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Crystal Structure of *Arabidopsis* Deg2 Protein Reveals an Internal PDZ Ligand Locking the Hexameric Resting State*[S]

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Background: The PDZ protease Deg2 is involved in chloroplast protein quality control through a yet unknown molecular mechanism.
Results: A novel PDZ domain with an internal ligand mediates hexamer formation and locks Deg2 into the resting state.
Conclusion: Formation of the resting hexamer may be a common strategy in a Deg protease subfamily.
Significance: We provide structural insights into the PDZ domain-mediated regulation of Deg proteases.

Eukaryotic organelles have developed elaborate protein quality control systems to ensure their normal activity, among which Deg/HtrA proteases play an essential role. Plant Deg2 protease is a homologue of prokaryotic DegQ/DegP proteases and is located in the chloroplast stroma, where its proteolytic activity is required to maintain the efficiency of photosynthetic machinery during stress. Here, we demonstrate that Deg2 exhibits dual protease-chaperone activities, and we present the hexameric structure of Deg2 complexed with co-purified peptides. The structure shows that Deg2 contains a unique second PDZ domain (PDZ2) following a conventional PDZ domain (PDZ1), with PDZ2 orchestrating the cage assembly of Deg2. We discovered a conserved internal ligand for PDZ2 that mediates hexamer formation and thus locks the protease in the resting state. These findings provide insight into the diverse modes of PDZ domain-mediated regulation of Deg proteases.

The Deg/HtrA proteases are key components of protein quality control systems of the cellular machinery (1, 2). Whereas bacteria such as *Escherichia coli* have developed a periplasmic protein quality control system utilizing the three well characterized Deg family members (DegS, DegP, and DegQ), eukaryotic organelles such as mitochondria and chloroplasts have more sophisticated protein quality control systems to maintain their functions inside the cells. The chloroplast protein quality control system is essential for the removal and repair of photodamaged proteins during light stress (3–5). Among the components of this system, several Deg proteases have been identified, including Deg1 and Deg2, the two prototypes of plant Deg proteases (6, 7). The recent Deg1 structure in an active hexameric state revealed a pH-dependent regulation mechanism and that the sole PDZ domain of Deg1 is necessary for both hexameric assembly and protease activation in a pH-dependent manner (8). In contrast, Deg2 contains two PDZ domains and is in the stromal side of the thylakoid membrane, where the pH is constantly higher than in the lumen, in which Deg1 is located, thus implying that Deg2 has different properties from Deg1. Deg2 was reported to be able to degrade the photosystem II reaction center D1 protein and the Lhcb6 protein (9–11).

Besides plant Deg1, the pH-dependent regulation of proteolytic activity has also been demonstrated for DegQ, a homologue of Deg2, although controversy exists as to the contribution of its two PDZ domains (12, 13). Results obtained with the DegQ-ΔPDZ2 12-mer indicated that PDZ2 is dispensable for oligomeric reassembly (12), whereas in structures of the full-length DegQ 12-mer and 24-mer, the PDZ2 domain was found to be critical for oligomerization (13–15). Another Deg protease whose activation requires large oligomeric assembly is DegP, whose two PDZ domains mediate reassembly of DegP from an inactive hexamer to an active 12-mer or 24-mer (16–19).

In this study, we show that Deg2 has a proteolytic activity with little pH dependence and exhibits chaperone-like activity, and we identify a previously uncharacterized internal ligand. This PDZ ligand mediates hexameric assembly, resulting in a sealed cage different from either the Deg1 hexamer (8) or DegP hexamer (DegP6) (16). These results suggest a novel strategy of proteolytic activity regulation by locking up the protease through interaction networks involving an internal PDZ ligand.

