Distinctive Types of ATP-dependent Clp Proteases in Cyanobacteria

Received for publication, January 10, 2007, and in revised form, February 12, 2007 Published, JBC Papers in Press, March 19, 2007, DOI 10.1074/jbc.M700275200

Tara M. Stanne1,2, Elena Pojidaeva1, Fredrik I. Andersson, and Adrian K. Clarke3

From the Department of Plant and Environmental Science, Gothenburg University, Box 461, 405 30 Gothenburg, Sweden

Cyanobacteria are the only prokaryotes that perform oxygenic photosynthesis and are thought to be ancestors to plant chloroplasts. Like chloroplasts, cyanobacteria possess a diverse array of proteolytic enzymes, with one of the most prominent being the ATP-dependent Ser-type Clp protease. The model Clp protease in Escherichia coli consists of a single ClpP proteolytic core flanked on one or both ends by a HSP100 chaperone partner. In comparison, cyanobacteria have multiple ClpP paralogs plus a ClpP variant (ClpR), which lacks the catalytic triad typical of Ser-type proteases. In this study, we reveal that two distinct soluble Clp proteases exist in the unicellular cyanobacterium Synechococcus elongatus. Each protease consists of a unique proteolytic core comprised of two separate Clp subunits, one with ClpP1 and ClpP2, the other with ClpP3 and ClpR. Each core also associates with a particular HSP100 chaperone partner, ClpC in the case of the ClpP3/R core, and ClpX for the ClpP1/P2 core. The two adaptor proteins, ClpS1 and ClpS2 also interact with the ClpC chaperone protein, likely increasing the range of protein substrates targeted by the Clp protease in cyanobacteria. We also reveal the possible existence of a third Clp protease in Synechococcus, one which associates with the internal membrane network. Altogether, we show that presence of several distinctive Clp proteases in cyanobacteria, a feature which contrasts from that in most other organisms.

Protein degradation is an essential component in the regulation of proteins and their quality control in all organisms (1–3). Many of the proteases that perform these tasks are highly selective in their targeted polypeptide substrates. This substrate specificity is achieved in certain proteases by the incorporation of a chaperone/regulatory component within the proteolytic complex. In such large multisubunit proteases, the proteolytic activity is sequestered within an internal chamber away from the surrounding cellular environment, thus preventing inadvertent degradation of non-targeted proteins. The associated regulatory component facilitates proteolysis by selectively binding protein substrates and unfolding them, often in an ATP-dependent manner. The unfolded protein is then translocated into the proteolytic chamber for degradation. This basic mechanism is shared by many well defined proteases including the 26S proteasome, Lon, Deg, PtsH, and Clp (4–8).

The model Clp protease in Escherichia coli is composed of the proteolytic subunit ClpP, the activity of which is dependent on an AAA+ HSP100 chaperone, either ClpA or ClpX (5, 9, 10). The proteolytic subunit ClpP forms a barrel-like structure comprised of two heptameric rings. These face-to-face rings enclose a proteolytic chamber which houses the catalytic triad Ser-His-Asp characteristic of a Ser-type protease (11). This chamber is capped on one or both ends by a hexameric ring of either ClpA or ClpX (12). These chaperone partners are responsible for substrate recognition, unfolding, and translocation into the proteolytic chamber and each confers distinct substrate specificities to the protease. Adding to the complexity of substrate recognition is the presence of modulators that alter the subset of proteins bound by the chaperone partners (13, 14). ClpS, for example, is essential for degradation of N-end rule substrates by the ClpAP protease in eubacteria. This small adaptor protein binds directly to N-terminal destabilizing residues on the substrate protein, targeting them to ClpAP for degradation (15, 16).

Cyanobacteria are a varied group of prokaryotes that inhabit a vast range of global habitats. They are unique for being the only bacteria that perform oxygenic photosynthesis and as such are considered the progenitors of plant chloroplasts according to the endosymbiotic theory (17–19). This complexity is mirrored by the diversity of proteases that exist in cyanobacteria (20). In the case of the Clp protease, cyanobacteria possess more than one paralog for several of the component subunits. Unlike E. coli, which has only a single ClpP, the model strain Synechococcus elongatus PCC 7942 (Synechococcus)4 has three distinct ClpP paralogs as well as a ClpP-like protein termed ClpR (21). Though similar in sequence and size to ClpP, ClpR lacks the catalytic triad enabling it to function as a Ser-type protease (22). Cyanobacteria also possess two HSP100 proteins, ClpX and ClpC that likely function as regulatory AAA+ components of the Clp protease. They also have two paralogs of the ClpS adaptor protein, which here we term ClpS1 and ClpS2.

Interestingly, the complexity in cyanobacterial ClpP subunits also occurs in plant chloroplasts but to a far greater extent. Five ClpP and four ClpR paralogs exist in chloroplasts of the model higher plant Arabidopsis thaliana (23). All nine proteins are found mixed together in a single 335-kDa complex, presumably

4 The abbreviations used are: Synechococcus, Synechococcus elongatus PCC 7942; Chl, chlorophyll; LSU, large subunit of Rubisco; PBS, phycobilisome; ATPyS, adenosine 5′-O-(thiotriphosphate).
in a double heptameric ring structure as for the similar-sized Clp proteolytic core in *E. coli* (24). Like those in land plants, chloroplasts of green algae also contain multiple ClpP and ClpR paralogs arranged in a single core complex (25). The significance of this complexity in the structure of the Clp proteolytic core in chloroplasts remains unknown as does the function of the ClpR variant.

