Heat activates the AAA+ HslUV protease by melting an axial autoinhibitory plug

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SUMMARY

At low temperatures, protein degradation by the AAA+ HslUV protease is very slow. New crystal structures reveal that residues in the intermediate domain of the HslU unfoldase can plug its axial channel, blocking productive substrate binding and subsequent unfolding, translocation, and degradation by the HslV peptidase. Biochemical experiments with wild-type and mutant enzymes support a model in which heat-induced melting of this autoinhibitory plug activates HslUV proteolysis.

Graphical Abstract
In Brief
Baytshtok et al. demonstrate that the activity of HslUV, a AAA+ heat shock protease, is regulated by thermal melting of its autoinhibitory axial plug, which activates ATP hydrolysis, substrate binding, and energy-dependent proteolysis and ensures that robust protein degradation by HslUV occurs only at elevated temperatures in the cell.

INTRODUCTION
By degrading incomplete, unfolded, or unneeded proteins, intracellular AAA+ proteases serve important roles in protein-quality control, often removing proteins damaged by heat or other destabilizing stresses (Sauer and Baker, 2011). HslUV consists of one or two AAA+ HslU_6 ring hexamers and the double-ring HslV_{12} peptidase (Figure 1A; Rohrwild et al., 1997; Sousa et al., 2000; Wang et al., 2001). In Escherichia coli, expression of the HslU and HslV enzymes increases ~10-fold at high temperatures (Rohrwild et al., 1996), and HslUV proteolysis is substantially faster at high than low temperatures in vitro (Burton et al., 2005). ATP-dependent proteolysis requires HslU_6 to bind the degrons of target proteins in its axial channel and then unfold and translocate the substrate into the proteolytic chamber of HslV_{12} (Sundar et al., 2012). To reach the axial channel of the HslU hexamer, substrates must transit a funnel-like structure formed by segments of its intermediate (I) domain (Figures 1A and 1B; Sousa et al., 2000; Wang et al., 2001). Both the I-domain and this funnel-like structure are unique to the HslU subfamily of AAA+ unfoldases (Sauer and Baker, 2011). An I-
domain L199Q mutation enhances degradation of some protein substrates and the rate of ATP hydrolysis (Baytshtok et al., 2016), possibly by relief of autoinhibition. However, the molecular mechanism by which Leu<sup>199</sup> and surrounding residues affect proteolysis and ATP hydrolysis is unknown, as amino acids 177–212 are disordered in known structures (Bochtler et al., 2000; Sousa et al., 2000; Wang et al., 2001). Here, we present evidence for a model in which HslU hexamers are autoinhibited at low temperatures by a trimeric I-domain plug that blocks the axial channel but melts at high temperatures to activate proteolysis.

RESULTS AND DISCUSSION

Autoinhibited structures of HslU and HslUV

We determined structures for three new crystal forms of <i>E. coli</i> HslU or HslUV by molecular replacement (Table 1).

Our new structures were similar to previous ones with the notable exception that a trimeric plug, formed by residues 183–206 of the I-domain, completely blocked the axial channel of the HslU ring (Figures 1C–1E). Because protein substrates must access the axial channel of HslU to be engaged, unfolded, and translocated into the peptidase chamber of HslV, this plug would inhibit substrate degradation. The plug consisted of amino acids from three of the six HslU subunits (Figures 1C–1E), each comprising an extended region (residues 183–188), a sharp turn (residues 189–191), and an α helix (residues 192–206). We could not reliably model connections between plug elements and other regions of the I-domain in specific subunits. Importantly, however, a crystallographic 3-fold axis related plug elements in the 6PX1 structure, indicating that I-domain elements from three alternating subunits of the HslU hexamer must form the plug.

