Activation of DegP chaperone-protease via formation of large cage-like oligomers upon binding to substrate proteins

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Cells use molecular chaperones and proteases to implement the essential quality control mechanism of proteins. The DegP (HtrA) protein, essential for the survival of *Escherichia coli* cells at elevated temperatures with homologues found in almost all organisms uniquely has both functions. Here we report a mechanism for DegP to activate both functions via formation of large cage-like 12- and 24-mers after binding to substrate proteins. Cryo-electron microscopic and biochemical studies revealed that both oligomers are consistently assembled by blocks of DegP trimers, via pairwise PDZ1–PDZ2 interactions between neighboring trimers. Such interactions simultaneously eliminate the inhibitory effects of the PDZ2 domain. Additionally, both DegP oligomers were also observed in extracts of *E. coli* cells, strongly implicating their physiological importance.

cryo-electron microscopy | protein quality control | HtrA | PDZ domain | oligomerization

Life depends to a great extent on the function of an extraordinarily large number of proteins, most of which have marginally stable structures and are thus subjected to continuous quality control to keep them in a functionally structural state; and the failure of this, leads to severe diseases (1, 2). Molecular chaperones and proteases, both binding to unfolded substrate (client) proteins, are the two families of proteins that cells generally employ to implement such quality control processes (3, 4). DegP, present in the periplasmic space of *Escherichia coli* cells, is a protein that functions as both, exhibiting the dual protease–chaperone activity in an ATP-independent manner (5–8), making it a unique case for understanding the quality control mechanism of proteins. Homologues of DegP (collectively named HtrA) have been identified in almost all organisms and are believed to function by protecting cells under stress conditions (9).

The DegP protein is composed of a protease domain (within which residues His 105, Asp 135, and Ser 210 make up the catalytic triad) and two PDZ (PDZ1 and PDZ2) domains (10–12). The protease domain is required for both the protease and chaperone activities, while the roles of the PDZ1 and PDZ2 domains are far less defined, although believed to respectively play a role in sequestering the substrate proteins and maintaining the hexameric status (13–15). The crystal structure of the DegP hexamer has been determined but found to be in an inactive form in which two trimeric units staggered together face to face, with the catalytic sites hidden in a central cavity almost completely inaccessible to substrate proteins (12). The crystal structures of bacterial DegS and human HtrA2, both members of the HtrA family and all lacking a PDZ2 domain, were determined in their trimeric forms with the active sites blocked by the surrounding loops or the PDZ domain (16–18). Despite several recent efforts to understand how such inactive hexamers or trimers of HtrA members become activated (16, 19–21), no model has yet achieved general acceptance.

This study was conducted in an attempt to clarify the structural status of DegP functioning as active protease and chaperone. Our combined biochemical and cryo-electron microscopic studies reveal that the DegP hexamers effectively convert to cage-like large oligomers via the pairwise PDZ1–PDZ2 interactions between neighboring trimeric units upon binding to substrate proteins, and such large oligomers represent the long-sought-after forms of DegP that exhibit both protease and chaperone activity. Such a manner of activity modulation allows the DegP protein to remain inactive until such time as substrate proteins become available, thus preventing the potential harmful effect it may cause to the cellular proteins under nonstressful conditions. This activation mechanism of protease, which depends on the homooligomerization triggered by substrate binding and is reversible in nature, is sharply different from those recognized so far, which depend on proteolytic cleavage of protease zymogen precursors and are irreversible in nature.

Results

Large Complexes Are Formed When DegP Is Binding to Substrate Proteins. The protease-deficient DegP(S210A) mutant is expected to form stable intermediates with substrate proteins, which might capture certain features of the active forms of DegP. Results of size-exclusion chromatography (SEC) (Fig. 1*A*) and SDS/PAGE (Fig. 1*B*) analysis reveal the presence of certain large complexes containing both DegP(S210A) and β-casein or unfolded lysozyme (via DTT treatment), the two most commonly used substrate proteins in analyzing the protease activity of DegP (22–24). Similar large complexes were also observed when DegP(S210A) was incubated with DTT-reduced α-lactalbumin or insulin (data not shown).

