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Multiple sequence signals direct recognition and degradation of protein substrates by the AAA+ protease HslUV

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Abstract

Proteolysis is important for protein quality control and for the proper regulation of many intracellular processes in prokaryotes and eukaryotes. Discerning substrates from other cellular proteins is a key aspect of proteolytic function. The *E. coli* HslUV protease is a member of a major family of ATP-dependent AAA+ degradation machines. HslU hexamers recognize and unfold native protein substrates and then translocate the polypeptide into the degradation chamber of the HslV peptidase. Although a wealth of structural information is available for this system, relatively little is known about mechanisms of substrate recognition. Here, we demonstrate that mutations in the unstructured N-terminal and C-terminal sequences of two model substrates alter HslUV recognition and degradation kinetics, including changes in V_{max}. By introducing N- or C-terminal sequences that serve as recognition sites for specific peptide-binding proteins, we also show that blocking either terminus of the substrate interferes with HslUV degradation, with synergistic effects when both termini are obstructed. These results support a model in which one terminus of the substrate is tethered to the protease and the other terminus is engaged by the translocation/unfolding machinery in the HslU pore. Thus, degradation appears to consist of discrete steps, which involve the interaction of different terminal sequence signals in the substrate with different receptor sites in the HslUV protease.

Introduction

AAA+ proteases are responsible for quality-control surveillance of the proteome and for regulating key intracellular processes in all kingdoms of life.[1,2] These ATP-fueled molecular machines recognize specific substrates, forcibly denature these proteins, and then spool the denatured polypeptide in a processive fashion through an axial channel and into a sequestered chamber for degradation.[3] In the HslUV protease, for example, ring hexamers of HslU perform the recognition, unfolding, and translocation functions, whereas the double-ring HslV dodecamer degrades polypeptides that are translocated into its proteolytic chamber.[4–7] Recent studies have shown that HslUV is an extremely powerful protein unfoldase when degrading proteins in the N-terminal to C-terminal direction but has modest denaturation activity when degradation initiates at the C-terminus of a substrate.[8]

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For all proteases, substrate recognition is critical in ensuring that the correct molecules are degraded. Exposed peptide sequences in specific substrates, called degrons or degradation tags, typically serve as recognition signals for bacterial AAA+ proteases.[3,9] Peptide sequences can also target proteins for HslUV degradation,[8,10,11] but relatively little is known about the underlying recognition events. Based on studies with related AAA+ proteases, it is clear that some degrons are bound by pore loops in the central translocation channel of the hexameric unfoldase, allowing these enzymes to grasp the attached substrate and to begin translocation-mediated unfolding.[12–14] This mechanism is also likely to be important for HslUV degradation, because mutations in the highly conserved GYVG pore loop of HslU diminish or block degradation of specific substrates.[15] Other degradation signals tether substrates to other parts of the protease, raising the effective concentration of the substrate relative to the enzyme and facilitating recognition of a weak degron elsewhere in the substrate. Indeed, there is growing evidence in both natural and engineered substrates that the existence of multiple degradation signals can play important roles in determining how well a protein is recognized and degraded by AAA+ proteases.[16–22]

Although a handful of substrates for HslUV have been identified,[8,10,11,15,23,24] relatively little is known about the ways in which sequence signals in these proteins influence degradation. To explore this issue, we employ two model substrates, the Arc repressor of phage P22 and the N-terminal domain of phage λ cI repressor. In solution, both proteins unfold in seconds but refold in milliseconds and thus maintain metastable structures.[25–26] As a consequence, unfolding of these substrates is not a serious impediment to HslUV degradation. We find that mutations in the unstructured N-terminal or C-terminal sequences of both proteins alter the steady-state kinetics of HslUV proteolysis, including changes in the maximal rate of degradation. By introducing N- or C-terminal sequences that serve as recognition sites for specific peptide-binding proteins, we also demonstrate that blocking either substrate terminus by protein binding interferes with HslUV degradation. Our results support a model in which one terminus of the substrate is tethered to the protease and the other terminus is engaged by the translocation/unfolding machinery in the HslU pore. This model explains how mutations in either N- or C-terminal sequence can affect the maximal degradation rate and suggests that substrate recognition by HslUV consists of discrete steps, with the efficiency of degradation ultimately depending on the interplay of multiple sequence signals in the substrate with appropriate receptor sites in the protease.

Results

Effects of terminal λcI^{N} sequences on HslUV degradation

By screening a library in which five unstructured C-terminal residues of the N-terminal domain of λ cI repressor (residues 1–102; λcI^{N}) had been randomized, Parsell *et al*. identified variants that were degraded rapidly *in vivo*.[27] To determine if some of these proteins were substrates for *Escherichia coli* HslUV, we recloned the parental domain and several variants to add an N-terminal $MGS_2H_6S_3H$ tag (called ext1), purified these proteins, and assayed HslUV degradation by SDS-PAGE. We identified one variant in which the C-terminal sequence was changed from RSEYE to ISVTL (ext1-λcI^{N}-ISVTL) that HslUV degraded faster than the parental protein (ext1-λcI^{N}-RSEYE; Fig. 1A), suggesting that the altered C-terminal residues of this mutant make it a better substrate.

Although substrate degradation by HslUV is a multi-step reaction, simple Michaelis-Menten kinetics are observed,[10–11] indicating that the overall reaction can be modeled by substrate binding and dissociation steps, and by a rate-limiting enzymatic step (k_{deg}), which could correspond to substrate engagement, unfolding, or translocation. Because degradation signals usually affect protease binding, we expected that the ISVTL sequence would reduce K_M for HslUV degradation when compared to degradation of the wild-type RSEYE

sequence. Indeed, when we determined steady-state kinetic parameters for degradation of ^{35}S-labeled proteins, a slight reduction in K_M was observed (Fig. 1B; Table 1). Surprisingly, however, the major effect on degradation of the ISVTL substrate was a ~2-fold increase in V_{max} (Fig. 1B; Table 1).

