Chapter 4: Modes of Action of Enzymatic Débridement

Over the years, various proteolytic enzymes have been employed (papain, ficin, streptokinase, streptodornase, trypsin-chymotrypsin, etc.) for the débridement of wounds. As mentioned in chapter 1, the only remaining (widely used in the US) commercially available enzymatic débrider is clostridial collagenase. One reason for this turbulent history may be related to an enzyme's ability to degrade collagen. Howes et al. (1959) and Rao et al. [1], have demonstrated that necrotic tissue (which itself is very rich in collagen and denatured collagen) is anchored to the wound surface by strands of undenatured and partially denatured collagen fibers. Until these fibers are severed, débridement cannot take place, granulation is slowed, and thus no supportive base is available for proper epithelialization. Another aspect may be the fact that most enzymes used historically have not been highly selective in their catalytic activity. Nonselective being the inability to distinguish between healthy and necrotic tissue. Other concerns exist around the safety (i.e., anaphylactic shock in the case of papain) and/or FDA rulings/drug classifications. All of these aspects have resulted in the removal of many topically applied enzymatic débriders from clinical use. The one exception would be clostridial collagenase, which is felt to be more selective than the enzymes mentioned, previously. In addition, the FDA has sited no real safety concerns or regulatory designation concerns for bacterial collagenase.

In this chapter we detail the form, function and mode of action (MoA) of clostridial collagenase, as well as, the catalytic MoA of endogenous collagenase. It should be noted that the following information is meant to be general, scientific in nature and not necessarily linked to any topically applied enzymatic débridement formulations (Figure 1).
Here we see a very simplified depiction of the catalytic mechanism of metalloproteinases (both endogenous and exogenous) on a single α-strand (from the triple helical structure of a collagen molecule). The reaction leads to the formation of a noncovalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of a glutamic acid proton to the leaving group. The mechanism is hydrolysis [2].

Figure 2 shows a more detailed depiction of the zinc catalyzed hydrolysis reaction proposed for MMPs, with the catalytic zinc ion as a sphere and hydrogen bonds as dashed lines (Figure 2). The three histidine ligands are represented by sticks. Proton transfer could hypothetically occur before or after scissile-bond cleavage [3]. Again, a single α-strand is ‘processed’ by the MMP. This mechanism will be addressed in much more detail in the following pages.

As a result of a rather extensive investigation into the MoA collagenase systems, the following summary can be made:

**Endogenous/mammalian collagenase (~ 64-75 KDa):**

Collagenase cleaves the triple stranded helix at a single point, (Gly$^{775}$–Ile$^{776}$ in the α$^1$ chains; Gly$^{775}$–Leu$^{776}$ in α$^2$ chain), which is located approximately three fourths of the distance from the N-terminus of the collagen molecule.

Results in 2 fragments a 225-kDa fragment (TC$_a$) and a 75-kDa fragment (TC$_b$).

Fragments denature spontaneously into randomly coiled gelatin peptides.

Gelatin peptides are attacked by a variety of enzymes (less specific), including the gelatinases (MMP-9 and 2).

These simple steps can be represented as follows (note, the arrow depicts the location of initial attack) (Figure 3):

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*Figure 2: Show a more detailed depiction of the zinc catalyzed hydrolysis reaction proposed for MMPs, with the catalytic zinc ion as a sphere and hydrogen bonds as dashed lines.*

*Figure 3: This description though accurate is simplistic and more recent research has greatly expanded our understanding of the MoA of endogenous collagenase.*
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The catalytic MoA of MMPs has been investigated for over 50 yrs. and there are a wide range of theories surrounding the MoA. A good place to start would be with Jeffrey in 1995 [4]. In this white paper, the MoA is described as follows and represents an accepted theory at the time. As in previous literature the type I collagen molecule is described as being comprised of two α1 chains and one α2 chain. Collagenase secreted as a proenzyme contains a zinc atom that is chelated in the molecule by cysteine residues [5]. Once activated, mammalian collagenase binds to collagen, which is typically aggregated into a fibril, and makes a single cleavage across all three chains of collagen (possibly, suggesting this occurs in a single step), resulting in two fragments: a larger 225 kDa fragment (known as TC A) and a 75 kDa fragment (TC B). Also, in this review it is noted that one area of research has centered on the crucial role played by water molecules in the process of collagenolysis [6,7]. Proteolysis is the hydrolysis of the peptide bond. A molecule of water is needed for every peptide bond to be hydrolyzed. In gelatin, access of water to a peptide bond in a random coil is easy. In collagen, the triple helix has a very hydrophobic center. The peptide bonds are arranged in a helical array (forming a cylinder), with the amino acid side chains arranged to the outside of the cylinder. Hence, the accessibility of water to the peptide bond is difficult and requires energy [4]. It seems clear that the aggregation of collagen molecules into fibrils is accompanied by a significant exclusion of water from between the molecules that make up the fibril. As a result of this hydrophobicity, the rate of collagenase activity is slowed considerably, which explains why collagen degradation is a very slow enzymatic process [8]. Furthermore, as the fibrils age and perfect their aggregated state, still more water is expressed from the interior of the fibril, slowing the rate of collagenase activity even more, perhaps by as much as 5-fold or even greater. The exclusion of water as collagen fibrils biologically age appears to present a formidable barrier to degradation \textit{in vivo}. It is even conceivable that some collagen fibrils are essentially non-degradable for this reason. Once bound to the molecule, the enzyme appears to move from molecule to molecule within that fibril without an intervening dissociation step [8]. This suggests that collagen in old skin would be more difficult to degrade than collagen in young skin, because it has probably lost GAGs and other molecules that keep the tissue hydrated. Clostridial collagenase hydrolyzes the bonds on the outside of the triple-stranded collagen helix, where water is most available. Mammalian collagenases catalyze the hydrolysis of the bonds closest to the center of the helix, where the environment is mostly hydrophobic and water is at a minimum. This spatial relationship of the water and bacterial collagenase’s MoA allows it to degrade collagen at a much faster rate than mammalian collagenase [4]. In a more recent \textit{in vitro} study [9], we see some very interesting adjustments to the earlier theories with respect to the catalytic MoA. One key aspect is the realization that the diameter of the active site of the collagenase molecule (~5Å) is too small to accommodate the triple helical collagen molecule (~15 Å), not to mention the collagen microfibril (~40Å) comprised of 5 individual collagen molecules [10].

