Structure and Function of a Novel Type of ATP-dependent Clp Protease

Fredrik I. Andersson, Anders Tryggvesson, Michal Sharon, Alexander V. Diemand, Mirjam Classen, Christoph Best, Ronny Schmidt, Jenny Schelin, Tara M. Stanne, Bernd Bukau, Carol V. Robinson, Susanne Witt, Axel Mogk, and Adrian K. Clarke

The Clp protease is conserved among eubacteria and most eukaryotes, and uses ATP to drive protein substrate unfolding and translocation into a chamber of sequestered proteolytic active sites. The main constitutive Clp protease in photosynthetic organisms has evolved into a functionally essential and structurally intricate enzyme. The model Clp protease from the cyanobacterium Synechococcus consists of the HSP100 molecular chaperone ClpC and a mixed proteolytic core comprised of two distinct subunits, ClpP3 and ClpR. We have purified the ClpP3/R complex, the first for a Clp proteolytic core comprised of heterologous subunits. The ClpP3/R complex has unique functional and structural features, consisting of twin heptameric rings each with an identical ClpP33ClpR4 configuration. As predicted by its lack of an obvious catalytic triad, the ClpR subunit showed that its presence in the core complex is not rate-limiting for the overall proteolytic activity of the ClpCP3/R protease. Altogether, the ClpP3/R complex shows remarkable similarities to the 20 S core of the proteasome, revealing a far greater degree of convergent evolution than previously thought between the development of the Clp protease in photosynthetic organisms and that of the eukaryotic 26 S proteasome.

Proteases perform numerous tasks vital for cellular homeostasis in all organisms. Much of the selective proteolysis within living cells is performed by multisubunit chaperone-protease complexes. These proteases all share a common two-component architecture and mode of action, with one of the best known examples being the proteasome in archaea bacteria, certain eubacteria, and eukaryotes (1).

The 20 S proteasome is a highly conserved cylindrical structure composed of two distinct types of subunits, α and β. These are organized in four stacked heptameric rings, with two central β-rings sandwiched between two outer α-rings. Although the α- and β-protein sequences are similar, it is only the latter that is proteolytic active, with a single Thr active site at the N terminus. The barrel-shaped complex is traversed by a central channel that widens up into three cavities. The catalytic sites are positioned in the central chamber formed by the β-rings, adjacent to which are two antechambers jointly built up by β- and α-subunits. In general, substrate entry into the core complex is essentially blocked by the α-rings, and thus relies on the associating regulatory partner, PAN and 19 S complexes in archaea and eukaryotes, respectively (1). Typically, the archaeal core structure is assembled from only one type of α- and β-subunit, so that the central proteolytic chamber contains 14 catalytic active sites (2). In contrast, each ring of the eukaryotic 20 S complex has seven distinct α- and β-subunits. Moreover, only three of the seven β-subunits in each ring are proteolytically active (3). Having a strictly conserved architecture, the main difference between the 20 S proteasomes is one of complexity. In mammalian cells, the three constitutive active subunits can even be replaced with related subunits upon induction by γ-interferon to generate antigenic peptides presented by the class 1 major histocompatibility complex (4).

Two chambered proteases architecturally similar to the protease also exist in eubacteria, HslV and ClpP. HslV is commonly thought to be the prokaryotic counterpart to the 20 S proteasome mainly because both are Thr proteases. A single type of HslV protein, however, forms a proteolytic chamber consisting of twin hexameric rather than heptameric rings (5). Also displaying structural similarities to the proteasome is the unrelated ClpP protease. The model Clp protease from Escherichia coli consists of a proteolytic ClpP core flanked on one or both sides by the ATP-dependent chaperones ClpA or ClpX (6). The ClpP proteolytic chamber is comprised of two opposing homo-heptameric rings with the catalytic sites harbored within (7). ClpP alone displays only limited peptidase activity toward short unstructured peptides (8). Larger native protein substrates need to be recognized by ClpA or ClpX and then translocated in an unfolded state into the ClpP proteolytic chamber (9, 10). Inside, the unfolded substrate is bound in an extended manner to the catalytic triads (Ser-97, His-122, and Asp-171) and degraded into small peptide fragments that can readily diffuse out (11). Several adaptor proteins broaden the array of...
substates degraded by a Clp protease by binding to the associated HSP100 partner and modifying its protein substrate specificity (12, 13). One example is the adaptor ClpS that interacts with ClpA (EcClpA) and targets N-end rule substrates for degradation by the ClpAP protease (14).

Like the proteasome, the Clp protease is found in a wide variety of organisms. Besides in eubacteria, the Clp protease also exist in mammalian and plant mitochondria, as well as in various plastids of algae and plants. It also occurs in the unusual plastid in Apicomplexan protozoan (15), a family of parasites responsible for many important medical and veterinary diseases such as malaria. Of all these organisms, photobionts have by far the most diverse array of Clp proteins. This was first apparent in cyanobacteria, with the model species *Synechococcus elongatus* having 10 distinct Clp proteins, four HSP100 chaperones (ClpB1–2, ClpC, and ClpX), three ClpP proteins (ClpP1–3), a ClpP-like protein termed ClpR, and two adaptor proteins (ClpS1–2) (16). Of particular interest is the ClpR variant, which has protein sequence similarity to ClpP but appears to lack the catalytic triad of Ser-type proteases (17). This diversity of Clp proteins is even more extreme in photosynthetic eukaryotes, with at least 23 different Clp proteins in the higher plant *Arabidopsis thaliana*, most of which are plastid-localized (18).

We have recently shown that two distinct Clp proteases exist in *Synechococcus*, both of which contain mixed proteolytic cores. The first consists of ClpP1 and ClpP2 subunits, and associates with ClpX, whereas the other has a proteolytic core consisting of ClpP3 and ClpR that binds to ClpC, as do the two ClpS adaptors (19). Of these proteases, it is the more constitutively abundant ClpCP3/R that is essential for cell viability and growth (20, 21). It is also the ClpP3/R complex that is homologous to the single type in eukaryotic plastids, all of which also have ClpC as the chaperone partner (16). In algae and plants, however, the complexity of the plastidic Clp proteolytic core has evolved dramatically. In *Arabidopsis*, the core complex consists of five ClpP and four ClpR paralogs, along with two unrelated Clp proteins unique to higher plants (22). Like ClpP3/R, the plastid Clp protease in *Arabidopsis* is essential for normal growth and development, and appears to function primarily as a housekeeping protease (23, 24).