The size of these large DegP(S210A)-substrate complexes was estimated, via negative staining electron microscopy (EM) (Fig. 1*C*), to be ≈14 nm (with lysozyme) and ≈19 nm (with β-casein), while that of the free DegP(S210A) hexamers was estimated to be ≈12 nm, consistent with what was estimated from crystal structure determination (12). Additionally, these forms of large

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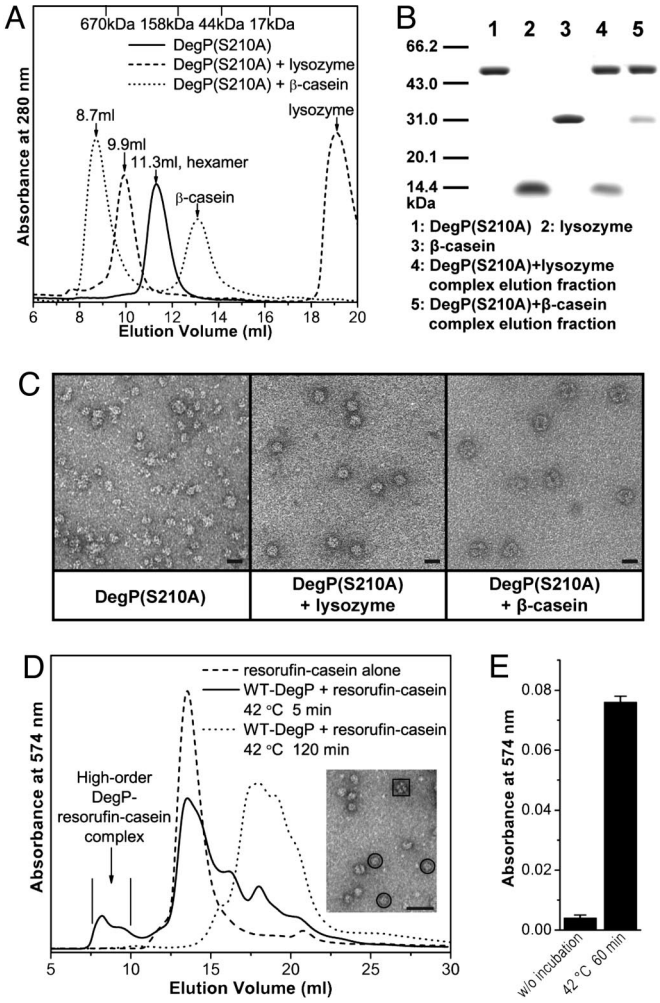

Fig. 1. DegP converts to large complexes in the presence of unfolded substrate proteins. (*A*) The SEC elution profiles for DegP(S210A) in the absence or presence of unfolded lysozyme and β-casein. The eluted positions of DegP hexamer (11.3 ml) and the two large complexes (9.9 ml and 8.7 ml) are indicated by the downward arrows. (*B*) SDS/PAGE analysis of the samples from fractions containing the large forms of DegP(S210A) in complex with lysozyme (lane 4) or β-casein (lane 5). (*C*) Electron micrographs of negatively stained DegP(S210A) hexamers and DegP(S210A)-substrate complexes corresponding to those indicated in *A*. (Scale bars: 20 nm.) (*D*) Transient formation of large complexes while DegP was degrading resorufin-labeled casein. The formation of large complexes containing the resorufin-labeled casein during the degradation process was indicated by the arrow. (*E*) Detection of degraded products from resorufin-labeled casein that was bound in the large complexes of DegP.

complexes were also observed by EM studies [supporting information (SI) Fig. S1] in reaction mixtures in which the aggregation of unfolded proteins was being suppressed by the presence of DegP(S210A) (25).

Such large DegP complexes were also detected when the wild-type DegP was cleaving the resorufin-labeled casein at 42°C for only a short period (5 min), both by SEC and EM analysis (Fig. 1*D*). The conclusion that such large DegP complexes represent the active protease form is strongly supported by the following: first, the large complex peak almost completely disappeared after a longer time (120 min) of incubation for a full degradation of resorufin-labeled casein (Fig. 1*D*); second, the resorufin-labeled casein proteins bound in the isolated large DegP complexes were degraded after an incubation at 42°C (right column, Fig. 1*E*).

The DegP Protein in the Large Complexes Exists as Cage-Like 12- or 24-mers both Being Composed of Identical Trimeric Units. The structures of large DegP(S210A)-substrate complexes were subsequently analyzed via cryo-EM and single particle reconstruction. A total of 11,998 and 7,686 particle images (representatives of which are shown in Fig. S2*A* and Fig. S2*B*) were used to reconstruct the DegP(S210A)-lysozyme and DegP(S210A)-β-casein complexes, respectively, achieving a resolution of 8.5 Å and 9.7 Å (0.5 Fourier shell correlation, Fig. S2*C*). The results reveal that they existed as 12-mers (tetrahedral symmetry) or 24-mers (octahedral symmetry), both being cage-like in appearance (Fig. 2*A*, Fig. S2*D* and Fig. S2*E*). The DegP trimers were found to be distributed on the shells of the cages but the bound substrate proteins (lysozyme and β-casein), which were expected to be located in the cavity, were not resolvable, likely being averaged out during the reconstruction process because of their disordered conformation and/or lack of symmetry.