Chung, focusing on the three-dimensional structure of a prototypic collagenase, MMP-1 (collagenase-1) indicates that the substrate-binding site of the enzyme is too narrow to accommodate triple-helical collagen. It is reported that collagenases bind and locally unwind the triple-helical structure before hydrolyzing the peptide bonds of each of the 3 α-chains (one at a time). In support of this theory a mutation of the catalytically essential residue Glu200 of MMP-1 to Ala resulted in a catalytically inactive enzyme, but in its presence non-collagenolytic proteinases digested collagen into the typical 3/4 and 1/4 fragments. From this finding Chung suggests that the MMP-1(E200A) mutant unwinds the triple-helical collagen. The study also shows that MMP-1 preferentially interacts with the α2(I) chain of type I collagen and cleaves the three α chains in succession, rather than all at once. 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. These MMPs cleave the three a 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.

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 were considered in the same year as Jeffrey [11]. The substratebinding 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 α1(I) chains and one α2(I) chain, is ~3,000 Å in length and ~15 Å in diameter [9]. Thus, the triple-helical collagen does not fit into the active site cleft of the enzyme. Molecular docking attempts to place the triple-helical model of Kramer et al. [12], 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. Chung et al. [9], concludes that either the active site of MMP-1 undergoes large conformational changes or that the triple-helical collagen needs to be unwound, so a single α-chain can fit into the active site of the enzyme. In an effort to demonstrate collagen unwinding by MMP-1, the researchers performed a series of in vitro experiments. Glu200, the residue essential for peptide hydrolysis, was mutated to Ala. They 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 non-collagenolytic enzymes. The MMP-1(E200A) mutant was essentially inactive and unable to cleave the α1(I) and α2(I) chains of collagen I. As demonstrated previously [13], the catalytic domain of MMP-1 lacking the C-terminal Hpx (hemopexin) like (attachment) domain (MMP-1(ΔC)) also could not cleave collagen I. However, when collagen I was incubated with MMP-1(E200A) and MMP-1(ΔC) at 25°C, it was cleaved into the typical 3/4 (TCα) and 1/4 (TCβ). So, essentially the authors concluded that with MMP-1(E200A)... thought to unwind the triple helical molecule and MMP-1(ΔC)...catalytic domain without attachment domain (Hpxhemopexin like) was able to produce the two initial fragments (TCa and b). NH2-terminal sequencing of the TCβ fragments indicated that MMP-1(ΔC) and MMP-3 (stromelysin-1, noncollagenolytic proteinases) cleaved the Gly775–Ile776 bond of the α1(I) chain(s) and the Gly775–Leu776 bond of the α2(I) chain in the presence of MMP-1(E200A). It was notable that the α1(I) chain(s) was cleaved more rapidly by non-collagenolytic proteinases in the presence of MMP-1(E200A) compared with the active MMP-1 alone. From this Chung suggests that the unwinder MMP-1(E200A) preferentially interacts with the α2(I) chain, which renders the α1(I) chains more exposed and susceptible to a cutter proteinase. This suggests that the unwinding of collagen by MMP-1 takes place only locally, and it does not affect the overall triple-helical structure. The requirement of higher concentrations of the unwinder and the cutter to de cleave collagen suggests that both components must simultaneously bind to the collagen substrate. In the case of HpxMMP-1 and MMP-1(ΔC), the ratio of the α1(I) to α2(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.

In general terms 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. 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. Chung goes on to mention that this is the first demonstration that a single polypeptide proteinase induces significant structural changes in the substrate prior to peptide bond hydrolysis. 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 [11,14,15]. This includes: a **proline zipper** model, 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 [11]. However, the intact linker region may not be necessary as the catalytic domain and the Hpx domain added together can cleave collagen, as per Chung. According to Chung the collagen trapping model is also inconsistent with the observation that non-collagenolytic proteinases can cleave α1(I) and α2(I) chains in the presence of MMP-1(E200A), whereas in the aforementioned model they would be protected by the Hpx domain. Chung also considered the following two other possible mechanisms:

Collagenase stabilizes the partially unwound state of collagen that may occur spontaneously around the collagenase-susceptible region.

Conformational changes occur within the collagenase molecule in such a way that it accommodates the triple-helical collagen in the active site.

As these two possibilities are also inconsistent with Chung’s results, Chung concluded that the critical aspects of the collagenolytic specificity rely on the structural changes in collagen, induced by interacting with collagenase.

