Biol. Chem., Vol. 390, pp. xxx-xxx, January 2009 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2009.XXX

Biochemical characterization of the catalytic domains of three different clostridial collagenases

Ulrich Eckhard[1,a], Esther Schönauer[1,a], Paulina Ducka[1,a], Peter Briza[2], Dorota Nüss[1] and Hans Brandstetter[1,*]

[1] Division of Structural Biology, Department of Molecular Biology, University of Salzburg, Billrothstraße 11, A-5020 Salzburg, Austria

[2] Division of Allergy and Immunology, Department of Molecular Biology, University of Salzburg, Hellbrunnerstraße 34, A-5020 Salzburg, Austria

* Corresponding author
e-mail: hans.brandstetter@sbg.ac.at

Abstract

Clostridial collagenases are used for a broad spectrum of biotechnological applications and represent prime target candidates for both therapy and diagnosis of clostridial infections. In this study, we biochemically characterized the catalytic domains of three clostridial collagenases, collagenase G (ColG) and H (ColH) from *Clostridium histolyticum*, and collagenase T (ColT) from *C. tetani*. All protein samples showed activity against a synthetic peptidic substrate (furylacryloyl-Leu-Gly-Pro-Ala, FALGPA) with ColH showing the highest overall activity and highest substrate affinity. Whereas the K_m values of all three enzymes were within the same order of magnitude, the turnover rate k_{cat} of ColG decreased 50- to 150-fold when compared to ColT and ColH. It is noteworthy that the protein N-terminus significantly impacts their substrate affinity and substrate turnover as well as their inhibition profile with 1,10-phenanthroline. These findings were complemented with the discovery of a strictly conserved double-glycine motif, positioned 28 amino acids upstream to the HEXXH zinc binding site, which is critical for enzymatic activity. These observations have consequences with respect to the topology of the N-terminus relative to the active site as well as possible activation mechanisms.

Keywords: *Clostridium histolyticum*; *Clostridium tetani*; enzymatic mechanism; FALGPA; inhibition; zinc metalloprotease.

Introduction

Clostridia comprise a family of more than 120 ubiquitously occurring, anaerobic, sporulating Gram-positive bacteria. Among them are serious pathogens causing diseases, such as botulism, gas gangrene, tetanus, and pseudomembranous colitis (Cato et al., 1986; Hatheway, 1990). To date, these toxigenic clostridia represent a threat to public health, as tetanus and clostridial myonecrosis have maintained double-digit mortality rates and pseudomembranous colitis is a known severe complication of antibiotic therapy (Burke and Opeskin, 1999; Bruggemann et al., 2003; Taubes, 2008a,b).

Furthermore, substantial amounts of pathogenic clostridia were cultured in the last 60 years for the use as bioweapons. As a prominent example, in the 1990s the Japanese cult Aum Shinrikyo (Sect of Supreme Truth) attacked the Japanese parliament and the wedding of the Japanese crown prince with botulinum toxin (Arnon et al., 2001).

While the histotoxicity of clostridia is primarily caused by specific toxins, many hydrolytic enzymes produced by pathogenic clostridia, such as collagenases (EC 3.4.24.3), are thought to be involved in host colonization by aiding their multiplication and spread into tissues; additionally, they might potentiate the histotoxicity by facilitating toxin diffusion (Bond and Van Wart, 1984a; Hatheway, 1990; Matsushita and Okabe, 2001). Therefore, clostridial collagenases represent prime targets for sensitive diagnosis and effective therapy against clostridial infections. They are of special interest since the outbreak of clinical symptoms of clostridial infections is – paradoxically – often triggered by antibiotic treatment (Burke and Opeskin, 1999; Modi and Wilcox, 2001; McFarland, 2005), thus preventing classical antibiotic treatment strategies.

With type I collagen being the most abundant protein of all higher organisms, it is not surprising that there exists a diverse spectrum of biotechnological applications of bacterial collagenases, including their use for meat tenderizing, islet cell isolation, wound healing, treatment of retained placenta, or their use as additives to laundry detergents (Sank et al., 1989; Hesse et al., 1995; Fecteau et al., 1998; Haffner et al., 1998; Yeh et al., 2002; Watanabe, 2004).

Clostridial collagenases are zinc metalloproteases and they belong to the MEROPS peptidase family M9, which comprises microbial metalloproteases with presumable collagenolytic activity (Rawlings et al., 2008). They are large mosaic proteins consisting of a signal peptide, a putative pro-domain, a catalytic domain, up to two PKD domains of unknown function and up to three collagen-binding domains (Matsushita and Okabe, 2001). Each of these accessory domains consists of approximately 110 amino acids with a molecular mass of approximately 12 kDa. The domain architecture of the three clostridial collagenases investigated in this study is shown in Figure 1.

All clostridial collagenases possess the consensus HEXXH sequence, a zinc binding motif, as well as a glutamate positioned 33–35 residues downstream the HEXXH motif, cf. Figure 2. This residue was identified as

[a] These authors contributed equally to this work.