Most notably, although these large DegP oligomers are different in size, their building blocks are trimeric units, each being surrounded by three other trimeric neighbors and interacting with each other side by side, instead of face to face as what was observed in the DegP hexamers (Fig. 2*B* and Fig. S3). As all observed oligomers of DegP are composed of trimeric units, conversion of oligomeric states might occur via the dissociation and reassociation of DegP trimers. This mechanism is supported by our observation of effective dissociation of DegP hexamers to trimers at elevated temperatures and reassociation at lowered temperatures (Fig. S4). The blocking of the active sites by the LA loops from the opposite trimers in the inactive DegP hexamers (12) would be effectively removed as a result of the disassociation event.

Another notable feature is the evenly distributed "holes" present on the shells of both types of the large DegP oligomers: 4 on the 12-mers, each being ≈2 nm in diameter and enclosed by 3 trimeric units; 8 on the 24-mers, each being ≈4 nm in diameter and enclosed by 4 trimeric units (Fig. 2*A*). Such holes might represent the entry and/or exit paths for substrate proteins but further investigation is required to verify this hypothesis.

Atomic Model Fitting Reveals the Critical Inter-Trimer PDZ1–PDZ2 Interaction in Forming the DegP 12- and 24-mers. The structure of the DegP trimer, as extracted from its hexamer crystal structure (PDB ID: 1KY9, "molecule B" conformation) (12), was then fitted into the reconstruction models of the large oligomers. Such an operation allowed only the protease domains but not the PDZ1 and PDZ2 domains to be fitted, hinting that certain conformational changes have occurred in the conversion from hexamers to large oligomers. Consequently, the protease, PDZ1 and PDZ2 domains were separately fitted into the models. A good match between the reconstruction models and the crystal structure was thus achieved, which also provided strong validation for the high quality of our reconstruction models. For example, the structural features of the helix α-helix E and β-sheet 1–2 of the protease domain were clearly observable on the reconstruction model of the 12-mer (Fig. 2*B* and Fig. S5).

The fitting results reveal that the 12- and 24-mer are very similar to each other in regard to both the structures of the trimeric units and the interaction pattern between the trimers (Figs. S3 *A* and *B*). The PDZ1 and PDZ2 domains, although considered to be highly flexible in the hexameric forms (12), become highly ordered with the PDZ1 domain of one trimeric unit interacting with the PDZ2 domain of a neighboring trimeric unit in a pairwise manner (as schematically illustrated in Fig. 2*C*). The almost identical inter-trimer PDZ1–PDZ2 interaction in both types of the large oligomers is apparently made possible by the variation of distances between the PDZ1 and PDZ2 domains within each subunit for the 12- and 24-mer because of the high-flexibility nature of the linker region.

