

# Covalent Linkage of Distinct Substrate Degrons Controls Assembly and Disassembly of DegP Proteolytic Cages

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## SUMMARY

Protein quality control requires careful regulation of intracellular proteolysis. For DegP, a periplasmic protease, substrates promote assembly of inactive hexamers into proteolytically active cages with 12, 18, 24, or 30 subunits. Here, we show that sensitive activation and cage assembly require covalent linkage of distinct substrate sequences that affect degradation (degrons). One degron binds the DegP active site, and another degron binds a separate tethering site in PDZ1 in the crystal structure of a substrate-bound DegP dodecamer. FRET experiments demonstrate that active cages assemble rapidly in a reaction that is positively cooperative in substrate concentration, remain stably assembled while uncleaved substrate is present, and dissociate once degradation is complete. Thus, the energy of binding of linked substrate degrons drives assembly of the proteolytic machine responsible for subsequent degradation. Substrate cleavage and depletion results in disassembly, ensuring that DegP is proteolytically active only when sufficient quantities of protein substrates are present.

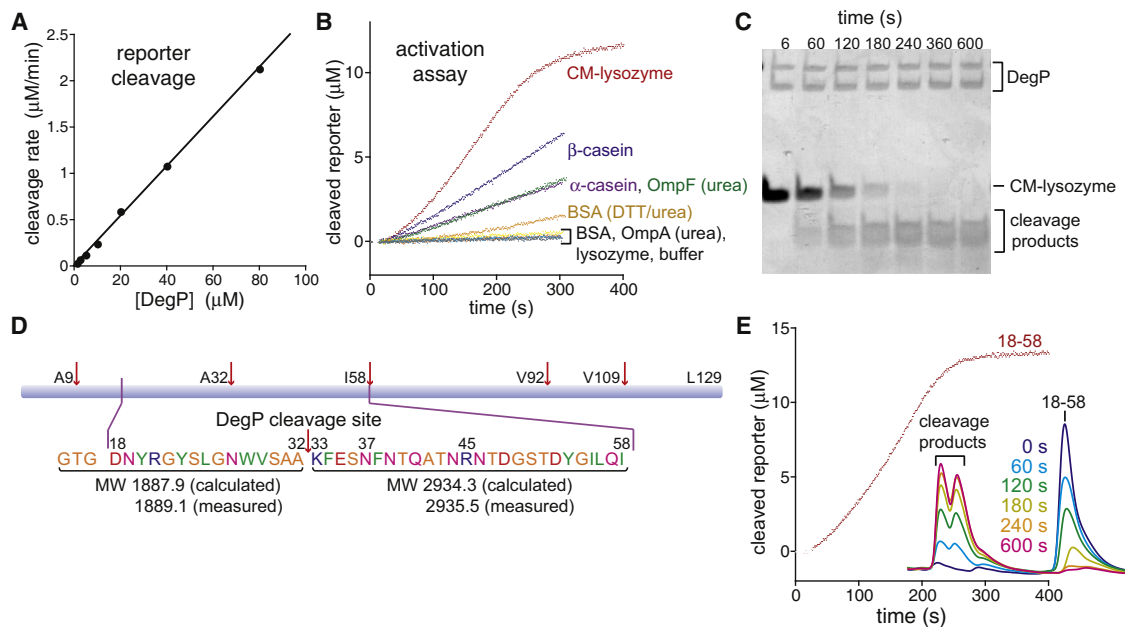
## INTRODUCTION

Intracellular protein quality control requires proteases to degrade and chaperones to refold misfolded proteins. The periplasm of Gram-negative bacteria presents a challenge for quality control because it lacks ATP, which is vital for the function of many cytoplasmic proteases and chaperones, and is exposed to environment stresses that denature native proteins and prevent protein folding and assembly de novo (Duguay and Silhavy, 2004). DegP, a major player in periplasmic protein quality control, functions as a protease and chaperone in *Escherichia coli*, is essential for cell viability at high temperatures, is implicated in the biogenesis of outer-membrane proteins (OMPs), and is up-regulated by the major periplasmic stress-sensing systems (Swamy et al., 1983; Lipinska et al., 1988, 1989, 1990; Strauch et al., 1989; Danese et al., 1995; Spiess et al., 1999; Rizzitello et al., 2001; Sklar et al., 2007). DegP is also important for the viru-

lence of many pathogenic bacteria (Raivio, 2005). Finally, DegP belongs to the HtrA family of PDZ-domain containing proteases, which includes human homologs implicated in Parkinson's disease, arthritis, and some cancers (Vande Walle et al., 2008).

DegP contains two PDZ domains and a trypsin-like protease domain, with a canonical His-Asp-Ser catalytic triad and oxyanion hole. It degrades denatured or non-native proteins in a relatively promiscuous fashion, generally cleaving substrates after Ile, Val, Met, Leu, Thr, Ser, and Ala (Kolmar et al., 1996; Krojer et al., 2008a). Thus, one element of degradation specificity involves DegP recognition of the substrate sequence around the cleavage site. We refer to this element as the cleavage-site degron. C-terminal substrate residues also bind to the PDZ1 domain of DegP (Iwanczyk et al., 2007; Krojer et al., 2008a), defining a second specificity element, which we call the PDZ1-binding degron. The  $\alpha$ -carboxylate and side chain of the C-terminal residue are especially important in determining binding affinity, and DegP cleavage frequently generates a product with a C-terminal residue that can bind PDZ1 (Krojer et al., 2008a). Thus, many cleavage fragments are themselves substrates for further DegP proteolysis.

Intracellular proteases must be regulated to avoid rogue degradation of improper substrates. The fundamental unit of DegP structure is a trimer, which is not typically populated (Jomaa et al., 2007). Instead, two trimers form a proteolytically inactive hexamer (Krojer et al., 2002). Intriguingly, addition of substrates results in formation of proteolytically active DegP dodecamers (12 mers) or icosatetramers (24 mers), which form hollow cage-like structures with interior active sites (Jiang et al., 2008; Krojer et al., 2008b). Crystal structures of the active icosatetramer and inactive hexamer are known, as are high- and low-resolution EM structures of a dodecamer, which suggest conflicting models of cage stabilization (Krojer et al., 2002, 2008b; Jiang et al., 2008). The mechanism by which substrate-mediated assembly of active DegP oligomers is regulated is poorly understood. One component involves the PDZ1-binding degron, as peptides containing this sequence activate DegP proteolysis and promote the hexamer to dodecamer transition (Krojer et al., 2008a, 2010; Merdanovic et al., 2010). However, the PDZ1-binding degron is not destroyed by DegP degradation, and it is unclear how disassembly of active dodecamers is achieved. Whether the cleavage-site degron is also important in activation is unknown. Moreover, the kinetics of formation and disassembly of DegP cages has not been carefully



**Figure 1. Efficient DegP Activation Requires an Intact Cleavage-Site Degron**

(A) DegP cleaved the reporter peptide (Abz-KASPVSLGY<sup>NO<sub>2</sub>D</sup>; 100  $\mu\text{M}$ ; Lee et al., 2010) slowly and in proportion to enzyme concentration ( $0.027 \text{ min}^{-1} \text{ DegP}^{-1}$ ). (B) Different proteins (5  $\mu\text{M}$ ) activated DegP (1  $\mu\text{M}$ ) cleavage of the reporter (100  $\mu\text{M}$ ) to varying extents. Higher activation results in a steeper slope of the fluorescence versus time curves and vice versa. Note that activation by CM-lysozyme was substantially reduced after 300 s, when most of this protein had been degraded (see C). See also Figure S1. (C) Cleavage of CM-lysozyme (5  $\mu\text{M}$ ) by DegP (1  $\mu\text{M}$ ) was monitored by SDS-PAGE. The lower DegP band results from autocleavage. (D) Major DegP cleavage sites in CM-lysozyme were determined by LC/MS (top). Sequence of the 18–58 polypeptide (middle). DegP cleaves 18–58 at a single site, as determined by HPLC purification and mass spectrometry of cleavage products. (E) The 18–58 polypeptide (5  $\mu\text{M}$ ) transiently activates DegP (1  $\mu\text{M}$ ) cleavage of reporter peptide (100  $\mu\text{M}$ ). Inset – HPLC analysis of DegP (1  $\mu\text{M}$ ) cleavage of 18–58 (5  $\mu\text{M}$ ). Activation slowed dramatically when 18–58 was completely cleaved.

investigated, and whether substrate-mediated assembly of cages allows degradation of only the initially encapsulated substrates or also of additional substrates that diffuse into the proteolytic chamber remains to be determined.