One of the most striking developments in the Clp protease in photosynthetic organisms and Apicomplexan parasites is the inclusion of ClpR within the central proteolytic core. Although this type of Clp protease has evolved into a vital enzyme, little is known about its activity or the exact role of ClpR within the core complex. To address these points we have purified the intact *Synechococcus* ClpP3/R proteolytic core by co-expression in *E. coli*. The recombinant ClpP3/R forms a double hexameric ring complex, with each ring having a specific ClpP3/R stoichiometry and arrangement. Together with ClpC, the ClpP3/R complex degrades several polypeptide substrates, but at a rate considerably slower than that by the *E. coli* ClpAP protease. Interestingly, although ClpR is shown to be proteolytically inactive, its inclusion in the core complex is not rate-limiting to the overall activity of the ClpCP3/R protease. In general, the results reveal remarkable similarities between the evolutionary development of the Clp protease in photosynthetic organisms and the eukaryotic proteasome relative to their simpler prokaryotic counterparts.

**EXPERIMENTAL PROCEDURES**

**Purification of ClpP3/R Complex**—The *Synechococcus clpP3* and clpR genes were amplified from genomic clones using Pfx DNA polymerase (Invitrogen) and cloned into the pACYC Duet vector (Novagen) for co-expression in *E. coli*. Another construct was made in which site-directed mutagenesis was used to replace the active site Ser residue in ClpP3 with Ala. A similar approach was used to prepare the chimeric form of ClpR, in which the central region of clpR coding for amino acids 38 to 212 was replaced with the corresponding region from clpP3. In all cases, a His6 tag was added to the 3’ end of the clpP3 gene to aid purification, whereas no such tag was included in the clpR gene. All gene constructs were confirmed by sequencing.

Co-expression of ClpP3 and ClpR was performed in *E. coli* BL21-STAR cells (Invitrogen) grown at 37 °C in 10 liters of LB cultures. Once in mid-exponential growth (*i.e.* A600 of 0.5), cultures were cooled to 16 °C and isopropyl 1-thio-β-d-galactopyranoside added (final concentration 0.5 mM) to induce protein expression. After 6 h, cells were pelleted and then resuspended in buffer A (20 mM Tris/Cl, pH 7.5, 400 mM NaCl, 40 mM imidazole, 1 mM DTT). Cells were ruptured using a French Press (1000 atm) followed by centrifugation to remove insoluble cell debris. The soluble protein fraction was loaded onto a Ni2+ affinity column (HisTrap HP, GE Healthcare). After washing the column with buffer A, bound proteins were eluted using buffer B (20 mM Tris/Cl, pH 7.5, 400 mM NaCl, 400 mM imidazole, 1 mM DTT). SDS-PAGE revealed that both ClpP3-His6 and ClpR were purified in the same fraction, indicating that the co-expressed proteins formed a single stable oligomer. After dialysis (Sild-A-Lyzer, Pierce) and equilibration in buffer C (20 mM Tris/Cl, pH 7.5, 75 mM NaCl, and 1 mM DTT), the ClpP3/R oligomer was further purified by gel filtration chromatography using a 16/60 Superdex column (GE Healthcare). Mass spectrometry (MS) of the sample revealed no contamination of the purified proteins with *E. coli* ClpP, as did a lack of peptidase activity as shown in supplemental Fig. S2. Proteins were stored in buffer C with 15% glycerol. Protein concentration was determined using the Bradford assay (Pierce), with the concentration of ClpP3/R used in each assay based on a monomeric conformation.

**Purification of ClpC and ClpS1**—Purification of *Synechococcus* ClpC and ClpS1 was performed as previously described (25). Protein concentration was determined using the Bradford assay, with the concentration of ClpC and ClpS1 used in each assay based on a monomeric conformation.

**Native PAGE**—Separation of purified ClpP3/R complexes under non-denaturing conditions was done using a Tris borate gel system (19). For recombinant ClpP3/R complexes, 25 μg of...
protein were resolved on 6–16% polyacrylamide gradient gels. 
To accurately separate proteins solely on their molecular mass, 
gels were run at constant current at 4 °C as described (26). Ferretin (440 kDa, monomer; 880 kDa, dimer), urease (270 kDa, 
trimer), and bovine serum albumin (132 kDa, dimer) were used 
as molecular mass markers. Protein complexes were visualized 
using Coomassie Blue staining (Invitrogen). When comparing 
the size of recombinant and native ClpP3/R complexes, 250 ng 
of recombinant protein was separated with 20 μg of soluble 
protein extracted from wild type Synechococcus. The ClpP3/R 
complex was then detected by immunoblotting using a ClpP3-
specific antibody (19).

**Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM)**—AFM was performed with a MultiMode 
NanoScope IIIa (Veeco) in a commercial glass fluid cell with 
SiC cantilevers (OMCL-TR-800, normal force constant 0.57 N 
m⁻¹, Olympus). For sample preparation, freshly cleaved mica 
was incubated with buffer solution (20 mM Tris/Cl, pH 7.5, 100 
mM NaCl, 0.5 mM DTT) containing 7 μg ml⁻¹ ClpP3/R for 10 
min. After several washing steps with buffer for removal of 
excess protein, imaging under native conditions was performed 
in TappingMode. For negative stain TEM, samples were pre-
pared on plasma-treated carbon-coated copper grids by incu-
bating a 5-μl droplet of the sample solution for 60 s. Excess 
suspension was removed by blotting with filter paper. After 
a washing step using 5 μl of buffer, the grid was blotted and 
stained with 5 μl of 2% uranyl acetate for 60 s. Images were 
recorded using a FEI CM20 FEG at a pixel size of 3.2 Å and a 
defocus of −2 μm. Candidate particles were selected automatic-
ically from electron micrographs using a blob detector. A subset 
of 1000 picks were subjected to unsupervised clustering based on 
a full similarity matrix derived by cross-correlating each par-
ticle with each other accounting for the rotational and transla-
tional degrees of freedom. Good particle views were then 
selected from the class averages of the resulting clusters, and 
the complete data set was classified against these templates. 
The highest scoring matches for each class were used to create 
a final set of class averages for visual inspection. The complete 
processing pipeline was implemented in the in-house Python/ 
C++ package “empi.”