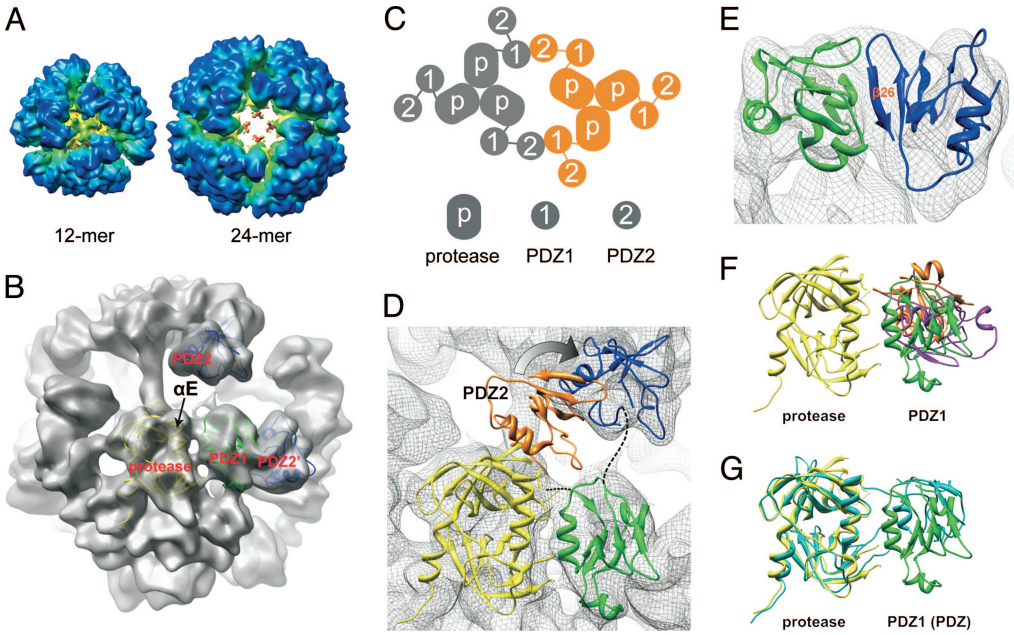

Fig. 2. Three-dimensional models of the large complexes of DegP. (*A*) The radially colored surface views on the reconstructions of the DegP(S210A)-lysozyme 12-meric and DegP(S210A)-β-casein 24-meric complexes. (*B*) The reconstruction of the 12-mer and the fitted domains (the protease, PDZ1 and PDZ2 domains of one molecule, and the PDZ2 domain [labeled as PDZ2′] from a neighboring trimer). If not otherwise indicated, the protease, PDZ1 and PDZ2 domains are respectively colored in yellow, green, and blue. (*C*) A schematic view of the pairwise inter-trimer PDZ1-PDZ2 interaction in the 12- or 24-mer. (*D*) The orientation of the PDZ2 domain (blue) in the 12-mer was altered notably in comparison with the ''molecule B'' conformation of the crystal structure (orange). (*E*) Closeup view on the inter-trimer PDZ1-PDZ2 interaction. The C-terminal β26 participating in the interaction was labeled. (*F*) The orientation of the PDZ1 domain (green) in the 12- or 24-mer was altered significantly in comparison with both the ''molecule A'' (purple) and ''molecule B'' (orange) conformations of the crystal structure. (*G*) The orientation of the PDZ1 domain (green) in the 12- or 24-mer is similar to that of the PDZ domain of the active form of DegS (cyan; PDB ID: 1SOZ).

Such pairwise PDZ1–PDZ2 interaction is made possible evidently because of a reorientation of both the PDZ1 and PDZ2 domains. The PDZ2 domain in the 12-mer was shifted for ≈2 nm toward the PDZ1 domain of the neighboring trimer apparently via a bending of the flexible region linking the PDZ1 and PDZ2 domains (residues 353–359, the long dashed line in Fig. 2*D*). As a result, each PDZ2 domain, through the C-terminal β-strand (residues 440–448, designated as β26 in Fig. 2*E*), interacts with a PDZ1 domain from a neighboring trimer. The PDZ1 domain at the same time underwent a rotation of ≈90° around the hinge residues Arg 262 and Gly 263 (Fig. 2*F*). Such pairwise PDZ1–PDZ2 interaction is the most noticeable association between two adjacent trimers, which generates the head-to-tail circular rings of PDZ1 and PDZ2 domains enclosing the holes on the large oligomers (Fig. S6). Apparently, interaction between the PDZ1 and PDZ2 domains plays the most important role for the assembly of both large DegP oligomers. We also noticed that the conformation of the protease-PDZ1 part of the 12- and 24-meric forms of DegP was highly similar to that of the active form of DegS protease trimers (Fig. 2*G*) (16, 21).

In contrast to the differences in ways of interaction between the trimeric units, the subunit interaction within each trimeric unit apparently remains unaltered through the DegP oligomers of various sizes, with all being mediated by the protease domains (12).

The PDZ2 Domain Plays an Important Modulatory Role for the Activity of DegP. Results of SEC reveal that the removal of the whole PDZ2 domain (residues 360–448), with the mutant protein designated as DegP(ΔPDZ2), results in the formation of only trimers that form neither the hexamers nor the 12- or 24-mers (Fig. S7*A*). Such a mutant trimeric form of DegP however exhibited both chaperone-like (Fig. 3*A*) and protease activities (Fig. 3*B*) at a level comparable to that of the wild-type protein, consistent with what was previously observed (13, 26).

Similar studies indicate that the removal of the β26 strand on the C terminus (residues 440–448), which was shown to directly interact with the neighboring PDZ1 domain as revealed by our EM studies (Fig. 2*E*), with the mutant designated as DegP(ΔB26), did not disrupt the formation of DegP hexamers but prevented their conversion to the 12- or 24-mers (Fig. S7*B*). Unexpectedly, the DegP(ΔB26) mutant protein exhibited significantly lower chaperone-like (≈65% lower, Fig. 3*A*) and protease (≈50% lower, Fig. 3*B*) activity. A similar decrease of protease activity was also observed when more sequences from the PDZ2 domain were truncated (Fig. S8). These observations suggest an inhibitory role of the PDZ2 domain for DegP to exhibit chaperone and protease activities.

