# Conformational dynamics accompanying the proteolytic degradation of trimeric collagen I by collagenases

Arjun S. Adhikari[‡], Emerson Glassey[‡] and Alexander R. Dunn*

Department of Chemical Engineering, Stanford University, Stanford, CA - 94305

***Supporting Information***

## Materials

Recombinant, post-translationally modified human collagen-I was purchased from Fibrogen (San Francisco, CA). Pyridoxal 5'-phosphate (PLP), biotin hydrazide, collagenase from *Clostridium histolyticum* (C1639), collagenase colorimetric substrate (N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala), 4-aminophenylmercuric acetate; APMA)) and bovine serum albumin (BSA; A2153) were purchased from Sigma Aldrich (St. Louis, MO). Dynabeads MyOne T1 and M280 superparamagnetic beads were purchased from Invitrogen (Carlsbad, CA). An antibody targeting a sequence near the collagen I *C*-terminus was purchased from Millipore (clone 5D8-G9; Billerica, MA). The MMP-1 gene was purchased from the Harvard Plasmid Database. A Zeiss Axiovert 100TV with a 10x objective and equipped with a CMOS camera (Thorlabs) was used for the single-molecule proteolysis experiments.

## MMP expression and magnetic tweezers calibration

MMP-1 was expressed, purified and activated as in Adhikari *et al*.[1] The magnetic tweezers were calibrated as described previously.[1]

## Collagen I biotinylation

Biotin was introduced at the collagen *N*-terminus by first converting the *N*-terminal amine into an aldehyde using PLP, and then reacting the introduced aldehyde with biotin hydrazide.[2-4] To

form the *N*-terminal aldehyde, human collagen type I (1.6 mg $ml^{-1}$; 240 µL) was mixed with phosphate buffer (25 mM sodium phosphate; pH 6.5; 360 µL) and pyridoxal 5'-phosphate (20 mM in 25mM phosphate buffer; 600 µL) to form 1.2 ml total volume. The pH was then adjusted to 6.5 with dilute sodium hydroxide solution. The reaction was incubated for 24 hours at 21 °C with gentle agitation. The functionalized collagen was then isolated from excess PLP via dialysis at 4°C. The aldehyde-functionalized collagen was biotinylated by adding biotin-hydrazide (10 mM in 25 mM phosphate buffer, 500 µL). The reaction was incubated at 21 °C for 8 h. Excess biotin hydrazide was removed via exhaustive dialysis against 1x PBS buffer at 4 °C. These specific temperatures were chosen to maximize labeling efficiencies[4] and because the collagen I triple helix is stable at these temperatures.[5] The resulting solution was used as the biotinylated collagen stock and estimated at 1 µM concentration. Since collagen gels at neutral pH, acetic acid was added to the biotinylated collagen to a final concentration of 0.2 M to ensure that the collagen remained soluble. The final solution was stored at 4 °C.

### *Clostridium* collagenase activity assay

The collagenase activity assay was performed using collagenase substrate (N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala) (F5135; Sigma Aldrich) according to the manufacturer's specifications.

### Single molecule proteolysis

Microfluidic flow chambers were assembled using 25x25 mm coverslips (#1.5) and 1x3 inch glass slides using double-sided Scotch-brand tape as spacers between the coverslip and slide surface. All dilutions are in 1x PBS unless otherwise specified. All experiments are done at room temperature. 15 µl anti-collagen[6] (50 µg $ml^{-1}$) was added to the flow cell and incubated for 30

minutes. This antibody was specifically chosen because it binds only trimeric collagen and not single collagen strands. BSA (5 mg $ml^{-1}$) was then added to the flow cell and incubated for 45 minutes. The flow cell was then washed with a BSA solution (5 mg $ml^{-1}$) and incubated for another 30 minutes to further passivate the slide surface.

The working sample of biotinylated collagen was prepared by diluting the 1 µM stock 1,000 fold in PBS. The diluted sample was spun at 10,000xG at 4°C for 10 minutes to remove any aggregated collagen. The supernatant was then diluted a further 10-fold to give a 100 pM working sample. 50 µl of the working sample was added to the flow cell and incubated for 45 minutes. The flow cell was then washed with PBS. Streptavidin coated beads (1 µm beads = 2 µg $ml^{-1}$, 2.8 µm beads = 20 µg $ml^{-1}$) were added to the flow cell and incubated for 45 minutes.

Prior to proteolysis, the flow cell was introduced into the magnetic trap under low force (~1-2 pN) to remove loose beads. Any beads that detached were gently washed out with 1x PBS. To initiate the proteolysis experiments, either MMP-1 (3 µM) or *Clostridium* collagenase (0.9 units $ml^{-1}$) was introduced into the flow cell. Four to seven fields of view were sampled at each time point in order to observe an adequate number of tethered beads. Measurements were taken until no further bead detachment was observed.

