Recognition and catabolism of synthetic heterotrimeric collagen peptides by matrix metalloproteinases

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Background: The general consensus is that interstitial collagens are digested by collagenases and denatured collagen by gelatinases, although processing of fibrillar and acetic-acid-soluble collagen by gelatinase A has also been reported. One of the main difficulties in studying the mechanism of action of these matrix metalloproteinases (MMPs) derives from the physicochemical properties of the natural triple-helical collagen, which makes it difficult to handle.

Results: Synthetic heterotrimeric collagenous peptides that contain the collagenase cleavage site of human collagen type I and differ in the thermal stability of the triple-helical fold were used to mimic natural collagen and gelatin, respectively. Results from digestion of these substrates by fibroblast and neutrophil collagenases (MMP-1 and MMP-8), as well as by gelatinase A (MMP-2), confirmed that the two classes of enzymes operate within the context of strong conformational dependency of the substrates. It was also found that gelatinases and collagenases exhibit two distinct proteolytic mechanisms: gelatinase digests the gelatin-like heterotrimer rapidly in individual steps with intermediate releases of partially processed substrate into the medium, whereas collagenases degrade the triple-helical heterotrimer by trapping it until scission through all three α chains is achieved.

Conclusions: The results confirm the usefulness of synthetic heterotrimeric collagenous peptides in the folded and unfolded state as mimics of the natural substrates collagen and gelatin, respectively, to gain a better a insight into the proteolytic mechanisms of matrix metalloproteinases.

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Introduction

Collagens are protein components of the extracellular matrix that provide mechanical strength and structural integrity to various tissues, and are also involved in cellular processes such as cell attachment, migration and differentiation. These abundant proteins are structurally unique molecules consisting of three identical, or two or three different α chains of primarily repeating Gly–Xaa–Yaa triplets where Xaa and Yaa are mainly proline and/or 4-*trans*-L-hydroxyproline (Hyp). This type of sequence favors a left-handed poly-Pro-II helix conformation and the intertwining of the three chains with a one-residue shift into a right-handed triple-helical coiled coil [1–4]. This triple helix imposes significant structural limitations on the complementarity of binding domains of the interacting proteins responsible for both the cell-adhesion and the collagen catabolism. Collagen turnover is required for remodelling of the connective tissue during growth and development, but is also associated with pathological processes accompanying arthritis, glomerulonephritis, atherosclerosis, tissue ulceration, periodontal disease, fibrotic lung disease, and cancer cell invasion and metastasis [5–10].

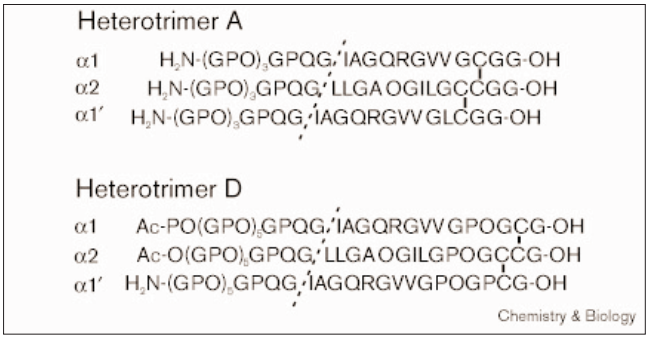
The fibrillar triple-helical type I, II and III interstitial collagens are cleaved by vertebrate collagenases at an appreciable rate under physiological conditions. The collagenases cleave their substrates in a highly specific manner, although with different k_{cat}/K_m values, through a single scission across all three α chains of the collagens at a specific sensitive locus, generating characteristic ¾ and ¼ fragments [11–15]. The resulting thermally unstable fragments unfold partially and are then digested further by other proteinases.

Enzymes capable of degrading collagen type IV also play an important role in tumor metastasis because this extracellular matrix protein constitutes the major component of the basement membrane. Indeed, metastasis and transformation are known to correlate with secretion of the gelatinases MMP-2 and MMP-9 (MMP, matrix metalloproteinase) [6]. MMP-2 is the most ubiquitous matrix metalloprotease in vertebrates and it acts not only on type IV collagen, but also on collagen types V, VII and X, fibronectin, elastin and all types of unfolded collagens [16]. The gelatinases apparently complete the enzymatic digestion of collagen by further degrading the α chains of collagen predigested by collagenases at specific loci.

The unique mode of cleavage of interstitial collagens by collagenases is one of the most specific and remarkable enzymatic processes. Studies of the cleavage of small peptides by collagenases have shown that sequence specificity is not sufficiently restrictive to account for the hydrolysis of native triple-helical collagens at a distinct locus, because numerous potential cleavage sites are present in the collagens [13,17]. The underlying structural basis for this selective recognition is, however, not well understood, but must be related to the local conformation of the collagen substrates at the cleavage site. In fact, the cleavage site is located in an apolar region of the collagens at the boundary between an imino-acid-rich, and therefore probably tightly packed, triple-helical segment, and an imino-acid-deficient section, which may unwind more easily and therefore adapt to the active site of the collagenases to allow the catalytic scission to proceed [17,18]. In humans, four homologous collagenases, the fibroblast collagenase (MMP-1), neutrophil collagenase (MMP-8), collagenase 3 (MMP-13) and MT1-MMP (MMP-14) from the large family of matrix metalloproteinases, have been characterized. They are capable of hydrolysing type I, II and III collagens at the Gly775–Ile776 or Gly775–Leu776 peptide bond of the corresponding α chains [19–22]. In addition, gelatinase A (MMP-2) was shown to digest native collagen type I at the same locus [14].

The collagenases consist of a catalytic subunit linked via a short flexible hinge peptide to a hemopexin-like domain, whereas the gelatinases also contain three consecutive fibronectin type-II modules inserted between the catalytic subunit and the hemopexin-like domain. Autocatalytic fragmentation of collagenases occurs in the linker region; the liberated catalytic domains, although retaining proteolytic activity against denatured collagen or small synthetic substrates, lose their ability to digest triple-helical collagen [15,23–25]. Several three-dimensional structures of the catalytic subunits of the human collagenases MMP-1 [26–28], MMP-8 [29,30], MMP-3 [31–33], MT1-MMP [34] and MMP-13 [35] are known. All have a highly homologous fold with a substrate-binding cleft too narrow to accommodate a triple-helical collagen molecule. The X-ray structure of the full-length porcine MMP-1 [36] and more recently of the human Pro-MMP-2 [37], as well as the known structures of various carboxy-terminal hemopexin-like domains [38–40], do not allow additional information to be derived about the mode of binding and proteolysis of the natural substrates. A possible role for the linker peptide was suggested by changes in substrate specificities obtained with chimeric MMPs and point mutations in the linker region [41–44].

Figure 1

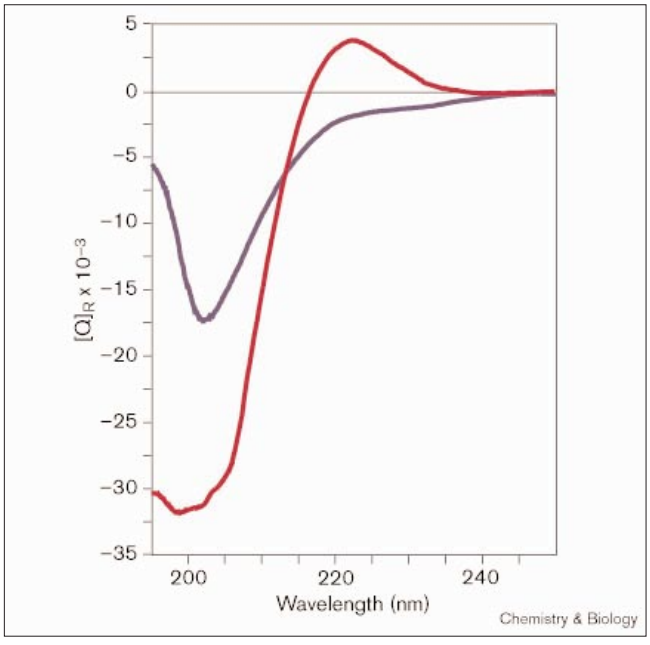


Primary structures of the synthetic collagenous heterotrimers A and D containing the sequences 772–784/785 of collagen type I, which correspond to the residues P4–P′9/P′10 of the cleavage site by collagenases. The scissile bonds are marked by a dotted line, and correspond to Gly775–Ile776 (α1 and α1′ chain) and Gly775–Leu776 (α2 chain).