The Specific Activity of DegP Protease Exhibits a Concentration Effect. Given that the DegP protein is active in its large oligomeric forms, a concentration effect is expected. The results presented in Fig. 3*C* (top curve) reveal that an 8-fold increase in the concentration of DegP resulted in an ≈2.4-fold increase in the specific protease activity. By contrast, there was no concentration effect detected when DegP(ΔPDZ2) and DegP(ΔB26), which were unable to form the large oligomers, were subjected to similar analysis (Fig. 3*C*).

This concentration effect was then examined by measuring the specific activity of a fixed amount of wild-type DegP proteins (1 μM) in the presence of increasing amounts of the protease-deficient DegP(S210A) protein. Strikingly, a similar concentration effect was also detected (top curve, Fig. 3*D*). Such a concentration effect however was not detectable when DegP(ΔPDZ2) and DegP(ΔB26), instead of the wild-type DegP protein, were analyzed (Fig. 3*D*). All these data strongly support our conjecture that the large DegP oligomers are the protease active form. It is conceivable that a concentration effect would

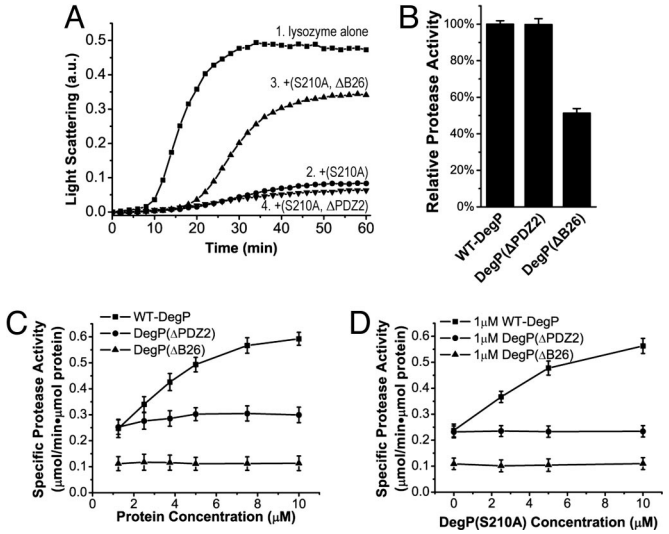

Fig. 3. The regulatory roles of the PDZ2 domain for DegP to exhibit activities. (*A*) Comparison of the chaperone-like activities of DegP(S210A), DegP(S210A, ΔPDZ2) and DegP(S210A, ΔB26) in suppressing the aggregation of lysozyme (0.1 mg/ml) at 42°C as reduced by 20 mM DTT. The final concentration for all of the DegP proteins was 0.1 mg/ml. The protein aggregation process was recorded by measuring the light absorption at 360 nm. Curve 1: lysozyme alone; Curve 2: lysozyme + DegP(S210A); Curve 3: lysozyme + DegP(S210A, ΔB26); Curve 4: lysozyme + DegP(S210A, ΔPDZ2). (*B*) Protease activities of DegP mutants (1 μM) having the whole or part of the PDZ2 domain truncated, measured at 42°C using resorunfin-labeled casein as the substrate. The relative protease activities were calculated by considering the activity of WT-DegP as 100%. (*C*) The concentration effect on the specific protease activities of WT-DegP, DegP(ΔPDZ2) and DegP(ΔB26). (*D*) The concentration effect of the protease-deficient DegP(S210A) on the protease activities of WT-DegP, DegP(ΔPDZ2) and DegP(ΔB26) (all present at 1 μM).

be displayed under stress conditions when the amount of DegP protein has been found to increase significantly (27).