Still, additional (and sometimes competing) theories on the MoA are described in a review paper by Duarte [16]. In this review more detail is added to the discussion. MMP-1 is described as having a C-terminal HPX-attachment domain that comprises a four-bladed βpropeller, and is linked to the catalytic domain via a flexible hinge region. It has been reported that this linker peptide has a critical role assisting collagen **binding/unwinding** before collagenolysis, either by direct binding of the substrate [11], or by allowing the proper alignment of the CAT (catalytic) and HXP (attachment) domains [17]. MMP-1, as well as the other “classic” collagenases (MMP-8 and MMP-13), hydrolyzes interstitial (fibrillar) collagens I, II and III into the characteristic 1/4 and 3/4 length fragments, at a region on collagen molecule more **susceptible** to conformational changes. It was also shown that the conformational arrangement of the hinge region of MMP-1 is crucial in the accurate positioning of HPX and CAT domains prior collagenolysis [18]. Note, in earlier work Chung pointed out that this ‘linker region’ may not be necessary for catalysis. As previously mentioned, crystalline structures of MMP-1 [19,20] and MMP-8 showed that the catalytic cleft of these enzymes is too narrow (~5 Å) to accommodate the collagen triple helix (~15 Å in diameter). The hypothesis that the triple-helical collagen needs to be **unwounded**, so that a single a chain can fit into the active site of the enzyme has experimental support, as well [21-23]. Using NMR and small angle X-ray scattering, Bertini and co-workers have observed an open/extended and a closed conformation of MMP-1 [21,22]. Also, the structures of MMPs and MMPpeptide complexes showed specific interactions between the collagenase and a triple-helical peptide (THP, composed by three chains: 1T, 2T, and 3T), used as a collagen model [18]. Bertini’s data suggest that collagenolysis relies on multiple exosites (secondary binding sites, remote from the active site) interactions, where MMP-1 domains interact cooperatively with the three different α-chains of collagen. These interactions allow the scissile bond to be correctly positioned at the active site, and, at the same time, the molecular **stretching** of the substrate promotes the local unfolding of collagen that is required for cleavage. The first proposition for a detailed mechanism describing the collagenolysis by MMP1 [18,21,22] considers four main steps:

In the extended (opened) conformation, MMP-1 binds to the triple-helical peptide 1T-2 T at Val23-Leu26 via the HPX domain: due to the flexibility of the linker region, the CAT domain is guided to the residues around the cleavage site (Gly16-Ile17 of chain 1T, corresponding to the Gly775–Ile776 bond on α1(I) collagen).
Back-rotation of the CAT and HXP domains leads to the closed MMP-1 conformation; this promotes unwinding of the triple-helical peptide and docking of the 1T chain into the metalloproteinase active site.

Hydrolysis occurs and, initially, both peptide fragments remain bounded to the active site.

The C-terminal region of the N terminal peptide fragment is released; afterwards, MMP-1 hydrolyzes the peptide bonds of each remaining chains in succession.

Most data concerning the mechanisms of collagenolysis, including data from the action of bacterial collagenases, have been interpreted based on this paradigm – that there is a local unfolding of collagen prior to cleavage [16,24], which is detailed in the next section, ‘bacterial collagenase’. However, as is common in scientific research, there are alternative theories. Hydrolysis at room temperature – a temperature well below the melting temperature of type I collagen, Tm <36°C [25] – is achieved without the HPX binding domain (non-catalytic) of MMPs that was known for being involved in the binding and the unwinding of triple-helical collagen [23,26]. Recall that Chung credits the unwinder MMP-1(E200A) mutant with the unwinding activity. In a break from the unwinding theory of collagenase activity it was also demonstrated that these collagenases preferentially recognize and hydrolyze partially unfolded states of collagen. These substrate-centered observations led the authors to propose an alternative mechanism of collagenolysis, in which collagenases do not act as triple-helicases. This proposal assumes that collagen is flexible in the vicinity of the cleavage site and in consequence, digestion occurs without collagenases actively unwinding the triple-helical collagen [26], an energetically costly task. It is possible to speculate that, within connective tissues, collagens and collagenases may interact with several other components of extracellular matrices like fibronectin, integrins, etc. Those interactions may induce conformational alterations on both enzyme and substrate which, in turn, impact the interaction between MMPs and their triplehelical substrates. So, clearly there are competing theories in the literature with respect to the details of the MoA of MMPs.

**Bacterial collagenase**

Bacterial collagenase, although a zinc metalloenzyme requiring calcium for its activity, bears little structural relationship to mammalian collagenase. Bacterial collagenase rapidly attacks and degrades human collagen into small peptides. In vitro, human types I and III collagen, extracted and purified from placental tissue, was digested by incubation with bacterial collagenase. After analysis on Superox-30 gel sieve chromatography, the breakdown products were shown to be of the size of di- and tri-peptides. The collagen-derived peptides were then added to rat fibroblast culture to evaluate the effects of these breakdown products on cell proliferation and biosynthetic activity. By means of the neutral red test, stimulated cell proliferation was demonstrated when collagen breakdown products, at a concentration of 5 to 50 ng/mL of medium, were added by Cortivo et al. [27], Postlethwaite et al. [28]. Other authors have observed migration of a variety of cell types (keratinocytes, endothelial vascular cells, fibroblasts, etc.) key for wound progression in response to exposure to clostridial and endogenous collagenases [28-33]. More recently it has been demonstrated (in vitro) that collagenase-driven digestion of human cellular-synthesized extracellular matrices yields several collagen and non-collagenous peptides with known and unexpected activities linked to wound progression and the cellular responses to injury, including cellular migration, proliferation and angiogenic activation. Pre-clinical cell based assays reveal that the bacterial collagenase elaborated and combinatorial peptides identified/synthesized possess significant growth-promoting, migration-enhancing and angiogenesis inducing activities when tested in the 10-100nM range. Also, in this work in vivo (murine) models were used to demonstrate cell migration/proliferation as a result of exposure to the aforementioned peptides. However, the peptides were much larger than those described by Cortivo et al, 1995 and Postlethwaite et al. 1978 [27,28]. In the more recent work the peptides ranged from 8 to 20 amino acids long [34]. Other literature suggests reduced scarring, acute/chronic wound progression, antiinflammatory properties, growth factor release, growth factor cascade
initiation, etc., as effects of bacterial collagenase interaction with wound matrix components. Though these aspects warrant much more investigation, it would appear that bacterial collagenase has the ability to rapidly digest collagen and promote other cellular functions beneficial in wound repair.