For inhibition experiments presented below, we changed the N-terminal residue of mature λcI^N from serine to leucine and/or changed the C-terminal sequence from RSEYE to RSYYF. Determination of the kinetic parameters for HslUV degradation of the S1LλcI^N-RSYYF and S1LλcI^N-RSEYE variants revealed a ~2-fold difference in V_{max} and ~3-fold difference in K_M (Fig. 1C; Table 1). Because this pair of substrates differs only at the final and antepenultimate amino acids, these C-terminal residues apparently affect both HslUV binding and the maximum velocity of degradation. λcI^N substrates with the same C-terminal sequences but different N-termini sequences were degraded by HslUV with similar kinetic parameters (Table 1).

We used another λcI^N variant to test if dimerization affected HslUV degradation. Monomers and dimers of λcI^N equilibrate rapidly in solution (K_D ~300 μM), but the I84S mutation reduces dimerization to undetectable levels.[28] We introduced the I84S mutation into ext1-λcI^N-ISVTL and determined kinetic parameters for HslUV degradation, which were within error of the parental protein (ext1-λcI^N-ISVTL; Table 1). We conclude that λcI^N dimerization is not a prerequisite for HslUV degradation.

Effects of terminal Arc sequences on degradation

Arc repressor and variants, including those with C-terminal sequences of H_6KNQHD (st11 tag) and/or AANDENYALAA (ssrA tag), are substrates for the *E. coli* and *Haemophilus influenzae* HslUV enzymes.[10–11] Using *E. coli* HslUV, we determined steady-state degradation parameters for Arc variants with a C-terminal ssrA tag (Arc-ssrA), the st11 and ssrA tags (Arc-st11-ssrA), or st11 and a mutant ssrA tag in which the terminal AA sequence was replaced by DD (Arc-st11-ssrA^{DD}) (Fig. 2; Table 1). K_M for degradation of these substrates varied over a ~2-fold range (Table 1; Fig. 2). Importantly, HslUV degraded Arc-ssrA and Arc-st11-ssrA with V_{max} values of 4.9 and 12 min^{-1} enz^{-1}, respectively. Thus, inserting the st11 tag before the ssrA tag increased the maximum degradation rate more than 2-fold. Changing the C-terminal residues of Arc-st11-ssrA from AA to DD reduced V_{max} from 12 to 7 min^{-1} enz^{-1}. Hence, the C-terminal sequences of both λcI^N and Arc substrates influence binding as well as the maximal rate of HslUV degradation.

Previous studies demonstrated that N-terminal residues of Arc were important for HslUV degradation.[11] Consistently, we found that addition of an 18-residue N-terminal extension (ext2; $LA_3SGAG_2SEG_2TSGAT$) to Arc-ssrA reduced V_{max} and increased K_M (Fig. 2; Table 1). Interestingly, extending the C-terminal region of Arc-ssrA by insertion of the st11 sequence increased V_{max}, whereas extending the N-terminal region decreased V_{max}. Thus, degradation efficiency is not a simple function of the total number of unstructured residues at the ends of a substrate.

Terminal sequences do not alter substrate stability

Sequences at the termini of λcI^N and Arc substrates might influence the kinetics of HslUV degradation by altering protein thermodynamic stability. However, no substantial differences in the melting temperatures of different λcI^N or Arc substrates were observed when thermal denaturation was monitored by changes in circular-dichroism ellipticity (Table 1). These results support a model in which interactions between the terminal sequences of these substrates and HslUV are responsible for the observed changes in degradation kinetics.

Protein binding to the C-terminus of substrates slows HslUV degradation

As noted above, λcI^{N} variants were engineered to contain a C-terminal YYF sequence, which can be bound with sub-μM affinity by the PDZ domain of *E. coli* DegS.[29–30] Saturating concentrations of the DegS PDZ domain inhibited HslUV degradation of $^{S1L}\lambda cI^{N}$-RSYYF to ~10% of the uninhibited rate but inhibited degradation of the $^{S1L}\lambda cI^{N}$-RSEYE control protein only marginally (Fig. 3A). Thus, inhibition by the PDZ domain is specific and depends on the C-terminal sequence of the substrate.

The *E. coli* SspB protein binds to the AANDENY segment of the ssrA tag with sub-μM affinity.[31–32] At saturating concentrations, SspB slowed HslUV degradation of ^{M1L}Arc-ssrA to ~15% of the uninhibited value (Fig. 3B). SspB addition had a much smaller effect on degradation of an otherwise identical protein with a mutant $ssrA^{N3A}$ tag that binds SspB poorly.[31] Again, these results show that SspB inhibits HslUV degradation in a substrate-specific fashion. Thus, blockade of the C-terminal sequences of λcI^{N} and Arc substrates by protein binding prevents efficient HslUV degradation.

ClpS binding to the N-terminus of substrates inhibits HslUV degradation

E. coli ClpS binds with μM affinity to proteins with an N-terminal leucine, phenylalanine, tyrosine, or tryptophan but binds substantially more weakly to proteins with N-terminal methionine or other amino acids.[33–35] To test if ClpS blockade of the N-terminus of λcI^{N} or Arc influenced HslUV degradation, we engineered variants of these substrates containing N-terminal leucines. Addition of ClpS to an Arc variant with an N-terminal leucine (^{M1L}Arc-ssrA) slowed HslUV degradation to ~20% of the uninhibited rate at saturation but did not inhibit degradation of an otherwise identical substrate with an N-terminal methionine (Fig. 4A). Thus, ClpS binding to the N-terminal leucine of ^{M1L}Arc-ssrA is responsible for slowing HslUV degradation.

We also observed strong ClpS inhibition of HslUV degradation of $^{S1L}\lambda cI^{N}$-RSEYE and somewhat weaker inhibition of $^{S1L}\lambda cI^{N}$-RSYYF (Fig. 4B). This difference may reflect the lower K_M for HslUV degradation of the latter substrate (Table 1).

Synergistic effects of N- and C-terminal blocking

If protein binding to the N- and C-terminus of substrates inhibits HslUV degradation by independent mechanisms, then blocking both termini should produce greater inhibition than blocking just one terminus. Indeed, inhibition of HslUV degradation of ^{M1L}Arc-ssrA by the combination of SspB and ClpS was greater than inhibition by either protein alone (Fig. 5A, top). Similarly, inhibition of HslUV degradation of $^{S1L}\lambda cI^{N}$-RSYYF was more severe in the presence of ClpS and the DegS PDZ domain than with either single protein (Fig. 5A, bottom).

