Reconstructed 19 kDa Catalytic Domain of Gelatinase A Is an Active Proteinase

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Received September 12, 1994; Revised Manuscript Received January 19, 1995[⊗]

ABSTRACT: Matrix metalloproteinases share high protein sequence homology and have defined domain structures. Gelatinases have a unique 19 kDa fibronectin-like insert in the catalytic domain. A synthetic gene was made to express the catalytic domain of human gelatinase A (GCD), in which two polypeptide fragments of the catalytic domain were joined with deletion of the insert. The synthetic gene was highly expressed in *Escherichia coli*, and the 19 kDa GCD was purified to homogeneity after in vitro refolding. The GCD showed activity at a pH range of 5.5−9 in cleavage of the thiopeptolide Ac-Pro-Leu-Gly-thioester-Leu-Leu-Gly-OEt with optimal activity at neutral pH (K_m = 134 μM and k_{cat} = 16 s^{-1} at pH 7.0). The activity required both zinc and calcium ions, but high concentration of zinc ion showed inhibition. Several stromelysin catalytic domain inhibitors inhibited the GCD with similar specificity. The GCD cleaved the fluorogenic peptides Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 and Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 with catalytic efficiency close to full length human gelatinase A. The reconstructed GCD cleaves not only thiopeptolide and peptide substrates but also protein substrates such as gelatin. These results are consistent with the notion that gelatinases have the same structure for the catalytic domain as other matrix metalloproteinases like stromelysins and collagenases.

Gelatinases participate in connective tissue remodeling (Woessner, 1991) by degrading protein substrates like denatured collagens (gelatins), type IV collagen (Morel et al., 1993), and elastin (Senior et al., 1991). Together with other members in the matrix metalloproteinase family, gelatinases may be responsible for several pathological processes involving connective tissue degradation, such as cancer cell invasion and metastasis (Stetler-Stevenson et al., 1993).

The potential utility of matrix metalloproteinase inhibitors in controlling these pathological processes has stimulated structure−function studies for this family of zinc-containing enzymes. All matrix metalloproteinases have a signal peptide for secretion, a propeptide containing a cysteine as zinc ligand for maintaining enzyme latency (Van Wart & Birkedal-Hansen, 1990), and a catalytic domain containing three histidines as zinc ligands for enzymatic activity. Collagenases, stromelysins, and gelatinases have a hemopexin-like C-terminal domain, while matrilysin lacks such a domain. Studies with C-terminally truncated stromelysin (Marcy et al., 1991; Ye et al., 1992a), collagenase (Schnierer et al., 1993), and gelatinase (Murphy et al., 1992) have shown that the catalytic domain is sufficient for enzymatic activity in cleaving peptide and protein substrates. However, the C-terminal domain is required for collagenase to degrade helical collagens (Hirose et al., 1993; Sanchez-Lopez et al., 1993; Schnierer et al., 1993) and involved in binding with TIMPs (Fridman et al., 1992; Murphy et al., 1992; Baragi et al., 1994). Both gelatinase A (72 kDa gelatinase, E.C. 3.4.24.24) and gelatinase B (92 kDa gelatinase, E.C. 3.4.24.35) have an insert (about 174 amino acid residues, 19 kDa) at the catalytic domain, which is homologous to the type II domain in fibronectin and has the ability to bind gelatin (Collier et al., 1992; Banyai et al., 1994). The gelatinase B has an extra collagen-like insert between the catalytic domain and the C-terminal domain.

The C-terminally truncated gelatinase A generated by Murphy et al. (1992), with presence of the 19 kDa fibronectin-like insert, had activity similar to the full length gelatinase A against peptide and protein substrates. With deletion of the fibronectin-like insert, the catalytic domain of gelatinases is homologous to that of other matrix metalloproteinases (Murphy et al., 1991). We reconstructed the catalytic domain of human gelatinase A (GCD)[1] by connecting the two peptide fragments for the catalytic domain with deletion of the insert (Figure 1). We demonstrate here that the reconstructed 19 kDa GCD, which we expressed in *Escherichia coli* from a synthetic gene, is a competent peptidase and proteinase.

