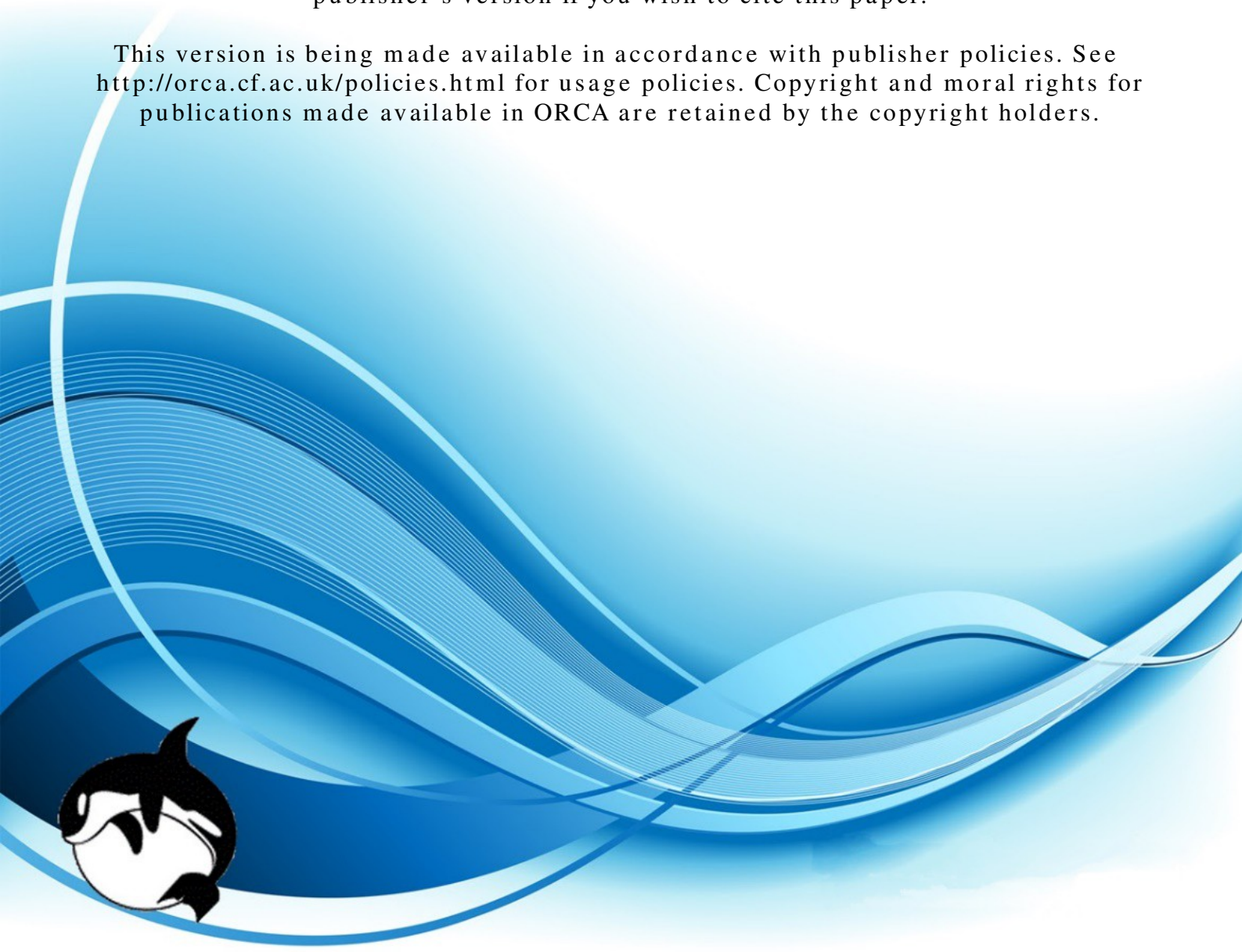


Substrate Occupancy

at the Onset of Oligomeric Transitions of DegP

Natalie J. Thompson[a,b], Melisa Merdanovic[c], Michael Ehrmann[c], Esther van Duijn[a,b], Albert J.R. Heck[a,b]

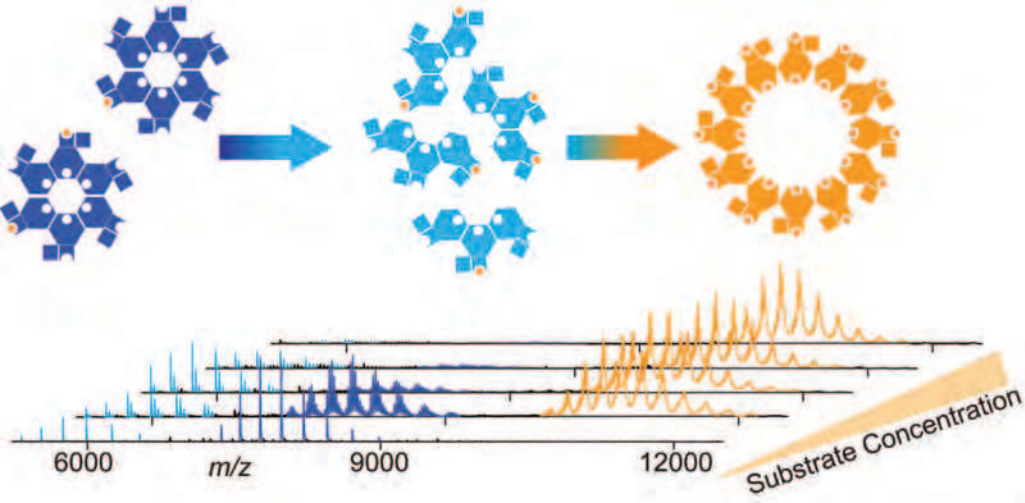
[a]Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute of Pharmaceutical Sciences, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

[b]Netherlands Proteomics Centre, Padualaan 8, 3584 CH Utrecht, The Netherlands

[c]Center for Medical Biotechnology, Faculty of Biology, University Duisburg-Essen, 45117 Essen, Germany

Correspondence to:

Albert J.R. Heck, email: a.j.r.heck@uu.nl, (t) +31 30 253 58 71, (f) +31 30 253 69 19


6000
m/z
9000
12000
Substrate Concentration

Abstract

The protease-chaperone DegP undergoes secondary through quaternary structural changes, regulating function and preventing indiscriminate proteolysis. Several structures of DegP oligomers have been observed, including the resting state 6-mer and the 12-mer and 24-mer active states. However, the precise events of the transition between the resting and active states still need to be elucidated. We used native mass spectrometry to demonstrate that binding of multiple substrate-mimicking peptide ligands to the DegP resting state occurs prior to the transition to an active conformation. This transition occurred at a 6-mer occupancy of 40% for each peptide ligand. We observed ligand-specific 9-mer formation with a maximum load of 9 peptides, whereas other substrates led to 12-mers accommodating 24 peptides. Based on these data, we present a model for the initial steps of substrate-induced transitions from the resting to active states of DegP.

Introduction

Protein misfolding and aggregation occurring under stress conditions is often counter-acted by molecular chaperones and proteases to prevent cell death.(Kim and Kim, 2005; Merdanovic et al., 2011; Tyedmers et al., 2010) These proteins frequently target the exposed hydrophobic regions of misfolded proteins thereby preventing aggregation. For the majority of proteases, function is regulated by ATP, which is used to control access to the proteolytic sites (Tyedmers et al., 2010). However, some proteases are ATP-independent, allowing them to function in an ATP-depleted environment, such as the bacterial periplasm. One such protease is the widely conserved extracytoplasmic protein quality control factor DegP (Merdanovic et al., 2011). *Escherichia coli* DegP is a periplasmic protein upregulated by the Cpx and σ^E pathways in response to conditions that result in protein folding problems, such as heat stress, and is required for cell survival above 37 °C.(Clausen et al., 2011; Kim and Sauer, 2012; Meltzer et al., 2009; Ortega et al., 2009) While it is generally accepted that the oligomeric state and function of DegP are directly related, a full, detailed structural model remains to be established.

DegP exists in multiple oligomeric forms with a 3-mer as the fundamental building block. The center of the DegP 3-mer consists of the three trypsin-like protease domains of the individual monomers arranged in a planar fashion with the PDZ1 and PDZ2 domains on the exterior.(Krojer et al., 2002) While digestion occurs at the catalytic triad in the protease domain, PDZ1 and PDZ2 domains have been shown to be necessary for substrate binding, allosteric regulation, and oligomer stabilization.(Iwanczyk et al., 2007; Jiang et al., 2008; Jomaa et al., 2007; Subrini and Betton, 2009) DegP forms multiple oligomers, composed of multiples of 3-mers, ranging from a 6-mer to a 24-mer that can be classified into three categories: face-to-face, cage-like, and bowl-shaped structures (Figure 1), each with the common feature of the active sites localized in the interior of the particle. The 6-mer is the only oligomer that exhibits a face-to-face structure where the two 3-mers are positioned parallel to each other.(Krojer et

al., 2002) The structure is stabilized by PDZ domain interactions across the exterior interface and the extension of the LA loop from a protease domain of one 3-mer into a protease domain of the opposite 3-mer.(Sawa et al., 2010) Two different DegP oligomers have been shown to have the cage-like structure: the 12-mer and the 24-mer. While the sizes of these cage-like structures are different due to the number of trimeric building blocks involved, a large interior cavity is common to both structures.(Sawa et al., 2010) The 12-mer adopts tetrahedral symmetry, and the 24-mer adopts octahedral symmetry, both containing large pores that can allow the entrance of unfolded substrates.(Jiang et al., 2008; Kim et al., 2011; Krojer et al., 2008b) The PDZ domains between neighboring 3-mers interact to stabilize these hollow shells. Multiple oligomers, including the 12-mer, the 15-mer, and the 18-mer, have been shown to form bowl-shaped structures on a lipid interface. These highly flexible structures exhibit similar PDZ domain interactions as those observed in the cage-like structures, suggesting that the bowl-shaped conformations may represent intermediates on the pathway to form large cages in the periplasmic space.(Shen et al., 2009)

The likely reason for the many different oligomeric states of DegP is to provide functional control of proteolytic activity. DegP is a highly efficient, relatively non-specific protease that binds and cleaves exposed hydrophobic residues. However, DegP has developed a mechanism through which to control its indiscriminate function.(Hauske et al., 2009; Huber and Bukau, 2008; Jones et al., 2002; Krojer et al., 2008a) In the resting state of DegP, i.e., 6-mer, the LA loops stabilize the face-to-face structure and distort the catalytic triad of an opposite monomer. Exposed hydrophobic regions of unfolded proteins can bind to the PDZ1 domain, initiating the transformation from the resting state to an active state, whereby the LA loops are extracted from the active site of their neighbors, and the activity of DegP is restored.(Hauske et al., 2009; Kim et al., 2011; Krojer et al., 2010; Meltzer et al., 2009; Merdanovic et al., 2010) During this process, the 6-mer rapidly transforms to higher-order oligomers, such as the 12-mer or 24-mer.(Jiang et al., 2008) The transition is initiated by the binding of the substrate to the PDZ1 domain

inducing a conformational change of the PDZ domains (Krojer et al., 2010) followed by dissociation from the 6-mer into 3-mers.(Jiang et al., 2008) The substrate-bound 3-mer then associates rapidly into higher-order oligomers that remove the unfolded substrate from solution via proteolysis. After DegP has performed its function and cleared unfolded proteins, it reverts back to the resting state.(Jiang et al., 2008; Kim et al., 2011; Krojer et al., 2008b) The changes in oligomeric states reflect an "on-off" switch of DegP, depending on the requirements of the cell. While structures of several oligomeric states are available, the exact transition from the 6-mer to the higher-order oligomers upon substrate interaction remains undefined.

