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## Adenosine Triphosphate-Dependent Degradation of a Fluorescent A N Substrate Mimic by Lon Protease

Irene Lee and Anthony J. Berdis

Lon is an ATP-dependent protease functioning independently of ubiquitin to degrade abnormal and transient cellular proteins (1-4). Lon eliminates abnormal polypeptides and unstable regulatory proteins in prokaryotes and eukaryotes. In yeast, Lon maintains the integrity of mitochondria and renders respiratory function in cells (5-7) that can be partially complemented by the *Escherichia coli* (*E. coli*) *Jon* homolog (8). In human, Lon is expressed in the nucleus of all tissues and is localized in mitochondria (9, 10). In *Brucella abortus*, Lon functions as a stress protease that is required for wild-type virulence during the initial stage of infection in mouse (11).

Lon represents a unique class of protease as ATP (3, 12-15) and DNA (16) activate its peptidase activity. However, the molecular mechanism of how these effectors mediate protein degradation is unclear. Several studies have demonstrated that the hydrolysis of small synthetic peptides requires only ATP binding, whereas the degradation of proteins requires additional ATP hydrolysis (4, 12, 14, 17-20). While the nonhydrolyzable ATP analogs ATP- $\gamma$ S, <sup>2</sup>AMPPCP, and AMPPNP support the cleavage of certain tetrapeptide substrates of chymotrypsin as effectively as ATP, these analogs are inferior to ATP in activating casein degradation by Lon (2).

The peptide substrate determinant responsible for peptidase and ATPase stimulation of Lon is also unclear. Until now, only protein but not synthetic peptide substrates stimulate the ATPase activity of Lon. The ability of protein substrates to stimulate ATP hydrolysis was originally attributed to structural features of the protein substrate that are recognition elements for the peptidase site. In this case, ATP hydrolysis is nee-

essary to unfold protein and to present the scissile peptide bond for Lon-mediated cleavage (2–4). While this hypothesis explains the requirement of ATP hydrolysis in degrading structured proteins such as casein (12) and CcdA (21), it fails to reconcile the observed ATPase stimulation during  $\lambda$  N degradation (20). Since  $\lambda$  N does not adopt detectable secondary structure in the absence of RNA (22, 23), its degradation should require very little if any ATP-mediated substrate unfolding and presentation.

It has also been proposed that the protein substrate-induced ATPase activity is a regulatory component of the proteolytic activity of Lon (2, 3). Protein substrates may interact with a putative allosteric site in Lon that is independent of the proteolytic site to promote ADP product release and facilitate ATPase turnover. The location of the allosteric site as well as the recognition determinants on substrate responsible for allosteric site interaction have yet to be determined. However, since Lon can also function as a chaperone (5, 24), it is plausible that the “putative allosteric site” constitutes part of the chaperone activity of Lon such that interaction of the potential protein substrate with the allosteric site activates the protease activity of Lon.

A detailed mechanistic characterization on the coordination of ATP binding and hydrolysis with peptide hydrolysis by Lon cannot be readily accomplished because of the lack of a suitable peptide substrate that interacts with Lon in a manner similar to protein substrate. Since the synthetic peptide substrate Glt-Ala-Ala-Phe-MNA inhibits ATP hydrolysis by Lon (2, 19), it is impossible to utilize this substrate to examine the functional role of the protein-induced ATPase activity of Lon. Although protein substrates stimulate ATP hydrolysis by Lon, they are not convenient peptidase substrates because they contain multiple Lon cleavage sites of varying sequences. Thus, it will be very difficult to directly correlate the binding and hydrolysis of ATP with a specific peptide bond cleavage event. In this study, we used a synthetic peptide mimic of  $\lambda$  N protein to develop a continuous peptidase assay to probe the relationship between substrate/allosteric site interaction with Lon catalysis. We demonstrate that a small synthetic peptide (FRETN 89-98) lacking detectable secondary structure stimulated *E. coli* Lon ATPase activity. Like its protein counterpart, this peptide was cleaved by *E. coli* Lon at a specific site with high efficiency only in the presence but not in the absence of ATP. The design, synthesis, and the kinetic properties of peptide degradation and Lon ATPase stimulation of this synthetic peptide are presented.

## EXPERIMENTAL PROCEDURES

**Materials.** Fmoc-protected amino acids, Boc-Abz, and Fmoc-protected Lys Wang resin were purchased

from Advance Chemtech and NovaBiochem. HATU was purchased from PerSeptive Biosystem. All solvents and reagents used for peptide synthesis were purchased from Acros, Aldrich, and Fisher. ATP, AMP-PNP, AMPPCP, and ATP $\gamma$ S were purchased from Sigma. Tris buffer, cell culture media, IPTG, and chromatography media were purchased from Fisher and Sigma. Electrospray ionization mass spectrometry (EIMS) of peptide substrate and Lon cleavage products was performed by the Cleveland Mass Spectrometry Facility (Cleveland State University) and amino acid analysis of *E. coli* Lon was performed by the Kerk Facility (Yale University). *E. coli* cells overexpressing *E. coli* Lon were kindly communicated by A. Goldberg (Harvard Medical School) and S. J. Benkovic (Pennsylvania State University). The human Lon overexpression vector was a gift from D. M. Markovitz (University of Michigan Medical Center) and C. Suzuki (New Jersey Medical School).

**Peptide synthesis.** Solid-phase peptide synthesis was conducted according to the procedure by Garner *et al.* (25) using an Advanced Chemtech Model 90 tabletop peptide synthesizer. Wang resin preloaded with Fmoc amino acid was swollen in DMSO for 12 h before the synthesis. The Fmoc-protecting group was deprotected with 2% DBU (v/v) in DMF. Fmoc-deprotected peptide resins were washed with DMF and NMP in between peptide coupling. Peptide products were cleaved from resin using reagent K (5% phenol/5% water/5% thioanisole/2.5% ethanediol/82.5% TFA) and then precipitated with cold diethyl ether. Crude peptide was purified by reverse-phase HPLC using a semi-preparative C18 column (Vydac). The concentrations of pure peptide were initially determined by amino acid analysis from which the extinction coefficient of each peptide was determined by their respective absorbances at 280 nm according to the Beer–Lambert law. The extinction coefficients were then used to determine peptide concentrations used in this study.

