Catalytic- and ecto-domains of membrane type 1-matrix metalloproteinase have similar inhibition profiles but distinct endopeptidase activities

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Membrane type 1-matrix metalloproteinase (MT1-MMP/MMP-14) is a major collagenolytic enzyme that plays a vital role in development and morphogenesis. To elucidate further the structure–function relationship between the human MT1-MMP active site and the influence of the haemopexin domain on catalysis, substrate specificity and inhibition kinetics of the cdMT1-MMP (catalytic domain of MT1-MMP) and the ecto domain Δ TM-MT1-MMP (transmembrane-domain-deleted MT1-MMP) were compared. For substrate **1** [Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂, where Mca stands for (7-methoxycoumarin-4-yl)acetyl- and Dpa for *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl], the activation energy E_a was determined to be 11.2 and 12.2 kcal/mol (1 cal = 4.184 J) for cdMT1-MMP and Δ TM-MT1-MMP respectively, which is consistent with k_{cat}/K_M values of 7.37 and $1.46 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. The k_{cat}/K_M values for a series of similar single-stranded peptide substrates were determined and found to correlate with a slope of 0.17 for the two enzyme forms. A

triple-helical peptide substrate was predicted to have a k_{cat}/K_M of $0.87 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for Δ TM-MT1-MMP based on the value for cdMT1-MMP of $5.12 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; however, the actual value was determined to be 2.5-fold higher, i.e. $2.18 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. These results suggest that cdMT1-MMP is catalytically more efficient towards small peptide substrates than Δ TM-MT1-MMP and the haemopexin domain of MT1-MMP facilitates the hydrolysis of triple-helical substrates. Diastereomeric inhibitor pairs were utilized to probe further binding similarities at the active site. Ratios of K_i values for the inhibitor pairs were found to correlate between the enzyme forms with a slope of 1.03, suggesting that the haemopexin domain does not significantly modify the enzyme active-site structure.

Key words: activation energy, catalytic domain, diastereomeric MMP inhibitors, ecto-domain, membrane type 1-matrix metalloproteinase (MT1-MMP), substrate specificity.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of related zinc endopeptidases known to play prominent roles during normal and pathological extracellular matrix remodelling events including cancer progression [1]. The first identified membrane type MMP, MT1-MMP, has been shown to play a key role in tumour cell invasion and metastasis by complex mechanisms, including activation of proMMP-2 and direct hydrolysis of interstitial collagens [2,3]. MT1-MMP is tethered to the cell membrane by a type 1 transmembrane region and is expressed as an active protease on the cell surface upon activation, primarily by proprotein convertases such as furin or furin-like serine proteinases [4]. In addition, consistent with other MMP family members, MT1-MMP has a propeptide, catalytic domain linker region and a haemopexin domain [5].

The haemopexin domain has been supposed to function mainly in protein-substrate recognition [6]. It is necessary for native type I collagen cleavage for reasons that are not completely understood [7,8]. Recently, new roles have been identified for the MT1-MMP haemopexin domain including enzymic regulation and subcellular localization [9,10]. Additionally, the linker region between the catalytic and haemopexin domains has been identified as having the ability to bind the collagen [11]. It is possible that this region is necessary for triple-helical substrate hydrolysis.

Although several groups have reported the cleavage of extracellular matrix constituents by MT1-MMP [12–14], a structural

comparison of the active site of the catalytic domain with and without the haemopexin domain has not been accomplished. In the present study, a comparison of the substrate specificity for two truncated forms of MT1-MMP was performed using single-stranded peptide substrates. The haemopexin domain was then found to facilitate the hydrolysis of a triple-helical substrate. To demonstrate that the haemopexin domain does not influence the architecture of the active site, diastereomeric inhibitor pairs were utilized to show identical K_i ratios.

