

Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis

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Breakdown of triple-helical interstitial collagens is essential in embryonic development, organ morphogenesis and tissue remodelling and repair. Aberrant collagenolysis may result in diseases such as arthritis, cancer, atherosclerosis, aneurysm and fibrosis. In vertebrates, it is initiated by collagenases belonging to the matrix metalloproteinase (MMP) family. The three-dimensional structure of a prototypic collagenase, MMP-1, indicates that the substrate-binding site of the enzyme is too narrow to accommodate triple-helical collagen. Here we report that collagenases bind and locally unwind the triple-helical structure before hydrolyzing the peptide bonds. Mutation of the catalytically essential residue Glu200 of MMP-1 to Ala resulted in a catalytically inactive enzyme, but in its presence noncollagenolytic proteinases digested collagen into typical 3/4 and 1/4 fragments, indicating that the MMP-1(E200A) mutant unwinds the triple-helical collagen. The study also shows that MMP-1 preferentially interacts with the $\alpha 2(I)$ chain of type I collagen and cleaves the three α chains in succession. Our results throw light on the basic mechanisms that control a wide range of biological and pathological processes associated with tissue remodelling.

The EMBO Journal (2004) 23, 3020–3030. doi:10.1038/sj.emboj.7600318; Published online 15 July 2004

Subject Categories: cell & tissue architecture; proteins

Keywords: collagenase; enzyme mechanism; matrix metalloproteinase; protein unfolding; triple helix

Introduction

Collagens are the major structural proteins of connective tissues such as skin, tendon, bone, cartilage, blood vessels and basement membranes. Interstitial collagens I, II and III are the most abundant and they provide the scaffolding of the tissue and guide cells to migrate, proliferate and differentiate.

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Received: 4 March 2004; accepted: 18 June 2004; published online: 15 July 2004

The degradation of these macromolecules is therefore an integral part of many biological processes such as embryogenesis, organ morphogenesis, tissue remodelling, angiogenesis and wound healing (Cawston, 1996; Woessner, 1998; Sternlicht and Werb, 2001). Recent studies have shown that collagenase-cleaved products of collagen I alter cellular activity by expressing cryptic biological functions: for example, activation and recruitment of osteoclasts during bone remodelling (Zhao *et al.*, 1999), epithelial cell migration during wound healing (Pilcher *et al.*, 1997) and apoptosis of amniotic fibroblasts at the term pregnancy (Lei *et al.*, 1996). Thus, collagenase does not simply function to degrade and remove collagen fibrils but also controls cellular behavior during tissue remodelling. Aberrant collagenolysis, on the other hand, is associated with progression of diseases such as arthritis, cancer, atherosclerosis, aneurysm and fibrosis (Woessner, 1998; Brinckerhoff and Matrisian, 2002).

Interstitial collagens consist of three α chains of approximately 1000 residues with repeating Gly-X-Y triplets, where X and Y are often proline and hydroxyproline, respectively. Because of the high imino-acid content and the tripeptide unit repeats, the α chain adopts a left-handed poly-Pro II-like helix, and three left-handed α chains intertwine with each other to form a right-handed superhelix (Ramachandran and Kartha, 1955; Rich and Crick, 1961; Fraser *et al.*, 1979; Kramer *et al.*, 2001). This triple-helical conformation makes interstitial collagens resistant to most proteinases. In vertebrates, enzymes that can cleave the triple-helical structure are collagenases (Visse and Nagase, 2003) and cathepsin K produced by osteoclasts (Garnero *et al.*, 1998). While cathepsin K cleaves collagen I in an acidic environment specialized primarily in bone resorption, all other collagenolytic enzymes act at neutral pH and are members of the matrix metalloproteinase (MMP) family, and are produced by many cell types including stromal cells, epithelial cells, macrophages and leukocytes (Sternlicht and Werb, 2001). The latter group includes collagenases (MMP-1, MMP-8, MMP-13 and MMP-18), MMP-2 (gelatinase A) (Aimes and Quigley, 1995; Patterson *et al.*, 2001) and membrane-type 1-MMP (MMP-14) (Ohuchi *et al.*, 1997). These MMPs cleave the three α chains of native triple-helical type I, II and III collagens after Gly in a particular sequence (Gln/Leu)-Gly#(Ile/Leu)-(Ala/Leu) (# indicates the bond cleaved) located approximately three quarters away from the N-terminus of the collagen molecule. The action of these enzymes is critical for the initiation of collagen breakdown, as once collagens are cleaved into 3/4 and 1/4 fragments they denature at body temperature and are degraded by gelatinases and other nonspecific tissue proteinases.

A typical collagenase is synthesized as a pre-proenzyme and secreted as an inactive proenzyme consisting of a pro-peptide, a catalytic domain, a short linker region rich in proline and a C-terminal hemopexin (Hpx) domain. Clark and Cawston (1989) first reported that the cleavage of triple-helical collagen by MMP-1 (collagenase 1) requires the C-terminal Hpx domain. The catalytic domain alone retains

proteolytic activities on noncollagenous proteins and peptides, but it fails to cleave collagen (Clark and Cawston, 1989; Murphy *et al*, 1992). The importance of the Hpx domain was also shown for other collagenolytic MMPs such as MMP-2 (Patterson *et al*, 2001), MMP-8 (Knäuper *et al*, 1993), MMP-13 (Knäuper *et al*, 1997) and MMP-14 (Ohuchi *et al*, 1997). Nevertheless, structurally similar MMP-3 (stromelysin 1) and MMP-10 (stromelysin 2) are unable to cleave collagen I or II (Woessner and Nagase, 2000). Replacement of the Hpx domain in MMP-3 with the Hpx of MMP-1 did not make MMP-3 collagenolytic, indicating that the Hpx is not the sole component responsible for the expression of collagenolytic activity (Murphy *et al*, 1992). The crystal structure of porcine MMP-1 indicated that the catalytic domain and the Hpx domain are tandemly connected through the linker peptide (Li *et al*, 1995), but understanding how the Hpx domain assists in the cleavage of collagen is elusive. Therefore, the structural basis for collagen-degrading specificity among certain members of MMPs is not clearly understood. An additional enigma is the mechanism by which collagenases cleave triple-helical collagens when the dimensions of the collagenase active site and the structure of interstitial collagens are considered (Bode, 1995). The substrate-binding site of MMP-1 forms a deep cleft with the catalytic zinc located at the bottom, and the entrance of this groove is only 5 Å wide, sufficient to accommodate only a single polypeptide chain. Type I collagen, on the other hand, consisting of two $\alpha 1$ (I) chains and one $\alpha 2$ (I) chain, is 3000 Å in length and 15 Å in diameter. Thus, the triple-helical collagen does not fit into the active site cleft of the enzyme. Our molecular docking attempts to place the triple-helical model of Kramer *et al* (2001) to the crystal structure of porcine MMP-1, indicated that the closest susceptible peptide bond is at least 7 Å away from the catalytic zinc atom (Figure 1). In addition, because each α chain forms a poly-Pro II-like helix, the spatial orientation of side chains in a single α chain is unique and dissimilar to that of a single-peptide chain substrate that fits in the substrate-binding cleft by forming β strand-like hydrogen bonds. This means that either the active site of MMP-1 undergoes large conformational changes or that the triple-helical collagen needs to be unwound so that a single α chain can fit into the active site of the enzyme.