Bacterial collagenase is made up of proteolytic enzymes that break collagen into small peptides of differing molecular weights. Seven collagenases (isoforms) have been identified in the purified culture filtrate of *C. histolyticum*, and all have been purified to homogeneity. It should be noted that only two genes, colG and colH transcribe for two metalloproteases. However, due to post-transcriptional autolytic events, seven truncated collagenases (isoforms) have been identified. The seven collagenases can be divided into two classes, I (α, β, γ, η) and II (δ, ε, ξ), which are classified based on their bacterial gene of origin (colG and ColH, respectively) and on their point of hydrolytic attack on the collagen molecule – class I enzymes act at loci near the ends of the collagen triple-helical domains, whereas class II enzymes make internal initial cleavages. These collagenases uniquely cleave the interstitial collagens and exhibit both endopeptidase and tripeptidylcarboxypeptidase activities. The combined activity of endo- and tripeptidyl-C-peptidase makes these enzymes ideally suited for rapid collagen degradation. Their combined action at many sites along the peptide chain results in the sequential cleavage into short segments. Both classes have specific binding domains that enable them to recognize and bind to triple helical collagen, in a variety of locations [35-38]. An interesting note, in the work by French who first studied the isoforms of collagenase described them as attacking at hyper-reactive cleavage sites suggesting that type I, II, and III collagens contain regions that have specific non-triple helical conformations [36]. In other words, there is no need to unwind the triple helix. So, again, we see competing theories with respect to the MoA of bacterial collagenase, as there are for endogenous collagenase.

As a reminder, in contrast to clostridium histolyticum collagenases, mammalian collagenases act differently by cleaving interstitial collagen at a single locus within the triple helical structure, giving rise to 2 large fragments, TCA and TCB [4,39]. These portions of the helix are then attacked by other nonspecific proteases, released by connective tissue cells, to be further degraded into small peptides [27].

An interesting thought with respect to bacterial collagenase and endogenous collagenase was put forth by Parks in 1995 [29]. The migration (obligatorily occurring within a viable part of the wound bed) of cells responsible for removing non-viable collagen is hindered by necrotic (non-viable) tissue. For this reason, little if any collagenase expression (from migrating cells) would be detected in necrotic regions. Alternatively, bacterial collagenase, when topically applied, attacks proteins within the superficial and necrotic (non-viable) areas of the wound [29]. It could be argued that bacterial collagenase treatment is not simply augmentation therapy, but rather provides an essential biochemical activity to areas where in the cells are not producing proteases. Thus, because of these critical and significant spatial differences and because of the marked differences in the matrix proteins that these proteinases can degrade, it is easy to conceive that the catalytic activity of these two collagenases can result in different effects in repair [29]. This is an interesting thought; however, it should be noted that collagenase-2 (MMP-8, neutrophil/PMNL collagenase) would still be present in chronic wounds halted in the inflammatory phase of wound healing. In addition, other sources suggest the presence of collagenase expressed by other cell types in necrotic tissue (due to the expression of pro-inflammatory cytokines by inflammatory cells- Parks, 1995) [29], whereas, other sources state the lack of collagenase expression in necrotic tissue [30].

It has been shown in diabetic ulcers that necrotic tissue is anchored to the wound surface by a layer of perpendicular strands of undenatured collagen [1]. However, it is likely that some of these collagen strands are partially denatured. Collagenases, by definition, are enzymes capable of solubilizing fibrous collagen (both native and denatured collagen) by peptide bond cleavage under physiologic pH and temperature conditions. Thus, collagenase attacks not only necrotic tissue, but also fibers of undenatured collagen. It
is suspected that the fibers of undenatured collagen anchor the eschar plug to the wound bed. With use of topically applied collagenase, the entire ‘necrotic plug’ could be released and the remaining anchoring fibers would be removed. This would tend to lead to a cleaner and more thoroughly prepared wound bed. It has also been documented that, at times, collagenase treatments have resulted in a decrease in visible scarring [40,41].

Since necrotic tissue is an important local cause of failure of wound progression, it would seem obvious that such binding must be severed, so that debridement and wound progression can occur. Otherwise, granulation is slowed and no supportive base is available for epithelialization. Collagenase is irreversibly inactivated in a low-pH environment. It functions best in the pH range of 6 to 8 and temperatures below 56°C. Chelating agents (EDTA, citric acid, sodium citrate, etc.) also inactivate the enzyme by interacting with Ca²⁺ ions and Zn²⁺ ions, essential constituents of the structure (and function) of collagenase. It is well known that four Ca²⁺ ions play a role in stabilizing the 3⁰ structure of the protein near the active site. It is also well known that a Zn²⁺ ion is located in the active site and is necessary for enzymatic activity. Collagenase hydrolyzes the peptide bonds in collagen. It does not attack other proteins such as hemoglobin or fibrin, important components in the formation of granulation tissue. In addition, collagenase does not attack growth factors, tissue inhibitors of metalloproteinase (TIMPs), and other critical components of the wound repair cycle [40], whereas, other, less specific enzymatic systems, such as papain–urea, were reported to have a negative effect on platelet-derived growth factor (PDGF), and perhaps (based upon its MoA) other growth factors, TIMPs, integrins, etc. One could postulate that this lack of selectivity is one reason these historical topical enzymatic formulations are no longer widely used or even available.

Collagenase has been reported as an effective agent for the débridement of thermal burns. Although any protease would thoroughly digest degraded matter in the center of the burn eschar, only collagenase would effectively attack necrotic edges of the eschar, including the perpendicular fibers of undenatured collagen. These perpendicular collagen fibers anchor the eschar plug to the wound bed, and their removal is likely to be key for optimal wound bed preparation.

Collagenase has also been found to be useful in the débridement of third-degree burns. Otteman and Stahlgren compared the lytic effects of a number of enzymes on experimentally induced burns. The enzymes studied included streptokinase–streptodornase, trypsin–chymotrypsin, papain, ficin, desoxyribonuclease–fibrinolysin, and collagenase. Of these enzymes, only collagenase and papain were more than 90% effective in the digestion of wound debris and necrotic material [42].

