A conserved activation cluster is required for allosteric communication in HtrA-family proteases

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Summary

In *E. coli*, outer-membrane stress causes a transcriptional response through a signaling cascade initiated by DegS cleavage of a transmembrane anti-sigma factor. Each subunit of DegS, an HtrA-family protease, contains a protease domain and a PDZ domain. The trimeric protease domain is autoinhibited by the unliganded PDZ domains. Allosteric activation requires binding of unassembled outer-membrane proteins (OMPs) to the PDZ domains and protein-substrate binding. Here, we identify a set of DegS residues that cluster together at subunit-subunit interfaces in the trimer, link the active sites and substrate-binding sites, and are crucial for stabilizing the active enzyme conformation in response to OMP signaling. These residues are conserved across the HtrA-protease family, including orthologs linked to human disease, supporting a common mechanism of allosteric activation. Indeed, mutation of residues at homologous positions in the DegP quality-control protease also eliminates allosteric activation.

INTRODUCTION

Allostery, in which binding of an effector molecule at one site alters activity at a distinct site, controls the biological activities of many enzymes, including proteases. In the periplasm of *Escherichia coli*, for example, heat and other environmental stresses activate the DegS protease, which is anchored to the inner membrane, and initiate an intramembrane proteolytic cascade that results in transcription of stress-response genes in the cytoplasm

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ACCESSION NUMBERS

The re-refined 1SOT, 1SOT, 1TE0, and 1VCW structures of DegS have been deposited in the Protein Data Bank with accession codes 4RQZ, 4RR1, 4RQY, and 4RR0, respectively.

(Ades, 2008). DegS is a trimeric HtrA-family protease, in which each subunit contains a trypsin-like protease domain and a regulatory PDZ domain (Fig. 1A). The trimer is stabilized by packing between the protease domains. The principal substrate of DegS is the periplasmic domain of RseA, a transmembrane protein with a cytoplasmic domain that binds and inhibits the σ^E transcription factor (Alba and Gross, 2004). Two stress-induced molecular signals are required for DegS cleavage of RseA *in vivo*. First, unassembled outer-membrane proteins (OMPs) accumulate in the periplasm during stress, and Tyr-Xxx-Phe (YxF) peptides at the C termini of these OMPs bind to the PDZ domains of DegS and stimulate proteolytic activity (Walsh et al., 2003). Second, lipopolysaccharides bind the RseB regulatory protein, releasing it from RseA, which allows cleavage of RseA at a single site by OMP-activated DegS (Lima et al., 2013). This initial cleavage event results in subsequent cleavage of RseA by other proteases, freeing σE to activate transcription of stress-response genes (Ades et al., 1999; Kanehara et al., 2002; Akiyama et al., 2004; Flynn et al., 2004; Chaba *et al*., 2007; Li et al., 2009).

In the absence of stress, DegS cleaves RseA at a low rate *in vivo* (Ades, 2008). Similarly, in the absence of OMP peptides *in vitro*, DegS cleaves the periplasmic domain of RseA >100-fold more slowly than in their presence (Walsh *et al*. 2003; Sohn *et al*., 2007; Sohn and Sauer, 2009). Crystal structures of DegS or DegS ^{PDZ} trimers show two distinct conformations, one inactive because of a malformed oxyanion hole and one with a functional His-Asp-Ser catalytic triad and oxyanion hole (Wilken *et al*., 2004; Zeth, 2004; Hasselblatt et al., 2007; Sohn et al., 2007; 2009; 2010). The PDZ domain has two major roles, keeping OMP-free DegS in an autoinhibited state (DegS ^{PDZ} has robust protease activity) and binding OMP peptide to allow activation. In structures of active DegS, the PDZ-bound OMP peptide is ~25 Å from the closest active site in the trimer. Allosteric activation over this distance is partially explained by remodeling of the protease-domain L3 loop, which is spatially adjacent to bound OMP peptide (Fig. 1A). This remodeling breaks autoinhibitory interactions between the L3 loop and the PDZ domain or other regions of DegS and allows formation of alternative contacts that appear to stabilize active DegS. Experiments using DegS enzymes with mixtures of full-length and PDZ subunits show that all subunits in the trimer experience a strongly coupled energetic landscape, with peptide binding to a single PDZ domain stimulating proteolytic cleavage both in the attached protease domain and in neighboring domains (Mauldin and Sauer, 2013).

Proteolytic activation of DegS largely conforms to a two-state model of MWC allostery, in which the preferential binding of RseA substrates and OMP peptides to active DegS provides ~2.2 and ~4.4 kcal/mol of stabilization, respectively, relative to the inactive enzyme (Fig. 1B; Monod *et al*., 1965; Sohn and Sauer, 2009). Thus, only ~0.3% of substrate-saturated DegS assumes the active conformation, whereas ~83% of substrate-saturated and OMP-saturated DegS is active. DegS ^{PDZ} still behaves as an allosteric enzyme with respect to RseA cleavage (Sohn and Sauer, 2009; Sohn *et al*., 2007; 2010), indicating that the protease-domain trimer still adopts inactive and active conformations and thus contains the basic machinery required for allostery. Interestingly, brief soaking of active DegS crystals to remove OMP peptide results in a transient state in which the protease domain remains in an "active" conformation but some autoinhibitory contacts between the

L3 loop and PDZ domain reform (Wilken et al., 2004). This hybrid state is metastable and assumes the inactive conformation after prolonged soaking. These results suggest that this hybrid conformation is unlikely to be substantially populated at equilibrium compared to inactive or active conformations, explaining why the two-state MWC model accounts well for proteolytic activation of DegS. The energetic basis for the relative stabilities of the active and inactive states is unclear, however, and dynamic factors and/or ensembles of states may play roles in the allosteric transition (Sohn et al., 2010; Motlagh et al., 2014).

Previous studies identified a handful of mutations that preferentially stabilize the inactive conformation compared to the active conformation of DegS or *vice versa*, and inspection of crystal structures suggested additional residues that may be involved in DegS allostery (Wilken *et al*., 2004; Hasselblatt et al., 2007; Sohn et al., 2007; 2010; Sohn and Sauer, 2009). However, there has been no comprehensive test of the importance of these residues in mediating the allosteric switch between DegS conformations. Here, we identify a critical set of amino acids needed for allosteric activation of DegS, providing a foundation for understanding regulation by substrate and OMP-peptide binding. These critical residues cluster at each subunit-subunit interface in the trimer and adjoin the active sites. Bound substrates would link these clusters and active sites around the trimer, providing a direct mechanism for allosteric activation by substrate. By contrast, bound OMP peptide appears to activate DegS indirectly by breaking autoinhibitory contacts. The cluster residues most important for DegS allostery are highly conserved within the HtrA-protease family, strongly suggesting that all of these enzymes function in similar fashions. Indeed, based on homology with DegS, we identify and characterize mutations that prevent allosteric activation of *E. coli* DegP, a trimeric HtrA quality-control protease that assembles into proteolytically active polyhedral cages in the presence of protein substrates (Jiang et al. 2008; Krojer et al., 2008; Kim et al., 2011). These results have broad implications, as mutant forms of human HtrA homologs have been linked to cancer, vascular disease, arthritis, macular degeneration, and neurodegenerative disease (Grau *et al*., 2005; Coleman *et al*., 2008; Milner *et al*., 2008; Vande Walle *et al*., 2008; Chien *et al*., 2009; Hara *et al*., 2009; Bowden *et al*., 2010).

RESULTS

Allosteric activation of DegS requires linkage between OMP-peptide binding to the PDZ domains, substrate binding to the protease domain, and the conformations of the active sites in each protease domain of the trimer (Sohn et al. 2007; Sohn and Sauer, 2009; Mauldin and Sauer, 2013). Based on interactions observed in DegS crystal structures and/or previous proposals, 16 side chains were selected for mutagenic analysis of allosteric activation (Table 1). Most of these amino acids lie between the binding sites for OMP peptide in the PDZ domains and the proteolytic active sites. In the sections below, we discuss evidence for or against these residues playing important roles in allosteric switching.

To monitor proteolytic activity *in vitro*, we purified wild-type or mutant variants of DegS without the membrane anchor and determined second-order rate constants (k_{cat}/K_M) for cleavage of sub-K_M concentrations of a ^{35}S-labeled protein fragment corresponding to the periplasmic domain of RseA in the presence of a high concentration of Tyr-Tyr-Phe (YYF) peptide (Sohn et al., 2007). The effects of different mutations were measured in otherwise

wild-type backgrounds and also in combination with the H198P mutation, which stabilizes active DegS (Sohn et al., 2009; 2010) and increased the proteolytic activity of otherwise wild-type DegS ~6-fold under our assay conditions (Fig. 2). The H198P mutation increased the relative proteolytic activities of some but not all mutations that diminish DegS activity (compare top and bottom panels in Fig. 2), allowing the relative importance of different mutations for proteolysis and allosteric switching to be determined.