In this report, using collagenase 1 (MMP-1) as a typical collagenase, we present evidence that collagenase locally unwinds triple-helical collagen before it hydrolyzes the peptide bonds. To our knowledge, this is the first demonstration that a single polypeptide proteinase induces significant conformational changes of the protein substrate before it cleaves specific peptide bonds.

Results

Unwound collagen chains are substrates of collagenase 1 (MMP-1)

It is generally considered that the triple-helical structure of collagen is critical for collagenases to cleave interstitial collagens (McCroskery *et al*, 1973). Based on the crystal structure of MMPs and our molecular modelling with a triple-helical peptide, we hypothesized that unwound collagen α chains, but not those in a triple-helical conformation, are the substrates of collagenases. We examined this possibility first by comparing the ability of MMP-1 to cleave native

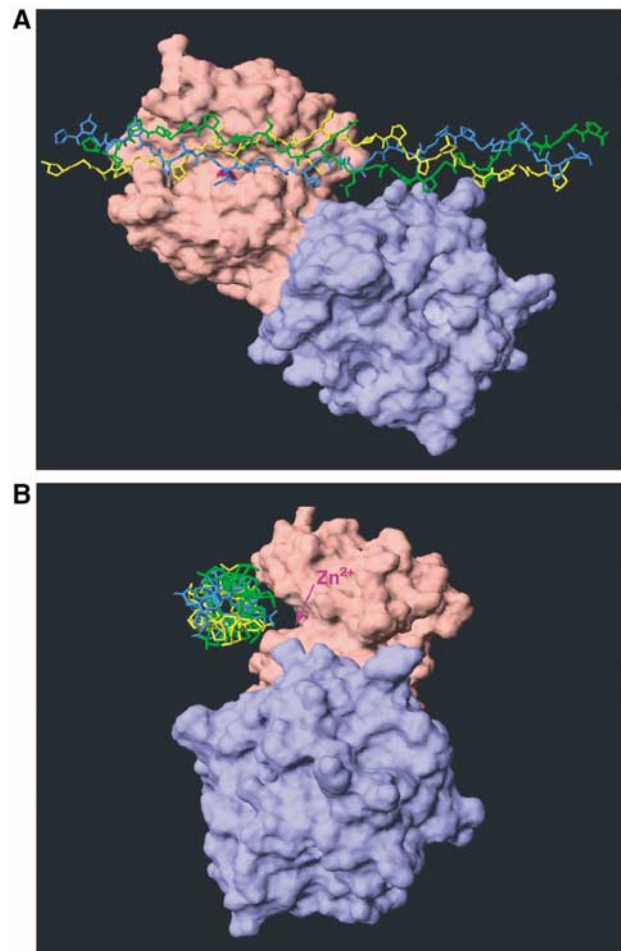


Figure 1 Alignment of the triple-helical peptide with the active site of MMP-1. (A) Collagen triple-helical peptides described by Kramer *et al* (2001) were manually aligned into the active site of the catalytic domain of porcine MMP-1 determined by Li *et al* (1995) using Insight II/Discover and the image was produced with Swiss PDB view (Guex and Peitsch, 1997). (B) The alignment model of MMP-1 and the triple-helical peptides in (A) were rotated 90° to the left. The location of the catalytic Zn^{2+} is indicated by an arrow. The active site shown as a cleft is unoccupied by the triple-helical peptide substrate. Pink, catalytic domain; blue, Hpx; purple, zinc ion.

collagen I and heat-denatured collagen I (gelatin I) at different temperatures. At 37°C, collagen I was readily cleaved into the typical three-quarter and one-quarter fragments (Figure 2A). Gelatin I was also cleaved in a similar manner, but was less susceptible. In contrast, when the reaction temperature was reduced, gelatin I was more susceptible to cleavage than collagen I: At 10 and 4°C little collagen I hydrolysis was observed, but the activity for gelatin I was retained (Figure 2C and D). NH₂-terminal sequencing of the 1/4 (TC^B) fragments generated from gelatin I by MMP-1 indicated that enzyme cleaved the Gly⁷⁷⁵-Ile⁷⁷⁶ bond of the $\alpha 1$ (I) chain and the Gly⁷⁷⁵-Leu⁷⁷⁶ bond of the $\alpha 2$ (I) chain, sites identical to those cleaved in native collagen I. These results indicate that the conformational state of the substrate significantly influences the activity of MMP-1 and that unwound collagen is a better substrate at temperatures lower than 25°C, conditions that decrease the backbone mobility of the triple-helical structure. Based on these observations, we

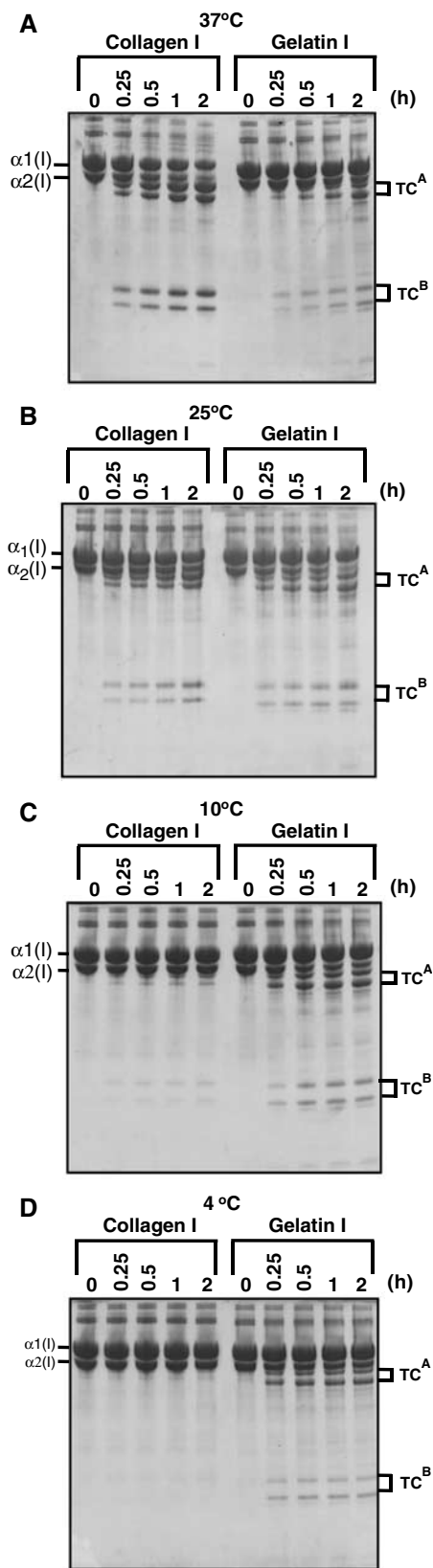


Figure 2 Digestion of collagen I and gelatin I by MMP-1. Collagen I (30 µg) and gelatin I (30 µg) were incubated with 6 nM (A, B) or 40 nM (C, D) active MMP-1 at various temperatures for up to 2 h. The reaction was terminated by addition of 20 mM EDTA and subjected to SDS-PAGE under reducing conditions. TC^A and TC^B are 3/4 fragments and 1/4 fragments of $\alpha 1(I)$ and $\alpha 2(I)$ chains, respectively.

considered that unwinding of the triple helices may be a prerequisite for collagenase to cleave interstitial collagens.

Demonstration of collagen unwinding by MMP-1

If MMP-1 has an ability to unwind collagen, such activity might be separated from the activity that hydrolyzes peptide bonds. To investigate this possibility, we mutated Glu200, the residue essential for peptide hydrolysis, to Ala. We postulated that such a mutant would locally unwind collagen upon interaction with collagen, but would not cleave peptide bonds, and that the unwound collagen would then be susceptible to cleavage by a noncollagenolytic enzyme.