The three α helices that comprise the plug center packed together via hydrophobic contacts involving the side chains of Met<sup>192</sup>, Met<sup>195</sup>, Leu<sup>199</sup>, and Phe<sup>203</sup>. Hence, replacing the nonpolar Leu<sup>199</sup> with a polar Gln<sup>199</sup> in the L199Q variant should destabilize helix-helix packing and plug stability, thereby reducing autoinhibition and providing a structural rationale for the ability of L199Q HslUV to degrade some protein substrates more rapidly than wild-type HslUV (WT HslUV; Baytshtok et al., 2016). In prior biochemical experiments, the subtilisin and chymotrypsin endoproteases were found to cleave sites within the plug region of L199QHslU faster than in WT HslU (Baytshtok et al., 2016), supporting a model in which destabilization of the mutant plug results in partial melting and increased susceptibility to proteolytic cleavage. Additional hydrophobic packing contacts within the plug included Val<sup>184</sup> and Ile<sup>186</sup> from the extended region, which occupy peripheral grooves between plug helices, and Met<sup>202</sup> from the helix. Notably, residues that form hydrophobic interactions within the plug are highly conserved in HslU orthologs (Figure 1F).

A Pro<sup>189</sup>-Pro<sup>190</sup>-Gly<sup>191</sup> tripeptide formed a sharp turn between the extended region and α helix of the plug. The negatively charged side chains of Glu<sup>193</sup> and Glu<sup>194</sup> following this turn were also close to the positively charged side chains of Arg<sup>101</sup> (94% conserved) and Lys<sup>293</sup> (92% conserved) in the HslU axial channel, suggesting that favorable electrostatic interactions also stabilize the plugged conformation. Conservation of the plug residues that form the turn and electrostatic interactions within the HslU channel (Figure 1F) provide
additional support for a model in which the autoinhibited conformation is functionally important.

**Electron microscopy of closed- and open-channel HslU structures**

To investigate channel accessibility in WT HslU and L199Q HslU in a non-crystalline environment, we used negative-stain electron microscopy. Both enzyme samples were equilibrated at room temperature prior to application to grids and staining. Class-average images of WT HslU, viewed approximately down the axis of the AAA+ ring, showed a shallow axial depression or blocked channel (Figure 2A, top row), similar to projections of plugged crystallographic HslU hexamers filtered to 15 Å (Figure 2A, bottom row). Most class averages for L199Q HslU hexamers (Figure 2B, top row), by contrast, resembled open-channel crystal structures filtered to 15 Å (Figure 2B, bottom row). These results provide further evidence that the axial channel of WT HslU at room temperature is plugged in most enzymes and that the L199Q mutation shifts the equilibrium to favor the open-channel conformation.

**Plug stability affects temperature-dependent activity**

We propose that high temperature destabilizes the plug and favors the active open-channel conformation of HslUV, whereas low temperature stabilizes the inactive plugged-channel conformation (Figure 3A). As a first test of this model, we measured the rates of ATP hydrolysis by WT HslUV and L199Q HslUV at 25°C, 35°C, 45°C, and 55°C without or with the 137A Arc-cp6GFP-st11-ssrA protein substrate (Figures 3B and 3C). These data fit well to melting curves calculated assuming that the high-temperature conformations of WT HslUV and L199Q HslUV had the same ATPase activity, whereas the low-temperature conformations were inactive in ATP hydrolysis. Without protein substrate, the fitted temperatures of half-maximal activity (T_M) were 42°C for L199Q HslUV and 52°C for WT HslUV (Figure 3B). Protein substrate reduced the T_M values by 10°C–12°C for both enzymes (Figure 3C), indicating that binding of the 137A Arc-cp6GFP-st11-ssrA substrate preferentially stabilizes the active enzyme conformation. The lower T_M values for L199Q HslUV than for WT HslUV, both with and without protein substrate, strongly support our model that plug melting results in temperature-dependent relief of autoinhibition.

As a second test of our temperature-dependent activation model, we used SDS-PAGE to measure HslUV degradation of an Arc-st11-ssrA substrate at 25°C or 55°C (Figure 3D). As expected from findings of a previous study (Burton et al., 2005), degradation by the WT enzyme was very slow at 25°C and much faster at 55°C. We also assayed degradation of the same substrate by R101A HslUV, constructed to remove favorable electrostatic interactions between Arg101 in the axial channel and glutamates in the autoinhibitory plug, and by I186N HslUV, which replaces a hydrophobic residue that normally stabilizes plug packing with a polar residue (Figures 1D and 1E). At 25°C, both the R101A HslUV and I186N HslUV enzymes degraded Arc-st11-ssrA substantially faster than did WT HslUV (Figure 3D), as expected by our model. At 55°C, all three enzymes degraded Arc-st11-ssrA more rapidly than at the lower temperature, with WT HslUV degradation being slower than degradation by the mutant enzymes. In each case, faster substrate degradation at the higher temperature probably results from a combination of reduced autoinhibition and reduced stability of the
native portion of the Arc-st11-ssrA substrate. Importantly, these results support a model in which the R101A and I186N mutations destabilize the autoinhibited conformation at low temperatures and thereby increase protease activity relative to the WT enzyme.