Single-turnover inhibition

In principle, protein binding to the N- or C-terminal sequences of substrates could block substrate sequences required for HslUV binding or engagement. Alternatively, the protein-bound substrate could be recognized, engaged, unfolded, and translocated like the free substrate, but steady-state degradation could be slowed because of the inability of the enzyme to initiate the next cycle of degradation. The latter possibility could occur, for example, if the protein that was bound at the terminus (ClpS, SspB, or the DegS PDZ domain) had to dissociate or be enzymatically stripped in order to complete the last steps of degradation. By this model, the initial release of ^{35}S-peptides from a substrate would occur faster than subsequent steps, and inhibition by bound protein might not be evident if enzyme were present in excess of substrate. Thus, we assayed single-turnover degradation of low concentrations of ^{35}S-labeled substrate in the presence of excess enzyme and inhibitor.

Under these conditions, specific inhibition by ClpS, SspB, and the DegS PDZ domain was still observed (Fig. 5B), suggesting that inhibition affects an early step in degradation.

Discussion

The simplest mechanism of targeting substrates for degradation by specific AAA+ proteases is exemplified by the ssrA-tagging system, in which a short peptide signal targets a wide variety of proteins for ClpXP or ClpAP degradation.[36–37] It is becoming evident, however, that substrate recognition mediated by a single tag or degron is generally the exception rather than the rule. Indeed, one substrate signal often mediates binding, whereas another signal ensures engagement by the translocation machinery of the AAA+ protease.[3] The results presented here show that sequences at both the N- and C-terminal ends of Arc and λcI^N substrates influence the rate of HslUV degradation and thus support a multi-degron model of recognition.

Unfolded monomers and native dimers of Arc equilibrate rapidly in solution, but the strong dimerization constant (~10 nM) ensures that most molecules are dimers at the concentrations used for degradation studies.[25,38] Thus, each Arc substrate has two N-terminal sequences and two C-terminal sequences that potentially could interact with HslU. By contrast, the dimerization constant of λcI^N is substantially weaker (~300 μM),[28] and a mixture of monomers and dimers are present in our degradation assays. Importantly, however, a mutation (I84S) that weakens λcI^N dimerization >30-fold did not cause significant changes in the steady-state kinetics of λcI^N degradation (Table 1), suggesting that HslUV recognizes monomers and dimers of λcI^N comparably.

HslUV degradation of Arc variants was initially suggested to depend exclusively on C-terminal sequences and was subsequently proposed to depend completely on N-terminal sequences.[10–11] Our present studies resolve this apparent conflict, as mutational and inhibitor experiments demonstrate that both termini of Arc substrates need to be recognized for efficient HslUV degradation. Similar results with λcI^N variants suggest that multi-degron recognition may be common for HslUV substrates. The terminal sequences of both Arc and λcI^N variants are unstructured in the free proteins and could mediate binding/engagement by the translocation machinery of the HslU pore or tethering to an independent receptor site elsewhere in the enzyme. At present, we do not know which termini serve which function or even if one specific terminus always binds the pore while the other always serves a tethering role, but the results presented here should allow future experiments to address this question.

Fig. 6 shows a model with three types of HslUV·substrate complexes and provides a framework for understanding how sequence changes at either terminus of a substrate can alter K_M and V_{max} for degradation. In complex P, one terminus of the substrate binds to the enzyme pore. In complex T, the other terminus of the substrate binds to a distinct tethering site. In complex TP, both termini of the substrate bind to their respective sites in HslUV. Only complexes P and TP are proteolytically active, because binding to the pore is required for degradation. The distribution of these species depends on the equilibrium constants for binding the tethering site in a bimolecular reaction (K_1), binding the pore in a bimolecular reaction (K_2), binding the pore in a unimolecular reaction (K_3), and binding the tethering site in a unimolecular reaction (K_4). Because $K_1K_3 = K_2K_4$, specifying three of these constants determines the fourth constant. Thus, the apparent K_M and the fraction of active enzymes at substrate saturation (f_{act}) can be expressed as a function of just K_1, K_2, and K_3 (Fig. 6). In principle, changing the sequence at either terminus could alter K_M and the maximal rate of degradation ($f_{act} \cdot k_{deg}$). In practice, however, substantial sequence-dependent changes in either kinetic parameter would only be observed if the T complex is present in excess of the P and TP complexes, which requires stronger bimolecular binding to the tethering site than

to the pore site ($K_1 < K_2$) and a K_3 value > 1. Under these conditions, mutations at either substrate terminus could alter both K_M and V_{max} by affecting binding to one of the enzyme sites (altering K_1 or K_2) and might also change these parameters by altering the length or flexibility of the terminal sequences (affecting K_3). Sequence changes at the substrate terminus that binds the pore of HslUV could also directly affect the efficiency of engagement by the translocation machinery and thus alter V_{max} by changing k_{deg} as well as f_{act}. Studies of adaptor-mediated delivery of substrates to the ClpXP protease provide general precedents for changing the steady-state kinetics of degradation via alterations in tethering sequences, pore-contact sequences, and the flexibility and geometry of intervening domains.[21,39]

From a biological perspective, multi-degron degradation permits combinatorial control of proteolysis through masking of one or both degrons in a substrate, as demonstrated by our observation that protein binding to the N- and C-terminal sequences of Arc and λcI^N substrates inhibits HslUV degradation. Such effects can be readily rationalized in terms of the model of Fig. 6, as substrates with proteins bound to their terminal sequences would be expected to have different K_1, K_2, and/or K_3 values. Many proteins are multimeric, and it is straightforward to envision regulatory strategies in which two degrons are properly displayed only in a monomer or only in a multimer, allowing just one species to be targeted for degradation.[9] Degrons could also be masked by a protein's native structure and only be revealed when the protein unfolds at high temperatures. In this regard, we note that HslUV is a heat-shock protease and that degradation signals in the interior of protein sequences have been shown to act as recognition signals for other AAA+ proteases.[19,22,40,41]

AAA+ proteases are allosteric machines that undergo continual cycles of ATP-powered conformational changes, even in the absence of substrate. As a consequence, it may be more difficult for the translocation machinery to bind and engage a degron in a single concerted step, but easier if the substrate is first tethered to the protease and engagement occurs subsequently. The latter situation could be viewed analogously to mid-air refueling, in which the speeds and general orientations of two airplanes are matched in one step and the fuel-transfer machinery is engaged in a subsequent step. We propose that HslUV carries out degradation in a similar multistep manner.

