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Distinct quaternary structures of the AAA+ Lon protease control substrate degradation

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Lon is an ATPase associated with cellular activities (AAA+) protease that controls cell division in response to stress and also degrades misfolded and damaged proteins. Subunits of Lon are known to assemble into ring-shaped homohexamers that enclose an internal degradation chamber. Here, we demonstrate that hexamers of *Escherichia coli* Lon also interact to form a dodecamer at physiological protein concentrations. Electron microscopy of this dodecamer reveals a prolate structure with the protease chambers at the distal ends and a matrix of N domains forming an equatorial hexamer–hexamer interface, with portals of ~45 Å providing access to the enzyme lumen. Compared with hexamers, Lon dodecamers are much less active in degrading large substrates but equally active in degrading small substrates. Our results support a unique gating mechanism that allows the repertoire of Lon substrates to be tuned by its assembly state.

Protein quality control is vital under stress conditions that promote protein unfolding and aggregation. *Escherichia coli* Lon degrades many unfolded proteins (1–3) and also degrades folded proteins, including SulA (suppressor of Lon protein) and the inclusion-body binding proteins A and B (IbpA and B) (4–6). In *E. coli* and many other bacteria, Lon is up-regulated under numerous stress conditions (7–10). In mitochondria, Lon helps combat oxidative stress (11–14), and human mitochondrial Lon was recently identified as a potential antilymphoma target (15). It is widely believed that a major role of Lon in all organisms is to degrade misfolded proteins (2, 10, 16).

Lon subunits consist of an N domain, a central ATPase associated with cellular activities (AAA+) ATPase module, and a C-terminal peptidase domain. Although early reports suggested that Lon might be a tetramer (17), it is now clear that six subunits of the *E. coli* enzyme assemble into a hexamer with an internal degradation chamber accessible via an axial pore in the AAA+ ring (18, 19). Lon substrates are recognized, unfolded if necessary by ATP-dependent reactions mediated by the AAA+ ring, and then translocated through the pore and into the peptidase chamber for degradation (20).

In many families of ATP-dependent proteases, the AAA+ unfolding/translocation ring and the self-compartmentalized peptidase are encoded by distinct polypeptides, which assemble into independent oligomers before interacting to form the functional protease (21, 22). For example, the ClpXP protease consists of AAA+ ClpX hexamers, which dock with the self-compartmentalized ClpP peptidase. This interaction suppresses the ATPase rate of ClpX and enhances the peptidase activity of ClpP (22). Lon activity cannot be controlled in this way because the ATPase and peptidase domains are always physically attached. Little is currently known about how Lon activity is regulated, although mutational studies show that the AAA+-peptidase domains influence each other’s activities (23–25). In some cases, the function of the two domains also appears to be linked via allosteric communication mediated by substrate binding (26, 27).

Here, we demonstrate that Lon forms dodecamers that equilibrate with hexamers at physiological concentrations. A structure determined by EM at low resolution reveals a unique protease architecture with the degradation chambers of each hexamer at opposite ends of a prolate ellipsoid. Near the equator of this structure, the arrangement of N domains creates portals, which could serve as entry sites for protein substrates. Formation of the dodecamer suppresses proteolysis of large but not small protein substrates, suggesting that the dodecamer uses a gating mechanism that allows the repertoire of Lon substrates to be tuned by its state of assembly.

Results

Lon Exists in Multiple Oligomeric Forms. In the process of characterizing *E. coli* Lon by size-exclusion chromatography (SEC) and multiangle laser light scattering (MALS), we observed enzyme complexes with properties similar to those expected for hexamers (calculated Mr 525 kDa) and dodecamers (calculated Mr 1,050 kDa). For example, SEC-MALS of the LonS679A variant, which had an active-site mutation in the peptidase domain to prevent autodegradation (28), revealed two major species corresponding to molecular weights of 565 ± 13 and 930 ± 5 kDa (Fig. 1A). Based on previous characterization of Lon as a hexamer (18, 19), the simplest interpretation of these results is that the larger species is a Lon dodecamer, which may dissociate to some extent during the SEC run.