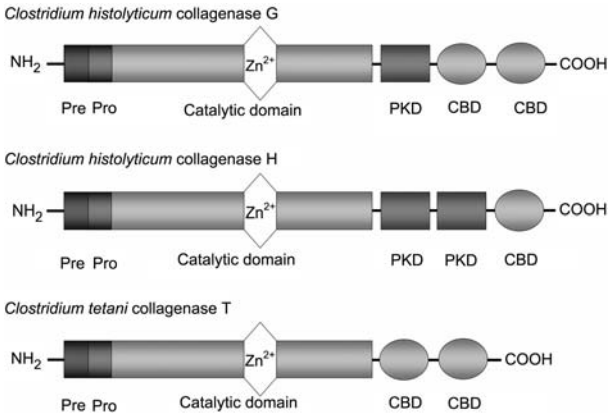

Figure 1 Schematic representation of the domain architecture of ColG, ColH, and ColT.
Pre, signal peptide; pro, putative prodomain; PKD, polycystic kidney disease-like domain; CBD, collagen binding domain.

the third zinc binding ligand in ColH and classifies all clostridial collagenases as members of the gluzincin subfamily (Jung et al., 1999).

On the basis of the ratio of the activities toward synthetic peptides and native collagen, clostridial collagenases are divided into two distinct classes: class I (like ColG) with high collagenolytic and low amidolytic activity, and class II (e.g., ColH) showing preferentially amidolytic activity (Bond and Van Wart, 1984b; Van Wart and Steinbrink, 1985). This enzymatic behavior correlates with a duplication of the calcium binding domain in ColG, which was hypothesized to be critical for collagen binding and presentation (Matsushita et al., 2001).

Contrasting the distinct substrate specificity of mammalian matrix-metalloproteases, clostridial collagenases are capable of degrading various types of collagen and gelatin, and can hydrolyze collagen at multiple sites within the triple helical regions. Generally, they cleave peptide bonds on the amino side of the glycine residue in the characteristic collagen motif PXGP. Notwithstanding the lack of any significant sequential conservation, human collagenases, such as MMP1 and MMP8 are also organized as mosaic proteins, and both enzyme families utilize Zn^{2+} for catalysis (Bond and Van Wart, 1984a,b; Jung et al., 1999; Ravanti and Kahari, 2000; Brandstetter et al., 2001; Matsushita and Okabe, 2001; Hu et al., 2002).

To exploit the mechanism of action of this enzyme family, it is of particular interest to understand the functional relevance of their individual domains. Along this line, Matsushita and colleagues could show calcium to significantly enhance the binding of the CBD to collagen and collagenous peptides (Matsushita et al., 2001). Subsequently, the crystal structure of the calcium binding domain (CBD) of collagenase G was determined in the presence and absence of calcium, setting a milestone in the structural and mechanistic understanding of the Ca^{2+}-dependence of collagen binding and presentation (Wilson et al., 2003). Beyond that, however, our mechanistic knowledge on bacterial collagenases in general and their catalytic domains in particular is still very limited. The situation is reflected and aggravated by the complete lack of structural data on their architecture.

In this article, we describe the first enzymatic characterization of the catalytic domains of three different clostridial collagenases: collagenase G (ColG) and H (ColH) from *Clostridium histolyticum*, and collagenase T (ColT) from *C. tetani*, a hitherto completely uncharacterized enzyme. Among other questions, this work clarifies to which extent catalytic properties are harbored within the catalytic domain alone rather than in the full-length protein.

Results

Expression and purification of recombinant catalytic domains

The catalytic domain constructs of ColG, ColH, and ColT were solubly expressed in *Escherichia coli* Bl21 DE3 cells with a proteolytically cleavable N-terminal hexahistidine tag. The expression typically yielded 10 mg of pure protein from 500 ml of culture. The recombinant proteins were purified by NiNTA affinity purification followed by size exclusion chromatography, as described in more detail elsewhere (Eckhard et al., 2008; ●●●Please provide initial●●● Ducka et al. ●●●Please provide full authors' names●●●, unpublished).

The recombinant proteins migrated on a denaturing SDS-PAGE gel with an apparent molecular mass of approximately 70–80 kDa and were found to be at least 95% homogenous (Figure 3).

Kinetic characterization of the purified catalytic domains

Consistent with reports in the literature (Bond and Van Wart, 1984b), the activity of all three clostridial collage-

```
ColA 470 DN GG IYI 476 ... 497 EELFR HEFTH YLQGRYVVPGMWGQGEFYQEG--VLTWYE E GTAEFFA 541
ColG 491 DN GG LYI 497 ... 518 EELFR HEYTH YLQARYLVDGLWGQGPFYEKN--RLTWFD E GTAEFFA 562
ColH 423 NN GG MYI 429 ... 450 EELFR HEYTH YLQGRYAVPGQWGRTKLYDND--RLTWYE E GGAELFA 494
ColB 449 DN GG IYI 455 ... 476 EELFR HEFTH YLQGRYLVPGLFNEGDFYKGNSGRITWFE E GSAEFFA 522
ColS 451 DN GG IYI 457 ... 478 EELFR HEFTH YLQGRYLIPGLFGQGDFYKGNSGRITWFE E GSAEFFA 524
ColT 433 DN GG IYI 439 ... 460 EELFR HEFTH YLQGRYLIPGLFNKGDFYKGNNGRITWFE E GSAEFFA 506
         :* ** :**             ***** **:** ***.** : * :..  :*. .   :**:: * * **:**

   Double glycine motif        Zinc binding motif                Third zinc ligand
```

Figure 2 Multiple sequence alignment of the catalytic center of six different clostridial collagenases.
ColA, collagenase A from *Clostridium perfringens*; ColG, collagenase G from *C. histolyticum*; ColH, collagenase H from *C. histolyticum*; ColB, collagenase B from *C. botulinum*; ColS, collagenase S from *C. sporogenes*; ColT, collagenase T from *C. tetani*. The zinc binding residues characterize the collagenases as gluzincins. The double-Gly motif is critical for enzymatic activity, as described in the results section.