Materials & Methods

Protein expression and purification

Genes encoding protein substrates were constructed by standard methods of molecular biology using the polymerase-chain reaction and/or site-directed mutagenesis. λcI^N substrates contained varied N-terminal sequences, a common core corresponding to residues 2–97 of λ cI repressor, and C-terminal sequences of RSEYE (residues 98–102 of λ cI repressor), RSYYF, or ISVTL.

The initiator methionine of wild-type λ repressor is removed post-translationally,[42] leaving Ser^1 as the N-terminal residue. Variants designated ext1-λcI^N had an additional N-terminal extension ($MGS_2H_6S_3H$; glycine at N-terminus after processing of initiator methionine), which was derived from the pACYCDuet-1 expression vector (Novagen). Variants designated $^{S1L}\ \lambda cI^N$ were initially cloned, expressed, and purified as fusions to an N-terminal H_6-tagged SUMO domain in a pET23b vector (Novagen), and the fusion protein was then purified and cleaved with Ulp1 protease to generate the mature substrate with Leu^1 at the N-terminus.[43] Arc substrates contained variable N-terminal sequences, a common core consisting of residues 2–53 of P22 Arc repressor, and C-terminal sequences including the st11 sequence (H_6KNQHD), ssrA tag (AANDENYALAA), $ssrA^{DD}$ tag (AANDENYALDD), or $ssrA^{N3A}$ tag (AAADENYALAA). The wild-type N-terminus of

Arc is $Met^{1.44\ M1L}$Arc variants were expressed as H_6-SUMO fusions from pET23b, purified, and cleaved to generate Leu^1 at the N-terminus. Substrates designated ext2-Arc contained the N-terminal extension $LA_3SGAG_2SEG_2TSGAT$ and were also purified as SUMO fusions and processed by Ulp1 cleavage.

H_6-tagged variants of *E. coli* $HslU_6$ and $HslV_{12}$ were purified essentially as described (Burton et al., 2005), except buffer B (pH 8.0) contained 50 mM $NaHPO_4$, 300 mM NaCl, and 250 mM imidazole, and buffer C (pH 7.5) contained 20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 10% (v/v) glycerol. Pooled $HslU_6$ or $HslV_{12}$ fractions after Ni^{++}-NTA chromatography were chromatographed on a Sephacryl-300HR26/60 gel filtration column (GE Healthcare) and then on an HR16/10 Q-Sepharose column (GE Healthcare), developed with a gradient from 0.1 M to 1 M NaCl in buffer C. Appropriate fractions were pooled, concentrated, and exchanged into buffer B using Amicon Ultra-15 centrifugation filters.

Arc and λcI^N proteins were expressed in *E. coli* strain X90 (λDE3). Cells were grown at 37 °C to an OD_{600} of ~0.8, and shifted to room temperature. Expression was induced by the addition of 1 mM IPTG, and cells were harvested 4 h later, lysed by sonication, and cleared lysates were applied to a Ni^{++}-NTA column as described for the $HslU_6$ and $HslV_{12}$ purifications The Arc-st11-ssrA, Arc-st11-$ssrA^{DD}$, ext1-λcI^N-ISVTL and ext1-λcI^N-RSEYE proteins were subjected to additional purification on a HiLoad 16/60 Superdex-100 gel-filtration column (GE Healthcare) equilibrated in buffer D (20 mM Tris (pH 7.5), 200 mM NaCl, 1 mM EDTA). Appropriate fractions were pooled and concentrated using Amicon Ultra-15 centrifugation filters. Following the Ni^{++}-NTA step, substrates expressed as H_6-SUMO fusions were exchanged into 50 mM $NaHPO_4$ (pH 8.0), 100 mM NaCl, and 20 mM imidazole and cleaved with Ulp1 protease (a gift from J. Davis and J. Kaplan, MIT) at 37 °C overnight. Cleaved samples were re-applied to a Ni^{++}-NTA column, and the flow-through fraction, which contained the desired product, was pooled and chromatographed on a HiLoad 16/60 Superdex-100 gel-filtration column (GE Healthcare) equilibrated in buffer D. Appropriate fractions were pooled and concentrated using Amicon Ultra-15 centrifugation filters. ^{35}S-substrates were expressed and purified as described (Burton et al., 2005), with additional steps of Ulp1 cleavage, Ni^{++}-NTA re-purification, and S100 purification for substrates expressed as H_6-SUMO fusions.

An *E. coli* SspB expression clone was a gift from Igor Levchenko (MIT) and an *E. coli* ClpS expression clone (pET23b-H_6-SUMO-ClpS) was a gift from G. Román-Hernández (MIT). SspB was purified as described.[31] ClpS was purified by the protocol used for all SUMO-fusion proteins. The purified *E. coli* DegS PDZ domain was a gift from Jungsan Sohn (MIT). Protein concentrations were determined by absorbance at 280 nm using extinction coefficients calculated from the amino-acid sequence.

Degradation assays

For degradation assays monitored by SDS-PAGE, substrates (10 μM) were incubated at 37 °C for different times with 300 nM *E. coli* $HslU_6$, 800 nM *E. coli* $HslV_{12}$, and an ATP regeneration system (5 mM ATP, 16 mM creatine phosphate, 10 μg/mL creatine kinase). Steady-state HslUV degradation of ^{35}S-labeled proteins at 37 °C was carried out as described,[11] using 100 nM $HslU_6$, 300 nM $HslV_{12}$, and the ATP regeneration system. Aliquots were quenched at appropriate time points by adding ice-cold trichloroacetic acid (TCA) to a final concentration of 10% (w/v) and were separated into pellet and supernatant fractions by centrifugation. Radioactivity in the acid-soluble fraction was used as a measure of degradation. For experiments probing the inhibitory effects of SspB, ClpS, or the DegS PDZ domain, each protein was mixed with ^{35}S-labeled substrate for 5 min at 37 °C before addition of 500 nM $HslU_6$, 1.5 nM $HslV_{12}$, and the ATP regeneration mix. At different times, degradation was assayed by acid-soluble radioactivity as described above. For

degradation of ^{35}S-labeled proteins under single turnover conditions, each reaction contained 2 μM substrate, 5 μM $HslU_6$, 6 μM $HslV_{12}$, and the ATP regeneration system, and degradation was assayed as described above for steady-state kinetic experiments.

