Characterization of the Phe-81 and Val-82 Human Fibroblast Collagenase Catalytic Domain Purified from Escherichia coli*

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Soluble recombinant human fibroblast collagenase catalytic domain was highly expressed and purified from Escherichia coli. The expression construct utilized the T7 gene 10 promoter for transcription of a two-cistron messenger RNA which encoded the ubiquitin-collagenase catalytic domain fusion protein as the second cistron. The ubiquitin domain was attached to the collagenase catalytic domain with the linker sequences Gly-Gly-Thr-Gly-Asp-Val-Ala-Gln (wild type) or Gly-Gly-Thr-Gly-Asp-Val-Gly-His (mutant) which served as cleavage sites for in vitro activation. The last four residues of the linker were included based on the crystal structure of human prostromelysin-1 catalytic domain. Soluble fusion proteins purified from E. coli retained the proteolytic activity of the collagenase catalytic domain. The collagenase catalytic domain was released by either autoproteolytic or stromelysin-1-catalyzed cleavage, purified to homogeneity, and separately possess Phe-81, Val-82, or Leu-83 as the amino-terminal residue. Very similar \( k_{cat}/k_m \) values were determined for the Phe-81 and Val-82 forms using continuous fluorogenic and chromogenic peptide cleavage assays.

Connective tissue cells are embedded within an extracellular matrix of glycoproteins. The integrity of this matrix is dependent upon a balanced rate of cell division, matrix synthesis, and degradation. Most connective tissue cells, including fibroblasts, chondrocytes, osteoblasts, and endothelial cells, have been reported to secrete matrix metalloproteinases (MMPs)\(^1\) (1). The MMP family of enzymes, acting synergistically, can break down all the components of the extracellular matrix at physiological pH. Uncontrolled matrix metalloproteinase activity may lead to the loss of connective tissue integrity and has been implicated in a variety of connective tissue disorders such as rheumatoid arthritis, tumor invasion, and metastasis. Thus, MMPs have become targets for therapeutic intervention in these pathological processes.

Fibroblast collagenase (MMP-1) belongs to the MMP family and has the unique ability of degrading the triple helical native type I, II, and III collagens (2, 3). MMP-1 is secreted as a proenzyme which can be activated by the removal of an auto-inhibitory prosequence containing a conserved cysteine switch region present in all MMPs (4). Mature MMP-1 is a 41-kDa protein composed of an NH\(_2\)-terminal catalytic domain containing the zinc-binding region of the active site and a COOH-terminal hemopexin-like domain. Activation in vitro can be facilitated by proteinases (e.g. trypsin, plasmin), oxidants, mercurials, or stromelysin-1 (MMP-3). Activation of MMP-1 by proteinases, mercurials, or oxidants produces a variety of active enzymes having either Met-72, Phe-81, Val-82, or Leu-83 as the NH\(_2\)-terminal residue (5–10). The Val-82 and Leu-83 forms accumulate only after extended incubation with p-aminophenyl mercuric acetate or proteinases. Activation with MMP-3, however, gives rise to a 41-kDa mature enzyme with Phe-81 as its NH\(_2\) terminus. This 41-kDa Phe-81 form, with an intact carboxyl-terminal hemopexin domain, has higher proteolytic activity against native substrate type I collagen as compared with the partially activated Val-82 and Leu-83 forms (10–13).

The MMP-1 catalytic domain (MMP-1cd) alone can cleave substrates such as casein, gelatin, and synthetic peptide substrates. However, unlike the full-length enzyme, MMP-1cd cannot cleave collagen (14). And, the missing COOH-terminal hemopexin domain is probably responsible for this difference. With peptide substrates, the catalytic activities of both MMP-1cd and the full-length enzyme are very similar; they differ by only 21% (14). This suggests that the active site structure of the catalytic domain is an appropriate template for the design of low molecular weight inhibitors. It is intriguing whether the structural differences between the Phe-81 and Val-82 or Leu-83 forms of MMP-1cd will be reflected by any changes in enzyme kinetics. Such understanding may provide additional insight in the design of inhibitors for therapeutic applications.