The Large Oligomeric Forms of DegP Were Detected in the Cell Extracts of *E. coli* Cells. The 12-meric form of DegP was reported before (5, 26, 28), and we also demonstrated their existence via EM in the extracts of *E. coli* cells heterologously expressing the DegP(S210A) protein. For this study, the protein was purified from the cell extracts by affinity chromatography and fractionated by size-exclusion chromatography (Fig. 4*A*) before being

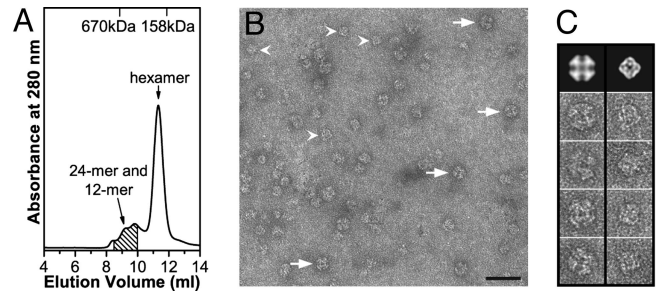

Fig. 4. Detection of the large complexes from cell extracts of *E. coli*. (*A*) SEC elution profile of DegP(S210A) purified from the cell extract of *E. coli*. The shaded fraction was subject to negative staining and EM analysis. (*B*) An electron micrograph of DegP(S210A) from the shaded fraction in *A*, representing the 24-mers (arrows) and 12-mers (arrowheads). (Scale bar: 50 nm.) (*C*) The representative images of the 24-mer (left column) and 12-mer (right column) of DegP(S210A) as picked up from *B*. The typical projections of the two oligomeric forms are shown on the top row. The side length for each image box is 31.2 nm.

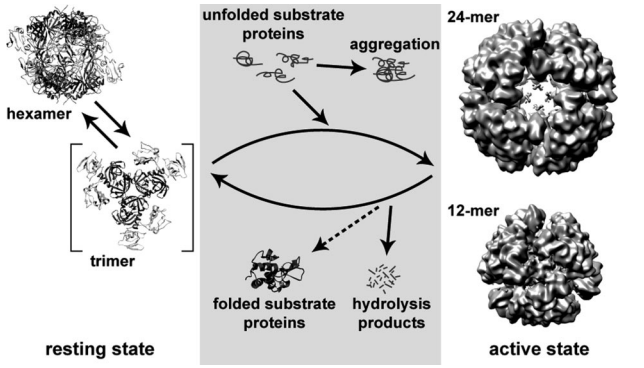

Fig. 5. A schematic illustration for the proposed transformation of the oligomeric status and the correlated activity status. Emphasized here are the substrate induced formation of the active cage-like 12- and 24-mers and the reversible nature of this activity modulation process.

subjected to EM analysis. The micrographs presented in Fig. 4 *B* and *C* unequivocally demonstrate the presence of both the 12-mers and 24-mers of DegP in the *E. coli* cells.

Discussion

Our most significant finding reported here is that the DegP hexamers are converted to the 12- and 24-mers upon binding to substrate proteins. Although the 12-mers were reported in one of the earliest studies of DegP (5) and later by others (15, 26, 28), our studies reveal an activity modulatory role for such large oligomers. This represents major progress not only in understanding the activation mechanism of DegP (12, 29), but also in revealing a unique mechanism for protease activation which is substrate dependent and reversible in nature.

In light of our observations, we propose a working model (Fig. 5) to explain how DegP activation may occur. Briefly, the hexameric forms of DegP, via trimeric intermediates, are convertible to 12-mers and 24-mers in binding to the substrate proteins. In this process, the pairwise inter-trimer PDZ1–PDZ2 interactions occur between the trimeric units, which simultaneously and elegantly eliminate the inhibitory effect of the PDZ2 domains. As a consequence, the DegP protein is converted to its active forms for both the protease and chaperone activities. The substrate protein is then subject to either protease degradation or refolding (13). Upon conclusion, such large oligomers might be able to convert back to their inactive hexameric form until being activated again by available substrate proteins.

This mechanism of activation, via homooligomerization, differs from most that were previously proposed. For example, the activation of the homologous DegS protein has been revealed to occur through the binding of a peptide ligand to its single PDZ domain (16, 19). Previous speculations on DegP activation were mostly focused on the ligand-binding activation and how the blocked active sites become accessible to substrate proteins (20, 21). The mechanism that we revealed would allow the protease activity of DegP to remain inert until such time as unfolded substrate proteins become available, which evidently signals that the cells are exposed to stress conditions. DegP function is then desired to cope with such environmental conditions. Such a means of activity modulation would avoid the potential harmful effects of DegP protease activity under stress-free conditions.

In our view, the trimeric forms of the wild-type DegP would exist only momentarily and immediately transform back into the inactive hexamers or into the large oligomeric forms upon binding to the substrate proteins (as illustrated in Fig. 5). According to our working model, such a transiently present trimeric form of DegP hardly exhibits protease or chaperone activity because of the inhibitory effect of the PDZ2 domain. It

follows that the full truncation of the PDZ2 domain exhibits both activities.

