Interplay of PDZ and protease domain of DegP ensures efficient elimination of misfolded proteins

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Aberrant proteins represent an extreme hazard to cells. Therefore, molecular chaperones and proteases have to carry out protein quality control in each cellular compartment. In contrast to the ATP-dependent cytosolic proteases and chaperones, the molecular mechanisms of extracytosolic factors are largely unknown. To address this question, we studied the protease function of DegP, the central housekeeping protein in the bacterial envelope. Our data reveal that DegP processively degrades misfolded proteins into peptides of defined size by employing a molecular ruler comprised of the PDZ1 domain and the proteolytic site. Furthermore, peptide binding to the PDZ domain transforms the resting protease into its active state. This allosteric activation mechanism ensures the regulated and rapid elimination of misfolded proteins upon folding stress. In comparison to the cytosolic proteases, the regulatory features of DegP are established by entirely different mechanisms reflecting the convergent evolution of an extracytosolic housekeeping protease.

allosteric regulation | chaperones | HtrA | protein quality control | molecular ruler

Proteases not only serve essential housekeeping functions by removing damaged or misfolded proteins but also play important regulatory roles (1). *Escherichia coli* contains several cytoplasmic proteases that recognize and degrade abnormally folded proteins. The biochemical and structural features of these ATP-dependent proteases have been studied extensively (2). However, relatively little is known about the molecular mechanisms of proteases that are responsible for the degradation of nonnative proteins in the periplasmic compartment of bacteria and that constitute the frontline of quality control factors dealing with protein folding stress. Such protective function has been attributed to the heat-shock protein DegP, which is an essential factor of *E. coli* for survival at elevated temperatures (3, 4). In addition to its protease activity, DegP has a general chaperone function and can switch between these dual functions in a tightly regulated manner (5, 6).

DegP is a member of the widely conserved family of HtrA proteins (7). HtrAs exhibit a modular architecture combining a protease domain adopting a chymotrypsin-like fold and one or more C-terminal PDZ domains, which are protein–protein interaction domains typically binding to the three to four C-terminal residues of target proteins (8, 9). When linked to a protease, PDZ domains often inherit important mechanistic functions, presenting specific substrate proteins to the catalytic domain, as observed for the tail-specific protease (Tsp) (10). The PDZ domain of Tsp recognizes the 11-aa SsrA tag, an earmark of misfolded proteins resulting from stalled translation, and subsequently initiates digestion of the captured protein (11). Similarly, the PDZ domain of *E. coli* DegP recognizes several C-terminal sequence motifs that are derived from the SsrA tag and the PapE pilin C terminus (12, 13). Although no clear consensus motif is evident, all recognized sequences have a small C-terminal residue such as alanine or serine. However, many protein substrates that are cleaved with high efficiency by DegP *in vitro* and *in vivo* do not fit into this scheme, and thus the absolute requirement of the PDZ domain for substrate recruitment remains to be understood.

Another important regulatory feature of protein quality control is to ensure that proteolytic sites only can be accessed by polypeptides destined for destruction. Well characterized examples are the proteasome, tricorn, and Clp proteolytic complexes (14–16). Although these proteases differ in sequence, structure, and mechanism, they structurally converged to form molecular cages. Their subunits associate into oligomeric rings, which stack upon each other, yielding a barrel-like complex with closely packed sidewalls and an axial channel. The proteolytic sites are located within the central cavity and are accessible through the narrow axial pores. Although DegP belongs to the family of cage-forming proteases, its quaternary architecture and multidomain organization are strikingly different (17). For example, the sidewall of the particle is formed by the protease domains, whereas the PDZ domains construct the entry/exit gates that are much wider as in related molecular cages. Furthermore, the proteolytic sites observed in the chaperone form of DegP are present in a distorted, inactive state where substrate binding and catalysis are abolished (17). To understand better how the digestive activity of DegP is restored and to elucidate the precise function of the PDZ domains, we carried out a comprehensive biochemical analysis of the protease function of DegP guided by its 3D structure.

Results

DegP Degrades Proteins Processively. Whereas classical proteases such as chymotrypsin release their substrates after a single proteolytic cut, most cage-forming proteases digest their substrates processively by introducing multiple cuts before they attack another protein molecule. Both types of proteases can be easily distinguished by monitoring their cleavage pattern. The product pattern of single-cut proteases changes with time; the pattern of processive proteases remains constant. To investigate the processivity of DegP, we used the denatured forms of α-lactalbumin (LA, 16 kDa), malate dehydrogenase (MDH, 36 kDa), and citrate synthase (CS, 52 kDa) as model substrates and tracked the appearance of cleavage products by reverse-phase HPLC (Fig. 1*A*). The HPLC profiles demonstrated that DegP generates a large number of different peptide fragments by cleaving substrates at multiple sites, even in the presence of large amounts of unprocessed protein. Furthermore, the relative heights of the individual product peaks did not change with time, indicating that DegP degrades misfolded substrate proteins in a processive manner. Consistently, SDS gel