The design/structure of the active site of bacterial collagenase allows it to cleave the triple helical collagen at many different points. Anywhere a Gly-X-Y (where X = proline and Y= hydroxyproline) exists, it is felt bacterial collagenase can attack [29,35]. Collagen is unique among proteins in that every third amino acid of the peptide chain is glycine, the smallest amino acid. Each of the 3 polypeptide chains contains about 1,000 amino acids, so the structure of each chain can be considered to be 330 repeating units of glycine–X–Y; where X = proline and Y= hydroxyproline [43]. More recent sources provide even more information as to the point of attack. One source describes 10.5% of the collagen molecule being comprised by the glycine-proline-hydroxyproline triplet [44]. Another source mentions that 23% of the molecule is comprised of a combination of proline and hydroxyproline [45]. Yet, a more recent source describes proline ~28%; hydroxyproline ~38% of the collagen molecule [46]. Given this, it is possible that on a single α-chain, there could be ~100 to ~330 locals where bacterial collagenase might attack.

As a result of a rather extensive investigation into the MoA collagenase systems, the following summary can be made:

Bacterial collagenase (~ 115-120 KDa):
1. MoA is very similar to that of mammalian collagenase with a few important distinctions.

2. The bacterial collagenase does not cleave the triple helical collagen in a single place, but attacks at many different points.

3. Anywhere a Gly-X-Y (where X = proline and Y = hydroxyproline) exists, it is felt bacterial collagenase can attack (in theory).

This (along with the aforementioned examples/theories of MoA) helps to explain the more rapid degradation of collagen via bacterial collagenase when compared to endogenous/mammalian collagenase.

These simple steps can be represented as follows (note, the arrows indicate that there are multiple points of initial attack) (Figure 4):

Below we see 7 points of initial enzymatic attack of bacterial collagenase (at hyper-reactive sites on the collagen molecule/α-chains) as previously mentioned (Figure 5) [35-38].

In wound debridement it has been suggested that bacterial collagenase migrates to the base of the eschar where it degrades the strands of undenatured collagen fibers, which hold the eschar plug to the wound bed. For this reason, it has been suggested that bacterial collagenase works from the “bottom-up”. However, it makes more sense that the collagenase works from the top and bottom of the necrotic tissue, as denatured and partially denatured collagen are present throughout necrotic tissue (Figure 6) [38].

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**Figure 4:** These simple steps can be represented as follows (note, the arrows indicate that there are multiple points of initial attack.

**Figure 5:** We see 7 points of initial enzymatic attack of bacterial collagenase (at hyper-reactive sites on the collagen molecule/α-chains) as previously mentioned.
The following are depictions of one aspect of this action:

This description though accurate is simplistic and more recent research has greatly expanded our understanding of the MoA of bacterial collagenase.

Much work has gone into understanding the form and function of these enzymes used so widely in research and wound care. Here we will go into much greater detail on the role of Ca$^{2+}$ in the structure and function of bacterial collagenase. *Clostridium histolyticum* collagenases ColG and ColH are segmental enzymes that are thought to be activated by Ca$^{2+}$-triggered domain reorientation. A β-bulge and the genesis of a Ca$^{2+}$ pocket in the archaeal PKD-like (polycystic kidney disease-like) domain suggest a close kinship between bacterial and archaeal PKD-like domains. The conserved properties of PKD-like domains in ColG and in ColH include Ca$^{2+}$ binding. Conserved residues not only interact with Ca$^{2+}$, but also position the Ca$^{2+}$-interacting water molecule. Ca$^{2+}$ aligns the N-terminal linker approximately parallel to the major axis of the domain. Ca$^{2+}$ binding also increases stability. The collagen-binding segment composed of the PKD-like domain and collagen-binding domain(s) (CBD) is not necessary to degrade gelatin (denatured, non-triple-helical collagen) and acid-solubilized collagen. However, this segment is necessary to degrade insoluble collagen fibers. Full-length ColH has been shown to undergo Ca$^{2+}$-dependent structural changes [47]. In ColG, Ca$^{2+}$ triggers the linker region (linking the binding and catalytic domains) to undergo secondary structure transformation from an α-helix to a β-strand to increase collagen affinity [48,49]. The N-terminal linker structure of the PKD-like domain is also thought to be Ca$^{2+}$-dependent.

Here we see a very useful depiction of the domains of collagenases ColG and ColH from *C. histolyticum* (Figure 7) [50].

The signal peptide (grey hatching) is cleaved from the mature enzyme and indicated by sequence numbering N1–N110 (ColG) and N1–N40 (ColH). The collagenase module is composed of an activator subdomain (olive) and peptidase subdomain (dark olive) that is accompanied by a helper subdomain. The PKD-like domain(s) (yellow for ColG; blue and green for ColH) connect the collagenase module to the C-terminal CBD(s) (red for ColG; salmon for ColH) that are responsible for collagen binding.

Ca$^{2+}$ plays a key role in structural modification of bacterial collagenase. Ca$^{2+}$ chelation appears to align the N-terminal linker approximately parallel to the major axis of the domain. In s2b Ca$^{2+}$ chelation could stabilize a 310-helix that aligns with the cylinder axis. In s2 and s2a, the N-terminal residues are positioned so that the N-terminal linker could also be positioned parallel to the major axis of the domain (Figure 8).

Ca$^{2+}$ coordination in s2a (a) and s2b (b). Seven O atoms from five residues and one water molecule coordinate to Ca$^{2+}$ in a pentagonal bipyramidal geometry.