**Single molecule control measurements**

The following control experiments confirmed the presence of specific attachments from bead to biotinylated collagen, collagen to antibody, and antibody to surface. In Table S1 below, one or more reagents necessary to link the beads to the surface was deliberately omitted. The remaining reagents (minus proteinase) were added as detailed above. Each measurement reports the number of beads observed per 80,000 $\mu m^2$.

**Table S1**. Controls to check single point bead attachments to collagen. Number of surface-attached beads observed per 80,000 $\mu m^2$ field of view.

| | |
|---|---|
| BSA + Beads | ~0 |
| Antibody + BSA + Beads | ~0 |
| Antibody + BSA + Collagen + Beads | ~0 |
| BSA + Collagen + Beads | ~0 |
| BSA + Biotinylated Collagen + Beads | 30-40 |
| Antibody + BSA + Biotinylated Collagen + Beads | 100-150 |

**Optimization of single-point bead attachments**

An initial optimization was performed to ensure that the attachments were predominantly single point, i.e. one collagen trimer was attached to a single bead (Table S2). At biotinylated collagen concentrations of 100 pM and below, we observed a trend in which the number of attached beads increased with the concentration of the biotinylated collagen solution used in flow cell assembly. Increasing the biotinylated collagen concentration from 100 pM to 200 pM did not result in further recruitment of beads. Performing the same biotinylated collagen I titration at increased antibody concentrations does not change bead recruitment levels, consistent with bead attachment limited by the number of collagen trimers and/or antibody molecules on the coverslip surface.

We used the optimized biotinylated collagen concentration of 100 pM, which yielded a high ratio of specifically attached beads (~70%; see Table S1). We note that the fraction of beads that attach non-specifically in our control experiments (~30%, Table S1) is similar to the fraction of beads remaining attached to the coverslip at the end of a proteolysis experiment. This observation suggests that the nonspecifically attached beads are also resistant to proteolytic release.

**Table S2.** Optimization of single-point attachments

| | |
|---|---|
| 50 pM Collagen | ~50-60 beads |
| 100 pM Collagen | ~100-150 beads |
| 200 pM Collagen | ~80-100 beads |

Importantly, we note that the MMP-1 proteolysis experiments consistently follow single exponential kinetics. This observation indicates that there is a single rate-limiting cutting step, thus implying that a large majority of the beads are attached via single collagen tethers. The presence of multiple collagen tethers per bead would result in a multistep bead detachment mechanism, which we do not observe.

**Effect of force on bead detachment in absence of proteinase**

Since non-covalent attachments (collagen–antibody and biotin–streptavidin) are used to tether to beads to collagen, it is important to determine if force plays a role in bead detachment in the absence of proteinase. Bead detachment was monitored in absence of a proteinase at various forces (Figure S1). In the absence of proteinase most of the beads remain attached to the

coverslip even at the maximum forces accessed in our experiment. We compared our results to those from a study of force-mediated biotin–streptavidin dissociation by Danilowicz *et. al*. Using the parameters derived in this study, the biotin-streptavidin dissociation rate at a force of 15 pN is predicted to be 0.03 $min^{-1}$, which is much slower than the proteolysis-mediated bead detachment rates (~1.0 $min^{-1}$) that we observe. Thus, proteolysis is the dominant kinetic process in our experiments, and not spontaneous bead detachment.

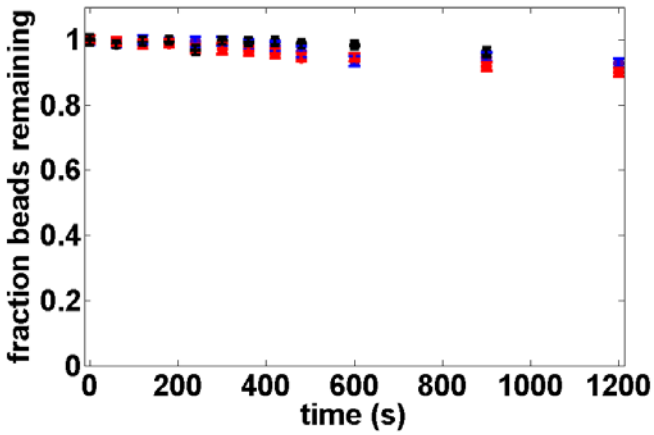


**Figure S1**. Bead detachment from surface tethered collagen in absence of proteinases. *Blue* – 0.25 pN, *black* – 6.2 pN, *red* – 16.8 pN. Very few beads detach over the course of the experiment in the absence of proteinase.