**Circular dichroism of FRETN 89-98 and  $\lambda$  N 89-98.** The CD spectra of 50  $\mu$ M peptide substrates in 10 mM potassium phosphate buffer containing 0.1 mM  $\beta$ -mercaptoethanol were recorded at 25°C using a Jasco J-810 spectropolarimeter. CD spectra were the average of eight scans from 190 to 280 nm.

**Purification of *E. coli* Lon.** *E. coli* Lon was purified from an overexpression cell strain using a modified protocol of Goldberg *et al.* (2). The enzyme was purified in 48 h to ensure optimal activity of the enzyme. Cell lysate was purified by phosphocellulose P11 and DE52 cellulose chromatography. Protein fractions containing Lon were analyzed by 12% SDS–PAGE (26) and fractions of apparent homogeneity were pooled and concentrated by 70% ammonium sulfate precipitation. Pro-

tein concentrations were determined by amino acid analysis and Bradford assay (27).

**Purification of human Lon protease.** Cultures of *E. coli* BL21(DE3) hosting the plasmid proEx-1/hLON were induced with 4 mM IPTG for 1 h at 37°C. Recombinant Lon was purified by phosphocellulose chromatography followed by Ni/NTA resin (28). The resulting protease was quantitated by Bradford assay and analyzed by 10% SDS-PAGE and Western blot analysis.

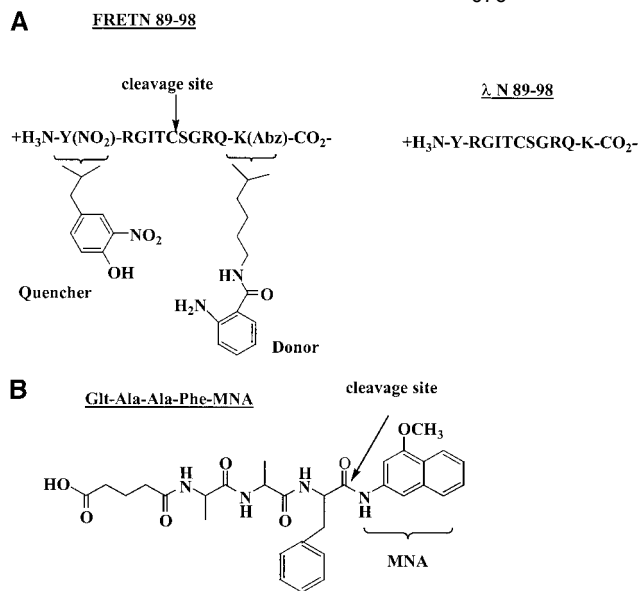
**ATP-dependent peptidase assay.** *E. coli* Lon (200 nM) was incubated with 2 μM of peptide substrate with and without 1 mM ATP and Mg(OAc)<sub>2</sub> (magnesium acetate) in 25 μL of 50 mM Tris-HCl, pH 8.1, 100 mM β-mercaptoethanol, at 37°C for 1 h. The reactions were quenched with 1 μL of 10% SDS and then subjected to LC/MS analysis (Cleveland Mass Spectrometer Facility, CSU).

**Fluorescent assay of ATP-dependent cleavage of FRETN 89-98.** *E. coli* Lon (125 nM) was incubated with varying concentrations of FRETN 89-98 in 60 μL of 50 mM Tris-HCl, pH 8.1 containing 1 mM ATP, 1 mM Mg(OAc)<sub>2</sub>, and 1 mM DTT at 37°C. Increase in fluorescence due to peptide cleavage was monitored by excitation at 320 nm and detecting emission at 420 nm using a Fluorolog 3 fluorometer (Spex Inc.). Background hydrolysis was obtained by incubation of all reaction components except Lon. The amount of peptide hydrolysis was calibrated by determining the amount of fluorescence generated per micromolar peptide due to complete digestion by trypsin under the same reaction conditions. Under our assay conditions, fluorescent signals resulting from complete FRETN 89-98 digestion by trypsin are linear up to 160 μM of peptide substrate. Initial rates of FRETN 89-98 cleavage by *E. coli* Lon were calculated from reaction time courses in which less than 5% of peptide substrate was cleaved. Steady-state kinetic parameters were determined from initial rates of peptide hydrolysis under varying substrate concentrations by fitting data using the computer programs KaleidaGraph (Synergy, Inc.) or Enzfitter (Biosoft, Inc.).

**ATPase of *E. coli* Lon.** ATP hydrolysis assays were carried out with 250 nM *E. coli* Lon, 1 mM Mg(OAc)<sub>2</sub>, 0.5 mM ATP, and 50 nM [ $\gamma$ -<sup>32</sup>P]ATP at 37°C. The ATPase activity of *E. coli* Lon was also measured in the presence of 20 μM casein or 100 μM peptide substrates under the same conditions. Time courses for each reaction were obtained by quenching and analyzing reaction aliquots as using thin-layer chromatography followed by PhosphorImaging as described by Berdis and Benkovic (29).

## RESULTS

**Peptide design and synthesis.** The Fmoc solid-phase peptide synthesis technique (30) was used to



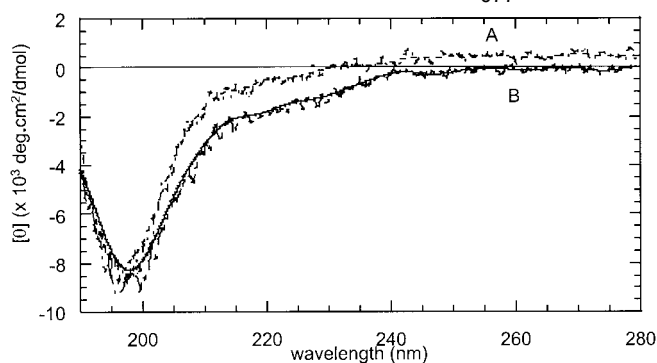
**FIG. 1.** Peptide substrates of *E. coli* Lon protease. (A) The 10-amino-acid synthetic peptide FRETN 89-98 is designed as an *in vitro* substrate mimic of the  $\lambda$  N protein. This substrate consists of residues 89–98 of  $\lambda$  N, a fluorophore anthranilamide (Abz) lysine and a fluorescence quencher 3-nitrotyrosine. The *E. coli* Lon cleavage site is located at Cys93 and Ser94. The FRETN 89-98 cleavage reaction by *E. coli* Lon is monitored by increase in fluorescence caused by separation of Abz from 3-nitrotyrosine. (B) The Lon cleavage site of Glt-Ala-Ala-Phe-MNA is located at the carboxyl end of the tetrapeptide. Peptidase activity is monitored by an increase in fluorescence due to release of 2-methoxynaphthylamine (MNA).