Ca$^{2+}$ plays a key role in structural stability of bacterial collagenase. As such, the conserved hydrogen-
bonding network may play a strong role in the overall stability of the domains. In the clostridial collagen-binding domain, Ca$^{2+}$-induced stability could be partially accounted for by a reduction in void volume and an increase in hydrogen bonds [51]. The apparent differences between the ColG-derived PKD-like domain and the ColH derived PKDlike domains may aid synergistic collagenolysis. Currently, it is not certain whether any of the clostridial PKD-like domains swell collagen fibers, though we will see that other authors feel this does occur. Both s3 and s3b share a common preference for under-twisted regions of collagen [52], although ColG and ColH initially cleave different sites in collagen [36]. When digesting the insoluble fiber, ColH is the workhorse [53]. The higher collagen affinity observed for s2b-s3 may be increase by the addition of s2a. The increased affinity could hold ColH close to the collagen fibril, so that it can slide along the fibril and find vulnerable regions [54]. Meanwhile, ColG has been proposed to adopt a compact structure in which the domains of the collagen-bonding segment are aligned by intermolecular $\beta$-sheet-type hydrogen-bond interactions [24]. The tandem CBDs of ColG may allow the enzyme to anchor itself to the most vulnerable region of the fibril. In this context, the ‘spring-like’ dynamics of s2 may allow it to swell the fibril. The swelled fibril would then expose the interior of the fibril and expose new sites for ColH collagenolysis.

Again, the collagenolytic mechanism differs between mammalian matrix metalloproteinases (MMPs) and bacterial collagenases [16,55]. Several authors have stated that unlike bacterial collagenases, MMPs are sequence-specific and are proposed to actively unwind the triple helix [6,18,39]. Meanwhile, each domain in bacterial collagenase is believed to play a unique role in collagenolysis [17]. Bacterial collagenase’s C-terminal CBD unidirectionally binds to under-twisted sites in the triplehelical collagenous peptide [52,56]. The CBD does not unwind mini-collagen, and hence targeting under-twisted regions of tropocollagen may circumvent the energy barrier required for unwinding the helix. Various roles have been proposed for the PKD-like domains. The PKD-like domain has been shown to swell, but not unwind, collagen fibrils [57].
Clostridial PKD-like domains do not bind tightly to collagen fibrils [58,50]. In an alternative theory, the N-terminal collagenase module has a two-domain architecture that disbands the collagen microfibril into monomeric triple helices and then cleaves the exposed peptide bond preceding the Gly residue [24,59]. It is clear that there are differing theories around the MoA of bacterial and endogenous collagenase, including differing theories with respect to the action of the enzyme on the collagen microfibril and the triple helical collagen molecule.

Vertebrate collagenases split collagen hydrolyzing the molecule at a single peptide bond across the three α chains organized in its native triple-helical conformation [60,61]. It is important to stress that a large number of bacterial proteases have the capacity to hydrolyze single-stranded and denatured collagen polypeptides. Those cannot be confounded with true bacterial collagenases, which are able to attack and degrade the triplehelical native collagen fibrils found in connective tissue. These clostridial collagenases are relatively large (~116 kDa). The high number of different active forms detected is related to (auto-) proteolysis events [62-64]. It has been suggested that truncated isoforms play important roles in the regulation of clostridial collagenases in vivo [65]. Previously, little was known about the true structure and hydrolysis mechanism of bacterial collagenases, in part due to the complexity of their multi-domain organization [50,62]. However, special efforts have been made to characterize the three-dimensional structure of ColG collagenase from *Clostridium histolyticum* by small angle X-ray scattering [51], by crystallographic analysis [24,48,59] and by single quantum coherence nuclear magnetic resonance titration [56]. Like other zinc peptidases, ColG contains a glutamate residue as the third zinc ligand. ColG catalytic zinc is tetrahedrally coordinated by His523, His527, Glu555 and a water molecule [24]. The collagen binding domain (CBD) of ColG [48,56], the PKD-like domain [24,59,66] and the collagenase unit [59] were the first structures of a bacterial collagenase to be analyzed, enabling the construction a full-length structural model of ColG [24]. From these studies, a chew and digest mechanism of bacterial collagenolysis arose [24]. Eckhard and co-workers concluded that, similarly to MMPs, collagenase G can switch between opened and closed states. In the closed state, the triple-helical collagen acts as a source of attraction between both domains of the collagenase module (the activator and the peptidase domains). Thus, the collagenase module gains a saddle-shaped architecture in an opened state that clamps the fibril, facilitating the peptidase domain accessibility to the monomeric triple-helices [24]. A closed conformation is achieved when collagen interacts with the activator domain (AD); the triple-helix α-chains are consequently unwound and progressively cleaved. The crystallized open structure has a cavity distance of ~42 Å flanked by the peptidase domain and AD; thus, it is acceptable to speculate that ColG can process collagen microfibrils (~35 Å in diameter).

Experimental evidence has increased understanding of the function of the different domains (subdomains) previously reviewed. ColG functional domains:

The CBD(s) locate and anchor the enzyme to collagen by specifically recognizing their triple-helical conformation (ColG CBDs promote interaction with fibrils, not with individual triple helices).

The PKD-like domain(s) swell and prepare the substrate without triple helix unwinding [57].

The collagenase unit degrades the prepared collagen molecules, digesting them from microfibrils of 35 Å diameter downwards [66]. Prior to collagenolysis, ColG follows a two-step mechanism similar to MMPs, in which unrolling collagen microfibrils and unwinding the triple-helical collagen are prerequisites for cleavage. The chew and digest mechanism is consistent with the existence of the five-stranded Smith microfibril, a minimal filamentous structure, with a diameter of approximately 40 Å [10,24].

Considering MMPs for a moment, it should be noted that (with respect to HPX-like domain of MMP 1, for example) such models (as above) assume that the cleavage region of the collagen molecule is as readily accessible in the fibrillar form as it is in a single isolated collagen molecule. The ‘sandwich model’ would require that the triple-helix region, carrying the cleavage site sequence, juts out of the fibril surface to allow
it to be surrounded by the N- and C-terminal domains of MMP 1. Some feel that it is more appropriate to assume that only selected parts of the triple helix will be accessible from the surface of the fibril, be it for enzymatic degradation, or the location of sites suitable for cellular interaction [23]. In this work (utilizing computational and molecular visualization methods) the extent of peptide chain disassociation from the center of the triple helix (which indicates vulnerability to proteolytic attack) was measured in the cleavage site region. This allows the viability and biological relevance of the ‘α2 chain first’ hypothesis of collagen cleavage, to be assessed within the natural, fibrillar context [9,23]. It was found that although there is no significant difference in the magnitude of triple-helix disassociation of the three peptide chains over the whole of the proposed enzyme interaction region, the α2 chain is more disassociated than the α1 chains at the actual cleavage position.