EXPERIMENTAL PROCEDURES

Expression, Purification, and Crystallization—The *deg2* gene from *Arabidopsis thaliana* was amplified by PCR and inserted into the pMCSG19 expression plasmid vector, yielding pMCSG19-His_6-Deg2. Deg2 expressed in *E. coli* was purified using a dextrin-Sepharose high performance affinity column (GE Healthcare). The maltose-binding protein-His_6 tag was

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[S] This article contains supplemental Figs. S1 and S2 and Table S1.
The atomic coordinates and structure factors (code 4FLN) have been deposited in the Protein Data Bank (http://wwpdb.org/).
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cleaved by tobacco etch virus protease at 4 °C. The purified protein was kept in buffer containing 200 mM NaCl with 20 mM Tris-HCl (pH 7.5) or with 50 mM MES (pH 6.0). The protein solution was shock-frozen in liquid nitrogen and stored at −80 °C. Both native and selenomethionine crystals of Deg2 were obtained at 16 °C using the sitting drop vapor diffusion method by mixing 0.2–1 μl of protein sample (9 mg/ml) with an equal volume of crystallization solution (0.1 M sodium malonate (pH 5.5) and 12% (w/v) polyethylene glycol 3350) taken from a 200-μl reservoir.

Data Collection and Structure Determination—Harvested crystals were cryo-protected in crystallization solution supplemented with 50% (v/v) ethylene glycol and then flash-frozen in liquid nitrogen. X-ray diffraction data were collected at beamline BL17U of the Shanghai Synchrotron Radiation Facility at a wavelength of 0.979 Å at 100 K and integrated and scaled using DENZO and SCALEPACK as implemented in HKL2000 (20). Selenium-labeled positions in the Deg2 crystal were determined using the program SHELXD from the CCP4 suite (21), and nine selenium sites were found corresponding to Met-148, Met-502, and Met-527 in each one of the three molecules in the asymmetric unit. The identified selenium sites were refined, and the initial phases were generated using the program AutoSol in the PHENIX suite (22). Additional missing residues in the auto-built model were manually added according to the $2F_o - F_c$ and $F_o - F_c$ electron density maps, and manual model building was performed in Coot (23). Structure refinement was done with CNS (24) and PHENIX (22). The overall quality of the final structural model was assessed by PROCHECK (25). In the final atomic model, 93.4, 6.6, and 0.0% of the amino acids are in the most favored, additionally allowed, and disallowed regions of the Ramachandran plots, respectively. Data collection and structure refinement statistics are summarized in supplemental Table S1. The protein structure figures were prepared using the program PyMOL.

Size Exclusion Chromatography—Size exclusion chromatography was performed on a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with buffer containing 200 mM NaCl with 50 mM MES (pH 6.0), with 50 mM Tris-HCl (pH 7.5), or with 50 mM Tris-HCl (pH 9.5) at 4 °C at a flow rate of 0.5 ml/min.

Proteolytic Activity Assay—A mixture of α- and β-caseins or pure β-casein was purchased from Sigma-Aldrich. The proteolytic activity of Deg2 was analyzed by incubation with the α- and β-casein mixture or with β-casein. The 200-μl reaction mixture was incubated at 37 °C, and a 25-μl sample was removed at the indicated time points and boiled with SDS loading buffer before analysis by SDS-PAGE.

Chaperone-like Activity Assay—A 1-ml mixture of Deg2 (0.1 mg/ml) or bovine serum albumin (0.1 mg/ml) with lysozyme (0.1 mg/ml) in buffer containing 50 mM PBS (pH 7.5) and 20 mM DTT was incubated at 37 °C. Aggregation of lysozyme was monitored by measuring the light absorption at 360 nm with a spectrophotometer as described (18, 26, 27).