Residues that Play Major Roles in Allosteric Switching

The side chains of Pro[161], Tyr[162], Leu[164], Thr[167], Thr[169], Gln[191], Asn[197], Ile[232], and Phe[234] in DegS play very important roles in allosteric switching by the following criteria. The P161A, Y162A, L164A, T167V, T169A, Q191A, N197A, I232A, and F234A mutants had less than 2% of YYF-stimulated wild-type cleavage activity, and combining these mutations with the H198P mutation did not rescue cleavage activity appreciably (Fig. 2; Table 1). The Arg[178] and Phe[220] side chains of DegS play somewhat important roles, as the R178A and F220A mutants had almost no cleavage activity, but the R178A/H198P and F220A/H198P mutants had activities between 9–24% of the parental enzyme. All of the mutant enzymes were soluble and well-behaved during purification, suggesting that the mutations had no substantial effects on folding or trimerization, and previously determined crystal structures of PDZ variants of Y162A, Y162A/H198P, T167V/H198P, and Q191A showed that these mutants form stable trimers with the protease domain in the proteolytically inactive conformation (Sohn et al., 2010).

By monitoring changes in the anisotropy of a fluorescein-labeled OMP peptide as a function of increasing concentrations of mutant DegS variants (Sohn et al., 2007), we determined binding affinities for the P161A, Y162A, L164A, T167V, T169A, R178A, Q191A, N197A, F220A, I232A, and F234A proteins in wild-type and H198P enzyme backgrounds. Each mutant bound OMP peptide with a K_D similar to or slightly stronger than wild-type DegS or DegS[H198P] (Table 1). Thus, the observed proteolytic defects of these mutants are not a consequence of an inability to bind OMP peptide. Rather, OMP-peptide binding to these mutants is not sufficient to shift most enzymes from the inactive to the active protease conformation.

Residues that Play Minor Roles or No Role in Allosteric Switching

Based on the crystal structures of active and inactive DegS, the side chains of Ile[179], Pro[183], Gln[187], Asp[221], and Glu[227] were proposed to play important roles in activation (Wilken et al., 2004; Hasselblatt et al., 2007). Both the P183A and E227A DegS mutants were previously found to have OMP-stimulated activities similar to or slightly higher than wild-type DegS (Sohn *et al.*, 2007), indicating that these side chains play no significant role in allosteric switching. Here, we found that I179A, Q187A, and D221A DegS displayed reduced YYF-stimulated cleavage activity compared to wild-type DegS (Fig. 2), but I179A/H198P, Q187A/H198P, and D221A/H198P DegS had YYF-stimulated cleavage activities that were 65% or more of the H198P parent (Fig. 2; Table 1). Thus, the Ile[179], Gln[187], and Asp[221] side chains play minor roles in stabilizing active compared to inactive DegS but are not critical determinants of allosteric activation.

Pro[229] and Glu[230] assume different conformations in active and inactive DegS structures (Sohn et al., 2010). We found that the P229A mutant was more active than wild-type DegS and H198P/P229A was more active than H198P (Fig. 2). Thus, the pyrrolidine ring of Pro[229] is not important for allosteric activation. The E230A mutant had ~10% of wild-type activity, whereas H198P/E230A had activity within error of H198P (Fig. 2). Hence, the Glu[230] side chain plays a minor role in DegS activation.

Structural Roles of Key Allosteric Residues

Prior to examination of the structural roles of DegS side chains that play important roles in allosteric switching, we sought to resolve apparent discrepancies among active and inactive crystal structures determined by different groups. For example, the side chains of Thr[167], Arg[178], and Gln[191] form a hydrogen-bond network in the all of the active DegS structures determined in our lab (Sohn et al., 2007; 2009; 2010), but a badly eclipsed rotamer of Thr[167] prevents hydrogen bonding in the 1SOZ structure determined by another group (Fig. 3A; Wilken et al., 2004; Hasselblatt et al., 2007). When we re-refined this structure, improving R factors and geometry (Table 2), the side chains of Thr[167], Arg[178], and Gln[191] formed a hydrogen-bonding network (Fig. 3B) similar to those in our previous DegS structures. We also re-refined several inactive or hybrid structures of DegS to improve geometry. As determined by MolProbity analysis (Chen et al., 2010), for example, the 1TE0 structure of inactive DegS (Zeth, 2004) had extremely poor geometry (23% poor rotamers; 7% Ramachandran outliers; 13% Cβ deviations; 3% bad backbone bonds; 3% bad backbone angles), suggesting a highly strained conformation or a poorly refined structure. By contrast, the 1SOT structure of full-length inactive DegS (Wilken *et al*., 2004) had somewhat better geometry (6% poor rotamers; 3% Ramachandran outliers; 0.4% Cβ deviations; 0.1% bad backbone bonds; 0.4% bad backbone angles), suggesting less strain or better refinement. The hybrid 1VCW structure of DegS after removal of bound OMP peptide by brief soaking (Wilken *et al*., 2004) also had geometric issues (8% poor rotamers; 4% Ramachandran outliers; 1% Cβ deviations; 0.2% bad backbone bonds; 0.8% bad backbone angles). Our re-refined 1TE0, 1SOT, and 1VCW structures had improved R factors and no poor rotamers, Ramachandran outliers, Cβ deviations, or bad backbone bonds or angles (Table 2). Thus, inactive or hybrid DegS does not assume an obviously strained conformation.

The residues we identified as playing the most important roles in allosteric activation cluster together at the subunit-subunit interfaces of the active conformation of the DegS trimer (Fig. 3C). The cluster is stabilized by hydrophobic packing between the side chains of Tyr[162], Leu[164], Phe[220], Ile[232], and Phe[234], and by hydrogen bonds involving side-chain atoms of Thr[167], Arg[178], and Gln[191] or main-chain atoms of Pro[161] Leu[164], Phe[220], and Ile[232]. OMP-peptide binding causes repositioning of the L3 loop, which includes Arg[178], on the outer edge of the cluster. Main-chain and side-chain atoms from Pro[161], Tyr[162], and Asn[197] on the inner edge of the cluster contact and stabilize the functional conformation of the active-site loop (residues 198–201) that forms the oxyanion hole and contains the catalytic Ser[201] (Fig. 4A). Importantly, these "activation clusters" are present in all crystal structures of DegS in the active conformation, including DegS with bound OMP peptides and DegS [PDZ] (Wilken *et al*., 2004; Zeth, 2004; Hasselblatt et al., 2007; Sohn et al., 2007; 2009; 2010). Moreover, activation clusters with very similar structural features are present in the functional

conformations of a DegS ortholog from *Mycobacterium tuberculosis* (2Z9I; Mohamedmohaideen et al., 2008) and in *E. coli* DegP (3OU0; 3OTP; Kroger et al., 2008; Kim et al., 2011).

In the inactive conformation of full-length DegS, represented by the re-refined 1TE0 structure, many residues of the activation cluster occupy substantially different positions than in the active conformation. For example, the side chains of Arg[178], Phe[220], and Tyr[162] move more than 10 Å, disrupting numerous packing and polar interactions. Indeed, all of the hydrogen bonds present in the functional activation cluster are missing in the inactive conformation. As a consequence of these structural rearrangements, the 198–201 active-site loop adopts an altered conformation which destroys the oxyanion hole. The R178A mutation has two effects. It destabilizes the activation cluster in active DegS by removing part of a hydrogen-bond network but also removes an autoinhibitory salt-bridge with Asp[320] in the unliganded PDZ domain in inactive DegS (Sohn et al., 2007), which is likely to ameliorate the severity of this mutation. Interestingly, after removal of OMP peptide from active DegS by brief crystal soaking (Wilken et al., 2004), the activation clusters remained intact, except for Arg[178], which again formed an autoinhibitory saltbridge with Asp[320] in the PDZ domain, and Phe[220], which became disordered. Like R178A, F220A was one of the least severe of the activation-cluster mutations (Fig. 2). By the principle of microscopic reversibility, the Arg[178] and Phe[220] side chains would be expected to be late additions to the activation cluster following OMP-peptide stimulation.