Here, we use a model substrate, containing a single cleavage-site degron and PDZ1-binding degron, to probe control of DegP assembly and proteolysis. This substrate bound to an active-site mutant of DegP in a positively cooperative reaction that promoted formation of dodecamers and larger cages. A crystal structure revealed four DegP trimers arranged in a distorted tetrahedron, with the C terminus of the substrate bound to PDZ1 and the cleavage-site degron bound to the DegP active-site cleft. Importantly, we discovered that covalent linkage of the cleavage-site and PDZ1-binding degrons was critical for sensitive activation of DegP proteolysis and cage assembly. Moreover, a fluorescence resonance energy transfer (FRET) assay for DegP assembly/disassembly demonstrated that cages assemble rapidly upon substrate binding, remain stably assembled as long as uncleaved substrate is available, and dissociate once intact substrate has been consumed. As a consequence, intact substrate binding drives formation of dodecamers and larger cages from inactive hexamers, and substrate cleavage promotes cage disassembly, providing a simple mechanism to ensure that DegP is proteolytically active only when sufficient quantities of protein substrates are present.

## RESULTS

### Identification of a Dual-Degron Activator/Substrate

We sought to identify a relatively small polypeptide that would serve as an efficient DegP activator and substrate. Initially, we assayed different proteins for their ability to accelerate cleavage of a small reporter peptide in which proteolysis results in increased fluorescence. DegP cleaved 100  $\mu\text{M}$  reporter at a very slow rate proportional to enzyme concentration (Figure 1A). Using the same reporter concentration, we monitored the ability of various proteins (5  $\mu\text{M}$ ), including known DegP substrates, to activate cleavage by 1  $\mu\text{M}$  DegP (Figure 1B). Some unfolded or intrinsically unstructured proteins (carboxymethylated [CM] lysozyme, denatured OmpF, denatured serum albumin,  $\beta$ -casein, and  $\alpha$ -casein) activated cleavage to different extents, but denatured OmpA, native lysozyme, and native serum albumin did not.

We focused on CM-lysozyme, which showed a high level of DegP activation. Comparing the kinetics of activation (Figure 1B) with those of DegP cleavage of CM-lysozyme (Figure 1C) revealed that reporter cleavage roughly paralleled CM-lysozyme cleavage and slowed markedly about the time or shortly after the intact substrate was consumed, despite the fact that  $\sim 90\%$  of the reporter was still uncleaved (Figure S1, available online, shows similar experiments for other substrates). These

results suggested that the presence of one or more uncleaved degrons is an important aspect of activation. To simplify experiments by reducing the number of cleavage sites, we initially mapped the sites of DegP cleavage in CM-lysozyme (Figure 1D), and then constructed and screened fragments that spanned one cleavage site. Notably, DegP cleaved a fragment comprising lysozyme residues 18–58 (with the cysteines mutated to serines) at a single site between Ala<sup>32</sup> and Lys<sup>33</sup> as monitored by HPLC and MALDI mass spectrometry (Figure 1D). The 18–58 fragment also activated reporter cleavage by DegP, with kinetics that closely paralleled 18–58 cleavage into two products (Figure 1E). Thus, the intact cleavage-site degron in the 18–58 peptide is critical for efficient DegP activation.

### Crystal Structure of DegP<sub>12</sub> with Bound 18–58

The DegP<sup>S210A</sup> active-site mutant copurifies with cellular substrates (Krojer et al., 2008b). To avoid this problem, we purified this mutant under denaturing conditions and allowed it to refold. When wild-type DegP was subjected to the same unfolding/purification/refolding protocol, no significant change in degradation activity occurred (Figure S2A). Refolded DegP<sup>S210A</sup> behaved as a hexamer in size-exclusion chromatography (Figure 2A) or sedimentation velocity centrifugation experiments (Figure 2B; Figure S2B). In gel filtration (Figure 2A), 18–58 addition resulted in a shift to a position expected for a 12 mer. In centrifugation (Figure 2B), 18–58 addition produced a mixture that was predominantly 12 mers (66% mole equivalents) but also contained 18 mers (24%), 24 mers (7%), and 30 mers (3%). The additional oligomeric species probably reflect higher average DegP concentrations in the centrifugation experiment but could also indicate reduced resolution in the gel-filtration experiment.

We crystallized DegP<sup>S210A</sup> with 18–58 and collected data from a crystal (space group C2) that diffracted to 3.76 Å resolution. Phases were obtained by molecular replacement using the isolated protease, PDZ1, and PDZ2 domains from the DegP<sub>24</sub> crystal structure (3CS0; Krojer et al., 2008b), which we re-refined to improve geometry (Table 1). Electron-density maps revealed two trimers in the asymmetric unit and a dodecamer formed by 2-fold crystallographic symmetry. The structure was refined to good statistics ( $R = 0.219$ ;  $R_{\text{free}} = 0.251$ ) and had excellent geometry (Table 1).

In each subunit of the dodecamer, one region of the 18–58 substrate was bound in the active-site cleft of the protease domain, which assumed the same active conformation observed in the icosatetramer, and another was bound to PDZ1 (Figure 2C). Although substrate main-chain density was clear, side chains were poorly resolved. Thus, we positioned the cleavage-site degron in the density near the active site, with the side chain and carbonyl oxygen of the P1 residue (Ala<sup>32</sup>) occupying the S1 pocket and oxyanion hole, respectively. For the C-terminal degron, the  $\alpha$ -carboxylate and side chain of Ile<sup>58</sup> were positioned in the binding pocket of the PDZ1 domain. After fixing these points of reference, residues 28–32 of the cleavage-site degron formed an antiparallel  $\beta$  sheet with DegP residues 226–230 in each subunit, and the side chain of the P4 residue (Val<sup>29</sup>) made a hydrophobic contact with DegP (Figure 2D). In each DegP subunit, residues 54–55 of the peptide were ordered and residues 56–58 formed an antiparallel  $\beta$  sheet with PDZ1 residues 267–269. In one crystallographically inde-