RESULTS

Dual Protease and Chaperone Activities—The stromal location of Deg2 suggests that the optimum pH for Deg2 proteolytic activity should be similar to that of the alkaline stroma. We first tested the pH dependence of Deg2 around its physiological range, and we found that the proteolytic activity of Deg2 was higher at pH 7.5 than at pH 6.0 (Fig. 1*A*). The purified Deg2 protein is an active protease and is able to specifically degrade β-casein but not α-casein. We then tested using the substrate β-casein at pH 9.5, at which the maximal proteolytic activity had been reported (9). Our result showed that the speed of β-casein degradation at pH 9.5 was slower than at pH 7.5 (Fig. 1*B*). Although pH 7.5 was more optimal than pH 6.0 or 9.5 for proteolytic activity and close to the physiological pH of the stroma, the overall activity was relatively low compared with DegP or DegQ. The low proteolytic activity of Deg2 could be accompanied by the chaperone-like activity, as indicated by DegP and DegQ proteases (14, 18). To test this possibility, we measured the chaperone-like activity of Deg2. As expected, the

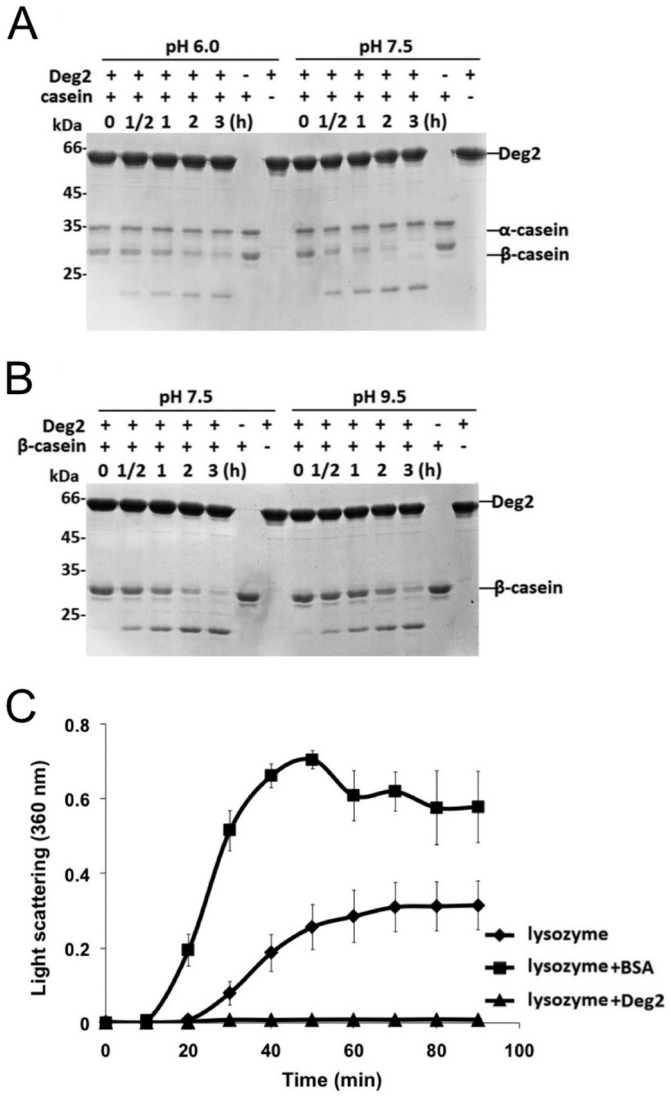

FIGURE 1. **Dual protease and chaperone-like activities of Deg2.** *A*, protease activity assay using a mixture of α- and β-caseins as the substrate at pH 6.0 or 7.5. Purified Deg2 and the casein mixture were incubated as indicated for 0–3 h. Controls containing only Deg2 or substrate were also included. *B*, protease activity assay using β-casein as the substrate at pH 7.5 or 9.5. *C*, chaperone-like activity upon suppression of 20 mM DTT-denatured lysozyme. The aggregation process was recorded by measuring the light scattering values at 360 nm. Data are means ± S.D. of three independent experiments.