generate a 10-amino-acid peptide FRETN 89-98 containing a fluorescent donor and a quencher (Fig. 1A). The sequence of FRETN 89-98 corresponds to residues 89–98 of  $\lambda$  N protein with a Lon cleavage site at Cys98 and Ser94 (20). Consequently the sulfhydryl moiety of this substrate can be derivatized by a variety of spectroscopic probes or crosslinkers to probe the peptide and Lon interaction. The peptide sequence of FRETN 89-98 is chosen because it contains a unique Cys at the Lon cleavage site. FRETN 89-98 differs from other Lon peptide substrates such as Glt-Ala-Ala-Phe-MNA (19) by its slightly larger size and by the location of the Lon cleavage site in the middle of the peptide sequence (Fig. 1A). FRETN 89-98 also differs from the  $\lambda$  N protein by a shorter chain length and the lack of multiple Lon cleavage sites. Due to internal quenching of anthranilamide (Abz) lysine by 3-nitrotyrosine (31, 32), FRETN 89-98 is minimally fluorescent until cleavage by Lon that separates the donor from the quencher. Consequently, the peptidase activity of Lon can be continuously monitored by fluorometry at excitation wavelength 320 nm and emission wavelength 420 nm. Furthermore, the increase in substrate size in relationship to the tetrapeptide in use (Glt-Ala-Ala-Phe-MNA) is designed to concomitantly probe the correlation of

peptide length with ATPase stimulation and efficiency of hydrolysis. Comparison of the ATPase stimulation by FRETN 89-98 with other unstructured substrates of varying length, such as  $\lambda$  N protein and Glt-Ala-Ala-Phe-MNA, allows for direct comparison on the efficacy of FRETN 89-98 as a substrate. Characterization of FRETN 89-98 degradation by *E. coli* Lon can be further compared directly with the degradation of  $\lambda$  N to assess the efficacy of FRETN 89-98 as a peptidase substrate as well as an ATPase stimulator.

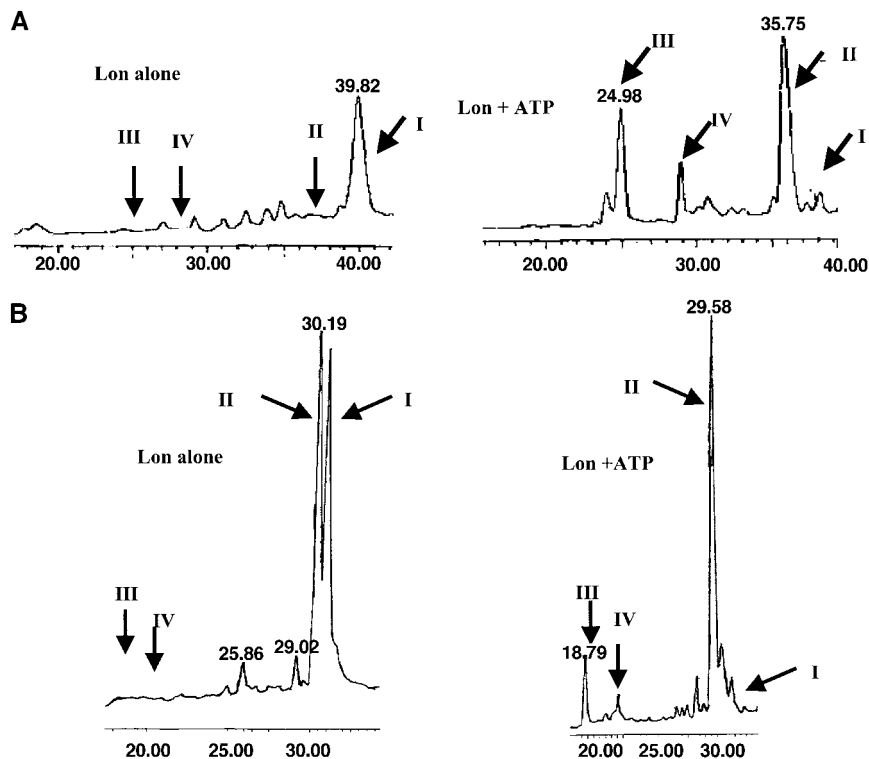
FRETN 89-98 was synthesized using the protocol of Garner *et al.* (25) in which the quencher 3-nitrotyrosine and the fluorophore BocAbz were introduced into FRETN 89-98 via HATU coupling (30). Target peptide of at least 95% pure was readily purified by C18 reverse-phase HPLC using a linear gradient of 0.05% TFA/H<sub>2</sub>O and 0.05% ACN/H<sub>2</sub>O. The molecular weight of FRETN 89-98 was 1432 as determined by ESIMS analysis (33) and agreed well with the predicted molecular weight of 1432.57. The nonfluorogenic peptide  $\lambda$  N 89-98 (Fig. 1A) which differs from FRETN 89-98 by replacement of the 3-(NO<sub>2</sub>)Tyr (fluorescence quencher) with Tyr and Abz-Lys (fluorophore) with Lys was also synthesized and used as a control to assess the contribution of ATPase stimulation caused by the quencher and the fluorophore. The respective absorption extinction coefficients for FRETN 89-98 and  $\lambda$  N 89-98 at 280 nm were 5650 and 1321 M<sup>-1</sup> cm<sup>-1</sup> as determined by amino acid analysis along with spectrophotometer using the Beer-Lambert law (27). Circular dichroism analysis (Fig. 2) of FRETN 89-98 and  $\lambda$  N 89-98 indicated that both synthetic peptides did not adopt defined secondary structure (34), consistent with the reported lack of secondary structure of  $\lambda$  N protein (23).