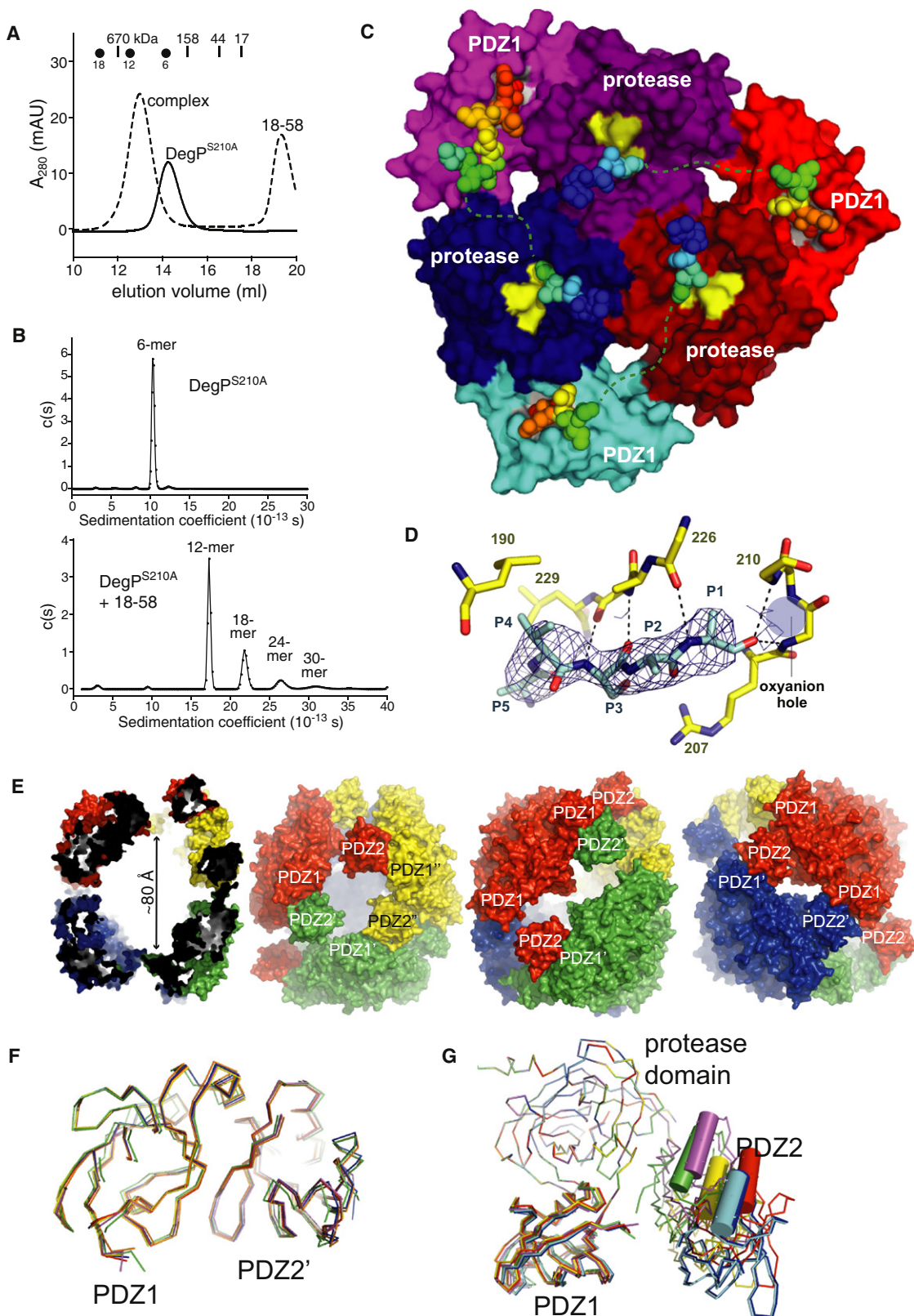
pendent subunit, electron density for residues 48–53 of peptide was also present, with the N-terminal residues of this segment contacting the C-terminal portion of PDZ2' in an adjacent trimer. Electron density for the linker region between the PDZ1-binding and cleavage-site degrons was absent. Based on geometric constraints, it seems most likely that this linker connects the cleavage-site and PDZ1-binding degrons between neighboring subunits in a trimer (Figure 2C).

In the dodecamer, four DegP trimers formed the faces of an asymmetric irregular tetrahedron, enclosing a proteolytic chamber (Figure 2E). Within the chamber,  $\sim 90$  Å separated the most distant active sites and  $\sim 65$  Å separated the most distant tips of the L2 substrate-binding loop (which project closest to the center of the dodecamer). The maximum distance between residues on the exterior of the dodecamer was roughly the same as observed in the high-resolution EM dodecamer (Jiang et al., 2008). There were openings or apertures at each vertex and near the center of each edge of the tetrahedron (Figure 2E). The only interactions between trimers occurred along the edges, where a PDZ1 domain from one trimer made extensive contacts with a PDZ2' domain in a neighboring trimer (Figure 2E). This interaction was highly conserved (rmsd 0.46 Å for 163 C $\alpha$  atoms after superposition of the six crystallographically independent pairs of interacting PDZ1.PDZ2' domains; Figure 2F) and was essentially identical to the corresponding interactions in the high-resolution EM structure of DegP<sub>12</sub> and the crystal structure of DegP<sub>24</sub> (Jiang et al., 2008; Krojer et al., 2008b). A symmetric dodecamer would form a regular tetrahedron with edges of equal length, but the crystallographic tetrahedron had edges varying by as much as 17 Å in length. This distortion resulted in variations in the dimensions of the oblong edge apertures, which were  $\sim 15$  Å across and varied from 20 to 34 Å in length (Figure 2E). The triangular apertures at each vertex had maximum dimensions of 13–17 Å. Either set of apertures might allow substrates and products to enter and leave the proteolytic chamber but could also be occupied by unstructured protein. For example, the expected mass of the disordered residues from the substrate and DegP LA loop would fill most of the internal volume of the chamber.

When the protease domains of the six crystallographically independent subunits in the dodecamer were aligned, the attached PDZ1 domains showed modest deviations ( $\sim 4$  Å between the most discrepant positions) but the position of the PDZ2 domain was highly variable ( $\sim 23$  Å deviations; Figure 2G). The latter result indicates that the PDZ1-PDZ2 linker is flexible. Indeed, except for one subunit, these linkers were not visible in the electron-density map. Moreover, the distance between residue 357 in PDZ1 and residue 368 in PDZ2 (roughly spanning the linker) varied by as much as 6 Å for different subunits. Such variation was not observed in orientation or distance between PDZ1 and PDZ2 in the crystallographic icosatetramer or high-resolution EM dodecamer, which are fully symmetric structures (Jiang et al., 2008; Krojer et al., 2008b).

### Linked Degrons Are Required for Efficient and High-Affinity DegP Activation

We constructed mutations in 18–58 to assess the role of each degron in DegP activation. To probe the importance of the



PDZ1-binding degron, we deleted Ile<sup>58</sup> ( $\Delta 58$ ), the C-terminal residue, which eliminated activation of DegP reporter cleavage at the concentration (5  $\mu\text{M}$ ) used in this assay (Figure 3A). Moreover, DegP did not detectably cleave the  $\Delta 58$  variant (Figure S3A). We also assayed DegP activation by an 18–58 variant containing mutations at the P2/P1 residues of the cleavage site (A31G/A32G). This double mutant supported substantially lower activation than 18–58 (Figure 3A). DegP also cleaved this substrate more slowly (Figure S3A), ruling out the possibility that poor activation results from faster DegP cleavage and consequent destruction of the activating species. Thus, both the cleavage-site degron and PDZ1-binding degron are important for activation.

To determine the importance of covalent linkage of these activation determinants, we synthesized one peptide containing the cleavage site (residues 18–37), another peptide with the PDZ1-binding degron (residues 45–58), and an 18–58 <sup>$\Delta 37-46$</sup>  variant to control for residues missing in the single-degron peptides. The C-terminal residues of the cleavage-site peptide were very polar (Glu-Ser-Asn) and should be an exceptionally poor PDZ1-binding sequence, based on the hydrophobic nature of the PDZ1 binding pocket. We then titrated 18–58, 18–58 <sup>$\Delta 37-46$</sup> , an equal mixture of 18–37 plus 45–58, 18–37 alone, or 45–58 alone against DegP and reporter and assayed activation (Figure 3B). Each curve showed an activation phase and all but 45–58 showed a subsequent inhibition phase, which we interpret as competition for reporter binding to the active site of DegP. Notably, maximal activation occurred at 2–5  $\mu\text{M}$  concentrations of 18–58 and 18–58 <sup>$\Delta 37-46$</sup> , at 100–200  $\mu\text{M}$  concentrations of 18–37 alone or mixed with 45–58 (although activity was higher for the mixture, demonstrating synergy), and at 2 mM or greater concentrations of 45–58 alone. These results show that covalent linkage of the cleavage-site and PDZ1-binding degrons is required for high-affinity activation. Krojer et al. (2008a) reported DegP activation using peptides terminating with YQV. When we replaced the C-terminal LQI tripeptide of 18–58 with YQV, we

observed maximal activation at slightly lower concentrations ( $\sim 1 \mu\text{M}$ ) but no activation by 1.3 mM concentrations of YQV peptides without a cleavage-site degron (Figure S3B). Thus, trans-activation of DegP appears to require very high concentrations of peptides with PDZ1-binding degrons alone. We conclude that each individual degron in the 18–58 substrate activates DegP weakly, a mixture of both degrons shows synergistic activation, and the most efficient activation requires covalent linkage of both degrons.