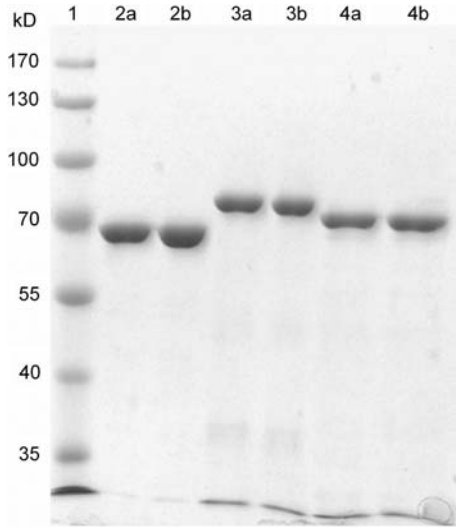

Figure 3 Coomassie-stained SDS-PAGE analysis of the catalytic domain constructs of ColG, ColH, and ColT.
Lane 1, molecular mass marker. From left to right: ColG (2), ColH (3), and ColT (4); (a) with His_6-tag, (b) without His_6-tag.

nases significantly decreased over time. Therefore, all enzymatic assays were performed immediately after purification. The heterologously expressed enzymes were subjected to kinetic measurements with FALGPA as substrate (Van Wart and Steinbrink, 1981). Apparent K_m and V_{max} values were determined for all three catalytic domains. Results are shown in Figure 4. A representative Michaelis-Menten plot is shown in Figure 5.

All recombinant protein samples showed activity against the synthetic peptidic substrate, albeit with substantial differences in their turnover rates (Table 1). The catalytic domain of collagenase H displayed the highest specific activity, approximately 12 U/mg, followed by collagenase T (approximately 2 times lower) and collagenase G (more than 140 times lower). By contrast, the variation in K_m values is less pronounced with approximately 3-fold deviations at the maximum, but reflects the trend observed in the turnover numbers. Consequently, the catalytic efficiencies (k_{cat}/K_m) vary over a factor of 450 between the extremes of ColH (highest) and ColG (lowest).

Influence of the N-terminal His_6-tag on enzyme activity

In the case of the human collagenase MMP8, the correctly processed N-terminus has a significant impact on enzyme activity, resulting in the so-called super-activation with Phe79, but not Met80 as the N-terminus (Reinemer et al., 1994; Farr et al., 1999). Therefore, we examined if the exact processing of the N-terminus also affects the enzymatic characteristics of the clostridial collagenases and compared their kinetics in the absence and presence of the N-terminal His_6-tag. We observed a significant influence of the His_6-tag on enzyme kinetics (Figure 4).

The presence of the N-terminal His_6-tag lowered both substrate affinity and maximum velocity consistently in all three enzymes. The Michaelis-Menten constant of ColG and ColH increased by approximately 50% and

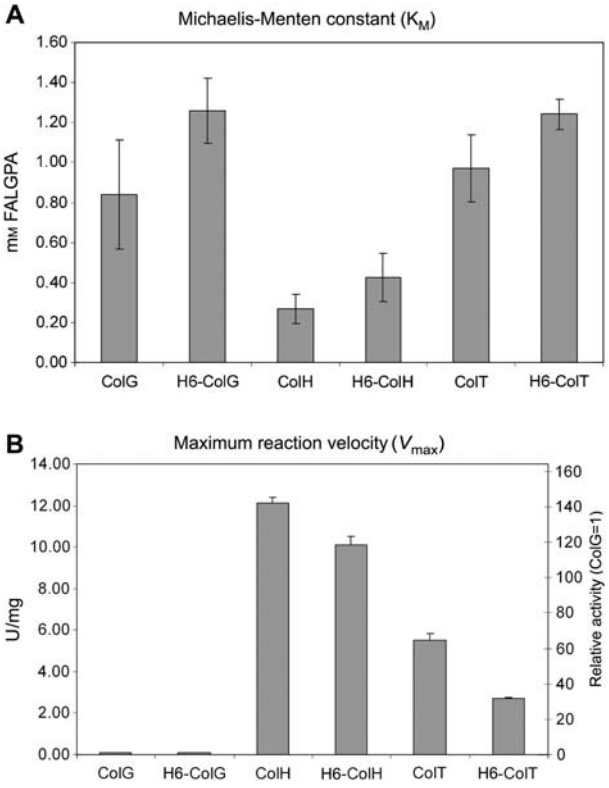

Figure 4 Michaelis-Menten constants K_m (A) and maximum velocities V_{max} (B) of the catalytic domains of ColG, ColH, and ColT with and without His_6-tag.
Error bars indicate the standard deviation error derived from triplicate measurements.

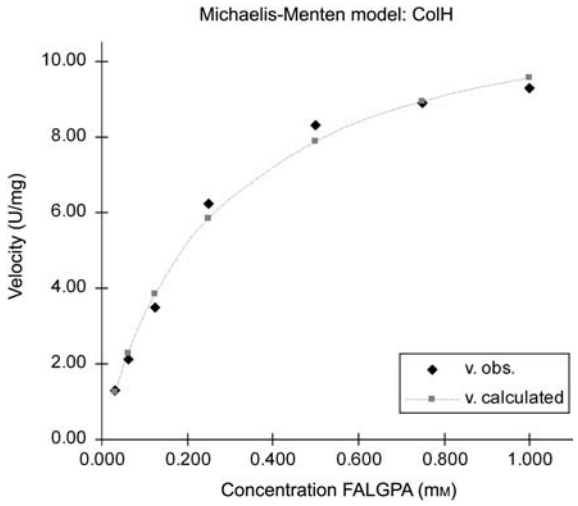

Figure 5 Michaelis-Menten model of the activity of the catalytic domain of collagenase H.
Black squares: velocity observed in the FALGPA assay (v. obs.), dashed line: velocity calculated by nonlinear least squares regression fitting (v. calculated).