*Nucleotide-dependent degradation of FRETN 89-98 by E. coli Lon.* Prior to spectroscopic analysis of FRETN 89-98 degradation, we determined the cleavage specificity of FRETN 89-98 by *E. coli* Lon in the presence of 1 mM ATP using LC/ESIMS analysis (Fig. 3). Reaction mixtures containing *E. coli* Lon and FRETN 89-98 in the presence and absence of ATP were resolved by reverse-phase HPLC from which the molecular mass of each peak was determined by ESIMS (Table 1). As seen in Fig. 3, FRETN 89-98 was specifically cleaved by *E. coli* Lon at Cys93 and Ser94 in the presence of ATP. An internal reference, Y(NO<sub>2</sub>)RGITC, was included to demonstrate that this peptide remained intact during FRETN 89-98 degradation, thereby demonstrating the specificity of FRETN 89-98. Based upon the aforementioned data, we conclude that the ATP-dependent cleavage of FRETN 89-98 by *E. coli* Lon was highly specific since cleavage was only observed between Cys93 and Ser94 and was dependent upon the presence of ATP.



**FIG. 2.** Circular dichroism of FRETN 89-98 and  $\lambda$  N 89-98. The spectra of 50  $\mu$ M of peptide samples were recorded in 10 mM potassium phosphate, pH 7, with 0.1 mM  $\beta$ -mercaptoethanol at 25°C as described under Experimental Procedures. Both  $\lambda$  N 89-98 (A) and FRETN 89-98 (B) display random coil structure as judged by the lack of  $\alpha$  helix (two negative absorptions near 222 and 208 nm) and  $\beta$  (a negative absorption near 217 nm) signals in the spectra. Analysis of the two spectra by the Jasco secondary structure estimation software (Jasco) also indicates random structures in both peptides. The raw data were overlaid with the noise-reduced data (smooth lines) processed by the noise-reduction program supplied by Jasco. The molar ellipticity values on the y axis are calculated from the raw data using the equation  $[\theta] = [\theta]_{\text{obs}} (\text{mrw})/10 lc$  (Ref. 25), where  $[\theta]_{\text{obs}}$  is the measured ellipticity in degrees, mrw is the molecular weight of the peptide divided by the number of amino acid residues,  $c$  is the peptide concentration in grams/milliliter, and  $l$  is the optical path-length of the cell in centimeters.

The presence of the FRET pair 3-nitrotyrosine and Abz-lysine allowed for the cleavage of FRETN 89-98 to be monitored by fluorescence spectroscopy. The usable range of FRETN 89-98 as a peptidase substrate was assessed by determining the change in fluorescence resulting from varying concentrations of FRETN 89-98 cleavage by trypsin. A linear increase in fluorescence change is observed for up to 160  $\mu$ M of FRETN 89-98 degradation (Fig. 4). At high peptide concentrations, the increase in fluorescence due to FRETN 89-98 cleavage is reduced due to inner filter effect (35) resulting from high concentration of nitrotyrosine present in the reactions. This inner filter effect is corrected for by determining the percentage of reduction in fluorescence at the indicated peptide concentrations. The corrected rate of peptide cleavage is obtained by dividing the observed fluorescence by the percentage of fluorescence reduction at the corresponding substrate concentration. Trypsin cleaves peptide substrate containing basic residues. Since FRETN 89-98 contains two arginine residues that are cleavage sites of trypsin, the fluorescent signals resulting from complete separation of the quencher and acceptor at a specific FRETN 89-98 concentration can be deduced by digesting the peptide with trypsin. Extensive digestion of FRETN 89-98 with *E. coli* Lon for 1 h at 37°C also generates the same end point result as trypsin (data not



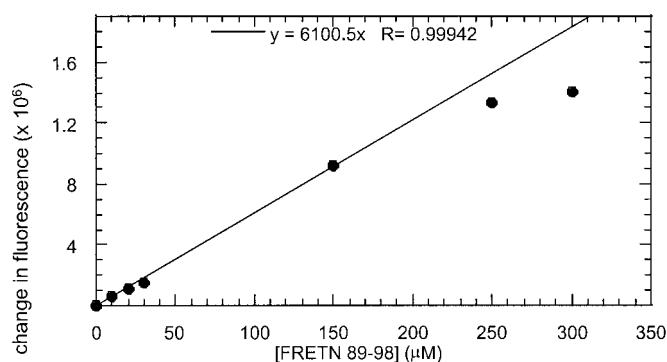
**FIG. 3.** Site-specific cleavage of FRETN 89-98 by *E. coli* Lon. (A) 2  $\mu\text{M}$  NFRET 89-98 (peak I) was incubated with 200 nM *E. coli* Lon in 25  $\mu\text{L}$  of 50 mM Tris-HCl, pH 8.1, 100 mM  $\beta$ -mercaptoethanol in the presence and absence of 1 mM  $\text{Mg}(\text{OAc})_2$  and ATP at 37°C for 1 h, quenched with 1  $\mu\text{L}$  of 10% SDS, and then stored at  $-20^\circ\text{C}$  until LC/ESIMS analysis. The molecular ion of the intact peptide detected was 1507.44 (peak I) while the molecular ions of the cleavage products were 833.69 (peak II) and 693.28 (peak III), respectively. Both peaks I and III were 76 mass units higher than the expected peptide fragment product (Table 1), suggesting that FRETN 89-98 was chemically modified during the analysis. (B) An internal peptide reference containing residues 3-nitrotyrosine and residues 89-93 of NFRET 89-98 (peak II) was included in the reaction. The presence of ATP leads to complete digestion of NFRET 89-98 as indicated by the absence of peak I in the right panel. The presence of peak II in both reactions indicated that *E. coli* Lon only cleaves NFRET 89-98 between Cys93 and Ser94 to generate peaks II and III. In the presence of 100 mM  $\beta$ -mercaptoethanol, the molecular ions of peaks I, II, and III were 1433.3, 757.7, and 693.35, respectively. Compared to A, the lack of peptide modification in the presence of highly reducing conditions (100 mM  $\beta$ -mercaptoethanol) indicates that the sulfhydryl moiety at Cys93 of FRETN 89-98 is susceptible to oxidation.

shown). Since complete digestion of FRETN 89-98 is readily accomplished within 1 min using trypsin, we chose to use trypsin degradation for routine calibration determination.