Thermal denaturation

Denaturation experiments were monitored by changes in circular-dichroism ellipticity at 222 nm using an AVIV Model 60DS spectrometer. Proteins (2 μM) in 10 mM KPO_4 (pH 6.8), 200 mM NaCl were heated from 25 to 95 °C in 2 °C steps, the sample was allowed to equilibrate for 1 min at each temperature, and the ellipticity was averaged for 30 s and recorded. Melting data were fit to a two-state model in which the intercepts and slopes of the native and denatured baselines, T_M, and ΔH_U were determined by a non-linear least squares fitting protocol.

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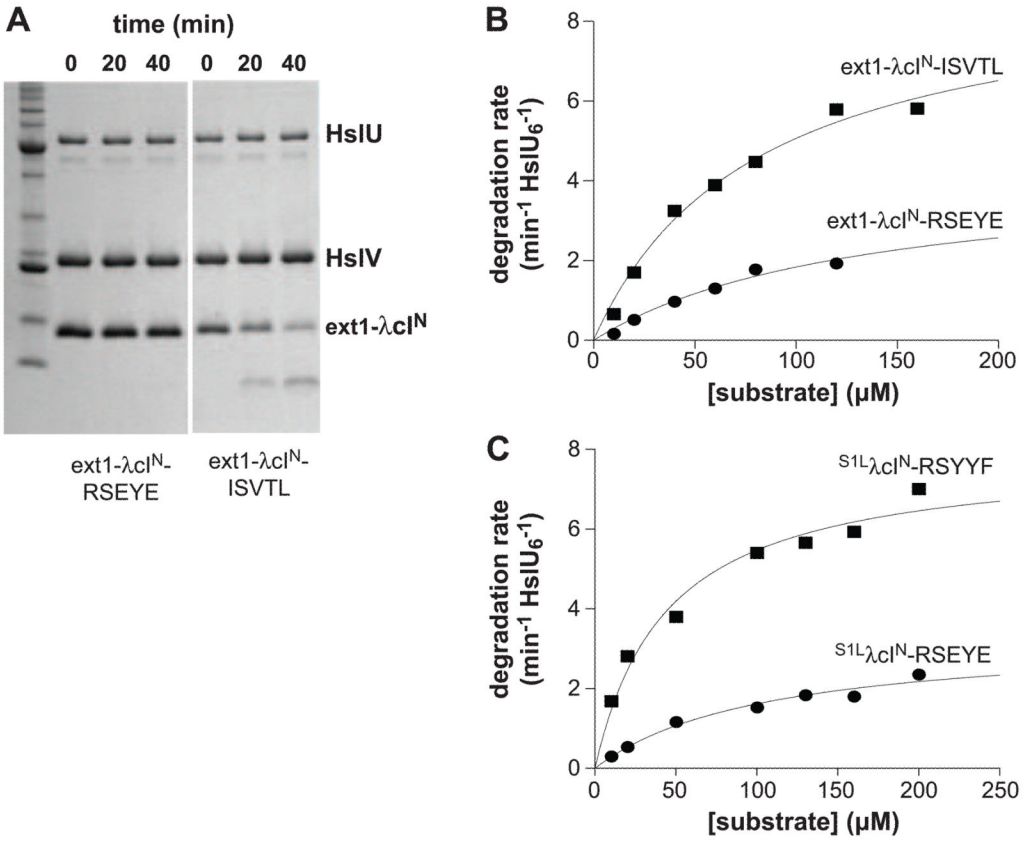

Figure 1.

C-terminal λcI^N sequences alter HslUV degradation. (**A**) Degradation of λcI^N variants (10 μM) bearing RSEYE or ISVTL C-terminal sequences by 300 nM $HslU_6$, 800 nM $HslV_{12}$ was analyzed by SDS-PAGE. (**B**, **C**) Rates of steady-state degradation of different concentrations of ^{35S}-λcI^N variants by 100 nM $HslU_6$, 300 nM $HslV_{12}$ were determined by assaying acid-soluble radioactivity. Lines represent non-linear-least-squares fits to the Michaelis-Menten equation: rate = $V_{max}\cdot[S]/([S]+K_M)$. Kinetic parameters are listed in Table 1.

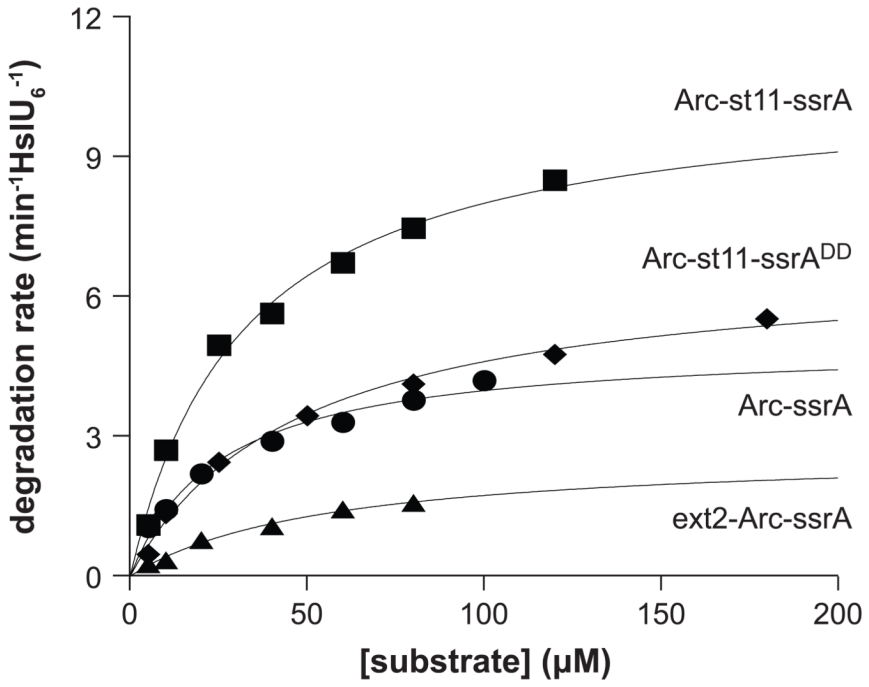

Figure 2.