**Mass Spectrometry**—To determine the stoichiometry of the 
ClpP3/R complex electrospray ionization (ESI), MS and tan-
dem MS (MS/MS) experiments were performed on a high mass 
Q-TOF type instrument adapted for a QSTAR XL platform (27, 
28). Prior to MS analysis 300 μl of a 0.6 mg ml⁻¹ solution of 
ClpP3/R in buffer C was concentrated 6-fold by using a 
Vivaspin (Vivascience) centrifugal device with a molecular 
mass cutoff of 5 kDa. The sample was then buffer exchanged 
twice into different concentrations of ammonium acetate solu-
tion (0.1, 0.2, or 1 M) using Bio-Rad Biopin columns. To induce 
dissociation of complex-containing solutions methanol was 
added from 5 to 20%. Nano-ESI capillaries were prepared in-
house from borosilicate glass tubes (29) and an aliquot of 2 μl of 
solution was loaded for sampling. The conditions within the 
mass spectrometer were adjusted to preserve non-covalent 
interactions. The following experimental parameters were 
used: capillary voltage up to 1.3 kV, declustering potential up to 
150 V, focusing potential 250 V, second declustering potential 
55 V, and focusing rod offset ranging from 20 to 100 V, MCP 
2350 V. For tandem MS experiments peaks centered at m/z 
5,650 or 5,850 were selected in the quadrupole and collision 
energy up to 200 V was employed. Argon was used as a collision 
gas at maximum pressure. All spectra were calibrated externally 
by using a solution of cesium iodide (100 mg ml⁻¹). Spectra are 
shown here with minimal smoothing and without background 
subtraction.

**Homology Modeling**—The monomeric structure of ClpR has 
been modeled based on the homologous template EcClpP (Pro-
tein Data Bank code 2fzs; Ref. 30) using MODELLER (31) fol-
lowing the alignment computed by hhssearch (32). Its N termi-
inus has been remodeled in SPDBV (33) using the EcClpP 
template 1yg6A (34). Accordingly, the C terminus was com-
pleted with coordinates from the human ClpP structure (1tg6E) 
(11). The insertions in ClpR were modeled in SPDBV as N-
terminal extensions to the helices of which they are part. The 
monomer of ClpP3 has been modeled using the template 
EcClpP (PDB code 2fzs). The alignment was computed using 
PSI-BLAST (35); the catalytic residues are conserved (Fig. 4A). 
The tetradecameric complex formed by two heptamer rings of 
ClpP3,ClpR (Fig. 4C) was built following a previous method 
(36). Additionally, the final model was selected from a number 
of alternative models recomputed using MODELLER and 
energy minimized in SPDBV. The model was analyzed using the 
iMolTalk server (37) and interface contacts in the complex 
were detected with a distance threshold of 3.4 Å. Images have 
been prepared using either Molscript (38) and Raster3D (39), or 
SPDBV and Povray.

**Peptidase Assay**—The peptidase activity of ClpP3/R and 
EcClpP was measured against the fluorogenic peptides N-suc-
cinyl (Suc)-Leu-Tyr-7-amido-4-methylcoumarin (AMC), Suc-
Val-Lys-Met-AMC, and Suc-Ile-Ile-Trp-AMC (Sigma). For 
each assay, 30 μM peptide and 1–5 μg of ClpP3/R or EcClpP 
were incubated in buffer D (25 mM Tris/Cl, pH 7.5, 75 mM NaCl, 
10 mM MgCl₂, 1 mM DTT) for 5–20 min at 37 °C. Peptide deg-
radation was measured as an increase in relative fluorescence 
(FluoSTAR; BWG) at 310–380 nm excitation and 460 nm emis-
sion wavelengths.

**Degradation of α-Casein and GFP Substrates**—For the pro-
teolytic assays, each of the Clp proteins used was diluted to 1 μM 
final concentration in buffer D together with an ATP-regener-
ation system (13). For each assay 1 μM α-casein was used, whereas 
100 nm was used for FITC-casein and the GFP sub-
strates (FR-GFP and MR-GFP). All reactions were performed at 
37 °C. Degradation of α-casein was monitored by SDS-PAGE 
and Coomassie Blue staining, whereas FITC-casein degrada-
tion was measured by fluorescence (FluoSTAR; BWG) at 490 
nm excitation and 525 nm emission wavelengths. Degradation of 
FR- and MR-GFP was determined either by immunoblotting 
using a GFP-specific antibody or by loss in fluorescence at 400 
nm excitation and 510 nm emission wavelengths.

**ATPase Activity**—The ATPase activity of ClpC was mea-
sured by the release of inorganic phosphate as previously 
described (25). In the assays, 0.5 μM ClpC was incubated either 
a lone or with 1 μM wild type or mutated ClpP3/R in buffer D 
(+4 mM ATP) for 20 min at 37 °C.
A Novel Clp Protease in Photosynthetic Organisms

RESULTS

Purification of Synechococcus ClpP3 and ClpR—We have shown that the two constitutively expressed ClpP3 and ClpR proteins in Synechococcus form an essential hetero-oligomeric complex in vivo (19). Initial attempts to reconstitute this core complex by mixing individually purified ClpP3 and ClpR proteins failed (data not shown), and so a new strategy was adopted (note: neither ClpP3 nor ClpR form a proteolytic core complex on their own). Given that both clpP3 and clpR genes in Synechococcus are arranged within a bicistronic operon (21), we used an E. coli expression system in which the genes were co-expressed within the same cell (Fig. 1A). Based on the premise that both proteins would readily oligomerize together once synthesized in E. coli, a His$_6$ tag was included at the C terminus of only ClpP3 to aid in purification of the core complex. Upon induction with isopropyl 1-thio-β-D-galactopyranoside (IPTG), ClpP3/R was first purified from soluble cell extracts by Ni$^{2+}$-affinity chromatography, then by gel filtration with ClpP3 (21 kDa) and ClpR (25 kDa) visualized by SDS-PAGE and Coomassie Blue staining. Size of the ClpP3/R complex (about 270 kDa) was determined from molecular mass standards as shown on the left, size of recombinant and native ClpP3/R complexes. Purified ClpP3/R (250 ng) was separated by native PAGE along with a soluble protein extract (20 µg) extracted from wild type Synechococcus. ClpP3/R complexes were detected by immunoblotting using a ClpP3-specific antibody.