60%, respectively, while the increase in K_m by approximately 30% was less pronounced in ColT. These changes were accompanied by the attenuation of V_{max}. The maximum velocity of ColG and ColH were moderately decreased by approximately 20%, whereas the activity of ColT was more than halved. The overall effect of the His_6-tag on the catalytic efficiency k_{cat}/K_m amounted to

Table 1 Enzymatic activity of the catalytic domains of collagenase G, H, and T, with and without His_6-tag.

	V_{max} (U/mg)	K_m (mmol/l)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
ColG	0.0852	0.840	0.110	130
H6-ColG	0.0700	1.26	0.092	73
ColH-	12.1	0.269	15.9	59 100
H6-ColH	10.1	0.425	13.6	31 900
ColT-	5.53	0.970	7.24	7470
H6-ColT	2.72	1.241	3.65	2940

an approximate 50% reduction in ColG and ColH and a more than 60% reduction in ColT (see Table 1).

Inhibitory effects of 1,10-phenanthroline

The specific metalloprotease inhibitor 1,10-phenanthroline exerted an inhibitory effect on all six constructs. A representative fit to the inhibition curve is shown in Figure 6. The N-terminal tag also affected the inhibition with 1,10-phenanthroline. The half maximal inhibitory concentrations (IC_{50}) of the catalytic domains for 1,10-phenanthroline were significantly decreased in the presence of the N-terminal His_6-tag (Figure 7).

Minimal catalytic domain

By Western blot analysis employing an anti-His antibody directed against the N-terminal His_6-tag, we could demonstrate the presence of a prominent, C-terminally degraded fragment of the catalytic domain of ColT migrating at approximately 55 kDa (data not shown). Therefore, we sought to investigate whether such a C-terminally truncated ColT construct retains catalytic activity. We designed a truncated ColT construct, which stops shortly after the third zinc binding residue. The construct (Tyr53-Ala506) with an approximate molecular mass of 55 kDa is consistent with the prediction of a minimal catalytic domain by the MEROPS web server (Rawlings et al., 2008). However, this construct showed no activity at all, despite containing all zinc binding residues including the catalytic glutamate that is supposed to serve as the general base.

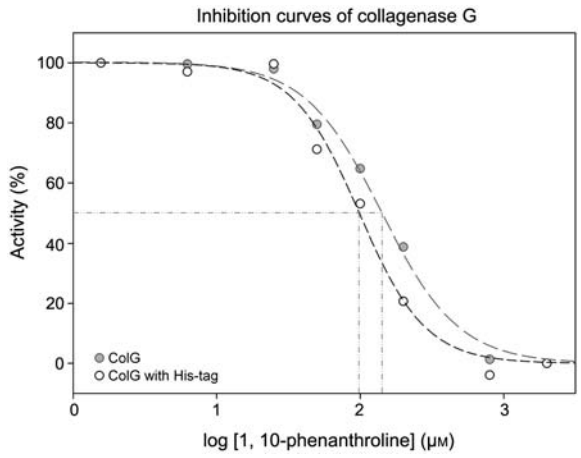

Figure 6 Representative 1,10-phenanthroline concentration-inhibition curves of enzyme activity of the catalytic domain of collagenase G with and without His_6-tag.

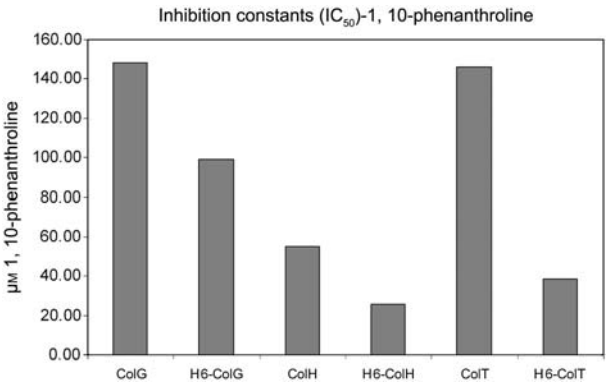

Figure 7 Inhibition constants (IC_{50}) of the catalytic domains of ColG, ColH, and ColT with/without N-terminal His_6-tag (H6).

Enzymatically critical double-glycine motif

We serendipitously introduced a single amino acid exchange (G426V) within a double-glycine motif of ColH, located 28 amino acids upstream to the HEXXH motif. This point mutation, verified by DNA and protein sequencing (ESI-TOF MS●●●Please clarify in full●●●), completely abolished enzymatic activity in the FALGPA assay. The double-glycine motif is strictly conserved throughout all available clostridial collagenase sequences (Figure 2). The finding implicates that this conserved residue is critical for the correct active site structure.

Discussion

The aim of the present study was to characterize the catalytic domains of three different clostridial collagenases, namely of collagenase G and H from *Clostridium histolyticum*, and ColT from *C. tetani*. Microbial collagenases are thought to play a key role in host infection and have been investigated in great detail for the enzymes from *C. histolyticum* (Bond and Van Wart, 1984b; Van Wart and Steinbrink, 1985; Yoshihara et al., 1994; Matsushita et al., 1999; Matsushita and Okabe, 2001). While these investigations represent major achievements in the field, work in particular with natively purified protein was challenged by the risk of contamination with related proteases; additionally, collagenases tend to (auto-)degrade, thus contributing another source of heterogeneity that may jeopardize accurate enzymatic analysis. Our approach addresses both of these concerns.