As shown in Figure 3A, the MMP-1(E200A) mutant was essentially inactive and unable to cleave the $\alpha 1(I)$ and $\alpha 2(I)$ chains of collagen I. As demonstrated previously (Clark and Cawston, 1989; Murphy *et al*, 1992), the catalytic domain of MMP-1 lacking the C-terminal Hpx domain (MMP-1(Δ C)) also could not cleave collagen I, even at high concentrations of the enzyme, whereas it readily cleaved gelatin I (Figure 3B and C). However, when collagen I was incubated with MMP-1(E200A) and MMP-1(Δ C) at 25°C, it was cleaved into the typical 3/4 (TC^A) and 1/4 (TC^B) fragments in a manner dependent on the concentration of MMP-1(Δ C) (Figure 3D) and MMP-1(E200A) (data not shown). MMP-3(Δ C) lacking the Hpx domain, full-length MMP-3 (data not shown) and human leukocyte elastase (HLE), all of which are unable to cleave the triple-helical region of collagen I, also generated 3/4 and 1/4 fragments in the presence of MMP-1(E200A) (Figure 4). In the case of HLE, most MMP-1(E200A) was split into catalytic and Hpx domains during incubation, but it retained unwinding activity. NH₂-terminal sequencing of the TC^B fragments indicated that MMP-1(Δ C) and MMP-3 cleaved the Gly⁷⁷⁵-Ile⁷⁷⁶ bond of the $\alpha 1(I)$ chain and the Gly⁷⁷⁵-Leu⁷⁷⁶ bond of the $\alpha 2(I)$ chain in the presence of MMP-1(E200A) (Figure 5). HLE cleaved the Val⁷⁸³-Gly⁷⁸⁴ bond of the $\alpha 1(I)$ chain in the same locus. The HLE cleavage site of $\alpha 2(I)$ chain was not identified, but potential sites are found in the same region. Incubation of collagen I with inactive full-length MMP-3(E202A) and MMP-1(Δ C) did not result in collagen cleavage, indicating that MMP-3 lacks the ability to unwind collagen. From these results, we have concluded that MMP-1(E200A) unwinds the triple-helical collagen I upon binding.

To determine the equilibrium-binding constant of MMP-1(E200A) with guinea-pig collagen I, various concentrations of MMP-1(E200A) (1–6 µM) were incubated with collagen I in the presence of a very high concentration of a 'cutter' proteinase MMP-1(Δ C), under the conditions in which the rate of collagen cleavage was dependent on the concentration of MMP-1(E200A). This allowed us to determine that the K_D of MMP-1(E200A) for collagen I was 1.6 µM. This value is similar to the K_m reported by Welgus *et al* (1981) for human MMP-1 and guinea-pig collagen I. This indicates that the mode of MMP-1(E200A) interaction with collagen I is essentially the same as the native MMP-1.

Preferential interaction of MMP-1 with the $\alpha 2(I)$ chain and the requirement of the active site for collagen unwinding

It was notable that the $\alpha 1(I)$ chain was cleaved more rapidly by noncollagenolytic proteinases in the presence of MMP-1(E200A) compared with the active MMP-1 alone (compare Figures 3D and 4 with Figure 2A and B). This suggests that

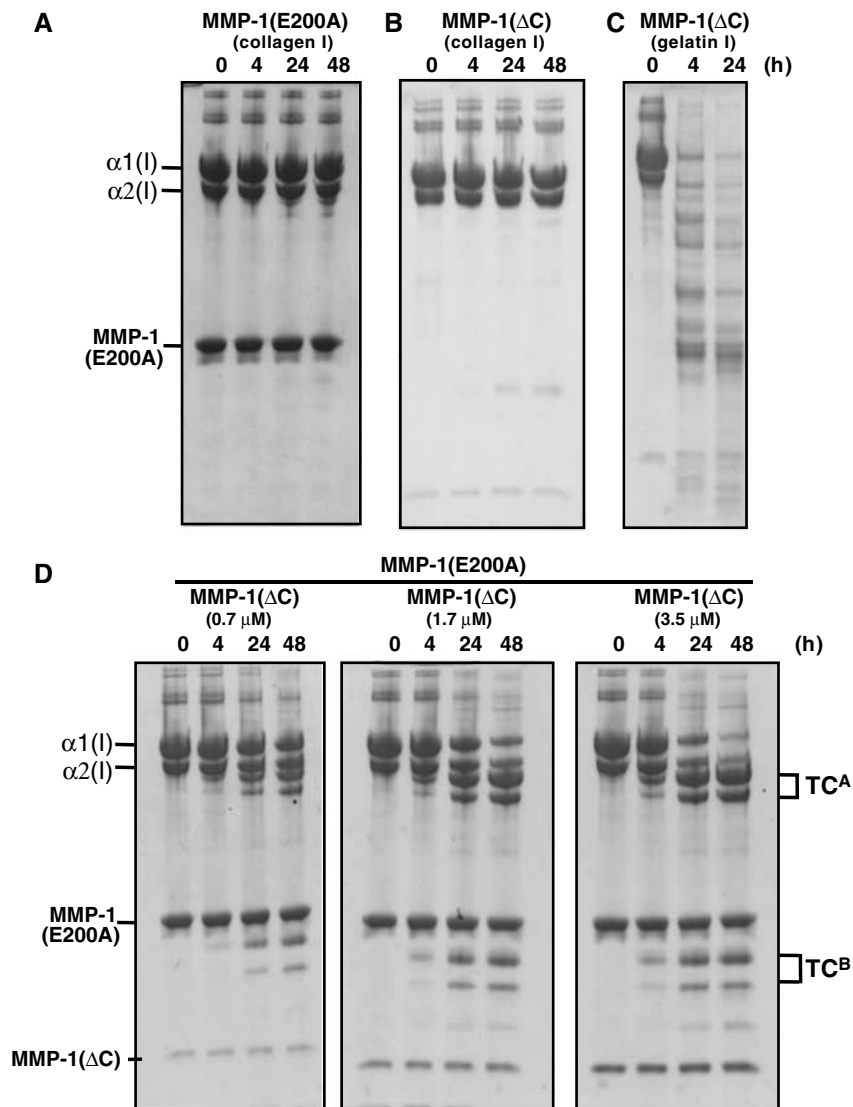


Figure 3 Digestion of collagen I in the presence of MMP-1(E200A) by MMP-1(Δ C). (A) Collagen I (30 μ g) was incubated with 6 μ M MMP-1(E200A) or (B) 0.7 μ M MMP-1(Δ C) at 25°C for the indicated time. (C) Gelatin I (30 μ g) was incubated with 0.7 μ M MMP-1(Δ C). (D) Collagen I (30 μ g) was made to react with an increasing amount of MMP-1(Δ C) in the presence of 6 μ M MMP-1(E200A). The reaction products were analyzed as in Figure 2.

the ‘unwinder’ MMP-1(E200A) preferentially interacts with the α 2(I) chain, which renders the α 1(I) chain more exposed and susceptible to a ‘cutter’ proteinase.

When MMP-1(E200A) and collagen were first incubated with 10 μ M GM6001X, an active site-directed synthetic MMP inhibitor, and then made to react with the serine protease HLE, no collagen digestion was detected (Figure 6). This indicates that the unwinding activity of MMP-1(E200A) requires an unoccupied active site which presumably accommodates the α 2(I) chain.