As a third test of our model, we determined steady-state degradation rates of different concentrations of the I37A-Arc-cp6GFP-st11-ssrA substrate at a temperature (37°C) at which both WT-HslUV and I186N-HslUV have substantial activity and fit the data to the Michaelis-Menten equation to determine $K_M$ and $V_{max}$ values (Figure 3E). At low substrate concentrations, degradation by I186N-HslUV was ~6-fold faster than by WT-HslUV, largely as a consequence of a tighter $K_M$ for the mutant (Figure 3E). By contrast, degradation of high concentrations of this substrate by I186N-HslUV was only ~1.2-fold faster than by WT-HslUV. These results support a model in which plug destabilization, as a consequence of reduced hydrophobic packing, makes the axial channel accessible in a larger fraction of the population of I186N mutant enzymes at low substrate concentrations, thereby resulting in tighter substrate binding and faster degradation. Very high substrate concentrations, in turn, appear to shift the equilibrium toward the open-channel conformation of WT-HslUV, thereby reducing the activity difference between the mutant and WT enzymes.

Conclusions and biological inferences

We have identified a new conformation of the HslUV protease in which part of the I-domain forms a plug that blocks the axial channel of the AAA+ HslU ring and prevents productive substrate engagement and thus degradation. This plugged-channel inactive conformation is in dynamic equilibrium with an open-channel active conformation, with higher temperature and/or higher protein-substrate concentrations favoring the active conformation.

In cells, direct thermal activation of HslUV by melting of the autoinhibitory plug at heat shock temperatures (Figure 3A) would increase protease activity almost immediately. Temperature-induced expression increases of HslU and HslV would then further amplify HslUV proteolytic capacity. Following a return to lower temperatures, levels of the HslUV enzyme would be expected to remain high for several generations before reduced expression and cell division allowed a return to the low-temperature steady state. During this transition from high to low temperatures, increased plug-mediated autoinhibition could contribute to cell fitness by minimizing rogue HslUV proteolysis and/or reducing excessive ATP hydrolysis.

**STAR METHODS**

**RESOURCE AVAILABILITY**

**Lead Contact**—Requests for information and resources should be directed to and will be fulfilled by the Lead Contact, Robert T. Sauer (bobsauer@mit.edu).

**Materials Availability**—This study generated new His$_6$-tagged mutants of *E. coli* HslU containing the R101A and I186N mutations. Plasmids expressing these mutant enzymes are available from the lead contact upon request without restriction.
Data and Code Availability—Coordinates, structure factors and electron-density maps for the 6PXI, 6PXL, and 6PXK crystal structures are available from the RCSB Protein Data Bank (https://www.rcsb.org/). The biochemical data and electron micrographs supporting the current study are available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Proteins were expressed in E. coli strain X90 (ADE3) slyD::kan hsUVE::tet). No additional biological strains were used in this work.

METHOD DETAILS

Genes encoding H6-tagged variants of E. coli HslU, E257QHslU, L199QHslU, I186NHslU, R101AHslU, HslV, I37A Arc-cp6GFP-st11-ssrA, and Arc-st11-ssrA were expressed from pET12b or pET21b vectors (Novagen) in E. coli strain X90 (λDE3) slyD::kan hslUV::tet and proteins were purified as described (Baytshtok et al., 2016).