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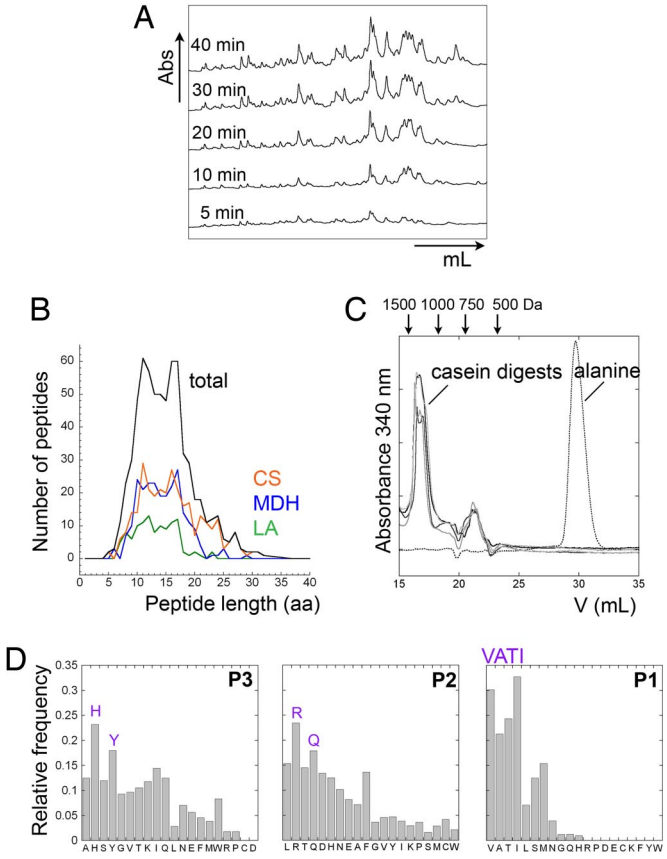

Fig. 1. Proteolytic properties of DegP. (*A*) Time course of the degradation of CS. Samples containing 10 μM CS and 2 μM DegP protease were incubated at 43°C, and the product pattern was analyzed by HPLC at the time points indicated. Analogous elution profiles were obtained for LA and MDH (data not shown). (*B*) Size distribution of peptide products generated by DegP. The completed digests of CS, MDH, and LA were analyzed by mass spectrometry. The sequence coverage was 76% for CS, 80% for MDH, and 83% for LA, and repeated digests yielded comparable results. (*C*) Analysis of cleavage products generated from methylated casein. The solid black and gray lines represent absorbance traces at 340 nm of four casein digests and the dotted line the alanine standard. Molecular markers are indicated. (*D*) Sequence analysis of the identified peptides resulting from CS, MDH, and LA degradation revealed the specificity of DegP for the P3, P2, and P1 positions. The bar diagram illustrates the relative frequencies of the 20 amino acids.

electrophoretic analysis of the degradation trials did not reveal significant amounts of high-molecular-mass intermediates at any time (Fig. 3*A*).

DegP Degrades Misfolded Proteins into Oligopeptides of 9–20 Residues. To determine the identity and size distribution of the degradation products, we prepared complete digests of CS, MDH, and LA and examined cleavage products by mass spectrometry. We identified 124 peptides of CS, 150 peptides of MDH, and 111 peptides of LA ranging in length from 6 to 22, 6 to 30, and 7 to 22 residues, respectively. The size distribution of product peptides was strikingly similar for all substrate proteins, displaying a mean size of 13–15 residues (Fig. 1*B*). Notably, the distribution of cleavage products appeared to be bimodal with maxima at 12 and 17 residues, possibly reflecting two alternative cleavage events as discussed later. Overall 80% of the identified products had a length between 9 and 20 residues, and only 7% of the peptides were shorter than 8 residues. Thus, DegP produces significantly longer degradation products than other processive proteases such as the proteasome and the Clp complexes (5–8 residues). The remarkable absence of short oligopeptides could be due to detection limitations of the mass spectrometry approach. To exclude this possibility, we degraded methylated casein and modified the newly formed N termini by trinitrophenylation. Size-exclusion chromatography of the labeled digestion products confirmed that hardly any peptide with less than five residues (≈500 Da) is generated by DegP (Fig. 1*C*).