EXPERIMENTAL METHODS

Gene Synthesis. The nature of the modification on the human gelatinase A makes it more appealing to synthesize the GCD gene than to construct the gene from cDNA. The synthetic gene coding for the reconstituted GCD protein (Figure 2) was assembled by a PCR-based gene synthesis method as described previously (Ye et al., 1992b). The sequences for the six long oligonucleotides (106-mers) are shown in Figure 2, and the two terminal oligonucleotides (20-mers) have the sequences AACGAAAGTGCTAGCTA-

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[⊗] Abstract published in *Advance ACS Abstracts,* March 15, 1995.

[1] Abbreviations: GCD, gelatinase A catalytic domain; SCD, stromelysin catalytic domain; TIMP, tissue inhibitor of metalloproteinase; BSA, bovine serum albumin; DTT, dithiothreitol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Bis-Tris propane, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane; APMA, 4-aminophenylmercuric acetate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NMR, nuclear magnetic resonance.

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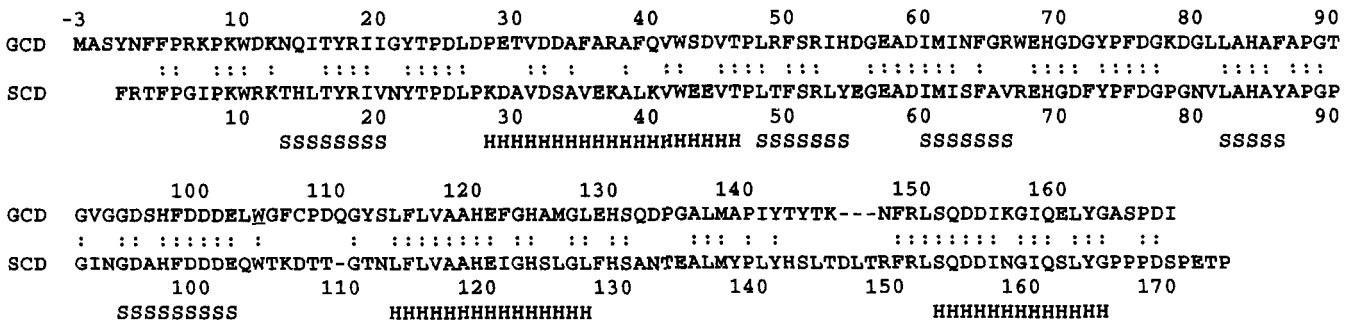

FIGURE 1: Protein sequence homology between GCD and SCD. Identical residues are indicated by colons. The secondary structures in SCD as determined by NMR (Van Doren et al., 1993) are indicated by H (α-helix) and S (β-sheet). The tryptophan (W104) in GCD connecting the two polypeptide fragments is underlined.

CAA and TGCATTGTTCAAGCTTAGAT. All oligonucleotides were synthesized on an ABI 394 DNA synthesizer using 40 nmol poly(styrene) columns and purified with OPC cartridges (Applied Biosystems, Foster City, CA). The oligonucleotides were assembled to form a synthetic gene in a PCR reaction with 30 cycles at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min. Six long oligonucleotides (106-mers) with 20-base overlapping sequences as the extension primers were assembled into a DNA fragment coding the GCD gene (Ho et al., 1989), and the DNA fragment was amplified by two terminal oligonucleotides (20-mers). The PCR-generated DNA fragment was purified with GLASSMILK using the GENECLEAN II kit (BIO101, La Jolla, CA), digested with *Nhe*I and *Hin*dIII, and ligated to the plasmid pGEMEX-1 (Promega, Madison, WI) which had been digested with the same two restriction enzymes and dephosphorylated with calf intestine alkaline phosphatase. The recombinant plasmid pGEMEX-GCD was transformed into *E. coli* strain DH5α (BRL, Gaithersburg, MD), and the sequence coding for the GCD gene was confirmed by DNA sequencing. In the synthetic gene, codons were optimized for *E. coli* expression, and restriction sites were introduced with even distribution for future mutagenesis studies. Extra codons for Met, Ala, and Ser were added to the N-terminus for translation initiation (Met) and restriction site *Nhe*I (Ala and Ser). The restriction site for *Hin*dIII was also added at the end of the gene after the termination codon for cloning.