Subunit Composition of the ClpP3/R Complex—Because the ClpP3/R core, unlike most other ClpP complexes described so far, consists of two distinct subunits, we set out to elucidate its structural arrangement using MS. Composition of the ClpP3/R complex was explored under conditions designed either to maintain the complex intact or to disrupt the complex in solution or gas phase (see “Experimental Procedures”). The major component in all mass spectra corresponds to a heptameric complex, consisting of four and three copies of ClpR and ClpP, respectively (ClpP$_3$ClpR$_4$) (Fig. 3A). In addition, a minor population corresponds to a double heptameric ring structure consistent with that of a Clp proteolytic core.

To define the subunit arrangement within the single ClpP3/R heptameric ring, we modeled all possible arrangements with a 3:4 subunit combination (Fig. 3B). From these initial structures we generated all potential subcomplexes formed by dissociation of one (Fig. 3C) and two subunits (Fig. 3D). The subcomplexes generated were then compared with those formed experimentally (supplementary Table). Only one of the five possible structural arrangements (structure 3, Fig. 3B) was consistent with the subcomplexes formed. Further support for this assignment comes from disruption of the complex in solution using subdenaturing quantities of organic solvent. In 20% methanol with an elongated cleft in the center, compatible with a diagonal view of two sandwiched rings (Fig. 2B). Taken together, the results suggest that the recombinant proteins form a complex matching that of native ClpP3/R in vivo, with a double layered heptameric ring structure consistent with that of a Clp proteolytic core.
the predominant species is the heterodimer together with a heterotrimer and other larger heteromers. The fact that no homodimers are formed under these conditions is consistent with our assignment of structure 3 in which the predominant dissociation products would be heteromers. Overall, by using MS approaches we could not only define the assembly state of the ClpP3/R complex but also define its specific subunit organization within a single heptameric ring.

**Modeling of the ClpR Monomer and the ClpP3/R Core Complex**—Given the high degree of amino acid similarity between ClpR and ClpP proteins in general, we next modeled Synechococcus ClpR based on the well defined homology (Fig. 4A) to the three-dimensional structure of EcClpP. Validation of the models did not show any residues in a disallowed region of the Ramachandran or psi/psi-angle plot. By superimposing the ClpR model (white) onto the known structure of EcClpP (green), a high degree of secondary and tertiary structure conservation could be observed between the two proteins (Fig. 4B). Moreover, the accuracy of the model enabled the more significant differences between ClpR and EcClpP to be highlighted. Besides lacking the three proteolytic active site residues, ClpR also varies from all ClpP proteins by having two amino acid extension regions located within the N-terminal half of the protein (Fig. 4A). Both insertions in ClpR, shown in magenta (Fig. 4B), were modeled as extensions to the helices they are part of, so the additional residues could be densely packed. From this model, it is clear that both insertions in ClpR would extend into the inner chamber space formed by a Clp core complex. Interestingly, by highlighting the catalytic residues in EcClpP (red) with a bound substrate (yellow), the second insertion in ClpR can be seen to extend over the specificity pocket and thus it would greatly limit, if not preclude access/binding of a protein substrate to the active site domain. This insertion is also adjacent to the Gly residue that in EcClpP supposedly stabilizes the oxyanion intermediate during proteolysis (7).

We next used the EcClpP structure to model ClpP3/R as a tetradecameric complex of two hetero-heptamers (Fig. 4C). Monomeric models of ClpR and ClpP3 were superimposed on selected monomers in the known EcClpP complex, with each heptameric ring consisting of the ClpP33ClpR4 arrangement determined by MS (Fig. 3). Two different ring configurations were made, the first a symmetric one in which the same type of monomer in each ring faces each other (Fig. 4C), and a second asymmetric assembly, where different types of monomers in each ring oppose each other (data not shown). The quality of each model was high, with Ramachandran analysis showing no unusual backbone conformations. Little deformation was observed after energy minimization, thus a favorable state can be assumed. One of the most interesting features of both ClpP3/R models was the conformation of the entrance pore at both ends of the core complex. Unusually, the pore is formed by an array of β-strands from the N-terminal region of both ClpP3 and ClpR (Fig. 4C). Although the actual diameter of the pore could not be determined, it appears much narrower than that for the EcClpP structure, a feature that can also be clearly seen in Fig. 2B. The N-terminal β-strands of both ClpP3 and ClpR fill the pore space formed by each heptameric ring, thereby closing the entrance aperture. Access to the inner proteolytic chamber would therefore almost certainly require some degree of dissociation of the N-terminal β-array, probably by the associating ClpC chaperone partner, for efficient translocation of the protein substrate.

**ClpP3/R Is Not the Typical ClpP Peptidase**—The first functional assay of the ClpP3/R complex was to test its activity against selected fluorogenic peptides (supplemental Fig. S2). The first peptide chosen was Suc-Leu-Tyr-AMC, a dipeptide substrate. The ClpC chaperone partner, for efficient translocation of the protein substrate.
AMC and Suc-Ile-Ile-Trp-AMC, were also tested but were similarly not degraded by the ClpP3/R complex (supplemental Fig. S2). For all peptides examined, prolonging the incubation time with ClpP3/R to 20 min did not result in any visible degradation (data not shown). Furthermore, addition of ClpC to the assays also failed to produce any degradation of the various peptides tested (data not shown).

ClpP3/R Core Associates with ClpC—Despite being unable to measure the peptidase activity of the ClpP3/R core, we next investigated whether the complex was proteolytically active when combined with its HSP100 chaperone partner ClpC (19). The first substrate tested was a fluorescently labeled variant of the unfolded model substrate /H9251-casein (FITC-casein). As shown in Fig. 5A, neither ClpC nor ClpP3/R alone degraded FITC-casein but they did when added together (it should be noted that no proteolytic activity was observed when ClpP3 alone was combined with ClpC, data not shown). A similar result was obtained for the non-fluorescent form of α-casein,
which was completely degraded by ClpC and ClpP3/R within 20 min (Fig. 5B). Although these results clearly demonstrate that the ClpP3/R core forms a proteolytic active complex with ClpC, the rate of α-casein degradation by ClpCP3/R was considerably slower than that by the E. coli ClpAP protease, which completely degraded the α-casein within the first 5 min of the assay. Moreover, EcClpA could not, despite high sequence similarity, substitute for ClpC to form an active complex with ClpP3/R (Fig. 5B). A similar lack of degradation activity was also observed when adding ClpC to EcClpP (data not shown).
Because of the relatively slow rate of α-casein degradation by the ClpCP3/R protease, we next tested if loss of the chaperone partner ClpC during the assay by autodegradation was one possible cause. It is known that either in the absence of substrate or when EcClpA is in excess of EcClpP that the ClpAP protease degrades EcClpA both in vitro and in vivo (42). To test for possible autodegradation of ClpC, the α-casein proteolytic assay was prolonged to 90 min, well beyond the time when all α-casein is degraded by the ClpCP3/R protease (i.e. 20 min). Performing the same assay with the E. coli ClpAP protease confirmed the autodegradation of EcClpA, with only trace amounts remaining after 90 min (Fig. 5C). In contrast, no significant loss of ClpC was observed after 90 min despite no protein substrate remaining after the first 20 min. Moreover, even with a 3 M excess of ClpC over ClpP3/R, no degradation of ClpC occurred after 90 min (data not shown). This suggests that ClpC is not affected by autodegradation as is EcClpA, and that this is not the underlying cause for the relatively slow activity observed for the ClpCP3/R protease.