MMP-1 unwinds collagen I only locally

To examine the extent of collagen unwinding by MMP-1, we measured the melting temperature, T_m , of guinea-pig collagen I in the absence and presence of MMP-1(E200A) or MMP-3(E202A). MMP-3(E202A) served as control since it does not unwind collagen I. As shown in Figure 7, there was no significant change in T_m (\sim 40°C) even in the presence of

MMP-1(E200A) or MMP-3(E202A). This suggests that the unwinding of collagen by MMP-1 takes place only locally, and it does not affect the overall triple-helical structure. To confirm this, we employed the property of chymotrypsin, which cleaves denatured collagens but not the native triple-helical collagens. As shown in Figure 8, chymotrypsin rapidly cleaved gelatin I into small fragments, but not collagen I. Incubation of collagen I with active MMP-1 at 25°C in the presence or absence of chymotrypsin generated the typical collagenase fragments, which are indistinguishable from those generated by collagenase alone. These results indicate that MMP-1 unwinds collagen only locally at the site where the collagenolytic cleavage takes place.

Reassociation of the catalytic domain and the Hpx domain of MMP-1 cleaves collagen I

The study with HLE showed that collagen-unwinding activity was retained even when MMP-1(E200A) was split into the

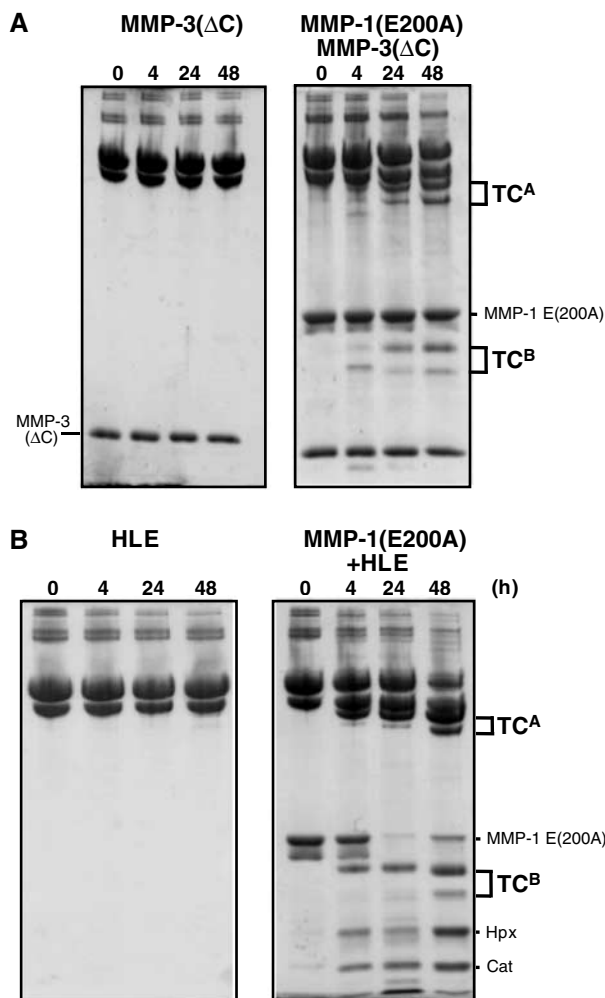


Figure 4 Digestion of collagen I by MMP-3(Δ C) or HLE in the presence of MMP-1(E200A). Collagen I (30 μ g) was incubated with (A) 7.6 μ M MMP-3(Δ C) or (B) 0.4 μ M HLE in the presence of 6 μ M MMP-1(E200A) at 25°C for up to 48 h. The reactions were terminated with 20 mM EDTA for MMP-3(Δ C) and 1 mM phenylmethylsulfonyl fluoride for HLE and the products were analyzed as in Figure 2.

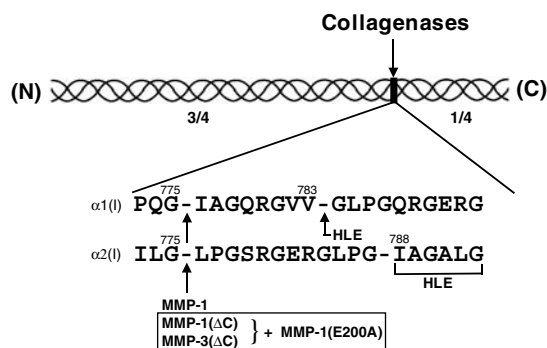


Figure 5 Cleavage sites in collagen I by MMP-1(Δ C), MMP-3(Δ C) and HLE detected in the presence of MMP-1(E200A). The N-terminal amino-acid sequence of the TC^B fragments of α 1(I) and α 2(I) chain was determined as described in Materials and methods. In the case of HLE, only the α 1(I) chain fragment was determined. The residues in the α 2(I) chain indicated by brackets are the predicted cleavage sites of HLE based on enzyme specificity.

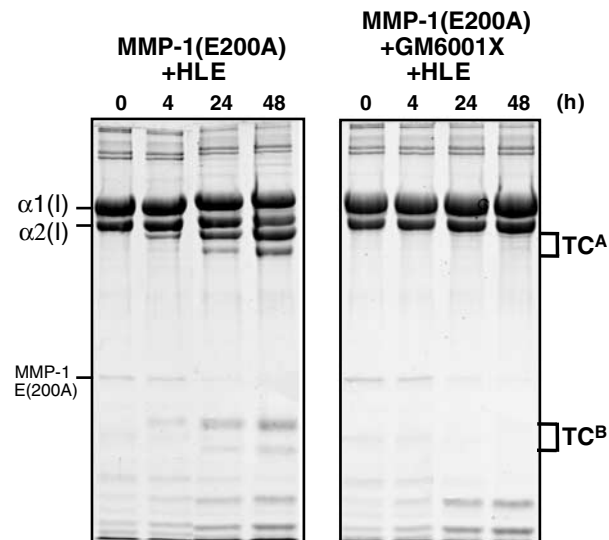


Figure 6 MMP-1(E200A) bound to GM6001X is unable to unwind collagen I. Collagen I (30 μ g) was incubated with 2.5 μ M MMP-1(E200A) and 0.4 μ M HLE in the absence or presence of 10 μ M GM6001X at 25°C for up to 48 h. The reaction of MMP-1(Δ C) and HLE was terminated by 1 mM phenylmethylsulfonyl fluoride and the products were analyzed as in Figure 4.