One of the main difficulties encountered in studying the mechanism of action of the collagenases derives from the substrate itself. In fact, the use of natural collagens for such a purpose is troublesome because of the difficulty in handling them. Using suitable fragments produced by enzymatic and chemical cleavage is an alternative approach, but generally the triple-helical structure of such fragments is thermally rather unstable and refolding is difficult [45,46]. Recently, we have developed a synthetic approach for the correct assembly of collagen peptides into heterotrimers using an artificial cystine knot [47,48]. With this method, heterotrimeric collagen peptides containing the collagenase cleavage sites 772–784 (P4–P′9) and 772–785 (P4–P′10) of the α1 chain and the α2 chain cleavage site 772–784 (P4–P′9) of collagen type I were assembled in the α1α2α1′ stagger as proposed for collagen type I [49], and stabilized in their triple-helical fold at the carboxyl terminus by the cystine knot and at the amino terminus by extensions of increasing chain length with $(Gly–Pro–Hyp)_n$ repeats. The enzymatic cleavage-site sequences incorporated into these model peptides start with the P_4 residue Gly772, because upstream of this sequence position the amino acid composition is more indicative of a stable triple helix. Mutations in the hydrophobic Val782–Val783 region of the α1(I) chain have clearly revealed significant effects on the enzymatic digestion of collagen type I [50,51]. Correspondingly, the natural sequence was extended carboxy-terminally beyond these positions to P′9/10. By optimizing the composition of the heterotrimers in terms of thermal stability of the triple helix, the two compounds shown in Figure 1 were obtained [52]. At room temperature the collagenous peptide A mimics denatured collagen, that is gelatin, with a melting temperature (T_m) of 9°C, whereas the heterotrimer D shows structural properties and thermal stabilities (T_m = 41°C) that correlate well with those of natural type I collagen (T_m = 38°C).

In the present study, a comparative analysis of the digestion of these two artificial heterotrimeric substrates by

Figure 2



CD spectra of the heterotrimer A (blue line) and heterotrimer D (red line) at 25°C and 50 μM concentration in 10 mM $CaCl_2$, 50 mM NaCl, 50 mM Tris/HCl (pH 7.4) as used for the enzyme assays; Rpn factor = 0.123 for heterotrimer D.

collagenase and gelatinase clearly confirmed the strong conformation dependency of the proteolytic activity of these enzymes. Furthermore, surface plasmon resonance experiments served to identify the domains of the enzymes responsible for specific recognition and binding of these collagenous peptides as conformational epitopes and therefore to gain a better insight into the different mechanism of action of these two classes of MMPs.

Results

Heterotrimeric collagen peptides as substrates of MMPs

The synthetic heterotrimers A and D have the same cleavage site for collagenases and gelatinases, and the three α chains are aligned in the identical α1α2α1′ stagger (Figure 1). Because of the different number of amino-terminal (Gly–Pro–Hyp) repeats, the two heterotrimers have well differentiated conformational properties under the conditions used in the enzyme assays. The circular dichroism (CD) spectrum of the heterotrimer A at 25°C is characteristic of a fully unfolded, gelatin-like structure (Figure 2), whereas the CD spectrum of the heterotrimer D confirms its triple-helical fold and therefore the collagen-like structure.

As shown in Figure 3a, the catalytic domain of MMP-8 is capable of degrading the gelatin-like trimer A and, as reported previously [46], monomeric peptide chains of these collagen-like constructs that contain the cleavage-site sequence 772–784 of collagen type I. Conversely, hydrolysis of the triple-helical heterotrimer D by this catalytic domain is very slow at room temperature, a finding that fully confirms the results obtained previously using a thermally less stable synthetic heterotrimer [47]. This finding also agrees with results obtained using the natural collagen substrate [24]. For the full-length MMP-8, the cleavage rates of these two synthetic substrates are reversed with a slow digestion of the unfolded gelatin-like heterotrimer A and a relatively fast hydrolysis of the collagen-like heterotrimer D (Figure 3b). Identical results were obtained with the full-length MMP-1, whereas

Figure 3

Digestion rates of the heterotrimers A (blue line) and D (red line) by **(a)** the catalytic subunit of MMP-8 and **(b)** the full-length neutrophil collagenase MMP-8 in 10 mM $CaCl_2$, 50 mM NaCl, 50 mM Tris/HCl (pH 7.4) at 25°C and a substrate/enzyme ratio of 1000. Degradation of the substrate was monitored by HPLC of aliquots of the incubation medium taken at time intervals, and quantitation of the substrate, expressed as percentage, was derived from the corresponding peak area.

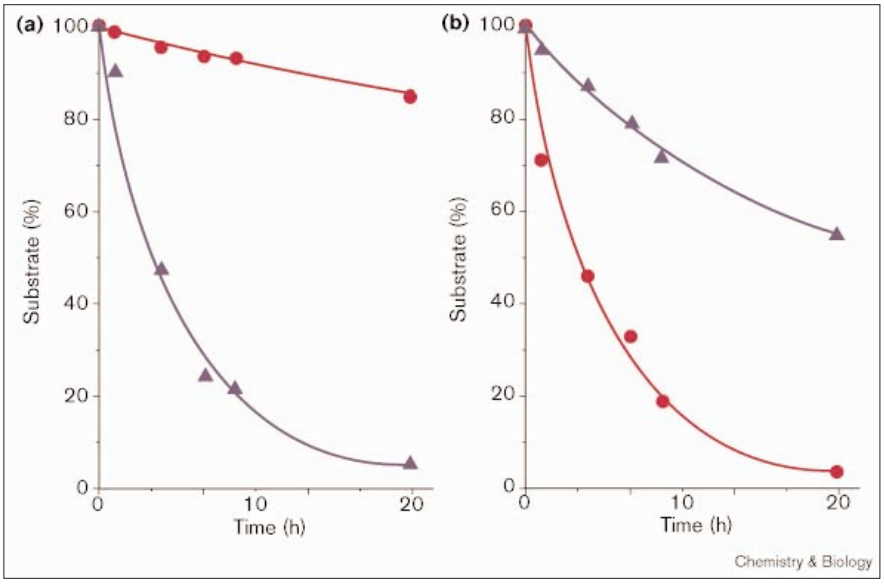
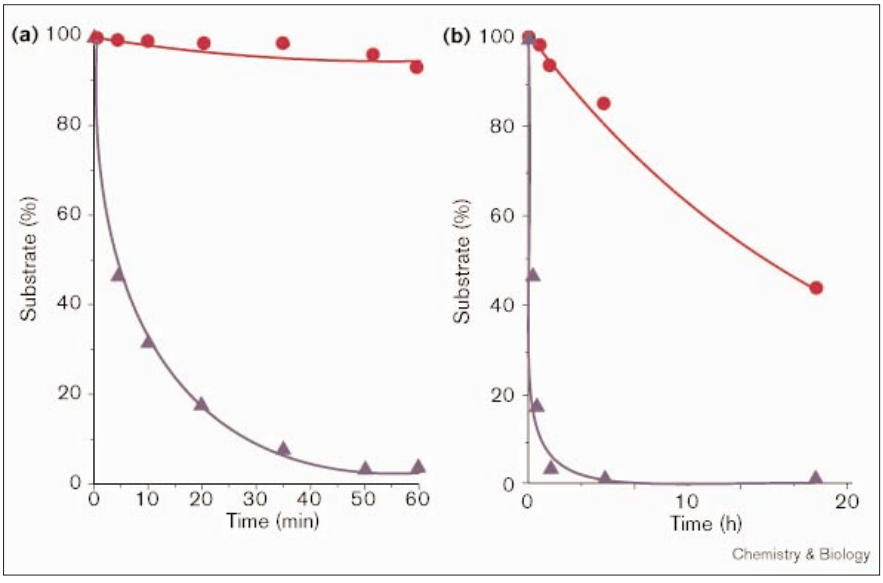