**Solution proteolysis measurements**

We used gel densitometry from our bulk proteolysis measurements to determine the proteolysis rates in solution at zero force.

***Clostridium* collagenase**

5 µL of 3.6 units $ml^{-1}$ *Clostridium* collagenase were added to 100 µL of 0.4 mg $ml^{-1}$ human collagen at room temperature, for a final collagenase concentration of 0.18 units $ml^{-1}$. Aliquots

were taken at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 minutes, quenched with SDS-PAGE loading buffer, boiled for 1 minute, and frozen. The aliquots were then analyzed via SDS-PAGE (Figure S2).

The amount of substrate remaining was plotted as a function of time to determine the rate of proteolysis (Figure S3). The substrate decay rate was determined as 0.10 ± 0.01 $min^{-1}$. Extrapolation to the *Clostridium* collagenase concentration used in the single molecule experiments (0.9 units $ml^{-1}$) yields a predicted bead detachment rate of 0.5 ± 0.03 $min^{-1}$, in good agreement with the rate of 0.58 ± 0.07 $min^{-1}$ actually observed at 0.25 pN.

**MMP-1**

The reaction contained 0.4 mg $mL^{-1}$ human collagen and 0.7 μM MMP-1, and was done at room temperature. Aliquots were taken at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, and 33 minutes, quenched with SDS-PAGE loading buffer, boiled for 1 minute, and frozen. Aliquots were then analyzed via SDS-PAGE (Figure S2). The extrapolated substrate decay rate was determined to be 0.15 ± 0.01 $min^{-1}$ (Figure S3), in good agreement with the extrapolated rate at zero force derived from single-molecule experiments (0.14 ± 0.01 $min^{-1}$)

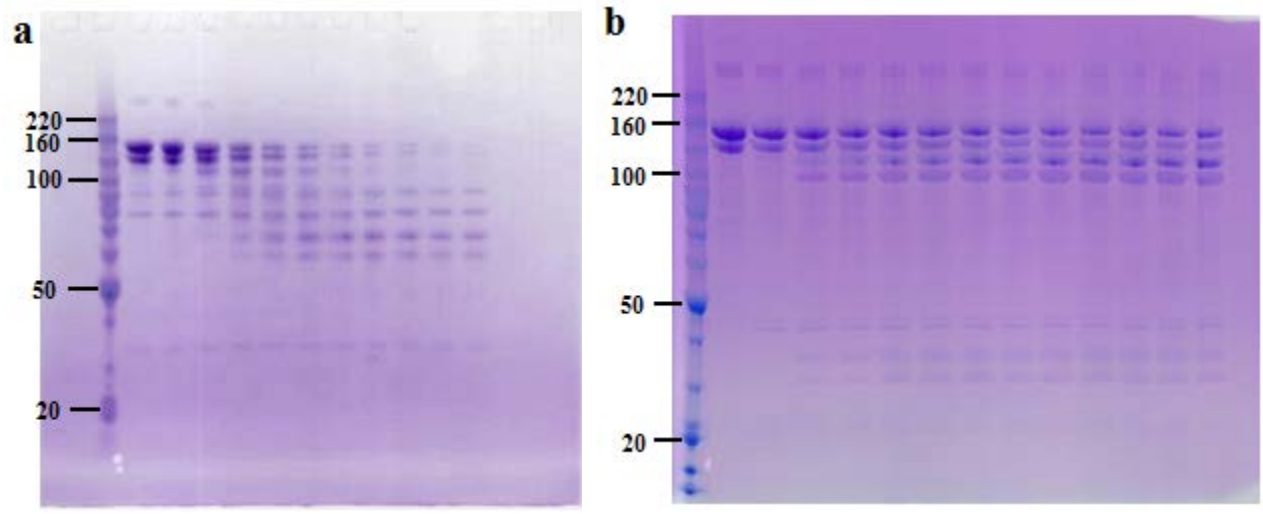


**Figure S2.** Bulk collagen proteolysis. Lane 1 is the molecular weight ladder (Invitrogen, #10748010), with weights marked in kDa. Each subsequent lane corresponds to aliquots at three minute intervals starting at zero minutes. The high molecular weight doublet that predominates at short times reflects the 2:1 stoichiometry of heterotrimeric collagen. (**a**) Proteolysis by *Clostridium* collagenase. *Clostridium* collagenase cuts the collagen with low sequence specificity, as evidenced by the multiple new bands appearing with time. (**b**) Proteolysis by MMP-1. Proteolysis results in the expected three-fourths and one-fourth molecular weight bands, with no new bands appearing with time.