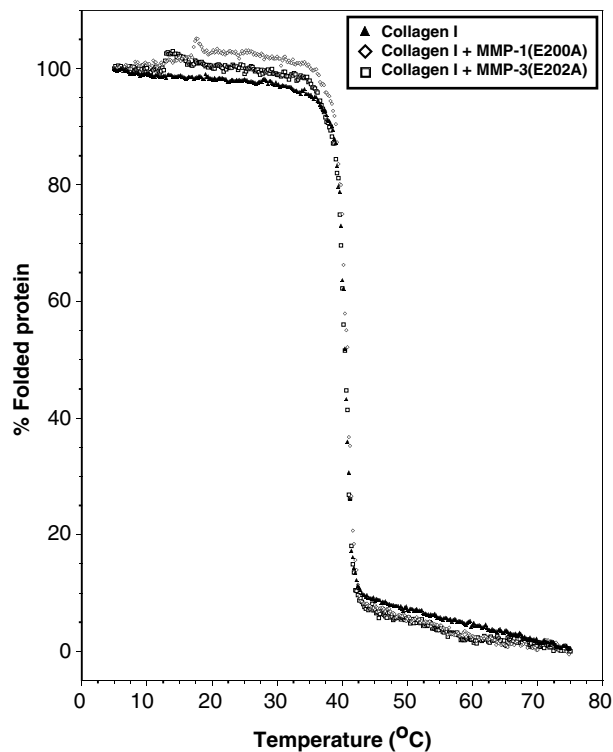


Figure 7 Thermal transition curve for guinea-pig type I collagen with or without MMP-1(E200A). Pepsin-treated guinea-pig type I collagen (3 μ M) was incubated with or without an equimolar concentration of MMP-1(E200A) or MMP-3(E202A), and molar ellipticities were recorded at 222 nm while the temperature increased from 5 to 70°C at a rate of 35°C/h.

catalytic domain and the Hpx domain (see Figure 4B), suggesting that the combination of MMP-1(Δ C) and the Hpx domain of MMP-1(Hpx_{MMP-1}) can unwind and cleave col-

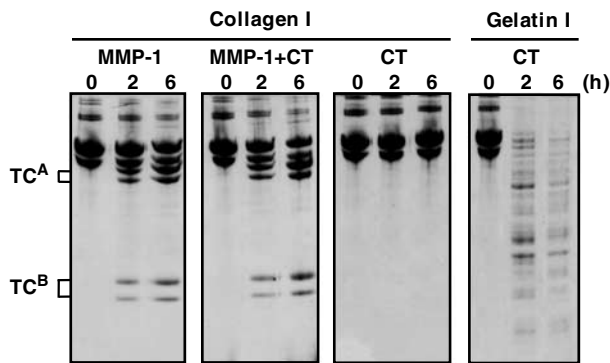


Figure 8 Unwinding of collagen by MMP-1 occurs only locally. Collagen I (30 μ g) was incubated with 0.05 μ M MMP-1 with or without chymotrypsin (CT) (20 μ g/ml) at 25°C for up to 6 h. CT alone was incubated with collagen I and gelatin I as controls.

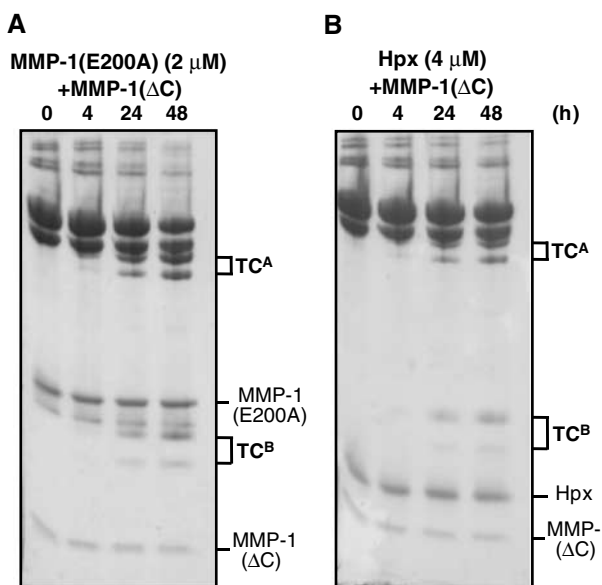


Figure 9 Collagenolytic activity expressed by reassociation of the catalytic domain and the Hpx domain. Collagen I (30 μ g) was made to react with (A) 2 μ M MMP-1(E200A) and 0.8 μ M MMP-1(Δ C), and (B) 4 μ M Hpx_{MMP-1} and 0.8 μ M MMP-1(Δ C) at 25°C for the indicated period of time. The reactions were terminated with 20 mM EDTA and the products were analyzed as in Figure 2.

lagen. This was confirmed by adding MMP-1(Δ C) to the isolated Hpx_{MMP-1} domain (data not shown). We then compared the collagen-unwinding ability of MMP-1(E200A) and Hpx_{MMP-1} by incubating collagen I with various concentrations of an 'unwinder' component (MMP-1(E200A) or Hpx_{MMP-1}) at various concentrations in the presence of a constant amount of a 'cutter' MMP-1(Δ C). Figure 9 shows that 2 μ M MMP-1(E200A) is approximately two-fold more effective than 4 μ M Hpx_{MMP-1}. A similar level of collagenolysis was observed with only 0.01 μ M of the full-length wild-type MMP-1 (data not shown). The combination of Hpx_{MMP-1} and MMP-3(Δ C) did not cleave collagen I, suggesting that the correct pairing of the MMP-1 catalytic domain and the Hpx domain is essential for collagenolysis. The requirement of higher concentrations of the unwinder and the cutter to cleave collagen suggests that both components must simultaneously bind to the collagen substrate. In the case of

Hpx_{MMP-1} and MMP-1(Δ C), the ratio of the α 1(I) to α 2(I) chain cleavage products was similar to that of full-length MMP-1, suggesting that together they behave like a full-length collagenase most likely by associating with collagen in a similar manner. The high efficiency of the active full-length collagenase is due to the fact that the two essential elements are linked in a single molecule, thus benefiting from entropic contributions.

Cleavage of a single α chain during collagenolysis

We investigated the steps involved in collagenolysis by taking advantage of the unwinding ability of MMP-1(E200A) and the peptide hydrolysis activity of MMP-1(Δ C). We employed nonpepsin-treated collagen I, which retains β components consisting of two crosslinked α chains (α 1(I)- α 1(I) and α 1(I)- α 2(I) crosslinks in a 1:2 ratio) and γ components consisting of three crosslinked α chains (α 1(I)- α 1(I)- α 2(I) crosslinks) through the noncollagenous telopeptide regions. Cleavage of one or two of these chains results in intermediate products containing cleaved α chains and intact α chains. When the nonpepsin-treated collagen I was incubated with MMP-1 and products were analyzed by SDS-PAGE with 5% total acrylamide, the crosslinked 3/4 fragments β^A and γ^A were observed, and there were no obvious intermediates (Figure 10A). On the other hand, digestion with MMP-1(Δ C) or MMP-3(Δ C) in the presence of MMP-1(E200A) did clearly show intermediate products (I_1 , I_2 , I_3 and I_4) that are larger than the β^A and γ^A products (Figure 10B). Intermediate I_3 is likely to be a cleaved α 1(I) chain crosslinked to an intact α 1(I) through N-terminal telopeptides, and I_4 is a product resulting from cleavage of either α 1(I) or α 2(I) chain of the α 1(I)- α 2(I) crosslinks. Intermediates I_1 and I_2 of γ chains are products resulting from cleavage of one and two of the three α chains, respectively. The detection of these intermediates was possible, probably because simultaneous binding of an unwinder and a cutter is required to cleave collagen and the dissociation of

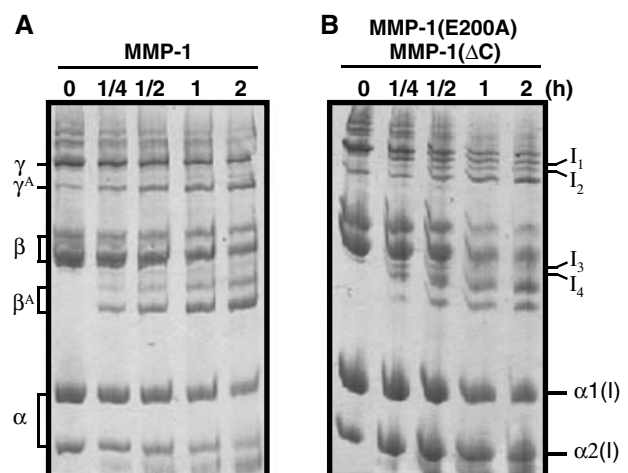


Figure 10 Detection of intermediate products during collagenolysis. Nonpepsin-treated collagen I was made to react with (A) 0.1 μ M full-length collagenase or (B) 0.8 μ M MMP-1(Δ C) and 6 μ M MMP-1(E200A). The reactions were stopped with 20 mM EDTA and the products were analyzed by SDS-PAGE with 5% total acrylamide under reducing conditions. γ^A and β^A are the 3/4 fragments of γ and β chains generated by MMP-1. I_1 , I_2 , I_3 and I_4 indicate intermediate products.

either one of these from the collagen interrupts the process. The active MMP-1, on the other hand, possesses both components in one molecule and therefore the three chains are cleaved more effectively at a rate faster than the detection method used to identify the intermediate products.