To characterize assembly further, we used sedimentation velocity-analytical ultracentrifugation (SV-AUC) at multiple concentrations of LonS679A in the presence of 100 μM ATPγS (Fig. 1B). Both the large (dodecamer) and smaller (hexamer) assemblies were clearly detectable at multiple LonS679A concentrations, as was a smaller species, which appeared to be a monomer. As expected, the dodecamer was more highly populated at higher concentrations, and the hexamer and presumed monomer populations increased at lower concentrations. The hexamer and dodecamer were both present at all concentrations, suggesting that the repertoire of Lon substrates can be tuned by its assembly state.

Significance

Lon protease degrades unfolded or damaged proteins as well as numerous cellular regulatory proteins. How these different classes of substrates are recognized is poorly understood. We find that Lon hexamers assemble via a matrix of N-domain interactions to form a dodecamer with altered substrate-degradation properties. Access of protein substrates to the degradation machinery in the dodecamer appears to require passage through equatorial portals. As a consequence, large substrates that are efficiently degraded by hexamers seem to be preferentially excluded from dodecamers. This gating mechanism allows the substrate repertoire of Lon to be adjusted by its assembly state.


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over both peaks suggests that \( \sim \mu_300 \) Lonpreferentially assumed a side-view orientation on the grid (Fig. 3B). Multiple views of hexamers were also observed. The side view of the hexamer corresponded to roughly half of the density observed in the side view of the dodecamer (Fig. 3B).

Following sixfold (C6) averaging, a 3D reconstruction of the dodecamer classes showed face-to-face hexameric rings connected by six strands of density (Fig. 3C). The dodecamer was \( \sim 250 \) Å long and \( \sim 160 \) Å wide and roughly the shape of a prolate ellipsoid. We created planar hexameric models of the ATPase and the protease domains from a crystal structure including these portions of a Bacillus subtilis Lon subunit (29) (Experimental Procedures) and manually placed them into the density map (Fig. 3D and E). The protease domains fit well into the density at the distal ends, with the adjacent ATPase domains closer to the equator. There were some clashes between the protease and ATPase domains, but the low resolution of the structure and uncertainty about the quality of our hexameric models precluded better fitting. The extra density near the equator was fit as a matrix of interacting E. coli Lon N domains, arranged as overlapping dimers through a coiled-coil region, which create bridges between the two halves of the structure (Fig. 3E). Strikingly, portals with diameters of \( \sim 45 \) Å were clearly visible between the N domains. As discussed

dodecamer were both populated at concentrations that are physiologically relevant (see below).

To confirm that wild-type Lon also formed dodecamers, we used analytical gel filtration (Fig. S1), which is rapid and minimizes autoproteolysis. In addition, because nucleotide can affect Lon assembly, experiments were performed with ATPγS or without nucleotide. Under the conditions tested, Lon appeared to chromatograph as a mixture of hexamers and dodecamers, and nucleotide had little effect on the distribution of these species (Fig. S1).

Dodecamers Should Exist at Intracellular Concentrations. To investigate the potential for Lon dodecamers to form in vivo, we determined intracellular concentrations using quantitative Western blots. A dilution series of purified Lon was analyzed on the same membrane as Lon from cells grown at 30 °C (Fig. 2A). The concentration of Lon in monomer equivalents ranged from 1.7 to 3.7 \( \mu \)M over four measurements and averaged 2.5 \( \pm 0.5 \) \( \mu \)M (SEM) for cells grown at 30 °C. Lon forms hexamers and dodecamers at these concentrations in vitro. Following a temperature increase to 42 °C, a modest increase in Lon levels was observed by Western blots (\( \sim 1.2 \)-fold) (Fig. 2B).

**EM Dodecamer Structure.** EM images of negatively stained Lon complexes showed two major populations (Fig. 3A). Classification of over 4,000 particles revealed that one major species was a dodecamer with roughly sixfold symmetry (Fig. 3B). These dodecamers preferentially assumed a side-view orientation on the grid (Fig. 3B). Multiple views of hexamers were also observed. The side view of the hexamer corresponded to roughly half of the density observed in the side view of the dodecamer (Fig. 3B).