ClpS1 Modulates the Substrate Specificity of the ClpCP3/R Protease—To further characterize the activity of the ClpCP3/R protease, we next examined if the ClpS1 adaptor modulated the substrate specificity of ClpCP3/R as it does for the E. coli ClpAP protease (14). Previously we have shown that Synechococcus ClpS1 binds to ClpC and does not affect its chaperone activities in vitro (25). Degradation of α-casein by the ClpCP3/R protease was assayed with and without the ClpS1 adaptor. Adding ClpS1 clearly inhibited α-casein degradation by the ClpCP3/R protease, with about 80% of the substrate remaining after 20 min (Fig. 6A). We next tested the activity of ClpCP3/R toward another substrate, a modified form of GFP with additional amino acids at the N terminus (FR-GFP). These destabilizing residues are recognized by EcClpS according to the N-end rule and target the GFP protein for degradation by the ClpAP protease (14). Degradation assays with ClpCP3/R were performed with FR-GFP, along with another GFP variant with non-destabilizing amino acids included at the N terminus (MR-GFP). The ClpCP3/R protease alone was unable to degrade either FR-GFP (Fig. 6B) or MR-GFP (data not shown). FR-GFP degradation did
occur, however, when ClpS1 was included in the assay. Interestingly, ClpCP3/R remained unable to degrade MR-GFP even in the presence of ClpS1 (Fig. 6B). FR-GFP degradation by the ClpCP3/R protease was also quantified during the assay, again clearly showing the need for ClpS1 (Fig. 6C). Overall, ClpS1 appears to alter the substrate specificity of the ClpCP3/R protease in a similar manner to the homologous proteins in E. coli.

The substrate FR-GFP was also used to again compare the activity of the ClpCP3/R protease with that of E. coli ClpAP. As shown in Fig. 6D, ClpCP3/R with ClpS1 took over 20 min to completely degrade FR-GFP, whereas the ClpAP protease degraded all FR-GFP within 2 min.

**Mutation of the Active Site Ser-101 in ClpP3 Inactivates the Entire ClpCP3/R Protease—**ClpR has long been considered an inactive variant of ClpP by its lack of a recognizable Ser-type catalytic triad, although no experimental evidence yet supports this conjecture. To address this point, we used site-directed mutagenesis to replace the active site Ser-101 residue in ClpP with an Ala (S101A). It is known from the E. coli ortholog that such a mutation to any one of the catalytic triad amino acids...
causes complete loss in proteolytic activity within that ClpP monomer (7, 43). By the S101A mutation, all ClpP3 subunits in the ClpP3/R core should in theory be inactive, whereas any possible proteolytic active site within the ClpR subunits should remain unaffected. Analyzing first the oligomeric structure, the S101A mutation in ClpP3 had no effect on the formation of the recombinant ClpP3/R core complex of 270 kDa (Fig. 7A). The mutated ClpP3/R complex also stimulated the steady-state ATPase activity of ClpC to the same extent as wild type ClpP3/R (Fig. 7B), suggesting the S101A mutation did not impair the association between the chaperone and proteolytic partners. Next we compared the proteolytic activity of the wild type and mutated ClpP3/R core complexes in association with ClpC, using α-casein as substrate. As shown in Fig. 7C, the mutated ClpP3/R core failed to degrade any of the α-casein. No degradation was also observed when the assay time course was extended to 40 min (data not shown). Performing the assay with the more sensitive FITC-casein as substrate also showed no activity from the mutated complex (data not shown). Overall, these results indicate that the ClpR subunit does not contribute to the proteolytic activity of the ClpP3/R core.

Inclusion of ClpR Is Not Rate-limiting for the ClpCP3/R Protease—Given that the ClpR subunit is proteolytically inactive, the question arises as to whether its inclusion in the ClpP3/R core complex limits the overall degradation activity of the ClpCP3/R protease. To address this point, we made selective modifications to ClpR in an attempt to restore its proteolytic activity. These modified forms of ClpR were then co-expressed with either the wild type or S101A-mutated ClpP3 protein. The first chimeric ClpR constructs had the three active site amino acids of ClpP restored, followed by the removal of the two ClpR-specific extension regions. Despite these modifications to ClpR, however, no additional proteolytic activity was observed when this protein was co-expressed with both forms of ClpP3 (data not shown). A more extensive modification was then made to ClpR in which the internal region from Met-38 to Arg-212 was replaced with the corresponding region from ClpP3 (Fig. 4A), with the N- and C-terminal regions left intact to facilitate intra-ring association with the ClpP3 subunit (ClpP3 alone does not form a stable heptameric ring).3 Co-expression and purification of this chimeric ClpR form with either the active wild type or inactive S101A ClpP3 produced a core complex of the expected oligomeric size (data not shown). Both core complexes containing chimeric ClpR also stimulated the steady-state ATPase activity of ClpC to the same extent as wild type ClpP3/R (data not shown), suggesting the chimeric form of ClpR did not interfere with the association to the chaperone partner.