Expression and Purification. The pGEMEX-GCD plasmid was transformed into *E. coli* strain BL21(DE3)/pLysS cells (Novagen, Madison, WI) for expression. The transformed *E. coli* cells were cultured in 2 × TY medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) supplemented with carbenicillin (50 μg/mL) and chloramphenicol (50 μg/mL) at 37 °C in a 2 L table top fermenter to OD_{600} = 1.7. GCD expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM), and incubation continued for 3 h at 37 °C. The cells were harvested by centrifugation at 10000*g* for 10 min at 4 °C. The cell pellet (8.3 g, wet weight) was washed once with 50 mM Tris·HCl (pH 7.6) and stored at −20 °C until use.

A portion of the cells (4.62 g of cell paste; line 1 in Figure 3) was resuspended in 20 mL of 50 mM Tris·HCl (pH 7.6) containing 0.1% Triton X-100. After a few minutes, a small amount of DNase I and $MgCl_2$ (5 mM) were added to digest the released DNA. Pellets were collected after centrifugation, washed with water twice, and mixed with 3.6 g of urea (final concentration 6 M) and water to a 10 mL final volume. The mixture was clarified by centrifugation and incubated at room temperature for 20 min after adding dithiothreitol (DTT) to 2 mM. Iodoacetamide (42 mg, final concentration 20 mM) was added to alkylate the cysteine's thiol group, and the mixture was incubated for 30 min at room temperature. DTT was added to 20 mM to inactivate residual iodoacetamide. The mixture was clarified by centrifugation, and a GCD solution was obtained (6.5 mL, 133 mg of protein; lane 2 in Figure 3).

The GCD solution was loaded on to a Hi-Load Q-Sepharose column (20 mL; Pharmacia, Piscataway, NJ) equilibrated previously with 50 mM Tris·HCl/6 M urea (pH 7.6) and eluted with a linear NaCl gradient (0−1 M) in the Tris/urea buffer. The combined active fractions (10 mL, 68 mg of protein; lane 3 in Figure 3) were dialyzed (membrane molecular weight cutoff 6−8 kDa) against 1 L of 50 mM Tris·HCl (pH 7.6) overnight at 4 °C without stirring. The buffer was changed the next morning. The dialysate contained homogeneous GCD apoprotein (10.5 mL, 61 mg of protein; lane 4 in Figure 3). Reducing SDS−PAGE analysis was carried out on precast 10−20% tricine gels (NOVEX, San Diego, CA).

Cleavage of Thiopeptolide by GCD. The ability of GCD to cleave the thiopeptolide Ac-Pro-Leu-Gly-thioester-Leu-Leu-Gly-OEt (Bachem Bioscience, King of Prussia, PA) was used to follow the enzymatic activity during purification and characterization as described previously for monitoring stromelysin catalytic domain (SCD) activity (Ye et al., 1992a). The assays were carried out on 96-well plates with a THERMOmax microplate reader (Molecular Devices, Menlo Park, CA) at room temperature (21−22 °C), and OD changes at 405 nm in each well were monitored continuously and simultaneously for 2−5 min. The 100 μL assay mixture typically contained 50 mM MOPS (pH 7.0), 1 mM DTNB, 100 μM thiopeptolide, 10 mM $CaCl_2$, 10 μM $ZnCl_2$, and enzyme.

For determining activity at different pHs, 50 mM MES buffer was used for pH 5.5−6.5 and 50 mM Bis-Tris propane buffer was used for pH 6.5−9.0, in place of 50 mM MOPS buffer. Activities at pH 8.0, 8.5, and 9.0 were corrected for nonenzymatic degradation of the thiopeptolide. Substrate concentrations used range from 37 to 742 μM, GCD concentration was 173 nM, and values for K_m and V_{max} were obtained from nonlinear regression of the plot of initial rates vs substrate concentrations. The determinations were repeated three times. As a control, GCD was incubated in buffers at pH 5.5−9.0 at room temperature, and then the activity was assayed at pH 7.0. Rate of the change in activity indicated that GCD was stable under the conditions for determining activity at pH 5.5−9.0.

FIGURE 2: Synthetic gene for GCD. The oligonucleotides used in gene synthesis are underlined. The translated protein sequence and the restriction sites are indicated.