Parts of the RseA substrate are likely to interact directly with the activation cluster. For example, the carbonyl oxygen of the scissile peptide bond would bind in the oxyanion hole, which is mimicked by fluorophosphate modification of the active-site Ser[201] side chain in many structures (Sohn et al., 2007; 2009; 2010). The S5-S1 positions of RseA are also likely to interact with parts of the DegS activation cluster, based upon structures of orthologs with peptide fragments bound to the active site (Mohamedmohaideen et al., 2008; Kim et al., 2011). Indeed, some effects of the F220A mutation on RseA cleavage may arise from weaker substrate binding. As shown in Fig. 4B, part of a bound substrate is predicted to contact the active-site loop and adjacent activation cluster. Substrate could not bind to the distorted oxyanion hole of inactive DegS and additional substrate contacts may also be weakened in inactive DegS, explaining allosteric activation by preferential substrate binding to active DegS.

Fig. 4B shows that bound OMP peptides are not in close physical proximity to the activation clusters. Moreover, mutations in L3-loop residues that lie between bound OMP peptide and the activation cluster do not have substantial defects in proteolytic activity, supporting a model in which OMP binding serves only to relieve autoinhibitory interactions that stabilize the inactive state and does not play a role in directly stabilizing the active conformation of the protease domain (Sohn et al. 2007; 2010). This loss-of-inhibition model also explains why the conformations of the residues of the active site and activation cluster are almost identical in DegS [PDZ] and in OMP-peptide bound DegS.

Mutations that Block Allosteric Switching in DegP

The DegP protease differs from DegS in several ways. First, the allosteric transition from inactive to proteolytically active DegP trimers is driven by binding of one degron in a protein substrate to the active site and by binding of a second C-terminal degron in the same substrate to a tethering site in the DegP PDZ1 domain (Kim et al., 2011). Second, proteolytic activation is accompanied by assembly of DegP trimers into polyhedral cages with 12, 18, 24, or 30 subunits (Jiang et al., 2008; Kroger et al., 2008; Kim et al., 2011), although cage formation is not required for proteolytic activation (Kim and Sauer, 2012). To test if DegP uses a pathway of allosteric activation similar to DegS, we focused on DegP residues Thr[176], Arg[187], and Gln[200], which form a hydrogen-bond network in active DegP (Fig. 5A) but not in the inactive enzyme (Kroger et al., 2002; 2008; Kim et al., 2011). These residues correspond to Thr[167], Arg[178], and Gln[191] in DegS, and the networks formed by each set of residues in the active structures are similar (cf. Figs. 3B and 5A). If these DegP interactions are important for allosteric switching, then mutation of these residues should prevent or greatly diminish proteolytic activity, weaken substrate binding, and reduce or eliminate the positive cooperativity of substrate binding. To test these predictions, we made individual T176V, R187A, and Q200A mutations in DegP. Each mutation severely compromised substrate degradation. For example, purified DegP[T176V], DegP[R187A], and DegP[Q200A] exhibited no detectable degradation of lysozyme denatured by carboxymethylation of cysteines after 40 min, whereas wild-type DegP degraded most of this substrate within 5 min (Fig. 5B). To assay degradation activity more quantitatively, we used a fluorescent 23-residue peptide (p23) derived from lysozyme at a concentration ~20-fold above the wild-type K_M. DegP[Q200A] cleaved p23 at a rate much lower than wild-type DegP, whereas the rate of cleavage of p23 by DegP[T176V] and DegP[R187A] was negligible (Fig. 5C).

To assay substrate binding, we monitored the change in fluorescence anisotropy of a fluorescent peptide derived from residues 18–58 of lysozyme (lys[18–58]) upon addition of DegP variants. In one set of experiments, equilibrium binding curves and apparent affinities were determined after incubation of a low concentration of fluorescent lys[18–58] with different concentrations of DegP[S210A] (a proteolytically inactive variant missing the catalytic serine), DegP[S210A/T176V], DegP[S210A/R187A], or DegP[S210A/Q200A]. In a second set of experiments, binding mixtures were supplemented with non-fluorescent lys[18–58] at one-half of the enzyme concentration, which should result in stronger binding for variants that display positively cooperative substrate binding (Kim et al., 2011). Addition of unlabeled substrate strengthened the apparent affinity of DegP[S210A] for fluorescent lys[18–58] from ~12 μM to ~1 μM (Fig. 5D). By contrast, DegP[S210A/T176V], DegP[S210A/R187A], and DegP[S210A/Q200A] displayed comparable affinities (~30 μM) in the presence and absence of unlabeled substrate (Fig. 4D). Thus, the T176V, R187A, and Q200A mutations almost completely eliminate degradation, weaken substrate binding, and abolish positive cooperativity, as expected if these variants are largely trapped in the inactive state, even when substrate is bound.

Formation of DegP cages was monitored by fluorescence resonance energy transfer (FRET) between trimers labeled with donor dyes and trimers labeled with acceptor dyes as described

(Kim et al., 2011). Mixtures of acceptor and donor labeled trimers were prepared in the S210A, S210A/T176V, S210A/R187A, and S210A/Q200A backgrounds and FRET was assayed as a function of increasing quantities of lys[18–58] substrate (Fig. 5E). Notably, we observed substrate-dependent increases in FRET for the T176V, R187A, and Q200A variants, although compared to the S210A experiment substantially more lys[18–58] was required to drive an equivalent level of cage assembly by the variants. As expected, no change in FRET was observed for a variant bearing the Y444A mutation, which removes contacts required for cage formation (Kim et al., 2011). These results support a model in which the T176V, R187A, and Q200A variants assemble into cages in which the component trimers remain in the "inactive" conformation, albeit requiring higher substrate concentrations because of weaker and non-cooperative substrate binding.

DISCUSSION

Our results identify a set of amino acids that play critical roles in allosteric activation of the *E. coli* DegS protease. Mutation of these residues almost completely eliminates proteolytic activity in the presence of an OMP peptide that robustly activates substrate cleavage by wild-type DegS. From an energetic perspective, if a single mutation in one activation cluster destabilizes active DegS by ~1–2 kcal/mol relative to inactive DegS, then the active trimer would be destabilized by 3–6 kcal/mol and little or no activation by substrate and OMP-peptide binding would be expected. The activation clusters of DegS and DegP are very similar. Indeed, we found that mutation of some of these "activation cluster" residues in DegP eliminated or dramatically decreased proteolytic activity, as well as reducing the affinity and eliminating the positive cooperativity of substrate binding. Strikingly, the amino acids that are critical determinants of allosteric activation of DegS are also highly conserved in homologous proteases from all kingdoms of life (Fig. 6). Thus, we propose that a conserved mechanism of allosteric activation is a fundamental feature of this entire family of proteases, including human HtrA enzymes linked to a wide spectrum of diseases.

Why is allosteric activation of HtrA proteases so important? The activities of all intracellular proteases must be carefully regulated to allow them to degrade the proper target substrates but to avoid degrading proteins required for cellular function. By linking proteolytic activation to assembly of specific enzyme-substrate or enzyme-substrate-activator complexes, these potentially dangerous enzymes can be maintained in an inactive state until the appropriate substrates and/or activators are present. Moreover, because allosteric activation is reversible, these proteases switch back to inactive conformations once protein substrates are degraded or activators are removed. Cage formation provides a second level of cellular protection for many HtrA proteases, as activation leads to assembly of complexes in which the proteolytic active sites are sequestered and only accessible to unfolded proteins that can enter the degradation chamber. Indeed, recent studies show that DegP mutations that prevent cage formation and facilitate allosteric activation result in rogue proteolysis that kills cells (Kim and Sauer, 2014).