Crystals of HslU were grown at 4°C by the hanging-drop method after mixing 0.5 μL of selenomethionine-labeled H6-tagged HslU (15 mg/mL) in 15 mM Tris (pH 7.5), 100 mM NaCl, 20 mM MgCl2, 0.2 mM EDTA, and 5 mM ATPγS with an equal volume of well solution containing 100 mM Tris (pH 7.5), 18% PEG-3350, and either 0.1 M (PDB code 6PXK) or 0.2 M (PDB code 6PXL) ammonium sulfate. For cryo-protection, PEG-3350 was increased to 20% and 10% MPD was added to the base-well solution. HslUV crystals were grown in the same way except the protein solution contained E257QHslU (10 mg/mL) plus HslV (9.6 mg/mL) and the well solution contained 0.1 M Bis-Tris (initial pH 5.5), 1.85 M ammonium sulfate, 5% glycerol, and 5 mM ATPγS. For cryo-protection, glycerol was increased to 25%. Diffraction data for the HslUV crystals were collected on our home source (Rigaku MicroMax-007HF with a Saturn 944 detector) and for the HslU crystals at the Advanced Photon Source (APS) beamline 24-ID-E and processed using HKL2000 (Otwinowski and Minor, 1997) or XDS (Kabsch, 2010). Structures were solved by molecular replacement with PHASER (McCoy et al., 2007) using search models consisting of HslU hexamers alone or HslUV (PDB code 5JI3; Baytshtok et al., 2016). Anomalous difference maps – showing the selenomethionine side chains of residues 187, 192, 195, and 202 – were used to assist model building into plug density. Phenix was used to refine structures (Adams et al., 2010). Coot was used for model building (Emsley et al., 2010), and MolProbity was used to assess model geometry (Chen et al., 2010).

Negative-stain EM experiments were performed as described (Baytshtok et al., 2016), using WT HslU or L199QHslU (1.5 μM) and ATPγS (5 mM) in 20 mM HEPES (pH 7.5), 5 mM MgCl2, 500 mM NaCl, 10% glycerol (v/v), and 0.032% Igepal CA-630. Particles with tilted six-fold symmetry axis were removed by two rounds of 2D classification. For WT HslU, 10927 particles from 23 micrographs were used to generate representative 2D class averages; for L199QHslU, 5567 particles from 20 micrographs were used. The e2pdb2mrc and e2project3d utilities in EMAN2 (Tang et al., 2007) were used to generate 2D projections from PDB files and Relion 3.0.8 (Scheres, 2012) was used for 2D classification.
Degradation and ATPase assays were performed in 25 mM HEPES (pH 7.5), 5 mM KCl, 20 mM MgCl₂, 10% glycerol, and 0.032% Igepal CA-630 as described (Baytshtok et al., 2016). ATPase rates at different temperatures were measured using a Spectramax M5 plate reader (Molecular Devices) by using an NADH-coupled assay (Nørby, 1988) with or without 50 μM I₃⁷A Arc-cp6GFP-st11-ssrA. Using the Solver tool of Microsoft Excel, the temperature dependencies of ATPase rates were globally fitted to the equation max/(1+exp(ΔH/R·(1/Tₘ-1/T))), where max is the maximum ATPase rate, T is the temperature in Kelvin, Tₘ is the temperature at 50% activity, ΔH is the enthalpy at Tₘ, and R is the universal gas constant. Degradation of I₃⁷A Arc-cp6GFP-st11-ssrA by WT HslUV or I₁₈₆N HslUV was monitored by loss of fluorescence (excitation 467 nm; emission 511 nm). For degradation of Arc-st11-ssrA (5 μM) by wild-type or mutant enzymes (0.3 μM HslU₆, 0.9 μM HslV₁₂), proteins were preincubated for 1 min at 25°C or 55°C before adding an ATP regeneration system (2.5 mM ATP, 7.5 mM phosphoenolpyruvate, 1 mM NADH, 18.8 U/ml pyruvate kinase, 21.5 U/ml lactate dehydrogenase) to initiate degradation. Aliquots were removed at different times, quenched by addition of Tricine sample buffer (Bio-Rad) with β-mercaptoethanol, and flash frozen in liquid nitrogen. Samples were then boiled for 5 min and electrophoresed on 15% Tris-Tricine SDS-PAGE gels. After electrophoresis, gels were stained for 10 min using a Coomassie-blue solution, destained overnight in water, scanned using a Typhoon FLA 9500 Imager (GE Healthcare), and the amount of substrate remaining at each time point was quantified using ImageQuant TL (GE Healthcare).