3 J. Schelin, unpublished result.

FIGURE 7. Mutation of Ser-101 in ClpP3 inactivates the ClpCP3/R protease. A, conversion of active site Ser-101 in ClpP3 to Ala (S101A) had no effect on the assembly of the recombinant ClpP3/R core complex (270 kDa) as visualized by native PAGE. B, stimulation of ClpC ATPase activity by addition of ClpP3/R or S101AclpP3/R as measured by the linear release of inorganic phosphate. C, degradation of α-casein by ClpP3/R protease containing either the non-mutated (O) or mutated (S101A) forms (C) of ClpP3 as visualized by SDS-PAGE/Coomassie Blue staining and quantified relative to the time 0 control set to 100%. Values shown in B and C are mean ± S.E. (n = 3).
DISCUSSION

In this study, we have described the biochemical characteristics of the first Clp proteolytic core complex to contain two distinct subunits, one of which is the unusual ClpP variant, ClpR. The ClpP3/R complex is the main constitutive Clp proteolytic core in cyanobacteria in vivo (19). Besides being essential in Synechococcus, it is homologous to the equally important Clp proteolytic core in eukaryotic plastids (23, 24). Phylogenetically, the ClpR variant appears to have evolved first in cyanobacteria and then through various endosymbiotic events retained in the sole Clp protease in plastids of photosynthetic eukaryotes and Apicomplexan protozoan. ClpR proteins in general have several signature characteristics; the apparent absence of a catalytic triad characteristic of Ser-type proteases and at least one extension within the N-terminal half that commonly make them larger (2–3 kDa) than ClpP (17). Despite the prevalence of ClpR in many different organisms, nothing was known about their structural or functional significance within the proteolytic core complex prior to this work.

Modeling of ClpP3/R using the known ClpP core structures from E. coli and Streptococcus revealed several key features. Besides the obvious lack of a catalytic triad, ClpR also has two N-terminal extensions that are absent in all ClpP forms. The second of these is the most highly conserved in terms of length and amino acid composition among all ClpR orthologs. According to the model, these additional amino acids extend the relevant α-helix in ClpR so that it protrudes further into the inner cavity space. Importantly, this region extends over the specificity pocket that houses the catalytic triad in ClpP proteins. In the EcClpP structure, the specificity pocket consists of two continuous parallel grooves formed by helices 4 and 9, along which the catalytic triads are arranged in each subunit (7). These grooves, which are also present in ClpP3, are covered in ClpR by the extended α-helix thereby severely restricting access to the pocket domain for unfolded protein substrates. In addition, the second extension in ClpR is in close proximity to the Gly residue proposed to stabilize the oxyanion intermediate (7), which would further disrupt the specificity pocket, a possibility also proposed for the corresponding extension in the Arabidopsis ClpR proteins (22). As a consequence, there would remain little selection pressure to retain a functional catalytic triad in ClpR, causing the active site residues to eventually be lost over time. Indeed, loss of the catalytic triad appears to be ongoing in certain cyanobacteria, with their ClpR ortholog still retaining one of the active site residues (i.e. His).

The other critical feature arising from the modeling of ClpP3/R was the structure of the region surrounding the entrance pore. Using recent ClpP structures that show more residues at the N terminus (34), we were able to obtain a detailed and reliable prediction of the conformation of the pore-forming residues in the ClpP3/R complex. Unstructured regions of the N terminus, particularly for ClpR, extend out of the main body of the core, more so than for the EcClpP core complex. Given the position of these extended “pin” regions, it is likely that they directly affect the association of ClpC to the ClpP3/R core. Moreover, they might well influence the specificity of the chaperone partner, possibly explaining why EcClpA
A Novel Clp Protease in Photosynthetic Organisms

is unable to function with ClpP3/R. Properties of the amino acids lining the pore channel also vary considerably in the ClpP3/R complex, with many more residues with hydrophobic side groups compared with the more hydrophilic ones in EcClpP. The increased hydrophobicity of the ClpP3/R pore might well influence the efficiency in which unfolded proteins are translocated into the degradative chamber. More importantly, however, is that these hydrophobic domains could contribute to the relatively narrow diameter to the ClpP3/R pore entrance, which appears effectively closed. Not only would this sealed entrance prevent inadvertent diffusion of proteins into the inner chamber, it would require significantly more “opening” presumably by the associating chaperone partner to enable translocation of the unfolded protein substrate. Indeed, the ability of the chaperone partner to efficiently perform such conformational changes to the core entrance might well be another difference between the various types of HSP100 proteins. Lateral openings in the complex barrel, as predicted by a previous study (22), cannot directly be deduced from our modeling attempts, probably due to closer docking of the monomers in the rings and between the two rings.

The Synechococcus ClpP3/R complex has proven to be an ideal model for this novel type of Clp protease. Dual expression of ClpP3 and ClpR was highly successful in purifying a single recombinant complex that matched the size of the native ClpP3/R complex (19). Surprisingly, the ClpP3/R core displayed no activity toward several short peptides that were efficiently cleaved by EcClpP, especially Suc-Leu-Tyr-AMC, which is routinely used to assay the peptidase activity of ClpP from many other organisms. Although the peptide specificity of this activity does not necessarily correlate to that for larger polypeptide substrates (44), it could well reflect a difference in the nature of the “specificity pocket” where protein substrates are bound inside the proteolytic core. Alternatively, the apparent lack of peptidase activity might instead be due to the relatively narrow entrance aperture of the core complex and thus being essentially closed to the diffusion of short peptides; a possibility that is supported by modeling of the ClpP3/R complex and positioning of the N-terminal regions of each subunit, particularly ClpR.

When associated to ClpC, the ClpP3/R complex was proteolytically active against several model protein substrates. Substrate specificity of the ClpCP3/R protease was also modulated by the ClpS1 adaptor, matching the characteristics of the ClpAP protease in E. coli (14). More surprising, however, was the relatively slow proteolytic activity displayed by ClpCP3/R compared with its E. coli counterpart irrespective of the substrate used. This reduced activity by ClpCP3/R was not due to ClpC autoregulation as occurs for EcClpA (42), nor was it limited by the unfolding activity of ClpC as shown by the use of the unstructured protein substrate α-casein. Moreover, ClpC has similar ATPase activity to that of EcClpA, and that α-casein addition stimulates this activity by about 2-fold for both HSP100 proteins (25). Addition of the wild type ClpP3/R core also stimulated ClpC ATPase activity 2-fold, identical to the effect by EcClpP on EcClpA (45), inferring similar associations between the two sets of chaperone and proteolytic complexes. When taken together, these observations suggest that the underlying cause for the slower activity of the ClpCP3/R protease lies either with the proteolytic core complex itself or in translocation of the protein substrate into the core complex by the associated chaperone partner.