Here, we apply native mass spectrometry (MS) (Loo, 2000; van Duijn et al., 2005) to study the transition of DegP upon binding of a variety of substrate-mimicking peptide ligands. Because native MS combines the ability to conserve large, non-covalent protein structures in the gas phase with high mass resolution and accuracy, it is well-suited for measuring both large and small changes in molecular mass of transitory protein complexes. Similar to this study, native MS has previously been used to characterize the DegP-related DegQ system, demonstrating the ability to retain multiple non-covalent complexes in the gas phase, and tandem MS was used to confirm the presence of a specific number of ligands bound to the DegQ oligomer.(Malet et al., 2012) In our study, we use native MS to monitor all oligomers present simultaneously, whereby the high mass resolving power allows us to quantify heterogeneous oligomer populations resulting from the variety of bound peptide ligands. We found that each substrate-mimicking peptide ligand induced a transition from the resting state 6-mer to a higher-order oligomer at the point where the 6-mer occupancy reached 40%. We also detected a transitory 9-mer that bound fewer peptides than the 12-mer "active" state. With native MS, we were able to probe the initial steps of substrate binding and oligomerization of DegP. The interactions between DegP and a variety of peptide ligands, as well as the transition to higher-order oligomers, allow us to contribute to the oligomerization model by characterizing the initial steps of activation by oligomerization.

Results

Selection of substrate-mimicking peptide ligands

In our experiments, a variety of substrate-mimicking peptide ligands were used as well as control peptides. Meltzer *et al.* previously demonstrated that DegP exhibits allosteric induction of proteolytic activity by peptides mimicking unfolded or mislocalized proteins.(Meltzer et al., 2008) Such activating peptides also induce a transition in oligomeric state.(Merdanovic et al., 2010) Therefore, peptides that increased proteolytic activity of DegP were treated as substrate-mimicking and assumed to induce an oligomeric transition; peptides that induced no such activity were used as negative controls. Two peptides, DPMFKLV and SPMFKGV have been shown to be activating and non-activating, respectively, with suggested DPMFKLV binding to both the protease domain and PDZ1 domain binding sites, and SPMFKGV unable to bind to either site.(Merdanovic et al., 2010) The observed factor of activation was compared between the peptide substrate and a known non-activating peptide substrate (SPMFKGV) to determine whether other peptides were substrate-mimicking or control (Table S1). The peptides DPMFKLV, DYFGSALLRV, CHHSAFPVFL, and SPMFKGVLDMMYGGMRGYQV were found to increase the proteolytic activity of DegP, and were thereby classified as substrate-mimicking with the implication of a peptide-induced oligomeric transition as well. SPMFKGVLDMMYGGMRGYQE, a derivative of the largest activating peptide used, along with SPMFKGV were previously shown to have little to no interaction with DegP.(Krojer et al., 2008a; Merdanovic et al., 2010) This protease assay revealed that these two peptides induced similarly low levels of proteolytic activity, and both were used as negative controls. The results from this protease assay agree with what has previously been observed, where the C-terminal sequence is crucial for substrate binding and degradation.(Krojer et al., 2008a)

Substrate-mimicking peptide ligands induce substrate-specific DegP oligomerization

To avoid auto-degradation, we used the proteolytically inactive mutant, DegP S210A, where the catalytic Ser210 residue is replaced by an Ala residue. DegP S210A has been shown to undergo oligomer transformations in the presence of substrates and substrate-mimicking peptide ligands.(Iwanczyk et al., 2011; Jiang et al., 2008; Kim et al., 2011; Krojer et al., 2010; Merdanovic et al., 2010) In addition, we used DegP samples that were purified under denaturing conditions (to reduce the amount of co-purified ligands) and subsequently refolded.(Merdanovic et al., 2010) Native MS of refolded DegP S210A revealed a mixture of oligomers, whereby the 6-mer and 3-mer were the most intense species (Figure 2A). The observation of these two species reflects the dynamic equilibrium between the 6-mer and 3-mer that has been suggested, (Jiang et al., 2008) though the population of the 3-mer may be enhanced due to the conditions required to transfer DegP oligomers to the gas phase and achieve high mass resolution. Subsequently, DegP S210A was mixed and incubated with the various peptide ligands. A clear transition from the resting state of DegP to "active" higher-order oligomers was observed for each substrate-mimicking peptide ligand as monitored by native MS. An example of this transition using the peptide DPMFKLV is shown in Figure 2 (B-E). At increased peptide concentrations, DegP forms higher-order oligomers (the 12-mer for this peptide) until none of the resting state (6-mer) remains (Figure 2E). The formation of higher-order oligomers was observed for each substrate-mimicking peptide ligand studied, however, the observed dominant higher-order oligomers were quite different. By native MS, the most abundant higher-order oligomers were the 9-mer and the 12-mer, showing a strong dependence on the specific peptide ligand incubated with DegP (Table 1 and Figure S1). Previous studies reported even larger higher-order oligomers; a reason for these apparent discrepancies may be due to the much lower peptide concentration used here compared to that used in previous studies.(Hasenbein et al., 2010; Jiang et al., 2008; Krojer et al., 2008a; Krojer et al., 2010; Krojer et al., 2008b; Merdanovic et al., 2010) Such high peptide concentrations, i.e., 100 μM – 500 μM, are not compatible with native MS as the signal from the unbound peptide would completely suppress that from the DegP oligomers. Since

the size of DegP higher-order oligomers has previously been linked to substrate size and concentration, (Iwanczyk et al., 2011; Krojer et al., 2008b) it is reasonable to attribute the observation of smaller higher-order oligomers by native MS to the use of low peptide concentrations. To confirm that these oligomeric transitions were not specific to the inactive DegP S210A mutant, the effect of peptide ligand binding to DegP WT was also tested, yielding similar transitions to the same higher-order oligomers as observed for DegP S210A (Figure S1).

The native mass spectra acquired during titration of small amounts of peptide suggest that a minimum amount of peptide is required to induce the formation of the higher-order oligomers. Upon the addition of peptide ligands to DegP, multiple peaks are observed for each charge state of both the 3-mer and 6-mer (Figures 2 and 3). The formation of additional peaks originates from DegP oligomers with various numbers of peptide ligands bound (Figure 3). Close inspection of a single charge state reveals that the mass difference between adjacent peaks corresponds to the mass of a single peptide. Due to the high mass resolving power, peaks corresponding to oligomers with different numbers of peptides were resolved (peptide masses ranging from 821 Da to 2297 Da compared to the DegP S210A 6-mer mass of 287,276 Da). As expected, the addition of greater amounts of peptide ligand results in increased numbers of bound peptides to both DegP 3-mer and 6-mer to a maximum of one peptide per monomer, i.e., 3 peptides per 3-mer and 6 peptides per 6-mer. A maximum occupancy for these oligomers implies specific binding of the peptide ligand to a DegP monomer. It has been shown that the PDZ1 domain is necessary for substrate binding (Iwanczyk et al., 2007; Krojer et al., 2008a; Merdanovic et al., 2010) and does indeed have a hydrophobic cleft as a suggested binding site.(Kim et al., 2011; Meltzer et al., 2008; Meltzer et al., 2009) An additional binding site exists in the active site of the protease domain.(Kim et al., 2011; Kim and Sauer, 2012; Krojer et al., 2010; Merdanovic et al., 2010) However, given that the protease domain is generally obstructed in the resting state structure(Krojer et al., 2002; Sawa et al., 2010; Subrini and Betton, 2009) and that the average number of peptides bound corresponds to a single

peptide per monomer at the highest peptide concentrations, it is most likely that the peptide ligands are bound to the PDZ1 domain.

To confirm the substrate-dependent oligomerization, DegP S210A and DegP WT were incubated with the control peptides, and the effect on the oligomeric state was investigated. No higher-order oligomers were formed, even at molar excess of the peptide (Figure S1, E and F). Both of the peptides used as negative controls have been shown to have limited binding to the PDZ1 domain of DegP yet retain the ability to bind to the protease domain site for cleavage.(Krojer et al., 2008a; Merdanovic et al., 2010) The severely reduced binding of these peptides to the DegP 6-mer indicates that the protease domain binding site of the protease domain is obstructed and that binding to the PDZ1 domain is necessary for the formation of higher-order oligomers.

Common occupancy found for oligomeric transitions induced by substrate-mimicking peptide ligands

To probe the initial steps of DegP's oligomeric transitions, we measured the occupancy of the 6-mer at the onset of higher-order oligomer formation because this value represents the number of occupied binding sites necessary to induce the transition from the resting state to the active state. Since the occupancy was calculated for the 6-mer, in which only one binding site is available, i.e., the PDZ1 domain binding site, (Krojer et al., 2002) only one site per monomer was considered in the calculation. This assumption is supported by the lack of binding of the control peptides, both of which have been shown to still bind to the protease domain binding site.(Krojer et al., 2008b; Merdanovic et al., 2010) The changes in occupancy as a function of peptide concentration are illustrated in Figure 4. The use of low peptide concentration afforded the observation of the initial steps of oligomerization, specifically peptide binding prior to higher-order oligomer formation, as evidenced by an increase in 6-mer occupancy upon the addition of peptide ligand. The peptide ligands DPMFKLV and DYFGSALLRV bind to DegP S210A strongly, demonstrated by the low [peptide]:[DegP 1-mer] ratio needed to induce the

transition to higher-order oligomers (indicated by the asterisks in Figure 4). The occupancy of the 6-mer increases slightly before a plateau is reached, indicating that a maximum number of peptides can be bound to the 6-mer. The occupancy of the 6-mer at the onset of higher-order oligomer formation is listed in Table 1 for each substrate-mimicking peptide ligand. The occupancy at the onset of oligomeric transition is approximately 40% for all of the substrate-mimicking peptide ligands, though the peptide concentration necessary for higher-order oligomer formation is different for each peptide. The similarities in occupancy upon higher-order oligomer formation imply that the initial steps of the oligomerization mechanism are similar for each peptide.