Cleavage Specificity of DegP. The specificity of classical serine proteases is mainly defined by the S3, S2, and S1 specificity pockets anchoring the P3, P2, and P1 residues of substrate proteins that are cleaved after P1. To elucidate the substrate specificity of DegP, we performed a sequence analysis of the identified cleavage products (Fig. 1*D*). The observed frequencies of amino acids in the P1 position indicated a strong preference for small hydrophobic residues (Val, Ala, Thr, or Ile), whereas the P2 position did not discriminate between polar or hydrophobic residues. Particularly arginine and glutamine were relatively enriched in comparison to their overall abundance. Similarly, at the P3 position tyrosine and histidine were present in a higher percentage than expected. On the basis of these findings, we aimed to design a model peptide that could be used in a colorimetric activity assay. We coupled the *p*-nitroaniline (pNA) chromophore to the C-terminal end of a series of oligopeptides, which varied in length from 7 to 18 residues. Although the great majority of peptide products generated by DegP had a length of 10–19 residues, surprisingly only the shortest peptide, a heptapeptide with the sequence SPMFKGV-pNA, was cleaved. Characterization of the kinetic parameters of the synthetic peptide (K_M = 3 mM, k_{cat} = 0.5 s^{-1}) (6) indicated that the pNA substrate was poorly cleaved by DegP as compared to other serine proteases.

PDZ and Protease Domain Collaborate During Peptide Cleavage. The failure of DegP to degrade synthetic pNA substrates that matched the observed cleavage products led to the presumption that the C terminus of peptide substrates is essential for efficient binding and/or hydrolysis. On the basis of the available biochemical and structural information for HtrA PDZ domains, we therefore hypothesized that the C terminus of substrates is bound by the first PDZ domain (PDZ1) of DegP, thereby facilitating cleavage of an upstream sequence at a distant active site. To test this hold-and-bite model, we synthesized various peptides with different combinations of P (cleavage) and Z (anchoring, PDZ1 binding) sequences (Fig. 2*A*). First, a model peptide was synthesized containing sequences at the P (ALV) and Z sites (YQV) that were expected to be recognized by the protease and PDZ1 domain of DegP, respectively. To account for the preferred peptide length of 16 residues, the P and Z sites were connected by the linker segment LDMMYGGMRG that corresponds to a noncleavable segment of CS. In addition, we synthesized peptides in which the valine residues in the P1 and Z1 positions were replaced by glutamate (Fig. 2*A*). The affinity of the individual peptides for the PDZ domain was measured by isothermal titration calorimetry (ITC). The ITC data suggested that binding to DegP entirely depends on the C-terminal Z motif. All peptides with the YQV C terminus bound to DegP with similar affinities, having K_D values between 10 and 30 μM (Fig. 2 *A* and *B*). In contrast, replacement of the valine at the C terminus (Z1) by glutamate abolished the interaction with DegP, showing the crucial importance of the terminal valine residue for peptide recognition. Variations in Z2 and Z3 weakened the interaction with DegP but still allowed anchoring of the substrate peptide, as was shown for peptides having a YAV, YEV, or AQV motif in the Z position (data not shown). Subsequently, we analyzed how the synthetic peptides were cleaved by DegP (Fig. 2*A* and 2*C*). When using the ALV sequence as the cleavable P site, only the peptide with the YQV C terminus was degraded. Mass spectrometry and HPLC analysis confirmed that the peptide was cleaved after the valine of the ALV P-site. Replacement of the valine at the P1

