentration of $MnCl_2$ showed an overlay of free plus bound Mn^{2+} (data not shown), indicating that only one metal binding site was present in MBP + Gel B/AS.

To exclude the possibility that Zn^{2+} or Mn^{2+} was nonspecifically bound to MBP + Gel B/AS, the effect of Zn^{2+} on the spectra of the Mn^{2+}−MBP + Gel B/AS complex was examined. We found that addition of 1 equivalent of Zn^{2+} to a solution of the Mn^{2+}−MBP + Gel B/AS complex changed the ESR signal of the complex to the one corresponding to a mixture of the bound and free Mn^{2+} (data not shown). The ESR spectrum of the complex completely reverted to that of free Mn^{2+} upon addition of a 20-fold excess of $ZnCl_2$, indicating that these metals specifically bind to the same site. That the observed ESR signal was specific and due to interaction of Mn^{2+} with the Gel B/AS component of the fusion protein was confirmed by the observation that the ESR spectrum of MBP was identical to that of free Mn^{2+} (data not shown). These data provided further conclusive evidence that the presence of the Gel B/AS is sufficient to endow a protein, MBP, with characteristics similar to MMP including the metal binding capabilities.

MBP + Gel B/AS, Similar to Gelatinase B, Binds to TIMP-1 and Its Activity Is Inhibited by TIMP-1. It is known that TIMP-1 is a specific inhibitor of MMPs. It interacts with the activated form of gelatinase B with a 1:1 stoichiometry and completely inhibits the enzyme activity (*17*). The X-ray crystal structure of the catalytic domain of the MMP-3−TIMP-1 complex showed that TIMP-1 binds to the active site of the enzyme and interacts with the catalytic Zn^{2+} (*49*). This prompted us to investigate the effect of TIMP-1 on the catalytic activity of the fusion protein. The inhibitory activity of 20 kDa recombinant TIMP-1 against MBP + Gel B/AS was measured by preincubating samples of TIMP-1 and the protease for 1 h at room temperature to allow binding to reach equilibrium. The remaining protease activity was then measured by addition of the peptide substrate to the reaction mixture. To achieve levels of protease activity that can be measured precisely, MBP + Gel B/AS was used at concentrations up to 9 μM. As shown in Figure 3, we found that TIMP-1 was able to inhibit the activity of MPB + Gel B/AS. The percentage inhibition was directly dependent on the concentration of the TIMP-1 in the reaction mixture. Activity of the enzyme (8.5 μM, 4.25 nmol) was decreased by 45% in the presence of 2.2 nmol of TIMP-1, and only <10% of its activity remained after stoichiometric addition of TIMP-1 (8.4 μM, 4.2 nmol) to the reaction mixture. The K_i value of MBP + Gel B/AS for TIMP-1, however, could not be determined accurately using steady-state kinetics. The low reagent concentrations that must be used to fulfill the requirements of [E] < K_i and [I] ≃ K_i resulted in a very slow rate of reaction for MBP + Gel B/AS with a half-life of several hours. Therefore, the nature of MBP + Gel B/AS−TIMP interaction was analyzed by determining the apparent dissociation constant (K_d) of TIMP-1 for the enzyme.

The K_d value for the MBP + Gel B/AS−TIMP-1 complex was determined by incubating either MBP + Gel B/AS or MBP with immobilized TIMP-1 on a microtiter plate and measuring the amount of the protein-bound TIMP-1 complex with anti-MBP antibody. As demonstrated in Figure 7A, TIMP-1 bound to MBP + Gel B/AS with an apparent K_d of 3.89 ± 0.94 μM, whereas interaction of MBP with immobilized TIMP-1 was undetectable. Addition of an increasing amount of free TIMP-1 to the MBP + Gel B/AS solution before incubation with immobilized TIMP-1 inhibited the binding of the MBP + Gel B/AS to the TIMP-1 coated plate (Figure 7B). The MBP + Gel B/AS−TIMP-1 interaction was specific, because no other proteins including soybean trypsin inhibitor were able to bind MBP + Gel B/AS (data not shown). These results suggested that, in addition to providing MBP with proteolytic activity, the inclusion of the Gel B/AS in the MBP molecule gave the protein the ability to bind to TIMP-1.