For DegS, an unresolved question is how OMP-peptide binding to the PDZ domains transmits a signal that results in remodeling the protease domain into an active conformation. An early model suggested that contacts between residues of the OMP peptide

and the L3 loop of DegS directly stabilized the active conformation (Wilken et al., 2004). This model, however, is inconsistent with numerous results, including the robust activity of DegS ^{PDZ} and crystal structures of OMP-bound active DegS that show no conserved contacts between the OMP peptide and the L3 loop (Sohn et al., 2007; 2010). Nevertheless, OMP-peptide binding is a key step in activation of full-length DegS and thus these binding events must either destabilize inactive DegS or stabilize active DegS by some mechanism. One possibility is that OMP-peptide binding causes conformational changes in the PDZ domain that then result in clashes with the L3 loop, initiating the conformational change in the protease domains (Sohn and Sauer, 2009). OMP-peptide binding to a single PDZ domain stimulates proteolytic activation of both the attached and neighboring protease domains in the DegS trimer (Mauldin and Sauer, 2013). The clusters of DegS residues that mediate allosteric switching are located at subunit-subunit interfaces in the protease domain, providing a simple mechanism by which binding of one OMP peptide could change the conformations of all subunits in the DegS trimer.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

Wild-type and mutant variants of *E. coli* DegS (residues 27–355) with an N-terminal His$_6$ tag replacing the wild-type membrane anchor and an ^{35}S-labeled variant of the periplasmic domain of *E. coli* RseA (residues 121–216) with a C-terminal His$_6$ tag were expressed in *E. coli* strain X90 from IPTG-inducible plasmids. Cultures were collected by centrifugation, resuspended in lysis buffer (50 mM sodium phosphate [pH 8], 300 mM NaCl, 10 mM imidazole), and lysed by sonication. Cleared lysates were applied to a pre-equilibrated Ni^{++}-NTA (Qiagen) affinity column, washed with lysis buffer, and eluted in elution buffer (50 mM sodium phosphate [pH 8], 300 mM NaCl, 300 mM imidazole) as described (Walsh et al., 2003; Sohn et al., 2007; Cezairliyan and Sauer, 2009). Proteins were then dialyzed against two changes of storage buffer (50 mM sodium phosphate [pH 8], 200 mM NaCl, 1 mM EDTA, 10% glycerol) before being stored at −80 °C. YYF peptide was synthesized by GenScript and purified by reverse-phase high-pressure liquid chromatography on a C$_{18}$ column. His$_6$-tagged variants of DegP were expressed in *E. coli* strain X90(DE3) bearing a deletion of the chromosomal *degP* gene, cells were lysed by sonication, and proteins were purified by Ni^{++}-NTA affinity chromatography, concentrated by Amicon filtration (Millipore), and purified by size-exclusion chromatography on a Superose-6 column (GE Healthcare) as described (Kim et al., 2011). Protein concentrations were determined by UV absorbance using extinction coefficients calculated from the amino-acid sequence.

Enzymatic and Biochemical Assays

DegS cleavage of RseA and OMP-peptide binding assays were performed at room temperature in buffer containing 150 mM NaHPO$_4$ [pH 8.3], 380 mM NaCl, 10% glycerol, and 4 mM EDTA as described (Sohn et al., 2007; Cezairliyan and Sauer, 2009). Cleavage was performed by incubating DegS$_3$ or variants (1 μM) with or without YYF tripeptide (230 μM) and three different sub-K_M concentrations of ^{35}S-RseA for different times. Reactions were terminated by quenching in 10% trichloroacetic acid, and acid-soluble radioactive cleavage products were quantified by scintillation counting to calculate cleavage rates.

Initial cleavage rates normalized by total enzyme were plotted as a function of the RseA concentration, and the second-order rate constant was determined from the slope of a linear fit. Binding assays were performed by titrating increasing DegS or variants against a fixed concentration of the fluorescent OMP peptide fluorescein-DNRDGNVYYF and monitoring changes in fluorescence anisotropy (excitation 480 nm; emission 520 nm) after correction for protein scattering. Binding curves were fitted to a hyperbolic equation using Prism (GraphPad). Substrate cleavage by DegP, assayed by fluorescence or SDS-PAGE, binding of fluorescent substrate to proteolytically inactive DegP, assayed by fluorescence anisotropy, and substrate-mediated cage assembly, assayed by Förster resonance energy transfer, were performed at room temperature in 50 mM sodium phosphate (pH 8), 100 mM NaCl as described (Kim et al., 2011; Kim and Sauer, 2012).

Structural Refinement

Structure factors for 1SOT, 1SOT, 1TE0, and 1VCW were obtained from the Protein Data Bank. Re-refinement was performed using COOT (Emsley and Cowtan, 2004) for model building, MolProbity (Chen et al., 2010) for assessment of model geometry, and PHENIX (Adams et al., 2010) for refinement.

Sequence Alignment

The sequences for the following HtrA proteins (with associated accession numbers) were downloaded from UniProt: *E. coli* DegS (P0AEE3), *E. coli* DegP (P0C0V0), *E. coli* DegQ (P39099), *Synechocystis* HtrA (HTRA_SYNY3), *Synechocystis* HhoA (P72780), *Synechocystis* HhoB (P73940), *H. sapiens* HtrA1 (Q92743), *H. sapiens* HtrA2 (O43464), *H. sapiens* HtrA3 (P83110), *H. sapiens* HtrA4 (P83105), *D. rerio* HtrA1a (Q6GMI0), *A. thaliana* DegP1 (O22609), *D. melanogaster* HtrA2 (Q9VFJ3), *C. reinhardtii* DegP-type protease (A8HQB3) *B. dendrobatidis* putative uncharacterized protein (F4P0R9), *A. pseudotrichonymphae* DegQ (B6YQJ5) and *K. cryptofilum* Serine protease Do (B1L493). Sequences were aligned in ClustalW2 (Larkin *et al.*, 2007). Alignments at positions highlighted in this paper were collected and submitted to WebLogo (Crooks *et al.*, 2004) for visualization.

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References

Adams PD, et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr.* D. 2010; 66:213–221. [PubMed: 20124702]

Ades SE, Connolly LE, Alba BM, Gross CA. The *Escherichia coli* sigma(E)-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti-sigma factor. Genes Dev. 1999; 13:2449–2461. [PubMed: 10500101]

Ades SE. Regulation by destruction: design of the σ^E envelope stress response. Curr. Opin. Microbiol. 2008; 11:535–540. [PubMed: 18983936]

Akiyama Y, Kanehara K, Ito K. RseP (YaeL), an *Escherichia coli* RIP protease, cleaves transmembrane sequences. EMBO J. 2004; 23:4434–4442. [PubMed: 15496982]

Alba BM, Gross CA. Regulation of the *Escherichia coli* sigma-dependent envelope stress response. Mol. Microbiol. 2004; 52:613–619. [PubMed: 15101969]

Bowden MA, et al. High-temperature requirement factor A3 (Htra3): a novel serine protease and its potential role in ovarian function and ovarian cancers. Mol. Cell. Endocrinol. 2010; 327:13–18. [PubMed: 20540986]

Chaba R, Grigorova IL, Flynn JM, Baker TA, Gross CA. Design principles of the proteolytic cascade governing the sigmaE-mediated envelope stress response in *Escherichia coli*: keys to graded, buffered, and rapid signal transduction. Genes Dev. 2007; 21:124–136. [PubMed: 17210793]

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

Chien J, Campioni M, Shridhar V, Baldi A. HtrA serine proteases as potential therapeutic targets in cancer. Curr. Cancer Drug Targets. 2009; 9:451–468. [PubMed: 19519315]

Coleman HR, Chan CC, Ferris F, Chew EY. Age-related macular degeneration. Lancet. 2008; 372:1835–1845. [PubMed: 19027484]

Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: A sequence logo generator. Genome Research. 2004; 14:1188–1190. [PubMed: 15173120]

Flynn JF, Levchenko I, Sauer RT, Baker TA. Modulating substrate choice: the SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation. Genes Dev. 2004; 18:2292–2301. [PubMed: 15371343]

Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D. 2004; 60:2126–2132. [PubMed: 15572765]

Grau S, et al. Implications of the serine protease HtrA1 in amyloid precursor protein processing. Proc. Natl. Acad. Sci. USA. 2005; 102:6021–6026. [PubMed: 15855271]

Hara K, et al. Association of HTRA1 mutations and familial ischemic cerebral small-vessel disease. N. Engl. J. Med. 2009; 360:1729–1739. [PubMed: 19387015]

Hasselblatt H, Kurzbauer R, Wilken C, Krojer T, Sawa J, Kurt J, Kirk R, Hasenbein S, Ehrmann M, Clausen T. Regulation of the sigmaE stress response by DegS: how the PDZ domain keeps the protease inactive in the resting state and allows integration of different OMP-derived stress signals upon folding stress. Genes Dev. 2007; 21:2659–2670. [PubMed: 17938245]

Jiang J, Zhang X, Chen Y, Wu Y, Zhou ZH, Chang Z, Sui SF. Activation of DegP chaperone-protease via formation of large cage-like oligomers upon binding to substrate proteins. Proc. Natl. Acad. Sci. USA. 2008; 105:11939–11944. [PubMed: 18697939]

Kanehara K, Ito K, Akiyama Y. YaeL (EcfE) activates the sigma(E) pathway of stress response through a site-2 cleavage of anti-sigma(E), RseA. Genes Dev. 2002; 16:2147–2155. [PubMed: 12183368]

Kim S, Sauer RT. Cage assembly of DegP protease is not required for substrate-dependent regulation of proteolytic activity or high-temperature cell survival. Proc. Natl. Acad. Sci. USA. 2012; 109:7263–7268. [PubMed: 22529381]

Kim S, Sauer RT. Distinct regulatory mechanisms balance DegP proteolysis to maintain cellular fitness during heat stress. Genes Dev. 2014; 28:902–911. [PubMed: 24736846]