In comparison to MMPs, interestingly, the most efficient collagenases are those found in clostridial bacteria. Focusing on the domain architecture of ColG, Clostridial, and other bacterial collagenases have an approximate size of 120 kDa (close enough to the ~116 kDa described by Duarte, previously). Based on naturally occurring isoforms and in vitro analysis, their domain organization was expected to be composed of a pre-domain (the pre-pro-peptide mentioned by Duarte) of variable length containing the export signal which is clipped in the mature protein; an N-terminal domain harboring the catalytic zinc. The crystalline structure indicates a distinct segmentation of the N-terminal collagenase module featuring a saddle-shaped two-domain architecture, as previously mentioned. The smaller N-terminal saddle flap serves as an activator domain and comprises an array of 12 parallel α-helices. Starting with a distorted helix pair, it continues with ten HEAT motifs (tandem repeat protein structural motif composed of two alpha helices linked by a short loop) ideally suited to generate a protein recognition interface [67]. A solvent-exposed glycine-rich linker is positioned at the twist of the saddle seat. The subsequent catalytic subdomain adopts a thermolysin-like peptidase (TLP) fold of mixed α and β type [68] and is accompanied by a catalytic helper subdomain. The flanking α-helix pairs of the activator domain and the catalytic subdomain combine to form a distorted four-helix bundle and thus comprise the seat of the saddle, which is completed by the glycine-rich linker. These structural elements latch the relative spatial arrangement of the peptidase domain and the N-terminal activator critical for collagen binding and triple-helix unraveling. Eckhart found that the peptidase domain was completely inactive towards collagen substrates. Full collagenolytic activity is, however, contained in the segment comprised of the activator and peptidase domains. The catalytic Zn²⁺ is tetrahedrally coordinated by the side chains of 3 amino acids and a water molecule. Eckhard also suggests that the substrate (collagen) contributes to the stabilization, and correct positioning of the catalytic Zn²⁺ [24,69].

As previously mentioned, the crystalline structure with the observed enzymatic properties of the different ColG variants suggests a two-step mechanism, whereby the N-terminal activator domain cooperates with the peptidase domain in both collagen triple helix and microfibril recognition and processing. The activator and peptidase domains, forming the two saddle flaps, have a distance of ~40 Å, whereas the diameter of the collagen triple helix is only approximately 15 Å. Eckhard proposes that clostridial collagenase can adopt two conformational states: In addition to the crystallized open state, there exists a closed state which allows the collagen triple helix to contact both the activator and peptidase domain. The closed state is latched by two major contacts at the bottom of the saddle and by an alternative 4-helix-bundle arrangement at the saddle seat. Only now are the activator HEAT-repeats able to interact with triple-helical collagen, and initiate the unwinding of the triple-helix α-chains which are cleaved one at a time [35,36]. Based on this MoA it is postulated that the activator and peptidase domains remain mostly closed during collagen cleavage, but relax to the open ground state, once the collagen is degraded. This allows the enzyme to accept the next section of the collagen molecule to be processed.

It should be noted that in the papers by French et al (just mentioned and referenced by Eckhard) describe collagenase acting at hyper-reactive cleavage sites suggesting that type I, II, and III collagens contain regions that have specific non-triple helical conformations.
Another interesting finding via crystalline structure analysis is that the ground state/open state conformation has dimensions similar to the dimensions of collagen microfibrils, ~40 Å in diameter. Though admittedly more speculative, this model provides an elegant mechanism of how collagen microfibrils are proteolytically processed. Upon transition from the microfibril-loaded open state to the closed state, ColG will crimp the microfibril with its pliers, with only one triple helix remaining within the collagenase pliers. Analogous to the triple-helical processing, one triple helix remains embraced by the activator and peptidase domains until it is completely processed, after which the collagenase will relax to the open conformation, allowing the remaining triple helices of the microfibril to enter the collagenase. Consistent with transition state theory of enzyme catalysis, the substrate is bound to the active site in a highly distorted conformation. A more efficient substrate distortion as compared to the MMPs may be achieved. If this is the case the activation energy for the catalysis would be lower for bacterial collagenase lending to a more efficient processing of collagen with respect to endogenous collagenase, as has been a common theme in the literature. As described here and elsewhere, unraveling collagen (micro)fibrils and unwinding triple-helical collagen are felt to be prerequisites for collagen cleavage, both for mammalian MMPs and clostridial collagenases. As we have seen clostridial collagenases were traditionally divided into classes, class I and class II. Whereas the latter group is highly active towards peptidic substrates, class I enzymes such as ColG have a particular preference for collagen substrate degradation in a processive manner [24,59]. Processing fibrillar collagen substrates includes two dimensions. First, the cutting of a (micro-) fibril at one site of the substrate. Second, (for the triple-helices), multiple cleavage events along the substrate, a process described as inch-worming by Overall and colleagues in reference to MMP-9 and -2 [70]. The structure suggests how the accessory domains assist in both aspects of the ‘processing’ of collagen. When degrading microfibrillar collagen, the activator and peptidase domains have to open to allow for the remaining microfibril to enter the collagenase active site. It is felt that the accessory domains prevent an inadvertent shift of the substrate. On triple helical collagen, but also on microfibrils, the accessory domains help to direct the collagenase module along the substrate. This directed movement implies directionality with respect to processing. Given the tri-carboxypeptidase activity of ColG, it is suggested that the collagen processing occurs from the C-terminus of the collagen substrate to its N-terminus.