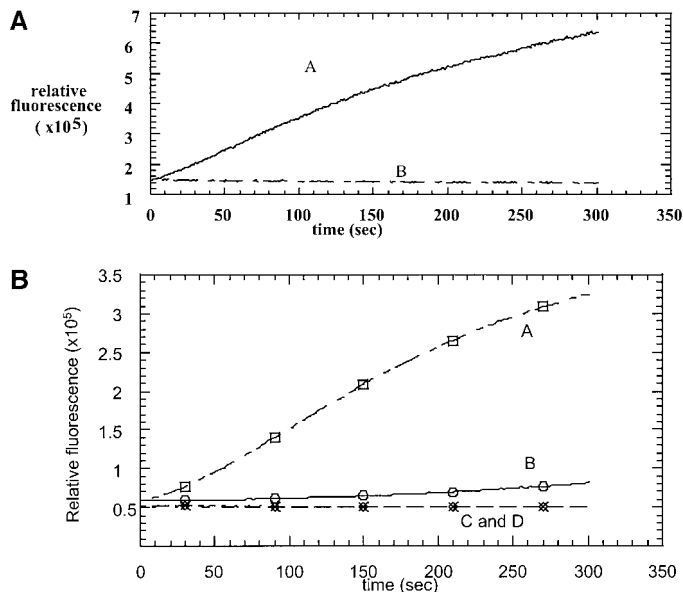
**TABLE 1**  
LC/ESIMS Analysis of FRETN 89-98 Degradation  
by *E. coli* Lon

Peak	Molecular ion (M + H) <sup>+</sup> /z, z = 1	Corresponding peptide sequence
I	1432.1	<sup>+</sup> H <sub>3</sub> N-Y(NO <sub>2</sub> )RGITCSGRQK(Abz)-CO <sub>2</sub> <sup>-</sup>
II	757.266	<sup>+</sup> H <sub>3</sub> N-Y(NO <sub>2</sub> )RGITC-CO <sub>2</sub> <sup>-</sup>
III	694.289	<sup>+</sup> H <sub>3</sub> N-SGRQK(Abz)-CO <sub>2</sub> <sup>-</sup>
IV	644.912	Unidentified

*Note.* FRETN 89-98 (2  $\mu\text{M}$ ) was digested by 200 nM *E. coli* Lon in the presence of 1 mM ATP. Reaction mixture was quenched by 0.5% SDS (final concentration) and then analysis by liquid chromatography/electrospray ionization mass spectrometry (LC/ESIMS). The LC-resolved molecular ions of the Lon cleavage products and intact FRETN 89-98 were determined by electrospray ionization mass spectrometry.



**FIG. 4.** Calibration curve of FRETN 89-98 digestion by trypsin. Each concentration of FRETN 89-98 was digested with 1.2  $\mu\text{M}$  trypsin at 37°C until no increase in fluorescence at 420 nm was detected. The net changes in fluorescence due to complete trypsin digestion are plotted against the respective substrate concentrations to establish a linear calibration curve for FRETN 89-98 degradation by *E. coli* Lon. The inner filter effects at the nonlinear range of the fluorescence intensities at 250 and 300  $\mu\text{M}$  peptide are corrected by determining the percentage decrease in fluorescence intensities at the respective peptide concentrations.

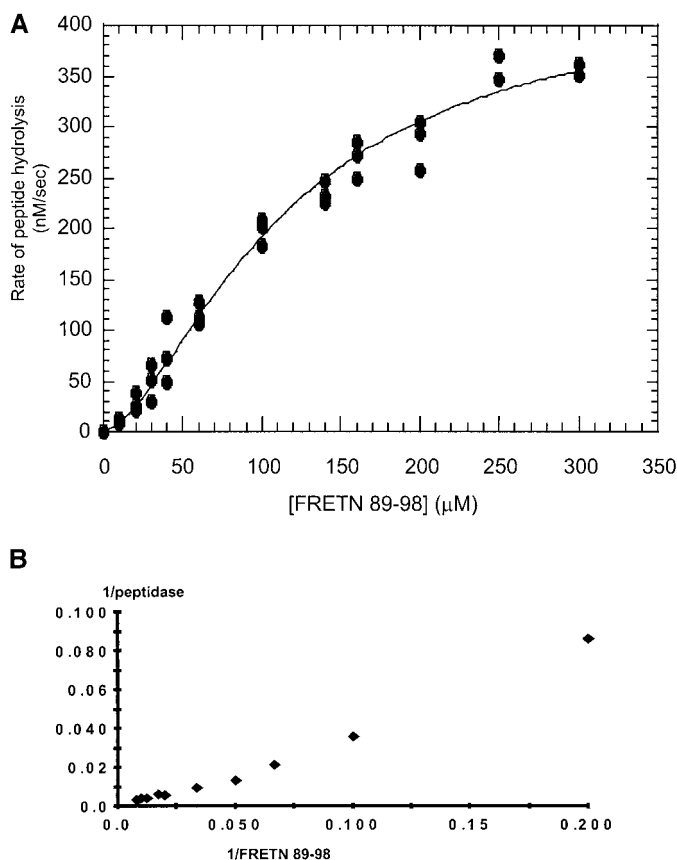


**FIG. 5.** (A) Spectrofluorometric detection of NFRET 89-98 cleavage by *E. coli* Lon. FRETN 89-98 (60  $\mu\text{M}$ ) was incubated with 250 nM of *E. coli* Lon in the presence (A) and absence (B) of 1 mM ATP as described under Experimental Procedures. Undigested FRETN 89-98 possess intrinsic fluorescence due to incomplete internal quenching of Abz by (NO<sub>2</sub>)Tyr. Fluorescence changes (excitation at 320 nm and emission at 420 nm) are only detected in the reaction with Lon and ATP. The ATP-dependent fluorescence time course is nonlinear. The initial rate of peptide hydrolysis is calculated from the first phase of the reaction. (B) Nucleotide-dependent cleavage of FRETN 89-98 by *E. coli* Lon. FRETN 89-98 (86  $\mu\text{M}$ ) was digested with 125 nM *E. coli* Lon at 37°C in the presence of ATP or nonhydrolyzable ATP analogs. Comparing the time courses of fluorescence change due to FRETN 89-98 digestion by *E. coli* Lon in the presence of (A) 0.5 mM ATP, (B) 0.5 mM AMPPNP, (C) ATP $\gamma$ S, and (D) AMPPCP indicates that peptide degradation is only supported by ATP and AMPPNP and ATP is eight times more efficient than AMPPNP in mediating FRETN 89-98 cleavage.