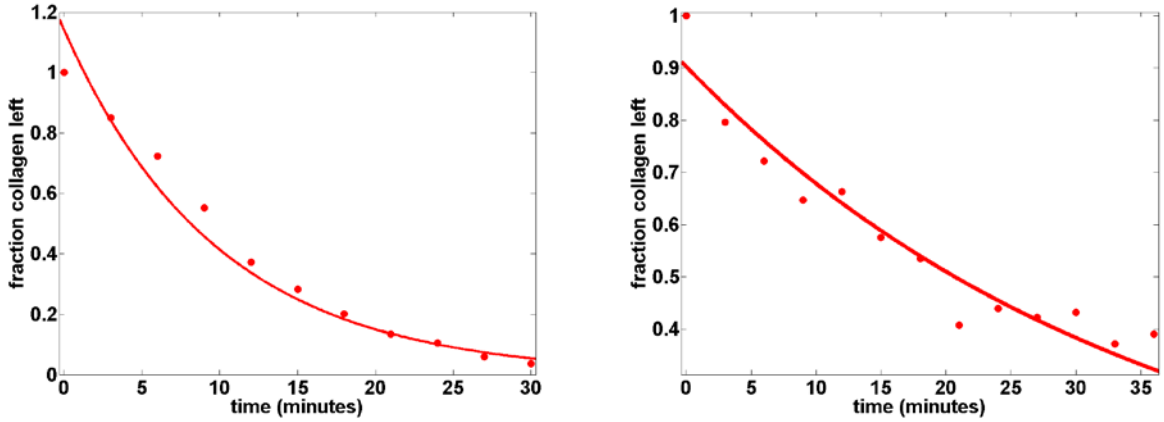


**Figure S3.** Bulk proteolysis of collagen by *Clostridium* collagenase (left) and MMP-1 (right). Exponential fits to the data gave rate constants of 0.10 ± 0.01 $min^{-1}$ for *Clostridium* collagenase and 0.029 ± 0.003 $min^{-1}$ for MMP-1. The extrapolated rates corrected for the concentration in the single molecule experiments are 0.5 ± 0.03 $min^{-1}$ for Clostridium collagenase and 0.15 ± 0.03 $min^{-1}$ for MMP-1 respectively.

**Proteinase action on single-molecule attachment components**

We investigated the effect of MMP-1 and *Clostridium* collagenase on the collagen antibody used as a single-molecule attachment and on streptavidin using SDS PAGE (**Figure S4, S5**). Simultaneous control experiments with collagen as the substrate confirmed the activity of both enzymes. Moreover, we replicated our single molecule experiment without the collagen linker and tethered the beads directly to the antibody and monitored bead detachment in presence of both MMP-1 and *Clostridium* Collagenase. We did not see any significant bead detachment during the course of the experiment (**Figure S6)**.