MATERIALS AND METHODS

Materials

Quenched fluorogenic peptide substrates (see Table 1) **1** and **3–5** [Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂, Mca-Pro-Lys-Pro-Leu-Ala-Leu-Dpa-Ala-Arg-NH₂, Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys-Dpa-NH₂ and Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Arg-NH₂, where Mca stands for (7-methoxy-coumarin-4-yl)acetyl, Dpa stands for *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl and Nva for norvaline] were purchased from Bachem and **2** (Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH₂) was from Calbiochem (San Diego, CA, U.S.A.). The triple-helical peptide substrate **6** [(GPP*)₅ GPK(Mca)GPQGLRGQK(Dnp)GVR(GPP*)₅-NH₂]₃, where Dnp stands for 2,4-dinitrophenyl and P* for 4-hydroxy-L-proline] was synthesized as described previously [15]. The mercaptosulphide

Abbreviations used: MMP, matrix metalloproteinase; MT1, membrane type 1; cdMT1-MMP, catalytic domain of MT1-MMP; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Mca, (7-methoxycoumarin-4-yl)acetyl; Nva, norvaline; RTTI, rat-tail tendon type I collagen; TIMP, tissue inhibitor of metalloproteinase; Δ TM-MT1-MMP, transmembrane-domain-deleted MT1-MMP.

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inhibitors were synthesized and characterized as described previously ([16,17]; Y. Jin and M. A. Schwartz, unpublished work). Hydroxamate inhibitors 444237 and 444238 were purchased from Calbiochem. The recombinant human cdMT1-MMP (catalytic domain of MT1-MMP; Ile¹¹⁴–Ile³¹⁸) was expressed in *Escherichia coli* and was activated by autocatalysis [18] and Δ TM-MT1-MMP (transmembrane-domain-deleted MT1-MMP), the recombinant human ectodomain of MT1-MMP (Tyr¹¹²–Glu⁵²³ consisting of the catalytic domain and the haemopexin domain) was expressed in *Pichia pastoris* and activated by yeast proteinases during maturation [19]. Human TIMP-2 (tissue inhibitor of metalloproteinase-2) was kindly provided by Professor Jack Windsor of Indiana University (Indianapolis, IN, U.S.A.). All standard chemicals were purchased from Fisher with the exception of 5,5'-dithio(bis-2-nitrobenzoate), which was from Sigma.

Enzyme kinetics

Kinetic assays were performed as described previously [20] in 50 mM Hepes buffer (pH 7.5), with 10 mM CaCl₂, 0.2 M NaCl and 0.05 % Brij-35 (polyoxyethylene lauryl ether) at 25 °C. The final substrate concentration was 1 μ M (1 % DMSO), which is at least 10-fold lower than K_M . Hence k_{cat}/K_M is calculated using the equation:

$$\frac{k_{cat}}{K_M} = \frac{v}{[E][S]}$$

where v is the initial rate and $[E]$ and $[S]$ are the enzyme and substrate concentrations respectively. The activation energy E_a was determined by the Arrhenius equation:

$$\ln v = -\frac{E_a}{RT} + \ln A[ES]$$

where R is the universal/ideal gas constant, T the absolute temperature in Kelvin, A the collision constant/Arrhenius A factor/pre-exponential factor and $[ES]$ the concentration of the enzyme–substrate complex. The initial rates were determined at 283 K and then at increments of 5 K up to 313 K. The fluorescence intensity was corrected for temperature using the standard Mca-Pro-Leu-OH (Bachem). For the inhibition assays, the inhibitors were incubated with enzyme for 15–30 min before adding substrate **1** to ensure equilibrium conditions. The inhibitor dissociation constant K_i was determined as described previously [21] using the Morrison equation [22]:

$$\frac{v_i}{v_o} = \frac{[E]_o + [I]_o - K_i^{app} + \sqrt{([I]_o + K_i^{app} - [E]_o)^2 + 4[E]_o K_i^{app}}}{2[E]_o}$$

where v_i and v_o are the initial rates with and without the inhibitor respectively, $[E]_o$ and $[I]_o$ are the initial enzyme and inhibitor concentrations respectively and K_i^{app} is the apparent K_i that is equal to the true K_i under the conditions of $[S] \ll K_M$ for competitive inhibition according to the equation:

$$K_i^{app} = K_i \left(1 + \frac{[S]}{K_M} \right)$$

The active mercaptosulphide inhibitor concentration was quantified with Ellman's reagent 5,5'-dithiobis-(2-nitrobenzoate) [23,24]. Active cdMT1-MMP and Δ TM-MT1-MMP concentrations were normalized by titration with standardized preparations

Figure 1 Arrhenius plot

E_a values were determined for the hydrolysis of substrate **1** with (A) cdMT1-MMP and (B) Δ TM-MT1-MMP as described in the Materials and methods section. Initial rates are expressed in nM/min. Fluorescence intensity was temperature-corrected. The slope of the line $-E_a$ (kcal/mol) was 11.2 and 12.2 for (A) and (B) respectively.

of recombinant TIMP-2. The titration was performed as described above for determination of K_i with an $[E]/K_i$ ratio > 100 to ensure proper titrating conditions.