Figure 5A shows the time course of 60  $\mu\text{M}$  FRETN 89-98 degradation by 250 nM *E. coli* Lon in which an increase in fluorescence over time was observed only in the presence but not the absence of 1 mM ATP. The time course of FRETN 89-98 degradation exhibited nonlinear behavior. The first phase corresponds to the initial rate of peptide hydrolysis, whereas the second phase might be attributed to inhibition of peptidase activity caused by the accumulation of ADP product (see ATPase assay below) as well as peptide substrate depletion. Using the FRET peptidase assay, we were able to further demonstrate that ATP was the preferred nucleotide in FRETN 89-98 cleavage by *E. coli* Lon (Fig. 5B). Comparing the relative fluorescence intensities as a function of time in the presence of 0.5 mM ATP or 0.5 mM nonhydrolyzable ATP analogs indicated that only ATP and AMPPNP support FRETN 89-98 degradation. Furthermore, ATP is eight times more effective than AMPPNP in mediating FRETN 89-98 cleavage. This result is consistent with the re-

ported observation that AMPPNP is less efficient than ATP in supporting  $\lambda$  N protein degradation (20).

**Kinetic characterization of FRETN 89-98 degradation.** The degradation of FRETN 89-98 by *E. coli* Lon in the presence of 0.5 mM ATP exhibited sigmoidal kinetics (Fig. 6). The kinetic parameter  $[S]_{0.5}$  corresponds to the concentration of peptide substrate required to achieve 50%  $V_{\text{max}}$  and is used as a  $K_m$  equivalent for comparison with other Lon substrates. Although FRETN 89-98 is a synthetic peptide containing only one Lon cleavage site, it exhibits a specificity constant ( $k_{\text{cat}}/[S]_{0.5}$ ) comparable to those of the protein substrates (Table 2). The data presented in Fig. 6A were performed in triplicate from which the average values were fitted to the Hill equation (36)  $v = V_{\text{max}}[S]^n/K' + [S]^n$ , where  $v$  is the observed rate of



**FIG. 6.** (A) Steady-state kinetics of FRETN 89-98 degradation by *E. coli* Lon. *E. coli* Lon (125 nM) was incubated with varying concentrations of FRETN 89-98. The initial rates of peptide hydrolysis were deduced by dividing the rate of fluorescence changes by the change in fluorescence per  $\mu\text{M}$  FRETN 89-98 degradation (see Fig. 4). The velocity curve of FRETN 89-98 degradation exhibits sigmoidal kinetics, indicating that the rate of peptide cleavage is faster at high substrate concentrations. (B) Double reciprocal plot of FRETN 89-98 degradation by *E. coli* Lon. The data presented in A are averaged to generate the double reciprocal plot. The observed concave plot is consistent with sigmoidal enzyme kinetics in FRETN 89-98 degradation.

**TABLE 2**  
Steady-State Kinetic Parameters of *E. coli*  
Lon Substrate Degradation

Substrate	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}/K_m$ ( $\times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ )
N protein <sup>a</sup>	1	13	7.7
FRETN 89-98 <sup>b</sup>	$3.2 \pm 0.3$	$106 \pm 21$	$3^d$
Casein <sup>a</sup>	0.03	2.5	1.3
Glt-Ala-Ala-Phe-MNA <sup>c</sup>	0.011	400–500	0.0027

<sup>a</sup> The kinetic constants were obtained from Ref. (20).

<sup>b</sup> The kinetic constants were reported as average values of three independent assays.

<sup>c</sup> The kinetic constants were obtained from Ref. (19).

<sup>d</sup>  $k_{\text{cat}}/[S]_{0.5}$  for FRETN 89-98 degradation.

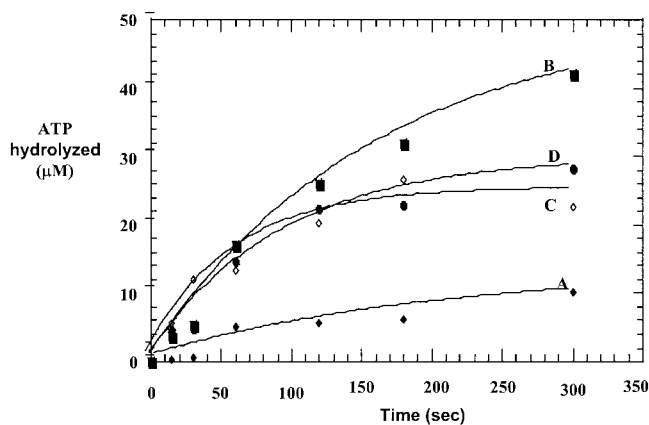
peptide hydrolysis,  $[S]$  is the peptide concentration,  $n$  is the Hill coefficient, and  $K'$  is a constant comprising the allosteric enzyme subunit interaction factors and the intrinsic substrate dissociation constant. When  $v = 0.5 V_{\text{max}}$ ,  $n \log[S]_{0.5} = \log K'$ . According to the data shown in Fig. 6A, the Hill constant is 1.6, the apparent  $K_m$  or  $[S]_{0.5}$  is  $106 \pm 21 \mu\text{M}$ , and the  $k_{\text{cat}}$  is  $3.2 \pm 0.3 \text{ s}^{-1}$ . Although the apparent  $[S]_{0.5}$  of FRETN 89-98 is 10 times higher than that of  $\lambda$  N, it is at least 4–5 times lower than that of Glt-Ala-Ala-Phe-MNA (Table 2). The double reciprocal of FRETN 89-98 degradation is shown in Fig. 6B. The concave plot is diagnostic of sigmoidal enzyme kinetics.