QUANTIFICATION AND STATISTICAL ANALYSIS
Crystallographic unit-cell parameters, data completeness and redundancy, CC₁/₂ values, and merging R-factors (Table 1) were obtained using HKL2000 (Otwinowski and Minor, 1997), which was used to index, integrate, and scale the diffraction data. Model-refinement statistics (Rwork, Rfree; Table 1) were obtained from the phenix.refine program in Phenix (Adams et al., 2010) and are shown in Table 1. Statistics for the quality of the final crystallographic models (Table 1) were determined using MolProbity (Chen et al., 2010). Biochemical experiments were performed using a minimum of three independent replicates. Biochemical data were plotted as averages ± one standard deviation (SD), as described in the Figure 3 legend, using Prism (GraphPad). The errors for Kᵊ₉ and Vₘₐₓ (Figure 3E) were calculated by non-linear least-squares fitting to the Michealis-Menten equation using Prism (GraphPad).

ACKNOWLEDGMENTS
This work was supported by NIH grant AI-15706. NECAT beamline studies at APS were supported by NIGMS (P41 GM103403) and DOE (DE-AC02–06CH11357) grants.

REFERENCES
Adams PD, Afonine PV, Bunkóczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, et al. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr 66, 213–221. [PubMed: 20124702]
Baytshtok V, Fei X, Grant RA, Baker TA, and Sauer RT (2016). A structurally dynamic region of the HslU intermediate domain controls protein degradation and ATP hydrolysis. Structure 24, 1766–1777. [PubMed: 27667691]

Bochtler M, Hartmann C, Song HK, Bourenkov GP, Bartunik HD, and Huber R (2000). The structures of HsIU and the ATP-dependent protease HsIU-HsIV. Nature 403, 800–805. [PubMed: 10693812]

Burton RE, Baker TA, and Sauer RT (2005). Nucleotide-dependent substrate recognition by the AAA+-HsUV protease. Nat. Struct. Mol. Biol 12, 245–251. [PubMed: 15696175]

Chen VB, Arendall WB 3rd, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, and Richardson DC (2010). MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr. D Biol. Crystallogr 66, 12–21. [PubMed: 20057044]

Emsley P, Lohkamp B, Scott WG, and Cowtan K (2010). Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr 66, 486–501. [PubMed: 20383002]

Goddard TD, Huang CC, Meng EC, Pettersen EF, Couch GS, Morris JH, and Ferrin TE (2018). UCSF ChimeraX: Meeting modern challenges in visualization and analysis. Protein Sci. 27, 14–25. [PubMed: 28710774]

Kabsch W (2010). XDS. Acta Crystallogr. D Biol. Crystallogr 66, 125–132. [PubMed: 20124692]

McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, and Read RJ (2007). Phaser crystallographic software. J. Appl. Cryst 40, 658–674. [PubMed: 19461840]

Milla ME, Brown BM, and Sauer RT (1993). P22 Arc repressor: enhanced expression of unstable mutants by addition of polar C-terminal sequences. Protein Sci. 2, 2198–2205. [PubMed: 8298465]

Mindell JA, and Grigorieff N (2003). Accurate determination of local defocus and specimen tilt in electron microscopy. J. Struct. Biol 142, 334–347. [PubMed: 12781660]

Nørby JG (1988). Coupled assay of Na⁺,K⁺-ATPase activity. Methods Enzymol. 156, 116–119. [PubMed: 2835597]

Otwinowski Z, and Minor W (1997). Processing of x-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–325.

Rohrwild M, Coux O, Huang HC, Moerschell RP, Yoo SJ, Seol JH, Chung CH, and Goldberg AL (1996). HsIV-HsIU: A novel ATP-dependent protease complex in Escherichia coli related to the eukaryotic proteasome. Proc. Natl. Acad. Sci. USA 93, 5808–5813. [PubMed: 8650174]

Rohrwild M, Pfeifer G, Santarius U, Müller SA, Huang HC, Engel A, Baumeister W, and Goldberg AL (1997). The ATP-dependent HsIV protease from Escherichia coli is a four-ring structure resembling the proteasome. Nat. Struct. Biol 4, 133–139. [PubMed: 903594]

Sauer RT, and Baker TA (2011). AAA+ proteases: ATP-fueled machines of protein destruction. Annu. Rev. Biochem 80, 587–612. [PubMed: 21469952]