C- and N-terminal sequences of Arc substrates affect HslUV degradation.Steady-state rates of HslUV degradation of ^{35}S-Arc variants were determined as described in the Fig. 1 legend. Lines are fits to the Michaelis-Menten equation; kinetic parameters are listed in Table 1.

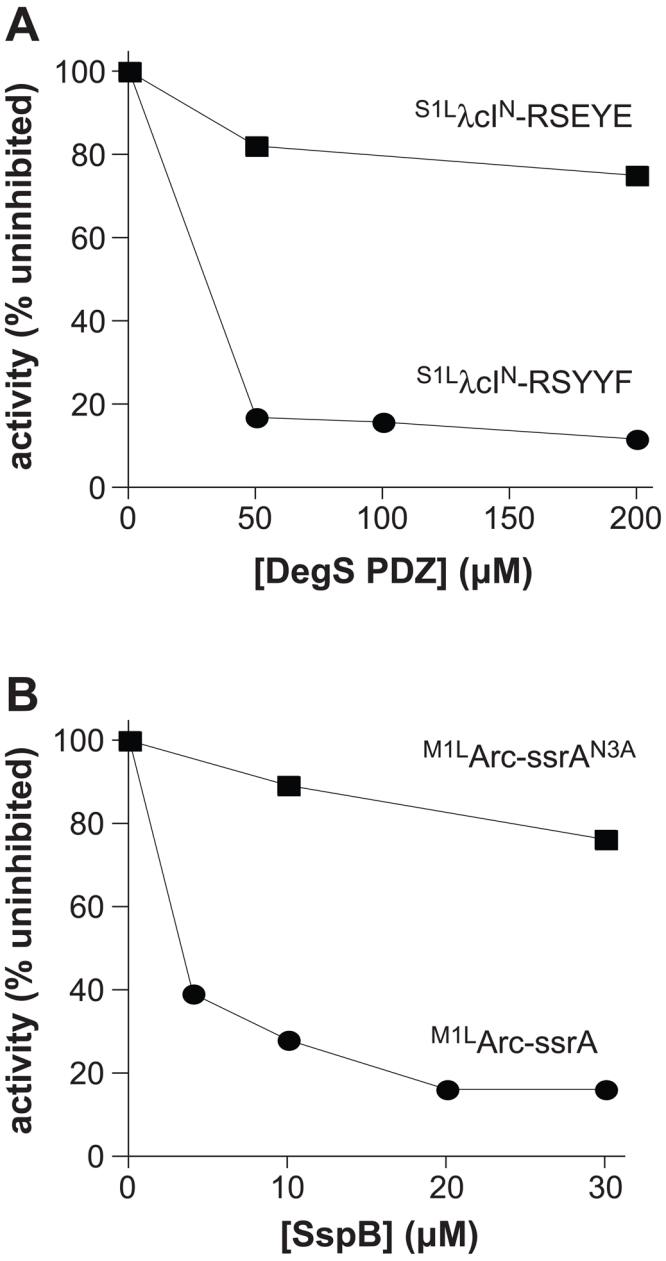

Figure 3.

Occluding the C-terminus of λcI^N and Arc substrates inhibits HslUV degradation. (**A**) Degradation of ^{35}S-labeled λcI^N substrates (50 μM) by 0.5 μM $HslU_6$, 1.5 μM $HslV_{12}$ was measured at different concentrations of the DegS PDZ domain, which binds strongly to the C-terminal tripeptide YYF but weakly to EYE. Rates are expressed as a percentage of the rate with no PDZ domain. (**B**) Degradation of ^{35}S-labeled Arc variants (4 μM) by 0.5 μM $HslU_6$, 1.5 μM $HslV_{12}$ was measured at different concentrations of SspB, which binds strongly to the wild-type ssrA tag but weakly to the $ssrA^{N3A}$ mutant. Rates are expressed as a percentage of the rate with no SspB.