Following sixfold (C6) averaging, a 3D reconstruction of the dodecamer classes showed face-to-face hexameric rings connected by six strands of density (Fig. 3C). The dodecamer was \( \sim 250 \) Å long and \( \sim 160 \) Å wide and roughly the shape of a prolate ellipsoid. We created planar hexameric models of the ATPase and the protease domains from a crystal structure including these portions of a Bacillus subtilis Lon subunit (29) (Experimental Procedures) and manually placed them into the density map (Fig. 3D and E). The protease domains fit well into the density at the distal ends, with the adjacent ATPase domains closer to the equator. There were some clashes between the protease and ATPase domains, but the low resolution of the structure and uncertainty about the quality of our hexameric models precluded better fitting. The extra density near the equator was fit as a matrix of interacting E. coli Lon N domains, arranged as overlapping dimers through a coiled-coil region, which create bridges between the two halves of the structure (Fig. 3E). Strikingly, portals with diameters of \( \sim 45 \) Å were clearly visible between the N domains. As discussed

![Fig. 1.](image1.png) Lon assembles into dodecamers as well as hexamers. (A) Catalytically inactive Lon_{5679A} (24-μM loading concentration) formed hexamers (expected \( M_w \sim 525 \) kDa) and dodecamers (expected \( M_w \sim 1,050 \) kDa) in SEC-MALS experiments. The variation in apparent \( M_w \) over both peaks suggests that dodecamers and hexamers are in equilibrium. Dashed lines represent an error of 5% in measurement of molecular weight. Chromatography was performed at room temperature in 50 mM Hepes (pH 7.6), 150 mM NaCl, 20 mM MgCl₂, 10% (vol/vol) glycerol, and 0.1 mM TCEP. (B) Concentration-dependent changes in the population of Lon_{5679A} dodecamers, hexamers, and monomers in SV-AUC c(s20,w) distributions. Traces at each concentration were offset on the y axis for clarity. Experiments were performed at 20 °C in 50 mM Hepes-KOH (pH 7.5), 150 mM NaCl, 0.01 mM EDTA, 0.1 mM TCEP, 1 mM MgCl₂, and 0.1 mM ATPγS.

![Fig. 2.](image2.png) Quantitative Western blot analysis of Lon concentrations in E. coli. (A) Western blot following SDS-PAGE of different concentrations of purified Lon and an E. coli lysate before and after heat shock. The intracellular concentration of Lon was 2.5 \( \pm 0.5 \) \( \mu \)M or \( \sim 1,500 \sim 300 \) Lon monomers per cell at 30 °C (n = 4). (B) Lon concentrations increased slightly in cells grown after a temperature increase to 42 °C compared with cells grown at 30 °C (n = 3). Values are averages \( \pm 1 \) SEM.
below, these portals may exclude entry of large substrates into the lumen of the enzyme, where the degradation machinery resides.

**Hexamers Have Higher Basal ATPase Activity than Dodecamers.** Because equilibration precluded isolation of pure hexamers or dodecamers for functional assays, we measured rates of ATP hydrolysis over the same range of Lon concentrations that altered the dodecamer/hexamer ratio in the SV-AUC studies. In control experiments, we confirmed that hexamers and dodecamers were still present as the major species in buffers and under conditions that mimicked our enzyme-assy conditions (Fig. S2). When rates of ATP hydrolysis were normalized for the total number of Lon subunits in each reaction, basal hydrolysis slowed substantially as the dodecamer/hexamer ratio increased (Fig. 4 and Fig. S3A). The concentration dependence of the ATPase activity was fitted best by a $K_D$ of 3.3 ± 1.5 μM for the hexamer–dodecamer interaction, a hydrolysis rate of 23 ± 3.6 subunit$^{-1}$ min$^{-1}$ for the hexamer, and a hydrolysis rate of 1.8 ± 1.3 subunit$^{-1}$ min$^{-1}$ for the dodecamer (Fig. S3A). Thus, the hexamer hydrolyzes ATP ~10-fold faster than the dodecamer in the absence of protein substrates.