Discussion

Substrate specificities of proteinases are normally dictated by a short stretch of amino-acid sequence and local secondary and tertiary structures of protein substrates. The regions susceptible to proteinases are usually exposed on the surface of molecules and they are often flexible, so that the scissile bond can readily be accommodated within the active site of the enzyme. Such flexibility may be an intrinsic property of the protein or influenced by post-translational modification, such as oxidation (Mehlhase and Grune, 2002), phosphorylation (Hershko and Ciechanover, 1998), ubiquitination (Hershko and Ciechanover, 1998) and proteolysis (Murphy *et al*, 1985; Nagase *et al*, 1990; Rawson, 2003; Haass, 2004). The interstitial collagens are long triple-helical structures consisting of three left-handed poly-Pro II-like helices stabilized by hydrogen bonds formed among the backbones of three α chains and they are highly resistant to most proteinases. In this report, we have demonstrated that in order for a collagenase to cleave collagen it first binds and then unwinds the rigid triple-helical substrate before it cleaves the Gly⁷⁷⁵-Ile⁷⁷⁶ and Gly⁷⁷⁵-Leu⁷⁷⁶ bonds of collagen I. As far as we are aware, this is the first demonstration that a single polypeptide proteinase induces significant structural changes in the substrate prior to peptide bond hydrolysis. For the large multi-subunit 26S proteasome, unfolding and ubiquitination of proteins are considered to be essential to digest proteins as the entrance of this complex is restricted (Voges *et al*, 1999). This reaction is accompanied by ATP hydrolysis (Voges *et al*, 1999; Goldberg, 2003), whereas collagenase activity does not require ATP. Owing to the structural constraint between collagenase and the collagen substrate, several hypotheses have been proposed to explain how collagenase may act on triple-helical collagens (Bode, 1995; de Souza *et al*, 1996; Gomis-Rüth *et al*, 1996; Otl *et al*, 2000; Overall, 2002). This includes: the 'proline zipper' model by de Souza *et al* (1996), proposing that the proline-rich linker region of collagenases interacts with and unwinds the triple-helical collagen, and a 'collagen-trapping' model in which the Hpx domain folds over the catalytic site sandwiching collagen (Bode, 1995; Gomis-Rüth *et al*, 1996). However, the intact linker region may not be necessary as the catalytic domain and the Hpx domain added together can cleave collagen. The collagen-trapping model is also inconsistent with our observation that noncollagenolytic proteinases can cleave $\alpha 1(I)$ and $\alpha 2(I)$ chains in the presence of MMP-1(E200A), whereas in the model they would be protected by the Hpx domain. We also considered the following two other possible mechanisms: (i) collagenase stabilizes the partially unwound state of collagen that may occur spontaneously around the collagenase-susceptible region; and (ii) conformational changes occur within the collagenase molecule in such a way that it accommodates the triple-helical collagen in the active site. These two possibilities are also inconsistent with our results. If collagen underwent spontaneous unwinding, then such a conformational state would be recognized and cleaved by MMP-1(ΔC),

and even by MMP-3(ΔC) or HLE, although the rate of these reactions could be much slower compared to full-length MMP-1. However, even with a very high concentration (3–7 μM) of MMP-1(ΔC) or MMP-3(ΔC), no collagenolytic activity was detected. It is also unlikely that the induction of a conformational change in the collagenase by collagen is the mechanism. If this were the case, MMP-1(ΔC), MMP-3(ΔC) and HLE would not be able to cleave collagen I in the presence of MMP-1(E200A), because the triple-helical collagen would be in the native conformation. Our experimental results support a mechanism in which collagenase locally unwinds the triple-helical collagen before it hydrolyzes the three peptide bonds as illustrated in Figure 11A.

Studies with synthetic substrates based on the collagen sequence have shown that the primary structure dictates the catalytic activity of MMP-1 (Nagase and Fields, 1996), but it is less likely so for collagenolysis, firstly because the synthetic substrates are cleaved also by other noncollagenolytic MMPs, such as MMP-3 and MMP-9, and secondly because the K_m values for these substrates are two to three orders of magnitude higher than those of collagen I (Welgus *et al*, 1981; Fields, 1991). The critical aspects of the collagenolytic specificity rely on the structural changes in collagen, induced by interacting with collagenase. This is evident from temperature-dependent collagenolysis versus gelatinolysis, as shown in Figure 2. At 37°C the rate of cleavage of collagen is much faster than that of gelatin, but at 10°C little or no collagenolytic activity of MMP-1 was observed compared with the gelatinolytic activity. Recent studies of Leikina *et al* (2002) indicated that human collagen I monomers denature at 37°C within a couple of days. Therefore, at 37°C collagen is unwound more readily by collagenase, and the configuration induced in the α chain by interacting with collagenase probably fits the active site and the specificity subsites of the enzyme optimally. Gross and Nagai (1965) postulated local structural instability of the triple helix around the collagenase cleavage site, and Brown *et al* (1977) proposed that it was due to a poor imino-acid content. Fields (1991) indicated that the collagenase-recognition sites in interstitial collagens are preceded by a tightly helical conformation with a high imino-acid content (>33%) and a low side-chain molar volume, followed by a loose triple-helical region with a low imino-acid content (<17%) and a low charged residue content for the entire 25-residue cleavage site region. A recent study of a synthetic heterotrimeric triple-helical peptide containing the collagen I collagenase cleavage site has also indicated that the structure around the collagenase cleavage site is less ordered (Fiori *et al*, 2002). Thus, it is most likely that the collagenase cleavage sites in interstitial collagens are more susceptible to changes in conformation. The cleavage of Val⁷⁸³-Gly⁷⁸⁴ bond of the $\alpha 1(I)$ chain near the collagenase cleavage site by HLE with the help of MMP-1(E200A) clearly supports this notion. It is also notable that mutation in the mouse $\alpha 1(I)$ chain around the collagenase cleavage site makes all three α chains resistant to collagenolysis (Wu *et al*, 1990). In those mutants, Ile776 (P1' subsite), or both Gln774 (P2 subsite) and Ala777 (P2' subsite), was replaced with Pro. Addition of one or two prolines in the $\alpha 1(I)$ chains around the collagenase cleavage site might have made collagen resistant to unwinding as the wild-type $\alpha 2(I)$ chain was also not cleaved by collagenase (Wu *et al*, 1990). Nonetheless, the T_m of the wild-type mouse collagen I and