Scheres SH (2012). RELION: implementation of a Bayesian approach to cryo-EM structure determination. J. Struct. Biol 180, 519–530. [PubMed: 23000701]

Sousa MC, Trame CB, Tsuruta H, Wilbanks SM, Reddy VS, and McKay DB (2000). Crystal and solution structures of an HsIV protease-chaperone complex. Cell 103, 633–643. [PubMed: 11106733]

Sundar S, Baker TA, and Sauer RT (2012). The I domain of the AAA+ HsIV protease coordinates substrate binding, ATP hydrolysis, and protein degradation. Protein Sci. 21, 188–198. [PubMed: 22102327]

Tang G, Peng L, Baldwin PR, Mann DS, Jiang W, Rees I, and Ludtke SJ (2007). EMAN2: an extensible image processing suite for electron microscopy. J. Struct. Biol 157, 38–46. [PubMed: 16859925]

Wang J, Song JJ, Seong IS, Franklin MC, Kamtekar S, Eom SH, and Chung CH (2001). Nucleotide-dependent conformational changes in a protease-associated ATPase HsIU. Structure 9, 1107–1116. [PubMed: 11709174]

Yakamavich JA, Baker TA, and Sauer RT (2008). Asymmetric nucleotide transactions of the HsUV protease. J. Mol. Biol 380, 946–957. [PubMed: 18582897]
Highlights

• A trimeric structure can plug the axial channel of the AAA+ HsIU ring hexamer
• This autoinhibitory plug is observed in crystal structures and by electron microscopy
• Temperature-induced melting of the plug activates ATP hydrolysis and proteolysis
• Low-temperature autoinhibition limits rogue degradation after recovery from heat shock
Figure 1. HslUV with open and closed axial channels
(A) Surface-representation side view of the HslUV protease (PDB: 5JI3).
(B) Surface-representation top view of open-channel HslUV structure (PDB: 5JI3).
(C) Surface-representation top view of plugged-channel HslUV structure (PDB: 6PXI).
(D) Top view of axial plug (PDB: 6PXI) in cartoon representation. The side chains of Arg$^{101}$, Ile$^{186}$, and Leu$^{199}$ are shown as spheres. The axial channel of HslU is shown in surface representation.
(E) Cutaway side view of axial plug (PDB: 6PXI) in cartoon representation with Arg$^{101}$, Ile$^{186}$, and Leu$^{199}$ shown as spheres.
(F) WebLogo representation of conservation of plug residues. ϕ indicates positions of hydrophobic residues (Val$^{184}$, Ile$^{186}$, Met$^{187}$, Met$^{192}$, Met$^{195}$, Leu$^{199}$, Met$^{202}$, and Phe$^{203}$).
that engage in packing within the plug; asterisk (*) indicates the positions of negatively charged residues (Glu$^{193}$ and Glu$^{194}$) that appear to form favorable electrostatic interactions with Arg$^{101}$ and Lys$^{293}$ in the axial channel of the HslU hexamer.
Figure 2. Effects of L199Q mutation on HslU channel size

(A) Class-average negative-stain electron microscopy (EM) images of WT HslU (top row) compared with projections of a plugged hexamer (PDB: 6PXK) filtered to 15-Å resolution (bottom row). Samples were prepared at room temperature.

(B) Class-average images of L199Q HslU (top row) compared with filtered images of an open-channel hexamer (PDB: 5JI3) or the plugged hexamer (PDB: 6PXK).
Figure 3. Activities of WT HslUV and mutants with destabilized plugs

(A) Model for activation of HslU by thermal melting of the autoinhibitory plug.

(B) Temperature dependence of ATP hydrolysis by WT HslUV or L199Q HslUV (0.3 μM HslU$_6$, 0.9 μM HslV$_{12}$). Values are averages (±SD) of three independent experiments. The dotted lines are fits to melting curves.

(C) Same as in (B) but in the presence of the I37A Arc-cp6GFP-st11-ssrA substrate (50 μM).

(D) SDS-PAGE was used to assay the kinetics of degradation of the Arc-st11-ssrA substrate (5 μM) at 25°C or 55°C by WT HslUV, R101A HslUV, or I186N HslUV (0.3 μM HslU$_6$, 0.9 μM HslV$_{12}$). In each subpanel, a representative gel is shown at the top, and the mean (±SD) of substrate remaining in three independent replicates is shown in the graph below.