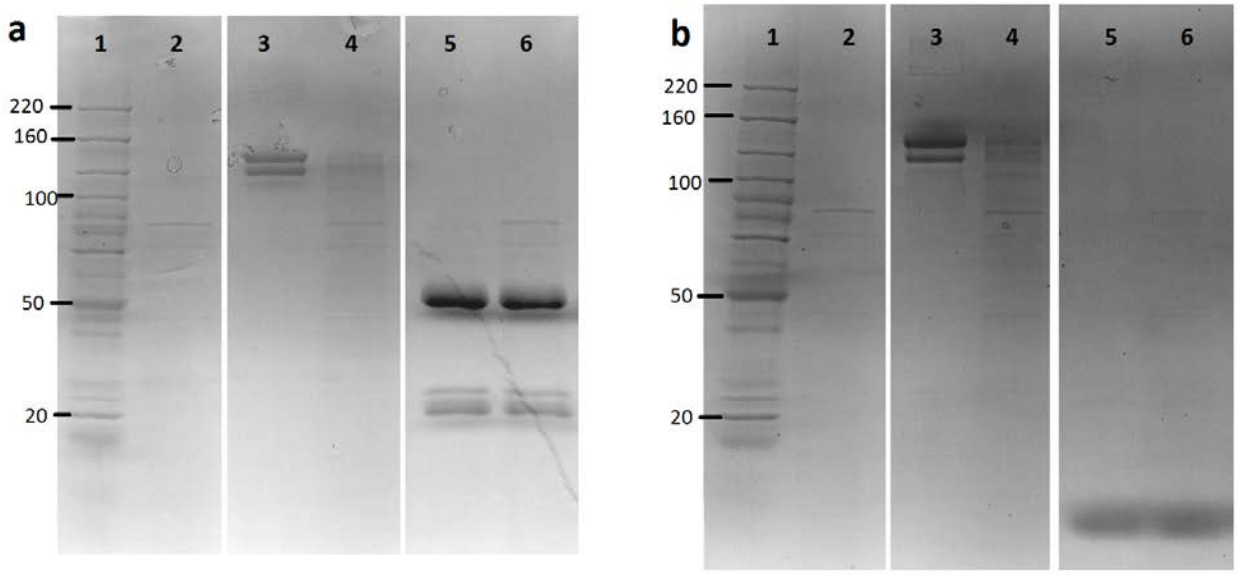


**Figure S4**. (**a**) Effect of *Clostridium* collagenase on anti-collagen antibody. Lane *1*: ladder; *2*: clos. collagenase; *3*: collagen; *4*: collagen + *Clos*. collagenase; *5*: antibody; *6*: antibody + *Clos.* collagenase. *Clos*. collagenase cuts collagen (lanes 3 and 4), but does not appreciably cleave the antibody under these conditions (lanes 5 and 6). (**b**) Effect of *Clostridium* collagenase on streptavidin. Lane *1*: ladder; *2*: *Clos*. collagenase; *3*: collagen; *4*: collagen + *Clos*. collagenase; *5*:streptavidin; *6*: streptavidin + *Clos*. collagenase. *Clos*. collagenase does not appreciably cleave streptavidin under these conditions (lanes 5 and 6). Concentrations used: *Clostridium* collagenase (0.18 units $ml^{-1}$), collagen (0.3 mg $ml^{-1}$), antibody (1 mg $ml^{-1}$), streptavidin (0.5 mg $ml^{-1}$).

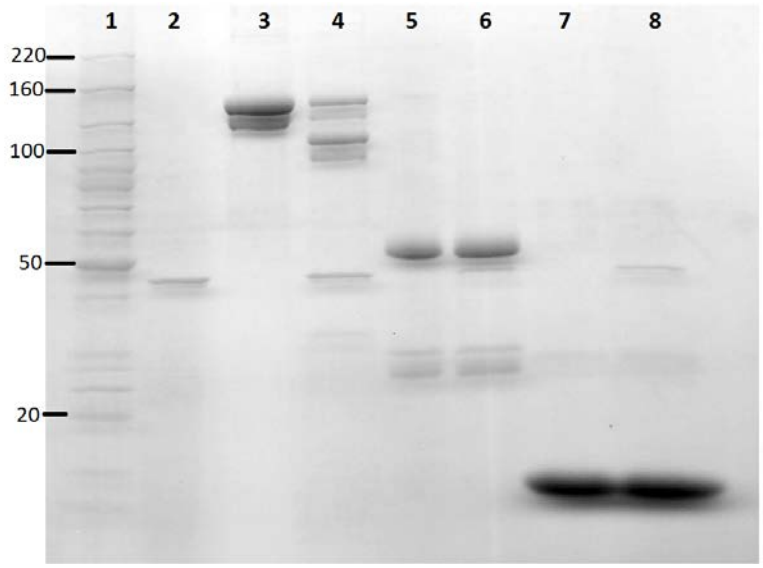


**Figure S5**. Effect of MMP-1 on linkage components. Lane *1*: ladder; *2*: activated MMP-1; *3*: collagen; *4*: collagen + MMP-1; *5*: anti-collagen antibody; *6*: antibody + MMP-1; *7*: streptavidin; *8*: streptavidin + MMP-1. Comparison of lanes 5 and 6 and lanes 7 and 8 shows that MMP-1 does not appreciably cleave either the antibody or streptavidin under these conditions. Concentrations used: MMP-1 (0.7 µM), Collagen (0.3 mg $ml^{-1}$), antibody (1 mg $ml^{-1}$), streptavidin (0.5 mg $ml^{-1}$).