This paper demonstrates high level Escherichia coli expression of soluble 19-kDa rMMP-1cd covering the region between Phe-81 and Gln-249 using the ubiquitin fusion-protein method. We also report in vitro activation methods used to generate homogeneous Phe-81, Val-82, or Leu-83 at the NH\(_2\) terminus as well as the purification and comparative kinetic analysis.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Expression

Oligonucleotides were synthesized based on the published human MMP-1 and ubiquitin cDNA sequences (15, 16) and were used as primers for PCR amplification. A human fibroblast cDNA pool (Clontech) and plasmid Pmuh-poly were used as DNA templates to amplify the coding region of MMP-1 and ubiquitin, respectively. Additional oligonucleotides were synthesized to encode the linker sequences spanning the KpnI and BstEII sites. All PCR amplifications were done with standard procedures (17).

The expression plasmid p\( \mu \)MCol1 was constructed by standard methods (18). The first step involved PCR amplification and cloning of ubiquitin into the final expression plasmid from an existing ubiquitin
plasmid pNmHub-poly. The 5'-ubiquitin PCR primer contained a unique NdeI site that also encoded the ATG for translational initiation. The 3'-ubiquitin PCR primer contained DNA sequence for a unique KpnI site and a downstream EcoRI cloning site. PCR mutagenesis was performed for the collagenase portion of the fusion protein. The 5’ primer introduced a silent mutation to create a BstEII site at Gly-86 codon in the MMP-1 coding region. The 3’ primer contained a unique EcoRI cloning site downstream of Gln-249 codon and the stop codon. This amplified fragment was digested and ligated with a synthetic cloning site downstream of Gln-249 codon and the stop codon. This introduced a silent mutation to create a KpnI site and a downstream BstEII linker. A unique EcoRI site was introduced at Gly-86 codon in the linker region and was provided by the ubiquitin coding region at the KpnI site. Plasmid constructs were confirmed by restriction enzyme mapping and dideoxynucleotide sequencing (19).

Fusion proteins were expressed in E. coli strain BL21(DE3) (Novagen, Ref. 20) which were grown at 37°C to mid-log phase and induced with 0.2 mM isopropyl-β-D-galactopranoside for protein production. Cells were harvested 3 h post-induction.

Purification of the rMMP-1cd Fusion Protein

Induced E. coli cultures were harvested and cell pellets were resuspended and lysed by microfluidization in the lysis buffer (100 mM Tris-HCl, pH 8.0, 5 mM CaCl₂, 0.5 mM zinc acetate) containing 0.5 mM phenylmethylsulfonyl fluoride, 1 mM each of aprotonin, pepstatin A, and leupeptin. All subsequent steps were performed at 4°C or on ice. The crude lysate was centrifuged at 340,000 x g for 45 min. The supernatant solution was recovered and applied to a Q-Sepharose FF column equilibrated in Buffer A. The column was washed with Buffer A and eluted with a linear gradient of 0–200 mM NaCl in Buffer A. Fractions containing the fusion protein were pooled and prepared for activation as described below.

Proteolytic Activation of the rMMP-1cd Fusion Proteins

Removal of the fusion protein and linker sequence could be performed by two different methods.

(i) Activation of Ubiquitin-Wild Type Linker-rMMP-1cd Fusion Protein by MMP-3 Cleavage to Form a Homogeneous Phe-81 or Val-82 NH₂ Terminus — The metalloproteinase inhibitor 1,10-phenanthroline was added to the pooled Q-Sepharose fractions to a final concentration of 10 μM. The sample was then concentrated with a 0–50% saturated ammonium sulfate precipitation and centrifugation at 26,900 x g for 20 min at 4°C. Pelleted protein was resuspended in Buffer B (20 mM Tris-HCl, pH 7.5 mM CaCl₂, 200 mM NaCl) containing 10 μM 1,10-phenanthroline and applied to a 5-100HR size exclusion column which was equilibrated in the same buffer. Fractions containing spontaneously autolysed or Val-82 MMP-1cd or intact fusion protein were pooled separately. Zinc acetate was added to the pooled intact fusion protein fractions to the final concentration of 0.5 mM and incubated with recumbent human MMP-3cd (expressed and purified in this laboratory) at an rMMP-3cd: fusion protein ratio of 1:50 (w/w) at 37°C for an hour.