### 18–58 Binds DegP with Positive Cooperativity

For binding studies, we added a cysteine near the N terminus of the 18–58 fragment, which was then modified by addition of a fluorophore (<sup>125</sup>I-18–58). In an initial experiment, we incubated 10  $\mu\text{M}$  DegP<sup>S210A</sup> with trace <sup>125</sup>I-18–58 and measured binding of the fluorescent molecule by changes in anisotropy in the presence of increasing concentrations of unlabeled 18–58. As shown in Figure 3C, <sup>125</sup>I-18–58 binding initially increased and then decreased as the concentration of 18–58 exceeded the concentration of DegP subunits. This behavior is expected for a positively cooperative allosteric system, in which low concentrations of the unlabeled competitor stabilize a DegP conformation with unoccupied high-affinity sites, and higher competitor concentrations fill these sites and prevent binding of the fluorescent substrate.

If the initial substrate-binding events result in more high-affinity sites, then binding should be stronger in the presence than absence of sub-saturating 18–58. To test this hypothesis, we used anisotropy to assay binding of different concentrations of DegP<sup>S210A</sup> to <sup>125</sup>I-18–58 alone or with unlabeled 18–58 added at one-half of the molar concentration of DegP for each point in the titration (Figure 3D). Fitting these data gave an apparent equilibrium dissociation constant ( $K_{\text{app}}$ ) of 0.88  $\mu\text{M}$  with unlabeled 18–58 present and 12  $\mu\text{M}$  in its absence. Thus, the unlabeled substrate increased the affinity of DegP for the fluorescent substrate >10-fold, demonstrating that 18–58 binding is positively cooperative.

### Figure 2. Crystal Structure of the DegP Dodecamer with Bound 18–58

- (A) In gel-filtration chromatography on a Superose 6 column, DegP<sup>S210A</sup> alone ran as a hexamer (solid line), whereas DegP<sup>S210A</sup> plus 18–58 ran as a dodecamer (dashed line). Two hundred microliter samples of DegP<sup>S210A</sup> (25  $\mu\text{M}$ ) with or without 18–58 (38  $\mu\text{M}$ ) were loaded, and chromatography resulted in dilution of  $\sim 10$ -fold. The elution positions expected for DegP 6 mers, 12 mers, and 18 mers based on protein standards are marked by dots.
- (B) c(s) distributions from sedimentation-velocity experiments of DegP<sup>S210A</sup> (20  $\mu\text{M}$ ; upper panel) or DegP<sup>S210A</sup> (20  $\mu\text{M}$ ) mixed with 18–58 (38  $\mu\text{M}$ ; lower panel) were determined using SEDFIT (Schuck, 2000). For DegP<sup>S210A</sup> alone, there was a single major species (10.4 S). The molecular weight of this species ( $\sim 310$  kDa calculated using  $f/f_0 = 1.4$ ) was consistent with a hexamer (calculated  $M_R$  289 kDa). For DegP<sup>S210A</sup> in the presence of 18–58, the hexamer largely disappeared and new species of 17.3, 21.8, 26.5, and 31 S were observed. Assuming a frictional ratio of 1.46, these species appear to be 12 mers ( $\sim 619$  kDa), 18 mers ( $\sim 878$  kDa; possibly a trigonal bipyramid), 24 mers ( $\sim 1.18$  MDa), and 30 mers ( $\sim 1.49$  MDa; possibly a pentagonal bipyramid). Figure S2B shows concentration versus radial distributions as a function of time for these experiments, as well as residuals of the fits.
- (C) The protease and PDZ1 domains from one trimer are shown in surface representation, with PDZ1 colored a lighter shade than the corresponding protease domain. The cleavage-site and PDZ1-binding degrons of 18–58 are shown in CPK representation and are colored from the blue to the red end of the spectrum in the N-terminal to C-terminal direction. The active-site region of each protease domain is highlighted in yellow; the pocket for binding the C-terminal residue of 18–58 is highlighted in gray. The dashed green lines depict plausible connections between cleavage-site and C-terminal degrons bound to neighboring subunits in the trimer.
- (D) Residues 28–32 (P5-P4-P3-P2-P1) of the cleavage-site degron are shown in stick representation (cyan) with  $2F_o - F_c$  electron density contoured at  $1\sigma$ . Protease-domain residues are also shown in stick representation (yellow), but only selected atoms are displayed for simplicity. Dashed lines represent hydrogen bonds between the substrate and enzyme.
- (E) Shown from left to right are views of a cross-section of the protease chamber, a vertex aperture, a large-edge aperture, and a small-edge aperture. Each trimer in the dodecamer (surface representation) is colored differently. Interactions between trimers are mediated by PDZ1-PDZ2' contacts near the ends of each edge.
- (F) Alignment of six pairs of interacting PDZ1-PDZ2' domains shows a highly conserved interface (rmsd 0.46 Å).
- (G) Superposition of the protease domains of six subunits reveals that PDZ1 assumes a relatively constant orientation, whereas PDZ2 is highly divergent. A single helix in PDZ2 is shown as a cylinder to emphasize the variation.

**Table 1. Crystallographic Statistics**

PDB Code	3OTP	3CS0 <sup>a</sup>	3OU0 <sup>b</sup>
Subunits in cage	12	24	
Space group	C2	F432	
Unit cell a =	215.4 Å	253.9 Å	
Unit cell b =	122.8 Å	253.9 Å	
Unit cell c =	138.7 Å	253.9 Å	
Unit cell $\alpha$ =	90°	90°	
Unit cell $\beta$ =	118°	90°	
Unit cell $\gamma$ =	90°	90°	
Refinement resolution	15–3.76	15–3.0 Å	
Wavelength	0.979 Å	0.979 Å	
R <sub>sym</sub> (%)	12.5 (68.8)	9.3 (57.9)	
Unique reflections	28,349	14,441	
Completeness (%)	88.3 (63.0)	99.7 (99.9)	
Data redundancy	2.9 (2.9)	5.1 (5.1)	
Average I/ $\sigma$ I	8.1 (1.5)	13.0 (2.9)	
R <sub>work</sub> (%)	21.9 <sup>c</sup>	21.2	19.6
R <sub>free</sub> (%)	25.1 <sup>c</sup>	27.4	22.9
Rmsd bond lengths (Å)	0.003	0.009	0.003
Rmsd bond angles (°)	0.61	1.53	0.67
Total protein atoms	17036	2893	2877
Solvent atoms	0	0	0
Ramachandran <sup>d</sup> favored/allowed/disallowed (%)	98.9/1.1/0	91.2/6.7/2.1	98.5/1.5/0
Favorable rotamers (%) <sup>d</sup>	100	89.7	100
Clash score <sup>d</sup>	0	16.25	0
Residues with bad angles <sup>d</sup>	0	4	0

Numbers in parentheses represent values for the highest resolution bin.  $R_{sym} = \sum_j |I_j(h) - \langle I(h) \rangle| / \sum_j I_j(h) \langle I(h) \rangle$ , wherein  $I_j(h)$  is the  $j$ th reflection of index  $h$  and  $\langle I(h) \rangle$  is the average intensity of all observations of  $I(h)$ .  $R_{work} = \sum |F_{obs}(h) - F_{calc}(h)| / \sum |F_{obs}(h)|$ , calculated over ~95% of the data in the working set.  $R_{free}$  is equivalent to  $R_{work}$  except calculated over ~5% of the data in the test set.