Figure 4



Digestion rates of the heterotrimers A (blue line) and D (red line) by gelatinase A (MMP-2) within 60 min **(a)** and 20 h **(b)** in 10 mM $CaCl_2$, 50 mM NaCl, 50 mM Tris/HCl (pH 7.4) at 25°C and a substrate/enzyme ratio of 1000. Degradation of the substrate was monitored by HPLC of aliquots of the incubation medium taken at time intervals, and quantitation of the substrate, expressed as percentage, was derived from the corresponding peak area.

stromelysin (MMP-3) was unable to digest the triple-helical heterotrimer D; similarly, this trimer is fully resistant to proteolysis by trypsin. The two synthetic substrates therefore mimic rather efficiently the conformational epitopes of the cleavage sites of collagen and gelatin, respectively, because these results correlate well with those reported for the natural substrates [9,11,53,54]. Moreover, a major role of the hemopexin-like domain and possibly even of the linker peptide in the collagenolytic activities of collagenases is clearly confirmed.

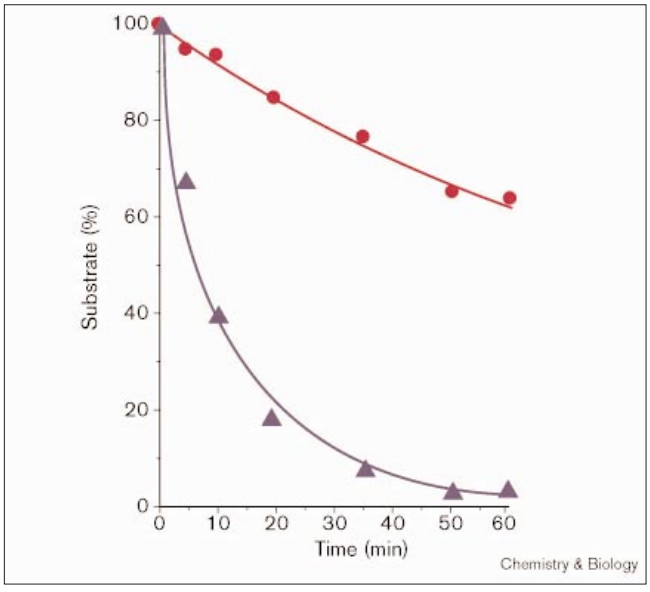
As expected, gelatinase A (MMP-2) has a quite different type of substrate preference. In fact, the gelatin-like heterotrimer A is quantitatively digested by the MMP-2 during a time period in which the collagen-like heterotrimer D was largely resistant towards this enzyme (Figure 4a). On the other hand, a mutant lacking the fibronectin type-II modules, MMP-2($\Delta_{V191\text{-}Q364}$), was found to degrade the heterotrimer A at remarkably lower rates than the wild-type enzyme (Figure 5), in full agreement with previous results obtained with this mutant and with gelatin as substrate [55].

When proteolysis of trimer D by MMP-2 was allowed to proceed for 20 hours (a period of time sufficient for MMP-8 and MMP-1 to quantitatively degrade this folded substrate) only about 55% digestion was observed (Figure 4b). MMP-2 can therefore process even the collagen-type substrate, although at rates significantly inferior to those of the collagenases. Kinetic data for comparative analysis were not derived, because quantification of the enzyme was performed when the enzyme was in the latent form, prior to activation, and not by titration of the active MMPs with tissue inhibitors of metalloproteinase (TIMPs). Nonetheless, a comparison of the $t_{1/2}$ values of substrate digestion (Table 1) clearly shows significant differences in the rates of the two classes of MMPs. The enzymatic hydrolysis of the gelatin-like substrate A by the gelatinase MMP-2 is a much faster process than that of substrate D by collagenases MMP-1 or MMP-8. MMP-2 was found to digest 50% of heterotrimer A in a few minutes under the assay conditions employed ($t_{1/2}$ = 4 min), whereas hydrolysis of the heterotrimer D by MMP-1 or MMP-8 requires several hours ($t_{1/2}$ = 3.5 h). It is well established that the enzymatic cleavage of collagen by collagenases is one of the slowest enzymatic processes known so far, but the mechanistic background for the very different enzyme kinetics of collagenases and gelatinases is not yet understood.

Product distribution upon enzymatic degradation of substrates A and D by MMPs

The water solubility of the synthetic collagenous peptides and their relatively low molecular weights allowed easy monitoring of enzymatic degradation using high-performance liquid chromatography (HPLC). Upon quenching the enzymatic proteolysis of the heterotrimeric substrates, the product distribution of the collagenases MMP-1 and MMP-8, analyzed at intervals by HPLC, was remarkably different from that of the gelatinase MMP-2. As previously reported for a thermally less stable heterotrimeric collagen peptide [47], cleavage of heterotrimer D by MMP-8 occurs by a single cut through all three α chains, yielding two distinct cleavage products: the amino-terminal peptide portions and the disulfide-bridged carboxy-terminal fragment (Figure 6). Intermediate cleavage products with a cut through only one or two strands of the heterotrimeric substrate were

Figure 5



Digestion rates of the heterotrimer A by the wild-type gelatinase A (MMP-2; blue line) and by the mutant MMP-2($\Delta_{V191-Q364}$) that lacks the fibronectin type-II modules (red line) in 10 mM $CaCl_2$, 50 mM NaCl, 50 mM Tris/HCl (pH 7.4) at 25°C and a substrate/enzyme ratio of 1000. Degradation of the substrate was monitored by HPLC of aliquots of the incubation medium taken at time intervals, and quantitation of the substrate, expressed as percentage, was derived from the corresponding peak area.

not detected using liquid chromatography–mass spectrometry (LC–MS) in the time course of the enzymatic digestion; a release of the partially processed triple-helical substrate from the enzyme into the medium was therefore not observed. Surprisingly, an identical degradation pattern was obtained when monitoring the slow digestion of the gelatin-like trimer A by MMP-8. Because the enzymatic proteolysis was performed at 25°C, that is at a temperature 16°C above the melting temperature of trimer A (T_m = 9°C [52]), only very small amounts of folded trimer A can be present at conformational equilibrium (see Figure 2). Although folded substrate would be continuously supplied by the fast establishment of the conformational equilibrium of the carboxy-terminally cross-linked collagenous peptides [52], its concentration would not suffice for the observed digestion rate. We speculate that the degradation pattern could be attributed to the artificial cystine knot that keeps the chains in proximity to one another, and therefore would not be of physiological significance.