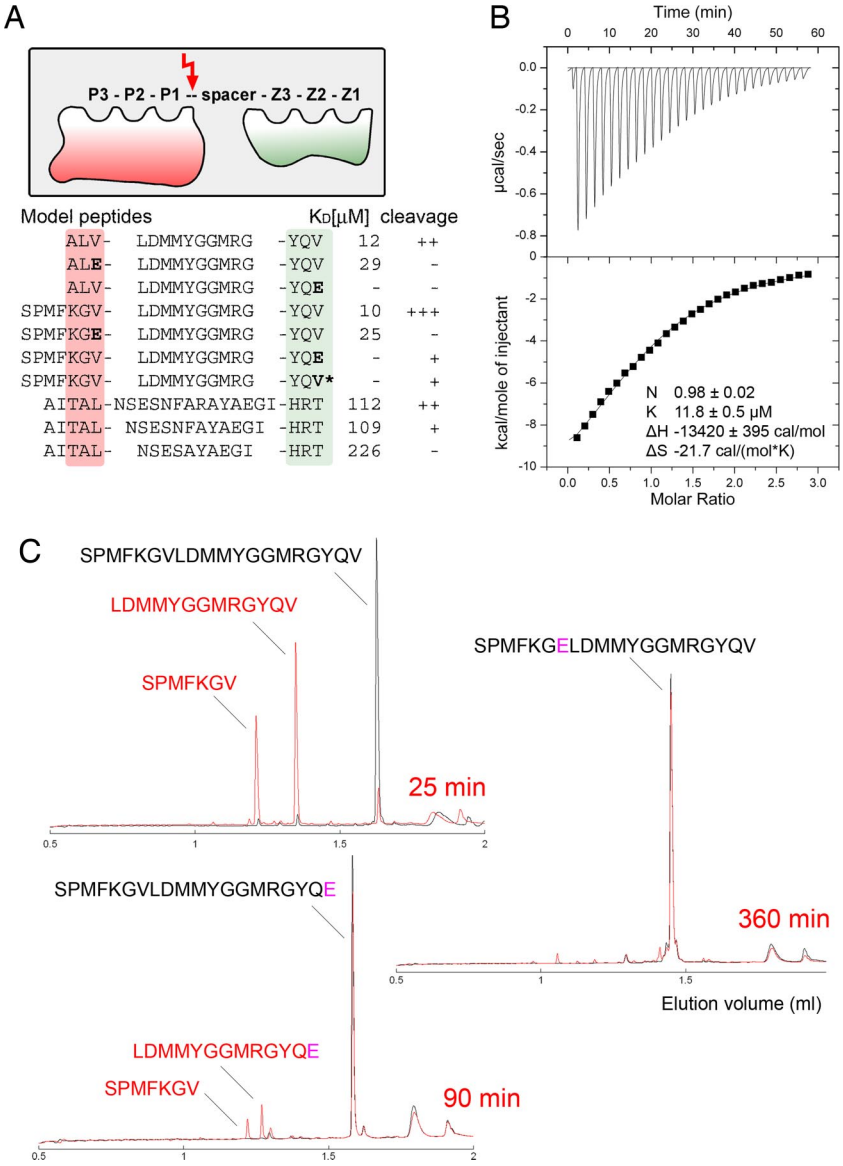

Fig. 2. Bipartite degradation system of DegP. (*A*) Schematic presentation of the bipartite degradation system and the nomenclature used. (*Upper*) As described in the text, we distinguish between the cleavable P motif (P3, P2, or P1; shown in red) and the anchoring Z motif (Z3, Z2, or Z1; shown in green). (*Lower*) A summary of the characterization of model peptides. K_D values were determined by ITC measurements, whereas cleavage susceptibility was estimated by HPLC. In cases where peptides were cut by DegP, the indicated cleavage sites were identified by mass spectrometry. (*B*) ITC measurement of the binding of the ALV-LDMMYGGMRG-YQV peptide to $DegP_{S210A}$. (*Upper*) Ten-microliter aliquots of the peptide (300 μM) were injected into the sample cell containing 30 μM DegP. (*Lower*) The area under each peak was integrated and plotted against the molar ratio of peptide/DegP inside the sample cell. The black line represents the fit to a binding isotherm assuming one binding site per protomer. (*C*) HPLC analysis of SPMFKGV/E-LDMMYGGMRG-YQV/E peptide cleavage by DegP (black, substrate; red, products after indicated time). Peptides yielding the distinct peak fractions were identified by mass spectrometry.

position by glutamate completely abolished cleavage, although the ALE peptide was still bound to DegP. Together these findings indicate that the P1 and Z1 positions are essential for the cleavage reaction and substrate binding, respectively.

Next, we fused the linker segment and the C-terminal Z motif (LDMMYGGMRG-YQV) to the SPMFKGV sequence that was initially identified as a poorly cleavable pNA substrate. In agreement with the hold-and-bite model, the elongated peptide was binding ≈35-fold tighter to DegP than the chromogenic substrate [$K_M \approx 3$ mM (6)] and was cleaved rapidly at the predicted P1 site (Fig. 2*C*). Similarly to the other model peptides, replacement of the P1 valine by glutamate abolished cleavage but still allowed binding of the peptide. The K_D value was slightly increased from 10 to 25 μM, indicating that the P-site contributes little to attaching peptide substrates to DegP. However, a remarkable difference was observed when replacing the valine in the Z1 position with a glutamate. Although binding to DegP could not be detected by ITC measurements, the peptide with the YQE C terminus was cleaved, although 30 times slower than the original peptide (Fig. 2*C*). Therefore, capturing of substrate peptides via their C-terminal Z motif is not an absolute requirement for the cleavage reaction but clearly facilitates proteolysis by DegP.

To analyze the impact of the linker region on the cleavage reaction, we synthesized a CS-like peptide and derivatives thereof that contained linker segments shortened successively by two residues, omitting initially the two central residues (see AITAL peptides in Fig. 2*A*). HPLC analysis of the cleavage reactions indicated that the shortening strongly impaired cleavage by DegP.