Peptide binding-induced higher-order oligomers exhibit an increased maximum loading of substrate-mimicking peptide ligands

The achieved resolving power of these native MS experiments reveals not only the number of peptide ligands bound to the DegP 6-mer but also the number of peptides bound to the higher-order structures, i.e., the 9-mer and the 12-mer. Close inspection of the signals for these two oligomers revealed that the 9-mer binds up to one peptide per DegP monomer, to a maximum of 9 peptides bound to the 9-mer, but, in sharp contrast, the 12-mer forms with two peptides per DegP monomer bound resulting in an average loading of 24 peptides per 12-mer. To confirm the correct assignment of the peaks in the native MS spectrum, simulated spectra of the higher-order oligomers were generated using SOMMS.(van Breukelen et al., 2006) The native MS spectra of both the 9-mer and the 12-mer were simulated with the addition of one or two peptides per monomer and compared to the experimental spectrum (Figure 5). Comparison of the same charge state between the two simulated spectra and the experimental data confirms the maximum loads of the 9-mer and 12-mer to be 9 and 24 peptides, respectively. Further evidence supporting this finding comes from the fact that two independent substrate-mimicking peptide ligands, i.e., DPMFKLV and CHHSAFPVFL, induce the formation of the 12-mer with 24 peptides bound,

whereas two other independent substrate-mimicking peptide ligands, i.e., DYFGSALLRV and SPMFKGVLVDMMYGGMRGYQV, induce the formation of 9-mers with only 9 peptides bound maximally. These data hint at a substrate-dependent fine regulation between enzyme and substrate.

As mentioned above, it was recently proposed that each DegP monomer possesses two binding sites: one on the PDZ1 domain and one in the protease domain.(Iwanczyk et al., 2007; Kim et al., 2011; Kim and Sauer, 2012; Krojer et al., 2008a; Krojer et al., 2010; Merdanovic et al., 2010) Therefore, the binding of up to two peptides per monomer can be explained by the presence of these two binding sites; however, the differences in maximum load between the 9-mer and the 12-mer imply inherent differences in structure. As observed earlier, the DegP 6-mer binds a maximum of one peptide per monomer, an observation that can be explained by the obstruction of the active site by interfacial loops in the 6-mer structure. Similar behavior of the 9-mer indicates that the 9-mer is not a complete active state conformation, but it may be a transitory oligomer. Examination of the distribution of bound ligands to the 9-mer and 12-mer at increasing peptide concentration revealed that the number of peptide ligands bound to the 9-mer increases with concentration, but this distribution on the 12-mer remains constant (Figure 6). These results indicate that the released 3-mers associate into the 9-mer prior to further binding of peptide ligands. The binding of two peptides to the 12-mer demonstrates the presence of two binding sites per monomer, both of which are available in this cage-like structure. From these results, it is most likely that the PDZ1 binding site is available in all DegP structures, but the protease domain substrate binding site becomes available upon the formation of the larger cage-like structures, and, therefore, multiple peptide binding is only observed for the DegP 12-mer and possibly for other higher-order oligomers, such as the previously reported 24-mer.(Krojer et al., 2008b)

Discussion

Here, we used native MS to monitor the initial steps in the activation of DegP, leading to the formation of higher-order oligomers. Native MS has provided excellent characterization of the small mass changes resulting from peptide ligand binding, which then induces dramatic oligomeric shifts. The high mass resolving power of native MS allows the observation and differentiation of not only multiple non-covalent DegP oligomers but also the heterogeneous population within each oligomeric species due to the variety of peptide ligands bound. Our data show that multiple peptides bind to the resting state DegP oligomers (3-mer and 6-mer only) prior to initiating higher-order oligomer formation. It was also observed that the maximum number of peptides bound is directly related to the oligomeric state of DegP, reflecting the possible differences in structures as well as demonstrating the differences in kinetics between released 3-mer association and subsequent ligand binding. Most interesting is the amount of peptide bound to the resting state 6-mer at the onset of higher-order "active" oligomer formation. It was observed that this amount, as calculated by the occupancy, is similar for all activating peptides, in that, at 40% occupancy, an oligomeric transition begins to occur within the DegP population. This common occupancy was only observed due to the use of low peptide ligand concentrations to probe the initial steps of oligomerization and the high resolving power of native MS to differentiate the heterogeneous population of each oligomer.

Previously, an oligomerization mechanism was proposed, (Jiang et al., 2008; Kim et al., 2011; Krojer et al., 2010) indicating that the binding of a substrate to the PDZ1 domain induces a conformational change of the PDZ1 and PDZ2 domains as well as in loops of the protease domain. This conformational change was represented as a transition from the closed 6-mer structure to the open 6-mer structure. The next step in this proposed mechanism is the dissociation of the 6-mer into two 3-mers, which subsequently function as building blocks for the large cage-like structures common to the active state of DegP. With current understanding, it is impossible to determine the level of PDZ conformational change necessary to disrupt 6-mer for progression to larger active structures.

The native MS data shed light onto the binding of a substrate and the transition from resting state to active state. With our observations of peptide binding (mimicking substrate binding), the occupancy at the onset of oligomerization, and the maximum load observed for multiple DegP oligomers, we can provide insight into the mechanism for higher-order oligomer formation (Figure 7). When in the resting state, i.e., no substrate present, the DegP exists predominantly as a 6-mer, as indicated by **(1)** in Figure 7. In this conformation, the only substrate-binding site available is on the PDZ1 domain (red squares) as the protease domain binding site is disrupted and inaccessible (blue hexagons). Upon the addition of a peptide ligand to the 6-mer **(2)**, we suggest it binds to a PDZ1 domain causing a conformational shift from the closed to the open conformation, thereby destabilizing the PDZ-PDZ interfacial interactions. Subsequent addition of peptide ligands induces conformation changes in other monomers of the 6-mer, progressively opening the 6-mer **(3)**. At an occupancy greater than 40%, the 6-mer conformation is destabilized to such an extent that it dissociates into two 3-mers bearing multiple substrates **(4)**. The reason for substoichiometric occupancy being sufficient to trigger oligomeric rearrangements is most likely due to positive cooperativity.(Merdanovic et al., 2010) The released 3-mers then rapidly associate with other 3-mers to form transitory oligomers, such as 9-mer **(5)**, before completing higher-order oligomer formation **(6)**. In Figure 7, transitory species are indicated by the gray brackets and include the released 3-mers and the 9-mer. We believe the transitory nature of the 9-mer causes the incomplete occupancy of the two peptide binding sites, rather than the 9-mer being a complete structure with an obstructed peptide binding site. This conclusion is supported by the increasing occupancy of the 9-mer with increased peptide concentration. The 12-mer, being a cage-like structure, is able to accommodate 24 peptides indicating that both the binding site on the PDZ1 domain and the protease domain are available.

The very rapid association of released 3-mers is deduced from the maximum load observed for the 9-mer and 12-mer structures and from the increasing occupancy of the 9-mer with increased peptide

concentration. In the native MS spectrum, formation of higher-order oligomers occurred with an occupancy of 40%, or approximately upon the addition of the third to fourth peptide, meaning that the released 3-mers typically have one or two peptides bound. The 9-mer exhibited up to 9 peptides bound, equating to one per monomer or three per trimer, which is increased over that of the released 3-mer. Therefore, during the formation of the transitory 9-mer, additional peptides bind to available binding sites, either in the PDZ1 or protease domains, as observed by increased number of peptides bound with increased concentration (Figure 6). However, the 12-mer exhibits a defined population dominated by the 12-mer with the addition of 24 peptides. It was recently shown that substrate binding in both the protease domain and PDZ1 domain binding sites enhances degradation over substrate binding in only one location.(Kim et al., 2011) This result combined with the formation of the 12-mer with two peptides per monomer implies that the 12-mer indeed reflects the active conformation and that the 9-mer is indeed a transitory structure. Given that 9-mer and 12-mer formation occur on similar time scales, and it is unlikely that peptide ligand addition would be faster for one ligand over another, it is highly likely that peptide ligand binding continues after cage-like higher-order oligomer formation. It is known that the cage-like structures of the 12-mer and 24-mer have large pores, (Jiang et al., 2008; Kim et al., 2011; Krojer et al., 2008b) and these results imply that the additional peptide can diffuse through these pores to the now-free protease domain binding site. Any more definite conclusions about the nature of the 9-mer or 12-mer structures cannot be deduced from our data, albeit that our MS-based binding assays define the ideal conditions for generating DegP 9-mers or 12-mers, amenable to X-ray crystallography or cryo-electron microscopy. Structural biology approaches may further reveal how the nature of the substrate-binding motif regulates the conformational changes in the resting state of DegP, inducing either 9- or 12-mer formation.

The oligomeric transitions of DegP upon the addition of intact substrate proteins or substrate-mimicking peptide ligands have been documented using techniques such as size exclusion chromatography (SEC)

and SDS-PAGE combined with cross-linking.(Merdanovic et al., 2010) However, these studies were unable to determine the substrate load in these different oligomers or observe the heterogeneity within a single oligomer population due to the low resolution of these techniques and the high concentration of substrate used. A single study has probed the substrate load of denatured lysozyme in a DegQ 12-mer utilizing native MS, (Malet et al., 2012) but the amount of substrate was determined for only the end point of the oligomeric transition rather than observing the transition in oligomeric state upon introduction of the substrate. While native MS has been used to probe oligomeric states of large protein complexes, the level of detail achieved for these transitory states of DegP is ground-breaking. We have been able to assess the amount of substrate necessary for DegP oligomeric transitions, and, with this novel information, have added crucial details to the already proposed oligomerization model.

For the DegP protease, the transition from the resting state to the active state involves a structural change through which proteolytic activity is governed by the oligomeric form. The exact mechanism of this transition is not known in detail, though the general shift from the small 6-mer structure to the large, cage-like structures of the active state has been well documented. Using a native MS approach, we have visualized the initial steps of resting-state destabilization and the formation of higher-order oligomers. The resolving power achieved with these native MS experiments enables the exact identification of the number of peptides bound to DegP. With this information, we developed a way to monitor DegP oligomers complexed with substrate-mimicking peptides by measuring the occupancy. It was observed that the onset of higher-order oligomer formation resulted from the binding of activating peptides. It was found that the occupancy of the 6-mer was similar for each of the activating peptides studied, being around 40%. We present a model of the transition from the 6-mer to higher-order oligomers for DegP.