(ii) Activation of In Vitro Mutant Linker-rMMP-1cd Fusion by Auto-activation to Form a Homogeneous Leu-83 NH₂ Terminus — Protein precipitated by 50% ammonium sulfate was resuspended at 20 mg/ml in 20 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 0.5 mM zinc acetate, and incubated at 37°C for 1 h.

Purification of Mature rMMP-1cd

After the ubiquitin domain was cleaved from rMMP-1cd fusion proteins by either autoproteolytic or rMMP-3cd digestion, the protein solution was centrifuged at 26,900 x g for 20 min to remove insoluble material. The supernatant solution was recovered and applied to a metal chelate affinity column (Pharmacia Biotech Inc.) which was saturated with zinc acetate and equilibrated with 3 column volumes of Buffer C (10 mM Na₂B₄O₇, 5 mM CaCl₂, 200 mM NaCl, 1 mM imidazole, pH 8.0) without prior imidazole saturation. rMMP-1cd was eluted with a 1–25 mM linear imidazole gradient in Buffer C.

Mass Spectrometry Analysis of Purified rMMP-1cd

Mass determination of MMP-3-activated rMMP-1cd was performed with an electrospray ionization mass spectrometer. A 110 ng MMP-1cd sample was denatured in 30 μl of 6 M urea and 40 mM EDTA, then diluted with 0.8 ml of 0.1% trifluoroacetic acid. This denatured protein sample was injected into a POROS R5 column (Perceptive BioScience) equilibrated in 0.1% trifluoroacetic acid and eluted with an acetonitrile gradient. A 0.5 ml fraction was collected and used directly for electrospray ionization mass spectrometer analysis.

Peptide-based Continuous Enzymic Assays and Determination of kcat/Km

The value of kcat/Km for rMMP-1cd was determined by both a resonance energy transfer fluorogenic assay and a chromogenic assay.

(i) Resonance Energy Transfer Fluorogenic Assay — We used a modified version of a procedure previously described by Weingarten and Fedor (22) with the chromogenic thioester-peptilide substrate Ac-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (McA-peptidase). When intact, the fluorescence of the Mc group is quenched by resonance energy transfer to the Dpa moiety. rMMP-1cd cleaves the McA-peptidase at the Gly-Leu bond and the released product-peptide containing the Mc group fluoresces 130 times greater than the intact substrate. Product formation was determined by measuring the cleavage-induced increase in fluorescence of the reaction mixture. Typical reaction mixtures for kcat/Km experiments contained TCS Buffer (0.5 mg/ml bovine serum albumin, 1 mM zinc acetate, 2% Me₂SO, 5 μM McA-peptide, and 500 mM rMMP-1cd). The reaction was conducted at 37°C using degassed buffer solutions and an Applied Photophysics stopped-flow spectrophotometer with an excitation wavelength of 328 nm. Incident light was filtered with the broad band-pass UGS cutoff filter to block stray and scattered light. The fluorescent band of emitted light centered at 393 nm was measured by monitoring all wavelengths greater than 335 nm by the use of the cutoff filter WG-335. 150 μl of the final reaction mixture was mixed rapidly with the stopped flow pneumatic drive and rapid peptide cleavage was followed for 10 min or until peptide cleavage exceeded 85%. The resulting progress curves were fitted by the instrumental nonlinear regression software to Equation 1 for a single exponential decay containing a linear term.

kcat/Km was calculated from the relationship kcat/Km = kcat/[rMMP-1cd], where [rMMP-1cd] is the active enzyme concentration determined by the active site titration described below.