**Dodecamers Degradate “Large” Substrates Poorly and “Small” Substrates Well.** To evaluate degradation of different substrates by the dodecamer, we assayed proteolysis using a range of Lon concentrations. The first substrate was an inclusion-body binding protein, specifically *E. coli* IbpB, which contains a native α-crystallin domain that is recognized by Lon (6). Although IbpB monomers are relatively small (~16 kDa), they assemble into large cage-like oligomers (30, 31). For example, IbpB runs at an apparent Mr > 670 kDa in gel-filtration chromatography (Fig. S4A) (6). We determined initial rates of Lon degradation of 35S-labeled IbpB by assaying the production of acid-soluble peptides and normalized these rates by the total concentration of Lon subunits (Fig. 5A). Importantly, IbpB was degraded more slowly at higher Lon concentrations, where more dodecamer was present. Fig. 5B shows Michaelis–Menten plots for IbpB degradation using 1.5 and 6 μM Lon. $K_M$ was similar at both Lon concentrations, but $V_{max}$ was substantially lower at the higher Lon concentration. Fitting the Lon-concentration dependence of IbpB degradation gave a $K_D$ of 1.8 ± 0.5 μM for the hexamer–dodecamer interaction, a maximum degradation rate of 0.057 ± 0.058 subunit$^{-1}$ min$^{-1}$ for the hexamer, and a rate of 0.00095 ± 0.0021 subunit$^{-1}$ min$^{-1}$ for the dodecamer (Fig. S3B). Thus, the Lon dodecamer degrades IbpB ~60-times more slowly than the hexamer.

Next, we examined degradation of fluorescein isothiocyanate (FITC)-conjugated β-casein (monomer Mr ~25 kDa) by different concentrations of Lon. β-Casein is unstructured but can form micelles and even as a monomer has a radius of gyration much larger than expected for a compact structure (Fig. S4A) (32, 33). Normalized initial rates of FITC-casein degradation were determined by changes in fluorescence, and decreased substantially as the Lon concentration and dodecamer/hexamer ratio increased (Fig. 5C). Fitting the Lon dependence of degradation gave a $K_D$
Fig. 5. High concentrations of Lon degrade IbpB and FITC-casein less efficiently. (A) Normalized initial rates of IbpB degradation (45 μM) decreased as the Lon concentration increased. (B) Michaelis-Menten plots of normalized rates of steady-state degradation of IbpB by 1.5 or 6 μM Lon. The lines are fits to the Michaelis-Menten equation. (C) Normalized initial rates of degradation of FITC-casein (50 μM) also decreased with increasing Lon concentration. In all panels, data are plotted as averages ± 1 SEM (n = 3), and reactions were performed at 37 °C and contained 4 mM ATP, 5 mM MgCl₂, 5 mM KCl, 2% (vol/vol) DMSO, 360 mM potassium glutamate, 12% (wt/vol) sucrose, and 50 mM Hepes-KOH (pH 8).

of 1.3 ± 0.6 μM for the hexamer-dodecamer interaction, a maximum degradation rate of 1.8 ± 0.4 subunit⁻¹ min⁻¹ for the hexamer, and a rate of 0.00001 ± 0.00003 subunit⁻¹ min⁻¹ for the dodecamer (Fig. S3C). Thus, as with IbpB, the Lon dodecamer degrades this substrate far more slowly than does the Lon hexamer.

Finally, we used the appearance of acid-soluble peptides to assay Lon degradation of [³⁵S]-labeled titin-I27 proteins (Mᵦ ∼12 kDa) with appended N- or C-terminal sul20 or β20 degrons (27, 34). We also assayed degradation of some titin-I27 substrates following cysteine carboxymethylation, which unfolds the protein (35). Notably, Lon concentration had little effect on degrontagged titin-I27 degradation, whether substrates were native or denatured or contained N- or C-terminal degradation tags (Fig. 6 A and B). Moreover, in the presence of carboxymethylated titin-I27–sul20, the rate of ATP hydrolysis by Lon showed a much smaller dependence on enzyme concentration (Fig. 6C), compared with the rate of ATP hydrolysis in the absence of a protein substrate (Fig. 4). We considered the possibility that binding of the sul20 degron to Lon might result in dodecamer dissociation. However, dodecamers were present in the EM experiments performed in the presence of sul20 peptide (Fig. 3), and addition of this peptide did not detectably change the dodecamer/hexamer ratio in gel-filtration experiments (distributions were similar to Fig. S1). In contrast to the case with the Ibps, the model degron substrates stimulate the rate of ATP-hydrolysis by Lon ∼3- to 10-fold (Fig. 6C). Nonetheless, the substrate-stimulated ATPase rate (normalized by Lon concentration) was lowest at the highest Lon concentrations (6 and 12 μM) (Fig. 6C), indicating that both degron-enzyme interactions and the hexamer-dodecamer equilibrium influence ATP hydrolysis.

