MMP–TIMP interaction depends on residue 2 in TIMP-4

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Abstract Extracellular matrix remodeling and degradation are of great importance in both physiological and pathological situations. Matrix metalloproteinases (MMPs) and their natural occurring inhibitors – tissue inhibitors of metalloproteinases (TIMPs) – are involved in matrix turnover. Among the TIMPs there is only little specificity for inhibiting individual MMPs. In this report we describe the mutational analysis of the interaction of human TIMP-4 with several MMPs. The effects of different substitutions of residue 2 (Ser^2) in the inhibitory domain of TIMP-4 were determined by kinetic measurements. Size, charge and polarity of residue 2 in the TIMP structure are key factors in MMP inhibition. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Matrix metalloproteinase; Tissue inhibitor of metalloproteinase; Matrix metalloproteinase–tissue inhibitor of metalloproteinase interaction; Tissue inhibitor of metalloproteinase specifity

1. Introduction

The matrix metalloproteinases (MMPs), a group of zinc-dependent metalloendopeptidases including collagenases, gelatinases, stromelysins and membrane-type metalloproteinases, play a pivotal role in the remodeling of the extracellular matrix and are thus implicated in many physiological and pathological processes concerning the connective tissue remodeling. By stoichiometric binding to MMPs, tissue inhibitors of metalloproteinases (TIMPs) mediate MMP activity and activation [1–4]. Currently, four members of the human TIMP family are known, of those TIMP-4 has been identified and cloned recently [5].

TIMPs are related in primary and secondary structure comprising 12 highly conserved cysteine residues forming six disulfide-bonded loops. TIMP-1, -2 and -4 are secreted proteins and are found in various body fluids and tissues. TIMP-3 is unique in that it appears bound to compounds of the extracellular matrix [6,7].

Besides their high degree of homology in the amino acid sequence especially in the first N-terminal amino acids, TIMP-4 and TIMP-2 show several similarities. They are non-glycosylated proteins and bind in a similar manner to human progelatinase A [8]. The effect of TIMP-4 on growth of human breast cancer cells in vivo was investigated by Wang et al. [9]. Cell invasiveness in vitro was inhibited by overexpression of TIMP-4. When tumor cells transfected with TIMP-4 cDNA were injected into nude mice tumor growth was significantly reduced [9]. TIMP-4 is able to inhibit several active MMPs [10,11].

TIMPs bind with their N-terminal domain into the entire active site cleft of the MMPs. In the case of TIMP-2 there is a considerable contribution of the AB β-hairpin loop to the association [12]. The five N-terminal amino acids of TIMP bind to the subsites of the MMP in a substrate-like manner, forming five intermolecular hydrogen bonds. The so called sC-connector loop including Val^{69}/Gly^{71} (TIMP-1) or Ser^{68}/Ala^{70} (TIMP-2) interacts in a substrate inverse manner [12,13]. A relative small number of intermolecular contacts are found between the C-terminal regions of the MMP and the TIMP. The C-terminal region of TIMP shows a high degree of flexibility [13]. Superposition with the structure of full-length porcine MMP-1 revealed that the C-terminal domain of TIMP and the hemopexin-like domain of the MMP would just touch each other. Thus, the C-terminal domain contacts do not contribute significantly to the TIMP binding of most MMPs [13,14].

Recombinant TIMPs without C-terminal domain, the so called inhibitory domains (idTIMPs), retain most of their activity towards MMPs [15]. They have been used in various inhibition studies and for analysis of MMP–TIMP interactions to determine binding specificities between TIMPs and MMPs [16–19].

The N-terminal domain of TIMP-4 (residues 1–127, id-TIMP-4) comprising three disulfide-bonded loops folds independently of the C-terminal domain. It contains the major site of interaction with the catalytic domain (cd) of MMPs and is therefore sufficient for inhibiting MMPs [11]. To examine the role of residue 2 of TIMP-4 in complex with MMPs several mutants of idTIMP-4 were constructed and expressed in the methylotrophic yeast *Pichia pastoris*. The interaction of those TIMP variants with MMPs was analyzed using kinetic measurements.