(ii) Continuous Chromogenic Assay — We used a modified version of a procedure previously described by Weingarten and Fedor (22) with the chromogenic thioester-peptilide substrate Ac-Pro-Leu-Gly-S-Leu-Leu-Gly-OEt (thioester-peptidase). rMMP-1cd cleaves this peptide at the Gly-Leu bond to form a free sulfhydryl group immediately reacts with 5,5'-dithiobis(2-nitrobenzoate) (DTNB) to form the colored product 3-carboxyato-4-nitrothiophenolate with an absorbance maximum at 412 nm. The absorbance described below was used to determine initial rates of product formation as well as the value of kcat/Km for the thioester-peptidase. Reaction mixtures of a final 1 ml volume contained TCS buffer, 0.5 mg/ml bovine serum albumin, 1 mM zinc acetate, 100 mM DTNB, 2% Me₂SO, 10 or 100 μM thioester-peptidase, and rMMP-1cd concentrations of 1 mM. For determination of initial rates of product formation, the concentration of thioester-peptidase and rMMP-1cd was 100 μM and 3 mM, respectively. For kcat/Km determination, the concentration of thioester-peptidase was 10 μM and the concentration of rMMP-1cd was 10 or 15 mM. Product formation was detected from the increase in absorbance at 412 nm on a Hewlett-Packard HP8452 diode array spectrophotometer using clear plastic disposable cuvettes. These cuvettes filter incident light by strongly absorbing wavelengths <300 nm. This circumvented low UV-dependent photodegradation of DTNB and hence minimized rMMP-1cd-independent blank rates. However, an important basal blank rate due to the spontaneous hydrolysis of the thioester-peptidase could not be diminished at this pH (22). The mixture was preincubated for 4 min at 37°C before rMMP-1cd was added to initiate the reaction. The progress was followed for 3 min to determine initial rates. The change in absorbance is linear over this time period and less than 5% of the substrate is consumed. During kcat/Km experiments, the reaction progress was followed for 20 min so that >85% of the thioester-peptidase had been cleaved. Progress curves were then fitted to an equation which describes a single exponential decay with a linear term similar to Equation 1. The linear term accounts for the small residual rMMP-1cd-independent blank rate described above. kcat/Km was calculated from the relationship kcat/Km = kcat/[rMMP-1cd].

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F = A \times k_{cat} \times [S] - V \times t + B
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The concentration of rMMP-1cd was determined by titration of the active site with a strong competitive MMP inhibitor BB-94 (23). Its Ki, of 50 pm against rMMP-1cd (determined in this laboratory) assures stoichiometric binding of BB-94 to the enzyme under the following conditions. rMMP-1cd was diluted to 1 µM with TCS buffer (50 mM Tricine-NaOH, pH 7.5, 10 mM CaCl2, 200 mM NaCl), 0.5 mg/ml bovine serum albumin, 1 mM zinc acetate and incubated at room temperature. BB-94 was added in small volumes so that the concentration of BB-94 increased by 100 nM increments. Binding was allowed to progress to completion at room temperature for 5 min for each addition. After each BB-94 addition, a small volume of the incubation mixture was assayed using the thioester-peptidase substrate to determine the residual enzymatic activity. During this assay period, dissociation of the bound inhibitor does not occur. The data were corrected for both dilution and the spontaneous hydrolysis of the thioester peptidase. The concentration of active rMMP-1cd was taken as the abscissal intercept of a linear fit on a plot of residual activity versus BB-94 concentration.