The presented data show, for the first time, the proteolytic activity of ColT from *C. tetani* and moreover its capability to cleave a substrate specific for microbial collagenases. By the inhibition with 1,10-phenanthroline, we further confirmed that ColT is indeed a zinc metalloprotease. Therefore, we propose that the function of a collagenolytic metalloprotease should be assigned universally to all gene products of the M09.002 (MEROPS database) family.

Surprisingly, the activity of the catalytic domain collagenase G against the synthetic peptide FALGPA was

approximately 50 times lower than published by Bond and Van Wart (1984b), whereas the activity of ColH was comparable. We propose two possible explanations: (1) slight variations in the amino acid sequence, including differences in the termini or the mentioned single site amino acid exchanges may cause a decrease in the catalytic activity of ColG; alternatively (2), there exists the possibility that the isolated ColG isoforms characterized by Bond and Van Wart were contaminated with ColH.

Significantly, all three enzymes have comparable substrate affinities, as reflected in less than 3.5-fold differences in their K_m values, suggesting that they share important active site features, such as substrate recognition pockets. Consequently, we consider major rearrangements at the substrate recognition site of ColG unlikely to explain its low activity. The striking difference between the enzymes relates to a k_{cat} effect with more than a 100-fold difference. The latter may be caused by subtle differences in the active site, such as variations in the local pK_a of the general base (E524 in ColG) or minute changes in the relative orientation of oxyanion hole and the zinc coordinating residues. Taking both kinetic parameters together, the catalytic efficiency (k_{cat}/K_m) differ most (500-fold) within the two *C. histolyticum* enzymes ColG and ColH. This differentiation may in fact be evolutionary driven allowing to develop different substrate specificities.

1,10-Phenanthroline has been shown to act by removing the catalytic metal from the metalloprotease (Angleton and Van Wart, 1988). Our inhibition constants for ColG and ColH support the published data by Angleton and Van Wart. They reported (1) an inhibition constant of approximately 0.040 mM for collagenase H, and (2) compared to ColH a more than 2-fold higher IC_{50} value for ColG (Angleton and Van Wart, 1988). Together with our findings from the FALGPA assay, these data indicate that the catalytic zinc in ColH is significantly better accessible and can be more easily extracted than in ColG and ColT. We conclude that the detailed zinc coordination geometry varies in the three enzymes.

The inhibitory effect of the N-terminal His_6-tag affected both the substrate affinity (K_m) and the turnover number (k_{cat} or V_{max}). There are at least three plausible mechanisms that may explain this behavior: (1) the His_6-tag acts as a non-competitive inhibitor of the enzyme, thereby reducing its V_{max}; in such a scenario, the His_6-tag could chelate and extract the catalytic Zn^{2+} from the active site, mimicking the effect of phenanthroline. (2) The His_6-tag acts as a competitive inhibitor of the enzyme, thereby increasing its apparent K_m; in this model, the tag partially shields and thus sterically hinders substrates to access the active site. (3) The un-tagged (physiological) N-terminus exhibits a stimulatory effect which may be both V_{max}- and K_m-related; addition of an artificial His_6-tag may suppress these effects. The latter model mirrors the activity enhancement described for human MMP8: only the correctly processed N-terminus (Phe79) induces activity stimulation, whereas variations in the N-terminus (e.g., Met80) lead to reduced enzymatic activity (Reinemer et al., 1994; Farr et al., 1999). Any of the three models (1) to (3) necessarily implies that the N-terminus is geometrically close to the active site in all three investigated enzymes. However, a detailed mechanistic understanding of the observed effects awaits further experiments.

In this context, it is particularly interesting that the presence of the His_6-tag decreases the IC_{50} value of phenanthroline. Within the proposed non-competitive inhibition mechanism (1), the His_6-tag would be expected to only reduce the number of active collagenases and, consequently, should behave neutral towards the inhibition profile of phenanthroline. Therefore, model (1) is inconsistent with the observed inhibition profile. The model of competitive inhibition by the His_6-tag (2) may, at first sight, appear counterintuitive with an IC_{50} decrease, given that the substrate K_m values increased with the His_6-tagged vs. untagged N-terminus. However, when compared with the substrate FALGPA, the smaller size of 1,10-phenanthroline might provide it with a competitive advantage in gaining access to the active site which is partly blocked by the His_6-tag. Therefore, part of the IC_{50} value reductions is consistently explained with model (2). Finally, also model (3) is consistent with the inhibition data: since a peptidic substrate and phenanthroline obey different interaction modes with the enzyme active site, the modulation exerted by the mature N-terminus may affect substrate and inhibitor affinities differently.

The effects observed in the presence and absence of the N-terminal His_6-tag strongly suggest a regulatory role of the N-terminal pro-domain, the functional relevance of which was so far unknown (Matsushita et al., 1999). Notably, neither signal peptide nor the putative pro-peptide is necessary for proper folding of the bacterial collagenases.

Intrigued by the presence of C-terminally truncated fragments of the three catalytic domains, we examined whether functionally active mini-proteases exist by cloning a minimal catalytic domain of ColT of approximately 50 kDa. However, the construct showed no catalytic activity in the FALGPA assay although it harbored the complete active site and was correctly folded, as judged by size exclusion chromatography (data not shown). We, therefore, postulate that the observed (auto-)degradations serve a regulatory role. We also wish to emphasize that there is no precedence of an active metalloprotease with a terminal residue in close sequential and spatial proximity to the active site; thus, the observed result is consistent with the current knowledge on the architecture of zinc proteases.