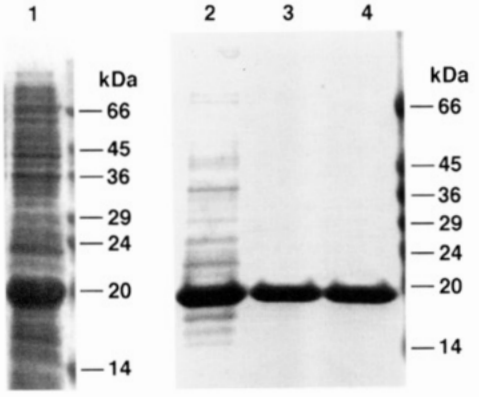

FIGURE 3: Purification of GCD. The protein samples were analyzed by SDS−PAGE on 10−20% tricine gel. Lane 1, *E. coli* cells before lysis; lane 2, GCD solution in Tris/urea before Q-Sepharose column; lane 3, combined active fractions after Q-Sepharose column; and lane 4, after dialysis against Tris buffer.

The effects of zinc and calcium ion concentrations on GCD activity were investigated using 173 nM GCD in MOPS buffer at pH 7.0. In the presence of 10 mM $CaCl_2$, the GCD activity as the initial rate was determined with $ZnCl_2$ concentration ranging from 10 nM to 10 mM. In the presence of 100 μM $ZnCl_2$, the GCD activity was determined with $CaCl_2$ concentration ranging from 100 μM to 100 mM. The determinations were repeated at least three times.

The compounds tested for GCD inhibition were obtained from Bachem. Assays were performed with 17.3 nM GCD, 10 mM $CaCl_2$, and 10 μM $ZnCl_2$ in MOPS buffer at pH 7.0. Inhibition was initially tested at inhibitor concentrations of 100 and 500 μM. IC_{50} values were determined when the compound showed 50% inhibition near or below 100 μM. K_i values were derived from IC_{50} values by using the equation $K_i = IC_{50}/(1 + [\text{substrate}]/K_m)$ (Cheng & Prusoff, 1973), where substrate concentration was 100 μM and K_m for the substrate was 134 μM. The determinations were repeated three times.

Cleavage of Fluorogenic Peptides by GCD and SCD. The activity of GCD in comparison with SCD in cleaving peptides Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 and Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 (both from Bachem) was assayed under the conditions described (Knight et al., 1992) with minor modifications. The cleavage of the fluorogenic peptide Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (1.6 and 4 μM) by GCD (10 nM) or SCD (100 nM) was monitored by measuring fluorescence at 393 nm with excitation at 328 nm at 25 °C continuously for 30 min in 50 mM MOPS buffer (pH 7.0) containing 10 mM $CaCl_2$ and 10 μM $ZnCl_2$. Similar conditions were used for the peptide Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 (7 and 10 μM) except excitation was at 283 nm and emission at 350 nm. The time course of the product generation was fitted exponentially as a first-order reaction, to give k_{app}. The k_{cat}/K_m value was derived from k_{app} with the equation $k_{cat}/K_m = k_{app}/[E]$, where [E] = 10 nM for GCD and 100 nM for SCD.

Digestion of Gelatin by GCD and Full Length Gelatinase A. Human progelatinase A was activated by incubation with 1 mM APMA at 37 °C for 60 min in 100 mM Tris buffer (pH 7.5). Type I collagen from rat tail (Sigma, Sant Louis, MO) was denatured by heating at 95 °C for 10 min. The denatured type I collagen (gelatin, 1.25 mg/mL) was incubated with 10 nM GCD or 10 nM activated full length gelatinase A at 37 °C in 50 mM MOPS buffer (pH 7.0) containing 10 mM $CaCl_2$ and 10 μM $ZnCl_2$. Aliquots were taken at 0, 5, 15, 30, 60, and 90 min and mixed with loading buffer and 2-mercaptoethanol for SDS-PAGE analysis on precast 10% tricine gels (NOVEX). The protein bands were visualized by Coomassie blue staining. The GCD and full length gelatinase A at the amount used were not visible by the staining. Under the same conditions without the enzymes, the denatured type I collagen showed no degradation during the 90 min incubation.

Determination of Protein Concentration. The Bradford method was used for determining protein concentration with dye reagent from Bio-Rad (Hercules, CA) and BSA as the standard. Concentration of the purified GCD was also determined by measuring UV absorption at 205 nm with the same BSA as the standard and the filtrate from the GCD solution through an ultrafiltration membrane (molecular weight cutoff 5 kDa) as the blank. The ratio of the GCD protein concentrations determined by using the Bio-Rad reagent and measuring OD_{205} was 1:1.18.