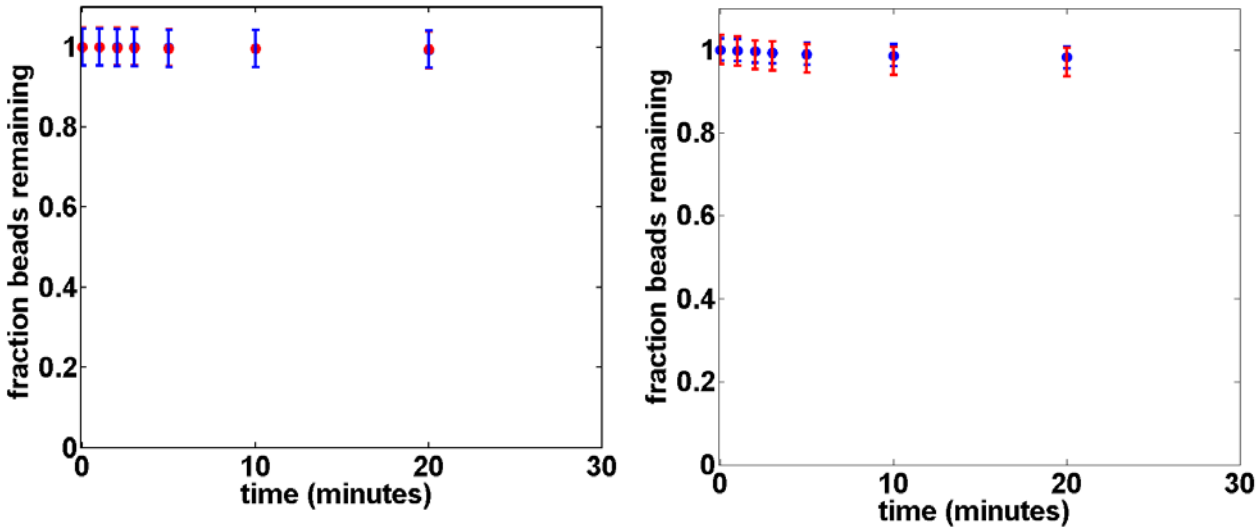


**Figure S6.** Single molecule measurement of effect of MMP-1(left) and *Clostridium* collagenase (right) on the linkage components. *Red*: 0.25 pN load, and *blue*: 12 pN load.

**MMP-1 and *Clostridium* Collagenase concentration determination**

When working with proteases it is essential to discriminate between the active and inactive protease. To determine the concentration of activated MMP-1, we measured the amount of activated protease against known standards using gel densitometry. This method allows us to separate the active protein from the degraded inactive protein. Total protein concentrations prior to activation were confirmed using a Bradford assay.

We observe a bulk cutting rate of 0.21 ± 0.03 $min^{-1}$ $\mu M^{-1}$ for MMP-1. Previously Lauer-Fields *et. al*[7] reported a value of 0.10 $min^{-1}$ $\mu M^{-1}$ for homotrimeric collagen triple helical peptide and Mallya *et. al*[8] reported a value of 0.87 $min^{-1}$ $\mu M^{-1}$ for collagen I (tissue derived). Han *et. al*[9] reported a cutting rate of 4.0 $min^{-1}$ $\mu M^{-1,}$ though with a different substrate (fibroblast derived Collagen I) and under different reaction conditions. MMP-1 catalytic efficiencies thus appear to differ substantially depending on the enzyme preparation, collagen source (e.g. recombinant, tissue derived or cell culture derived) and experimental conditions.

For *Clostridium* collagenase we used a nominal concentration of 1.5 μM for the single molecule proteolysis and find that the half-life of the reaction at 0.25 pN is 69 seconds. This value is consistent with the half-life of the reaction at "low force," which was 17 seconds at an enzyme concentration of 5.56 μM as reported by Camp et. *al*,[10].

**Data fitting and error analysis**

**Data fitting**: The average number of beads per field of view was determined by recording several (3 to 7) fields of view at each time point. Time points were collected until no further proteolysis was observed. The remaining beads were deemed to be non-specifically stuck to the surface. The number of non-specifically attached beads to the surface was determined by fitting

the data to an exponential plus a constant. The constant was then subtracted from the average number of attached beads at each time point prior to further data analysis. Following subtraction of the background beads, the normalized fraction of beads remaining was plotted as a function of time and fit to a single exponential using nonlinear least squares fitting implemented in Matlab.

**Error analysis**: The error at each data point was attributed to Poisson-distributed counting noise[1]. Thus, at each time point the error in *n* counted beads is $n^{1/2}$. This error was propagated to derive the error in the calculated fraction of beads remaining at each time point. The error in the exponential fit was then calculated using the parametric bootstrap method implemented using custom routines written and executed in Matlab.[11,12]

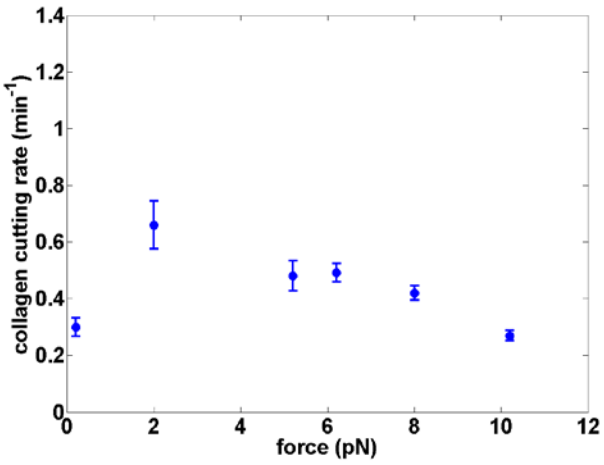


**Figure S7**. Supplemental data showing the effect of force on collagen proteolysis by *Clostridium* collagenase (0.36 units $ml^{-1}$).