Collagen hydrolysis and electrophoresis

Samples containing 280 μ g/ml purified RTTI (rat-tail tendon type I collagen) [20] were incubated with 80 nM enzyme at 37 °C in 50 mM Hepes (pH 7.5), 10 mM CaCl₂, 0.2 M NaCl and 0.05 % Brij-35. The reactions were stopped with 50 mM EDTA, 100 mM dithiothreitol and boiled for 5 min. The samples were analysed by SDS/PAGE (7 % gel) with silver staining. The cdMT1-MMP and Δ TM-MT1-MMP samples were visualized by a Western blot (12 % SDS/polyacrylamide gel) using a previously characterized antibody [3].

RESULTS

Substrate specificity

The catalytic domain alone is predicted to hydrolyse small peptide substrates more efficiently than Δ TM-MT1-MMP if the active sites are identical, possibly due to flexibility, rotational freedom or substrate exosite binding. The activation energy for enzyme hydrolysis of peptides should therefore be lower for cdMT1-MMP when compared with Δ TM-MT1-MMP. The E_a values for substrate **1** (see Table 1) with cdMT1-MMP and Δ TM-MT1-MMP were determined to be 11.2 and 12.2 kcal/mol respectively (Figure 1). Consistently, the k_{cat}/K_M values were 7.37 and $1.46 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively (Table 1). If the peptide binds to a haemopexin exosite region, the effective substrate concentration would be reduced, subsequently lowering the apparent k_{cat}/K_M .

Table 1 Comparison of substrate specificity

Fluorogenic peptide substrates	$k_{\text{cat}}/K_{\text{M}} (\times 10^4 \text{ s}^{-1} \cdot \text{M}^{-1})$	
	cdMT1	ΔTM
1 Mca-Pro-Leu-Gly ↓ Leu-Dpa-Ala-Arg-NH ₂	7.37	1.46
2 Mca-Pro-Leu-Ala ↓ Nva-Dpa-Ala-Arg-NH ₂	2.27	0.25
3 Mca-Pro-Lys-Pro-Leu-Ala ↓ Leu-Dpa-Ala-Arg-NH ₂	7.82	1.04
4 Mca-Arg-Pro-Lys-Pro-Tyr-Ala ↓ Nva-Trp-Met-Lys-Dpa-NH ₂	1.81	0.15
5 Mca-Pro-Leu-Ala ↓ Gln-Ala-Val-Dpa-Arg-Ser-Ser-Arg-NH ₂	0.18	0.02
6 $\{(\text{GPP}^*)_3\text{GPK}(\text{Mca})\text{GPQG} \downarrow \text{LRGQK}(\text{Dnp})\text{GVR}(\text{GPP}^*)_3\text{-NH}_2\}_3^*$	5.12	2.18

* Single-letter amino acid abbreviations are used here. P* stands for 4-hydroxy-L-proline. The arrow indicates the putative cleavage site.

Figure 2 Correlation of specificity constants

The $k_{\text{cat}}/K_{\text{M}}$ values from Table 1 for the single-stranded peptides **1–5** were plotted (●) and found to have a slope of 0.17. The triple-helical substrate **6** was predicted to have a $k_{\text{cat}}/K_{\text{M}}$ value of $0.87 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for ΔTM -MT1-MMP (□) based on the value of $5.12 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cdMT1-MMP. The actual value was determined to be 2.5-fold higher, i.e. $2.18 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (■).

Since the E_{a} and $k_{\text{cat}}/K_{\text{M}}$ values correlate, exosite binding is probably not a valid reason for differences in hydrolytic efficiency. These results suggest that cdMT1-MMP is slightly more efficient than ΔTM -MT1-MMP for hydrolysing single-stranded peptides as predicted.

This trend was also noted with other similar peptides and a correlation curve was constructed with the $k_{\text{cat}}/K_{\text{M}}$ values giving a slope of 0.17 (Table 1 and Figure 2). A prediction of $k_{\text{cat}}/K_{\text{M}}$ values for ΔTM -MT1-MMP may then be found by multiplying the $k_{\text{cat}}/K_{\text{M}}$ values for cdMT1-MMP by 0.17. The fluorescently labelled triple-helical peptide that was characterized previously [15] was tested with cdMT1-MMP and was found to have a $k_{\text{cat}}/K_{\text{M}}$ value of $5.12 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. Assuming this substrate is hydrolysed similarly to the single-stranded peptides, the predicted $k_{\text{cat}}/K_{\text{M}}$ value would be $0.87 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for ΔTM -MT1-MMP (Figure 2, open square); however, the actual value was determined to be $2.18 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, a value 2.5-fold higher (Figure 2, closed square).