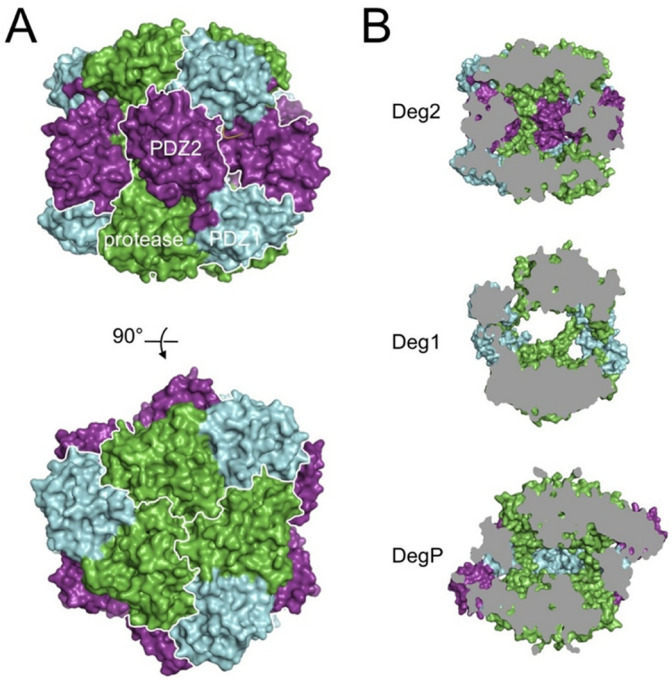

FIGURE 2. **Deg2 structure and comparison with Deg1 and DegP6.** *A*, side and top views of the Deg2 hexamer. The structure is colored-coded (*green*, protease domain; *light blue*, PDZ1; *purple*, PDZ2; *yellow*, co-purified peptide), and each protomer is indicated by *white lines*. *B*, clipped views of Deg2, Deg1 (Protein Data Bank code 3QO6 (8)), and DegP6 (code 1KY9 (16)).

presence of Deg2 reduced the aggregation of the DTT-denatured lysozyme (Fig. 1*C*). This chaperone-like activity was so effective that it entirely suppressed aggregation from the very beginning of the incubation. Used as a control for unspecific prevention of aggregation, the presence of BSA was not effective in suppressing lysozyme aggregation and even resulted in simultaneous aggregation.

Hexameric State and Large Oligomeric State—Hexameric Deg2 in solution was observed by size exclusion chromatography and dynamic light scattering methods. The hexameric state was independent of pH. At three pH values (pH 6.0, 7.5 and 9.5), Deg2 existed predominantly as a hexamer with the coexistence of a large oligomer, but without a trimer or monomer. This was different from Deg1, DegP, and DegQ. In Deg1, the oligomeric state relating to proteolytic activity is pH-dependent (8). In DegP and DegQ, the predominant state is the 12-mer or 24-mer when incubated with substrates (13, 14, 17, 18). The low efficiency of β-casein degradation by Deg2 prompted us to study the rate of large oligomer formation (supplemental Fig. S1). The amount of Deg2 in the large oligomeric state increased with the incubation time with β-casein, accompanying the increase in the β-casein degradation product. This result indicated that the Deg2 hexamer existing predominantly during substrate incubation is in a resting state and suggested that the large oligomeric state is the active state.

The hexameric structure of mature Deg2 (Fig. 2*A*) was determined by the selenium-based, single-wavelength anomalous method. Mature Deg2 contains 468 amino acids (positions 110–577), including a protease domain (positions 110–313), followed by PDZ1 (positions 314–422) and PDZ2 (positions 423–577). A co-purified peptide binds to PDZ1 of each protomer. The Deg2 hexamer is reminiscent of Deg1 (8) and DegP6 (16), but it differs in the arrangement of the individual domains. The cavity size of the Deg2 hexamer (Fig. 2*B*) is comparable with that of Deg1, but PDZ2 blocks the entrance and is more spacious than the narrow cavity of DegP6.

The Deg2 protease domain consists of a catalytic triad comprising His-159, Asp-190, and Ser-268 (Fig. 3*A*). The loops are named according to chymotrypsin nomenclature (28). The electron density of loop L2, the specificity-determining surface loop, is not completely observed due to its flexibility, but the remaining part indicates that loop L2 hinders binding of the substrates to the active site. Active site blocking has been reported for DegP6, whose loop trio LA*-L1-L2 (the asterisk denotes the contribution from a neighboring protomer) completely blocks the active site (16). In the Deg2 hexamer, the active site is only partially blocked by loop L2, but the assembly is sealed by multiple interactions between opposite trimeric units. Therefore, hexamerization confines the proteolytic activity to inside the sealed cage.