DISCUSSION

We have recently shown that an FC chimera in which the active site domain of the collagenase has been replaced with that of the gelatinase B, FC + Gel B/AS, had significantly higher catalytic efficiencies toward gelatin and the peptide substrate than that of the wild-type enzyme (*34*). This finding suggested that unique amino acids in the active site of gelatinase B are important for enhancing the catalytic efficiency of the collagenase toward these substrates. Site-directed mutagenesis studies indicated that Leu 397 and Ala 406 are important for general catalytic activity, while Asp 410 and Pro 415 specifically contribute to enhancing the gelatinase B ability to cleave type V collagen and gelatin, respectively (*34*). In addition, we found that the catalytic activity of gelatinase B depends on the presence of Glu 401 in the active site of the enzyme (unpublished data). To investigate whether the Gel B/AS in itself is sufficient to provide a nonproteolytic protein with peptidase activity, we affixed it to the carboxyl terminus of MBP and studied substrate specificity of the generated fusion protein, MBP + Gel B/AS. These studies clearly demonstrated for the first time that Gel B/AS by itself is capable of transforming a nonproteolytic protein to a metalloprotease. This is based on the observation that MBP + Gel B/AS cleaved MMP peptide substrates Mca-PLGL(Dpa)AR-NH_2 and DNP-PLGLWA-(D)-R-NH_2 and EDTA, hydroxamate, and TIMP-1 inhibited its activity.

To study the structure and function relationship of MMP, we, as well as other investigators, previously generated several MMP chimeric proteins by exchanging domains among the members of the MMP family (*34*, *42*, *50*−*53*). All of the chimeras showed proteolytic activity toward the peptide substrate. This result was expected, because, like their wild-type counterparts, they contain a catalytic domain consisting of an amino-terminal and the active site fragment, hinge region, and a carboxyl-terminal domain. MBP + Gel B/AS, on the other hand, is the only MMP fusion protein generated to date that consists of a nonproteolytic protein (MBP) and the active site fragment of the catalytic domain of gelatinase B and that has peptidolytic activity. Several β-galactosidase fusion proteins containing different repeats of the fibronectin-like domain of either MMP-2 (*54*) or gelatinase B (*55*) have been constructed and demonstrated to bind to denatured collagen substrates. However, no data regarding their ability to degrade peptide substrate are available.

The substrate specificity of MBP + Gel B/AS was similar to that of gelatinase B. MBP + Gel B/AS hydrolyzed Mca-PLGL(Dpa)AR-NH_2 and DNP-PLGLWA-(D)-R-NH_2 peptides by 3 and 2 orders of magnitudes faster than DNP-PQGIAGQ-(D)-R-OH peptide, respectively. The catalytic efficiency of MBP + Gel B/AS toward the peptide substrate Mca-PLGL(Dpa)AR-NH_2 as determined by the k_{cat}/K_m value (1×10^6 M^{-1} h^{-1} at 25 °C) was about the same as that reported for MMP-3 (6.5×10^6 M^{-1} h^{-1}) and MMP-1 (Gln185-Thr193)-MMP-3 chimera (9.76×10^5 M^{-1} h^{-1}) at 37 °C (*42*) and was only 1 order of magnitude lower than that of FC + Gel B/AS (*34*; Table 1). This is reasonable considering that amino acid residues 183−191 in the catalytic domain of FC have been shown to play a significant role in the catalytic activity of the enzyme (*42*). The catalytic activity of MBP + Gel B/AS toward this substrate was, however, 2 orders of magnitudes lower than that of wild-type gelatinase B (Table 1). This can be attributed to the low affinity of MBP + Gel B/AS for the substrate that resulted from its lack of interaction with the Pro residue of the peptide substrate. This conclusion is based on the crystal structure of the catalytic domain of the MMP-8-hydroxamate inhibitor complex, demonstrating that the Pro, Leu, and Gly residues of the inhibitor mimic the unprimed residues of the productively bound Mca-PLGL(Dpa)AR-NH_2 peptide substrate and that the Pro residue of the inhibitor interacts with His 173 and Phe 175 residues of the enzyme (*28*). These residues are in the amino-terminal region of the catalytic domain of gelatinase B. The fact that MBP + Gel B/AS is deficient in these residues supports the notion that lack of interaction of MBP + Gel B/AS with the Pro residue of the substrate is responsible for the observed differences between the catalytic efficiency of the fusion protein and gelatinase B. This is supported by the observation that the affinity of MBP + Gel B/AS for the substrate analogue, Z-Pro-Leu-Gly-hydroxamate, was ~15 times lower than that of gelatinase B (Figure 4). These data indicate that the active site structure of MBP + Gel B/AS resembles the active site of gelatinase B.