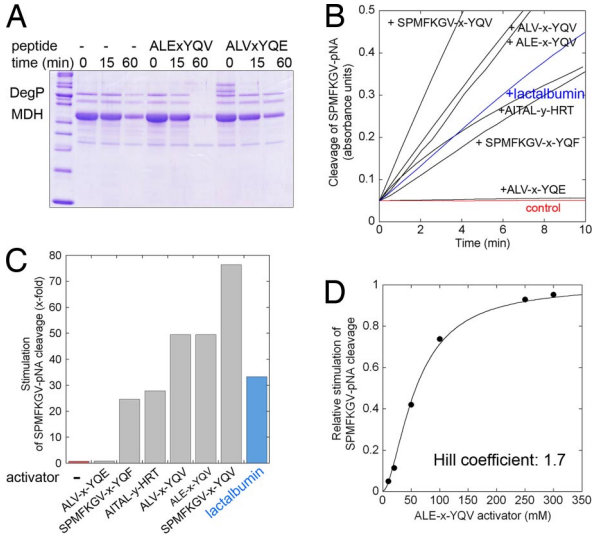

Fig. 3. Allosteric activation of DegP. (*A*) SDS/PAGE analysis of the cleavage of 20 μM concentration of unfolded MDH by 1 μM DegP in the presence of different model peptides at 200 μM (with the linker *x*, LDMMYGGMRG). (*B*) Cleavage of 0.5 mM SPMFKGV-pNA by 2 μM DegP in the presence of different model peptides at 0.3 mM and denatured lactalbumin. Kinetics were recorded by measuring the change of absorbance at 405 nm. The individual peptides are represented by their N-terminal sequence, the conserved linker segment (*x*, LDMMYGGMRG; *y*, NSESNFARAYAEGI), and their C-terminal motif. (*C*) Activation potential of different allosteric activators in relation to the control reaction, where cleavage of the pNA substrate was carried out in the absence of activator (same nomenclature as in *B*). (*D*) Stimulation of DegP-catalyzed substrate cleavage by different amounts of the ALE-*x*-YQV activator.

Only the peptide that was shortened by two residues was degraded, although the rate of cleavage was reduced by a factor of five. Shortening the same linker segment from 14 to 10 residues completely abolished proteolysis, pointing to the existence of a molecular ruler in the DegP protease that is required to efficiently bind and cleave substrates.

To study the direct involvement of PDZ1 in substrate presentation, we synthesized the most efficiently cleaved peptide substrate SPMFKGV-LDMMYGGMRG-YQV with an amide $CONH_2$ group as its C terminus. As it is known that PDZ domains capture their peptide ligands by coordinating the C-terminal carboxylate group, such a peptide was not expected to bind to DegP. Although the corresponding COOH-containing peptide was bound with a K_D of 10 μM, ITC binding studies could not detect any interaction between protein and $CONH_2$-containing ligand. However, when we incubated the amide substrate with DegP and investigated the reaction mixture by HPLC analysis, we could observe a slow digestion of the substrate. The cleavage reaction was ≈20 times slower than that of the COOH-containing substrate and thus in the same order of magnitude as the peptide with the YQE C terminus, which also did not bind to DegP. Together these observations suggest that PDZ1 functions as the primary substrate binding site that captures the C-terminal Z-motif of misfolded proteins and reaction intermediates and presents the N-terminal segment to the protease domain.

Allosteric Coupling Between Substrate Binding and Proteolysis. On the basis of the fact that substrate tethering to PDZ1 is essential to form a productive enzyme–substrate complex, peptides with hydrophobic C termini and noncleavable N termini were expected to function as competitive inhibitors of DegP. However, the ALE-LDMMYGGMRG-YQV peptide that fulfills these criteria had a mild stimulatory effect on the rate of degradation of misfolded protein substrates (Fig. 3*A*). This finding suggests that the noncleavable ALE peptide might function as an allosteric activator like in the homologous DegS protease, where peptide binding to the PDZ domain triggers a series of conformational changes in the catalytic domain that stimulate peptidase activity (18, 19). To explore further the activating effect of the ALE peptide, we preincubated DegP with saturating amounts of the potential activator and measured the rate of degradation of the "poor" SPMFKGV-pNA substrate that lacks a C-terminal anchoring motif. Remarkably, the ALE peptide accelerated proteolysis 50-fold (Fig. 3 *B* and *C*). Dose–response experiments with the ALE peptide revealed sigmoidal kinetics and a Hill coefficient of 1.7, implying positive cooperativity in the proteolytic reaction (Fig. 3*D*). Hence, binding of the ALE peptide not only stimulates the protease activity of one subunit but also facilitates processing of peptide substrates in neighboring subunits of the DegP oligomer. When we tested the stimulating effect of peptides having a YQF, YQV, or HRT C terminus, we observed that peptides binding to PDZ1 generally increased the rate of degradation by a factor of 25–75. In contrast, peptides that did not bind to the PDZ domain did not affect cleavage of the pNA peptide. To test the activation potential of misfolded proteins, we measured the digestion of SPMFKGV-pNA in the presence of denatured lactalbumin. Remarkably, lactalbumin exhibited an activation potential similar to that of the model peptides and stimulated cleavage 35-fold. Together these findings demonstrate the dual role of PDZ1 tethering substrates to DegP as well as allowing allosteric activation of protease function.