HPLC monitoring of the product distribution that resulted from hydrolysis of the gelatin-like trimer A by MMP-2 revealed a completely different degradation pattern (Figure 6). In the time course of the enzymatic process a heterogeneous product mixture was formed that consists of partially digested substrate with one or two intact α chains,

Table 1

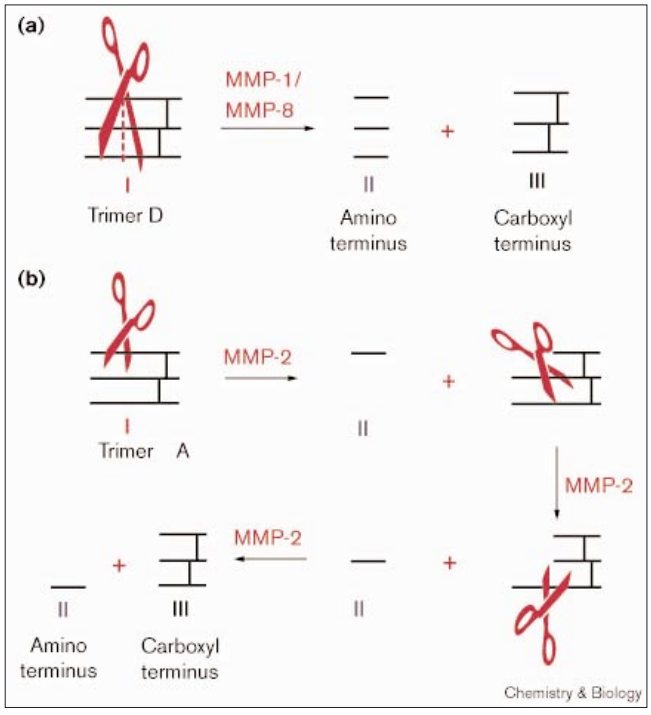
Rates of digestion ($t_{1/2}$) of the heterotrimers A and D.

Substrate	MMP-1/MMP-8	MMP-2	MMP-8 catalytic domain	MMP-2 ($\Delta_{V191-Q364}$)
Trimer A	>>20 h	4 min	3 h	2.5 h
Trimer D	3.5 h	> 13 h	>>20 h	n.d.

n.d., not determined.

as well as the starting material and the two expected end products (Figure 7). The intermediates are released from the enzyme and then further processed in subsequent steps finally to produce the expected amino-terminal portions of the α chains, that is H-(Gly–Pro–Hyp)$_3$–Gly–Pro–Gln–Gly-OH and the heterotrimeric carboxyl terminus of the substrate. The identical intermediate product

Figure 6



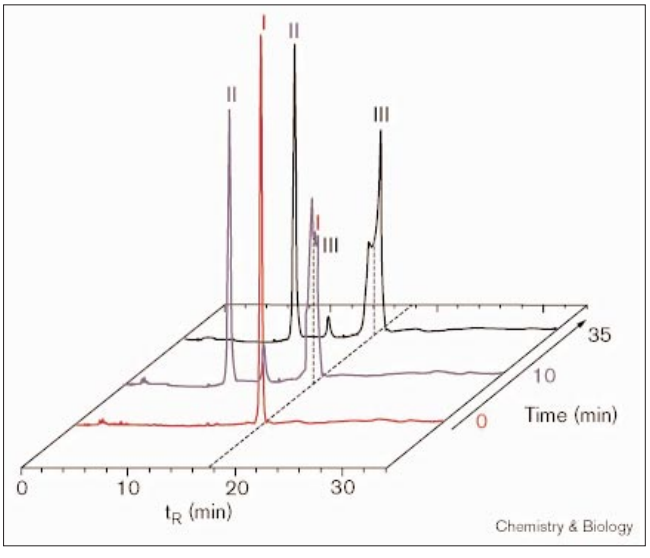
Product distribution of proteolytic degradation of the collagen-like heterotrimer D and the gelatin-like heterotrimer A by MMPs. **(a)** Digestion of the triple-helical collagen-like substrate D by the collagenases MMP-1 and MMP-8 occurs with a single cut across the three α chains at the Gly–Ile (α1 and α1′ chain) and Gly–Leu bonds (α2 chain) without release of intermediates into the medium as monitored by HPLC and with production of the amino-terminal monomeric fragments and the carboxy-terminal cystine-knot fragment. **(b)** Digestion of the unfolded gelatin-like substrate A by gelatinase A (MMP-2) occurs in successive steps at the Gly–Ile (α1 and α1′ chain) and Gly–Leu bonds (α2 chain) with intermediate release of partially digested heterotrimeric substrate into the medium as monitored by HPLC (see Figure 7); upon completion of proteolysis again only the amino-terminal monomeric fragments and the carboxy-terminal cystine-knot fragment are detected in the incubation medium.

distribution was obtained when substrate A was digested with the gelatinase mutant MMP-2($\Delta_{V191–Q364}$), and, even more interestingly, when the triple-helical trimer D was cleaved by MMP-2. As the enzymatic degradation of the trimer D was performed at 25°C, 16°C below its T_m value, only minute amounts of unfolded trimer D should be present at conformational equilibrium. As discussed above for trimer A, even in this case the slow digestion of the trimer D by the gelatinase MMP-2 has to occur in the triple-helical state.

Binding of collagenous peptides by MMPs: surface plasmon resonance measurements

The conformational dependency of recognition and binding of heterotrimeric collagen peptides by MMPs and their subunits was analyzed using the BIAcore technique [56] to quantitate related bimolecular interactions. Using this technique, refractive index changes are monitored and converted into resonance units in a sensorgram that directly reflects protein binding and dissociation, allowing kinetic data of interactions to be derived [57]. The heterotrimer D acetylated at two α chains was grafted selectively at the single underivatized terminal amino group of the α1′ chain (Figure 1) to the sensorchip via EDCI/ HOSu preactivation of the carboxydextrane matrix. This selective immobilization of the collagen peptide D was preferred over a statistical immobilization at the three amino termini to prevent a possible partial unfolding of the triple helix. In the case of heterotrimer A, which is already denatured at room temperature, this precaution was not adopted. In all experiments, as a control for nonspecific analyte/carboxydextrane matrix interaction, one of the four flow cells was left without immobilized peptide. To avoid partial digestion of the immobilized collagen peptides, catalytically inactive pro-MMPs or inactive enzyme mutants were used.

Figure 7



Product distribution upon digestion of the heterotrimer A by gelatinase A (MMP-2) as monitored using HPLC at different time intervals: (I) heterotrimer A, (II) amino-terminal monomeric fragment and (III) carboxy-terminal heterotrimeric cystine-knot fragment.

Binding of heterotrimers A and D by MMP-1 and related subunits

To measure the binding affinities of collagenases for the collagenous peptides the MMP-1(E200A) mutant was used. This mutant was expected to retain fully the substrate-binding affinity, although it is proteolytically inactive. In fact, storage of the trimer D with MMP-1(E200A) at 15°C over a period of days showed no detectable substrate degradation.

As expected from the blockade of the substrate-binding site by the propeptide, pro-MMP-1(E200A) was unable to recognize and bind to the gelatin-like heterotrimer A or the collagen-like heterotrimer D at 22 and 12°C. Identical results were obtained with the wild-type pro-MMP-1, thus excluding significant substrate interactions at exosites. Upon removal of the propeptide, a strong binding affinity of the collagenase mutant MMP-1(E200A) for the triple-helical substrate D and a reduced affinity for the heterotrimer A were measured (Table 2). From a fitting of the experimental interaction curves for substrate D, K_D values of 3.7 μM at 22°C and of 2.0 μM at 12°C were measured, which correlate well with the K_m values (0.80–0.90 μM) of fibroblast collagenase determined for rat, guinea pig and human collagen type I [11,17,53]. The relatively small temperature effect fully agrees with the stability of the triple-helical fold of trimer D in this temperature range [52]. Conversely, for the trimer A the identical temperature drop led to a greater increase in affinity for MMP-1(E200A), reflecting the increased triple-helical content. The K_D values of 13.0 μM at 22°C and of 2.75 μM at 12°C determined for the gelatin-like substrate A are consistent with the K_m values of 3.7–9.8 μM of collagenases for gelatin [17,53,58].

Both heterotrimers A and D were analyzed for their binding to the hemopexin-like domain of MMP-1. Although this domain is essential for the collagenolytic activity of MMPs, no interaction with both the collagen- and gelatin-like substrate could be detected (Table 2).