PDZ Domains Complexed with Their Ligands—Deg2 PDZ1 maintains the canonical HtrA-like PDZ fold (2), except for an additional β-sheet consisting of strands β17 and β18 that protrudes outward from the peptide-binding groove (Fig. 3*A*). The peptide-binding groove of PDZ1 is situated between helix αF and strand β13. Like Deg1 (8), a co-purified and co-crystallized peptide binds to the groove of each Deg2 protomer. The N-terminal "tail" of the longest peptide stretches out of the small pore of the hexameric Deg2 cage. Each co-purified peptide forms an antiparallel β-strand to β13, with the side chain of the third residue on the β-strand accommodated in the −1 position of the PDZ1 groove, which selects specifically for tryptophan (Fig. 3*B*).

Deg2 PDZ2 has an unusual topology that differs from conventional PDZ domains. Compared with known structures of PDZ domains, PDZ2 is most similar to PDZ1 of DegQ (Fig. 3*C*). Interestingly, Deg2 PDZ2 has three unique features. First, an uncharacterized β-strand (β21) immediately preceding β22 by a sharp turn occupies the peptide-binding groove between αI and β22 (Fig. 4*A*). β21 resembles a pseudo-peptide motif that mimics one type of the canonical peptide ligand of the PDZ domain (29, 30) by the way that β21 acts as an intramolecular internal PDZ ligand. Second, between β22 and β23, there is an additional helix (αH) that directly interacts with αH from the neighboring subunit in the hexamer. This αH pair seems to gate the substrate access, for the N-terminal tail of the co-purified peptide is just around it (Fig. 3*B*). Third, following the last β-strand (β27), there are two additional α-helices (αJ and αK). These two helices and the loop between (loop JK) partially shield β21 from exposure to the solvent. Loop JK and helix αG constitute the outside edges of a concave surface with β21-β22-β23 as the bottom sheet, forming a loop LA-docking surface (Fig. 4*B*). Because loop JK lies outside the hexamer shell, it provides protection for the loop LA-PDZ2 interface from solvents and may play a role in changes in the oligomeric Deg2 state.

Unlike DegP6, whose PDZ2 is quite flexible, so only three of the six PDZ2 domains are partially visible (16), in the Deg2 hexamer, PDZ2 is well positioned. In each Deg2 protomer, loop LA from the protease domain is docked onto the PDZ2 concave