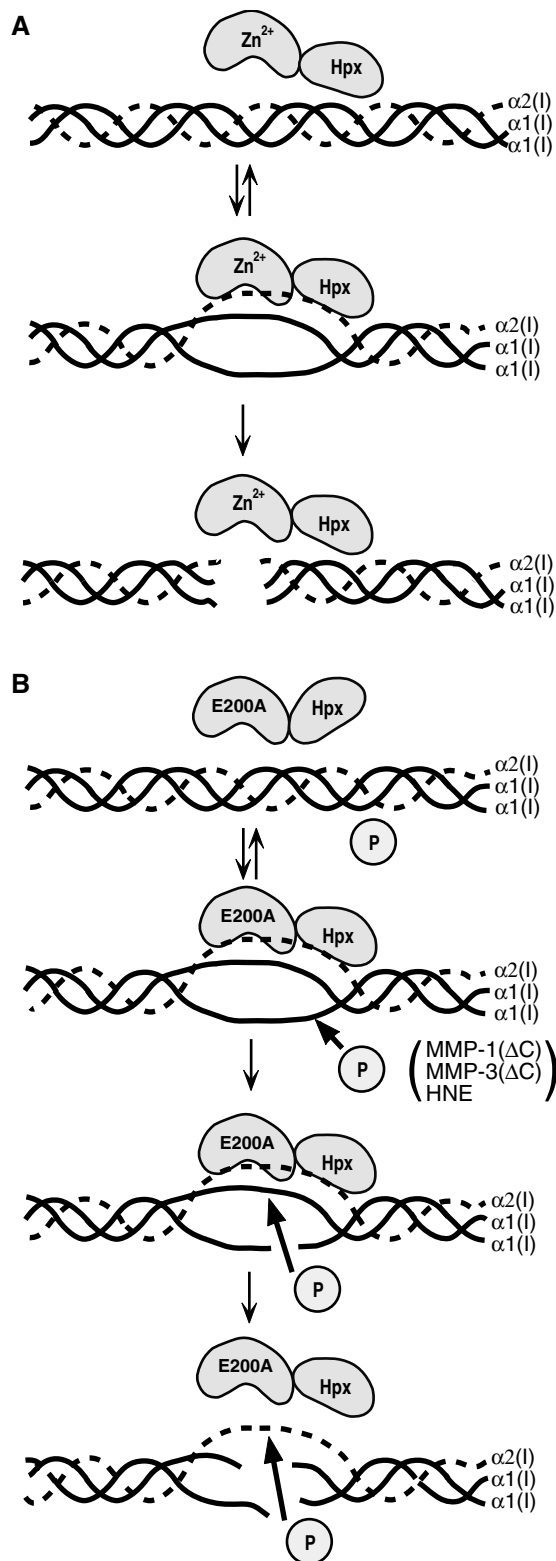


Figure 11 Steps involved in collagenolysis. (A) Collagenase binds to and locally unwinds collagen before it cleaves the triple-helical interstitial collagen. (B) MMP-1(E200A) binds preferentially to the $\alpha 2(I)$ chain and unwinds the triple-helical collagen, but is unable to cleave collagen. The unwound collagen becomes susceptible to noncollagenolytic proteinases indicated as 'P' (e.g., MMP-1(ΔC), MMP-3(ΔC) and HLE), and the $\alpha 1(I)$ chains are initially cleaved. This reaction requires the trimolecular complex formation of the unwinder (MMP-1(E200A)), a cutter proteinase and collagen.

those of mutants were also indistinguishable, indicating that these structural changes are too small to observe changes in the overall T_m of collagen I. These results agree well with our observation that binding and unwinding collagen I by MMP-1(E200A) did not alter the T_m . These observations support the conclusion that the structural changes induced in collagen by MMP-1 are local. On the other hand, structures of gelatin are probably not optimal for the substrate-enzyme interaction in the case of collagenase, which may be evident from the K_m values of collagen I for human MMP-1 which are about $1 \mu M$, whereas those of gelatin I are $4-7 \mu M$ at $37^\circ C$ (Welgus *et al*, 1985).

In our experiments with MMP-1(E200A), a relatively high concentration of an unwinder protein and a cutter proteinase are required, and the efficiency of collagenolysis in this system is extremely low. This is probably because all three components (i.e., an unwinder, a cutter proteinase and the collagen substrate) need to assemble simultaneously (see Figure 11B). Our observation that the combination of Hpx_{MMP-1} and MMP-1(ΔC) cleaved collagen I was unexpected, as our earlier work indicated that the collagenolytic activity was lost when MMP-1 is cleaved into the catalytic domain and the Hpx domain (K Suzuki and H Nagase, unpublished work). A similar loss of collagenolysis was reported for MMP-8 (Knäuper *et al*, 1993). When the catalytic domain and the Hpx domain are combined, they express collagenolytic activity, but a high concentration of these components is required to form a productive trimolecular complex. Such trimolecular interaction is apparently transient, explaining the low efficiency of the activity. This concentration-dependent association would also explain the earlier observation that MMP-1 and MMP-8 lost collagenolytic activity when the catalytic domain and the Hpx domain were split. When the two components are linked by the linker, it is a far more effective enzyme, as the interaction with collagen would be increased analogous to an increased free energy of binding when two low-affinity binding molecules are linked together (Jencks, 1981).

Our studies have also revealed that collagenase cleaves the three α chains one by one. Early studies of Sunada and Nagai (1983) showed that one or two α chains of collagen III were cleaved by collagenase. In the case of collagen I, we show that collagenase preferentially interacts with the $\alpha 2(I)$ chain and this is the first chain to be cleaved. This conclusion is based on the observation that the $\alpha 1(I)$ chain is much more readily cleaved than the $\alpha 2(I)$ chain by MMP-1(ΔC), MMP-3(ΔC) or HLE in the presence of MMP-1(E200A). An initial cleavage of $\alpha 2(I)$ chain by MMP-8 was reported also with a synthetic heterotrimeric triple-helical peptide (Muller *et al*, 2000). However, it is not known which part of the collagenase molecule makes the first contacts with collagen. Our molecular modelling suggests that one possible site is formed by the catalytic domain and the Hpx domain together, in a region rather distal to the active site of the enzyme (R Visse and H Nagase, unpublished results). This possibility and how unwinding of the triple helix occurs are currently being investigated by mapping the critical sites involved in unwinding activity by mutagenesis studies. It appears that several subsites of MMP-1 are involved in this activity. Our current hypothesis is that these sites are located both in the catalytic and Hpx domains of MMP-1 and they make contacts with the triple-helical collagen cooperatively in succession. One of the

sites is the active site of the enzyme, as demonstrated here with an active site-directed synthetic inhibitor. Once these regions are clarified, it may provide new insights into generating inhibitors that specifically block unregulated collagenolysis.

Materials and methods

Materials

Chemicals were from the following sources: 4-aminophenylmercuric acetate (APMA) from ICN Biochemicals, Cleveland, OH; chymotrypsin and phenylmethylsulfonyl fluoride from Sigma, St Louis, MO; human leukocyte elastase from Athens Research, Athens, GA. The hydroxamate inhibitor GM6001X (HONHCOH₂CH (isobutyl) CO-Tyr(OMe)-NHMe (Grobely *et al*, 1992) was a gift from Dr J Oloksyszyn of OsteoArthritic Sciences Inc. Type I collagen was purified from guinea-pig skin (Glimcher *et al*, 1964). Pepsin-treated collagen I was prepared according to Trentham *et al* (1977). Type I gelatin was formed by heating type I collagen to 65°C for 20 min. Restriction enzymes and T4 DNA ligase were from New England Biolabs and *Pfu* polymerase was from Stratagene. *Escherichia coli* BL21(DE3) and the pET3a expression vector were from Novagen. Recombinant MMP-3(ΔC) was expressed in *E. coli* as an active form and purified by using a hydroxamate affinity column as described by Moore and Spilburg (1986).