Although the number and types of Clp proteins in cyanobacteria have been well established, nothing is yet known about the composition of the Clp protease(s) they form. In particular it is unclear whether the various ClpP and ClpR subunits in cyanobacteria oligomerize into a single heterogeneous core complex as do their counterparts in plant chloroplasts, or form several, more simpler structures. Moreover, the identity of the HSP100 chaperone partners for whatever Clp proteolytic core complexes exist in cyanobacteria remains unknown, as does the possible interaction with the two ClpS adaptor proteins. In this study, we have examined the oligomeric structure of the various Clp proteins in *Synechococcus*. We report the identity of two distinct Clp proteolytic core complexes in the soluble fraction of the cell. Each core complex is a mixture of two separate subunits, and each associates with a specific HSP100 chaperone partner. We also provide evidence for the possible existence of a third Clp proteolytic core attached to the inner membrane network.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—Cultures of *S. elongatus* PCC 7942 wild type and the two mutant strains, ΔclpP1 (26) and ΔclpP2 (21), were grown in BG-11 inorganic medium (27) buffered with 10 mM 3-(N-morpholino)propanesulfonic acid (pH 7.5) and bubbled with 5% CO2 in air at 37 °C with continuous, white light of 70 μmol photons m−2 s−1 (28). Experimental cultures were inoculated from liquid precultures to a concentration of 3.0–4.5×105 cells ml−1. Chl content was determined using whole cell spectra and the equations as previously described (29). For both mutant strains, the selection antibiotic (kanamycin for ΔclpP1, chloramphenicol for ΔclpP2) was included in the BG-11 plates and in liquid precultures but excluded from all experimental cultures to avoid possible antibiotic-induced changes in the phenotype.

**Preparation of Clp-specific Antibodies**—In addition to those already existing for certain Clp proteins in *Synechococcus*, polyclonal antibodies specific for ClpR, ClpS1, and ClpS2 were prepared in this study. Antibodies for *Synechococcus* ClpR and ClpS1 were made against full-length recombinant proteins produced in *E. coli*. The recombinant ClpS1 protein was expressed with an N-terminal His tag using the pQE-80L vector (Qiagen) and ClpR as a fusion with the maltose-binding protein using the pMAL-c2 overexpression plasmid (New England Biolabs). Expression of both proteins was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to actively growing *E. coli* cells. Both proteins were then purified according to the manufacturer’s instructions. The ClpS2 antibody was made using the peptide CRREGTVTAPEAAE conjugated to BSA at the N terminus via a disulfide bridge. All purified proteins were injected into rabbits intramuscularly and subcutaneously to produce the specific antibodies (AgriSera). The specificity of the antibodies for all *Synechococcus* Clp proteins used in this study has been shown either previously (21, 33) or in the supplementary figure.

**Denaturing-Polyacrylamide Gel Electrophoresis (PAGE)—**

Total cell proteins were isolated from *Synechococcus* cultures by first lysing cells using a French Press (1000 psi; Thermo Spectronic) in isolation buffer containing 25 mM HEPES-NaOH (pH 7.0), 330 mM sucrose, 10 mM MgCl2, and 5 mM ATP. Following a low-speed centrifugation (1,000 × g for 10 min at 4 °C) to remove cell debris, internal membrane and soluble protein fractions were separated by centrifugation at 35,000 × g for 45 min at 4 °C. Soluble protein fractions were concentrated as necessary (Microsep 3K Omega; Pall Life Sciences), while the membrane protein fractions were washed twice with isolation buffer and repelletted at 35,000 × g for 20 min at 4 °C. Prior to electrophoresis, NuPAGE sample buffer was added to the soluble protein fraction, and used to resuspend the membrane protein fraction as recommended (Invitrogen). Samples containing equal protein content were separated on 12% polyacrylamide Bis-Tris NuPAGE gels (or 3–8% Tris-acetate gels when detecting ClpC by immunoblotting) according to manufacturer’s instruction (Invitrogen).

**Native-PAGE**—For separation of native soluble protein complexes the Tris borate-based PAGE system was used (30). Soluble proteins were isolated as previously described (31) with the following modifications: *Synechococcus* cells were lysed in 50 mM HEPES-KOH (pH 7.0), 0.5 mM sucrose, 10 mM NaCl, 5 mM MgCl2, and 2 mM ATP·γS by vortexing with glass beads. Membranes were separated from the soluble protein fraction by centrifugation at 21,000 × g for 45 min. Soluble proteins were concentrated (10K Nanosep, Pall Life Sciences), and then loaded (60 μg of total protein) on large (20 × 20 cm), 6–13% gradient polyacrylamide gels. Samples were electrophoresed at 4 °C for 20 h at a constant current to ensure proteins reach their pore size limitation within the gel matrix as previously described (32). Native molecular mass standards included ferritin (440 kDa monomer, 880 kDa dimer), urease (272 kDa trimer), and bovine serum albumin (66 kDa monomer, 132 kDa dimer).