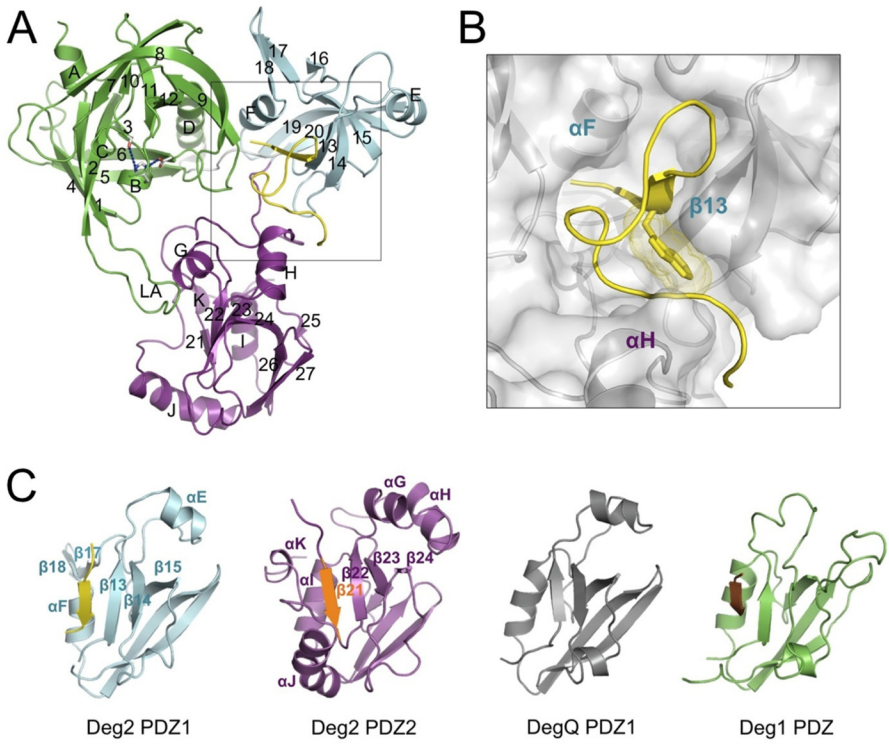

FIGURE 3. **Deg2 domains and comparison with Deg2 PDZ1 and PDZ2, DegQ PDZ1, and Deg1 PDZ.** *A*, ribbon presentation of the Deg2 protomer. All secondary structure elements are labeled. The catalytic triad is shown as a *stick* model, the two conserved hydrogen bonds are indicated by *dotted blue lines*, and the co-purified peptide is shown as a *yellow ribbon*. *B*, the co-purified peptide-binding site overlaid with a transparent surface of Deg2. The tryptophan at the −1 position is shown as a *stick* model surrounded by a *dotted* surface. *C*, comparison of Deg2 PDZ1 and PDZ2, DegQ PDZ1 (*gray*; Protein Data Bank code 3PV5 (13)), and Deg1 PDZ (*light green*; code 3QO6 (8)). The internal PDZ ligand (β21) is shown in *orange*, and the co-purified peptide bound to Deg1 PDZ is shown in *brown*.

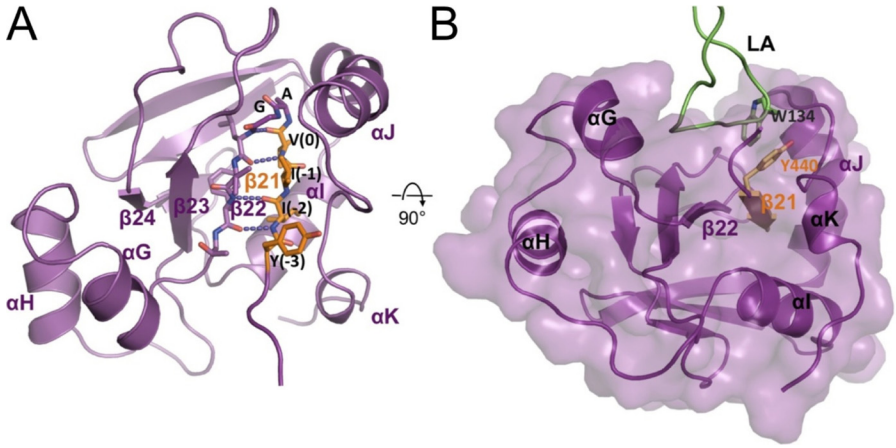

FIGURE 4. **Internal PDZ ligand and loop LA-PDZ2 interface.** *A*, PDZ2 (*purple*) in complex with the internal PDZ ligand (*orange*). Residues of the internal PDZ ligand are indicated by the conventional PDZ ligand positions (0 to −3). Ala-444 and Gly-445 from the β-turn are also indicated. Hydrogen bonds between β21 and β22 are shown as *dotted blue lines*. *B*, ribbon presentation of the loop LA-PDZ2 interface with a transparent surface of PDZ2. Loop LA is shown as a *green ribbon*. Trp-134 and Tyr-440 are shown as *stick* models.

surface (Fig. 4*B*). Trp-134 on loop LA and Tyr-440 on strand β21 are in a *t*-stacking position. Interactions between loop LA and PDZ2 not only fix the protease domain onto PDZ2 but also restrict PDZ1 through coupled PDZ2-PDZ1* interactions as described below. Therefore, the overall structure of Deg2 in its hexameric state is quite rigid. As two compatible jigsaw pieces, two trimers are fitted fairly well and leave only six small pores to restrict the access and egress of the substrate to the interior.