#### Stimulation of *E. coli* Lon ATPase by FRETN 89-98.

The ability of FRETN 89-98 to stimulate ATP hydrolysis by *E. coli* Lon was assessed using a radioactive assay that detected  $^{32}\text{P}_i$  resulting from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis. The hydrolyzed ATP products were separated by TLC from which the production of  $^{32}\text{P}_i$  over time was quantitated by PhosphorImaging. Figure 7 shows the time courses of ATP hydrolysis by *E. coli* Lon alone and in the presence of  $20 \mu\text{M}$  casein,  $100 \mu\text{M}$  FRETN 89-98, or  $100 \mu\text{M}$   $\lambda$  N 89-98. *E. coli* Lon exhibited intrinsic ATPase activity that was stimulated by either peptide or protein substrates. All the time courses shown in Fig. 7 are nonlinear, presumably caused by accumulation of ADP product inhibiting ATP hydrolysis as well as by depletion of peptide or casein substrate (in time courses B, C, and D). Inclusion of an ADP-regenerating enzyme system (3 mM phosphoenolpyruvate and 3 units/ml of pyruvate kinase) alleviated the ADP inhibition process and extended the linear range of ATP hydrolysis (data not shown). According to Fig. 7, both FRETN 89-98 and  $\lambda$  N 89-98 exhibit the same ATPase stimulation under identical reaction conditions. This result excludes the possibility that the FRET moieties stimulate Lon ATPase. In the presence of  $100 \mu\text{M}$  peptide substrates, the ATPase activity of *E. coli* Lon was elevated by 2.5-fold. Casein, on the other hand,

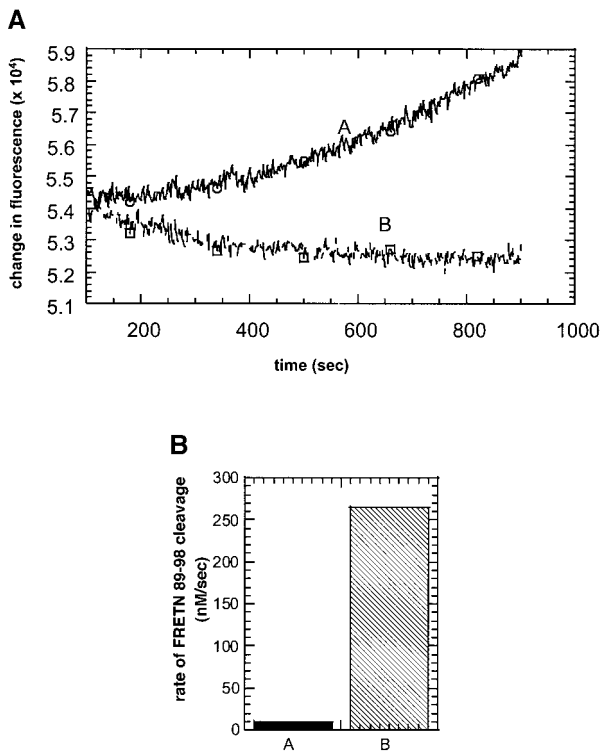
stimulated ATP hydrolysis of Lon by 4.5-fold at  $20 \mu\text{M}$ . The latter observation is consistent with published data (19) and thus serves as a positive control in our experiment. Comparison of the time courses in Fig. 7 also shows that casein differs from peptide substrates by its relative resistance to ADP inhibition. This result is consistent with previous report on protein substrates stimulate ATP hydrolysis by promoting ADP release (12, 13). In this case, more ATP is hydrolyzed by *E. coli* Lon in the presence of casein because the protein substrate is more effective in promoting ADP release.

*ATP-dependent degradation of FRETN 89-98 by human Lon.* The FRETN 89-98 peptide is also a substrate of the truncated recombinant human Lon (10, 28). This recombinant enzyme has been demonstrated to bind to mitochondrial DNA at specific sites (28) and exhibit ATP-dependent casein degradation activity (10). As shown in Fig. 8A, FRETN 89-98 is also degraded by 83 nM human Lon in the presence but not in the absence of 0.5 mM ATP. Under this reaction condition, the rate of FRETN 89-98 cleavage is 2 nM/s. Direct comparison of the rates of FRETN 89-98 cleav-



**FIG. 7.** Stimulation of *E. coli* Lon ATPase by casein and peptide substrates. ATP hydrolysis assays were carried out with 250 nM *E. coli* Lon, 1 mM  $\text{Mg}(\text{OAc})_2$ , 0.5 mM ATP, and 50 nM  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  at  $37^\circ\text{C}$  (A). The effects of casein (B), FRETN 89-98 (C), and  $\lambda$  N 89-98 (D) on *E. coli* Lon ATPase activity were assessed by monitoring the ATPase time courses of *E. coli* Lon in the presence of each protein or peptide substrates. Reaction aliquots were quenched at the indicated times from which the hydrolyzed products were analyzed by thin-layer chromatography. The time courses of ATP hydrolysis were quantitated by the increase in  $^{32}\text{P}$  liberation over time. All four ATPase reactions display complex time courses. The initial phases of the time courses are linear. The slow phases of the time course are partially contributed by ADP product inhibition since removal of ADP by inclusion of phosphoenolpyruvate kinase and phosphoenolpyruvate in the reaction extends the linearity of the first phases of the reactions (data not shown). In time courses B, C, and D, the nonlinear time courses are also contributed by peptide or protein substrate depletion as the reaction progresses. The initial rates of ATPase stimulation by casein, FRETN 89-98, and  $\lambda$  N 89-98 are identical. Compared to the casein and peptide induced ATPase activity, the intrinsic *E. coli* Lon ATPase activity is two- to fourfold lower after 300 s of reaction.





**FIG. 8.** (A) ATP-dependent degradation of FRETN 89-98 by recombinant human Lon. FRETN 89-98 ( $86 \mu\text{M}$ ) was digested with  $83 \text{ nM}$  recombinant human Lon at  $37^\circ\text{C}$  using the reaction conditions for *E. coli* Lon. An increase in fluorescence is detected only in the presence of  $0.5 \text{ mM}$  ATP, indicating that the degradation of FRETN 89-98 by human Lon exhibits ATP-dependency. (B) Comparison of the rate of FRETN 89-98 cleavage by *E. coli* and truncated human Lon. FRETN 89-98 ( $160 \mu\text{M}$ ) was digested with  $125 \text{ nM}$  human Lon (A) or  $125 \text{ nM}$  *E. coli* Lon (B) in the presence of  $0.5 \text{ mM}$  ATP at pH 8.1 at  $37^\circ\text{C}$ . The data presented in A are an average of two assays with less than 10% experimental variation. The data presented in B are an average of three assays with less than 16% experimental variation. The rate of FRETN 89-98 cleavage was monitored as described under Experimental Procedures using *E. coli* Lon enzyme. The rate of FRETN 89-98 cleavage by human Lon is 25-fold slower than that by *E. coli* Lon.

age by the human and *E. coli* lon homologs indicates that FRETN 89-98 is a better substrate for the bacterial enzyme because under identical reaction conditions the rate of FRETN 89-98 cleavage is  $11 \pm 0.7 \text{ nM/s}$  by human Lon and  $265.3 \pm 19.2 \text{ nM/s}$  by *E. coli* Lon (Fig. 8B).