The cage-like structures of the DegP 12-mers and 24-mers are reminiscent of their highly structural analogy with GroEL and proteasome, well-studied molecular chaperone and protein degradation machinery respectively (30, 31). The dual protease and chaperone function of the DegP protein suggests that their similarity in structure is not coincidental but a result of evolutionary selection.

Our data clearly demonstrate that the 12- and 24-mers of DegP are all assembled via a similar pattern of interaction mediated by the PDZ1 and PDZ2 domains (Fig. 2*C*). The nature of this interaction pattern would allow the trimeric units of DegP to assemble into larger cage-like oligomers as 60-mers in icosaheral symmetry. Whether such large forms of DegP actually exist under certain conditions and what function they may serve, merits further investigation. Given that the DegP protein, located in the periplasmic space of *E. coli* cells, has been believed to play a role in the folding, assembly, and translocation of the outer membrane proteins (32, 33), such super-large oligomeric forms of DegP might be needed for DegP to function in the assembly of large oligomeric membrane proteins.

Largely unanswered questions regarding the structure and function of DegP include the following: What determines whether DegP functions as a protease or as a chaperone? Could the protein function repeatedly in such a large oligomeric form or does it have to undergo the disassembly–reassembly process each time it functions? How are the refolded substrate proteins released from the large oligomeric complexes? Understanding the function and action mechanism of any protein in a living cell content, which is of highest significance, presents correspondingly greater challenges.

At the submission of this paper, a *Nature* advance online publication appeared, reporting the structural studies of the two large oliogmers of DegP by a competing group (34). There are four main differences between our results reported here and those reported in the Krojer *et al.* article. First, Krojer *et al.* determined the crystal structure of the 24-meric form, which revealed similar PDZ1–PDZ2 interactions between the trimeric units. However, the structure of the 12-meric form was determined only at a very limited resolution of 28 Å by cryo-EM, from which they proposed that the contact between the trimeric units of the 12-mers occurs via PDZ1–PDZ1 interaction. To the contrary, our higher (8.5 Å) resolution cryo-EM studies unequivocally revealed that the interactions mediating the formation of both 12-mers and 24-mers are similar, both via PDZ1–PDZ2 interactions, thus an almost identical strategy is actually used for DegP to form both types of large oligomers. Second, our structure-based mutagenesis and functional studies showed a significant inhibitory effect of the PDZ2 domain on the chaperone–protease activity, suggesting an elegant mechanism through the participation of PDZ2 in mediating the formation of the large oligomers to simultaneously eliminate its inhibitory effect on the protease–chaperone activity. Third, we observed a correlation between high DegP concentration and higher specific protease activity, which was also observed by adding an increasing concentration of the protease-deficient mutant DegP(S210A) to a system containing a fixed amount of the wild-type DegP, supporting the notion that large oligomers are the active form. Finally, our study provides significant insights into the mechanism of activation of the protease. Our proposed model explains a mechanism for protease activation by protein homooligomerization, which is substrate inducible, and more significantly, reversible in nature.

Materials and Methods

Chemicals. Ni-NTA agarose and β-casein were purchased from Sigma, resorufin-labeled casein from Roche, lysozyme from Amresco, and DTT from Merck. The other chemicals were of analytically pure grade.

Plasmid Construction. The wild-type *degP* gene carried on the pTdeg plasmid (14) was amplified by PCR and inserted into the pET-28a expression plasmid vector, after both being cleaved with *Nco*I and *Xho*I restriction enzymes to generate pET-28a-degP. A 6-histidine tag was added to the C terminus of the DegP protein from this plasmid. Mutagenesis was performed using this plasmid to generate the DegP mutant proteins.

Protein Expression and Purification. Proteins were heterologously expressed in the BL-21 (DE3) *E. coli* strain transformed with the pET-28a-degP or its derivative plasmid and purified by affinity chromatography (13). Briefly, cells were grown at 37°C in the Luria–Bertani medium (containing 50 μg/ml kanamycin), induced (at A_{600} of 0.8–1.0) with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h, before being harvested by centrifugation at 5,000 rpm for 10 min. The buffer (buffer A) used in the purification is 50 mM Na_2HPO_4-NaH_2PO_4, 50 mM NaCl, pH 7.6. The cell pellet was then resuspended in the buffer A (containing 20 mM imidazole and 1 μg/ml pepstatin A), lysed by sonication, before being centrifuged for 50 min at 15,000 rpm. The supernatant was then loaded into a 2 ml Ni-NTA agarose column, washed sequentially with 200 ml buffer A (100 ml containing 0.5 M NaCl and 20 mM imidazole and another 100 ml containing 1 M NaCl and 20 mM imidazole) before being eluted with buffer A (containing 250 mM imidazole). The purity (>95%) of the fractions was confirmed by SDS/PAGE analysis. Protein concentration was determined by the protein assay kit (from Pierce).