While this current study has focused on the initial steps of DegP oligomerization, the results found here apply to homologs of DegP, including many members of the HtrA family. Also, this technique is applicable to many other systems where oligomeric transitions are crucial to function, including most molecular chaperones. Native MS has already been used to study substrate-binding to complexes as large as CRISPR-associated complexes as well as probing the structure and stability of virus particles.(Shoemaker et al., 2010; van Duijn et al., 2012) This study has highlighted the applicability of native MS to these large heterogeneous protein complexes, demonstrating the ability to measure both small and large mass differences in these complexes. These capabilities of native MS demonstrate its complementarity to techniques such as X-ray crystallography and cryo-EM, making it a powerful tool in the field of structural biology.

Experimental Procedures

DegP purification

DegP purification was carried out under non-denaturing conditions as previously described.(Spiess et al., 1999) Purified DegP were repurified using nickel tris-carboxymethyl ethylene diamine affinity columns. Equilibration and binding with 50–100 mM NaH_2PO_4 pH 8 was followed by thorough washing with 8 M urea and 50 mM NaH_2PO_4 pH 8. The denatured protein was recovered with 150 mM imidazole, and protein concentration was adjusted to 2 mg ml^{-1}. Refolding was performed by 50-fold dilution of the denatured protein sample in the refolding buffer (in 500 mM sodium phosphate buffer, pH 7). After refolding, DegP 6-mer formation was confirmed by size-exclusion chromatography (Superdex 200 26/60).

DegP activity assay

DegP activity was assessed via protease assays using the synthetic p-nitroanilin substrate, SPMFKGV-pNA. The pNA-peptide has been previously shown to have very little interaction with the PDZ1 domain but undergo proteolytic cleavage to remove the absorbent pNA. The assays were performed in 100 mM ammonium acetate (pH 7.5) with 10 µM DegP (monomer equivalent) and 0.5 mM pNA-substrate by measuring the changes in OD_{405} (Tecan GENios Pro reader) continuously for 1 hr at 37°C. Substrate-mimicking peptides were preincubated with DegP for 10 minutes at 37°C before adding the pNA-substrate. The factor of activation was calculated via the comparison of the pNA substrate turnover with and without the additional substrate-mimicking peptide.

Native mass spectrometry

Purified, refolded DegP S210A and DegP WT samples were exchanged to 100 mM ammonium acetate buffer, pH 7.5, using 10 kDa molecular weight cutoff spin-filter columns (Millipore, Carrigtwohill, Co. Cork, Ireland). Peptides were synthesized in the M. Kaiser lab (University Duisburg-Essen) and dissolved in water. DegP S210A or WT was mixed with peptide solutions to the various molar ratios used in these experiments. The concentration of DegP (1-mer) was approximately 10 µM for each mixture. Mixtures of DegP and substrate-mimicking peptide were sprayed on a nano-electrospray time-of-flight (nanoESI-TOF) mass spectrometer (LCT, Waters, Manchester, UK) using gold-coated borosilicate needles prepared in house. Needles were generated using a Sutter P-97 puller (Sutter Instruments Co., Novato, CA, USA) and coated using an Edwards Scancoat six sputter-coater (Edwards Laboratories, Milpitas, CA, USA). Source backing pressure was increased to 6.2 mbar.(Tahallah et al., 2001) Capillary and cone voltages were set to 1300 V and 200 V respectively. Mass calibration was performed using aqueous CsI solution (25 mg/mL). All measurements were performed in triplicate.

Data analysis

MassLynx V4.1 (Waters, Manchester, UK) was used for experimental mass determination. Oligomers and the number of attached peptides were assigned based on the theoretical mass of the purified DegP 1-mer (with His tag, 47879.26 Da) and the theoretical mass of the peptides. Peak area for the occupancy calculation of the 6-mer was determined using Igor Pro V6.22. Individual peaks were fit using a Lorentzian function with the peak width determined according to the raw spectrum and a constant baseline (Figure S2). All peaks corresponding to the 6-mer were used for the occupancy calculations.

DegP occupancy calculation

$$\text{Occupancy} = \left(\frac{\sum_{n=0}^{N} M_n \times A_n}{\sum_{n=0}^{N} A_n} - M_{n=0}\right) \div M_{peptide} \div N_{sites} \times 100 \qquad \textbf{Equation 1}$$

DegP occupancy was used to assess the amount of peptide ligands bound to each DegP oligomer. Since the focus was the transition from resting to active state, the occupancy was calculated for the 6-mer and 3-mer. From the crystal structure of the 6-mer, it is suggested that only one binding site is available, i.e., the PDZ1 domain binding site.(Krojer et al., 2002) To calculate the occupancy, the average mass of peptides bound to a DegP oligomer was calculated by finding the weighted average mass of that oligomer and subtracting the mass of the free oligomer ($M_{n=0}$). The weighted average mass of an oligomer was derived from the mass of each peptide:DegP complex (M_n) and the peak area corresponding to that complex (A_n). For these sums, n indicates the number of peptide substrates bound, and N is the maximum number of bound peptide substrates observed in the spectrum. A_n is the sum of the peak area for all charge states observed for a given complex, e.g., the DegP 6-mer with 3 substrate-mimicking peptides bound. The mass of the free DegP oligomer, calculated from the theoretical mass of the DegP 1-mer (47879.26 Da), was subtracted from the weighted average mass to yield the average mass of peptide bound to the DegP oligomer. The mass of peptide bound was then normalized by the theoretical mass of the peptide ($M_{peptide}$) and the number of possible binding sites (N_{sites}), i.e., the number of PDZ1 domains, to yield a percentage of binding sites occupied.

Acknowledgements

The authors would like to thank the Netherlands Proteomics Center embedded in the Netherlands Genomics Initiative for funding. This work was supported by the Netherlands Organization for Scientific Research (NWO) (VENI 700.58.402) to E.v.D. and Deutsche Forschungsgemeinschaft to M.E.

References

Clausen, T., Kaiser, M., Huber, R., and Ehrmann, M. (2011). HTRA proteases: regulated proteolysis in protein quality control. Nat Rev Mol Cell Bio *12*, 152-162.

Hasenbein, S., Meltzer, M., Hauske, P., Kaiser, M., Huber, R., Clausen, T., and Ehrmann, M. (2010). Conversion of a Regulatory into a Degradative Protease. J Mol Biol *397*, 957-966.

Hauske, P., Meltzer, M., Ottmann, C., Krijer, T., Clausen, T., Ehrmann, M., and Kaiser, M. (2009). Selectivity profiling of DegP substrates and inhibitors. Bioorgan Med Chem *17*, 2920-2924.

Huber, D., and Bukau, B. (2008). DegP: a protein "Death Star". Structure *16*, 989-990.

Iwanczyk, J., Damjanovic, D., Kooistra, J., Leong, V., Jomaa, A., Ghirlando, R., and Ortega, J. (2007). Role of the PDZ domains in Escherichia coli DegP protein. J Bacteriol *189*, 3176-3186.

Iwanczyk, J., Leong, V., and Ortega, J. (2011). Factors Defining the Functional Oligomeric State of Escherichia coli DegP Protease. Plos One *6*.

Jiang, J.S., Zhang, X.F., Chen, Y., Wu, Y., Zhou, Z.H., Chang, Z., and Sui, S.F. (2008). Activation of DegP chaperone-protease via formation of large cage-like oligomers upon binding to substrate proteins. P Natl Acad Sci USA *105*, 11939-11944.

Jomaa, A., Damjanovic, D., Leong, V., Ghirlando, R., Iwanczyk, J., and Ortega, J. (2007). The inner cavity of Escherichia coli DegP protein is not essential for molecular chaperone and proteolytic activity. J Bacteriol *189*, 706-716.

Jones, C.H., Dexter, P., Evans, A.K., Liu, C., Hultgren, S.J., and Hruby, D.E. (2002). Escherichia coli DegP protease cleaves between paired hydrophobic residues in a natural substrate: the PapA pilin. J Bacteriol *184*, 5762-5771.

Kim, D.Y., and Kim, K.K. (2005). Structure and function of HtrA family proteins, the key players in protein quality control. J Biochem Mol Biol *38*, 266-274.

Kim, S., Grant, R.A., and Sauer, R.T. (2011). Covalent Linkage of Distinct Substrate Degrons Controls Assembly and Disassembly of DegP Proteolytic Cages. Cell *145*, 67-78.

Kim, S., and Sauer, R.T. (2012). Cage assembly of DegP protease is not required for substrate-dependent regulation of proteolytic activity or high-temperature cell survival. P Natl Acad Sci USA *109*, 7263-7268.

Krojer, T., Garrido-Franco, M., Huber, R., Ehrmann, M., and Clausen, T. (2002). Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine (vol 416, pg 455, 2002). Nature *417*, 455-459.

Krojer, T., Pangerl, K., Kurt, J., Sawa, J., Stingl, C., Mechtler, K., Huber, R., Ehrmann, M., and Clausen, T. (2008a). Interplay of PDZ and protease domain of DegP ensures efficient elimination of misfolded proteins. P Natl Acad Sci USA *105*, 7702-7707.

Krojer, T., Sawa, J., Huber, R., and Clausen, T. (2010). HtrA proteases have a conserved activation mechanism that can be triggered by distinct molecular cues. Nat Struct Mol Biol *17*, 844-852.

Krojer, T., Sawa, J., Schafer, E., Saibil, H.R., Ehrmann, M., and Clausen, T. (2008b). Structural basis for the regulated protease and chaperone function of DegP. Nature *453*, 885-890.