Discussion

Because protein misfolding and aggregation represent a severe hazard to all cells, molecular chaperones and proteases have to carry out protein quality control in each cellular compartment. In contrast to the energy-dependent quality control factors of the cytosol, the mechanistic properties of proteases and chaperones operating in other compartments are largely unknown. Biochemical analysis of the periplasmic DegP protease uncovered an intriguing bipartite degradation system that allows efficient degradation of misfolded proteins in a tightly regulated manner. The present findings suggest that DegP represents a "simple" version of the proteasome that in contrast to its cytosolic counterpart exclusively acts on misfolded proteins and follows unique regulatory and mechanistic strategies.

Molecular Ruler. Several proteases perform fairly precise measurements of their substrates. For example the proteasome (20) and ERAP1 (21) trim peptides for antigen presentation, whereas Clp proteases from *E. coli* (22) generate short oligopeptides for further rounds of digestion. The DegP protease is another member of the diverse family of ruler enzymes. Its molecular features have been elucidated in this study, leading to a simple working model of how the PDZ1 domain and the proteolytic site cooperate to shape cleavage products (Fig. 4*A*). We show that polypeptides are recognized by PDZ1, which anchors the hydrophobic C terminus of misfolded substrate proteins or cleavage intermediates. This binding event allows the presentation of the substrate's downstream segment to the proteolytic site while, in parallel, the protease activity is switched on. The active site mainly recognizes substrates via the P1 residue that is preferentially a valine, alanine, threonine, or isoleucine. These four amino acids belong to the most abundant residues in proteins, and thus the promiscuity of DegP is well suited to introduce multiple cuts in a protein chain. The geometric arrangement of protease and PDZ1 domains matches the observed size distribution of product peptides remarkably well. The shortest distance to reach the P1 from the Z1 site is ≈55 Å within one DegP protomer and 40 Å to the neighboring subunit, distances that could be bridged by peptides with 16 and 12 residues, respectively. Therefore, substrates seem to be bound in precise orientations to the PDZ1 and protease domain, requiring linkers of specific lengths (Fig. 4*A*). The bimodal product length distribution with peaks at 12