RESULTS

Production of human proMMP-1 full-length enzyme in E. coli has been reported to be problematic as it could only be accumulated in E. coli either at relatively low levels or as an insoluble protein sequestered within inclusion bodies (4, 24). Expression levels and solubility of proMMP-1 were improved when the heat shock chaperone protein GroESL or DnaK was co-expressed in E. coli (24). Lowry et al. (25) expressed a recombinant 19 kDa MMP-1cd without the prosequence in E. coli. Again, it existed only as inclusion bodies and required refolding to gain solubility and proteolytic activity (25). When the collagenase catalytic domain was initially expressed as a proenzyme (proMMP-1cd) in E. coli in this laboratory, it was also observed that only low amounts of insoluble protein could be produced. Clearly a better system was needed to improve the expression and solubility of proMMP-1cd. Therefore, expression constructs such as pUMColl.1 (Fig. 1) were made. pUMColl.1 contains the T7 gene 10 promoter for efficient transcription. To ensure efficient translation, we applied a two-cistron approach. The ribosome binding site and the first 20 codons of the dihydrofolate reductase gene from a trimethoprim-resistant E. coli strain were used as the first cistron (26) followed by the ubiquitin-MMP-1cd fusion as the second cistron. Yeast ubiquitin was chosen since it had been used as the NH2-terminal leader to produce various soluble fusion proteins at high levels (16). The Gly-Gly-Thr-Gly sequence was chosen as the first half of the linker sequence between the ubiquitin and rMMP-1cd domains to introduce both flexibility and distance between the two protein domains and to create a unique KpnI restriction enzyme site for cloning purposes. Based on the solved x-ray structure of proMMP-3cd,2 it was seen that the last four amino acid residues from the prosequence were exposed to solvent and susceptible to proteolytic attack. Assuming three-dimensional structures around this region of MMP-1 and MMP-3 are similar, we chose to include only the last four residues from the prosequence as the second half of the deavage site linker sequence. It was anticipated that the linker region between two protein domains would be flexible and susceptible to either autoprocessing or proteolytic attack by MMP-3cd at the His-Phe bond. As shown in Fig. 2, the ubiquitin fusion protein was indeed expressed at a higher level than proMMP-1cd in E. coli. Furthermore, the vast majority of fusion protein was found in the soluble fraction of crude E. coli lysates.

Initial purification of the fusion protein containing the wild type linker sequence showed that in the absence of 1,10-phe-
The second fusion protein, with the last four amino acid residues from the prosequence of MMP-3, was made to determine if the chimeric cleavage site became resistant to autoproteolytic cleavage and thus could result in higher yields of the Phe-81 form after the MMP-3 activation. During purification, more intact fusion protein was indeed observed in pooled Q-column fractions indicating a reduced level of autoproteolysis (data not shown). However, only 80% homogeneity of the Phe-81 form was attained after MMP-3 activation. On the other hand, NH$_2$-terminal sequencing showed that the purified rMMP-1cd possessed Leu-83 homogeneously when allowed to autoprocess at 37°C. The yield of the Leu-83 form was slightly higher than 6 mg/liter of E. coli culture, since its purification did not require the S-100 column chromatographic step needed for the Phe-81 form. Therefore, with these two fusion proteins, we have generated three forms of rMMP-1cd: Phe-81, Val-82, and Leu-83. The degree of homogeneity for each form was determined by NH$_2$-terminal sequence analysis. The data are summarized in Table I.

The carboxyl-terminal integrity of the Phe-81 form of rMMP-1cd was confirmed by mass spectroscopy. The main component of this sample had a molecular mass of 18,896.2 Da, which is essentially identical to the calculated rMMP-1cd (Phe-81 to Gln-249) molecular mass of 18,895.7 Da. However, two minor components comprising less than 5% of the total mass had molecular masses of 18,750 and 18,653 Da. These two minor components correspond to the Val-82 and Leu-83 forms of rMMP-1cd, respectively. Both the mass spectroscopy and NH$_2$-terminal sequence analysis data indicated that purified rMMP-1cd protein possessed a high degree of homogeneity. This purified rMMP-1cd protein is stable and can be stored at 12 mg/ml and 4°C for at least 6 weeks.

Accurate determination of $k_{cat}/K_m$ requires an accurate measurement of the active enzyme concentration. To address this, enzyme samples were titrated, under conditions which promote stoichiometric binding, with the strong competitive inhibitor BB-94. For stoichiometric binding, it is essential that the enzyme concentration be kept high and the $K_i$ of the titrating ligand low, since low enzyme concentrations and/or weaker ligands results in falsely high estimates of the active site concentration. The activity titration of rMMP-1cd was linear as shown in Fig. 5. The abscissal intercept of a linear fit gave a cuvette concentration for the Val-82 form of rMMP-1cd of 1009 nM. The stock concentration of rMMP-1cd was then calculated to be 778 μM. After three determinations, the stock concentration of the Val-82 form was 764 ± 25 μM and that of the Phe-81 form was 448 ± 30 μM.