Probing the active site

The difference in the predicted and actual values for triple-helical substrate hydrolysis by ΔTM -MT1-MMP may be due to structural differences at the active site. To probe the active-site structure, the inhibition profiles for cdMT1-MMP and ΔTM -MT1-MMP were compared with diastereomeric inhibitor pairs (Figure 3 and Table 2). The correct stereochemistry of these inhibitors is

Figure 3 Inhibitor structures

The inhibitors are shown as pairs of diastereomers with the more potent inhibitor on the left. K_{i} values are shown in Table 2.

Table 2 K_{i} values of the inhibitor

Inhibitor	cdMT1		ΔTM -MT1	
	K_{i} (nM)	K_{i} ratio of the isomer	K_{i} (nM)	K_{i} ratio of the isomer
MAG 133	19	4	26	6
MAG 128	70		150	
MAG 182	24	11	29	19
MAG 181	259		540	
YHJ 294-2	13	231	24	242
YHJ 294-1	3000		5800	
444237	0.5	90	0.5	110
444238	45		55	

important for potency towards MT1-MMP as noted by the large differences in K_{i} values between inhibitor pairs. The K_{i} values were generally lower for cdMT1-MMP when compared with those for ΔTM -MT1-MMP, which is consistent with the peptide studies. Interestingly, the ratios of K_{i} values for diastereomers correlated with a slope of 1.03 between cdMT1-MMP and ΔTM -MT1-MMP (Figure 4). These results demonstrate that the haemopexin domain has no direct influence on the structure of the active site of the enzyme.

To ensure that ΔTM -MT1-MMP is a fully functional enzyme (i.e. ability to cleave collagen), RTTI was utilized as a substrate. Figure 5(B) shows the ability of this enzyme form to degrade native RTTI at 37 °C (three-quarter and one-quarter fragments not shown). The RTTI was not hydrolysed by cdMT1-MMP under the same conditions (reaction was also followed overnight with the same results; not shown). The same assay was performed with RTTI preparations that were first boiled for 5 min. Both the enzymes rapidly degraded the denatured RTTI (gelatin) samples. These results demonstrate that both enzyme forms maintain hydrolytic characteristics consistent with published reports.

Figure 4 Correlation of diastereomer K_i ratio

The ratios from Table 2 were plotted and found to have a correlation of 1.03 ($r^2 = 0.9961$).

DISCUSSION

The haemopexin domain in MMPs is necessary for the cleavage of native interstitial type I–III collagens by mechanisms that are not completely understood [7,8]. In this study, the substrate specificity and inhibition of cdMT1-MMP was compared with Δ TM-MT1-MMP. The enzyme concentrations were normalized by titration with TIMP-2. Both forms of the enzyme were found to be fully active, as demonstrated by hydrolysis of either native or denatured RTTI, in addition to showing a single band on a Western blot. Although collagenolytic assays are typically performed at 25 °C,

no cleavage was detected after incubation for up to 2 days at room temperature (23 °C). This is consistent with an earlier study that showed MT1-MMP does not hydrolyse type I collagen at 25 °C [13], although other groups have demonstrated cleavage [12,14]. The assay temperature was therefore increased to 37 °C to allow more flexibility in the collagen strands that are more readily hydrolysed. Δ TM-MT1-MMP alone is not stable after prolonged incubation at 37 °C; however, in the presence of a substrate (or an inhibitor), the stability is significantly improved (results not shown).