While addition of Gel B/AS to MBP was enough to confer proteolytic activity to the protein, it was not sufficient to provide MBP with the ability to cleave gelatin. This result is consistent with our previous data demonstrating that the Fib domain of gelatinase B is required for the gelatinolytic and type V collagenolytic activity of the enzyme (*33*, *34*). The Fib-deleted mutant of gelatinase B and the FC chimera containing Gel B/AS were not able to efficiently cleave gelatin (*33*, *34*).

The presence of the Gel B/AS in MBP not only endowed the protein with peptidolytic activity but it gave MBP other characteristics similar to that of MMP. Like gelatinase B, the activity of MBP + Gel B/AS was dependent on the presence of Zn^{2+} in the reaction mixture (Figure 5A). The activity of the enzyme was completely inhibited by EDTA and Z-Pro-Leu-Gly-hydroxamate (Figure 3). The ESR analysis demonstrated that MBP + Gel B/AS bound one molecule of Zn^{2+}, indicating that a metal ion plays a critical role in the reaction mechanism of the MBP + Gel B/AS. According to the crystal structure of the catalytic domain of MMP-8 (*28*) and FC (*45*) in complex with the hydoxamate inhibitor, the hydroxyl and the carbonyl oxygen atoms of the hydroxamate group of the inhibitor interact with the catalytic Zn^{2+}. This finding suggested that, similar to the collagenases, the hydroxamate inhibited the activity of MBP + Gel B/AS by interacting directly with its catalytic Zn^{2+}. A reaction mechanism for the cleavage of peptides by FC has been proposed on the basis of the FC-hydoxamate interaction (*45*). The key feature of this mechanism is the coordination of a water molecule by Glu -219 and the Zn^{2+} near the carbonyl of the scissile bond. The hydroxyl group of the water molecule generated by the general base catalyst, Glu 219, apparently attacks the carbonyl group of the substrate initiating cleavage of the peptide bond. This is consistent with our previous observation that Glu 401 in gelatinase B, corresponding to Glu 219 in FC, is required for the catalytic activity of the enzyme. The fact that the MBP + Gel B/AS molecule containing Glu 401 binds to and requires metal ion(s) for activity supports the idea that this enzyme has a

reaction mechanism similar to that of FC and MMP-8.

Interestingly, similar to the other MMPs, MBP + Gel B/AS stoichiometrically bound to TIMP-1, and its activity was inhibited by TIMP-1 (Figures 3 and 7). The K_d value of MBP + Gel B/AS−TIMP-1 complex (3.89 ± 0.94 μM) was found to be similar to the value reported for the low-affinity TIMP-1 binding site of gelatinase B (3.15 + 0.51 μM) (*56*). It is worth mentioning that gelatinase B has a high-affinity (K_d = 45.8 ± 4.3 nM) and a low-affinity (K_d = 3.15 ± 0.51 μM) binding site for TIMP-1 (*56*, *57*). However, the relationship between the active site of gelatinase B and the TIMP-1 binding sites is not clear. According to the crystal structure of the TIMP-1−MMP-3 complex (*49*), the N-terminal segment of TIMP-1, Cys 1-Thr-Cys-Val-Pro 5, binds to the active site of the enzyme, and the α-amino and carboxyl groups of Cys 1 bidentately coordinate the catalytic Zn^{2+}. Similarity between the active site structures of gelatinase B and MMP-3 suggests that TIMP-1 also inhibited the activity of MBP + Gel B/AS by interacting with its "catalytic" Zn^{2+} in the Gel B/AS fragment. Since TIMP-1 binds only to a correctly folded protein, this suggests that the "active site" of MBP + Gel B/AS is folded properly and its structure is similar to that of the other MMPs. Further structure/function study of MBP + Gel B/AS should identify specific amino acid residues that interact with the inhibitor.

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