<sup>a</sup>The 3CS0 structure and data collection statistics are from Krojer et al. (2008b) or the 3CS0 PDB file.

<sup>b</sup>Our rerefinement of the 3CS0 structure.

<sup>c</sup>Refinement statistics with tightly restrained individual B factors. With one B factor per residue,  $R_{work}$  was 22.8% and  $R_{free}$  was 25.7%.

<sup>d</sup>Favorable/allowed/disallowed Ramachandran angles, favorable rotamers, residues with bad angles, and the clash score (number of steric overlaps  $\geq 0.4$  Å per 1000 atoms) were calculated using MolProbity (Davis et al., 2007).

### DegP Assembly Assayed by FRET

Formation of dodecamers and higher-order DegP cages requires association of hexamers and should give rise to increased fluorescence resonance energy transfer (FRET) upon mixing of donor and acceptor-labeled hexamers, allowing the equilibrium properties and real-time kinetics of cage assembly to be monitored. To facilitate these experiments, we mutated both wild-type cysteines in DegP to serines and introduced the N296C mutation into PDZ1 to provide a site for labeling (we refer to this triple mutant as DegP\*). Initial experiments were per-

formed with DegP\*<sup>S210A</sup> to eliminate degradation. We labeled one batch of DegP\*<sup>S210A</sup> with a donor dye and another batch with an acceptor dye. Adding 18–58 to an equimolar mixture of these proteins resulted in a dose-dependent increase in the ratio of acceptor to donor fluorescence emission (Figure 4A) and to a shift to the dodecamer position in gel-filtration chromatography (Figure S4A). Control experiments confirmed that the increased emission ratio was not a result of subunit exchange among hexamers (Figure S4B) or principally a consequence of FRET-independent changes in the fluorescent properties of donor- or acceptor-labeled DegP\*<sup>S210A</sup> alone (Figure S4C). To confirm that the fluorescence change upon 18–58 binding arises from assembly, we constructed a variant (Y444A) in which a critical side chain in the PDZ1-PDZ2' interface of the crystallographic dodecamer was mutated. Upon addition of the 18–58 substrate, a mixture of donor and acceptor labeled DegP\*<sup>S210/Y444A</sup> showed no increase in FRET (Figure 4A). We also confirmed that DegP\*<sup>S210/Y444A</sup> bound 18–58 but eluted as a hexamer in gel filtration in both the presence and absence of 18–58 (Figure S4D).

The 18–58 assembly curve (Figure 4A) was fit well by the Hill equation ( $K_{app} = 3.1 \mu\text{M}$ ;  $n = 1.9$ ), showing that this substrate promotes formation of DegP cages in a positively cooperative fashion. Consistent with the activation results presented above, peptides containing just the cleavage-site degron or PDZ1-binding degron also facilitated cage assembly, but only at substantially higher concentrations than 18–58 (Figure 4B).

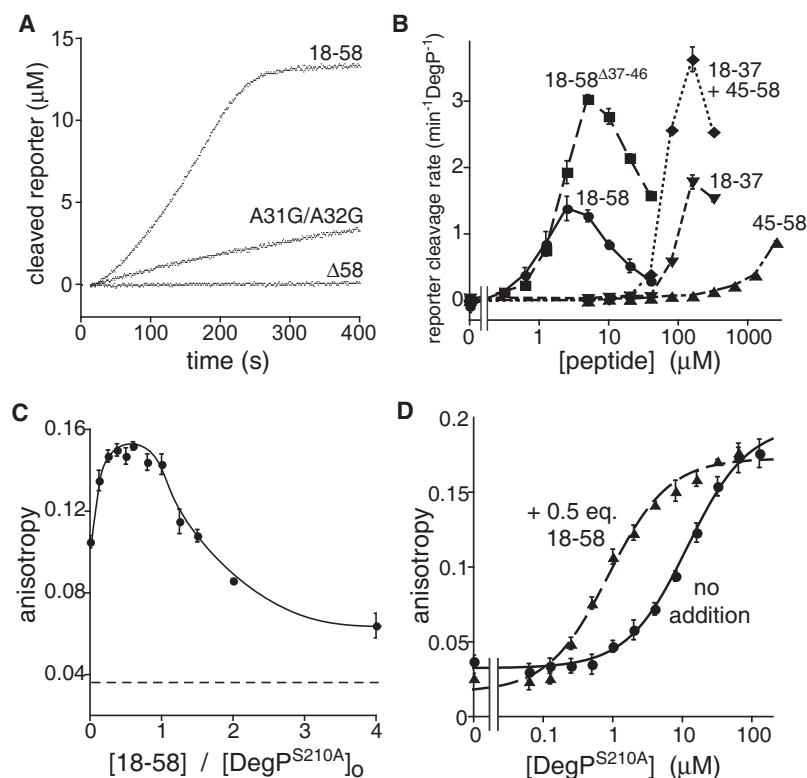
### Kinetics of Cage Assembly and Disassembly

To monitor the kinetics of assembly/disassembly of DegP cages during proteolysis, we prepared a mixture of donor-labeled and acceptor-labeled proteolytically active DegP\* (500 nM each) and initiated assembly by addition of 5, 10, or 20  $\mu\text{M}$  concentrations of 18–58 (Figure 4C; left panel). In each case, the FRET signal increased over the course of ~1 min, remained relatively constant for a time period (5–15 min) that increased for higher substrate concentrations, and then decreased to the initial level. In a parallel experiment (Figure 4C; right panel), we monitored the kinetics of 18–58 degradation by SDS-PAGE, revealing that disassembly occurred when substrate degradation was largely complete.

The initial phases of assembly, monitored by FRET, fit well to exponential functions with shorter half-lives at each increasing 18–58 concentration (Figure 4D). Averages ( $\pm$ SD) of replicate experiments gave assembly half-lives of  $10.8 \pm 1.1$  s (20  $\mu\text{M}$ ),  $13.5 \pm 0.7$  s (10  $\mu\text{M}$ ), and  $19.8 \pm 1.9$  s (5  $\mu\text{M}$ ). The average assembly rate constants fit well to a hyperbolic function (Figure 4D, inset), suggesting that substrate binding is rate limiting for assembly at low concentrations, whereas another step limits this rate at saturating substrate.

### Cages Are Stable until Substrate Is Depleted

Two models could explain our assembly/disassembly results. Dodecamers and larger cages could form upon initial 18–58 binding and then remain stably associated until most substrate molecules were cleaved. By this model, cleavage products would need to be released from the proteolytic cage, and new substrates would need to enter the cage chamber for



**Figure 3. Linked Degrons Promote Efficient Activation and Positively Cooperative Binding**

(A) Wild-type cleavage-site and PDZ1-binding degrons are required for efficient activation. Compared to 18–58 (5  $\mu\text{M}$ ), the same concentration of variants with mutations in the cleavage-site degron (A31G/A32G) or PDZ1-binding degron ( $\Delta 58$ ) activated DegP (1  $\mu\text{M}$ ) cleavage of reporter (100  $\mu\text{M}$ ) poorly. Figure S3A shows DegP degradation of the A31G/A32G and  $\Delta 58$  variants.

(B) Peptides carrying the cleavage-site degron (18–37) and PDZ1-binding degron (45–58) show synergistic activation of DegP (1  $\mu\text{M}$ ) cleavage of reporter (100  $\mu\text{M}$ ), but only at substantially higher concentrations than observed for intact 18–58 or 18–58 $\Delta 37-46$ . Error bars represent averages  $\pm 1$  SD from three independent experiments. Also see Figures S3B and S3C.