One possible explanation for the comparatively slow degradation activity of ClpCP3/R is the inclusion of ClpR in the proteolytic core, particularly because we have now demonstrated that it is catalytically inactive and does not contribute to the activity of the ClpP3/R core. Because ClpR constitutes over half of the subunits in the tetradecameric ClpP3/R core, it would therefore not be surprising if the ClpCP3/R protease displays slower activity than that of the E. coli Clap protease that contains a core with 14 active ClpP subunits. Despite this possibility, however, the results using the proteolytically active chimeric ClpR subunit clearly showed that the inclusion of ClpR in the wild type ClpP3/R core complex is not rate-limiting for the overall activity of the ClpCP3/R protease and that available ClpP3 subunits are sufficient for the required degradation. Given that the unstructured substrate α-casein was used in these assays, the translocation rate of substrate into the core complex now appears to be the more likely limiting factor for the overall activity of the ClpCP3/R protease. Reduced translocation efficiency of unfolded substrate by the associating chaperone partner into the ClpP3/R core would again be consistent with the apparently closed nature of its entrance pore.

In a broader context, ClpR is now shown to have characteristics similar to the subunits comprising the 20 S proteasome. Proteasomal α- and β-type subunits are remarkably similar in regards to their sequence and fold, which is mirrored in the ClpP3/R core. Like the α-subunit, ClpR is inactive despite having protein sequence similarity to its catalytically active partner within the core complex. Moreover, entrance to the catalytic cleft is sealed in ClpR as well as in the proteasomal α-subunit by an additional α-helix missing in the respective active counterparts, ClpP3 and β-subunits. Although the exact function of the α-subunit remains unknown, as it does for ClpR, both types of subunits might play a pivotal role in the association of the proteolytic core complex with its regulatory partner. Inclusion of ClpR also causes the entrance aperture of the core to be essentially sealed, which is another characteristic of the α-ring of the proteasome (3, 46). As yet, the reason for this closing of the entrance pores in both the ClpP/R core and 20 S proteasome remains unknown. One possibility is that the gated entrance of the ClpP3/R complex might facilitate selective degradation of substrates that are able to open the narrow orifice without the aid of the chaperone partner, as has been described for the 20 S proteasome (47). Such a substrate-specific entry mechanism would also enable proteolytic specificity without the need for an internal tagging system such as ubiquitin. Alternatively, the gated entrance of ClpP3/R might be related to the presence of signaling peptides in these cell types and the need to avoid their inadvertent degradation. Indeed, limited access to the inner proteolytic chamber might well be responsible for the lack of peptidase activity displayed by the ClpP3/R core in this study. Intriguingly, cyanobacteria and higher plants produce secondary metabolites, including small peptides, which are bioactive (48). The gated entrance to the ClpP3/R complex could there-
fore have evolved to prevent uncontrolled degradation of such functionally important peptides.

We also see marked similarities between ClpR and the development of the proteasome β-subunits in eukaryotes. Like the β-rings that form the proteolytic chamber of the archaeal proteasome, the ClpP core in *E. coli* and other non-photosynthetic eu-bacteria consists of a single active subunit arranged in two heptameric rings. The inclusion of the inactive ClpR subunit in the cyanobacterial Clp core complex matches the change in the eukaryotic 20 S core from its archaeal counterpart. In eukaryotes, only three of the seven β-subunits in each ring have the Thr N-terminal nucleophile (3), and thus only six of the 14 subunits in the 20 S core complex are proteolytically active. This number of active β-subunits is identical to that of ClpP3 subunits in the ClpP3/R complex, although the arrangement of the active/inactive subunits in each proteolytic core does differ. Further similarities can be seen in the evolution of the plastid ClpP/R core complex in plants from its endosymbiotic ancestor in cyanobacteria. Typically, the archaean 20 S proteasome consists of a single type of α- and β-subunits, whereas seven distinct paralogs of each comprise the 20 S core in eukaryotes. In comparison, the Clp proteolytic core in *Arabidopsis* chloroplasts consists of five distinct ClpP and four ClpR subunits (48), whereas the homologous complex core in cyanobacteria consists of only a single type of ClpP and ClpR subunit. Indeed, only one of the two rings within the chloroplast core appears to contain the homologous Clp proteins (*i.e.* ClpP1, ClpR1–4) to those in the *Synechococcus* ClpP/R core complex, with the other ring comprised of four nuclear-encoded ClpP paralogs (24).

Overall, it appears that the essential constitutive Clp protease in cyanobacteria and plastids of algae, plants, and Apicompl-exan parasites has evolved from its simpler and often functionally less important equivalent in eubacteria. Moreover, the changes in the ClpP/R type of protease mirror much of the development of the 20 S proteasome from the archaeal prototypetype to its more intricate counterpart in eukaryotes, and as such both enzymes show a remarkable degree of convergent evolution. Despite these similarities, the key question remains: why have the proteolytic cores of ClpP/R and eukaryotic proteasome evolved to include inactive subunits? Notably, both types of core complexes show the same ratio of active to inactive subunits (*i.e.* 3:4), although this ratio does appear to vary somewhat in the chloroplast Clp core complex (24). As shown in this study, incorporation of ClpR does not significantly reduce the activity of the protease, suggesting that translocation of protein substrate into the core complex might instead be the rate-limiting step. Such reduced translocation efficiency could be due to the narrower and possibly sealed entrance to the protease chamber relative to that in other bacterial ClpP core complex such as for EccPp. However, although a more restrictive aperture might have an important role for the ClpP/R protease, similar to that performed by the α-ring in the proteasome, it does not explain the loss of the catalytic function on ClpP and the unprocessed β-subunits in the eukaryotic proteasome. Given that the N-terminal extensions of ClpR protrude into the degradative chamber, with one in close proximity to the specificity pocket, it seems likely that they affect the binding properties of the protein substrate to the adjacent catalytically active ClpP3 subunits. As a consequence, this might affect the diversity of substrates degraded by the Clp protease, possibly in response to the different types of proteins targeted for degrada-tion in plastids and cyanobacteria. Elucidating the biological function of ClpR and its selective advantage remains a major challenge, as it does for the inactive β-subunits of the eukaryotic 20 S proteasome.

Acknowledgment—We thank Kerstin Lindgren at Biovitrum (Göteborg) for generously allowing access to their FluorSTAR optima for assays.