Kim S, Grant RA, Sauer RT. Covalent linkage of distinct substrate degrons controls assembly and disassembly of DegP proteolytic cages. Cell. 2011; 145:67–78. [PubMed: 21458668]

Krojer T, Garrido-Franco M, Huber R, Ehrmann M, Clausen T. Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine. Nature. 2002; 416:455–459. [PubMed: 11919638]

Krojer T, Sawa J, Schafer E, Saibil HR, Ehrmann M, Clausen T. Structural basis for the regulated protease and chaperone function of DegP. Nature. 2008; 453:885–890. [PubMed: 18496527]

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. ClustalW and ClustalX version 2. Bioinformatics. 2007; 23:2947–2948. [PubMed: 17846036]

Li X, Wang B, Feng L, Kang H, Qi Y, Wang J, Shi Y. Cleavage of RseA by RseP requires a carboxyl-terminal hydrophobic amino acid following DegS cleavage. Proc. Natl. Acad. Sci. USA. 2009; 106:14837–14842. [PubMed: 19706448]

Lima S, Guo MS, Chaba R, Gross CA, Sauer RT. Dual molecular signals mediate the bacterial response to outer-membrane stress. Science. 2013; 340:837–841. [PubMed: 23687042]

Mauldin RV, Sauer RT. Allosteric regulation of DegS protease subunits though a shared energy landscape. Nat. Chem. Biol. 2013; 9:90–96. [PubMed: 23201899]

Milner JM, Patel A, Rowan AD. Emerging roles of serine proteinases in tissue turnover in arthritis. Arthritis Rheum. 2008; 58:3644–3656. [PubMed: 19035508]

Mohamedmohaideen NN, Palaninathan SK, Morin PM, Williams BJ, Braunstein M, Tichy SE, Locker J, Russell DH, Jacobs WR, Sacchettini JC. Structure and function of the virulence-associated high-temperature requirement A of *Mycobacterium tuberculosis*. Biochemistry. 2008; 47:6092–6102. [PubMed: 18479146]

Monod J, Wyman J, Changeux JP. On the nature of allosteric transitions: A plausible model. J. Mol. Biol. 1965; 12:88–118. [PubMed: 14343300]

Motlagh HN, Wrabl JO, Li J, Hilser VJ. The ensemble nature of allostery. Nature. 2014; 508:331–319. [PubMed: 24740064]

Sohn J, Sauer RT. OMP peptides modulate the activity of DegS protease by differential binding to active and inactive conformations. Mol. Cell. 2009; 33:64–74. [PubMed: 19150428]

Sohn J, Grant RA, Sauer RT. Allosteric activation of DegS, a stress sensor PDZ protease. Cell. 2007; 131:572–583. [PubMed: 17981123]

Sohn J, Grant RA, Sauer RT. OMP peptides activate the DegS stress-sensor protease by a relief of inhibition mechanism. Structure. 2009; 17:1411–1421. [PubMed: 19836340]

Sohn J, Grant RA, Sauer RT. Allostery is an intrinsic property of the protease domain of DegS: implications for enzyme function and evolution. J. Biol. Chem. 2010; 403:420–429.

Vande Walle L, Lamkanfi M, Vandenabeele P. The mitochondrial serine protease HtrA2/Omi: an overview. Cell Death Differ. 2008; 15:453–460. [PubMed: 18174901]

Walsh NP, Alba BM, Bose B, Gross CA, Sauer RT. OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. Cell. 2003; 113:61–71. [PubMed: 12679035]

Wilken C, Kitzing K, Kurzbauer R, Ehrmann M, Clausen T. Crystal structure of the DegS stress sensor: How a PDZ domain recognizes misfolded protein and activates a protease. Cell. 2004; 117:483–494. [PubMed: 15137941]

Zeth K. Structural analysis of DegS, a stress sensor of the bacterial periplasm. FEBS Lett. 2004; 569:351–358. [PubMed: 15225661]

Highlights

- Residues critical for DegS-protease activation cluster at the trimer interfaces

- Activation-cluster residues adjoin the active sites but not OMP-peptide binding sites

- Activation-cluster residues are conserved throughout the HtrA-protease family

- Mutation of "cluster" residues in DegP prevents allosteric activation by substrates
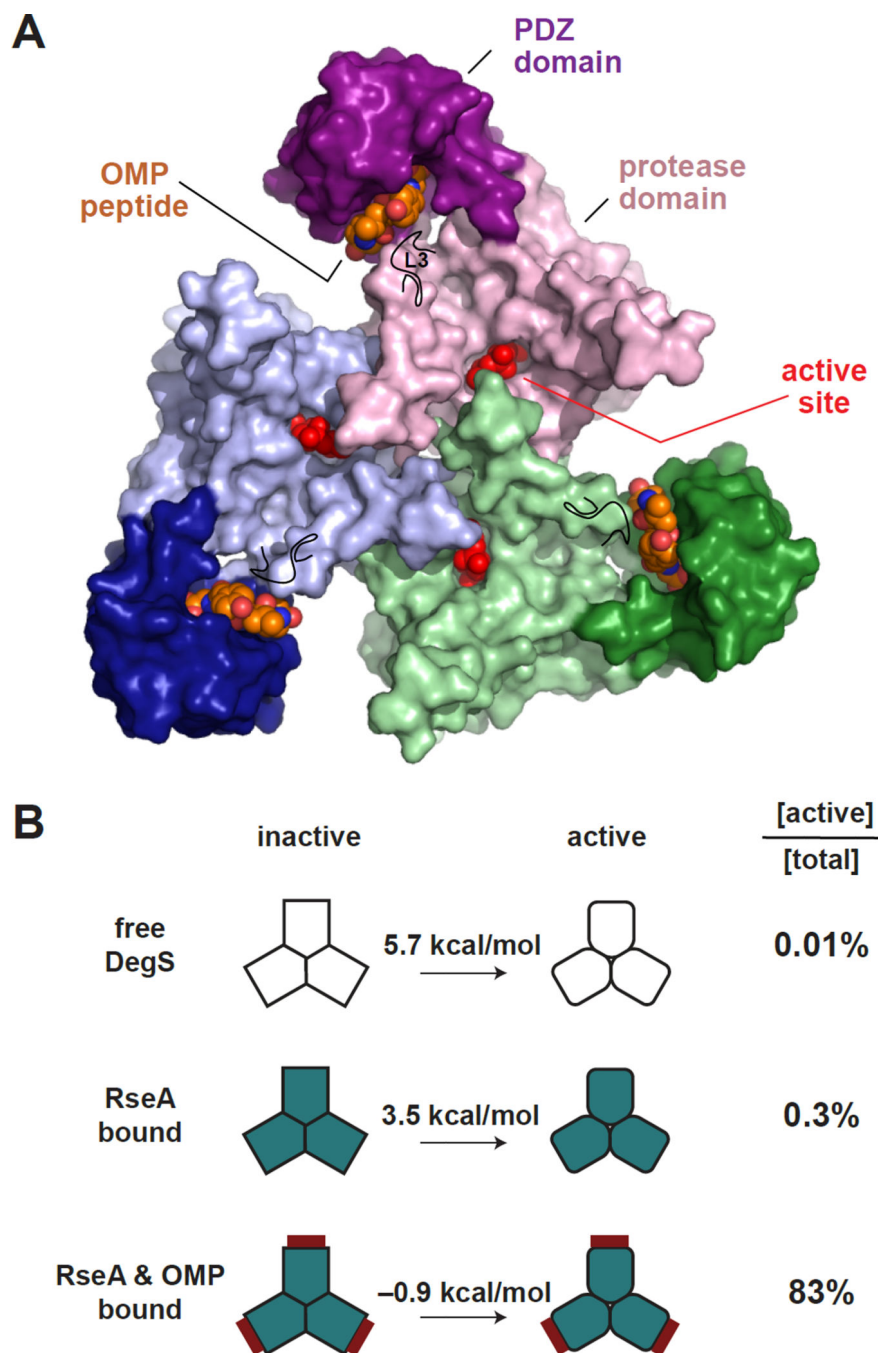
Figure 1. The DegS Trimer Equilibrates Between Inactive and Active Structures
(**A**) Structure of a DegS trimer in the active conformation with OMP peptides bound to the PDZ domains (pdb code 3GDS). Each DegS subunit (surface representation colored green, purple, or blue) consists of a protease domain (lighter colors), which pack together to form the trimer, and a peripheral PDZ domain (darker colors). Bound OMP peptides are shown in CPK representation with orange carbons. An isopropyl phosphate group covalently bound to the active-site serine is shown in CPK representation and colored red. The position of the L3 loop is marked by a black line.