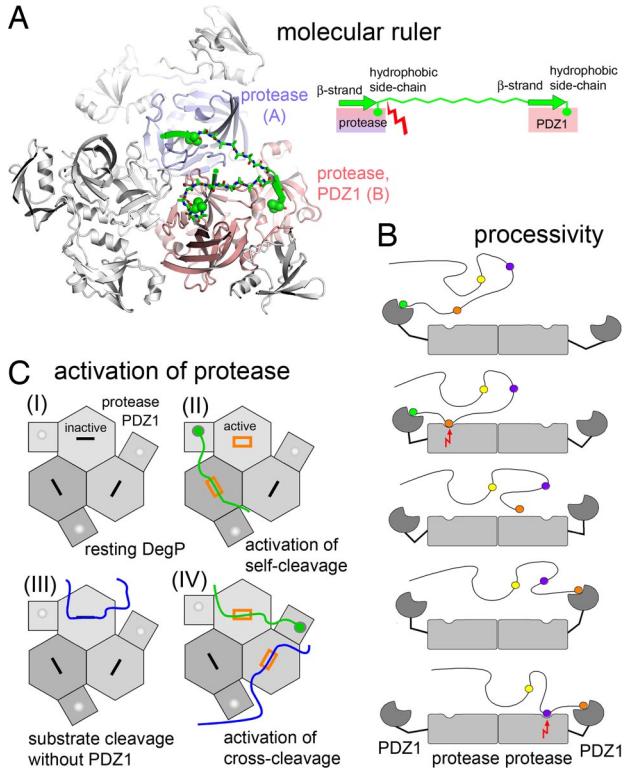

Fig. 4. Mechanistic features of DegP. (*A*) Molecular ruler of DegP. (*Left*) Shown is a DegP trimer with two modeled peptide ligands (green) bound to two different active sites. Protease and PDZ1 domains of one subunit are shown in red (required linker length, 13 residues) and the protease domain of a neighboring subunit in blue (required linker length, 8 residues). (*Right*) Binding to the active site as well as to PDZ1 depends on β-sheet augmentation and the presence of a hydrophobic P1/Z1 residue. (*B*) Model of how PDZ1 and protease domains cooperate to degrade substrate proteins in a processive manner. Illustrated is a schematic side view of one DegP trimeric ring showing two subunits (protease, light gray; PDZ1, dark gray). A substrate protein with several potential cleavage sites (colored spheres) is first bound and cleaved by one subunit. As the cleavage (protease) and docking (PDZ1) sites obtain similar binding specificities, the introduced cut generates a substrate for the next digestive cycle that can bind to a nearby PDZ domain (the orange sphere illustrates possible binding to the adjacent subunit). (*C*) Allosteric activation of the protease. Illustrated is a schematic top view of one DegP trimeric ring (protease, hexagon; PDZ1, square). Proteolytic sites (inactive, black; activated, orange) and PDZ binding sites are indicated. (*I*) Resting DegP, where proteolytic sites occur in a rather inactive state. (*II*) Peptides tethered to PDZ1 activate DegP and thus autostimulate their own cleavage. (*III*) Binding of substrate peptides without involvement of PDZ1 will not activate DegP, and thus proteolysis will proceed highly inefficiently (e.g., pNA substrate or priming cleavage of protein substrates). (*IV*) The DegP protease functions cooperatively. Binding of activating peptides to one PDZ1 domain stimulates cleavage of other peptides at a remote active site. The Hill coefficient of 1.7 suggests that two of three subunits are activated by one PDZ binding event.

and 17 residues and the weak cooperativity with a Hill coefficient of 1.7 further support such an alternating processing by neighboring catalytic domains. However, it should be noted that DegP is also capable of attacking internal peptide segments without the support of PDZ1 as suggested by the cleavage of the pNA-heptapeptide and the endoproteolytic activity against model peptides that are not recognized by the PDZ domains. Peptide products generated by this initial cut might use in further degradation cycles the more effective bipartite degradation system.

Processive Degradation of Misfolded Proteins. Analysis of the degradation pattern demonstrated that DegP degrades misfolded proteins in a processive fashion, retaining substrates until they are cleaved into small oligopeptides. In contrast to the proteasome and Clp proteolytic complexes, which function like a molecular sieve measuring the physical size of polypeptides that can be released (23, 24), DegP multimers contain wide channels for substrate entry and product release. Thus, DegP has to employ a different processive mechanism. Notably, the peptide binding sites of the PDZ1 and catalytic domains exhibit strikingly similar substrate specificities. Both sites anchor the protein main chain of the ligand by β-sheet augmentation and have a clear preference for small hydrophobic residues at the C terminus. This consensus might enable the PDZ domains to capture peptide fragments released from the proteolytic sites via their newly generated C termini and to prepare them for the next degradative cycle (Fig. 4*B*). This "hold-bite-and-rebind" processive mechanism, in which the PDZ domains exert an equivalent function as the regulatory subunits of energy-dependent proteases, would prevent the premature release of "toxic" degradation intermediates that could accumulate as large aggregates in the periplasm and interfere with cellular structure and function.

Allosteric Activation by Substrates. In addition to its substrate tethering function, the PDZ1 domain is critical for protease activation. This regulatory principle was first established for the DegS protease, the central regulator of the bacterial unfolded protein response (25). Upon folding stress, DegS is activated by the C termini of mislocalized outer membrane proteins (26), which interact with the PDZ domain and induce the remodeling of the catalytic domain, leading to protease activation (18, 19) and subsequent cleavage of the anti-sigma factor RseA (27). Our data suggest that the DegP activity is controlled by a similar activation mechanism given that binding of an allosteric peptide to PDZ1 enhances the peptidase activity of the catalytic domain by switching the observed distorted active site (17) into an enzymatically competent conformation (Fig. 4*C*). However, in contrast to DegS, the activating allosteric ligand also represents a potential substrate molecule and thus enhances its own degradation. We propose that efficient protein degradation by DegP is accomplished by a positive feedback mechanism, where the initial cut of a substrate accelerates further degradation of the generated peptide fragments. Protein digestion continues as long as peptide fragments are bound to DegP. Upon release of the final cleavage products, DegP returns to the resting state. Furthermore, our data unveil that not only oligopeptides but also misfolded proteins can act as allosteric activators that dramatically stimulate protease activity. Such an activation procedure would ensure the tight regulation of DegP because the switch from the resting to the activated protease would only occur under folding stress conditions.

Implications for HtrA Proteases. HtrA proteases can be divided into two functional classes linked to regulatory proteolysis and protein quality control. The functional divergence is reflected by distinct substrate preferences. Regulatory proteases such as DegS have a clearly defined substrate specificity, whereas housekeeping proteases such as DegP act on a wide range of misfolded proteins. Our data uncovered an important regulatory feature that might be a common characteristic of the entire HtrA family. Regulatory as well as housekeeping HtrA proteases seem to rest in a rather inactive state until they become activated by binding of an allosteric ligand to their PDZ domains. However, the nature of the allosteric activator is different and mirrors the degree of protease specificity. The DegS regulator is selectively activated by ligands with a ϕXF C-terminal motif, a characteristic motif of the C termini of outer membrane proteins signaling the onset of folding stress (19). In contrast, a broad palette of peptide ligands with a rather unspecific XXϕ C-terminal consensus can activate the housekeeping DegP protease. Misfolded proteins and generated cleavage products are therefore capable of keeping DegP active as long as the folding stress persists and target proteins are entirely digested. This regulatory mechanism ensures the rapid and tightly regulated elimina-

