Supporting Information

Discontinuous Steady-State Peptide Hydrolysis Assay. Reactions containing 50 mM Tris (pH 8.1), 10 mM $Mg(OAc)_2$, 2 mM DTT, 150 mM NaCl (human Lon reactions only), 100-200 nM *S.* Typhimurium Lon monomer or 900 nM human Lon monomer, and varying concentrations of the fluorescent peptide substrate (from 0-3 mM) were initiated by the addition of 1 mM ATP and incubated at 37˚C. At different time points (from 0-10 min), aliquots were quenched in Buffer A (100 mM EDTA (pH 8), 1% SDS, and 700 mM Tris (pH 8.1)). The quenched reaction time points were then further diluted in Buffer A such that the final peptide concentration did not exceed 100 μM. The fluorescent signal at each time point was determined by excitation at 320 nm and monitoring at 420 nm using a Fluoromax 3 spectrophotometer (Horiba Group). The amount of peptide cleaved was calibrated by determining the change in fluorescence/μM peptide cleaved after complete trypsin digestion under identical reaction conditions. The steady-state velocities were then determined from the linear phase of a plot of the amount peptide cleaved versus time using KaleidaGraph (Synergy, Inc.) and multiplying by the dilution factor used to reduce the peptide concentration to at least 100 μM. The steady-state kinetic parameters associated with peptide cleavage were determined by fitting the k_{obs} data with eq 1 using the nonlinear regression program Prism 4 (GraphPad Software, Inc.).

$$k_{obs} = \frac{k_{cat}[S]}{K_m^{\,n} + [S]^n} \qquad (1)$$

where k_{obs} is the observed rate constant, k_{cat} is the maximal k_{obs}, S is peptide substrate, n is the Hill coefficient, and K_m is the Michaelis-Menton constant. All experiments were preformed at least in triplicate.

Figure 1A

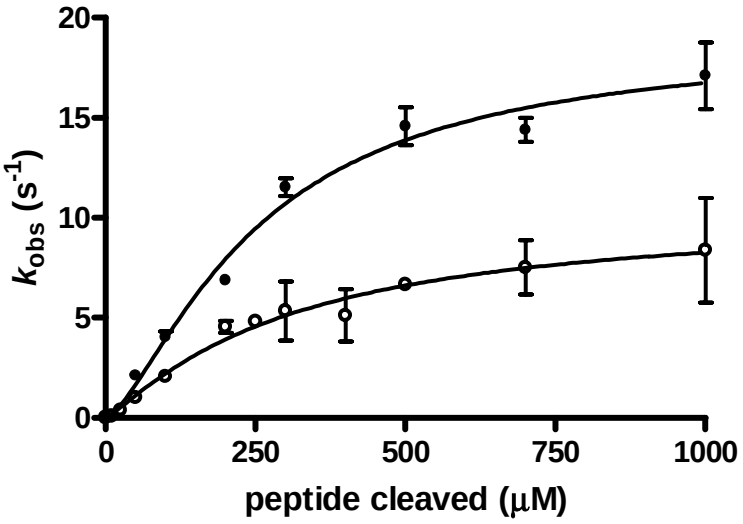

Figure 1B

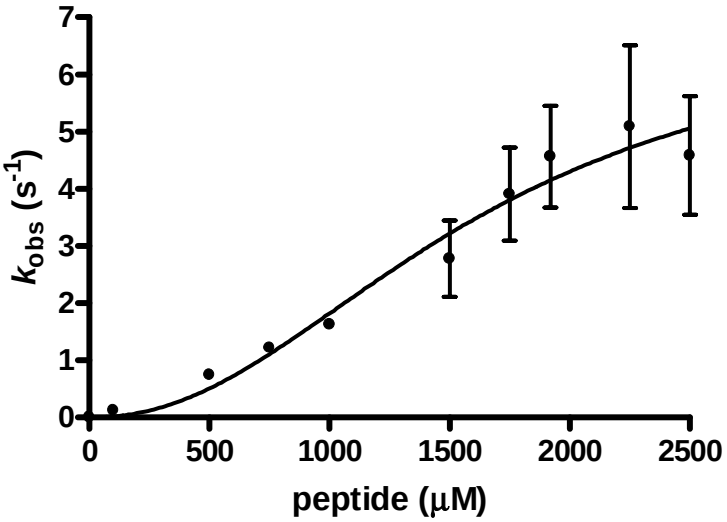

Figure 1. *Steady-state peptide cleavage by* S. *Typhimurium and human Lon.* Reactions containing *S.* Typhimurium (A) or human Lon (B) were preincubated with varying concentrations of **1** (●) or **2** (○) prior to the addition of 1 mM ATP. All experiments were preformed at least in triplicate and the averaged k_{obs} values (±1 SD) were plotted against the corresponding peptide concentration. The data were best fit with the Hill equation (eq 1) as described above. For *S.* Typhimurium Lon, k_{cat} = 19 ± 2 s^{-1}, K_m = 251

± 46 μM, and n = 1.4 ± 0.3 for **1** and k_{cat} = 10 ± 2 s^{-1}, K_m = 287 ± 70 μM, and n = 1.2 ± 0.2 for **2**. For human Lon, k_{cat} = 7 ± 2 s^{-1}, K_m = 1.6 ± 0.5 mM, and n = 2.2 ± 0.6 for **1.**