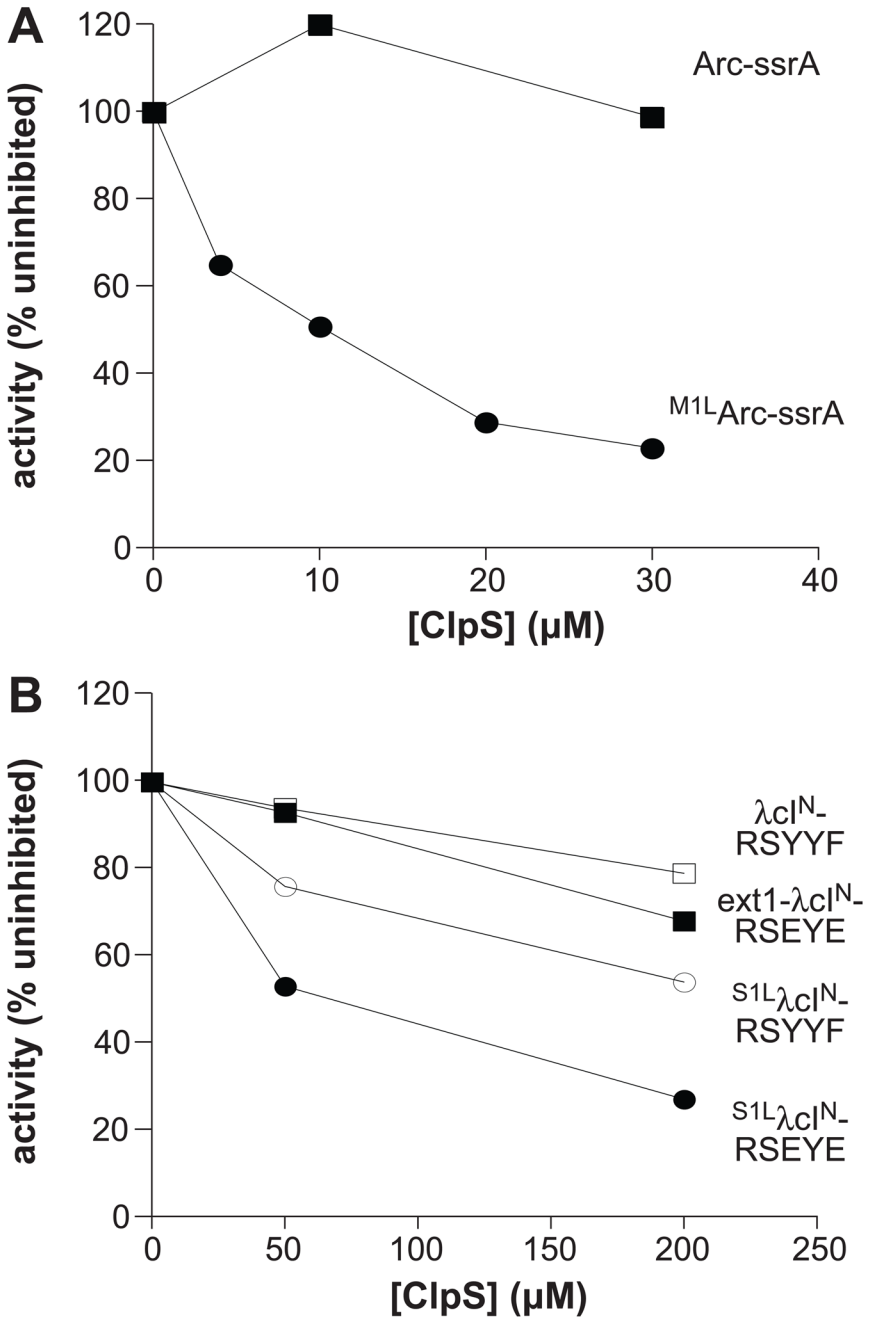

Figure 4.

ClpS binding to the N-terminus of Arc or λcI^{N} substrates slows HslUV degradation. (**A**) Proteolysis of ^{35}S-labeled Arc variants (4 μM) by 0.5 μM $HslU_6$, 1.5 μM $HslV_{12}$ was measured at different concentrations of ClpS, which binds strongly to the N-terminal leucine of ^{M1L}Arc-ssrA but weakly to the N-terminal methionine of Arc-ssrA. (**B**) ClpS inhibits HslUV (0.5 μM $HslU_6$, 1.5 μM $HslV_{12}$) degradation of λcI^{N} substrates (50 μM) with N-terminal leucines (^{S1L} λcI^{N}-RSYYF – open circles; ^{S1L} λcI^{N}-RSEYE – closed circles) more efficiently than variants with an N-terminal serine (λcI^{N}-RSYYF – open squares) or glycine (ext1-λcI^{N}-RSEYE – closed squares).

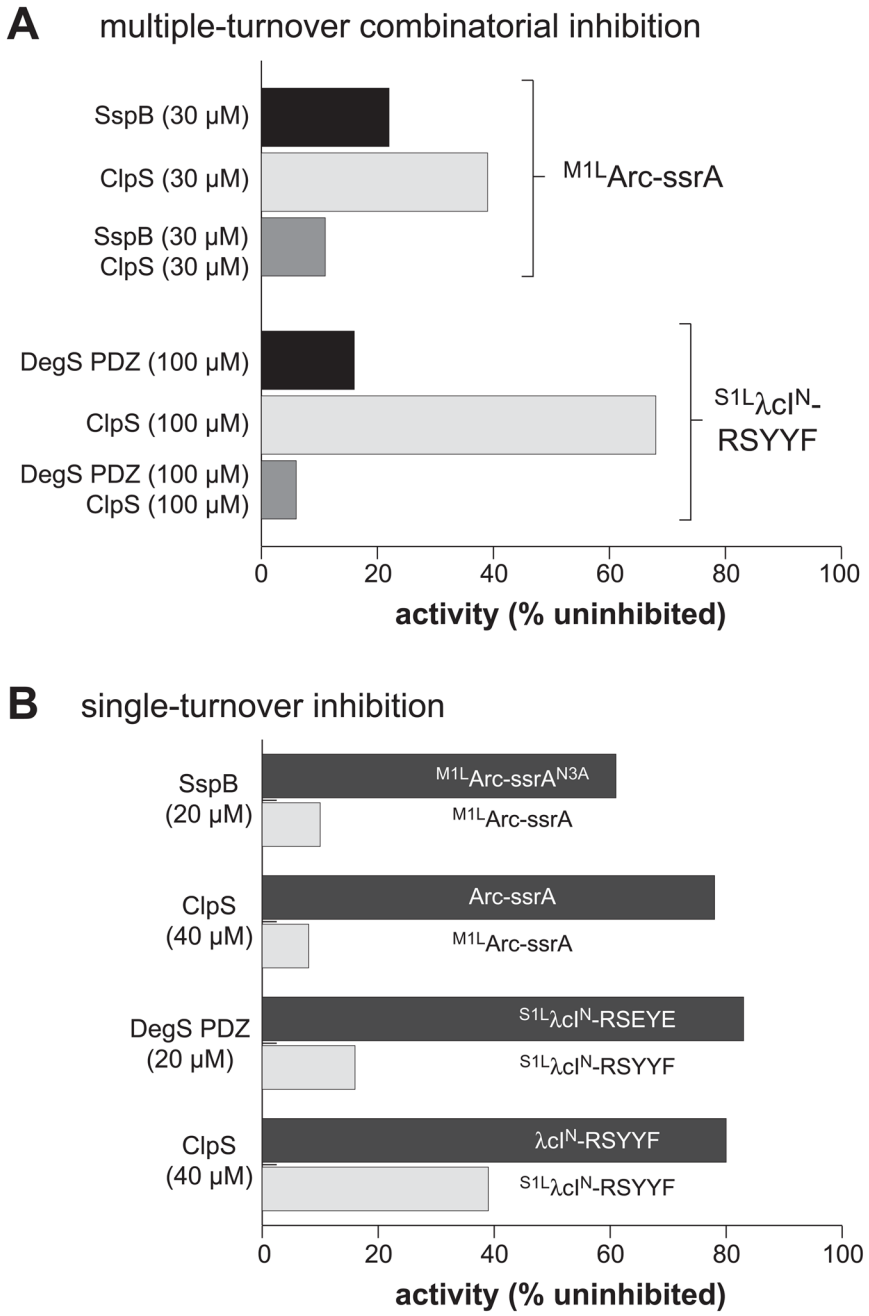

Figure 5.