Construction of MMP-1(E200A) and MMP-3(E202A)

Cloning of wild-type proMMP-1 and proMMP-3 cDNA into the expression vector pET3a was carried out as described previously (Chung *et al*, 2000). To generate pro-MMP-1(E200A) mutant, the pET3a-proMMP-1 vector, whose *Bam*HI site in the original MMP-1 cDNA was removed by mutating GGATCC to GGACTT without affecting the codon for Asp 389, was used as a template for polymerase chain reaction (PCR) with a sense pET3a primer (5'-ACTTTAAGAAGGAGATATACATATG-3'), which includes the *Nde*I site (underlined), and an antisense primer with a mutation changing Glu200 to Ala (5'-AGAATGGCCGAGTGCATGAGCCG CAAC-3'). A second PCR was carried out with a sense primer containing the codon E200A mutation (5'-GTTGCGGCTCATG CACTCGGCCATTCT-3') and an antisense pET3a primer (5'-GCTTTGTTAGCAGCCGGATCC-3') containing the *Bam*HI site (underlined). The two PCR fragments were combined and a third PCR carried out with the sense and antisense pET3a primers. The resulting fragment was digested with *Nde*I and *Bam*HI and ligated into pET3a. For all PCRs, *Pfu* polymerase was used, and the sequence was confirmed by DNA sequencing. To generate MMP-3(E202A), the same strategy was used but with MMP-3-specific sense (5'-GTTGCTGCTCATGCAATTGGCCACTCC-3') and antisense (5'-GGAGTGGCCAATTGCATGAGCAGCAAC-3') primers where Glu202 was mutated to Ala. The resulting PCR fragment was digested with *Nde*I, and ligated into the *Nde*I-cleaved pET3a-proMMP-3 vector. The orientation of the fragment was checked with PCR, and the sequence was confirmed by DNA sequencing.

Expression of mutant proteins

The pET3a vector harboring wild-type or mutant MMP cDNA was transformed into *E. coli* BL21(DE3) cells and protein synthesis was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactoside at 37°C for 4 h. Inclusion bodies were harvested as described previously (Chung *et al*, 2000) and dissolved in 8 M urea, 20 mM Tris-HCl (pH 8.6), 20 mM dithiothreitol and 50 μM ZnCl₂, and passed over a High Q Support anion exchange column (Bio-Rad). Recombinant proteins were diluted to <0.3 mg/ml and 20 mM cystamine was added. For folding, proteins were dialyzed against a buffer containing 50 mM Tris-HCl (pH 8.6), 0.15 M NaCl, 5 mM CaCl₂, 100 μM ZnCl₂, 5 mM β-mercaptoethanol, 1 mM 2-hydroxyethyl disulfide and 0.02% NaN₃, and then against 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 5 mM CaCl₂, 0.02% NaN₃ (TNC buffer) containing 50 μM ZnCl₂ at 4°C. Folded proMMPs were purified by chromatography on a Green A Dymatex gel column (Amicon) and a Sephacryl S-200 column (Pharmacia). ProMMP-1, proMMP-1(E200A) and proMMP-3(E202A) were activated by incubating with 1 mM APMA and one-tenth molar amount of MMP-3(ΔC) at 37°C for 2 h. ProMMP-3(ΔC) lacking residues 248–460 and proMMP-3 were activated by the addition of 1 mM APMA at 37°C

for 2 h. The activated MMPs and MMP mutants were applied to a Sephacryl S-200 column to remove MMP-3(ΔC), APMA and the cleaved propeptide. The hemopexin domain of MMP-1 (Hpx_{MMP-1}) and the Hpx of MMP-3 (Hpx_{MMP-3}) were made by incubating either proMMP-1 or proMMP-3 with 1 mM APMA and one-tenth molar amount of MMP-3(ΔC) at 37°C for at least 16 h. The protein was run over a hydroxamate affinity column to remove the full-length MMP, and the catalytic domain of MMP and the unbound material containing the Hpx domain was collected.

Digestion of collagen and gelatin

Proteins and enzymes of indicated concentrations were used to digest 30 μg pepsin-treated type I collagen or gelatin in TNC buffer at 25°C for up to 48 h, unless otherwise specified. Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA. Products were analyzed by SDS-PAGE with 7.5% total acrylamide under reducing conditions, and the gels were stained with Coomassie brilliant blue R-250. Collagen digestion was examined for the generation of 3/4 and 1/4 fragments. Nonpepsin-treated type I collagen was used to detect intermediate products generated during collagenolysis and analyzed on SDS-PAGE with 5% total acrylamide under reducing conditions.

N-terminal sequencing

The 1/4 fragments generated by MMP-1(ΔC), MMP-3(ΔC) and HLE in the presence of MMP-1(E200A) were separated by SDS-PAGE, and transferred to a poly(vinylidene difluoride)-Millipore Immobilon transfer membrane. The proteins were located by staining with Coomassie brilliant blue R-250 and the bands of interest were excised and sequenced by an Applied Biosystem 447A pulse liquid sequencer.

Measurement of collagen melting temperature T_m

Pepsin-treated guinea-pig type I collagen (3 μM) was incubated with or without an equimolar concentration of MMP-1(E200A) or MMP-3(E200A) in TNC buffer. Thermal transition curves were obtained with a JASCO J-600 circular dichroism spectrometer with 0.1 cm path length quartz cell, by recording the molar ellipticity at wavelength 222 nm, while the temperature was continuously increased from 5 to 70°C at a rate of 35°C/h. Temperature was controlled using the JASCO PTC-348WI temperature control unit. The inflection point of a sigmoidal melting curve was used to define the melting temperature T_m .

Determination of K_D of MMP-1(E200A) for collagen I

MMP-1(E200A) at various concentrations (0–6 μM) was incubated with 4.5 μM guinea-pig collagen I and 6 μM MMP-1(ΔC). Samples removed at different incubation times up to 24 h were analyzed by SDS-PAGE (7.5% total acrylamide) for generation of 3/4 and 1/4 collagen fragments. Proteins were stained with Coomassie brilliant blue R-250, and the percentage cleavage was determined by densitometry and analyzed using Phoretics 1D (Nonlinear Dynamics), from which the initial rate of cleavage was calculated. The equilibrium dissociation constant (K_D) was determined by double reciprocal plots of the rate of product formation versus the concentration of MMP-1(E200A).

Molecular modelling

The full-length crystal structure of porcine MMP-1 (1FBL; (Li *et al*, 1995) and the crystal structure of triple-helical peptide comprising part of the MMP-1-specific cleavage site of type III collagen (Kramer *et al*, 2001) were used to model the interaction of collagenase and interstitial collagen. For this purpose, the hydroxamate inhibitor that was co-crystallized with porcine MMP-1 was removed. The collagen triple-helical peptide positioning was performed manually with InsightII/Discover, and images were produced with SwissPDB viewer (Guex and Peitsch, 1997).

Acknowledgements

We thank Professor John White for critical reading of the manuscript. This work was supported by the Wellcome Trust grant no. 057508 and National Institutes of Health grants AR39189 to HN, and CA77402 and CA98799 to GBF. JLL-F was supported by a Glenn/American Foundation for Aging Research Scholarship.

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