Binding of heterotrimers A and D by MMP-2 and related subunits

Although interstitial collagens and, as shown above, even the triple-helical heterotrimer D are relatively poor substrates of MMP-2 compared with gelatin and the gelatin-like trimer A, MMP-2 has been reported to bind strongly to triple-helical portions of collagen type I. There is experimental evidence that the fibronectin type-II modules of

Table 2

Kinetics and binding affinities of MMP-1 and MMP-1 subunits for the collagenous heterotrimers A and D.

Peptide	Enzyme/domains	T (°C)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (μM)
Trimer A	pro-MMP-1	22/12			No interaction
	pro-MMP-1(E200A)	22/12			No interaction
Trimer D	pro-MMP-1	22/12			No interaction
	pro-MMP-1(E200A)	22/12			No interaction
Trimer A	Hemopexin-domain (MMP-1)	22/12			No interaction
Trimer D	Hemopexin domain (MMP-1)	22/12			No interaction
Trimer A	MMP-1(E200A)	12	1.77×10^3	4.33×10^{-3}	2.75 ± 1.5
Trimer D	MMP-1(E200A)	12	3.14×10^3	5.92×10^{-3}	2.0 ± 0.9
Trimer A	MMP-1(E200A)	22	4.5×10^2	5.94×10^{-3}	13.0 ± 0.1
Trimer D	MMP-1(E200A)	22	1.56×10^3	5.35×10^{-3}	3.7 ± 1.1

Data were derived from bimolecular interaction parameters determined by the BIAcore technique. Binding assays were performed at 4–5 different protein concentrations and in triplicate; the values are averages from these measurements.

the enzyme are involved in this interaction [55,59–63]. In contrast to pro-MMP-1 and pro-MMP-1(E200A), which were devoid of any affinity for substrates A and D, the mutant pro-MMP-2(E375A) (Table 3) and pro-MMP-2 were found to recognize and bind with high affinity both trimers A and D. As this recognition of the collagenous peptides is not affected by occlusion of the enzyme active site by the propeptide, the binding sites have to be located at exosites, that is on other surfaces of the enzyme, most probably on the fibronectin type-II modules. This is further confirmed by the observation that inhibition of MMP-2 with an excess of CT1847 or with TIMP-2 did not affect the binding affinity for both substrates, although in the MMP-2–TIMP-2 complex a large surface of the catalytic domain is occupied by the inhibitor (data not shown). Conversely, a pro-MMP-2 mutant lacking the fibronectin domain, that is pro-MMP-2($\Delta_{V191–Q364}$), did not bind the trimers A and D, confirming the major role of the fibronectin type-II modules in binding trimers A and D. The ranking order of binding affinity of pro-MMP-2(E375A) is trimer D > trimer A > α1′D(StBu) (Table 3).

Binding of heterotrimers A and D by the fibronectin type-II modules

The fibronectin type-II modules of MMP-2 (Arg190–Tyr366) show a slightly different behavior in terms of temperature dependency than the pro-MMP-2(E375A). The binding constants at 22°C are comparable with those measured with pro-MMP-2-(E375A), but the ranking order of the binding affinities is monomer α′1D(StBu) chain > trimer A > trimer D, with less pronounced differences in the K_D values (Table 4). By lowering the temperature (12°C), again an increase in affinity is obtained. The observed differences in the affinities of the fibronectin-like domain and of the parent gelatinase for the three different substrates suggest the presence of a second or a larger binding site in the full-length MMP-2 that also involves the catalytic domain as can be envisaged by inspection of the X-ray structure of pro-MMP-2 [37]. The structure shows a putative binding cleft extending from the catalytic domain to the fibronectin type II modules. A direct involvement of the fibronectin-like domain in binding MMP-2 to native collagen and gelatine

Table 3

Binding affinities of MMP-2 and related subunits for the heterotrimeric collagenous peptides A and D.

Peptide	Enzyme/subunit	T (°C)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (μM)
Trimer A	pro-MMP-2($\Delta_{V191–Q364}$)	22			No interaction
Trimer D	pro-MMP-2($\Delta_{V191–Q364}$)	22			No interaction
Trimer A	Hemopexin-domain (MMP-2)	22			No interaction
Trimer D	Hemopexin-domain (MMP-2)	22			No interaction
Trimer A	pro-MMP-2(E375A)	22	2.6×10^3	2.1×10^{-3}	0.81 ± 0.36
Trimer D	pro-MMP-2(E375A)	22	3.1×10^3	2.5×10^{-3}	0.67 ± 0.02
α1D(StBu)	pro-MMP-2(E375A)	22	1.4×10^3	2.3×10^{-3}	1.54 ± 0.83
Trimer A	pro-MMP-2(E375A)	12	4.2×10^3	2.0×10^{-3}	0.49 ± 0.06
Trimer D	pro-MMP-2(E375A)	12	5.6×10^3	2.4×10^{-3}	0.43 ± 0.05
α1D(StBu)	pro-MMP-2(E375A)	12	3.1×10^3	2.9×10^{-3}	0.91 ± 0.05

Binding affinities were derived from bimolecular interaction parameters measured by the BIAcore technique. Binding assays were performed at 4–5 different protein concentrations and in triplicate; the values are averages of these measurements.

Table 4

Binding affinities of the three fibronectin type-II modules (Arg190–Tyr366) for the heterotrimers A and D.

Peptide	T (°C)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (μM)
Trimer A	22	7.6×10^3	4.9×10^{-3}	0.67 ± 0.08
Trimer D	22	7.5×10^3	6.0×10^{-3}	0.80 ± 0.02
α1′D(StBu)	22	9.4×10^3	5.0×10^{-3}	0.56 ± 0.20
Trimer A	12	5.9×10^3	2.8×10^{-3}	0.47 ± 0.12
Trimer D	12	6.7×10^3	4.8×10^{-3}	0.73 ± 0.16
α1′D(StBu)	12	9.6×10^3	4.7×10^{-3}	0.50 ± 0.09

Binding affinities were derived from bimolecular interaction parameters measured by the BIAcore technique. Binding assays were performed at 4–5 different protein concentrations and in triplicate; the values are averages of these measurements.

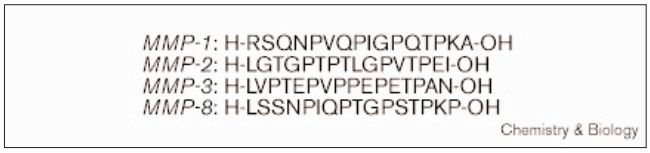
has previously been reported [62,64]. Conversely, the hemopexin-like domain does not act as autonomous binding site, because by itself it is unable to bind to the collagenous peptides.

MMP-2 is known to bind to collagen type I with significant affinity, but with a lower affinity than to gelatin type I [59–61]. The binding sites are located in the CB-7 and CB-8 fragments, and in the CB-7 fragment the binding site is apparently positioned close to the collagenase cleavage site [65]. With the two conformationally well defined heterotrimers, different affinities between a triple-helical and gelatin-like state of the collagenous peptides were not clearly detected, but there was a slight preference for the folded state. Taking into account the relatively high affinity of the pro-MMP-2 for trimer A, the absence of binding of trimer A to pro-MMP-2($\Delta_{V191–Q364}$) and the lower digestion rate of the fibronectin domain-deleted mutant than that of the wild-type MMP-2, a functional role of the fibronectin II like-domain in processing gelatin has to be advocated.

Linker peptides of MMPs

The flexible hinge region that links the catalytic subunits of MMPs to the carboxy-terminal hemopexin-like domains differs in size and composition among the MMPs and apparently is essential for full expression of the substrate specificities by these enzymes [15,43,44]. A hypothetical mechanistic contribution of these linker peptides to the collagenase activity has been postulated, by which the linker peptides, rich in proline residues and thus possibly folded into a poly-Pro-II helix, are inserted into the triple helix of the collagen substrate with concomitant unwinding of the tightly packed supercoil [66,67]. The synthetic linker peptides of MMP-1, MMP-2, MMP-3 and MMP-8 (Figure 8) were analyzed for their conformational preferences in the MMP assay buffer (pH 7.4) at 4°C. The related CD spectra showed a strong negative maximum at 196 nm, but no positive dichroism around 220–224 nm, thus excluding a tendency to fold into a poly-Pro-II helical

Figure 8



Sequences of the synthetic linker peptides of MMP-1, MMP-2, MMP-3 and MMP-8.