(E) Michaelis-Menten plots and steady-state kinetic parameters for I37A Arc-cp6GFP-st11-ssrA degradation at 37°C by WT HslUV or I186N HslUV (0.3 μM HslU$_6$, 0.9 μM HslV$_{12}$). Values plotted are averages (±SD) of three independent replicates.
Table 1.

Crystallographic statistics

| PDB entry | 6PXI | 6PXL | 6PXK |
|-----------|------|------|------|
| Protein   | HslUV| HslU | HslU |
| Resolution (Å) | 3.45 | 3.74 | 3.65 |
| Space group | P321 | C2   | P2   |
| Unit cell  |      |      |      |
| A (Å)      | 168.60 | 414.59 | 200.03 |
| B(Å)       | 168.60 | 92.33  | 91.20  |
| C(Å)       | 162.89 | 200.85 | 201.80 |
| α          | 90°   | 90°   | 90°   |
| β          | 90°   | 99.43° | 99.43° |
| γ          | 120°  | 90°   | 90°   |
| No. of unique reflections | 29,212 | 68,314 | 15,427 |
| Redundancy | 2.2  | 3.8   | 5.0   |
| Completeness (%) | 81.7 (59.3) | 95.3 (77.1) | 98.4 (92.5) |
| CC-1/2     | 0.985 (0.593) | 0.981 (0.669) | 0.998 (0.609) |
| R_{sym}    | 0.114 | 0.121 | 0.105 |
| R_{pin}    | 0.081 | 0.068 | not calculated |
| R_{work}/R_{free} | 0.257/0.285 | 0.220/0.265 | 0.223/0.271 |
| MolProbity score (percentile) | 1.04 (100) | 1.31 (100) | 1.30 (100) |
| Clash score (percentile) | 2.54 (100) | 5.71 (100) | 5.9 (100) |
| Ramachandran outliers (%) | 0 | 0 | 0 |
| Ramachandran favored (%) | 98.58 | 98.15 | 99.26 |
| Bad bonds/angles | 0/0 | 0/0 | 0/0 |
| Cβ deviations | 0 | 0 | 0 |

Completeness and CC-1/2 values in parentheses are for the highest resolution shell.
**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial Strains** |        |            |
| X90 (λDE3) slyD::kan hslU::tet | Baytshtok et al., 2016 | N/A |
| **Proteins and Enzymes** |        |            |
| HslU (His<sub>6</sub> tagged) | Bochtler et al., 2000 | N/A |
| L199QHslU (His<sub>6</sub> tagged) | Baytshtok et al., 2016 | N/A |
| R101AHslU (His<sub>6</sub> tagged) | This study | N/A |
| i186NHslU (His<sub>6</sub> tagged) | This study | N/A |
| e257QHslU (His<sub>6</sub> tagged) | Yakamavich et al., 2008 | N/A |
| HslV (His<sub>6</sub> tagged) | Bochtler et al., 2000 | N/A |
| i187AARC<sup>-<i>a</i>+</sup>GFP-st11-ssrA | Baytshtok et al., 2016 | N/A |
| Arc-st11-ssrA | Milla et al., 1993 | N/A |
| pyruvate kinase | Sigma-Aldrich | N/A |
| lactate dehydrogenase | Sigma-Aldrich | N/A |
| **Deposited Data** |        |            |
| e257QHslU-HslV-ADP, plugged | This study | PDB: 6PXI |
| WT<sup>+</sup>HslU- ADP, plugged | This study | PDB: 6PXL |
| WT<sup>+</sup>HslU- ADP, plugged | This study | PDB: 6PXK |
| **Software and Algorithms** |        |            |
| Coot | Emsley et al., 2010 | N/A |
| ChimeraX | Goddard et al., 2018 | N/A |
| Cttfind3 | Mindell and Grigorieff, 2003 | N/A |
| EMAN2 | Tang et al., 2007 | N/A |
| HKL2000 | Otwinowski and Minor, 1997 | N/A |
| Phenix | Adams et al., 2010 | N/A |
| PyMOL | Schrodinger, LLC | N/A |
| Relion | Scheres, 2012 | N/A |
| XDS | Kabsch, 2010 | N/A |