(C) As assayed by fluorescence anisotropy, binding of DegP<sup>S210A</sup> (10  $\mu\text{M}$ ) to <sup>125</sup>I-18–58 (50 nM) was initially enhanced and then inhibited by addition of unlabeled 18–58, suggesting that binding is positively cooperative. The dashed line shows the anisotropy of <sup>125</sup>I-18–58 alone. Error bars represent averages  $\pm 1$  SD from three independent experiments.

(D) As expected for a positively cooperative system, DegP<sup>S210A</sup> bound <sup>125</sup>I-18–58 (50 nM) more tightly in the presence ( $\blacktriangle$ ;  $K_{\text{app}} = 0.88$   $\mu\text{M}$ ) than absence ( $\bullet$ ;  $K_{\text{app}} = 12$   $\mu\text{M}$ ) of unlabeled 18–58 added at half the DegP<sup>S210A</sup> concentration. Error bars represent averages  $\pm 1$  SD from three independent experiments.

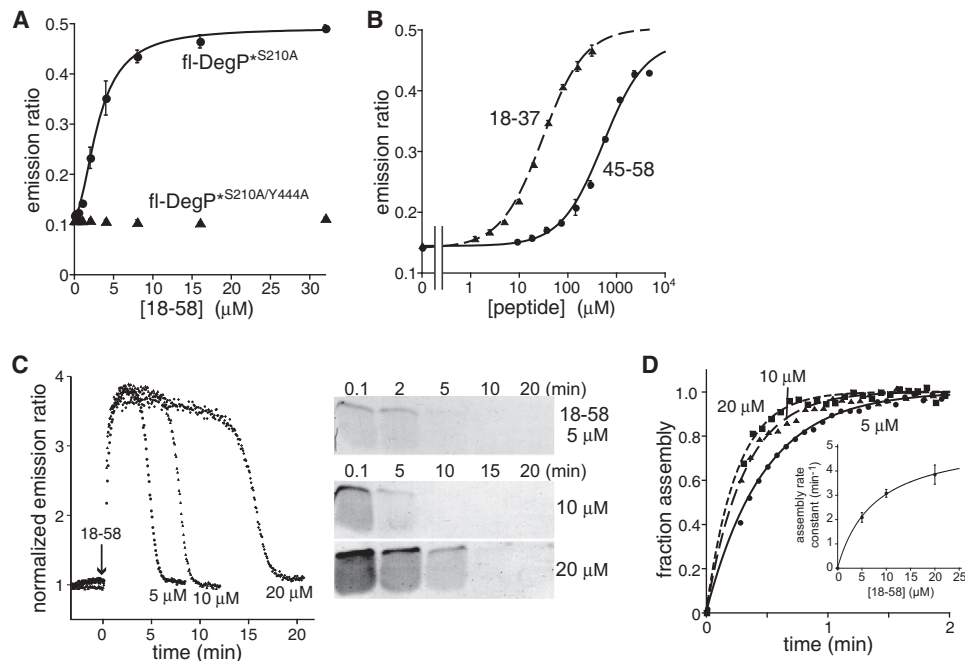
degradation. Alternatively, dodecamers and larger cages could form, degrade the initially encapsulated substrates, dissociate, release the proteolytic products, and then rebind new 18–58 substrates and reform cages. In this model, the FRET levels observed during degradation would represent a population-weighted average determined by the fraction of time spent in each oligomeric conformation.

Two experiments were performed to distinguish between these models. In the first, we incubated 18–58 (10  $\mu\text{M}$ ) separately with either donor-labeled or acceptor-labeled DegP\* (500 nM), waited  $\sim 3$  min (a time sufficient to form cages but not to deplete substrate), and then mixed both samples and monitored FRET (Figure 5A). The continuous assembly/disassembly model predicts an immediate increase in FRET, whereas the stable-cage model predicts little increase in FRET. As shown in the upper panel in Figure 5A, only a small increase in FRET was observed in the  $\sim 8$  min following mixing. Notably, however, a large FRET increase was observed when additional 18–58 was added after this period, when most of the original substrate had been degraded. The lower panel in Figure 5A shows the same experiment except the second batch of 18–58 was added  $\sim 3$  min after mixing, when cages were still assembled because some original substrate was still present. Importantly, no substantial FRET increase was observed under these conditions. In a second experiment, we mixed donor- and acceptor-labeled DegP\* (500 nM each) with 18–58 (10  $\mu\text{M}$ ) to facilitate assembly, and then added unlabeled 5  $\mu\text{M}$  DegP<sup>S210A</sup> (Figure 5B). The continuous assembly/disassembly model predicts

a rapid decrease in FRET, which was not observed. By contrast, preincubation of donor- and acceptor-labeled DegP\* with unlabeled DegP<sup>S210A</sup> resulted in no significant FRET increase upon subsequent addition of substrate (Figure 5B). In combination, these results are inconsistent with a model in which DegP cages assemble and disassemble continually and provide strong support for a model in which dodecamers and larger cages, stabilized by the binding of multiple substrate molecules, remain assembled and active in degradation until bulk substrate is depleted and disassembly occurs (Figure 5C).

## DISCUSSION

Our results demonstrate that a model unstructured polypeptide with a single cleavage-site degron and PDZ1-binding degron is a good activator of DegP proteolysis, a good substrate for DegP cleavage, and a good promoter of the association of inactive hexamers to form dodecamers and larger cages. Importantly, at low concentrations, all of these properties require the cleavage-site degron, the PDZ1-binding degron, and covalent linkage of both elements. Peptides containing just one or the other degron mimicked these activities but only at substantially higher concentrations. Thus, binding of low concentrations of substrate drives assembly of active DegP cages and substrate cleavage depletes the activating signal, resulting in disassembly and a return to the inactive state (Figure 5C). This mechanism provides an elegant solution to the problem of regulation, as the energy of substrate binding is used to construct the



**Figure 4. Efficient DegP Cage Assembly Requires Linked Degrons and Is Reversed upon Cleavage**

(A) To monitor dodecamer assembly, donor and acceptor labeled DegP<sup>S210A</sup> (50 nM each) were mixed, increasing 18–58 was added, and the FRET ratio was measured. Data were fitted to the Hill equation ( $K_{app} = 3.1 \mu\text{M}$ ;  $n = 1.9$ ). Performing the same experiment with DegP<sup>S210A/Y444A</sup>, which does not form dodecamers or larger cages, showed no FRET increase upon 18–58 addition. Error bars represent averages  $\pm 1$  SD from three independent experiments. Also see Figure S4.

(B) The isolated cleavage-site degron (18–37;  $K_{app} = 31 \mu\text{M}$ ) or PDZ1-binding degron (45–58;  $K_{app} = 540 \mu\text{M}$ ) also supported cage assembly, but only at substantially higher concentrations than intact 18–58. Same conditions as (A), but curves were fitted to a hyperbolic isotherm. Error bars represent averages  $\pm 1$  SD from three independent experiments.

(C) Cages disassemble when substrate cleavage is complete. Donor and acceptor labeled proteolytically active DegP<sup>\*</sup> (500 nM each) were mixed, 18–58 was added at different concentrations to promote assembly, and FRET was monitored as a function of time. One representative set of reaction is shown in left panel. The time spent in the assembled cage state was roughly proportional to the initial 18–58 concentration. Samples were assayed for DegP cleavage of 18–58 by SDS-PAGE (right panel), showing that cage disassembly coincides with substrate depletion.