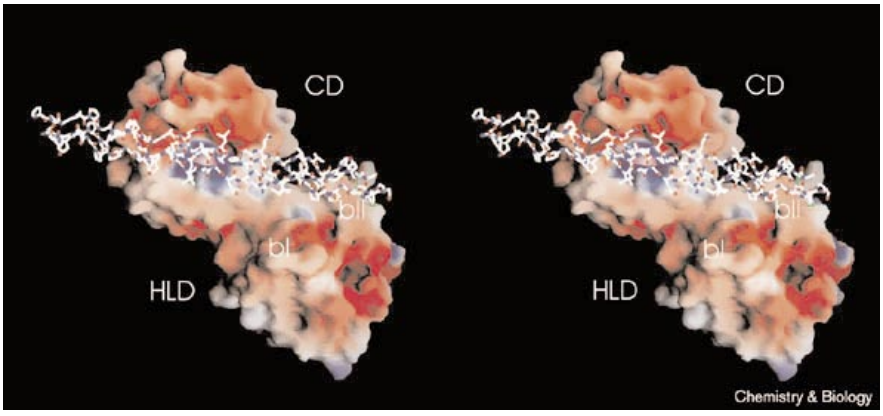
conformation as theoretically predicted. Similarly, addition of the MMP-1 linker peptide to the heterotrimer D did not affect its triple-helical structure at all. The lack of interaction of the linker peptides with the triple-helical cleavage site of collagen was fully confirmed by BIAcore experiments, in which interaction of the linker peptide with the immobilized heterotrimer D could not be detected. Finally, addition of excesses of the various linker peptides to native type I collagen did not affect the ability of MMP-1 to cleave the collagen.

Modelling of the MMP-1–heterotrimer D complex

On the basis of the 2.0 Å triple-helical crystal structure of the type III collagen cleavage site reported by Kramer *et al.* [68] (PDB; accession no. 1bkv), the heterotrimer D was modeled using TurboFRODO [69]. The rod-like molecule was placed along the active-site cleft of full-length porcine MMP-1 [36] so that the α1-chain segment Pro–Gln–Gly–Ile–Ala–Gly–Gln–Arg was approximately directed towards the central groove, with the scissile Gly–Ile pair flanking the catalytic zinc. This octapeptide was slightly freed from the triple-helical association and aligned (under considerable rearrangement of the Ile–Ala peptide backbone) to the active-site cleft in a substrate-like manner as described by Grams *et al.* [70], that is under formation of several inter-mainchain hydrogen bonds to the enzyme, with the carbonyl group of the scissile bond squeezing the zinc-bound water molecule against the catalytic glutamate residue, and with the P1′ sidechain extending into the S1′ pocket (Figure 9). The complex has been energy-minimized using X-PLOR [71] to avoid steric clashes.

Upstream of the bound Pro–Gln–Gly segment, another 1½ Pro–Hyp–Gly repeats stay in close contact to the enzyme, making van der Waals but possibly also a few polar interactions with the sIV–sV loop of the catalytic domain (for nomenclature of secondary structure elements of the catalytic and hemopexin-like domain see [33,70], respectively). The residual 3½ Pro–Hyp–Gly repeats required for stabilization of the triple-helical fold protrude into bulk water. Downstream of the scissile bond, the rod-like substrate nestles against the catalytic domain before extending towards the adjacent hemopexin-like domain. The disulfide knot and the carboxy-terminal end are in

Figure 9



Stereoview of the truncated heterotrimer D docked to porcine MMP-1. The heterotrimer (white stick model, with nitrogen, oxygen and sulfur atoms shown in blue, red and green) lacks the first two Pro–Hyp–Gly repeats at its amino terminus (towards the left). The enzyme is represented as a solid surface, in red and blue according to its negative and positive electrostatic surface potential, respectively (GRASP [90]). The catalytic domain (CD, top) is oriented in such a manner that the substrate runs across the catalytic zinc (pink sphere) along the active-site cleft from left to right, with the scissible α1 strand slightly pulled out of the triple-helical association to make optimal substrate-like interactions with cleft residues. The carboxy-terminal part (right) of the bound heterotrimer D extends into a surface depression of the hemopexin-like domain (HLD, bottom) found between blades I (bI) and II (bII). The blue surface spot indicates the position of the surface-located guanidino group of Arg291. The surface hole (to the right centre in HLD) represents the exit side of HLD's central tunnel.

close contact with sidechains of the projecting s3–s4 loop (around Gln354) of propeller blade II, whereas the Gln–Arg–Gly–Val–Val–Gly segments of the substrate are placed just within a shallow depression of the exit surface of the hemopexin-like domain formed between blades I and II.

The heterotrimer D was also docked to the active site of MMP-2 [37]. Similar close contacts and interactions are possible with the catalytic and hemopexin-like domain without creating clashes with the third fibronectin type-II subdomain. The general picture is surprisingly similar to that of the modelled heterotrimer-D–MMP-1 complex. MMP-2 has an additional cleft based on the first and flanked by the second and third fibronectin type II subdomains, and extending to the β-sheet surface of the catalytic domain, aside the S-shaped double loop. This groove, which does not cross the active-site cleft, could represent a second binding site for both triple-helical and single-stranded substrates.

Discussion

There is a general consensus that rod-shaped collagen is digested by collagenases and gelatin by gelatinases, but degradation of fibrillar and acetic acid-soluble type I collagen by MMP-2 has also been reported [14]. With the unfolded heterotrimer A and the triple-helical heterotrimer D as mimics of gelatin and collagen, respectively, at room temperature, this issue was re-examined. Although the gelatin-like trimer A is readily digested by the gelatinase MMP-2, it was found to be highly resistant to proteolysis by MMP-1 and MMP-8. Conversely, the triple-helical trimer D is degraded by MMP-1 and MMP-8 at rates typical for collagenases, but its digestion by MMP-2 occurs with remarkably reduced efficiency. These results would indicate that gelatinases *per se* are capable of processing triple-helical substrates. The low rates observed with the synthetic mimic of collagen type I would not be physiologically relevant, however. Moreover, the proteolytic process involved differs considerably from that of the collagenases.

The mechanism of substrate digestion by gelatinase

HPLC analyses of the product distribution upon digestion of the gelatin-like substrate A by MMP-2 clearly revealed that this unfolded heterotrimer is degraded at rates comparable to those of substrate digestion by other proteinases and by a standard mechanism that consists of binding and processing of single peptide chains which satisfy the selectivity requirements of the S and S′ subsites of the substrate-binding cleft (Figure 6). The unfolded substrate A is also degraded by the catalytic domain of MMP-8, but with remarkably reduced efficiency. Similarly, the MMP-2 mutant that lacks the three fibronectin type-II modules, MMP-2($\Delta_{V191–Q364}$), degrades the heterotrimer A at rates comparable to those of the catalytic domain of MMP-8 and therefore at significantly lower rates than those of the wild-type enzyme, as previously reported using gelatin as substrate [55]. Thus the fibronectin type-II modules present in MMP-2 play an essential role in processing gelatin. In fact, BIAcore experiments showed a high affinity of the fibronectin subdomains for single-chain collagenous peptides as well as for the unfolded trimer, which suggests an exosite binding of these domains to the large network of the gelatin chains. This would sensibly increase the stability of the enzyme–substrate complex through bivalent substrate binding. For heterotrimer A, a similar interaction with the freely fluctuating three chains cross-linked at the carboxyl terminus can be envisaged. The X-ray structure of pro-MMP-2 [37]

reveals a large groove extending from the fibronectin-like domain towards the catalytic domain as a putative exosite binding cleft. Such a bivalent binding of substrate A, which is not possible with the catalytic domain of MMP-8 and with the mutant MMP-2($\Delta_{V191–Q364}$), could account for their low efficiency in processing the gelatin-like substrate compared with the full-length MMP-2.