tion of misfolded proteins. Functionally related HtrA proteases that also maintain protein homeostasis in extracytosolic compartments might be regulated similarly. For example, it is known that human HtrA1 and HtrA2, which are implicated in protein folding diseases (28, 29), exhibit a consensus in cleavage and PDZ binding specificity (30–32) that would allow a similar interdomain co-working as reported here for DegP. The present work provides excellent leads to address the molecular mechanisms of these human HtrA proteases and to clarify the roles of regulatory and housecleaning HtrAs in general.

Materials and Methods

Protein Purification. Wild-type DegP and $DegP_{S210A}$ were purified through a three-step purification procedure using Ni-NTA, hydroxyapatite, and size-exclusion chromatography as described in ref. 17.

Identification of Small Product Peptides Generated by DegP. Before the cleavage assay, bovine β-casein was reductively methylated (33). Subsequently, the methylated β-casein (0.3 mM) was incubated for 3 h with 6 μM DegP in 25 mM Hepes/NaOH (pH 7.5) and 5 mM $MgCl_2$, and the newly formed peptide N-termini were trinitrophenylated as described in ref. 34. The modified cleavage products were separated on a Superdex peptide column (GE Healthcare) by using 0.2 M Na_2SO_4/25% acetonitrile (pH 3.0) as the mobile phase. The column was calibrated with single amino acids and peptides of different lengths, which were all trinitrophenylated before injection.

Assay of DegP Peptidase Activity by Using the pNA-Chromogenic Substrate. Peptidase activity of DegP was measured in 0.8-ml reaction mixtures containing 0.5 mM SPMFKGV-pNA, 100 mM $NaPO_4$ (pH 7.5), 150 mM NaCl, 4% DMSO, and the respective activating peptides/proteins. The samples were preincubated for 15 min at 37 °C, then DegP was added to a final concentration of 2 μM, and the increase in the absorbance at 405 nm and 37 °C was continuously monitored.

HPLC Analysis of the Cleavage Pattern of Model Peptides and Proteins. The processive degradation of substrate proteins by DegP was analyzed by incubating a solution containing 2 μM DegP in 25 mM Hepes/NaOH (pH 7.5), 5 mM $MgCl_2$ with 3.5 mg/ml LA, 1 mg/ml CS, or 2 mg/ml MDH at 43°C. The degradation of synthetic peptides was monitored by dissolving peptides (300 μM) in a solution containing 100 mM $NaPO_4$ (pH 7.5), 150 mM NaCl, and 3% DMSO. DegP was added to the assay to a final concentration of 7 μM, and the reaction mixture was incubated at 37°C. Samples were taken at certain time points and mixed with an equal volume of 1% TFA to stop the cleavage reaction. The samples were injected onto a 5-μm Jupiter C18 column equilibrated with 0.06% TFA and eluted with a linear gradient of 80% acetonitrile and 0.05% TFA.

Peptide Analysis by Mass spectrometry. The model substrates CS, MDH, and carboxymethylated LA were digested for 3 h as described above. Subsequently, undigested proteins were removed by precipitation with six volumes of cold acetone. After incubation for 12 h at −20 °C, samples were centrifuged for 30 min at 4 °C and 20,000 × *g*. The pellet was discarded, and the acetone was removed from the supernatant in a SpeedVac. Peptides generated by DegP were subjected to sequencing by mass spectrometry on a hybrid linear ion trap/Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT Ultra, (ThermoFisher). For peptide identification, a database of all respective substrate proteins was generated, and the identified masses were searched against this database. The analysis was performed with the probability-based MASCOT software suite, version 2.2.0 (Matrix Science) without specifying any cleavage specificity.

ITC. The thermodynamic values of the interaction between the proteolytically inactive $DegP_{S210A}$ and the different activating peptides were determined by ITC (MCS-ITC; Microcal). All experiments were conducted in overflow mode at 30°C. Approximately 1.4 ml of a solution of 30 μM DegP was placed in the temperature-controlled sample cell and titrated with different model peptides (300 μM), which were loaded in the 300-μl mixing syringe. For all experiments, 100 mM $NaPO_4$ buffer (pH 7.5) supplemented with 150 mM NaCl and 3% DMSO was used as the buffer. Injections of 10 μl of peptide were dispensed into the sample cell by using a 120-s equilibration time between experiments and stirring at 300 rpm. Control experiments employing the identical experimental setup were carried out to measure and to correct the heat of dilution. Ultimately, the data were analyzed with ORIGIN software by following the instructions of the manufacturer.

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