$k_{cat}/K_m$ values for both the Phe-81 and the Val-82 forms of rMMP-1cd were determined by first order progress curve analysis with both the Mca-peptide and the thioester-peptide.

$rMMP-1cd$ was fully stable during the collection of the progress curves. Table II shows the $k_{cat}/K_m$ values obtained for the Mca-peptide and the thioester-peptide substrates with the two forms of rMMP-1cd. The $k_{cat}/K_m$ for the Mca-peptide shows that Val-82 rMMP-1cd is roughly 19% more active than the Phe-81 form. With the thioester-peptide, Val-82 is only 7% more active than Phe-81. These data show that the activity of the Phe-81 and Leu-82 forms of rMMP-1cd are very similar. Initially, an attempt was made to determine the individual values of $k_{cat}$ and $K_m$ for the Mca-peptide by saturation analysis. Independent determination of such may have shown more significant but counteracting effects on these parameters. However, this was not possible, since the $K_m$ significantly exceeded its solubility of 250 μM. For the same reason, $K_m$ could not be determined through progress curve analysis using the integrated Michaelis-Menten equation. In the chromogenic assay,
the thioester-peptilide $K_m$ could not be determined at pH 7.5, since this molecule was quite unstable and underwent significant spontaneous hydrolysis at this pH. At greater than 500 $\mu$M thioester-peptilide, the rate of enzyme independent hydrolysis was large, and therefore, partial saturation could not be reached. It is clear that the $K_m$ for both these substrates with rMMP-1cd is large. Hence, the condition $[\text{substrate}] < < K_m$ is satisfied and progress curves are pseudo-first order.

**DISCUSSION**

This report demonstrates that rMMP-1cd could be produced abundantly as part of a soluble ubiquitin fusion protein. The increased solubility precludes the need to refold out of urea or other denaturants and therefore results in a more stable protein. The fusion protein retains proteolytic activity as shown by its ability to catalyze its own release from fusion protein as well as to cleave peptide substrates. This is not surprising since both the cysteine switch region and the upstream prosequence were omitted from the fusion proteins. The presence of these two regions is crucial for maintaining the latency of MMPs in general (4). High homogeneity of the purified Phe-81 and Val-82 forms of the rMMP-1cd allowed us to perform comparative kinetic studies.

It is important to note that the $k_{cat}/K_m$ values obtained with Phe-81 and Val-82 MMP-1cd are very similar. But, they differ significantly from the values previously reported. Knight et al. (21), using a full-length MMP-1 purified from NSO myeloma cells, reported a $k_{cat}/K_m$ for the Mca-peptide of 14,800 $M^{-1} s^{-1}$. This is roughly half the value we determined for the Phe-81 and Val-82 forms. However, it has been shown that the MMP-1 catalytic domain is 21% less active than full-length MMP-1 against the peptide substrate 2,4-dinitrophenyl-Pro-Leu-Gly-Leu-Trp-Ala-Arg (14). Although the MMP-1 preparations were titrated with tissue inhibitor of metalloproteases for effective in-study comparative purposes, tissue inhibitor of metalloprotease concentration was not standardized. Comparison of resulting data with independent active site titrations must be made with caution.

Other factors contributing to the disparity were that the enzyme used by Weingarten and Feder (22) was full-length and partially activated by trypsin digestion. Other authors have shown this activation method to result in partially activated...
MMP-1 with an NH₂-terminal extension that is detrimental to activity (9–11). It is commonly observed that full-length MMP-1, as well as other MMPs, are relatively unstable (27). An estimate of active enzyme concentration based on protein content can introduce significant errors in \(k_{cat}/K_m\) calculations. Concentrations of the active enzymes in the present report were more accurately determined by active site titration with BB-94, a small molecular weight synthetic tight binding metalloproteinase inhibitor. These points together could explain the 20-fold difference in the \(k_{cat}/K_m\) values determined by Weingarten and us. It is quite possible that the abundant expression and solubility of our rMMP-1cd has allowed higher purity, stability, and proteolytic activity when measured with peptide substrates.