MMP-2 degraded the triple-helical substrate D with release of partially digested intermediates, a mechanism similar to that observed for the unfolded substrate A. From the X-ray structure of MMP-2 [37] and modelling experiments, it can be seen clearly that the triple-helical substrate can be docked to the active-site cleft without steric clashes, in a mode similar to that shown in Figure 9 for the MMP-1–trimer-D complex. This type of binding would lead to interactions with the protein surface of both the catalytic and the hemopexin-like domain. In the modelled complex, the fibronectin-type-II modules, in particular the third subunit, would not preclude binding of a rod-shaped substrate. The carboxy-terminal hemopexin domain would act as a 'helping hand' in terms of additional binding or orienting the substrate (as discussed below for the collagenases), whereas the role of the fibronectin domain that has high affinity for the folded substrate remains unclear. These domains might serve more like a 'sticky hand' to accumulate gelatinases on partially processed and denatured collagen components of the extracellular matrix, as previously proposed [62].

The mechanism of substrate digestion by collagenases

The collagenases degrade the triple-helical substrate with high specificity, and release of the products from the enzyme apparently occurs only when all three α chains are cleaved at the peptide bonds defined by the S and S′ subsite specificities. Multiple proteolytic cuts through substrates are known to occur in multicatalytic enzyme complexes such as the proteasome or tryptase, the recently resolved X-ray structures of which clearly reveal a geometric distribution of identical or of different active sites in their cavities [72,73]. For this type of enzyme, a multiple alignment of the substrate to the different active sites followed by simultaneous or successive cleavage of the substrate with final release of the digestion mixture can be envisaged. Conversely, in the case of the collagenases, the multiple cut has to be performed by a single active site of the enzyme.

A tight triple-helical structure distal to the collagenase cleavage site and a loose conformational state around the cleavage site of type I collagen were proposed, although experimental evidence for a less constrained conformational space in this portion of native collagen was not available [17,58]. This hypothetical mode of substrate binding was supported by the inhibitory effect observed with a triple-helical $(Gly–Pro–Hyp)_n$-homotrimer cross-linked at the carboxyl terminus by a di-lysine moiety [46]. Our conformational analysis of the heterotrimer D yielded strong evidence for a triple-helical fold extending from the carboxyl to the amino terminus, with a cooperative two-state thermal transition from the folded to the unfolded heterotrimer [52]. This would also be in agreement with the recently solved X-ray structure of a homotrimeric collagen peptide containing the sequence 785–796 of collagen type III, which corresponds to the site cleaved by collagenases [74]. This homotrimer is folded into a rod-like triple helix, although the cleavage site itself contains various non-imino acids.

As the heterotrimer D fulfills the requirements of a specific substrate of collagenases, it was docked in the triple-helical structure to the known X-ray structure of full-length MMP-1 [36]. As shown in Figure 8, both upstream and downstream from the scissile Gly–Ile bond there are contacts of one peptide chain to the active-site cleft and the carboxyl terminus interacts with the hemopexin-like domain as well. By means of a slight (few degrees) upwards movement of the hemopexin-like domain along the contacting edge between the domains and/or a slight rotation/bending of the carboxy-terminal substrate portion, these intermolecular interactions would be intensified. In particular, the projecting guanidino groups of Arg291 (s1→s2, blade I) and Arg304 (central s3, blade I) could favorably interact with carbonyl groups extending from the substrate, in this way contributing to its further unfolding and facilitating adaptation to the active site. Such a putative interaction mode would indicate a large and continuous collagen-binding surface in which the hemopexin-like domain could play the role of a 'helping hand'. However, a reasonable explanation for the successive cleavage of the three chains without intermediate release of the partially digested substrate cannot be derived from this model, as it is surprisingly similar to the structural model obtained by docking the trimer D to MMP-2, although MMP-2 was shown to degrade the trimer D in successive steps. An upward movement of the hemopexin-like domain for trapping the collagen molecule between the two subunits was previously proposed [40,75,76]. In this movement the linker peptide could play an important collagenase/gelatinase discriminating role, as the results of mutational studies in the linker region suggest [41–44]. Such movement of the hemopexin-like domain seems not to be impeded in MMP-2, however, and therefore can not be advocated for the distinct mechanisms of proteolysis.

As an alternative hypothesis, a dimerization of the collagenases, hence entrapping of the triple-helical substrates could be envisaged, whilst this process could be impeded in the gelatinase by steric clashes with the fibronectin domains. With this speculative enzyme dimer model, a concomitant digestion of at least two chains by the two

adjacent active sites would be possible. Such a model, however, would raise the question of how collagenases could dimerize on fibrillar collagen. An answer to these questions and a better insight into the mechanism of the single-cut digestion of collagen by collagenases can only be expected from X-ray analysis of a collagenase–triple-helical substrate complex. Even with this technique, however, only a static picture of the substrate-binding mode would be accessible and not the full dynamic of the concerted rotational motion of the enzyme-trapped molecule for successive alignment of the chains to the active sites.

Significance

Synthetic heterotrimeric substrates cross-linked with a cystine knot offer great advantages over the natural collagen substrate. Their triple-helix stability can be designed to mimic both gelatin and collagen, the compounds are easy to handle and monitoring of their enzymatic digestion with collagenases or gelatinases is readily obtained by using high-performance liquid chromatography. Such properties allowed the identification of two distinct proteolytic mechanisms for the two classes of matrix metalloproteinases (MMPs). Moreover, with the selective immobilization of these folded and unfolded substrates on sensor chips, the binding affinities of inactive mutants of the enzymes and their subunits were determined and the combined set of new data provided a better understanding of the differentiated modes of action of these MMPs. Docking experiments with the substrates and the known X-ray structures of the collagenase MMP-1 and gelatinase MMP-2 allowed models of their differentiated proteolytic substrate digestion to be proposed as working hypothesis for further studies of the unique mode of proteolysis of fibrillar or water-soluble interstitial collagens by collagenases.

Materials and methods

Materials

All reagents and solvents used in the synthesis were of the highest quality commercially available. Amino acid derivatives were purchased from Alexis (Grünberg, Germany) or were prepared according to standard protocols; Tentagel-S-PHB-resin was from Rapp (Tübingen, Germany). Analytical HPLC was carried out on a Waters 717 HPLC system (Eschborn, Germany) equipped with a photodiode array using Nucleosil 300/C8 columns (Machery & Nagel, Düren) and eluting with a linear gradient of acetonitrile/2% H_3PO_4 from 5:95 to 80:20 in 30 min. ESI–MS was performed on PE Sciex API 165 (Perkin Elmer) and amino acid analyses of the acid hydrolysates (6 M HCl containing 2.5% thioglycolic acid, 110°C; 72 h) on a Biotronic analyzer (LC 6001).

Trypsin and BPTI were purchased from Boehringer Mannheim (Roche Diagnostics) and *p*-aminophenyl-mercuric acetate (APMA) from Sigma. The catalytic domain of MMP-8 was a gift from Boehringer-Mannheim (Roche Diagnostics). The expression and isolation of the MMPs and inhibitors used in the present study were reported elsewhere: pro-MMP-2 [77], pro-MMP-2(E375A) with an alanine residue replacing Glu375 [78], pro-MMP-2($\Delta_{V191-Q364}$) with deleted fibronectin domain [55], MMP-2/CT1847 [79], pro-MMP-8 [79], pro-MMP-2/TIMP-2 [80], catalytic domain of MMP-3 and full-length MMP-3 [81]. Pro-MMP-1 and pro-MMP-1(E200A) with a substitution of alanine for Glu200 were expressed as inclusion bodies in *E. coli* using the pET3a vector and refolded and purified as described previously [81]. Pro-MMP-1 exhibited a similar specific activity on collagen as the natural pro-MMP-1 isolated from human rheumatoid synovium when activated with APMA. The hemopexin domains of MMP-1 and MMP-2 were isolated by gelfiltration on Sephacryl S-200 after treating pro-MMP-1 and pro-MMP-2 with 1 mM APMA for 24 h at 37°C. Both hemopexin domains were homogeneous on SDS–PAGE. Expression and isolation of the fibronectin domain (R190–Y366) was reported elsewhere [82].