Taken together, our results support a model in which Lon hexamers and dodecamers are both active proteases. Importantly, however, the dodecamer only efficiently degraded the degrontagged titin-I27 substrates, which behaved as much smaller species than the IbpB and β-casein substrates (Fig. S4). As we discuss below, the portals created by dodecamer assembly may provide a “gating” mechanism that prevents larger substrates from entering the luminal chamber and being degraded.

Discussion

Our results show that hexamers of E. coli Lon assemble into a dodecamer that displays different enzymatic properties. Hexamers and dodecamers are both populated at low micromolar concentrations in vitro, and the Lon concentration in vivo is ∼2.5 μM. Thus, the hexamer-dodecamer equilibrium is likely to be a physiologically relevant factor in controlling Lon activity in cells.

Our EM structure of the Lon dodecamer reveals a face-to-face association of hexamers in which the N domains appear to be largely responsible for stabilizing the complex. This architecture positions the degradation chambers of each hexamer at the distal ends of the complex and has not been observed in other AAA+ proteases. As observed in the crystal structure of an archaeal LonB hexamer (36), the degradation chambers in the dodecamer appear to be sequestered from bulk solution. Thus, degradation still requires substrate unfolding/translocation by the Lon AAA+ ring, an apparently universal feature of AAA+ proteases (20).

The Lon N domains have been implicated in substrate recognition (37–39). Here we show an additional role for the N domain in dodecamer assembly. Thus, formation of the dodecamer may alter the substrate-recognition properties of the enzyme by creating or occluding substrate-binding sites. Furthermore, we find that when the N domains interact with each other, and/or with the ATPase domain of the opposite hexamer in the dodecamer, the rate of ATP hydrolysis by Lon can be suppressed. A similar suppression of ATPase activity is observed when CspX interacts CspP (22). Understanding the molecular basis of the suppression of ATPase activity upon dodecamer assembly, and the activation of ATP hydrolysis by some Lon substrates is likely to provide important insight into the allosteric mechanisms that are used by substrates to control Lon’s enzymatic activities (26, 27).

A notable feature of the dodecamer is the presence of six portals, each ∼45 Å in diameter, spaced around the equator of the structure. The size of these portals should prevent entry of large substrates into the lumen of the dodecamer (Fig. 7). Indeed, we found that IbpB and β-casein substrates, which have large radii of...
Lon hexamers and dodecamers degrade degron-tagged titin-I27 substrates with similar efficiencies. (A) Degradation of native substrates with C-terminal su20 degrons (gray), C-terminal p20 degrons (blue), and N-terminal p20 degrons (orange). (B) Degradation of denatured titin-I27 substrates. Deagon colors are the same as in A. (C) In the presence of carboxymethylated titin-I27-sul20 (40 μM), ATPase rates decreased only marginally at high Lon concentrations. In all panels, data are plotted as averages ±1 SEM (n = 3). Reactions were performed at 37 °C and contained 25 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, and 2 mM ATP.

Fig. 6. Lon hexamers and dodecamers degrade degron-tagged titin-I27 substrates with similar efficiencies. (A) Degradation of native substrates with C-terminal su20 degrons (gray), C-terminal p20 degrons (blue), and N-terminal p20 degrons (orange). (B) Degradation of denatured titin-I27 substrates. Deagon colors are the same as in A. (C) In the presence of carboxymethylated titin-I27-sul20 (40 μM), ATPase rates decreased only marginally at high Lon concentrations. In all panels, data are plotted as averages ±1 SEM (n = 3). Reactions were performed at 37 °C and contained 25 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, and 2 mM ATP.