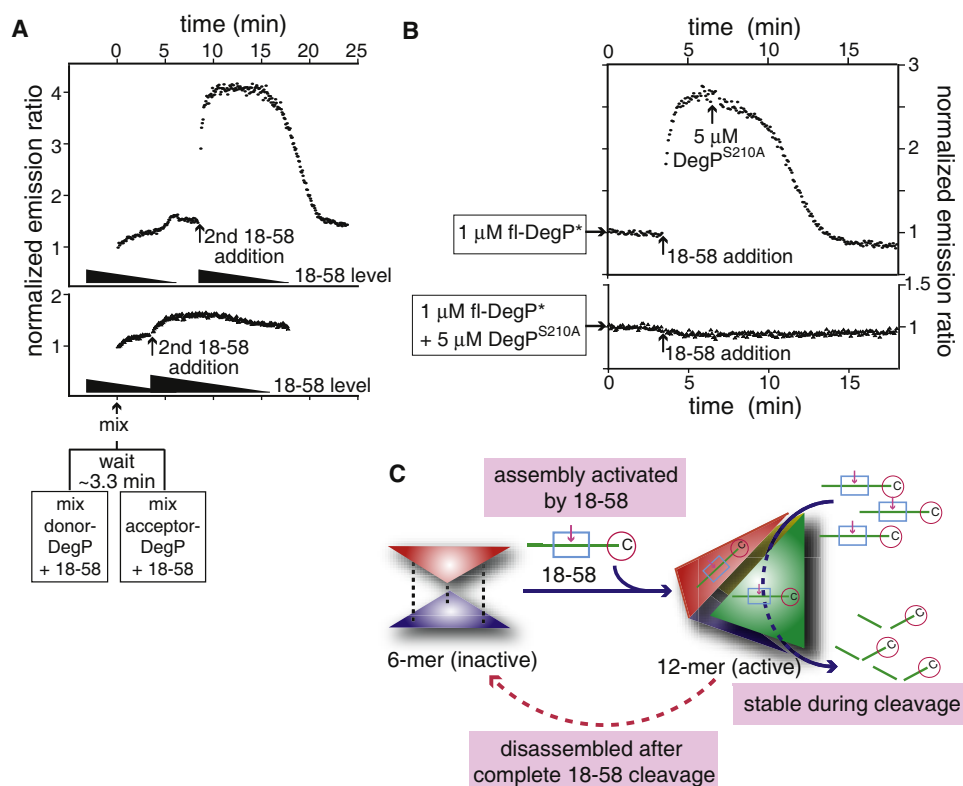
(D) Cage assembly occurs faster at higher 18–58 concentrations. Initial FRET increases in Figure 3C were normalized by dividing by the total amplitude and fitted to single-exponential functions. Half-lives from four independent measurements were  $10.8 \pm 1.1$  s (20  $\mu\text{M}$ ),  $13.5 \pm 0.7$  s (10  $\mu\text{M}$ ), and  $19.8 \pm 1.9$  s (5  $\mu\text{M}$ ). The inset shows a fit of average assembly rate constants to a hyperbolic equation ( $y = \max_a[18-58]/(K_{app} + [18-58])$ ;  $\max = 5.3 \pm 0.2 \text{ min}^{-1}$ ;  $K_{app} = 7.6 \pm 0.5 \mu\text{M}$ ;  $R = 0.9998$ ). Error bars represent averages  $\pm 1$  SD from four independent experiments.

proteolytic machine responsible for its degradation. Coupling of weak interactions to provide enhanced binding energy and specificity is a recurring theme in biological recognition, macromolecular folding and assembly, and allosteric systems (Creighton, 1983; Pabo and Sauer, 1992; Baker and Sauer, 2006; Kuriyan and Eisenberg, 2007). Tethering of one degron to its binding site in DegP increases the “effective concentration” of the second degron relative to its site, generating enough energy to drive assembly of active cages at low substrate concentrations.

Activation of DegP proteolysis/assembly by PDZ1-binding peptides has been reported, leading to the proposal that PDZ1 binding is an essential step in remodeling of the active site to allow proteolysis (Krojer et al., 2008a, 2010; Merdanovic et al., 2010). However, we find that a peptide with a cleavage-site degron but no PDZ1-binding degron can also activate DegP proteolysis and cage assembly. Thus, PDZ1 binding cannot be a mandatory component of activation. Moreover,

for the 18–58 substrate, the isolated cleavage-site degron is a more potent activator than the PDZ1-binding degron. Indeed, some DegP substrates may contain multiple cleavage-site degrons but no PDZ1-binding degron. For substrates of this type, the coupled binding of different cleavage-site degrons to different active sites in a DegP multimer could drive activation/assembly at low substrate concentrations. Subsequent cleavage would uncouple and destroy some degrons, making the active protease less stable, but would also generate cleavage intermediates with C-terminal PDZ1-binding degrons. Binding of these intermediates could then maintain the stability of the active cage until most cleavage sites present in the initial substrate had been proteolyzed. Indeed, our kinetic experiments demonstrate that DegP cages remain stably associated and proteolytically active, as long as sufficient uncleaved 18–58 substrate is available. By eliminating the need to disassemble and reassemble cages continuously, this mechanism would speed bulk degradation but also require products to





**Figure 5. DegP Cages Remain Stably Assembled until Substrate Is Depleted**

(A) To promote cage assembly, donor and acceptor labeled DegP\* (500 nM each) were incubated in separate tubes with 18–58 (10  $\mu$ M). When these samples were mixed, no substantial FRET increase occurred, demonstrating that substrate-engaged dodecamers do not dissociate and reform rapidly. A large FRET increase, indicating mixing of the two populations, was observed when a second addition of 10  $\mu$ M 18–58 was made after a time ( $\sim$ 8 min) sufficient to deplete the original substrate (top panel). By contrast, when the second addition of 10  $\mu$ M substrate was made before depletion of the initial substrate ( $\sim$ 3 min), only a modest increase in FRET was observed (bottom panel).

(B) In the top panel, donor- and acceptor-labeled DegP\* (0.5  $\mu$ M each) were mixed and 18–58 (10  $\mu$ M) was added after 3.3 min, resulting in cage assembly as assayed by FRET. Subsequent addition of unlabeled DegP<sup>S210A</sup> (5  $\mu$ M) at 6.6 min resulted in little change in FRET until substrate cleavage was complete (see Figure 4C). In the bottom panel, donor- and acceptor-labeled DegP\* (0.5  $\mu$ M each) were mixed with unlabeled DegP<sup>S210A</sup> (5  $\mu$ M), allowed to incubate for 3.3 min, and 18–58 (10  $\mu$ M) was added. In this experiment, no significant FRET increase was observed, suggesting that very few cages contained both an acceptor-labeled DegP\* hexamer and a donor-labeled DegP\* hexamer.

(C) Cartoon model. The coupled binding energies of distinct degrons in multiple substrate molecules promote assembly of proteolytically active dodecamers and larger cages from inactive hexamers. After cleavage of initially bound substrates, products leave and new substrates enter the cage chamber. This cycle maintains cage stability as long as uncleaved substrate at a sufficiently high concentration is available. Once substrate falls below this level, the cage dissociates into inactive hexamers.

exit and new substrates to enter the proteolytic cage, presumably through vertex and/or edge apertures similar to those observed in the dodecamer and icosatetramer structures. These apertures would exclude denatured proteins bound to chaperones and disordered segments or loops attached to otherwise native proteins, protecting these sequences from degradation.

The majority of final products of DegP cleavage will contain PDZ1-binding degrons but not cleavage-site degrons. Our results indicate that these molecules will be poor activators and poor competitors. By contrast, if cage assembly and activation were driven principally by binding to the PDZ1 domain as suggested (Krojer et al., 2010), then these final proteolytic products would keep DegP activated when substrates were absent and would also compete for binding of new substrates.