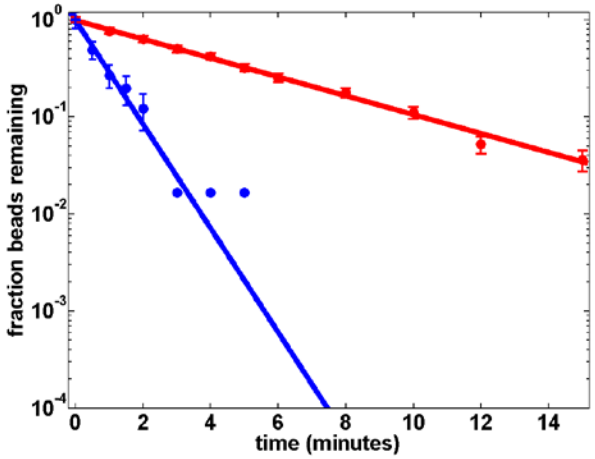


**Figure S8**. Fraction of beads remaining as a function of time in the proteolysis of collagen model peptide homotrimer, as reported in Adhikari *et al.*[1] The data are shown fit to a single exponential. *red*: 1 pN force and 3 μM MMP-1. *blue*: 12 pN and 100 nM MMP-1. The points without error bars correspond to 0% beads remaining, but are set to 0.01 in order to be included on the semilog plot.

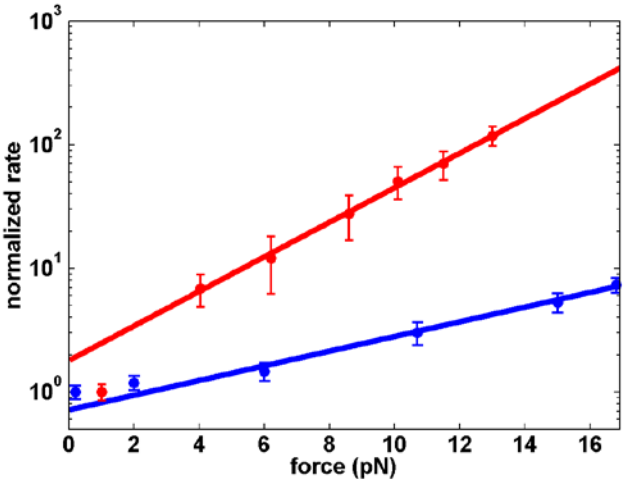


**Figure S9**: Comparison of the effect of force on the proteolysis of collagen model peptide homotrimer (*red*, data from Adhikari *et al.*[1]) and on the proteolysis of post-transitionally modified collagen heterotrimer (*blue*, present study).

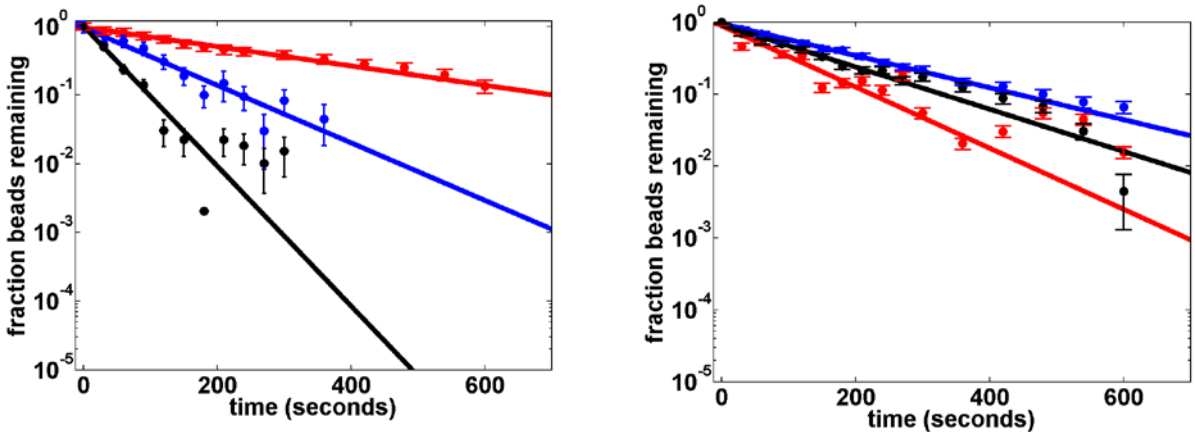


**Figure S10**: Semilog plots corresponding to figure 2 of the main text. Left: MMP-1(red: 0.25 pN, blue: 10.7 pN, black: 16.7 pN). Right: *Clostridium* collagenase (red: 0.25 pN, blue: 5.2 pN, black: 15 pN).