The critical role of the double-glycine motif 28 aa upstream to the active site came as unexpectedly. Glycine residues often serve as hinge residues, since they have unique conformational degrees of freedom. Consequently, we assume that the exchange of a glycine in this conserved motif disrupts the structure or dynamics of the protease that may be necessary for breathing-like motions during catalysis. Clearly, the elucidation of the exact mechanism awaits further experiments.

In summary, we succeeded in the first biochemical comparison of three distinct catalytic domains of clostridial collagenases. We found that the activity of this protease family is starkly differentiated and tightly regulated on different levels, including the influence of the N-terminus, auto-degradation, and conformational dynamics

near the active site. The findings pave the way for engineering more specific variants of these biotechnologically relevant proteases.

Materials and methods

Enzymes: restriction enzymes and T4 ligase were purchased from Fermentas (St. Leon-Rot, Germany); *Pfu* polymerase was from Stratagene (Amsterdam, The Netherlands). Primers and sequence analysis: custom made primers were obtained from Eurofins MWG Operon (Ebersberg, Germany); sequence analyses were performed at MWG Operon (Martinsried, Germany). All reagents were of the highest standard available from Sigma-Aldrich Co. (München, Germany) or AppliChem (Darmstadt, Germany).

Bioinformatics analysis

On the basis of the catalytic domain of ColG (Tyr119-Ala790), a protein BLAST search for related protein sequences was performed (Altschul et al., 1997). We found significant sequence similarity (scoring value E better than 1e-75) only with clostridial and bacilli collagenases. The catalytic domains of ColH (Val41-Leu717) and ColT (Tyr53-Leu727; sequence numbering according to Swiss-Prot entry Q9X721, Q46085, and Q899Y1 respectively) showed sequence identities of 48% and 46%, respectively. For the active site (±50 amino acids with respect to the HEXXH motif), a sequence identity between 60% and 69% was calculated.

The particular choice, as outlined above, reflects three information (●●●Please clarify meaning of information in this sentence●●●) on naturally occurring isoforms of ColG and ColH from *C. histolyticum* and ColA from *C. perfringens* (Bond and Van Wart, 1984a,b; Matsushita et al., 1994, 1999), on the N-terminal maturation of ColG and ColH (Matsushita et al., 2001), and bioinformatical analysis on domain boundaries that include sequence alignment, conservation, and domain decomposition algorithms (Liu and Rost, 2004).

The molecular mass of the three catalytic domains was computed to be approximately 80.5 kDa, with a p*I* of approximately 6.0 using the ProtParam Web applet (Wilkins et al., 1999). Multiple sequence alignments were performed by ClustalW (Larkin et al., 2007). Sequence numbering of ColA, ColB, ColG, ColH, ColS, and ColT in Figure 2 follows the Swiss-Prot entries P43153 (Matsushita et al., 1994), A5I290 (Sebaihia et al., 2007), Q9X721 (Matsushita et al., 1999), Q46085 (Yoshihara et al., 1994), Q84IM4 (Matsushita, 2002), and Q899Y1 (Bruggemann et al., 2003), respectively.

Cloning, expression, and purification

The catalytic domains of ColG and ColH were PCR-amplified from cloning vectors kindly provided by Roche Diagnostics GmbH (●●●City? Country?●●●) using *Pfu* Ultra Polymerase (Stratagene). For the catalytic domain of collagenase T, genomic DNA kindly provided by the laboratory of Gerhard Gottschalk (Göttingen Genomics Laboratory) was used as PCR template. The primers used were:

ColG_for: 5′-ACG T**GG TAC C**AT GTA TGA TTT TGA GTA TTT AAA TG-3′;
ColG_rev: 5′-ACG T**GG ATC C**TT ACC CAT TAT CTG TTA AAA CCC-3′;
ColH_for: 5′-ACG T**GG TAC C**AT GGT ACA AAA TGA AAG TAA GAG-3′;
ColH_rev: 5′-ACG T**GG ATC C**TT ATA AAT ATC CGT GGA ATA CTA-3′;
ColT_for: 5′-CCG **CTC GAG** TCA TAA TAA TCC ATG GAA AAC TAT ATC ATA-3′;
ColT_rev: 5′-CCG **CTC GAG** TTA ATT TAT TAT TAC TGA ATA ATT ACC-3′.

Cloning restriction sites are represented by underlined and bold letters.

Compared to the Swiss-Prot and NCBI (●●●Please clarify in full●●●) entries several amino acid exchanges were observed: 14 in the catalytic domain of ColH and 3 in the catalytic domain of ColG.

All constructs (as specified in the bioinformatics analysis section) were cloned with an N-terminal His_6-tag, followed by the TEV-protease recognition site (ENLYFQ↓GGT), followed by the enzyme-specific starting sequence MYDF (ColG), MVQN (ColH), and YKT (ColT). Proteins were expressed in *E. coli* BL21(DE3) cells at 20°C. All purification steps were carried out at 4°C. The two-step purification included Ni^{2+}-affinity purification and size exclusion chromatography. Removal of the N-terminally His_6-tag was obtained by enzymatic digestion using TEV-protease and samples were repurified by IMAC (●●●Please clarify in full●●●). The quantitative removal of the affinity tag was verified by Western blot analysis using a monoclonal anti-His_6-tag antibody (data not shown).