(**B**) Free energies for conversion of inactive to active DegS in the free enzyme, the substrate-saturated enzyme, and the substrate and OMP-peptide saturated enzyme (Sohn and Sauer, 2009). These values would be decreased by ~4 kcal/mol for H198P DegS (Sohn et al., 2009; 2010). The column on the right lists the percentage of total DegS that assumes the active conformation for the unbound and ligand bound states.
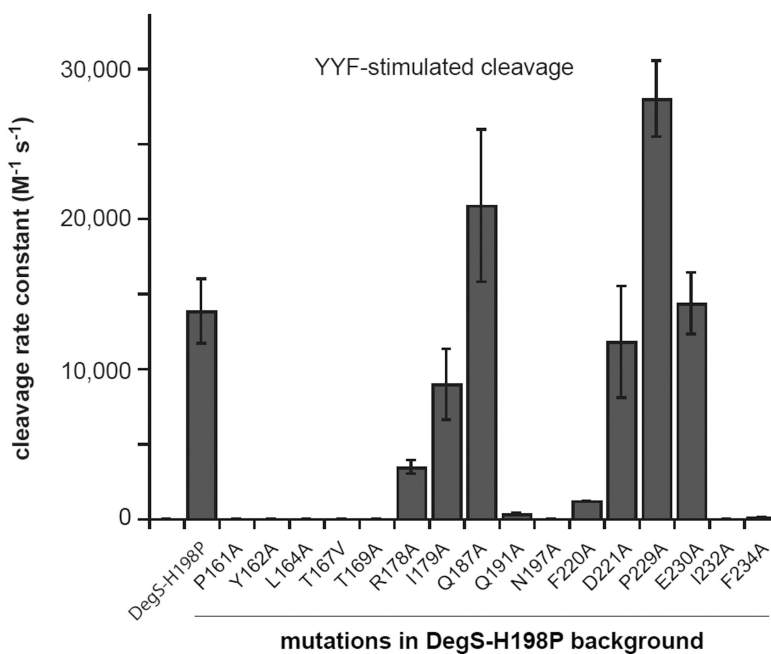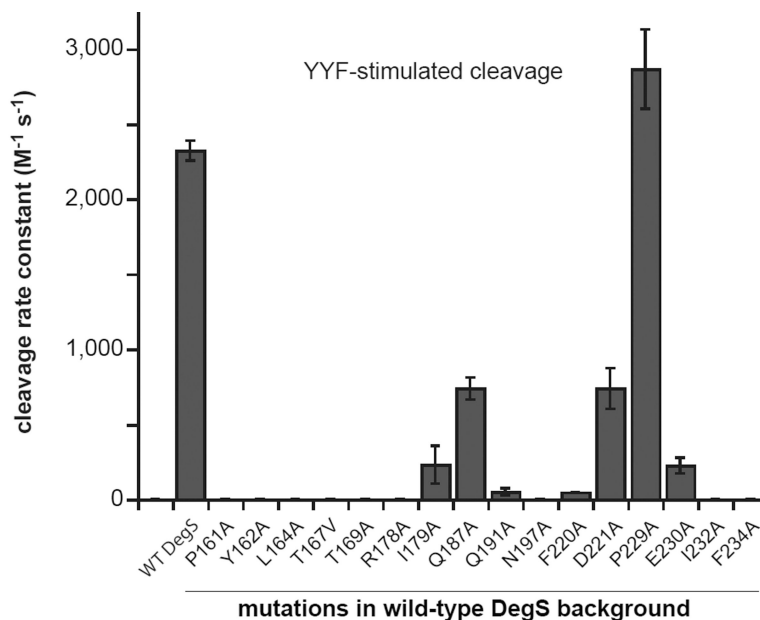
Figure 2. Mutational Effects on DegS Cleavage of RseA

Effects of DegS mutations in an otherwise wild-type background (upper panel) or in an H198P background (lower panel) on OMP-peptide stimulated proteolytic activity, expressed as the second-order rate constant (k_{cat}/K_M) for RseA cleavage. Values are averages of two or more independent trials ± SEM. Cleavage reactions contained different sub-K_M concentrations of ^{35}S-labelled RseA, DegS or variants (1 μM trimer), and YYF OMP peptide (230 μM). Initial cleavage rates normalized by total enzyme were plotted as a

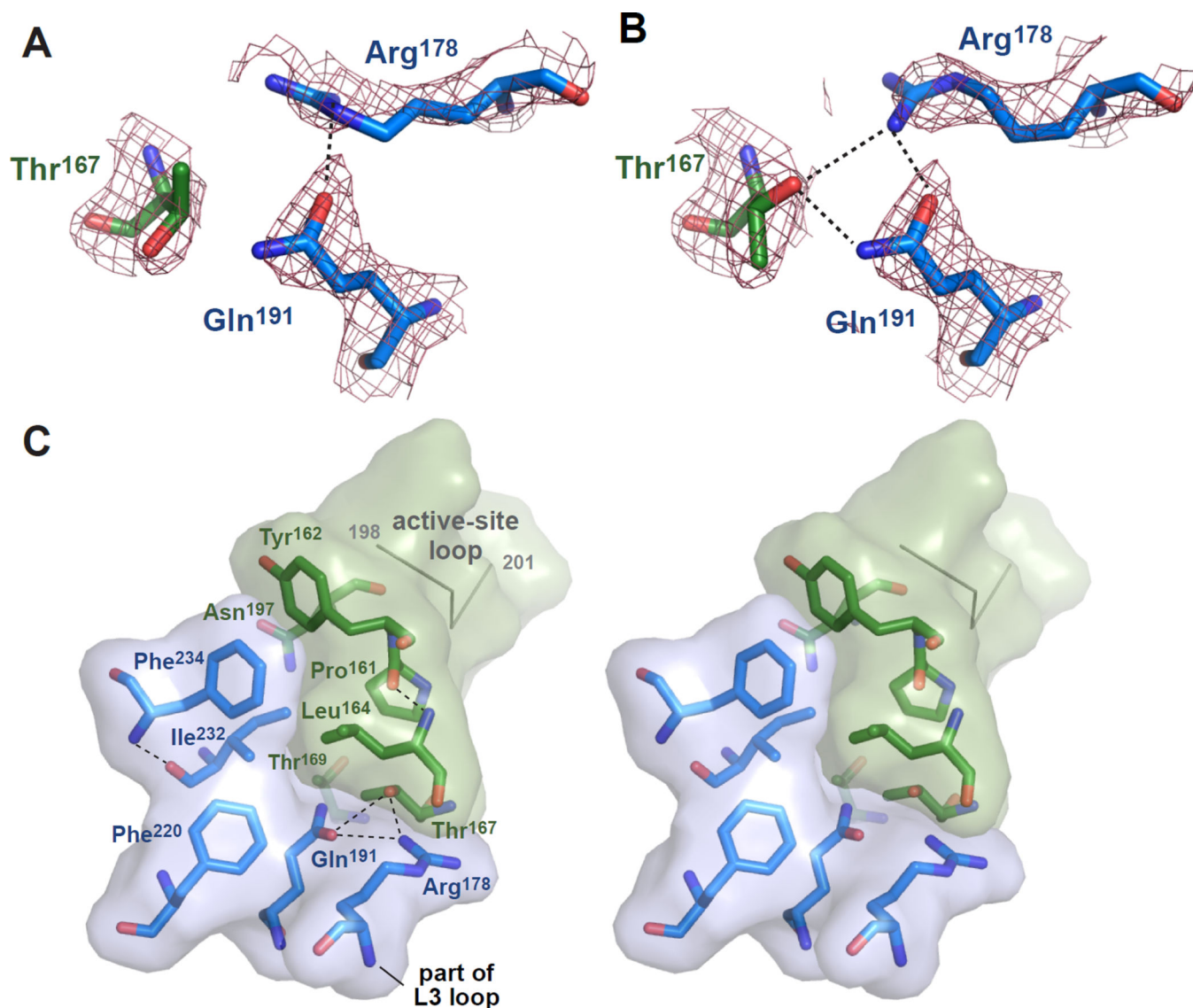function of the RseA concentration, and the second-order rate constant was determined from the slope of a linear fit.

Figure 3. Structural Determinants of DegS Activation

(**A**) Electron-density map (2F$_O$–F$_C$; contoured at 1.3 σ) for Thr[167] in chain B (dark green carbons) and Arg[178] and Gln[191] in chain C (marine carbons) in the 1SOZ DegS trimer. Thr[167] is modeled in an eclipsed conformation (χ 116°) found in only 0.1% of high-resolution structures. A hydrogen bond is shown as a dashed black line.