glyation, were degraded very slowly by Lon dodecamers compared with hexamers. In fact, given the confidence of data fitting, we cannot exclude the possibility that the dodecamer cannot degrade IbpB or β-casein. In contrast, we found that smaller degron-tagged titin-I27 substrates in both folded and unfolded states were degraded at similar rates by Lon hexamers and dodecamers. These results support a model in which a major determinant of substrate degradation by the Lon dodecamer is the ability of a substrate to diffuse or be pulled through the portals to allow engagement by the degradation machinery. In principle, substrate size, charge, hydrophobicity, stability, or the length of the disordered degron tag could all contribute to determining whether specific substrates could enter the lumen and be degraded by Lon dodecamers.

At present, we can only speculate about possible biological functions for the Lon dodecamer. One possibility is that cellular stress results in very high concentrations of misfolded proteins that form large aggregates or bind the IbpA/IbpB chaperones, with degradation of such substrates by Lon hexamers swamping the enzyme’s ability to degrade regulatory proteins and therefore inhibiting the recovery from stress. These regulatory proteins include SulA (19 kDa, C-terminal degron), which is rapidly degraded during recovery after DNA damage to allow cell division to resume (40). Similarly, SoxS (13 kDa, N-terminal degron), a transcriptional activator of the superoxide-response regulon (41), and UmuD (30-kDa dimer, N-terminal degron), a subunit of the lesion-bypass DNA polymerase (42) need to be degraded by Lon specifically as cells recover from stress. In this scenario, Lon dodecamers could carry out regulatory degradation, whereas hexamers triage and perform quality-control degradation of damaged/unfolded proteins. It is also possible, that dodecamers are more highly populated under stress conditions, reducing the rate of IbpA/IbpB degradation and increasing their concentration to suppress aggregation and promote refolding in cooperation with ClpB and DnaK (43, 44). Alternatively, equatorial interfaces in Lon dodecamers, which are absent in hexamers, might allow recognition and degradation of additional important substrates. ATP is abundant during exponential growth but scarce when nutrients are lacking. In principle, Lon dodecamer formation might serve to conserve ATP, as we find that basal hydrolysis by the dodecamer is much lower than by the hexamer. Thus, if Lon were predominantly dodecameric during stationary-phase growth, when substrates became available they could still be degraded to provide amino acids or for regulatory purposes but at a lower cost in terms of overall ATP consumption. Testing these models will require the design of Lon variants that are exclusively hexameric or dodecameric. We are pursuing higher resolution structures of the dodecamer to guide these efforts. B. subtilis and Mycobacterium smegmatis Lon have also been reported to form oligomers larger than hexamers (23, 29, 45). Thus, hexamer-dodecamer equilibria may be a relatively conserved feature of this important intracellular protease.

Experimental Procedures

Protein Purification. Wild-type E. coli Lon (9) and the LonS679A variant (28) were purified as described previously (34) with minor changes. After lysis by sonication or French press, lysates were incubated with benzonase (Merck) for 1 h at 4 °C. Following elution from P11 phosphocellulose (Whatman, GE Healthcare), the sample was concentrated and buffer exchanged to 2 mL either in 25 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-
KOH (pH 7.5), 2 M NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid), 0.1 mM TCEP [tris(2-carboxyethyl)phosphine], and 1% (vol/vol) glycerol, or 50 mM Hepes-KOH (pH 7.5), 2 M NaCl, and 0.1 mM TCEP, passed through a 0.45-μm filter, and purified on a HR 10/30 Superose 6 gel-filtration column (GE Healthcare). Fractions containing Lon at >95% purity, as judged by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and 280/260 nm absorbance, were combined, dialyzed against Lon storage buffer [50 mM Hepes-KOH (pH 7.5), 150 mM NaCl, 10% (vol/vol) glycerol, 1 mM EDTA, and 0.1 mM TCEP], concentrated, flash frozen in aliquots, and stored at −80 °C. Escherichia coli ipbp8 was purified as previously described (16), dialyzed against E. coli storage buffer [50 mM Hepes-KOH (pH 8), 600 mM potassium glutamate, 20% (wt/vol) sucrose, and 0.1 mM TCEP], flash frozen in aliquots, and stored at −80 °C. His-tagged titin-I27 proteins with sul20 or j20 degrons were purified as previously described (34).