RESULTS

Reconstruction of GCD. Matrix metalloproteinases are a family of homologous enzymes. The gelatinases are unique in that both gelatinase A and gelatinase B have a fibronectin-like insert of approximately 19 kDa in the catalytic domain, separating the catalytic domain of gelatinases into two pieces. By sequence alignment among matrix metalloproteinases, we identified in human gelatinase A (Collier et al., 1988a) two tryptophan residues at the two junctions of the insert and connected the two pieces for the catalytic domain into a single polypeptide chain by merging the two tryptophans into one. The reconstructed GCD has 58% amino acid identity with SCD (Figure 1), the C-terminally truncated stromelysin we expressed and characterized previously (Ye et al., 1992a).

Expression and Purification of GCD. The expression system utilizing T7 RNA polymerase (Tabor & Richardson, 1985; Studier et al., 1990) was used for GCD expression in *E. coli*. The GCD synthetic gene replaced the *Nhe*I/*Hin*dIII fragment containing T7 gene 10 in the plasmid pGEMEX-1 and was situated behind a T7 promoter. GCD was expressed in high yield as an insoluble protein, and its refolding in vitro was rapid and efficient in both the presence and absence of calcium and zinc ions. However, the refolding does not require either of the ions since GCD could refold in the presence of 1,10-phenanthroline or Chelex-100 resin (Bio-Rad). High yield of apoenzyme was obtained after dialysis against 50 mM Tris·HCl at pH 7.6. The apoenzyme was stable at 4 °C and showed instant activity when zinc and calcium were provided. The rapid refolding and the instant activity make it possible to monitor enzyme activity during purification even for column fractions containing 6 M urea. The reconstruction of the GCD gene left one cysteine residue in the protein molecule, and early attempts in GCD purification showed that the cysteine caused dimerization as analyzed by electrospray mass spectrometry. Since the cysteine is not present in other matrix metalloproteinases, iodoacetamide was used to block the cysteine thiol group before purification. After purification in the presence of 6 M urea on a Q-Sepharose column and subsequent dialysis, GCD was homogeneous when analyzed by reducing SDS−PAGE (Figure 3).

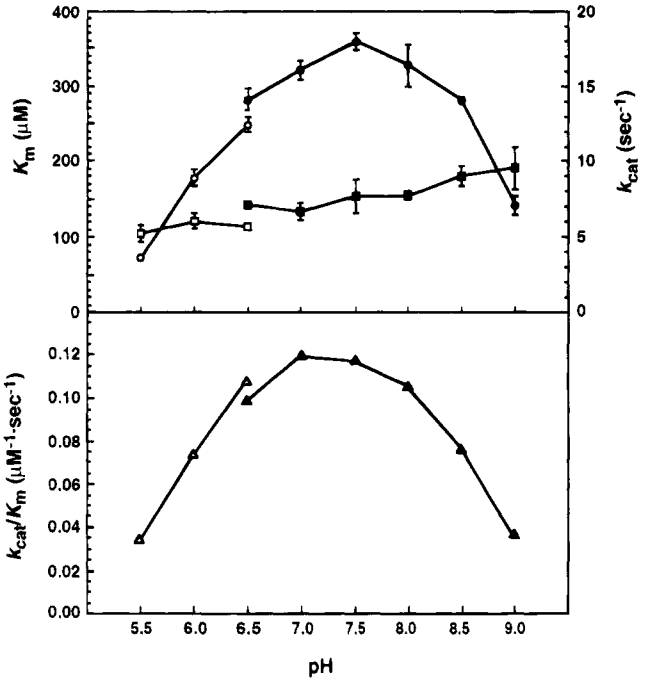

FIGURE 4: pH effects on GCD activity. Hydrolysis of the thiopeptolide was monitored in MES buffer (pH 5.5−6.5; open circles, squares, and triangles) or Bis-Tris propane buffer (pH 6.5−9.0; closed circles, squares, and triangles). Values for k_{cat} are shown as circles, those for K_m are shown as squares, and those for k_{cat}/K_m are shown as triangles.