(**B**) Electron-density map (same residues and parameters as panel A) but for the 4RQZ re-refined structure of 1SOZ. Thr[167] is modeled as a rotamer (χ 290°) found in 18.7% of high-resolution structures and forms hydrogen bonds (dashed black lines) with Arg[178] and Gln[191].

(**C**) Stereo view of residues identified as being very important or somewhat important for DegS allostery cluster between the active-site loop containing the catalytic Ser[201] and oxyanion hole (top right) and the L3 loop (bottom center). Residues with marine-colored carbons are from one subunit of the 4RQZ trimer and those with dark-green carbons are from another subunit; transparent surfaces are shown for both subunits to emphasize the
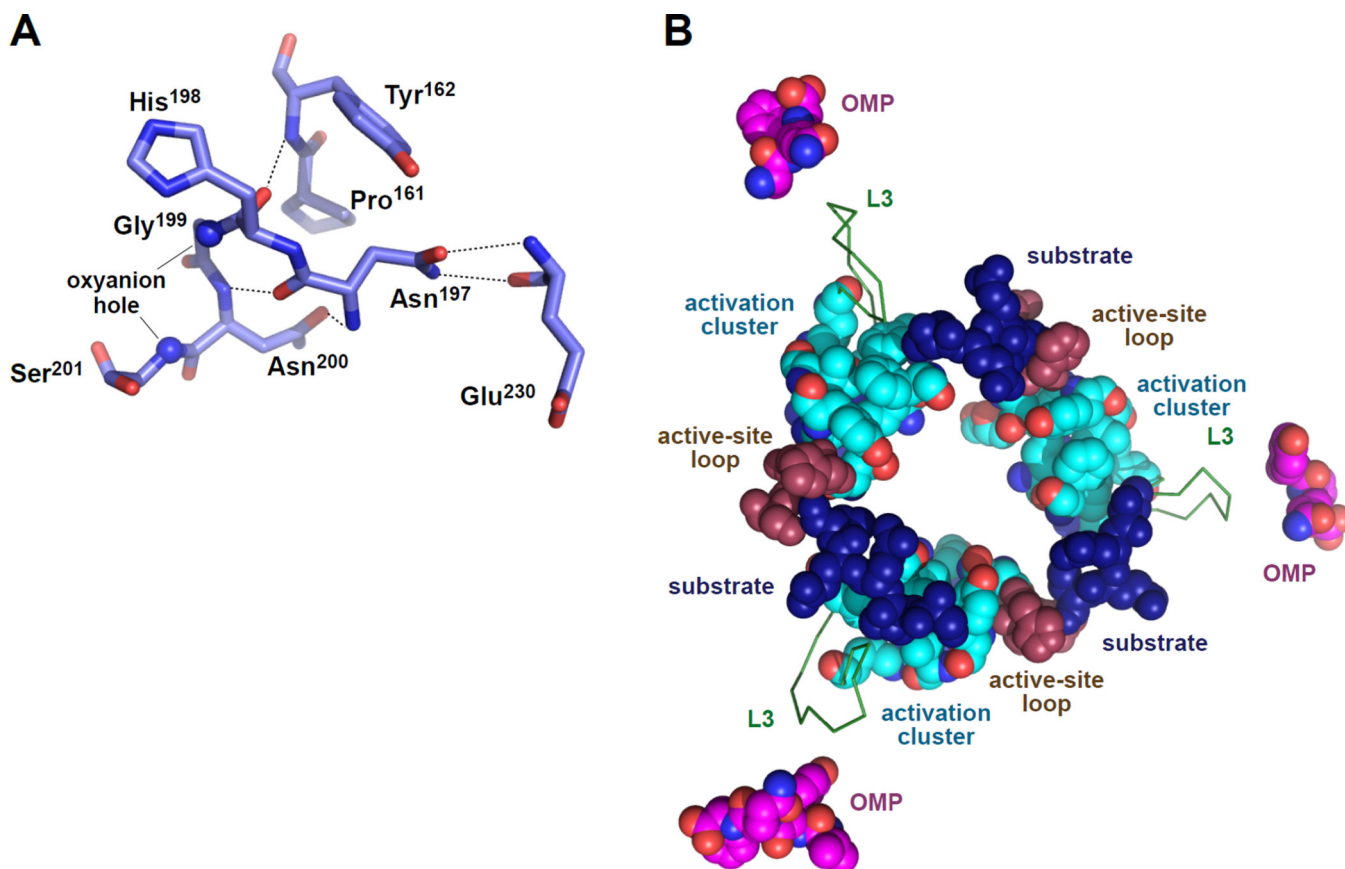
position of the interface. The residues shown represent one of the three activation clusters in the DegS trimer. Hydrogen bonds are represented as dashed black lines.

Figure 4. Allosteric Ligands and Catalytic and Regulatory Elements in Active DegS

(**A**) Main-chain and side-chain atoms from activation-cluster residues Pro[161], Tyr[162], and Asn[197] stabilize the functional conformation of the active-site loop, including the catalytic Ser[201] and the oxyanion-hole nitrogens (small spheres). Hydrogen bonds are shown as dashed lines.

(**B**) Positions of OMP peptides, L3 loops, activation clusters, active-site loops, and modeled substrate in an active DegS trimer (4RQZ). The position of substrate was modeled by aligning 4RQZ with a DegS ortholog from *Mycobacterium tuberculosis* with a bound substrate-cleavage product (2Z9I; Mohamedmohaideen et al., 2008). Although the PDZ-bound OMP peptides are adjacent to the L3 loops, there are no critical residues between the OMP peptide and the activation clusters, suggesting that OMP-peptide binding destabilizes autoinhibitory interactions rather than directly stabilizing the active conformation. Substrate binding ties together all of the active-site loops and activation clusters in the trimer, providing a mechanism for allosteric substrate stabilization of the active conformation.
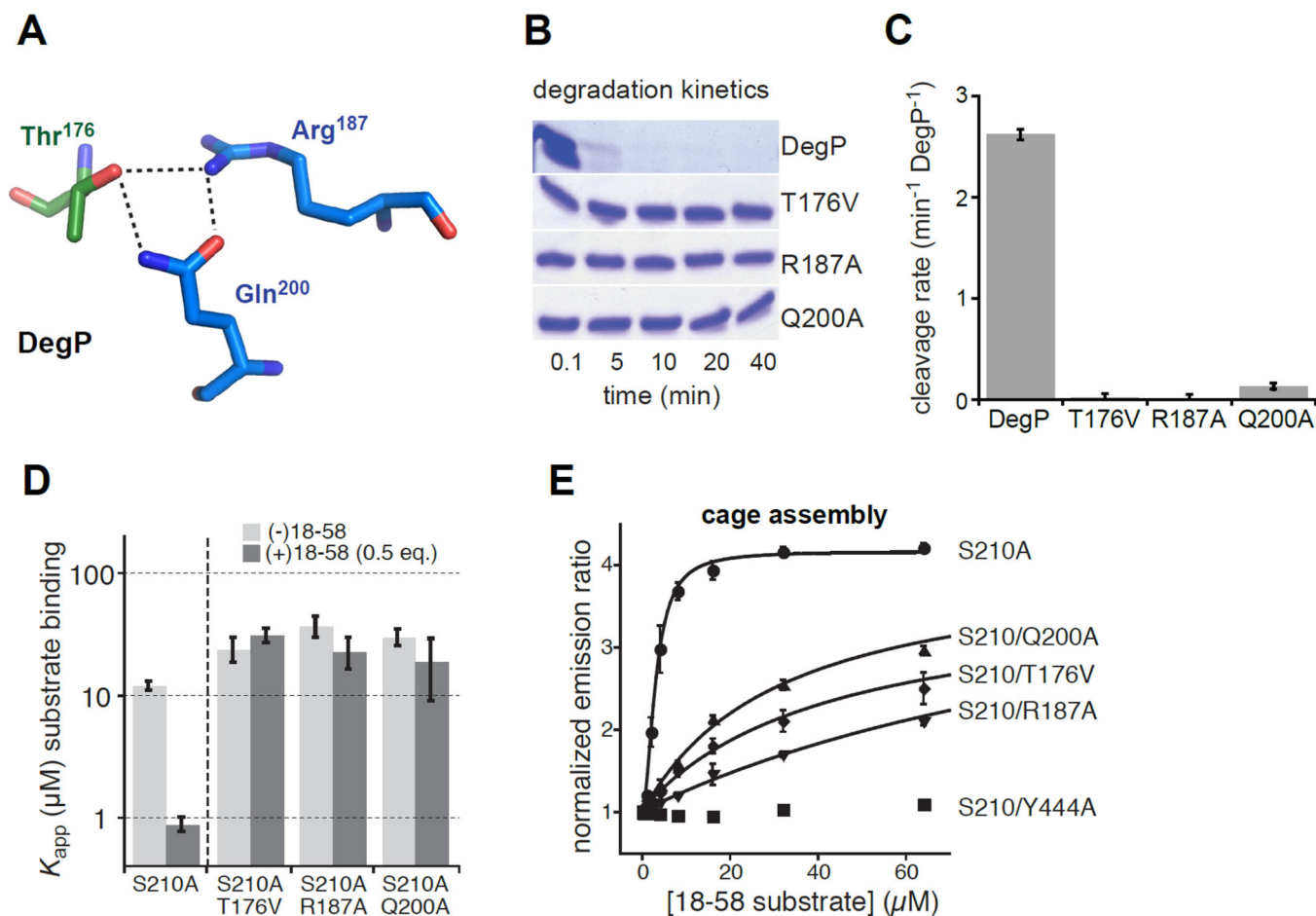
Figure 5. Effects of activation-cluster mutations on DegP activity and cage assembly