Many environmental stresses increase rates of protein unfolding and decrease rates of folding, causing an increase in the steady-state concentration of denatured polypeptides. We find that both binding to DegP and assembly of active cages is positively cooperative in substrate concentration. Thus, small increases in the concentration of unfolded substrates would be sufficient to trigger efficient assembly of proteolytically active DegP cages. Positive cooperativity is a hallmark of allosteric enzymes that operate by the classical MWC mechanism, equilibrating between a low-affinity inactive state that predominates in the absence of substrate or stabilizing effectors and a high-affinity active state that is stabilized by substrate/effector binding (Monod et al., 1965). For example, DegS, a close relative of DegP, is an MWC enzyme for which binding of substrate sequences to its protease domain and binding of effector

peptides to its PDZ domain synergistically stabilize the proteolytically active conformation (Sohn et al., 2007; Sohn and Sauer, 2009). The amino acids that regulate the transition between the active and inactive DegS are largely conserved in DegP, suggesting that regulation of both enzymes occurs by a conserved mechanism. However, the principal substrate of DegS does not contain a PDZ-binding degron and activation *in trans* by C-terminal sequences of unassembled OMPs is the biologically relevant mechanism of regulation of this stress-sensor protease (Walsh et al., 2003). Our results suggest that *trans* activation of DegP is also likely to occur in the cell, but that the best activators will be substrates that contain linked cleavage-site and PDZ1-binding degrons.

It would be deleterious if DegP efficiently degraded any unfolded protein. For example, most newly synthesized proteins enter the periplasm in a denatured state via secretion by the SecYEG system. Moreover, many native proteins unfold transiently and then refold. Our results suggest that denatured proteins that fold rapidly would be spared from DegP degradation by two factors. From an equilibrium perspective, rapid folding would keep the steady-state concentration of unfolded species low, and thus result in inefficient DegP binding and cage assembly. From a kinetic viewpoint, the assembly of DegP cages occurs rapidly enough to be biologically meaningful (~1 min) but slowly enough to produce a lag, which increases with decreasing substrate concentration. We propose that this lag may serve as a fail-safe mechanism, ensuring that transient binding of unfolded proteins to DegP does not result in assembly of active cages and degradation.

Trimers, stabilized by tight packing between protease domains, are the building blocks of higher DegP oligomers. In proteolytically active DegP dodecamers and icosatetramers, the protease domains assume the same functional conformation, the PDZ1 orientation with respect to the protease domain is relatively constant, trimers form the faces of a tetrahedron or octahedron, and conserved PDZ1-PDZ2' edge contacts between neighboring trimers stabilize the cage. Nevertheless, the crystallographic dodecamer is asymmetric, whereas the high-resolution EM dodecamer and crystallographic icosatetramer are symmetric (Jiang et al., 2008; Krojer et al., 2008b). However, symmetry was assumed in solving the EM structure, and DegP dodecamers and icosatetramers probably sample a wide range of solution structures. The irregularities in the crystallographic dodecamer arise from substantially different orientations of linked PDZ1 and PDZ2 domains, some of which resemble those in the EM dodecamer, whereas others resemble the crystallographic icosatetramer. This variation results in a broader range of aperture sizes, which might be important in facilitating substrate entry, in adjusting to variable degron spacing in the binding of diverse substrates, or in forming the 18 mers and 30 mers observed in our centrifugation experiments. There is evidence that larger substrates drive assembly of larger cages (Jiang et al., 2008; Krojer et al., 2008b), but degron number and/or spacing probably also play a role in determining cage size and substrate-binding stoichiometry.

In the cytoplasm, protein degradation is largely executed by AAA+ proteases, which employ an ATP-fueled hexameric ring

to unfold native proteins and to translocate the denatured polypeptide into the chamber of a barrel-shaped compartmental protease (Baker and Sauer, 2006). These compartmental proteases can assemble stably in the absence of the partner unfoldase and have almost no proteolytic activity by themselves, even against unfolded substrates. In the periplasm, there is no obvious way to forcibly denature proteins, and substrate unfolding prior to DegP degradation is presumably driven by high temperature or other stresses. These denatured substrates play an active role in promoting formation of proteolytically active DegP cages, which disassemble and return to an inactive state once substrate is depleted. Although the cytoplasmic and periplasmic degradation systems are very different, both rely on coupling of weak interactions to ensure specificity. Efficient degradation of many substrates by AAA+ proteases requires coupled recognition of two or more weak degrons, often assisted by adaptor proteins. Similarly, we find that coupling between weakly recognized degrons plays a key role in substrate-promoted activation and assembly of DegP cages and also in cleavage-mediated disassembly. The need to couple the binding energies of inherently weak degrons allows simple sequences to serve as individual degradation signals, allows facile evolutionary mixing and matching of degrons, and allows proteolysis to be regulated by controlling the way in which these signals are displayed in native proteins or unfolded polypeptides.

## EXPERIMENTAL PROCEDURES

Purification of peptides and proteins is described in the [Supplemental Information](#).

### Crystallography

DegP<sup>S210A</sup> was purified in 8 M urea, allowed to refold on a Ni<sup>2+</sup>-NTA column after washing with native buffer, eluted, concentrated, mixed with a 1.5 molar excess of 18–58, and chromatographed on a Superose-6 column (Figure 2A). This material was concentrated to 200 μM and crystallized by hanging drop vapor diffusion. Crystals grew at 18°C after 1:1 mixing with a well solution containing 65 mM citric acid, 35 mM Bis-Tris propane (pH 3.6), 8% PEG3350. Crystals were transferred for cryoprotection to a solution containing additional 7% PEG3350 and 25% PEG400, flash cooled in liquid nitrogen, and screened for diffraction on our home source. A data set from one crystal that diffracted to 3.76 Å resolution was collected at the NE-CAT 24-ID-C beamline of the Advanced Photon Source, Argonne National Laboratories (Table 1). Diffraction data were processed using HLK-2000 (Otwinski and Minor, 1997). Prior to using domains from the 3SC0 icosatetramer (Krojer et al., 2008b) in molecular replacement, we re-refined this structure to correct mediocre geometry (Table 1). The original 3SC0 structure contained a PDZ1-bound peptide, which apparently was a contaminant in the DegP<sup>S210A</sup> used for crystallization (Krojer et al., 2008b); there was also obvious electron density for a cleavage-site peptide, which we added in the re-refined structure. We then used the protease, PDZ1, and PDZ2 domains from the re-refined structure as search models for molecular replacement using PHASER (Storoni et al., 2004). This procedure allowed initial placement of all protease, PDZ1, and PDZ2 domains of DegP in the asymmetric unit of the new structure, and revealed electron density for the cleavage-site and PDZ1-binding degrons of the substrate. The model was improved by rigid-body refinement, positional refinement with NCS restraints, and tightly restrained individual B factor refinement using PHENIX (Adams et al., 2002), iterated with model building in COOT (Emsley and Cowtan, 2004). The final model had excellent refinement statistics and geometry (Table 1).

### Assays and Analysis

Activation, degradation, and assembly assays were performed at 23°C in 50 mM sodium phosphate (pH 8), 100 mM NaCl. DegP activation was monitored by increased fluorescence following cleavage of the reporter peptide (100 μM; excitation 320 nm; emission 420–430 nm) using a QM-2000-4SE spectrofluorimeter (Photon Technology International) or a SpectraMax M5 micro-plate reader (Molecular Devices). Fluorescence anisotropy was measured in the presence of 50 nM <sup>18</sup>C-18–58 (excitation 493 nm; emission 520 nm; cutoff filter 515 nm) using a micro-plate reader. FRET assays were performed with equimolar mixtures of donor- and acceptor-labeled DegP, using the spectrofluorimeter or micro-plate reader (excitation 520 nm; emission 570 or 670 nm). Error bars represent averages ±1 SD from three or four independent experiments.

DegP-cleavage sites in lysozyme were determined by LC/MS analysis (MIT Proteomics core facility) following cleavage of 45 μM CM-lysozyme by 3 μM DegP for 2 hr. The DegP-cleavage site in the 18–58 polypeptide was determined by HPLC isolation of the cleavage products and molecular-weight determination using an OminiFlex MALDI-TOF mass spectrometer (Bruker).

### ACCESSION NUMBERS

Coordinates have been deposited in the Protein Data Bank with accession codes 3OTP and 3OU0.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at [doi:10.1016/j.cell.2011.02.024](https://doi.org/10.1016/j.cell.2011.02.024).

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