Peptide synthesis

The syntheses of the heterotrimers A and D were reported previously [52,83]. The linker peptides of MMP-1, MMP-2, MMP-3 and MMP-8 were synthesized by standard protocols according to Fmoc/tBu chemistry. Cleavage from the resin and deprotection was performed with trifluoroacetic acid/triethylsilane/water (95:3:2) in 1.5 h and purification of the peptides was achieved using HPLC on Nucleosil 5 C18 PPN with a linear gradient of acetonitrile (0.08% TFA)/0.1% TFA from 15:85 to 60:40 in 70 min.

H-Arg–Ser–Gln–Asn–Pro–Val–Gln–Pro–Ile–Gly–Pro–Gln–Thr–Pro–Lys–Ala-OH (MMP-1): 40% yield; HPLC: $t_R = 10.20$ min; ESI-MS: $m/z = 1699.9$ $[M]^+$; calcd. 1698.8; amino acid analysis: Asp 1.03 (1), Thr 0.97 (1), Ser 0.94 (1), Glu 3.00 (3), Pro 3.57 (4), Gly 1.05 (1), Ala 0.98 (1), Val 0.99 (1), Ile 0.99 (1), Lys 0.99 (1), Arg 1.00 (1); peptide content: 67.9 %.

H-Leu–Gly–Thr–Gly–Pro–Thr–Pro–Thr–Leu–Gly–Pro–Val–Thr–Pro–Glu–Ile-OH (MMP-2): 30% yield; HPLC: $t_R = 14.63$ min; ESI-MS: $m/z = 1548.8$ $[M]^+$; calcd. 1547.8; amino acid analysis: Thr 3.83 (4), Glu 1.05 (1), Pro 3.61 (4), Gly 2.96 (3), Val 1.00, Ile 1.03 (1), Leu 1.98 (1); peptide content: 75.5 %.

H-Leu–Val–Pro–Thr–Glu–Pro–Val–Pro–Pro–Glu–Pro–Gly–Thr–Pro–Ala–Asn-OH (MMP-3): 43% yield; HPLC: $t_R = 11.65$ min; ESI-MS: $m/z = 1596.8$ $[M]^+$; calcd. 1595.7; amino acid analysis: Asp 1.00, Thr 1.88 (2), Glu 2.03 (2), Pro 5.24 (5), Gly 1.06 (1), Ala 0.99 (1), Val 1.84 (1), Leu 1.00 (1); peptide content: 74.5%.

H-Leu–Ser–Ser–Asn–Pro–Ile–Gln–Pro–Thr–Gly–Pro–Ser–Thr–Pro–Lys–Pro-OH (MMP-8): 30% yield; HPLC: $t_R = 10.77$ min; ESI-MS: $m/z = 1602.8$ $[M]^+$; calc'd 1601.6; amino acid analysis: Asp 0.95 (1), Thr 1.90 (2), Ser 2.82 (3), Glu 1.00, Pro 4.38 (4), Gly 1.04 (1), Ile 0.98 (1), Leu 1.02 (1), Lys 0.98 (1); peptide content: 68.02 %.

Circular dichroism

CD spectra were recorded on a Yobin–Yvon dichrograph Mark IV equipped with a thermostated cell holder and connected to a data station for signal averaging and processing. All spectra are averages of ten scans and the spectra were recorded employing quartz cells of 0.1 cm optical path length. The concentrations were determined by weight and peptide content as determined by quantitative amino acid analysis of the peptides. Solutions of the peptides (0.2 mg/ml) were prepared in the collagenase assay buffer (10 mM $CaCl_2$, 50 mM NaCl, 50 mM Tris/HCl, pH 7.4). The Rpn factor as index of triple-helical conformation of collagenous peptides [84,85] was determined by the ratio of the dichroic intensity of the positive band over the dichroic intensity of the negative band.

Activation of the MMPs

Pro-MMP-1 and pro-MMP-1(E200A) were converted to MMP-1 and MMP-1(E200A) essentially as described by Suzuki *et al.* [86]. The latent enzymes were incubated in 10 mM $CaCl_2$, 50 mM NaCl, 50 mM Tris/HCl (pH 7.4) with 0.02 equivalents of the catalytic domain of MMP-3 and 0.2 mM APMA at 25°C for 15 h. Pro-MMP-2 and pro-MMP-3 were activated according to Murphy *et al.* [77] by incubation in 10 mM $CaCl_2$, 50 mM NaCl, 50 mM Tris/HCl (pH 7.4) with 1 mM APMA at 25°C for 1 h. Pro-MMP-8 was activated as described

by Knäuper *et al.* [87] by incubation in 10 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris/HCl (pH 7.5) with 0.05 equivalents of trypsin at 37°C for 2 h; then five equivalents (relative to trypsin) of BPTI were added. Concentrations of the stock solutions of activated enzymes were determined according to Bradford [88].

Enzyme assays

To a 50 µM solution of the collagen peptides in 10 mM $CaCl_2$, 50 mM NaCl, 50 mM Tris/HCl (pH 7.4) at 25°C aliquots of the stock solutions of the MMPs were added to reach a concentration of 50 nM. At time intervals 20 µl aliquots of the assay-solution were taken and quenched with 15 µl of 40% aqueous H_3PO_4. The quenched probes were analyzed by HPLC on Nucleosil 300/C8 using a linear gradient of acetonitrile/2% H_3PO_4 from 5:95 to 80:20 in 30 min.

Surface plasmon resonance

The heterotrimers A and D and the single-chain Cys(StBu)-protected peptide α1′(B) were immobilized each on one flow-cell of the sensorchip CM5 (BIAcore) according to known procedures [89]. The cells were activated with 35 µl of an aqueous solution of EDCI (0.4 M)/HOSu (0.1 M), 1:1, at a flow rate of 5 µl/min. Then 20 µl aliquots of the respective peptide solution (3 mg/ml in 10 mM sodium acetate, pH 4.0) were injected into the single cells of the sensorchip to reach the desired loading; with repeated injections of octyl-β-glucopyranoside detergent (30 µl aliquots of a 40 mM aqueous solution) at a flow rate of 50 µl/min noncovalently bound peptide was eluted. Residual activated matrix carboxyl functions in the four cells were inactivated with ethanolamine (35 µl of a 1 M solution) at a flow rate of 5 µl/min. Different peptide loadings (400–4000 RU) were not found to affect the interaction analysis. The fourth flow cell which was not loaded with peptide served as control for unspecific binding.

The analyte probes were dissolved in MMP-assay buffer (10 mM $CaCl_2$, 50 mM NaCl, 50 mM Tris.HCl; ph 7.4) at concentrations varying from 0.1 to 10 µM for the enzymes and from 0.1 to 20 µM for fibronectin. The binding assays were performed on a Biosensor BIAcore 2000 (BIAcore, Uppsala, Sweden) injecting 35 µl protein analyte with the Kinject-mode and eluting at flow rates of 20 µl/min with the buffer 10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20 (pH 7.4). Binding experiments were performed at 12°C and 22°C and after each cycle the residual bound analyte was removed with octyl-β-glucopyranoside (40 µl of a 40 mM solution) at 50 µl/min and the system was reequilibrated for 20 min at 20 µl/min with the eluting buffer for complete refolding of the peptides after denaturation with detergent. Evaluation of the interaction parameters was carried out with the BIAcore software Evaluation 3.0.

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