Dimerization Interface between Deg2 Trimers—Two interfaces are formed for each Deg2 protomer, and they mediate trimer dimerization. Interface 1 consists of the protease domain and PDZ2, and interface 2 is consists of PDZ1 and PDZ2 (Fig. 5). In interface 1, inter-protomer interactions are observed between two extended surfaces facing each other in a 2-fold symmetric manner, with Gln-135–Gln-135* as the pivotal axis. The extended surface is composed of Gln-139 on loop LA, Lys-168 on strand β4, and residues flanking strand β21, including Val-490, Ala-492, Glu-500, and Tyr-561 (Fig. 5*A*). The Gln-139 side chain amide forms hydrogen bonds with the backbone carbonyl oxygen of Val-490* and the backbone amide

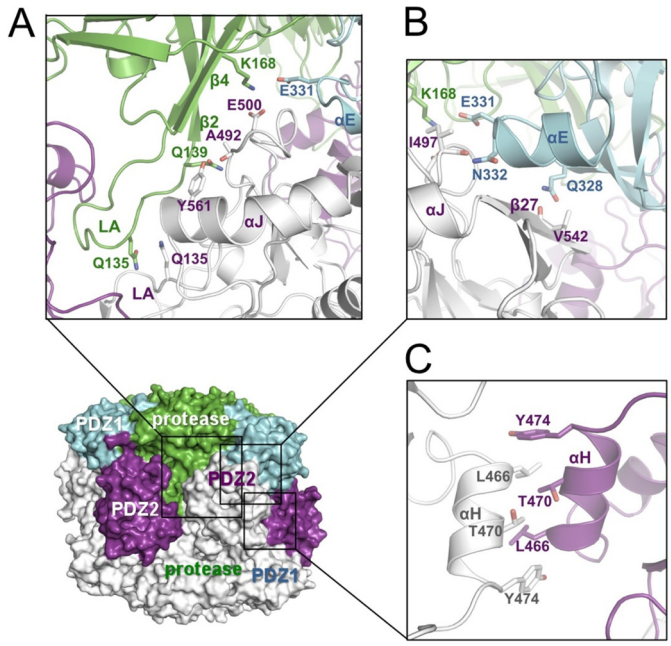

FIGURE 5. **Dimerization interface between Deg2 trimers.** The *upper* trimer is colored according to the scheme described in the legend to Fig. 2, and the *lower* trimer is colored *gray*. *A*, residues involved in interactions on interface 1. *B*, residues involved in PDZ1-PDZ2* interactions. *C*, residues involved in PDZ2-PDZ2* interactions.

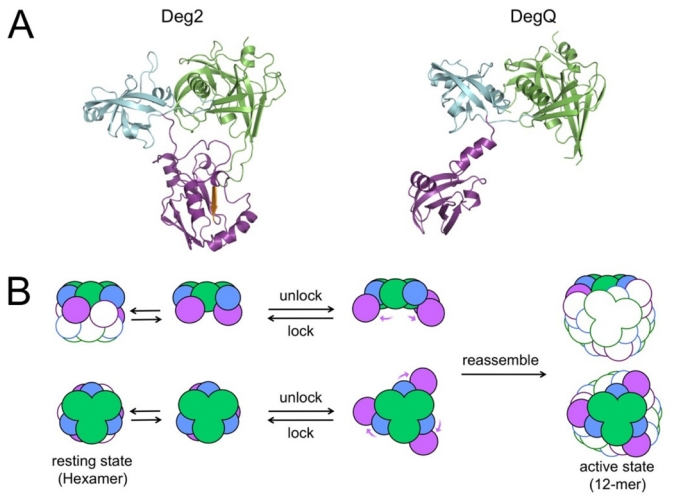

FIGURE 6. **Model of oligomeric state change.** *A*, comparison of protomers from the Deg2 hexamer and DegQ 12-mer (Protein Data Bank code 3PV5 (13)). *B*, schematic representation of the Deg2 oligomeric state change. Side and top views of the Deg2 hexamer are illustrated according to the corresponding colors. During the unlocking process, the loop LA-PDZ2 interface breaks up, and PDZ2 flings away from the protease-PDZ1 module. The unlocked trimers then reassemble into the higher order oligomeric active state (shown as a 12-mer in this model).