2. Materials and methods

All chemicals used were purchased from Merck, Darmstadt, Germany, or ICN, Eschwege, Germany. Oligonucleotides were from Life Technologies, Karlsruhe, Germany. T4-DNA-ligase and TA-cloning vector pGEMT were from Promega, Mannheim, Germany. The *P. pastoris* expression kit was from Invitrogen, Groningen, The Netherlands. All restriction enzymes were purchased from New England Biolabs, Beverly, MA, USA. Chromatography materials were from Pharmacia, Freiburg, Germany.

2.1. Construction and expression of idTIMP-4 mutants

With the appropiate oligonucleotides (Table 1) mutations and restriction sites were introduced into the idTIMP-4 cDNA by polymer-

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Abbreviations: TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinase; cd, catalytic domain; id, inhibitory domain

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Table 1
Primer sequences for the introduction of mutations in idTIMP-4 cDNA, codon exchanges are marked by bold characters

idTIMP-4 variant	Primer sequence
idTIMP-4 Ser^2	5′-CTCGAGAAAAGATGC**AGC**TGCGCCCCGGCG-3′
idTIMP-4 $Ser^2 \rightarrow$ Gly	5′-CTCGAGAAAAGATGC**GGC**TGCGCCCCGGCG-3′
idTIMP-4 $Ser^2 \rightarrow$ Ala	5′-CTCGAGAAAAGATGC**GCC**TGCGCCCCGGCG-3′
idTIMP-4 $Ser^2 \rightarrow$ Glu	5′-CTCGAGAAAAGATGC**GAA**TGCGCCCCGGCG-3′
idTIMP-4 $Ser^2 \rightarrow$ Asp	5′-CTCGAGAAAAGATGC**GAC**TGCGCCCCGGCG-3′
idTIMP-4 $Ser^2 \rightarrow$ Pro	5′-CTCGAGAAAAGATGC**CCC**TGCGCCCCGGCG-3′
idTIMP-4 rev	5′-ATATAGCGGCCGCCTAGGGCTGAACGATGTCA-3′

ase chain reaction (PCR) using the Expand High fidelity system (Roche Diagnostics, Penzberg) and the pPICza-idTIMP-4 expression vector as template [11]. The PCR product was subcloned into the TA-cloning vector pGEMT, digested with *Xho*I and *Not*I, and inserted into the pPICza vector. The correct insertion of the cDNA of idTIMP-4 in frame with the α-mating factor was confirmed by DNA sequencing.

Electrocompetent cells of the *P. pastoris* strain X-33 were transformed with the *Sac*I-linearized plasmid DNA by electroporation according to the manufacturer's manual. The cells were plated at low density on plates of yeast, peptone, dextrose and sorbitol containing 100 μg/ml zeocin for selection of transformants. Transformants were cultured in buffered minimal glycerol medium for 24 h to an OD_{578} of 2–6 at 28°C with vigorous shaking. Cells were harvested by centrifugation at 2000×*g* and resuspended in one volume expression medium (buffered minimal methanol). Expression was carried out at 28°C for 6–7 days and was monitored by SDS–PAGE with silver staining. The N-terminal amino acid sequence was verified by automated Edman degradation [20].

2.2. Purification

Culture broth was centrifuged at 2000×*g* to separate carefully from cells and once again at 10 000×*g* supernatant was filtered and $(NH_4)_2SO_4$ was added to a final concentration of 1 M. Then the solution was chromatographed on HIC material with 100 mM phosphate buffer and 1 M $(NH_4)_2SO_4$ as starting buffer. The content of $(NH_4)_2SO_4$ was reduced stepwise to zero and finally the protein was eluted. After concentration by ultrafiltration, dialysis against running buffer (50 mM Tris–HCl pH 7.5, 0.5 M NaCl, 5 mM $CaCl_2$, 0.05% Brij 35), and S200 FPLC size exclusion chromatography homogenous protein could be obtained.