SEC-MALS. SEC was performed on a Wyatt WTC-03055 size-exclusion column using an Agilent HPLC. MALS was measured in line using a Wyatt DAWN HELOS instrument; concentrations were determined using a Wyatt Optilab rEx instrument. Standard Zimm-plot analysis was performed with the ASTRA software 5.3.4 (Wyatt Technology). Lon679A samples (loading concentrations 24, 12, and 6 μM) were run in 50 mM Hepes-KOH (pH 7.6), 150 mM NaCl, 20 mM MgCl₂, 10% (vol/vol) glycerol, and 0.1 mM TCEP at room temperature.

Ultrafiltration. SV-AUC experiments for Lon679A were performed using a Beckman OptimaXL-I analytical ultrafiltration (Biophysical Instrumentation Facility, Massachusetts Institute of Technology, Cambridge, MA). Samples were dialyzed overnight against 50 mM Hepes-KOH (pH 7.5), 150 mM NaCl, 0.01 mM EDTA, and 0.1 mM TCEP. Before loading the cells, 1 mM MgCl₂, and 0.1 mM ATP-S were added to the samples. Samples were loaded in dual-sector centrifugal filter units (Amicon) in a Beckman A50 Ti rotor at 20 °C. SEDFIT (46) was used to calculate the continuous distribution of sedimentation coefficients from 15 to 60% at resolutions of 200 or 100 scans per second. Calculations were performed using a density of 1.00831, a viscosity of 0.010475, and a Lon partial specific volume of 0.7431 (SEDMTERP, J. Philo; www.jphilo.mailway.com).

Western Blotting. E. coli W3110 cells were grown at 30 °C in M9 medium supplemented with 0.4% (wt/vol) glucose, 100 μM CaCl₂, 2 mM MgSO₄, 0.2% (wt/vol) thiamin, and 0.2% (wt/vol) casamino acids. At an OD₆₀₀ of 0.3, cultures were split, additional medium at 54 °C was added to the heat-shock samples, and additional medium at 30 °C was added to the control. The final temperature of the heat-shock sample was 42 °C. Aliquots of 1 mL were taken at each time point from both samples, OD₆₀₀ was recorded, cells were pelleted by centrifugation, and the supernatant was removed. Pellets were stored at −20 °C until resuspended to 2.5 OD₆₀₀ equivalents with 5x SDS loading dye. Resuspended samples were heated at 99 °C for 10 min while shaking, and then cooled. The samples (10 μL) were loaded on Mini-PROTEAN TGX 4–20% (wt/vol) polyacrylamide precast gels (Bio-Rad). The gels were transferred onto filter paper using a wet-transfer apparatus (Bio-Rad), probed with anti-Lon polyclonal antibody (provided by Covance Research Products) at a 1:2,000 dilution for 4 h at room temperature, incubated with goat anti-rabbit IgG-AP conjugate (Bio-Rad) at a 1:10,000 dilution for 1 h, and washed, and then developed with alkaline phosphatase dephosphor- ylation and filter paper using a wet-transfer apparatus (Bio-Rad), and stained with a 1:10,000 dilution of rabbit anti-Lon antiserum (1:10,000 dilution), blebbistatin conjugated to horse radish peroxidase (1:10,000 dilution), as previously described (35).

Degradation Assays. Ipbp8 degradation reactions contained 60% (vol/vol) Ipbp storage buffer, 5% (vol/vol) Lon buffer, 5 mM MgCl₂, 5 mM KCl, and 2% (vol/vol) DMSO (dimethyl sulfoxide), 360 mM potassium glutamate, 12% (wt/vol) sucrose, and 50 mM Hepes-KOH (pH 8).

Degradation of titin-I27 constructs was carried out at 37 °C in buffer containing 25 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM MgCl₂, 1 mM DTT (dithiothreitol), 2 mM ATP, 20 mM phosphoenolpyruvate, and 10 μM pyruvate kinase. Kinetics were determined using a mixture of 5% (mol/mol) 35S-labeled substrate and 95% (mol/mol) unlabeled substrate, as previously described (51).

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