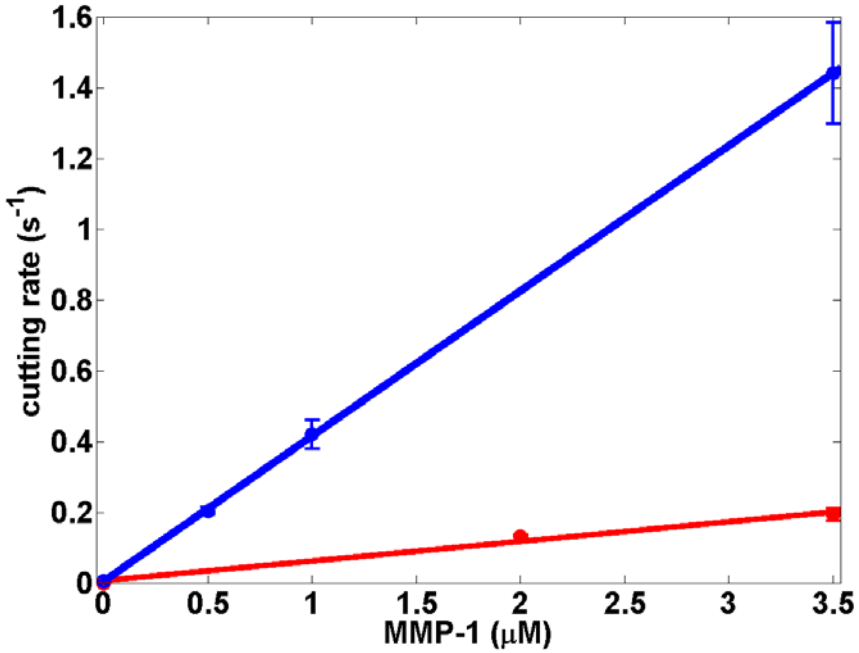


**Figure S11**. Effect of MMP-1 concentration on the proteolysis of heterotrimeric collagen. Data were collected using the single-molecule magnetic tweezers apparatus, as described above. *blue*:

16.8 pN (slope = 0.41 ± 0.004 $\mu M^{-1} s^{-1}$, intercept = 0.004 ± 0.0043 $s^{-1}$). *Red*: 0.25 pN (slope = 0.055 ± 0.009 $\mu M^{-1} s^{-1}$, intercept = 0.006 ± 0.01 $s^{-1}$). Bead detachment rates are linear with MMP-1 concentration under the conditions used in this study.

## References


(1) Adhikari, A. S.; Chai, J.; Dunn, A. R. *J. Am. Chem. Soc.***2011,** *133*, 1686.

(2) Christman, K. L.; Broyer, R. M.; Tolstyka, Z. P.; Maynard, H. D. *J. Mater. Chem.* **2007**, *17*, 2021.

(3) Gilmore, J. M.; Scheck, R. A.; Esser-Kahn, A. P.; Joshi, N. S.; Francis, M. B. *Angew. Chem. Int. Edit.* **2006**, *45*, 5307.

(4) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem. Int. Edit.* **2009**, *48*, 6974.

(5) Leikina, E.; Mertts, M. V.; Kuznetsova, N.; Leikin, S. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 1314.

(6) Werkmeister, J. A.; Ramshaw, J. A.; Ellender, G. *Eur. J. Biochem.* **1990**, *187*, 439.

(7) Lauer-Fields, J. L.; Tuzinski, K. A.; Shimokawa, K.; Nagase, H.; Fields, G. B. *J. Biol. Chem.* **2000**, *275*, 13282.

(8) Mallya, S. K.; Mookhtiar, K. A.; Gao, Y.; Brew, K.; Dioszegi, M.; Birkedal-Hansen, H.; Van Wart, H. E. *Biochemistry* **1990**, *29*, 10628.

(9) Han, S.; Makareeva, E.; Kuznetsova, N. V.; DeRidder, A. M.; Sutter, M. B.; Losert, W.; Phillips, C. L.; Visse, R.; Nagase, H.; Leikin, S. *J. Biol. Chem.* **2010**, *285*, 22276.

(10) Camp, R. J.; Liles, M.; Beale, J.; Saeidi, N.; Flynn, B. P.; Moore, E.; Murthy, S. K.; Ruberti, J. W. *J. Am. Chem. Soc.* **2011**, *133*, 4073.

(11) Efron, B. *The jackknife, the bootstrap, and other resampling plans*; Society for Industrial and Applied Mathematics: Philadelphia, Pa., 1982.

(12) Efron, B.; Tibshirani, R. *An introduction to the bootstrap*; Chapman & Hall: New York, 1993.