Loo, J.A. (2000). Electrospray ionization mass spectrometry: a technology for studying noncovalent macromolecular complexes. Int J Mass Spectrom *200*, 175-186.
Malet, H., Canellas, F., Sawa, J., Yan, J., Thalassinos, K., Ehrmann, M., Clausen, T., and Saibil, H.R. (2012). Newly folded substrates inside the molecular cage of the HtrA chaperone DegQ. Nat Struct Mol Biol *19*, 152-157.
Meltzer, M., Hasenbein, S., Hauske, P., Kucz, N., Merdanovic, M., Grau, S., Beil, A., Jones, D., Krojer, T., Clausen, T.*, et al.* (2008). Allosteiric activation of HtrA protease DegP by stress signals during bacterial protein quality control. Angew Chem Int Edit *47*, 1332-1334.
Meltzer, M., Hasenbein, S., Mamant, N., Merdanovic, M., Poepsel, S., Hauske, P., Kaiser, M., Huber, R., Krojer, T., Clausen, T.*, et al.* (2009). Structure, function and regulation of the conserved serine proteases DegP and DegS of Escherichia coli. Res Microbiol *160*, 660-666.
Merdanovic, M., Clausen, T., Kaiser, M., Huber, R., and Ehrmann, M. (2011). Protein Quality Control in the Bacterial Periplasm. Annu Rev Microbiol *65*, 149-168.
Merdanovic, M., Mamant, N., Meltzer, M., Poepsel, S., Auckenthaler, A., Melgaard, R., Hauske, P., Nagel-Steger, L., Clarke, A.R., Kaiser, M.*, et al.* (2010). Determinants of structural and functional plasticity of a widely conserved protease chaperone complex. Nat Struct Mol Biol *17*, 837-843.
Ortega, J., Iwanczyk, J., and Jomaa, A. (2009). Escherichia coli DegP: a Structure-Driven Functional Model. J Bacteriol *191*, 4705-4713.
Sawa, J., Heuck, A., Ehrmann, M., and Clausen, T. (2010). Molecular transformers in the cell: lessons learned from the DegP protease-chaperone. Curr Opin Struc Biol *20*, 253-258.
Shen, Q.T., Bai, X.C., Chang, L.F., Wu, Y., Wang, H.W., and Sui, S.F. (2009). Bowl-shaped oligomeric structures on membranes as DegP's new functional forms in protein quality control. P Natl Acad Sci USA *106*, 4858-4863.
Shoemaker, G.K., van Duijn, E., Crawford, S.E., Uetrecht, C., Baclayon, M., Roos, W.H., Wuite, G.J.L., Estes, M.K., Prasad, B.V.V., and Heck, A.J.R. (2010). Norwalk Virus Assembly and Stability Monitored by Mass Spectrometry. Mol Cell Proteomics *9*, 1742-1751.
Spiess, C., Beil, A., and Ehrmann, M. (1999). A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell *97*, 339-347.
Subrini, O., and Betton, J.M. (2009). Assemblies of DegP underlie its dual chaperone and protease function. Fems Microbiol Lett *296*, 143-148.
Tahallah, N., Pinkse, M., Maier, C.S., and Heck, A.J.R. (2001). The effect of the source pressure on the abundance of ions of noncovalent protein assemblies in an electrospray ionization orthogonal time-of-flight instrument. Rapid Commun Mass Sp *15*, 596-601.
Tyedmers, J., Mogk, A., and Bukau, B. (2010). Cellular strategies for controlling protein aggregation. Nat Rev Mol Cell Bio *11*, 777-788.
van Breukelen, B., Barendregt, A., Heck, A.J.R., and van den Heuvel, R.H.H. (2006). Resolving stoichiometries and oligomeric states of glutamate synthase protein complexes with curve fitting and simulation of electrospray mass spectra. Rapid Commun Mass Sp *20*, 2490-2496.
van Duijn, E., Bakkes, P.J., Heeren, R.M.A., van den Heuvell, R.H.H., van Heerikhuizen, H., van der Vies, S.M., and Heck, A.J.R. (2005). Monitoring macromolecular complexes involved in the chaperonin-assisted protein folding cycle by mass spectrometry. Nat Methods *2*, 371-376.
van Duijn, E., Barbu, I.M., Barendreg, A., Jore, M.M., Wiedenheft, B., Lundgren, M., Westra, E.R., Brouns, S.J.J., Doudna, J.A., van der Oost, J.*, et al.* (2012). Native Tandem and Ion Mobility Mass Spectrometry Highlight Structural and Modular Similarities in Clustered-Regularly-Interspaced Shot-Palindromic-Repeats (CRISPR)-associated Protein Complexes From Escherichia coli and Pseudomonas aeruginosa. Mol Cell Proteomics *11*, 1430-1441.

Figure Legends

Figure 1 Classification of the different shapes of DegP oligomers with each color representing an individual 3-mer subunit. In face-to-face structures, the planar faces of the 3-mers are parallel to each other, and structural stabilization results from loops extending from one 3-mer into the opposite 3-mer as well as PDZ domain interactions across the interface (closed structure). Bowl-shaped structures have similar curvature to the cage-like structures but do not form a closed sphere. Cage-like structures are comprised of multiple 3-mers to form a hollow sphere with large pores on the surface. These structures were derived using the 1KY9 and 3OU0 PDB structures.

Figure 2 Native MS spectra simultaneously monitoring peptide-binding and oligomerization of DegP S210A induced by the binding of the substrate-mimicking peptide DPMFKLV. The different oligomers are color-coded, with the 12-mer being the "active" form. The masses of each oligomer without additional peptide substrate are: 143,638 Da for the 3-mer, 287,276 Da for the 6-mer, and 574,551 Da for the 12-mer. Increasing amounts of DPMFKLV were added to a constant amount of DegP S210A (b-e). The [DPMFKLV]:[DegP 1-mer] ratios were 0.00 (A), 0.58 (B), 1.16 (C), 1.74 (D), and 2.32 (E). For DegP oligomeric transition to the 9-mer, see Figure S1.

Figure 3 Native MS spectra revealing the binding of multiple peptide ligands prior to higher-order oligomer formation. These spectra show the DegP S210A 6-mer with increasing amounts of the peptide DPMFKLV. [DPMFKLV]:[DegP 1-mer] ratios of 0.00 (A), 0.13 (B), 0.26 (C), and 0.50 (D). In these spectra, the 34+ through the 39+ charge states of the 6-mer are shown. The gray number above each peak indicates the number of peptide ligands bound to the DegP 6-mer. The higher-order oligomer (12-mer) formed only at [DPMFKLV]:[DegP 1-mer] ratios above 0.25 (C and D).

Figure 4 Occupancy of the DegP 6-mer (maximum loading of 6 peptides) with increasing amounts of the peptide ligand. Panels A-D show the occupancy for those peptides that induced higher-order oligomer formation measured by native MS. The asterisk indicates the ratio at which the higher-order oligomer first appears in the mass spectrum. Panels E and F show the lack of response for the negative control peptides. The error bars are the standard deviation over three replicates. Individual peak fitting is illustrated in Figure S2.

Figure 5 Overlays of experimental and simulated spectra used to validate the number of peptide ligands bound to each higher-order oligomer. Native MS spectra were simulated using SOMMS. The charge state distribution for the oligomer with one peptide per monomer is shown in red, and the distribution for the oligomer with two peptides per monomer is shown in blue. The experimental data is black with the number of substrate-mimicking peptides bound indicated in gray. For each spectrum, a single charge state is highlighted by a circle. A) Native MS spectra highlighting the 46+ through 48+ charge states of the DegP 9-mer formed after incubation with DYFGSALLRV at a [peptide]:[DegP 1-mer] ratio of 1.49:1. The alignment of the simulated and experimental spectra for each charge state confirms the addition of 9 peptides to the DegP 9-mer. The slight increase in mass is most likely due to incomplete desolvation. B) Native MS spectra highlighting the 57+ through 60+ charge states of the DegP 12-mer formed after incubation with CHHSAFPVFL at a [peptide]:[DegP 1-mer] ratio of 7:1. The alignment of the simulated and experimental spectra for each charge state confirms the addition of 24 peptides to the DegP 12-mer.

Figure 6 Native mass spectra reveal transitory nature of 9-mer. These spectra show the bound ligand distribution of DegP S210A higher-order oligomers with increasing peptide concentration (top to bottom: [peptide]:[DegP 1-mer] of 0.26, 0.50, 0.74, 1.0, and 1.49). The DegP S210A 9-mer (left) was formed via binding with the peptide DYFGSALLRV, and the DegP S210A 12-mer (right) was formed via

binding with the peptide DPMFKLV. The distribution of peptides bound to the 12-mer shifts only slightly with increased peptide concentration whereas the distribution of peptides bound to the 9-mer shifts dramatically (from an average of 4 peptides to 8 peptides). These results indicate that the 12-mer forms with both binding sites occupied while the 9-mer is transitioning between the occupancy of the 6-mer and the 12-mer. The charge states are labeled above each distribution (in black), and the number of peptides bound to each oligomer is listed above each peak (in gray).

Figure 7 Proposed mechanism for the substrate-mimicking peptide-induced transition from resting to active state. In this model, the structure of DegP is represented graphically, whereby the blue hexagon is the protease domain, the red square is the PDZ1 domain, and the green square is the PDZ2 domain. In the resting state **(1)**, the binding site on the PDZ1 domain available for binding, but the protease domain binding site is blocked, each represented by white areas. The substrate-mimicking peptide (orange circle) binds to the PDZ1 domain **(2)**, inducing a conformational change, destabilizing that interfacial PDZ-PDZ interaction. Additional binding of substrates to other PDZ1 domains induces further destabilization of the 6-mer **(3)**. The destabilized 6-mer structure dissociates into 3-mers with mixed numbers of peptide ligands bound **(4)**. Free 3-mers then associate, through transitory oligomers such as the 9-mer **(5)**, to form higher-order oligomers, such as the 12-mer **(6)**. The gray brackets around the released 3-mer and the 9-mer indicate that these species are transitory. The 9-mer is shown as binding 9 peptide ligands, but given the transitory nature, is unable to reach 100% occupancy of both binding sites. The cage-like 12-mer, however, is capable of accommodating 24 peptides, indicated by peptide binding to both PDZ1 and protease domain.

Tables

Peptide Sequence	Induced	[Peptide]:[DegP 1-mer]	Occupancy of the DegP

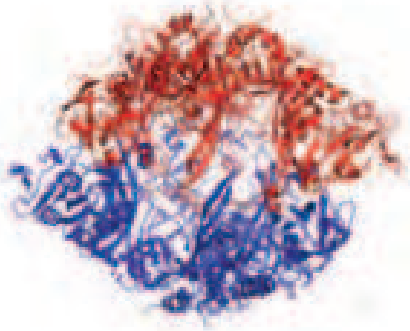
	Oligomer	ratio at higher-order oligomer formation	6-mer at higher-order oligomer formation
DPMFKLV	12	0.25:1	37%
DYFGSALLRV	9	0.25:1	30%
CHHSAFPVFL	12	5:1	41%
SPMFKGVLDMMYGGMRGYQ**V**	9	4:1	44%
SPMFKGV	-	N/A	N/A
SPMFKGVLDMMYGGMRGYQ**E**	-	N/A	N/A

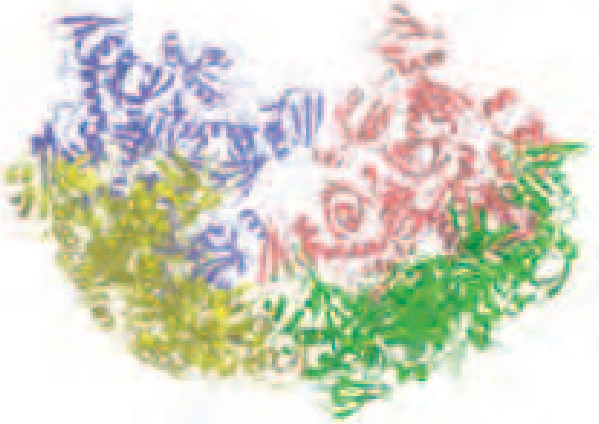
Table 1 Summary of observed oligomerization of DegP S210A induced by peptide ligands. Induced oligomer indicates the predominant higher-order oligomer formed with sufficient peptide concentration, which is any concentration greater than the [peptide]:[DegP 1-mer] ratio listed. "-" indicates that no higher-order oligomer formed, even at molar excess of peptide. The peptide-binding occupancy of the DegP 6-mer is listed at the onset of higher-order oligomer formation. For the factor of activation used to classify a peptide as "substrate-mimicking" or "control", see Table S1. Formation of the 9-mer and the lack of higher-order oligomer formation for a control peptide are illustrated in Figure S1.