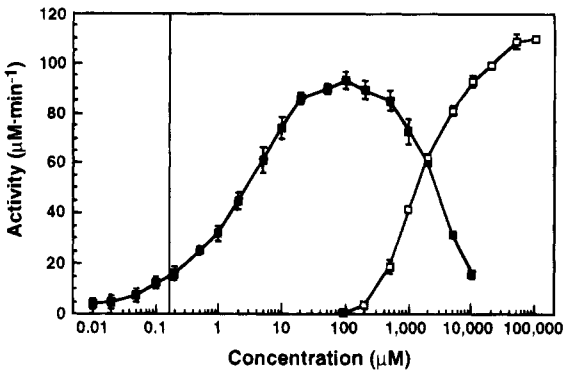

FIGURE 5: Effects of zinc and calcium concentration on GCD activity. Hydrolysis of the thiopeptolide was monitored in MOPS buffer (pH 7.0). Activities at different $ZnCl_2$ concentrations in the presence of 10 mM $CaCl_2$ are shown as closed squares, and activities at different $CaCl_2$ concentrations in the presence of 100 μM $ZnCl_2$ are shown as open squares. GCD concentration at 173 nM is indicated by a vertical line.

Table 1: Inhibition of GCD and SCD by Amino Acid Derivatives

		GCD		SCD[a]	
no.	compd[b]	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
1	Cbz-L-Trp-OH	6.1 ± 0.2	3.5	2.5 ± 0.9	2.1
2	Cbz-D-Trp-OH	128 ± 15	73	86 ± 34	71
3	H-L-Trp-OH	>500		>500	
4	H-D-Trp-OH	>500		>500	
5	Boc-L-Trp-OH	57 ± 4	33	10 ± 4	8
6	Boc-D-Trp-OH	>500		100−500	
7	Cbz-L-Tyr-OH	10.4 ± 0.4	6.0	24 ± 10	20
8	Cbz-D-Tyr-OH	>500		432 ± 215	356
9	Cbz-L-Phe-OH	21 ± 1	12	40 ± 8	33
10	Cbz-D-Phe-OH	100−500		>500	

[a] Data for SCD were taken from Ye et al. (1994). [b] Abbreviations: Cbz, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl.

The purified GCD was subjected to amino acid sequencing. The N-terminal sequence was determined as ASYNFFPRKPKWDKNQITYRIIGYTPDLDP as predicted from the DNA sequence with methionine removed during *E. coli* expression (Flinta et al., 1986; Hirel et al., 1989). The molecular weight of 19 157 Da as determined by electrospray mass spectrometry was consistent with a full length protein starting at Ala at position −2 and ending at Ile at 168 with a CH_2CONH_2 group attached on the cysteine thiol (calculated molecular weight 19 154 Da). The iodoacetamide modification was complete since no dimer or nonalkylated species was detectable by the electrospray mass spectrometry. The protein sample contained 0.34 atom of zinc/GCD molecule according to atomic absorption analysis.

pH Effect on GCD Activity. We observed previously that SCD prefers slightly acidic conditions for activity (Ye et al., 1992a), which is consistent with the pH profile for full length stromelysin (Harrison et al., 1992; Wilhelm et al., 1993). For comparison, we tested GCD in hydrolyzing the thiopeptolide at a pH range from 5.5 to 9.0 (Figure 4). The K_m for the thiopeptolide increased gradually with increasing pH (from 105 μM at pH 5.5 to 192 μM at pH 9.0), while k_{cat} showed an optimum at pH 7.5 (18 s^{-1}). The k_{cat}/K_m had an optimum at pH 7.0 (0.12 μM^{-1} s^{-1}). The preference of GCD for expressing activity at neutral pH is consistent with the previous report that gelatinase is a neutral protease (Selzer et al., 1981).

Effect of Zinc and Calcium Concentrations on GCD Activity. The availability of apo-GCD offered an opportunity to study the structural and functional roles of zinc and calcium ions. We did not vigorously exclude metal ions during protein purification, so it is not surprising to see a small amount of zinc (0.34 atom/GCD molecule) in the GCD sample. Both calcium and zinc ions are required for activity, since either metal ion alone is not sufficient for GCD to show activity. In the presence of 10 mM $CaCl_2$, the optimal activity was achieved around 100 μM $ZnCl_2$ (Figure 5), which is more than 500 equiv of zinc with only 173 nM GCD present. This is in sharp contrast with C-terminally truncated stromelysin, for which only 1 equiv of zinc was required for maximal activity (Salowe et al., 1992). Higher zinc concentration (>100 μM) inhibited GCD activity. With 100 μM $ZnCl_2$ present in solution, GCD showed increased activity with the increase of calcium concentration up to 100 mM.