Protein concentrations were assayed using the Bradford method with bovine serum albumin as standard (Bradford, 1976).

Enzyme assays

Unit definition: one unit hydrolyzes 1.0 μmol of furylacryloyl-Leu-Gly-Pro-Ala (FALGPA) per minute at 25°C at pH 7.5 in the presence of calcium ions.

Enzyme assays were performed as described by van Wart and Steinbrink (Van Wart and Steinbrink, 1981) and detailed in the manufacture's protocol (Sigma-Aldrich). The FALGPA concentrations were varied from 0.031 mM to 1.0 mM. Absorbance was monitored at 324 nm (0.031–0.062 mM FALGPA; $\Delta\varepsilon_{324}$=2.250 mM^{-1} cm^{-1}) and 345 nm (0.125–1.00 mM FALGPA; $\Delta\varepsilon_{324}$=0.530 mM^{-1} cm^{-1}). Triplicate data sets were analyzed by nonlinear least squares regression fitting to the Michaelis-Menten equation (Hernandez and Ruiz, 1998). The catalytic efficiency k_{cat} is calculated by V_{max}/E_{tot}.

A stock solution of 10 mM 1,10-phenanthroline was prepared in ddH_2O. The half-maximal inhibitory concentrations (IC_{50}) were calculated in the presence of 0.25 mM FALGPA by nonlinear regression analysis using Sigma Plot (SPSS, Chicago, IL, USA). Protein samples were pre-incubated for at least 1 h with the respective inhibitor concentration.

Concentrations of FALGPA and 1,10-phenanthroline were determined in solution via UV absorbance at 305 nm (ε=24.70 mM^{-1} cm^{-1}) and 272 nm (ε=30.20 mM^{-1} cm^{-1}), respectively.

All measurements were carried out at 25°C and pH 7.5.

Acknowledgments

We would like to thank Prof. Gottschalk (Göttingen Genomics Laboratory) and Dr. Hesse (Roche Diagnostics GmbH) for kindly providing the genetic material and Land Salzburg, GEN-AU and the Wissenschaftsfonds (FWF project P20582) for their financial support.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389–3402.

Angleton, E.L. and Van Wart, H.E. (1988). Preparation and reconstitution with divalent metal ions of class I and class II *Clostridium histolyticum* apocollagenases. Biochemistry *27*, 7406–7412.

Arnon, S.S., Schechter, R., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Hauer, J., Layton, M., et al. (2001). Botulinum toxin as a biological weapon: medical and public health management. J. Am. Med. Assoc. *285*, 1059–1070.

Bond, M.D. and Van Wart, H.E. (1984a). Purification and separation of individual collagenases of *Clostridium histolyticum* using red dye ligand chromatography. Biochemistry *23*, 3077–3085.

Bond, M.D. and Van Wart, H.E. (1984b). Characterization of the individual collagenases from *Clostridium histolyticum*. Biochemistry *23*, 3085–3091.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.

Brandstetter, H., Grams, F., Glitz, D., Lang, A., Huber, R., Bode, W., Krell, H.W., and Engh, R.A. (2001). The 1.8-Å crystal structure of a matrix metalloproteinase 8-barbiturate inhibitor complex reveals a previously unobserved mechanism for collagenase substrate recognition. J. Biol. Chem. *276*, 17405–17412.

Bruggemann, H., Baumer, S., Fricke, W.F., Wiezer, A., Liesegang, H., Decker, I., Herzberg, C., Martinez-Arias, R., Merkl, R., Henne, A., et al. (2003). The genome sequence of Clostridium tetani, the causative agent of tetanus disease. Proc. Natl. Acad. Sci. USA *100*, 1316–1321.

Burke, M.P. and Opeskin, K. (1999). Nontraumatic clostridial myonecrosis. Am. J. Forensic Med. Pathol. *20*, 158–162.

Cato, E.P., George, W.L., and Finegold, S.M. (1986). Genus Clostridium. Prazmowski, 1880, 23^{AL}. In: Bergey's Manual of Systematic Bacteriology, Vol. 2, P.H.A. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt, eds. (Baltimore, MD: The Williams & Wilkins Co.), pp. 1141–1200.

Eckhard, U., Nuss, D., Ducka, P., Schonauer, E., and Brandstetter, H. (2008). Crystallization and preliminary X-ray characterization of the catalytic domain of collagenase G from *Clostridium histolyticum*. Acta Crystallogr. F Struct. Biol. Cryst. Commun. *64*, 419–421.

Farr, M., Pieper, M., Calvete, J., and Tschesche, H. (1999). The N-terminus of collagenase MMP-8 determines superactivity and inhibition: a relation of structure and function analyzed by biomolecular interaction analysis. Biochemistry *38*, 7332–7338.

Fecteau, K.A., Haffner, J.C., and Eiler, H. (1998). The potential of collagenase as a new therapy for separation of human retained placenta: hydrolytic potency on human, equine and bovine placentae. Placenta *19*, 379–383.

Haffner, J.C., Fecteau, K.A., Held, J.P., and Eiler, H. (1998). Equine retained placenta: technique for and tolerance to umbilical artery injections of collagenase. Theriogenology *49*, 711–716.

Hatheway, C.L. (1990). Toxigenic clostridia. Clin. Microbiol. Rev. *3*, 66–98.

Hernandez, A. and Ruiz, M.T. (1998). An EXCEL template for calculation of enzyme kinetic parameters by non-linear regression. Bioinformatics *14*, 227–228.