of Ala-492*, and it forms a polar interaction with Tyr-561*. Lys-168 forms electrostatic interactions with Glu-500* and Glu-331* from another neighboring protomer. A characteristic feature of the interface 1 interaction is that in the Gln-135–Gln-135* pivotal area, only weak contacts are observed. The two opposite PDZ2 domains have no direct interactions even in close proximity. Interactions in interface 2 can be attributed to two sources: PDZ1-PDZ2* and PDZ2-PDZ2*. PDZ1-PDZ2* interactions are between a consensus sequence in PDZ1 (residues 328–332, corresponding to the loop between β13 and αD) and a patch on PDZ2* that includes a turn (residues 497–499) and β27 (residues 541–546) (Fig. 5*B*). Hydrogen bonds are formed between the Asn-332 side chain and the Ile-497 backbone and between the Gln-328 side chain and the Val-542 backbone. The consensus sequence (residues 328–332) also provides the link to interface 1 with electrostatic interaction between Glu-331 and Lys-168* as mentioned above. PDZ2-PDZ2* interactions are between helix αH of each PDZ2. The side chains of Leu-466, Thr-470, and Tyr-474 are interlocked (Fig. 5*C*). Similar to the Gln-135–Gln-135* pair of interface 1, the αH-αH* pair contains only weak interactions. The lack of strong interactions in these areas suggests the possibility of the opening of the sealed cage and hence an oligomeric state change such as the assembly of the 12-mer or 24-mer.

DISCUSSION

In *A. thaliana*, 16 Deg proteases (Deg1–Deg16) have been found, and five of these (Deg1, Deg2, Deg5, Deg7, and Deg8) are in chloroplasts and participate in the removal and repair of photodamaged photosystem II in response to light stress. Deg1, Deg5, and Deg8 are located inside the thylakoid lumen and possess only one or no PDZ domain (31–33). For the other 13 family members, seven have two PDZ domains. As an internal PDZ2 ligand, strand β21 of Deg2 is conserved in these seven members (supplemental Fig. S2). In addition to β21, the β-turn ((A/G)G) between β21 and its pairing β-strand (β22) is also conserved, as well as loop LA. According to the Deg2 structure, this internal PDZ ligand plays two critical roles in hexameric assembly. First, it contributes to the formation of a loop LA-binding concave surface to which loop LA is fixed, therefore making the Deg2 protomer a rigid module. This differs from both the DegQ 12-mer and 24-mer (13–15) in that DegQ PDZ2 has no interaction with the protease domain and flings away from the protease-PDZ1 module (Fig. 6*A*) and DegP6, whose long LA loop is directly involved in hexamer formation (16). Second, the lack of a strong interaction surrounding the loop LA-PDZ2 docking site provides the possibility to unlock the sealed cage by dissociation of this internal PDZ ligand from PDZ2 (Fig. 5) and therefore disruption of the LA-PDZ2 interaction so that the trimeric units will be rearranged into the higher oligomeric state for protease or chaperone-like activity (Fig. 6*B*).

These seven Deg proteases all contain sequences similar to Deg2 PDZ1 and PDZ2 (supplemental Fig. S2), including the C-terminal extra region (helices αJ and αK and loop JK) of PDZ2; and except for Deg13, their LA loops are similar in size to Deg2, and all have the tryptophan (Trp-134 for Deg2) that may stack with the tyrosine (Tyr-440 for Deg2) of the internal PDZ ligand (Fig. 4*B*). These data suggest that a subfamily of plant Deg proteases, including Deg2, Deg3, and Deg9–Deg12, employs a similar oligomerization strategy to regulate their protease-chaperone activities.

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