REFERENCES

1. Pickart, C. M., and Cohen, R. E. (2004) Nat. Rev. 5, 177–187
2. Löwe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R. (1995) Science 268, 533–539
3. Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997) Nature 386, 463–471
4. Tanaka, K., and Kasahara, M. (1998) *Immunol. Rev.* 163, 161–176
5. Bochtler, M., Ditzel, L., Groll, M., and Huber, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 6070–6074
6. Grimaud, R., Kessel, M., Beuron, F., Steven, A. C., and Maurizi, M. R. (1998) *J. Biol. Chem.* 273, 12476–12481
7. Wang, J., Hartling, J. A., and Flanagan, J. M. (1997) *Cell* 91, 447–456
8. Thompson, M., and Maurizi, M. (1994) *J. Biol. Chem.* 269, 18201–18208
9. Kim, Y. I., Levchenko, I., Fraczkowska, K., Woodruff, R. V., Sauer, R. T., and Baker, T. A. (2001) *Nat. Struct. Biol.* 8, 230–233
10. Ortega, J., Lee, H. S., Maurizi, M. R., and Steven, A. C. (2002) *EMBO J.* 21, 4938–4949
11. Kang, S. G., Maurizi, M. R., Thompson, M., Mueser, T., and Alhvazi, B. (2004) *J. Struct. Biol.* 148, 338–352
12. Levchenko, I., Seidel, M., Sauer, R. T., and Baker, T. A. (2000) *Science* 289, 2354–2356
13. Dougan, D. A., Reid, B. G., Horwich, A. L., and Bukau, B. (2002) *Mol. Cell* 9, 673–683
14. Ehrbe, A., Schmidt, R., Bornemann, T., Schneider-Mergener, J., Mogk, A., Zahn, R., Dougan, D. A., and Bukau, B. (2006) *Nature* 439, 753–756
15. Waller, R. F., Keeling, P. J., Donald, R. G. K., Striepen, B., Handman, E., Lang-Unnasch, N., Cowman, A. F., Besra, G. S., Roos, D. S., and McCadden, G. I. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 12352–12357
16. Clarke, A. K., MacDonald, T. M., and Sjögren, L. L. E. (2005) *Physiol. Plant.* 132, 403–412
17. Porankiewicz, J., Wang, J., and Clarke, A. K. (1999) *Mol. Microbiol.* 32, 449–458
18. Adam, Z., Adamska, I., Nakabayashi, K., Ostersetzer, O., Haussuhl, K., Manuell, A., Zheng, B., Vallon, O., Rodermerl, S. R., Shinozaki, K., and Clarke, A. K. (2001) *Plant Physiol.* 125, 1912–1918
19. Stane, T. M., Pojdaeva, E., Andersson, F. I., and Clarke, A. K. (2007) *J. Biol. Chem.* 282, 14394–14402
20. Clarke, A. K., and Eriksson, M. J. (1996) *Plant Mol. Biol.* 31, 721–730
21. Schelin, L., Lindmark, F., and Clarke, A. K. (2002) *Microbiol. 148, 2255–2265
22. Peltier, J. B., Ripoll, D. R., Friso, G., Rudella, A., Cai, Y., Ytterberg, I., Giacomelli, L., Pillardy, J., and van Wijk, K. J. (2004) *J. Biol. Chem.* 279, 4768–4781
23. Shikanai, T., Shimizu, K., Ueda, K., Nishimura, Y., Kuroiwa, T., and Hashimoto, T. (2001) *Immunol. Rev.* 161, 161–176
24. Sjögren, L. L. E., Stanne, T. M., Zheng, B., Sutinen, S., and Clarke, A. K. (2006) *Plant Cell* 18, 2635–2649
25. Andersson, F. I., Blaktyr, R., Kirstein, J., Turgay, K., Bukau, B., Mogk, A., and Clarke, A. K. (2006) *J. Biol. Chem.* 281, 5468–5475
26. Clarke, A. K., and Critchley, C. (1992) *Plant Physiol.* 100, 2081–2089
27. Sobott, F., Hernandez, H., McCammon, M. G., Tito, M. A., and Robinson, C. V. (2002) *Anal. Chem.* 74, 1402–1407
A Novel Clp Protease in Photosynthetic Organisms

28. Chernushevich, I. V., and Thomson, B. A. (2004) *Anal. Chem.* 76, 1754–1760
29. Nettleton, E. J., Sunde, M., Lai, Z., Kelly, J. W., Dobson, C. M., and Robinson, C. V. (1998) *J. Mol. Biol.* 281, 553–564
30. Szyk, A., and Maurizi, M. R. (2006) *J. Struct. Biol.* 156, 165–174
31. Sali, A., and Blundell, T. (1993) *J. Mol. Biol.* 234, 779–815
32. Soding, J. (2005) *Bioinformatics* (Oxf). 21, 951–960
33. Guex, N., and Peitsch, M. (1997) *Electrophoresis* 18, 2714–2723
34. Bewley, M. C., Graziano, V., Griffin, K., and Flanagan, J. M. (2006) *J. Struct. Biol.* 153, 113–128
35. Altschul, S., Madden, T., Schaffer, A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. (1997) *Nucleic Acids Res.* 25, 3389–3402
36. Diemand, A., and Lupas, A. (2006) *J. Struct. Biol.* 156, 230–243
37. Diemand, A., and Scheib, H. (2004) *Nucleic Acids Res.* 32, W512–W516
38. Kraulis, P. (1991) *J. Appl. Crystallogr.* 24, 946–950
39. Merritt, E. A., and Bacon, D. J. (1997) *Methods Enzymol.* 277, 505–524
40. Schiener, J., Witt, S., Hayer-Hartl, M., and Guckenberger, R. (2005) *Biochem. Biophys. Res. Commun.* 328, 477–483
41. Gottesman, S., and Maurizi, M. R. (1992) *Microbiol. Rev.* 56, 592–621
42. Gottesman, S., Clark, W., and Maurizi, M. (1990) *J. Biol. Chem.* 265, 7886–7893
43. Maurizi, M. R., Clark, W. P., Kim, S. H., and Gottesman, S. (1990) *J. Biol. Chem.* 265, 12546–12552
44. Wang, J., Hartling, J. A., and Flanagan, J. M. (1998) *J. Struct. Biol.* 124, 151–163
45. Unno, M., Mizushima, T., Morimoto, Y., Tomisugi, Y., Tanaka, K., Yasuoka, N., and Tsukihara, T. (2002) *Structure* 10, 609–618
46. Liu, C. W., Corboy, M. J., DeMartino, G. N., and Thomas, P. J. (2003) *Science* 299, 408–411
47. Namikoshi, M., and Rinehart, K. L. (1996) *J. Ind. Microbiol.* 17, 373–384
48. Peltier, J. B., Ytterberg, J., Liberles, D. A., Roepstorff, P., and van Wijk, K. J. (2001) *J. Biol. Chem.* 276, 16318–16327