2.3. Kinetic measurements

ProMMP-9 was isolated from human plasma buffy coat as described by Tschesche et al. [21]. Following activation by treatment with trypsin at 37°C for 30 min, activated gelatinase B was stored for two additional hours at 0°C. The recombinant cd of MMP-8 was expressed in *Escherichia coli* as inclusion bodies, refolded, and purified as described by Kleine et al. [22].

Enzyme concentrations were determined by active-site titration using Batimastat (BB-94) as inhibitor. All kinetic measurements were performed in fluorimetry assay buffer (FA buffer; 5 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM $CaCl_2$, 0.05% PEG-6000) at 25°C. MMP and idTIMP-4 were preincubated at 25°C in 100 μl FA buffer for 30 min and then diluted with FA buffer containing substrate to a final volume of 200 μl. Enzyme activity was quantified by measuring the rate of hydrolysis of the quenched fluorescent substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitro-phenyl]-L-2,3-diamino-propionyl)-Ala-Arg-NH_2) using the plate reader Spectra-Fluor Plus (Tecan, Austria) with excitation at 330 nm and emission at 405 nm with the gain set to 155. Each measurement was done in triplicate.

The degree of inhibition was determined for each inhibitor concentration by plotting the steady state rates against the concentration of inhibitor. Using the GraFit 4.0 software (Erithacus Software Limited) a four parameter logistic fit to the dose–response data was employed to calculate the corresponding IC_{50} values.

Assuming competitive inhibition K_i values were calculated for each enzyme based on known K_m values, which were determined at identical conditions [11] according to the equation described by Copeland et al. [23]:

$$K_i = (IC_{50} - [E_t]/2)/(1 + [S]/K_m).$$

3. Results and discussion

In complexes with MMPs TIMPs bind with their five N-terminal residues to the active site cleft in a substrate-like manner. A key feature of the protein–protein interface is the bidentate coordination of the catalytic zinc of the MMP by the α-amino and the carbonyl groups of the N-terminal cysteine of the TIMP molecule. The Thr/Ser side-chain of the second residue extends into the S'_1 pocket of the cognate MMP. The Ser^2 side-chain hydroxyl of TIMP-2 is hydrogen-bonded to the other carboxylate oxygen of the catalytic Glu of the MMP displacing a water molecule from the active site that normally plays an essential role in catalysis [12]. Therefore this residue is important for inhibiting MMPs. Analysis of its function in complexes of idTIMP-4 and MMPs is possible by substitution of this residue and determining the dissociation constants of these TIMP variants in complexes with MMPs.

The mutations for the exchange of residue 2 were introduced into the cDNA of idTIMP-4 by PCR using the idTIMP-4 expression vector as template. After successful transformation of *P. pastoris* strain X-33 the expression of a 14.6 kDa protein could be detected after 3 days reaching a maximum at day 7 (Fig. 1). We were not able to express idTIMP-4 $Ser^2 \rightarrow$ Pro. This substitution seems to be incompatible with the TIMP structure, as proline is an imino acid. After several purification steps including hydrophobic interaction chromatography and size exclusion chromatography using a S200 column the homogenous protein was obtained and analyzed by SDS–PAGE and N-terminal sequencing.

Using the quenched fluorescent substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitro-phenyl]-L-2,3-diamino-propionyl)-Ala-Arg-NH_2) MMP activities were measured [24]. cdMMP-8 and MMP-9 were preincubated with different concentrations of idTIMP-4 mutants and then diluted with substrate-containing buffer to reach final concentrations of 0.8 nM (cdMMP-8) and 1.6 nM (MMP-9) and an enzyme:substrate ratio of at least 1:1000. The remaining

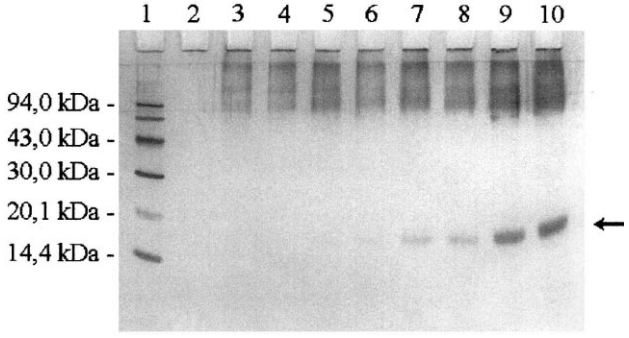

Fig. 1. Expression of idTIMP-4 $Ser^2 \rightarrow$ Gly (←). Analysis of *P. pastoris* culture supernatants by tricine–PAGE. Lane 1: LMW marker, lanes 3–10: days 0–7 of expression.