(**A**) The side chain of Thr[176] from one DegP subunit (dark-green carbons) forms hydrogen bonds with the side chains of Arg[187] and Gln[200] in an adjacent subunit (marine carbons) in the protease domain of an active DegP trimer (3OTP).

(**B**) Kinetics of degradation of carboxymethylated lysozyme (5 μM) by wild-type DegP and the T176V, R187A, or Q200A variants (1 μM) at room temperature was assayed by SDS-PAGE and staining with Coomassie Blue.

(**C**) Rates of cleavage of the p23 substrate (40 μM) by wild-type DegP or variants (1 μM) were determined at room temperature. Error bars are averages ±1 SD (N = 3).

(**D**) Apparent equilibrium dissociation constants (K_{app}) for binding of different concentrations of proteolytically inactive $DegP^{S210A}$ or variants to a fluorescent substrate ($^{flC}lys^{18-58}$; 50 nM) were determined in the absence or presence of unlabeled lys^{18-58} at one-half of the DegP concentration by changes in fluorescence anisotropy (Kim et al., 2011; Kim and Sauer, 2012). Positive cooperativity makes binding stronger in the presence of unlabeled lys^{18-58} for variants, like $DegP^{S210A}$, that can switch between the inactive and active conformations. The T167V, R187A, and Q200A mutations weakened binding and eliminated positive cooperativity. Bars represent the error of fitting of individual binding curves to a hyperbolic binding equation.

(**E**) Polyhedral-cage assembly of DegP variants as a function of added lys[18–58] substrate was monitored by increases in FRET between trimers labeled with donor and acceptor dyes (Kim et al., 2011). Emission ratios were normalized to data in the absence of substrate and fitted to a Hill equation ($y = y_0 + \text{max} \bullet [\text{lys}^{18-58}]^h/(K_{app}^h + [\text{lys}^{18-58}]^h)$) for DegP[S210A] or to a hyperbolic equation ($y = y_0 + \text{max} \bullet [\text{lys}^{18-58}]/(K_{app} + [\text{lys}^{18-58}])$) for variants containing the T176V, R187A, or Q200A mutations. No assembly of the DegP[S210A/Y444A] variant was observed, as expected because the Y444A mutation removes a critical interaction that stabilizes cages (Kim et al., 2011). Error bars are averages ±1 SD (N = 3).

Figure 6. Side Chains Important for DegS Allostery Are Conserved in the HtrA Family
Residues shown here to be very important (red labels) or somewhat important (orange labels) for allosteric activation of DegS are largely conserved in HtrA proteases from all kingdoms of life. Residues that play minor roles (blue labels) or no role (black label) in DegS activity are far less conserved. HtrA sequences from *E. coli* (3), *H. sapiens* (4), *Synechocystis* (3), *D. rerio*, *A. thaliana*, *C. reinhardtii*, *D. melanogaster*, *B. dendrobatidis*, *A. pseudotrichonymphae*, and *K. cryptofilum* were aligned using ClustalW2 (Larkin *et al.*, 2007) and submitted to WebLogo (Crooks *et al.*, 2004) for visualization of conservation information. A higher number of bits indicates greater conservation for a given position.

Table 1

Second-order rate constants for RseA cleavage and K_D's for OMP-peptide binding are listed for DegS and variants in otherwise wild-type (WT) backgrounds or in the H198P DegS (HP) background.

variant enzyme	activity WT background ($M^{-1}\,s^{-1}$)		activity HP background ($M^{-1}\,s^{-1}$)		K_D for OMP peptide (µM)	
	basal	with YYF	basal	with YYF	WT background	HP background
WT DegS	<4.5	2300 ± 60	540 ± 42	13900 ± 2100	4.0 ± 0.4	2.5 ± 0.3
P161A	<4.5	<4.5	<4.5	<4.5	1.5 ± 0.1	2.5 ± 0.4
Y162A	<4.5	<4.5	<4.5	<4.5	1.9 ± 0.4	n.d.
L164A	<4.5	<4.5	<4.5	<4.5	2.8 ± 0.6	2.5 ± 0.5
T167V	<4.5	<4.5	<4.5	<4.5	1.8 ± 0.1	1.9 ± 0.7
T169A	<4.5	<4.5	<4.5	<4.5	1.8 ± 0.1	2.0 ± 0.2
R178A	<4.5	<4.5	160 ± 28	3400 ± 470	3.6 ± 0.2	4.0 ± 0.1
I179A	<4.5	230 ± 130	260 ± 150	9000 ± 2400	2.3 ± 0.4	1.6 ± 0.1
Q187A	<4.5	740 ± 74	800 ± 330	21000 ± 5100	2.3 ± 0.1	1.5 ± 0.1
Q191A	<4.5	<4.5	<4.5	<4.5	3.4 ± 1.3	1.4 ± 0.1
N197A	<4.5	49 ± 24	<4.5	300 ± 68	1.4 ± 0.1	0.7 ± 0.1
F220A	<4.5	46 ± 1	50 ± 11	1200 ± 11	2.1 ± 0.1	1.6 ± 0.1
D221A	<4.5	740 ± 140	1400 ± 390	12000 ± 3700	3.2 ± 0.2	1.7 ± 0.1
P229A	<4.5	2900 ± 270	640 ± 420	28000 ± 2500	1.6 ± 0.2	2.0 ± 0.1
E230A	<4.5	230 ± 50	300 ± 150	14000 ± 2000	5.7 ± 1.3	1.8 ± 0.4
I232A	<4.5	<4.5	<4.5	<4.5	2.2 ± 0.3	2.7 ± 0.3
F234A	<4.5	<4.5	<4.5	70 ± 15	5.9 ± 0.6	2.9 ± 0.1

(n.d.) not determined

Table 2

Statistics for refinement and geometry of original structures (1SOZ, 1SOT, 1TE0, and 1VCW) and the same structures re-refined here (4RQZ; 4RR1, 4RQY, and 4RR0).

PDB ID	ref.	ligand	resolution (Å)	R_{work} / R_{free}	clash percentile	poor rotamers %	Rama. outliers %	Rama. favored %	bad backbone bonds	bad backbone angles	Cβ deviations >0.25 Å	MolProbity score (rank percentile)
1SOZ	(1)	OMP peptide	2.4	0.231 / 0.272	40	5.3	3.5	87	3	7	30	3.05 (21)
4RQZ [a]	(2)	OMP peptide	2.4	0.196 / 0.226	100	0	0	98	0	0	0	0.96 (100)
1SOT	(1)	apo	2.3	0.198 / 0.248	29	8.9	3.3	86	1	1	9	3.34 (13)
4RR1 [b]	(2)	apo	2.3	0.184 / 0.217	100	0	0	100	0	0	0	0.75 (100)
1TE0	(3)	apo	2.2	0.245 / 0.295	8	23	7	85	73	140	186	3.81 (1)
4RQY [c]	(2)	apo	2.2	0.179 / 0.223	100	0	0	99	0	0	0	0.75 (100)
1VCW	(1)	OMP peptide removed	3.05	0.233 / 0.301	42	8.2	4.4	82	9	11	73	3.55 (36)
4RR0 [d]	(2)	OMP peptide removed	3.05	0.226 / 0.256	100	0	0	98	0	0	0	0.89 (100)

[a] re-refinement of 1SOZ

[b] re-refinement of 1SOT

[c] re-refinement of 1TE0

[d] re-refinement of 1VCW

[1] Wilken et al. (2004)

[2] This work

[3] Zeth (2004)

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