SEC. SEC was performed at 4°C with an AKTA FPLC system using a prepacked Superdex 200 10/300 GL column (GE Healthcare Life Sciences). A sample of 100 μl was loaded into the column and eluted with the buffer containing 50 mM Na_2HPO_4-NaH_2PO_4, pH 7.6 and 50 mM NaCl.

Preparation of the DegP-Substrate Complexes. To obtain the DegP-substrate complexes, the purified hexameric forms of DegP(S210A) (at 1.0 mg/ml) were added to the unfolded substrate proteins (β-casein, lysozyme) in a molar ratio of 1:2 (DegP monomer:substrate) and incubated at 42°C for 30 min, centrifuged to remove aggregates. DTT was added to lysozyme in a final concentration of 20 mM to unfold it.

Transient formation of large complexes while DegP was degrading resorufin-labeled casein was detected as following. The mixture of 0.16 mg DegP and 0.6 mg resorufin-labeled casein was incubated at 42°C for 5 min, divided into two halves, with one being immediately applied to SEC analysis and the other continuing to be incubated at 42°C for 120 min before SEC analysis. The elution curves were recorded as the distinctive absorbance of resorufin at 574 nm to detect the formation and breakdown of large complexes containing the resorufin-labeled casein during the degradation process. The transiently formed large complexes were pooled and either incubated further at 42°C for another 60 min or directly applied for the detection of degradation products.

Electron Microscopy and Image Processing. Negatively stained samples were prepared with 1% uranyl acetate and then imaged on a Philips CM120 BioTWIN transmission electron microscope operated at 100 kV and a magnification of 52,000×. The frozen samples of DegP(S210A)-substrate complexes were imaged on an FEI Polara cryo-electron microscope equipped on a 4k × 4k CCD camera, operated at 300 kV with a dosage of ≈20 $e^-/Å^2$ at a magnification of 130,000×. The defocus was set to −0.5 to −2.5 μm.

Single particle reconstructions of the DegP(S210A)-substrate complexes were performed using the EMAN software package (ver. 1.8) (35) following the procedures essentially as described in the manual. Briefly, the particle images were manually extracted from the micrographs with the *boxer* program and their CTF parameters were carefully determined with the *ctfit* program. The phase-corrected images were then used to generate reference-free class averages, which were subsequently used to make the initial models. The symmetries of the models were determined by trial and error and then applied to the following projection-matching refinements. The models were iteratively refined during the reconstruction process until the resolutions could no longer be improved. The final reconstruction models were Gaussian-lowpass filtered to 8.5 Å (12-mer) or 9.7 Å (24-mer), respectively, for fitting and display purposes.

The fitting of the crystal structure onto the 3-D EM map was carried out with the *colores* program of the Situs software package (36). The images of the reconstructions and crystal structures were prepared with the UCSF Chimera program (37).

Light Scattering Assay. Mixtures (400 μl) of the DegP protein (0.1 mg/ml) and lysozyme (0.1 mg/ml), containing 50 mM Na_2HPO_4-NaH_2PO_4, 20 mM DTT, pH 7.6, were incubated at 42°C in quartz cuvettes. DegP protein was preheated at 42°C before being added into the mixtures. Aggregation of lysozyme was

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monitored by measuring the apparent light absorption at 360 nm with a spectrophotometer.

Protease Assay. The protease activity of DegP was measured using resorufin-labeled casein as the substrate protein. A volume of 50 μl of resorufin-labeled casein [0.4% (wt/vol) in H_2O] was added into 150 μl sample buffer (50 mM Tris-HCl, pH 7.6, containing a specified amount of DegP), and incubated at 42°C. Every 5 min, a 40-μl sample was removed and added into 96 μl of 5% TCA to stop the reaction and incubated for another 10 min at 37°C before being centrifuged for 5 min at 20,000 g. An 80-μl sample of the supernatant was then mixed with a 120-μl assay buffer (0.5 M Tris·HCl, pH 8.8) before the absorbance at 574 nm was immediately measured. The specific protease activity was calculated from the slope of the linear range of the absorbance curve.

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