Domain organization and architecture of ColG (a) Schematic of the domain organization of ColG together with a functional annotation as depicted by Bauer et al. 2015 [54]. The catalytic Zn²⁺ ion (yellow dot) and the catalytic residues (red stars) within the peptidase domain are indicated. (b) Ribbon representation of the collagenase module, with identical color code as in a. The position of the PKD-like domain (yellow ribbon) at the rear of the peptidase domain is indicated in surface representation, reflecting a positional variance of up to 10 Å. The saddle-shaped collagenase is composed of an activator and a peptidase domain. The catalytic Zn²⁺ and the catalytic residues are highlighted by ball and stick representation. The seat of the saddle is formed by the distorted four-helix bundle, represented by four cylinders, and completed by the glycine-rich hinge, shown in green. (c) Full-length model of ColG in complex with a collagen microfibril. The collagenase module (colored as in a) bound to a modeled collagen microfibril (in surface representation; grey) is shown in ribbon representation. The accessory domains are shown as surface representation. The two collagen binding domains (orange). Direction of collagenase processivity is indicated at the right top (Figure 9).

Unified processing model of triple-helical and microfibrillar collagen (a) A collagen triple helix (green) initially ‘docks’ to the peptidase domain of collagenase. In the open state, the activator (dark blue) cannot interact with the substrate (i.e., no hydrolysis can occur). (b) Step 2, closed conformation, showing the activator HEAT repeats interacting with the triple helix, which is a prerequisite for collagen hydrolysis. (c) Step 3, semi-opened conformation, allowing for exchange and processive degradation of all three α-chains, one at a time [35,36]. Once the triple helix is completely cleaved, the collagenase can relax back to the open ground state conformation. (d) Collagenase with a ‘docked’ collagen microfibril (grey). The micro-fibril typically consists of five triple-helical molecules; the triple helix analogous to (a) is indicated.
in green. (e) Step 2, closed conformation with all triple helices but one (green) being expelled from the collagenase. The microfibril ‘wound’ caused by the triple-helix stripping is indicated in purple. (f) Step 3, semi-opened conformation allowing for the complete processing of the triple helix, indicated in green. Then the collagenase will relax back to the ‘open state’ allowing the remaining part of the microfibril to enter the collagenase for processing of the next triple helix. This will occur 3 additional times to process a total of 5 triple helices making up the microfibril (Figure 10).

Figure 9: Domain organization and architecture of ColG (a) Schematic of the domain organization of ColG together with a functional annotation as depicted by Bauer et al, 2015 [54]. The catalytic Zn2+ ion (yellow dot) and the catalytic residues (red stars) within the peptidase domain are indicated. (b) Ribbon representation of the collagenase module, with identical color code as in a. The position of the PKD-like domain (yellow ribbon) at the rear of the peptidase domain is indicated in surface representation, reflecting a positional variance of up to 10 Å. The saddle-shaped collagenase is composed of an activator and a peptidase domain. The catalytic Zn2+ and the catalytic residues are highlighted by ball and stick representation. The seat of the saddle is formed by the distorted four-helix bundle, represented by four cylinders, and completed by the glycine-rich hinge, shown in green. (c) Full-length model of ColG in complex with a collagen microfibril. The collagenase module (colored as in a) bound to a modeled collagen microfibril (in surface representation; grey) is shown in ribbon representation. The accessory domains are shown as surface representation. The two collagen binding domains (orange). Direction of collagenase processivity is indicated at the right top.

Figure 10: Unified processing model of triple-helical and microfibrillar collagen (a) A collagen triple helix (green) initially ‘docks’ to the peptidase domain of collagenase. In the open state, the activator (dark blue) cannot interact with the substrate (i.e., no hydrolysis can occur). (b) Step 2, closed conformation, showing the activator HEAT repeats interacting with the triple helix, which is a prerequisite for collagen hydrolysis. (c) Step 3, semi-opened conformation, allowing for exchange and processive degradation of all three α-chains, one at a time [35,36]. Once the triple helix is completely cleaved, the collagenase can relax back to the open ground state conformation. (d) Collagenase with a ‘docked’ collagen microfibril (grey). The microfibril typically consists of five triple-helical molecules; the triple helix analogous to (a) is indicated in green. (e) Step 2, closed conformation with all triple helices but one (green) being expelled from the collagenase. The microfibril ‘wound’ caused by the triple-helix stripping is indicated in purple. (f) Step 3, semi-opened conformation allowing for the complete processing of the triple helix, indicated in green. Then the collagenase will relax back to the ‘open state’ allowing the remaining part of the microfibril to enter the collagenase for processing of the next triple helix. This will occur 3 additional times to process a total of 5 triple helices making up the microfibril.
Summary

From this chapter one gets a sense of the amount of investigative work performed over the past 50 to 60 years in the area of wound débridement via topical application of bacterial collagenase as a debriding agent. From this and previous chapters one also gets a sense of the variety of differing viewpoints on mechanisms of action of both endogenous and bacterial collagenases, as well as, on the effects on viable tissue of the various enzymatic débriders used clinically in the past and present. Eckhard et al., provide an elegant and easy to follow model (i.e., addresses the geometric aspects of active site cleft vs. collagen microfibrils/triple helix; open/closed conformation of enzymes; unwinding of microfibrils/helices; cleavage of individual α chains). This theory is applied in explaining the MoA of endogenous collagenases, as well. Here we see a similar MoA including unique functional 'domains', unwinding of microfibrils/helices, open/closed enzyme geometries, cleaving individual α chains (one at a time). However, as demonstrated in this and in previous chapters, bacterial collagenase is far more efficient in its MoA for a variety of reasons.

As is the nature of scientific research, old ideas make way for new ideas generated as analytical technologies/techniques improve. However, we should not discount the work of so many others over the yrs. out of hand. It is likely that the 'true' MoA is a conglomeration of bits and pieces of the theories discussed thus far (and those yet to come). In time, and as more studies using modern analytical methods are performed, perhaps we will see more agreement in the literature. Although the mechanisms of action and the substrates upon which various enzymes act are interesting from an academic standpoint, they more importantly provide an insight into their effects on the wound bed and subsequent wound progression.

References


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