Inhibition of GCD by SCD Inhibitors. We identified several amino acid derivatives as SCD inhibitors, and they also inhibited full length human stromelysin with the same potency rank order (Ye et al., 1994). SCD and GCD share high protein sequence homology (Figure 1), so it was reasonable to test the SCD inhibitors on GCD. The benzyloxycarbonyl or *tert*-butyloxycarbonyl moiety was required for GCD inhibition (Table 1), since L-tryptophan itself was not active. L-Isomers of these amino acid derivatives always showed better inhibitory activity than D-isomers. The closely related specificity for inhibitors displayed by GCD and SCD revealed the similarity as well as the subtle differences at the active sites of GCD and SCD.

Comparison of Activity of GCD with Other Matrix Metalloproteinases. Both SCD and GCD cleave efficiently the fluorogenic peptides Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 and Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 and

Table 2: Catalytic Efficiency[a] in Cleaving Fluorogenic Peptiddes and Thiopeptolide by Matrix Metalloproteinases

enzyme	Mca/Dpa peptide[b]	Dnp/Trp peptide[b]	thiopeptolide[b]
72 kDa gelatinase	629 000[c]	58 000[c]	
stromelysin	23 000[c]	2200[c]	
GCD	184 000	112 000	119 400
SCD	10 000	13 700	7840

[a] Catalytic efficiency is expressed as k_{cat}/K_m (M^{-1} s^{-1}). [b] Mca/Dpa peptide, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2; Dnp/Trp peptide, Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2; and thiopeptolide, Ac-Pro-Leu-Gly-thioester-Leu-Leu-Gly-OEt. [c] Values reported by Knight et al. (1992).

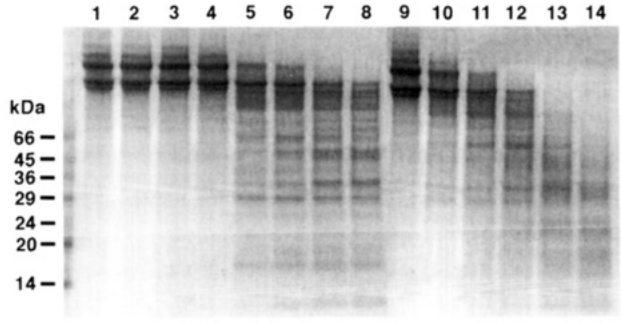

FIGURE 6: Digestion of denatured type I collagen (gelatin) by GCD and full length gelatinase A. Protein samples were analyzed by SDS−PAGE on 10% tricine gel. Lane 1, gelatin alone at 0 min; lane 2, gelatin alone at 90 min; lanes 3−8, gelatin with GCD at 0, 5, 15, 30, 60, and 90 min; and lanes 9−14, gelatin with activated human full length gelatinase A at 0, 5, 15, 30, 60, and 90 min.

the thiopeptolide Ac-Pro-Leu-Gly-thioester-Leu-Leu-Gly-OEt (Table 2). GCD is approximately 10−20 times more active in the cleavages than SCD, which is consistent with previous reports that gelatinase A is a more efficient enzyme in digesting synthetic substrates than stromelysin (Netzel-Arnett et al., 1991; Knight et al., 1992). The efficiency in cleaving the fluorogenic peptides by GCD was close to the reported values for full length gelatinase A (Knight et al., 1992). GCD was capable of cleaving not only thiopeptolide and peptide substrates but also protein substrates such as gelatin (Figure 6), the natural substrate for gelatinases. The rate of gelatin digestion by GCD is apparently slower than the full length gelatinase A, but the cleavage patterns displayed on the gel are similar, indicating the similar substrate specificity between GCD and full length gelatinase A.