## DISCUSSION

*E. coli* Lon exhibits varying degrees of energy requirement towards hydrolysis of different protein and peptide substrates. The degradation of protein substrates stimulates ATP hydrolysis while the degradation of small peptides such as Glt-Ala-Ala-Phe-MNA inhibits ATP hydrolysis (19). It is impossible to unambiguously define the reason for the difference in nucleotide requirement in peptide degradation because the  $\lambda$

N protein and Glt-Ala-Ala-Phe-MNA differ by size, amino acid sequence, and the location of Lon cleavage sites. One or a combination of these attributes may contribute to the observed difference exhibited by the two Lon substrates. To examine this issue, we synthesized FRETN 89-98 based upon the degradation profile of  $\lambda$  N protein (20) and examined the specificity of Lon-mediated cleavage as well as its effect on ATP hydrolysis by *E. coli* Lon. This peptide contains a single Lon cleavage site, it is substantially larger than the traditionally used tetrapeptide substrates, and thus it can be used to initially address the effect of peptide length toward ATPase stimulation. Nevertheless, Glt-Ala-Ala-Phe-MNA also differs from FRETN 89-98 by its peptide sequence and the Lon cleavage site sequence (Fig. 1). Therefore we cannot rule out the possibility that the observed difference in ATPase stimulation and peptide degradation between FRETN 89-98 and Glt-Ala-Ala-Phe-MNA degradation is attributed to peptide sequence rather than peptide length. Examination of the changes in Lon activities using truncated FRETN 89-98 peptide substrates will be required to further address this issue.

By incorporating a long-range fluorescence resonance energy transfer pair, Abz and Y(NO<sub>2</sub>), into a peptide substrate, the ATP-dependent cleavage of FRETN 89-98 by Lon can be continuously monitored. Since other peptidase assays of Lon are discontinuous (2), the FRETN 89-98 peptide provides a convenient alternative toward characterizing Lon activity. The quenching mechanism of Abz by Y(NO<sub>2</sub>) has been demonstrated to involve a long-range resonance energy transfer type in which the quenching efficiency varies linearly with the distance separating the quencher Y(NO<sub>2</sub>) and the fluorophore (Abz) (31, 35). As demonstrated by Meldal and Breddam (31), complete fluorescence quenching is observed over distances of more than  $20 \text{ \AA}$  that separate the donor and the quencher, and only 18% of the quenching efficiency is lost when the donor and acceptor are separated by  $50 \text{ \AA}$  in a 16-amino-acid peptide. Therefore utilization of peptide substrates containing the Y(NO<sub>2</sub>) and Abz functionalities will allow for kinetic characterization of Lon degrading longer synthetic peptides.

Although FRETN 89-98 contains only part of the  $\lambda$  N protein sequence, it is cleaved effectively by *E. coli* Lon in the presence of ATP (Table 2). LC/ESIMS analysis (Fig. 3) indicates that *E. coli* Lon specifically cleaves FRETN 89-98 at Cys93 and Ser94, which is also the same cleavage site in the native  $\lambda$  N protein. Furthermore, one unique feature of FRETN 89-98 is its ability to stimulate ATP hydrolysis by *E. coli* Lon with concomitant degradation. Employing this technique, values of  $k_{\text{cat}}$ ,  $[S]_{0.5}$ , and  $k_{\text{cat}}/[S]_{0.5}$  were readily determined (Table 2). Our data indicate sigmoidal kinetics in FRETN 89-98 degradation by *E. coli* Lon using  $0.5$

mM ATP. This study reports the first sigmoidal behavior in peptide degradation by Lon. The appearance of sigmoidal kinetics in enzyme velocity curves suggests cooperativity of the substrate binding events that precede the catalytic steps at the active sites. However, it has been noted that a two-substrate enzyme that follows a random-ordered mechanism can display sigmoidal kinetics without true cooperativity (37, 38). Therefore FRET<sub>N</sub> 89-98 will be an important tool to further define the observed sigmoidal kinetic behavior of Lon.

The degradation of FRET<sub>N</sub> 89-98 by *E. coli* Lon is most effective using ATP as the cosubstrate. In fact, ATP analogs such as AMPPCP and ATP $\gamma$ S which generally support peptide cleavage by Lon (2, 19) exert no stimulatory effect on FRET<sub>N</sub> 89-98 degradation. Furthermore, substrates capable of stimulating ATP hydrolysis in general display high specificity constants as peptidase substrates of Lon (Table 2). While the catalytic efficiency ( $k_{\text{cat}}/[S]_{0.5}$ ) of FRET<sub>N</sub> 89-98 degradation by *E. coli* Lon approximates that of protein substrates, it is 1000-fold higher than the tetrapeptide substrate Glt-Ala-Ala-Phe-MNA (Table 2). Since both FRET<sub>N</sub> 89-98 and protein substrates stimulate while Glt-Ala-Ala-Phe-MNA inhibits the ATPase activity of *E. coli* Lon, FRET<sub>N</sub> 89-98 will serve as a valuable substrate for probing the molecular mechanism of peptide-induced ATPase activity of Lon.

The ability of FRET<sub>N</sub> 89-98 to serve as a substrate of the truncated human Lon has also been assessed. Compared to *E. coli* Lon, the human enzyme is 25-fold slower in cleaving FRET<sub>N</sub> 89-98 under identical reaction condition (Fig. 8B). The reason for the observed difference in the rate of FRET<sub>N</sub> 89-98 cleavage cannot be deduced from this study. Further kinetic characterization of FRET<sub>N</sub> 89-98 degradation by human Lon, however, should provide interesting insight into the fundamental substrate discrimination mechanism among *lon* homologs.

In conclusion, we have demonstrated that ATP hydrolysis contributes to the efficiency of FRET<sub>N</sub> 89-98 peptide cleavage. The *E. coli* Lon cleavage profile of FRET<sub>N</sub> 89-98 is nearly identical to that of the native  $\lambda$  N protein, thereby validating the utilization of FRET<sub>N</sub> 89-98 as an *in vitro* substrate mimic to probe the mechanism of ATP-dependent proteolysis. Since human Lon also cleaves FRET<sub>N</sub> 89-98 in an ATP-dependent fashion, we propose that the FRET peptidase assay will be a very useful tool in kinetic characterization Lon catalysis.

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