Table 2
Results of kinetic measurements

Tested idTIMP	cdMMP-8 (0.8 nM) K_i (nM)	MMP-9 (1.6 nM) K_i (nM)
idTIMP-4 Ser^2	9.7	14.5
idTIMP-4 $Ser^2 \rightarrow Gly$	n.m.	n.m.
idTIMP-4 $Ser^2 \rightarrow Ala$	1039.3	473.5
idTIMP-4 $Ser^2 \rightarrow Glu$	979.3	431.5
idTIMP-4 $Ser^2 \rightarrow Asp$	2667.6	1132.8
idTIMP-4 $Ser^2 \rightarrow Pro$	n.d.	n.d.

Determination of K_i values. Concentrations of MMPs were determined by active-site titration with Batimastat (S.D. were less than 10%). n.m. = no useful measurement results. n.d. = not determined.

MMP activity was measured and plotted against the inhibitor concentration. The calculated K_i values are shown in Table 2.

The exchange of residue 2 in TIMP-4 leads to an inhibitor with altered specificities. Whereas the wild type TIMP-4 shows a slight preference for collagenase-2 (MMP-8) the mutants obviously bind tighter to gelatinase B (MMP-9). All mutants of idTIMP-4 are inhibitors of lower affinity to the analyzed MMPs. The idTIMP-4 $Ser^2 \rightarrow Gly$ is the weakest inhibitor for the analyzed MMPs. The absence of a side-chain in this mutant results in the loss of a major fraction of free binding energy. Concentrations of 30 μM neither inhibited MMP-8 nor MMP-9 more than 30%. Therefore K_i values could not be determined for this variant.

Thus, the substitution of Ser by Gly results in a protein that is inactive as inhibitor. The side-chain of residue 2 is therefore a second marker of binding capacity. A methyl group as side-chain is sufficient to recover inhibitory activity as was clearly shown by the use of the alanine substitution. Charged amino acids like aspartate and glutamate also reduce the TIMP/MMP affinity significantly. Besides the polarity of the amino acid the steric consequence of the large side-chain seems to play an important role. Compared to other MMPs, the S'_1 pocket of collagenase-2 is of medium size because Arg^{243} of the specificity loop extends into this pocket. In contrast the S'_1 pocket of gelatinase B is believed to be deep and narrow, so that the effects of polar or bulky substituents are of lower influence [25,26]. Several studies concerning amino acid exchanges in TIMP-1 and TIMP-2 resulted in comparable variations of binding specificities [18,19]. Meng et al. analyzed the influence of residue 2 in idTIMP-1 for inhibition of MMP-1, -2 and -3 and came to the result that gelatinase A binds mutants of idTIMP-1 with polar substituents in position 2 tighter than does collagenase-1 [18]. The experiments of Butler et al. showed a clear preference of idTIMP-2 mutants containing amino acids with polar or bulky side-chains as residue 2 to other MMPs than collagenase-3 [19]. So the size and the shape of the S'_1 pocket of the cognate MMP and the nature of the residue 2 of TIMP are of clear influence for the inhibition mechanism.

The ability of TIMP to displace solvent molecules in a free MMP depends on the capacity to interact with hydroxyl groups. This is the major determinant of a high affinity and therefore substituents like serine (see TIMP-2 and TIMP-4) or threonine (see TIMP-1 and TIMP-3) are favorable and conserved amino acids at this position. There is a clear structure–function relationship of these amino acids for the inhibition of the cognate MMPs.

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