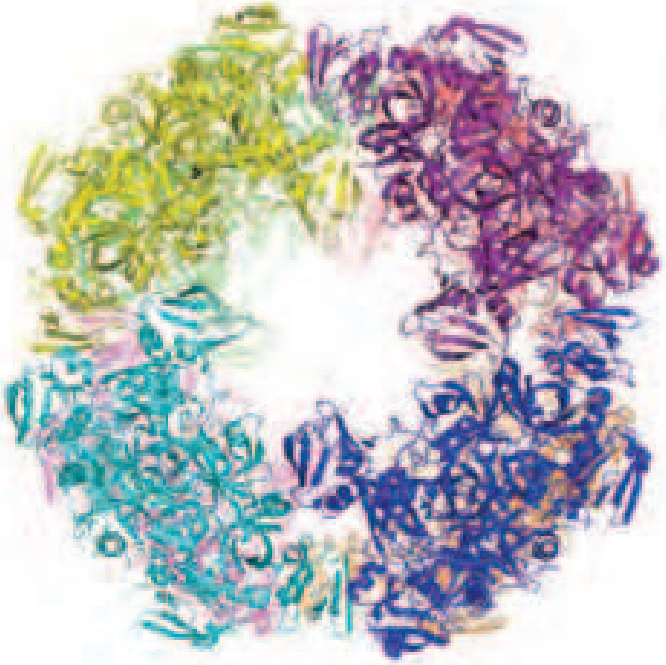
Face-to-Face

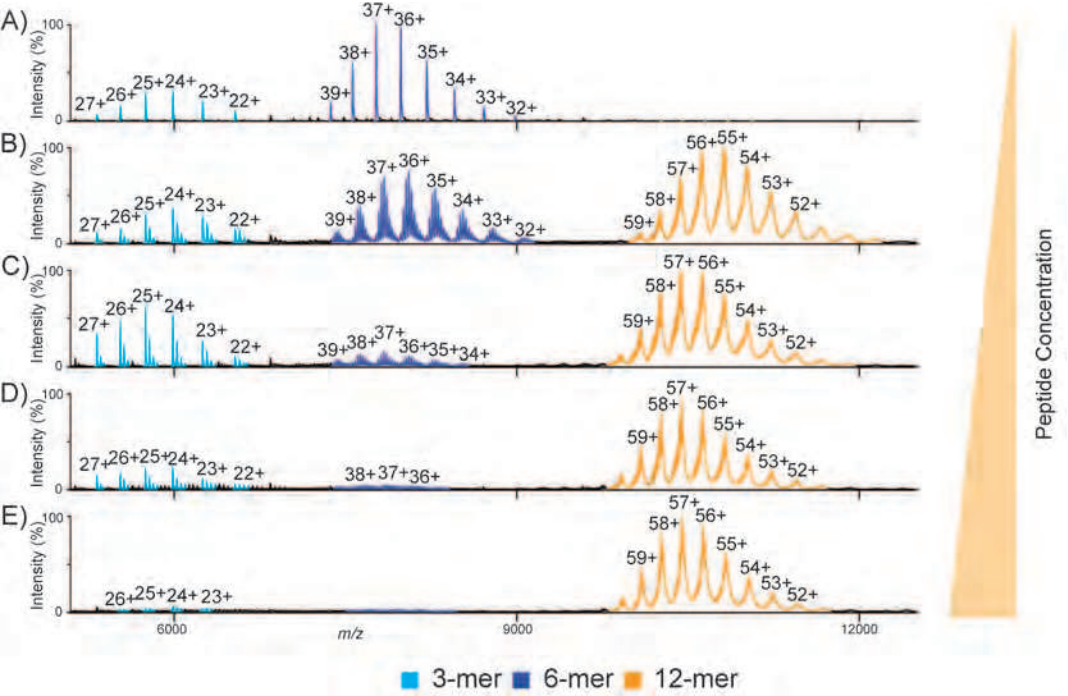
Bowl-Shaped

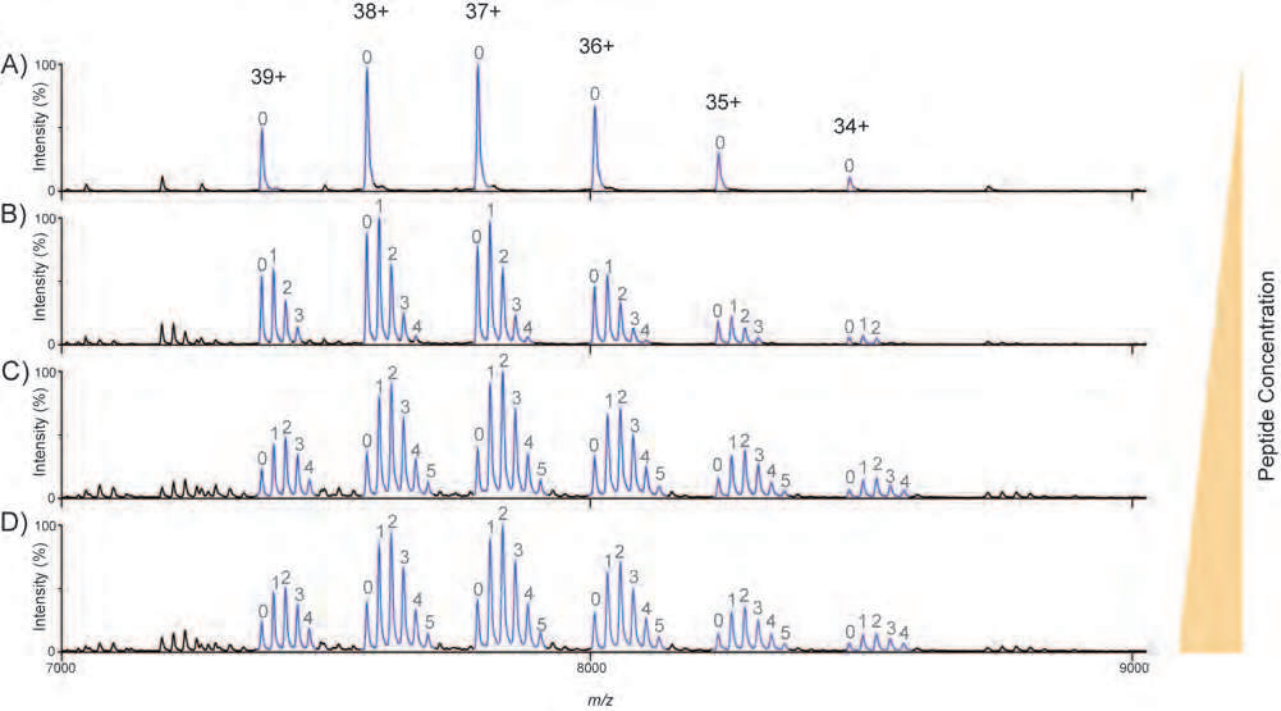
Cage-like

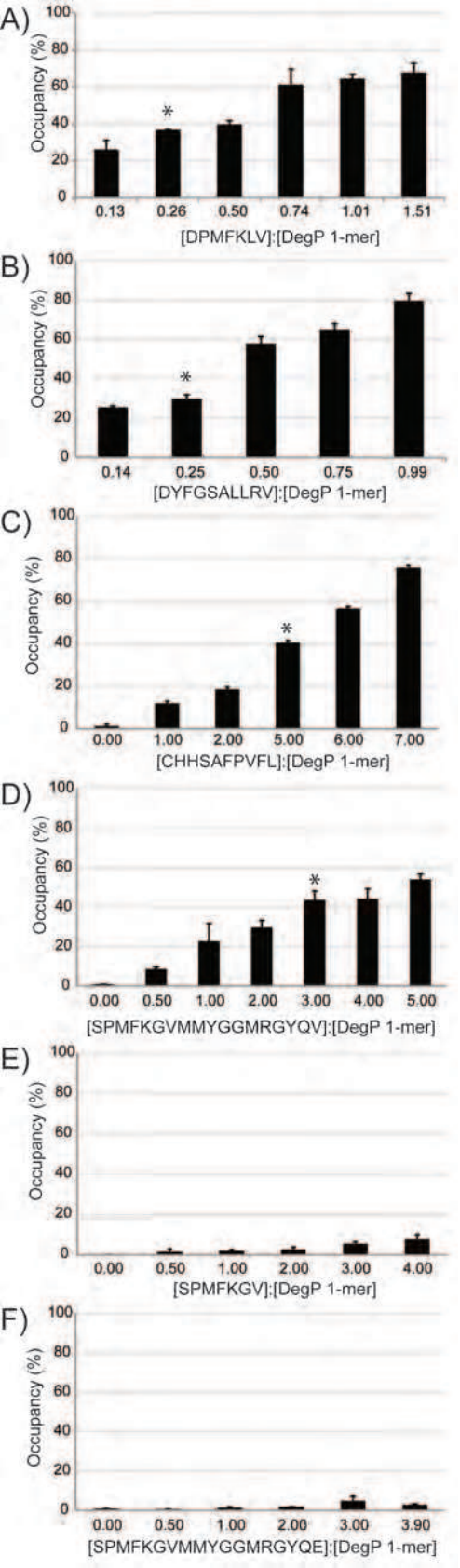


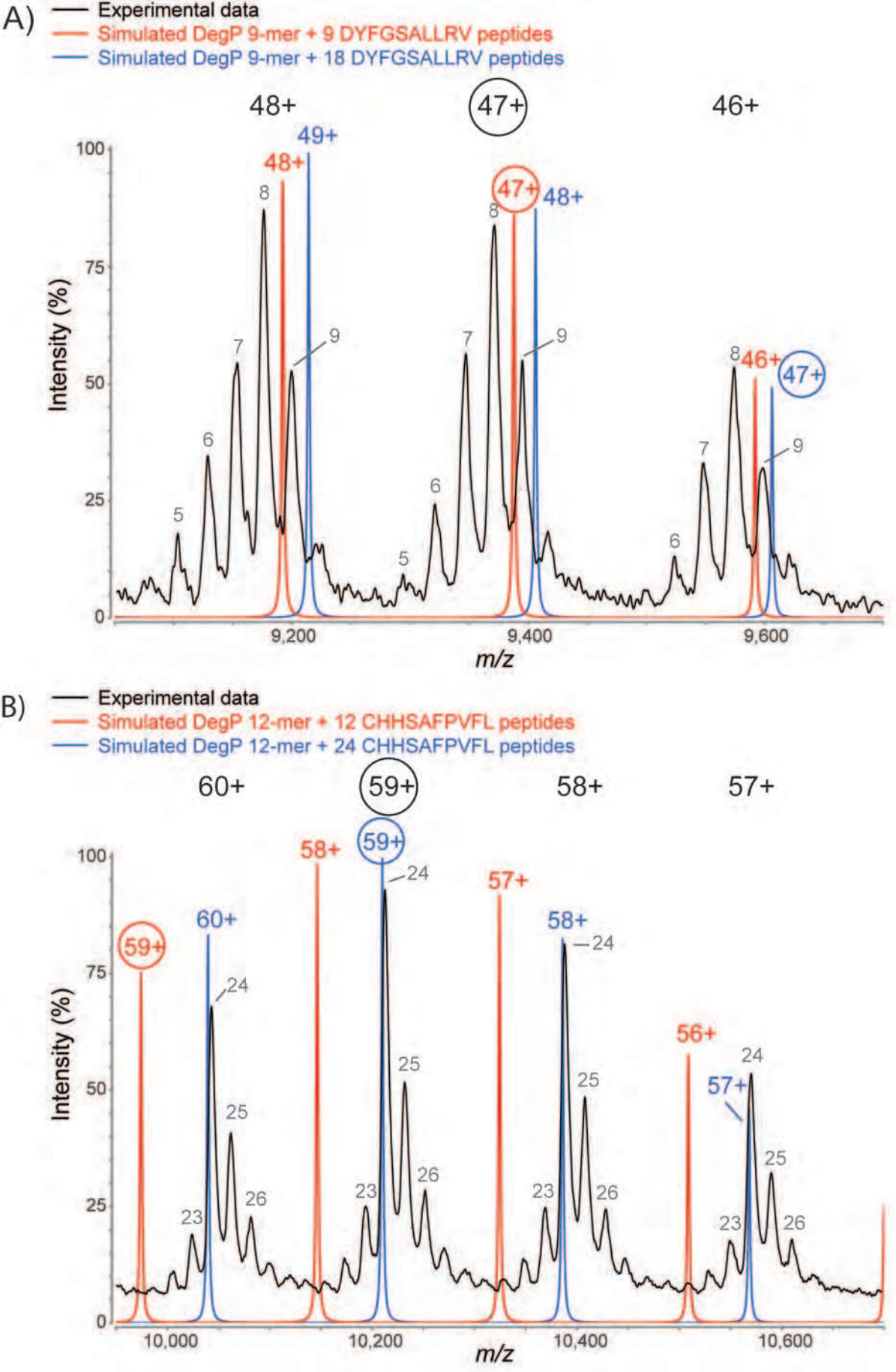


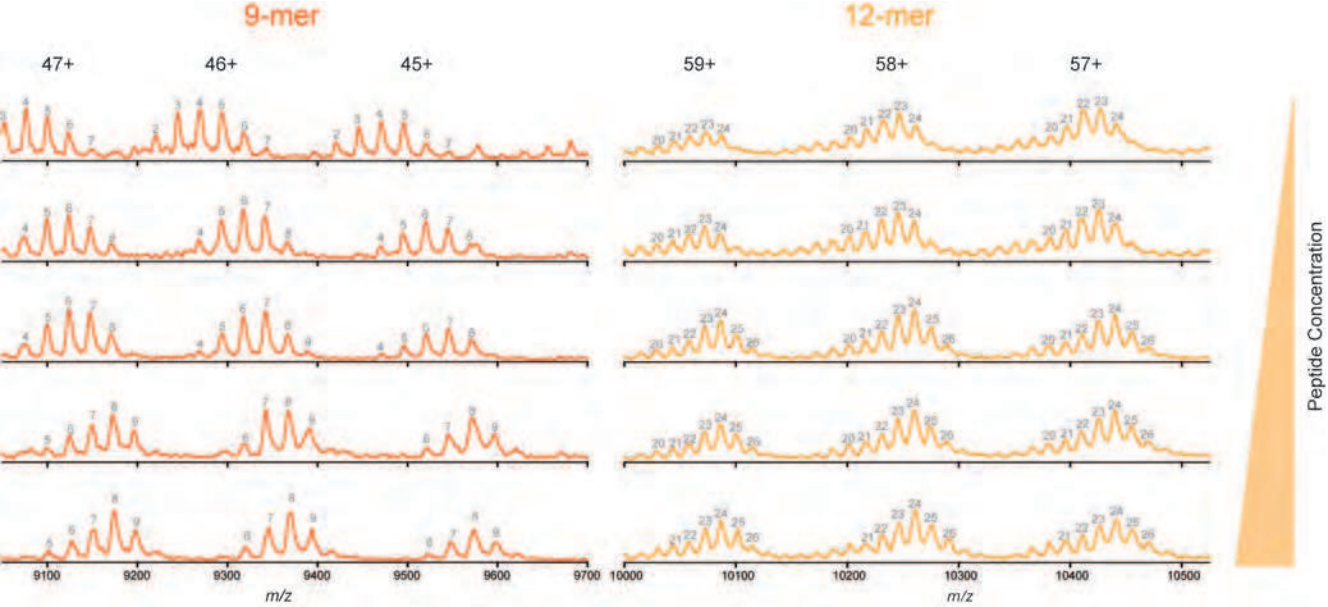


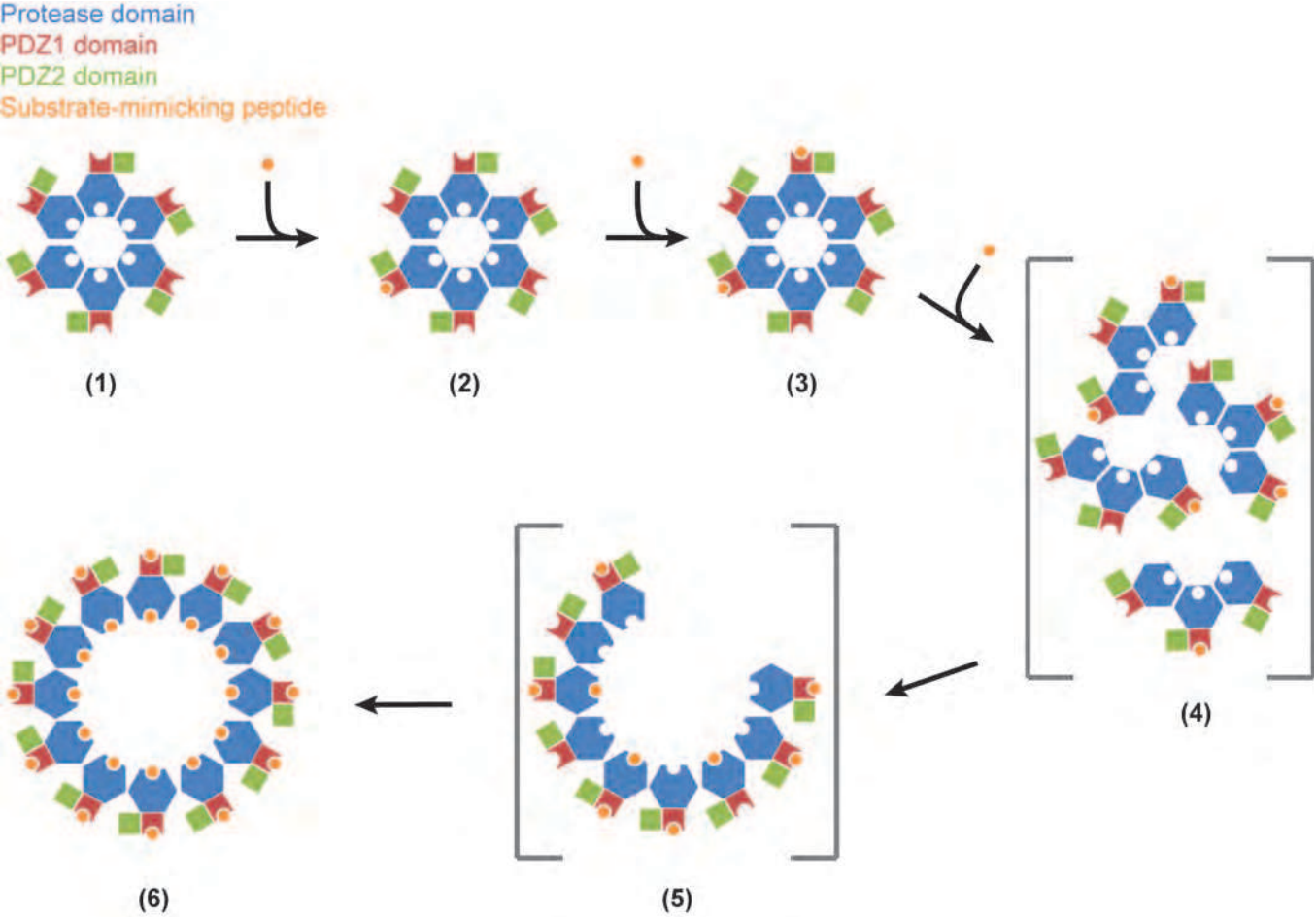

A)
B)
C)
D)
E)
Intensity (%)
100
0
27+ 26+ 25+ 24+ 23+ 22+
39+ 38+ 37+ 36+ 35+ 34+ 33+ 32+
59+ 58+ 57+ 56+ 55+ 54+ 53+ 52+
6000
9000
12000
m/z
Peptide Concentration
3-mer
6-mer
12-mer


38+
37+
36+
39+
35+
34+
A)
B)
C)
D)
Intensity (%)
100
0
7000
8000
9000
m/z
Peptide Concentration


A)
Occupancy (%)
100
80
60
40
20
0
*
0.13
0.26
0.50
0.74
1.01
1.51
[DPMFKLV]:[DegP 1-mer]
B)
Occupancy (%)
*
0.14
0.25
0.50
0.75
0.99
[DYFGSALLRV]:[DegP 1-mer]
C)
Occupancy (%)
*
0.00
1.00
2.00
5.00
6.00
7.00
[CHHSAFPVFL]:[DegP 1-mer]
D)
Occupancy (%)
*
0.00
0.50
1.00
2.00
3.00
4.00
5.00
[SPMFKGVMMYGGMRGYQV]:[DegP 1-mer]
E)
Occupancy (%)
0.00
0.50
1.00
2.00
3.00
4.00
[SPMFKGV]:[DegP 1-mer]
F)
Occupancy (%)
0.00
0.50
1.00
2.00
3.00
3.90
[SPMFKGVMMYGGMRGYQE]:[DegP 1-mer]