DISCUSSION

We have constructed the catalytic domain of human gelatinase A with deletion of the 19 kDa fibronectin homologous insert. The reconstructed 19 kDa GCD showed activity as a peptidase and proteinase with optimal activity at neutral pH. C-Terminally truncated progelatinases with the insert present have been generated from mammalian cells (Fridman et al., 1992; Murphy et al., 1992) and shown the ability of cleaving peptide and protein substrates with activity similar to the full length enzyme (Murphy et al., 1992). Recently, Murphy et al. (1994) also constructed a mutant of the full length gelatinase A with deletion of the fibronectin-like insert, and the deletion mutant had a similar activity in degrading a fluorogenic peptide but a reduced activity against gelatin and casein. Our GCD lacks both the C-terminal hemopexin-like domain and the fibronectin-like insert. Compared to full length gelatinase A, the GCD has a similar activity against peptide and thiopeptolide substrates and a reduced activity against the protein substrate gelatin. This is consistent with the findings from the C-terminally truncated gelatinase A with the insert present (Murphy et al., 1992) and the full length gelatinase A with deletion of the insert but with the C-terminal domain (Murphy et al., 1994). Our results demonstrate that the 19 kDa catalytic domain from gelatinase A is sufficient for enzymatic activity.

The gene structure of human gelatinase A (Huhtala et al., 1990), in comparison with those of gelatinase B, collagenase, and matrilysin (Collier et al., 1988b; Huhtala et al., 1991; Gaire et al., 1994), showed that the insert came from three exons, each of which codes for a complete homologous unit of type II domain of fibronectin. The possibility of acquisition of the extra exons by gelatinase for unique substrate specificity has been suggested (Huhtala et al., 1990). Our GCD cleaves fluorogenic peptides with efficiency similar to full length gelatinase A, indicating that the insert does not participate in hydrolysis of peptides and can be removed with retention of the peptidase activity. Since is has the ability to bind gelatin (Collier et al., 1992; Banyai et al., 1994), the insert may be involved in binding and hydrolysis of protein substrates. Our GCD was able to digest gelatin with retention of specificity but reduction in catalytic efficiency, suggesting the partial involvement of the insert in catalysis.

Recently, several structural studies on the catalytic domain of collagenases by X-ray analysis (Borkakoti et al., 1994; Lovejoy et al., 1994; Reinemer et al., 1994; Stams et al., 1994) and on the catalytic domain of stromelysin by NMR (Gooley et al., 1993, 1994; Van Doren et al., 1993) have appeared. On the basis of protein sequence alignment, the insert should be located at the loop region between the fifth β-strand and the second α-helix (Figure 1), which is on the surface of the protein molecule. It is possible for the insert to form an independent domain away from the active site of the enzyme. With deletion of the insert, the reconstructed GCD has a protein sequence homologous to other matrix metalloproteinases and could assume the same structure as the catalytic domain in other matrix metalloproteinases. GCD has the same activity in hydrolyzing thiopeptolide, peptide, and protein substrates as the full length gelatinases, indicating the integrity for the catalytic machinery. The shared inhibitor specificity between GCD and SCD further confirmed that GCD has the same structure for the catalytic domain as other matrix metalloproteinases, and the deletion of the insert does not cause a major structural disturbance in the catalytic domain.

Our observation that GCD required both zinc and calcium ions for activity suggests that the ions play important roles in GCD structure and catalysis. Okada et al. (1986) reported that both zinc and calcium ions were required for human stromelysin to show activity and high zinc concentration was inhibitory. Seltzer et al. (1976) showed that calcium ion was required not only for thermostability but also for activity of human and rat collagenases. X-ray structures of collagenases showed two zinc atoms, one catalytic and the other structural, and one (Lovejoy et al., 1994) or two (Reinemer et al., 1994) calcium atoms. The requirement of a 500-fold excess of zinc for maximal GCD activity (Figure 5) was unexpected. In addition to supplying zinc for the catalytic site, zinc and calcium ions may change protein conformation through

binding on the surface. It may be the conformational change that is necessary for a functional arrangement at the enzyme active site. The exact roles of calcium and zinc in structure and catalysis need further investigation.

ACKNOWLEDGMENT

We thank Dr. William Stetler-Stevenson for generously providing the human full length progelatinase A, Lan-Hsin Liang for synthesizing oligonucleotides, Margaret Whitton for sequencing the GCD protein, and Dr. Pei-Feng Hu for determining the molecular weight of the GCD protein by electrospray mass spectrometry.

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BI942159S