Hesse, F., Burtscher, H., Popp, F., and Ambrosius, D. (1995). Recombinant enzymes for islet isolation: purification of a collagenase from *Clostridium histolyticum* and cloning/expression of the gene. Transplant. Proc. *27*, 3287–3289.

Hu, Y., Webb, E., Singh, J., Morgan, B.A., Gainor, J.A., Gordon, T.D., and Siahaan, T.J. (2002). Rapid determination of substrate specificity of *Clostridium histolyticum* β-collagenase using an immobilized peptide library. J. Biol. Chem. *277*, 8366–8371.

Jung, C.M., Matsushita, O., Katayama, S., Minami, J., Sakurai, J., and Okabe, A. (1999). Identification of metal ligands in the *Clostridium histolyticum* ColH collagenase. J. Bacteriol. *181*, 2816–2822.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., et al. (2007). Clustal W and Clustal X version 2.0. Bioinformatics *23*, 2947–2948.

Liu, J. and Rost, B. (2004). CHOP proteins into structural domain-like fragments. Proteins *55*, 678–688.

Matsushita, O. (2002). Reiterated domain duplication in clostridial collagenases. Direct submission of the nucleotide sequence of collagenase S from *Clostridium sporogenes* to the EMBL/GenBank/DDBJ databases. ●●Journal? volume? pages?●●

Matsushita, O. and Okabe, A. (2001). Clostridial hydrolytic enzymes degrading extracellular components. Toxicon *39*, 1769–1780.

Matsushita, O., Yoshihara, K., Katayama, S., Minami, J., and Okabe, A. (1994). Purification and characterization of *Clostridium perfringens* 120-kilodalton collagenase and nucleotide sequence of the corresponding gene. J. Bacteriol. *176*, 149–156.

Matsushita, O., Jung, C.M., Katayama, S., Minami, J., Takahashi, Y., and Okabe, A. (1999). Gene duplication and multiplicity of collagenases in *Clostridium histolyticum*. J. Bacteriol. *181*, 923–933.

Matsushita, O., Koide, T., Kobayashi, R., Nagata, K., and Okabe, A. (2001). Substrate recognition by the collagen-binding domain of *Clostridium histolyticum* class I collagenase. J. Biol. Chem. *276*, 8761–8770.

McFarland, L.V. (2005). Alternative treatments for *Clostridium difficile* disease: what really works? J. Med. Microbiol. *54*, 101–111.

Modi, N. and Wilcox, M.H. (2001). Evidence for antibiotic induced *Clostridium perfringens* diarrhoea. J. Clin. Pathol. *54*, 748–751.

Ravanti, L. and Kahari, V.M. (2000). Matrix metalloproteinases in wound repair. Int. J. Mol. Med. *6*, 391–407.

Rawlings, N.D., Morton, F.R., Kok, C.Y., Kong, J., and Barrett, A.J. (2008). MEROPS: the peptidase database. Nucleic Acids Res. *36*, D320–325.

Reinemer, P., Grams, F., Huber, R., Kleine, T., Schnierer, S., Piper, M., Tschesche, H., and Bode, W. (1994). Structural implications for the role of the N terminus in the 'superactivation' of collagenases. A crystallographic study. FEBS Lett. *338*, 227–233.

Sank, A., Chi, M., Shima, T., Reich, R., and Martin, G.R. (1989). Increased calcium levels alter cellular and molecular events in wound healing. Surgery *106*, 1141–1147; discussion 1147–1148.

Sebaihia, M., Peck, M.W., Minton, N.P., Thomson, N.R., Holden, M.T., Mitchell, W.J., Carter, A.T., Bentley, S.D., Mason, D.R., Crossman, L., et al. (2007). Genome sequence of a proteolytic (Group I) *Clostridium botulinum* strain Hall A and comparative analysis of the clostridial genomes. Genome Res. *17*, 1082–1092.

Taubes, G. (2008a). The bacteria fight back. Science *321*, 356–361.

Taubes, G. (2008b). Collateral damage. The rise of resistant *C. difficile*. Science *321*, 360.

Van Wart, H.E. and Steinbrink, D.R. (1981). A continuous spectrophotometric assay for *Clostridium histolyticum* collagenase. Anal. Biochem. *113*, 356–365.

Van Wart, H.E. and Steinbrink, D.R. (1985). Complementary substrate specificities of class I and class II collagenases from *Clostridium histolyticum*. Biochemistry *24*, 6520–6526.

Watanabe, K. (2004). Collagenolytic proteases from bacteria. Appl. Microbiol. Biotechnol. *63*, 520–526.

Wilkins, M.R., Gasteiger, E., Bairoch, A., Sanchez, J.C., Williams, K.L., Appel, R.D., and Hochstrasser, D.F. (1999). Protein identification and analysis tools in the ExPASy server. Methods Mol. Biol. *112*, 531–552.

Wilson, J.J., Matsushita, O., Okabe, A., and Sakon, J. (2003). A bacterial collagen-binding domain with novel calcium-binding motif controls domain orientation. EMBO J. *22*, 1743–1752.

Yeh, C.M., Yang, M.C., and Tsai, Y.C. (2002). Application potency of engineered G159 mutants on P1 substrate pocket of subtilisin YaB as improved meat tenderizers. J. Agric. Food. Chem. *50*, 6199–6204.

Yoshihara, K., Matsushita, O., Minami, J., and Okabe, A. (1994). Cloning and nucleotide sequence analysis of the colH gene from *Clostridium histolyticum* encoding a collagenase and a gelatinase. J. Bacteriol. *176*, 6489–6496.

Received August 11, 2008; accepted September 22, 2008