A)
Experimental data
Simulated DegP 9-mer + 9 DYFGSALLRV peptides
Simulated DegP 9-mer + 18 DYFGSALLRV peptides
48+
47+
46+
49+
48+
47+
48+
46+
47+
Intensity (%)
100
75
50
25
0
9,200
9,400
9,600
m/z
B)
Experimental data
Simulated DegP 12-mer + 12 CHHSAFPVFL peptides
Simulated DegP 12-mer + 24 CHHSAFPVFL peptides
60+
59+
58+
57+
59+
58+
59+
60+
57+
58+
56+
57+
Intensity (%)
100
75
50
25
0
10,000
10,200
10,400
10,600
m/z


9-mer
12-mer
47+
46+
45+
59+
58+
57+
Peptide Concentration
9100
9200
9300
9400
9500
9600
9700
10000
10100
10200
10300
10400
10500
m/z
m/z


Protease domain
PDZ1 domain
PDZ2 domain
Substrate-mimicking peptide
(1)
(2)
(3)
(4)
(5)
(6)

Supplemental Information

Peptide	Factor of Activation
DPMFKLV	219
DYFGSALLRV	165
CHHSAFPVFL	7
SPMFKGVLDMMYGGMRGYQV	43.4
SPMFKGV	1.3
SPMFKGVLDMMYGGMRGYQE	1.7

Table S1, related to Table 1. Substrate-mimicking peptide ligands determined via observation of increased proteolytic activity compared to a known non-substrate. The factor of activation was determined by the comparison of pNA substrate turnover with and without the additional substrate-mimicking peptide. Comparison of activation factors with that of a known non-activating peptide substrate (SPMFKGV) was used to assign each peptide. Those peptides that exhibited increased proteolytic activity were classified as substrate-mimicking and assumed to induce oligomeric transitions (DPMFKLV, DYFGSALLRV, CHHSAFPVFL, and SPMFKGVLDMMYGGMRGTQV). The peptide that did not show increased proteolytic activity was classified as a control (SPMFKGVLDMMYGGMRGYQE). The factors of activation listed here are for solutions with a [peptide]:[DegP 1-mer] ratio of 10.

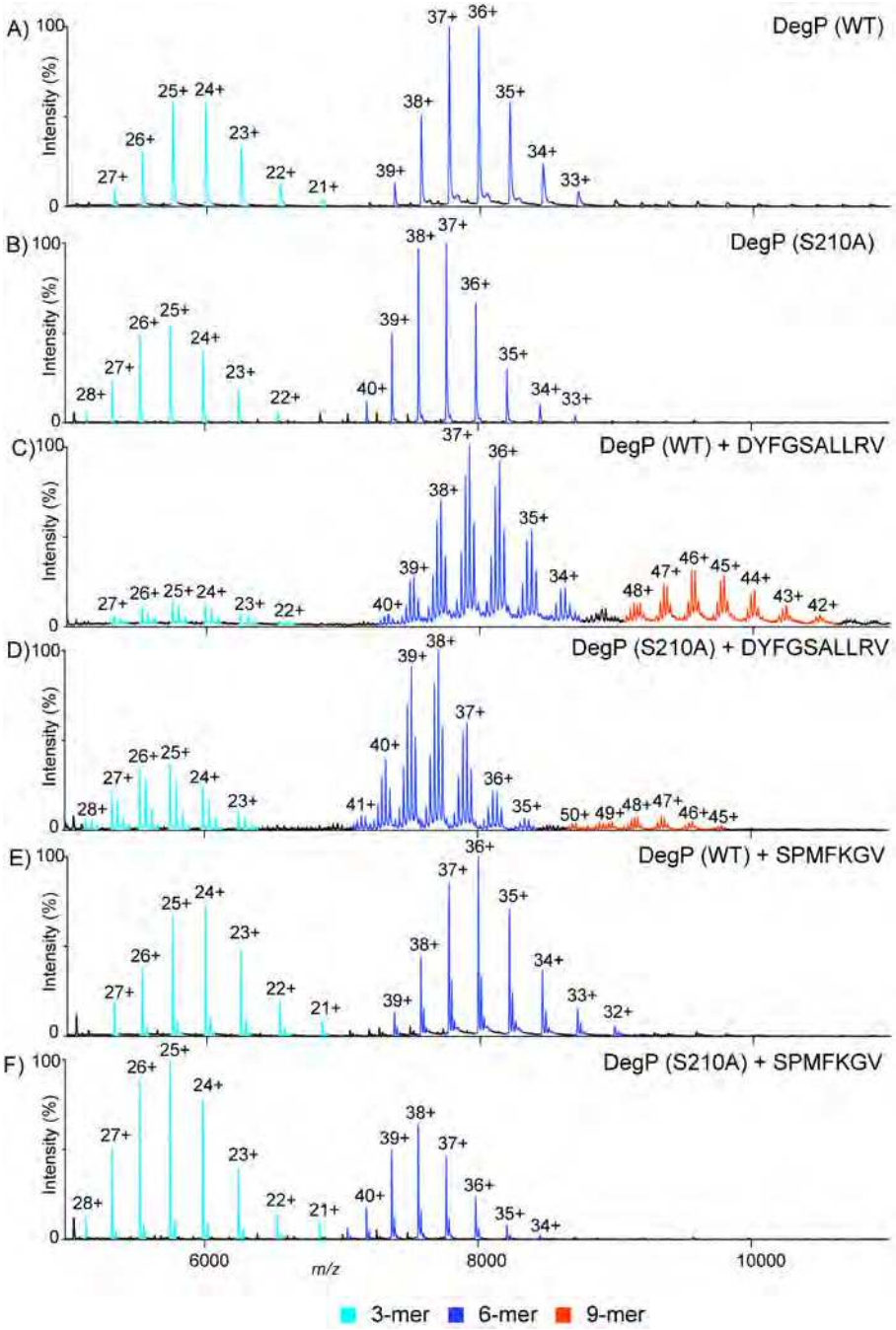


Figure S1, related to Figure 2 and Table 1 Native MS spectra of DegP WT (a, c, e) and DegP S210A (b, d, f) with no peptide substrate added (A, B), with the substrate-mimicking peptide ligand DYFGSALLRV at a [peptide]:[DegP 1-mer] ratio of 0.98 (C, D), and with the control peptide SPMFKGV at a [peptide]:[DegP 1-mer] ratio of 5.04 (E, F). The various DegP oligomers are color-coded according to the legend, and the masses of the unbound DegP oligomers are: 143,686 Da (WT) and 143,638 Da (S210A) for the 3-mer, 287,372 Da (WT) and 287,276 Da (S210A) for the 6-mer, and 431,057 Da (WT) and 430,913 Da (S210A) for the 9-mer. Similar activity is observed for both the inactive mutant and the wild type.

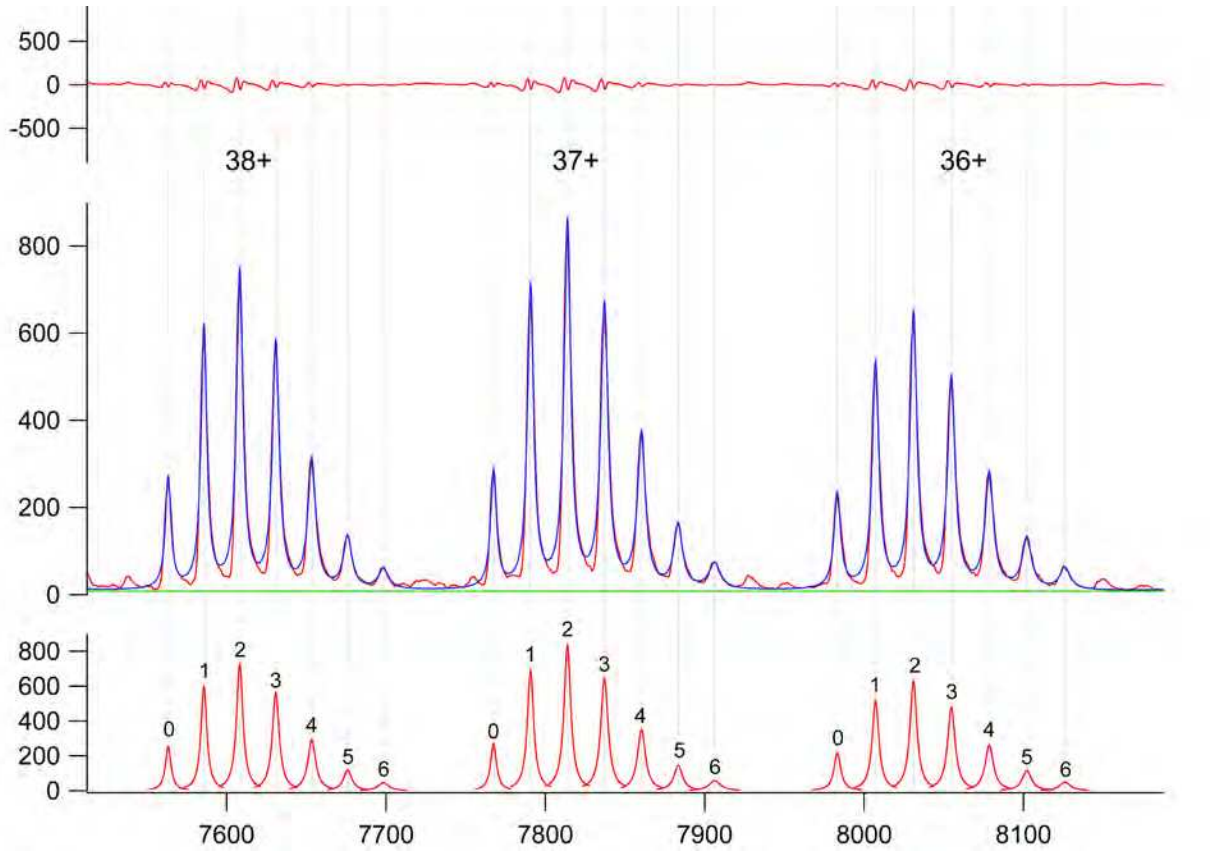


Figure S2, related to Figure 4 Illustrative peak fitting of a native MS spectrum using Igor Pro V6.22. In this example, the fitting of the 36+ through the 38+ charge states of the DegP 6-mer with the peptide ligand DPMFKLV is shown. The top spectrum represents the residuals between the experimental and fitted spectrum. The middle spectrum is an overlay of the composite fitted peaks (blue) and the experimental data (red). The bottom spectrum shows the individual Lorentzian peaks used to create the fitted spectrum. The charge states are labeled, and the number of peptides bound is indicated above each individual Lorentzian peak.