Previous reports have shown that MMP-1 with Phe-81 as the NH₂-terminal residue has higher collagenolytic activity than the other forms (9–13). In some cases, NH₂-terminal sequencing has identified the NH₂ terminus to be Val-82 or Leu-83 (10, 27). In others, there was a 15-amino acid extension on the NH₂ terminus (10). All these MMP-1 proteins were full-length 41- or 43-kDa species containing the hemopexin domain and collag- enolytic activity. Murphy et al. (14) showed that the hemopexin domain is responsible for binding collagen and that the short form is unable to do so. Not only is the hemopexin domain important for binding but also must be responsible for local denaturation or unwinding of the collagen triple helix. The x-ray crystal structure clearly shows that only one strand of the collagen can fit into the active site of MMP-1 (28). The short forms of MMP-1 generated in this report do not retain collagenolytic activity. The loss of collagenolytic activity is not due to a change in the active site but to a loss of its distant binding site within the hemopexin domain.

Higher activity was observed with the Phe-81 form of full-length MMP-1 and primarily with collagen as substrate. An explanation of the cause of superactivation of MMP-1 has been proposed based on observed salt bridge of Phe-79 and Asp-232 which stabilizes the NH₂ terminus in neutrophil collagenase (29). Similar interaction between the NH₂-terminal Phe-81 residue and the conserved aspartic acid has been observed in solved structures of MMP-1cd, MMP-3cd, and MMP-7 (30). The loss of this salt bridge results in the disorder of the NH₂ terminus, which, in turn, may lead to less efficient catalysis. We considered that if Phe-81 were missing, the Val-82 residue might contact the catalytic zinc and decrease the \(k_{cat}\) value. However, the disordered NH₂-terminal section is too short to do so. Besides, positively charged amine cannot form a stable interaction with the catalytic zinc. The mobility of the disordered NH₂ termini may perturb the architecture of the S3 and S4 pockets, thus causing less favorable contacts with extended substrates which will be reflected by the elevated \(K_m\) value. Knäuper et al. (9) did show 2-fold higher activity of the Phe-81 form of full-length MMP-1 with a 2,4-dinitrophenyl octapeptide substrate, which has a binding site presumably reaching the S5 pocket. Clark and Cawston (13) have shown that the MMP-1 catalytic domain does cleave casein and gelatin. However, they are not in vivo substrates for MMP-1 (13). We expect the Phe-81 form of MMP-1cd may have a slightly higher cleavage rate for these extended substrates than the Val-82 form. However, the experimental determination of the Michaelis constant and the \(K_m\) and \(k_{cat}\) values for these substrates will be very difficult and so will further interpretation of the data.

Willeenbrock et al. (31) have recently reported that full-length MMP-3, and MMP-9 contains only one catalytic zinc atom and not the additional structural zinc atom detected in the catalytic domain of MMP-1, MMP-3, MMP-7, and MMP-8 (28, 30, 32, 33). This indicates that the hemopexin domain may stabilize the catalytic domain in a different manner, thus eliminating the need for the second zinc. Although the reason for this difference in metal requirements is unclear, the catalytic activities of the full-length and truncated MMP-1 with small peptide substrates are similar as are the inhibition constants of small molecular weight inhibitors. Collagenolytic activity data and the difference in zinc content indicate that there can be structural differences between the full-length enzyme and its truncated form. However, with currently available kinetic and active-site structural information we feel that the catalytic domain does provide us an adequate model for the design of low molecular weight inhibitors. Ultimately, the structural study of full-length MMP-1 will be needed for a full comprehension of this enzyme. Instability of full-length MMPs has so far hampered such studies.

Information from kinetic, small molecule inhibition, and structural observations indicate that the active site of rMMP-1cd is not significantly different from that of the full-length form. High level expression of soluble rMMP-1cd as a cleavable ubiquitin fusion protein has allowed for detailed kinetic and crystallographic studies, and a similar expression approach may be used for other proteins that are either poorly expressed or insoluble in E. coli.

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Purification and Characterization of Recombinant MMP-1

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