It may be expected that if the active sites were the same, the catalytic domain alone would be a more efficient enzyme to hydrolyse single-stranded peptides due to hindrance by the haemopexin domain. This domain may limit enzyme flexibility, diffusion of peptide into the enzyme-active site or alternatively decrease effective substrate concentration by exosite binding. The activation energies for substrate **1** are consistent with k_{cat}/K_M values which may suggest enzyme flexibility or diffusion as possible reasons for the difference. The k_{cat}/K_M values for five similar single-stranded peptides were correlated between both forms of the enzyme. The k_{cat}/K_M value for triple-helical substrates will correspond to this fit if the enzyme hydrolyses these substrates in a manner similar to the single-stranded substrates. Although the absolute k_{cat}/K_M values were slightly higher for cdMT1-MMP, the values did not correlate with the single-stranded substrates. Therefore the haemopexin domain of MT1-MMP facilitates the hydrolysis of triple-helical peptide substrates, consistent with previous studies, indicating that the haemopexin domain of

Figure 5 Enzyme titration and collagen cleavage

(A) The concentrations of active cdMT1-MMP and Δ TM-MT1-MMP were normalized by titration with TIMP-2 as described in the Materials and methods section: $[E] = [I]$ at the x -intercept. (B) Collagen cleavage was verified for Δ TM-MT1-MMP with RTTI. Either Δ TM-MT1-MMP (lanes 2 and 4) or cdMT1-MMP (lanes 3 and 5) at 80 nM was incubated with 280 μ g/ml RTTI (lanes 1–3 native; lanes 4–6 denatured by first boiling for 5 min) at 37 °C for 8 h. The reactions were stopped with 50 mM EDTA, 100 mM dithiothreitol and boiling for 5 min. The samples were analysed by SDS/PAGE (7% gel) with silver staining. Lane 1 is native RTTI only and lane 6 is denatured RTTI only. (C) Western blot (SDS/PAGE, 12% gel) of the purified enzymes shows one band for the Δ TM-MT1-MMP sample.

MT1-MMP modulates activity towards proteinaceous substrates [25].

Diastereomeric inhibitor pairs were utilized to probe the architecture of the enzyme-active site. Structural differences between the enzyme forms would be expected to be shown by different K_i ratios for inhibition by the pairs of stereoisomeric compounds tested. A correlation of 1 between the enzymes demonstrates that no significant differences were found at the active site. Although several groups have published the requirement of the haemopexin domain to hydrolyse native type I–III collagens, no previous report has addressed an active-site comparison for MT1-MMP with and without the haemopexin domain.

The triple-helical peptidase activities of collagenolytic enzymes (MMP-1, MMP-13 and MT1-MMP) exhibit noteworthy differences. MT1-MMP shows triple-helical peptidase activity much greater than either MMP-1 or MMP-13 [15]. The haemopexin domain of MMP-1 appears to have virtually no influence on triple-helical peptidase activity [15,26], unlike MT1-MMP. MMP-13 shows a far greater difference between single-stranded and triple-helical substrate hydrolysis compared with either MMP-1 or MT1-MMP [15,27]. Thus, although a number of MMP family members are classified as ‘collagenolytic’, their precise mechanisms for binding, distorting and hydrolysing triple-helical structures are almost certainly different in subtle, but as yet undefined, ways.

One important question remaining is how does the haemopexin domain influence the catalytic activity of MT1-MMP? It has been proposed that triple-helical peptidase and collagenase activity are distinguishable, as the catalytic domains of collagenases have the ability to cleave triple-helical peptides but not collagens [26]. The haemopexin domain may therefore be required for proper orientation and distortion of the collagen. Overall and co-workers [11] recently found that it is the linker region between the catalytic and haemopexin domain that is necessary for native type I collagen binding. The linker region may influence triple-helical activity. Without knowing the three-dimensional structural organization of the linker and haemopexin domain, it is difficult to address specifically how the activities of MMPs are modulated by their structural motifs and domains.

We thank Professor L. Jack Windsor of Indiana University for providing us with purified human TIMP-2 protein and Janelle Lauer-Fields of Florida Atlantic University for synthesizing the triple-helical substrate. We are grateful to Dr Yunge Zhao for isolation of RTTI, and Robert Newcomer, Dr Hyun I. Park and Dr Tiebang Kang for a critical reading of the manuscript and valuable discussions. This work was supported by a predoctoral fellowship award from D. O. D./U.S. Army Breast Cancer Research Program DAMD17-00-1-0243 (to D. R. H.), and grants from D. O. D./U.S. Army Prostate Cancer Research Program DAMD17-02-1-238, National Institutes of Health (NIH) CA78646 (to Q.-X. A. S.), CA77402 and CA98799 (to G. B. F.), a grant from the Molecular Design and Synthesis (MDS) Research Foundation (to M. A. S.), and by the Deutsche Forschungsgemeinschaft (DFG), Bonn (SFB 549, project A05 and DFG grant Ts 8-35/3) to H. T.

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