Combinatorial and single-turnover inhibition.(**A**) (*top*) A combination of SspB and ClpS inhibited degradation of ^{35}S-^{M1L}Arc-ssrA (15 μM) by 0.5 μM $HslU_6$, 1.5 μM $HslV_{12}$ to a greater extent than either individual protein. (*bottom*) A combination of the DegS PDZ domain and ClpS also inhibited degradation of ^{35}S-^{S1L} λcI^{N}-RSYYF (50 μM) by 0.5 μM $HslU_6$, 1.5 μM $HslV_{12}$ more than either single protein alone. (**B**) Degradation of different ^{35}S-labeled substrates (2 μM) by 5 μM $HslU_6$, 6 μM $HslV_{12}$ was assayed in the presence the indicated concentrations of SspB, ClpS, or the DegS PDZ domain.

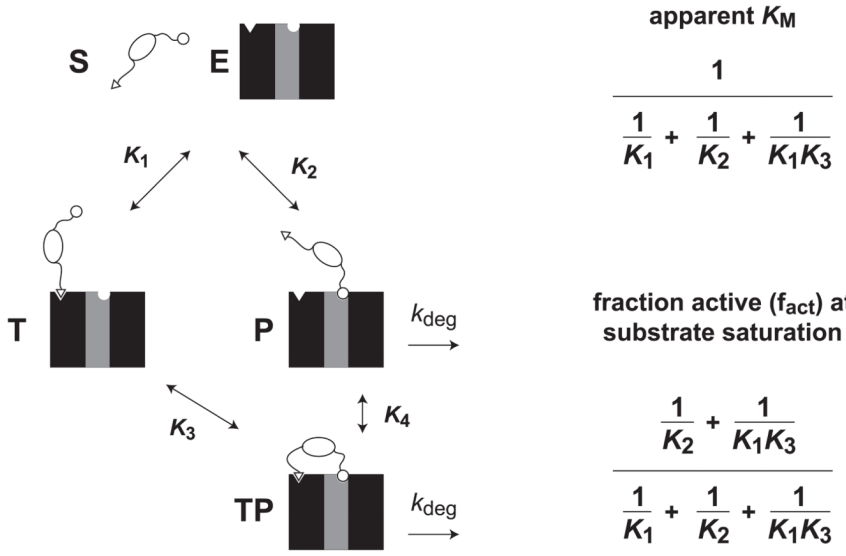

Figure 6.

Model of HslUV recognition of a substrate (S) with degrons at each terminus. One degron (shown as a triangle) binds to a tethering site on the HslUV enzyme (E). The other degron (shown as a circle) binds to a site in the translocation pore of HslU. Because binding to the pore is a prerequisite for degradation, the enzyme·substrate complexes marked P and TP are proteolytically active but the T complex is inactive. K_1 ([E][S]/[T]), K_2 ([E][S]/[P]), K_3 ([T]/[TP]), and K_4 ([P]/[TP]) are equilibrium dissociation constants. Note that $K_1 \cdot K_3 = K_2 \cdot K_4$. If the rate of substrate dissociation is fast compared to k_{deg}, then the apparent K_M ([S] at half maximal velocity) is $1/(1/K_1+1/K_2+1/(K_1 \cdot K_3))$. At substrate saturation, the fraction of active enzymes (f_{act}) = ([P]+[TP])/([T]+[P]+[TP]) = $(1/K_2+1/(K_1 \cdot K_3))/(1/K_1+1/K_2+1/(K_1 \cdot K_3))$. $V_{max}/[E_{total}]$ equals $f_{act} \cdot k_{deg}$. The K_3-k_{deg} pathway of degradation corresponds to single-degron recognition.

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Table 1

Properties of λcI^{N} and Arc substrates.

					epitopes for binding to		
substrates	**V_{max} (min^{-1})**	**K_M (μM)**	**V_{max}/K_M $min^{-1}\mu M^{-1}$**	**T_m (°C)**	**SspB**	**DegS PDZ**	**ClpS**
ext1-λcI^{N}-RSEYE	4.3 ± 0.1	120 ± 19	0.04	56.7	no	no	no
ext1-λcI^{N}-ISVTL	9.9 ± 1.1	89 ± 12	0.11	56.1	no	no	no
ext1-λcI^{N}(I84S)-ISVTL	9.9	79	0.13	50.5	no	no	no
$^{S1L}\lambda cI^{N}$-RSEYE	3.5 ± 0.3	120 ± 24	0.03	56.5	no	no	**yes**
$^{S1L}\lambda cI^{N}$-RSYYF	7.1 ± 1.0	40 ± 5.0	0.18	55.5	no	**yes**	**yes**
λcI^{N}-RSYYF	5.7 ± 0.6	54 ± 6.7	0.11	54.5	no	**yes**	no
Arc-ssrA	4.9 ± 0.1	24 ± 1.4	0.20	58.6	**yes**	no	no
Arc-st11-ssrA	12 ± 1.9	31 ± 0.9	0.38	56.5	**yes**	no	no
Arc-st11-$ssrA^{DD}$	7.0 ± 0.3	51 ± 4.8	0.14	59.3	**yes**	no	no
ext2-Arc-ssrA	3.0 ± 0.5	55 ± 1.3	0.06	58.2	**yes**	no	**yes**
^{M1L}Arc-ssrA	4.7 ± 1.5	21 ± 4.0	0.22	56.9	**yes**	no	**yes**
^{M1L}Arc-$ssrA^{N3A}$	5.7 ± 0.5	23 ± 7.7	0.25	56.3	no	no	**yes**

V_{max} values are expressed as turnover numbers (the degradation rate at substrate saturation divided by the total enzyme concentration). V_{max} and K_M values are generally averages of two determinations (error estimated as $\sqrt{\sum_{1}^{2}(value - mean)^2}$). Values with no error are from a single experiment.