**Native/Denaturing Two-dimensional PAGE**—For the separation of individual Clp proteins from native complexes, a combination of native and denaturing PAGE was used as described (32). After separation of protein complexes by native PAGE, the gel lanes were excised and immersed in equilibration buffer (100 mM sucrose, 40 mM Tris-HCl, pH 8.8, 40 mM dithiothreitol, 1 mM EDTA, and 5% LDS) for 30 min. The gel piece was then layered on top of a second dimension denaturing 15% linear polyacrylamide gel (20 cm × 20 cm) and electrophoresed for 16 h under constant current at 4 °C.

**Immunoblotting**—After electrophoresis, separated proteins were transferred to supported nitrocellulose membranes using either the NuPAGE blotting system (Invitrogen) or Trans-Blot Cell (Bio-Rad) according to the manufacturer’s directions. Besides those made in this study, polyclonal antibodies specific for *Synechococcus* ClpP1–3, ClpC, or ClpX have been described elsewhere (21, 26, 33). All primary antibodies were detected with a horseradish peroxidase-conjugated, anti-rabbit secondary antibody and visualized by enhanced chemiluminescence in isolation buffer containing 25 mM HEPES-NaOH (pH 7.0), 330 mM sucrose, 10 mM MgCl2, and 5 mM ATP.
Cyanobacterial Clp Proteases in Vivo

(ECL Plus, GE Healthcare). When necessary, chemiluminescent signals were detected and quantified from triplicate samples using the ChemiGenius Imaging system (Syngene) and associated software.

**Gel Filtration Chromatography**—Separation of native soluble protein complexes by gel filtration chromatography was performed at 4 °C using a calibrated Superose 6 10/30 GL column (GE Healthcare) attached to an automated low pressure chromatography and fraction collection system (ÄKTAPrime, GE Healthcare). The column was pre-equilibrated in 25 mM HEPES-NaOH (pH 7.4), 10 mM NaCl, 10 mM MgCl₂, and 5 mM ATP. The size range of each collected fraction was calculated from the separation of molecular mass standards thyroglobulin (669 kDa), ferritin (440 kDa), catalase (237 kDa), and aldolase (158 kDa). Soluble proteins were extracted from cultures as described above and concentrated (10K Nanosep, Pall Life Sciences) to 4 mg ml⁻¹. Samples (2 mg of total protein) were then filtered (0.45 μm filter) and immediately applied to the size exclusion column. Proteins were separated at a flow rate of 0.3 ml min⁻¹ and collected in 0.5-ml fractions. Each fraction was concentrated (10K Nanosep, Pall Life Sciences) to a volume of 60 ml, separated on denaturing 12% polyacrylamide Bis-Tris or 3–8% polyacrylamide Tris-acetate NuPAGE gels (Invitrogen) and then analyzed by immunoblotting using the suite of Clp-protein specific antibodies.

**Immunoprecipitation**—Soluble proteins from wild-type Synechococcus were isolated in 20 mM HEPES-NaOH (pH 8.0), 150 mM KCl, 0.1 mM EDTA, 10 mM MgCl₂, 0.2% bovine serum albumin, and 0.2% glycerol. Immunoprecipitations were performed using a kit (SeizeX, Pierce) according to the manufacturer’s directions, firstly to prepare columns for ClpC and ClpX antibodies as well as their respective pre-serums for controls. For each column 500 μg of purified IgG was used and 200–400 μg of total soluble protein was loaded. After incubation for 1 h at room temperature followed by overnight at 4 °C, the collected elute was concentrated (10K Nanosep, Pall Life Sciences), separated by denaturing-PAGE, and then analyzed by immunoblotting with antibodies specific for Synechococcus ClpC, ClpX, ClpR, ClpP1–3, ClpS1, and ClpS2. In all experiments when necessary, protein concentrations were determined using the BCA method (Pierce).

**RESULTS**

**Localization of Clp Proteins**—The first characteristic we examined for the numerous Clp proteins in Synechococcus was their intracellular localization. Most unicellular cyanobacteria like Synechococcus contain an internal membrane system, which unlike the thylakoid membranes in plant chloroplasts consists of several concentric rings positioned just inside the cell membrane. Using our suite of antibodies, we examined the distribution of Clp proteins in soluble and internal membrane fractions extracted from wild-type Synechococcus and both ΔclpP mutants (Fig. 1). The quality of the fractionations was verified using antibodies to exclusively soluble (large subunit of Rubisco-LSU) or membrane (D1 protein of photosystem II) proteins.

Localization analysis revealed that all Clp proteins in wild-type Synechococcus were primarily or exclusively localized in the soluble fraction (Fig. 1). ClpP2, ClpP3, ClpS1, and ClpX were found only in soluble protein fraction. In comparison, a minor proportion (20–40%) of ClpC, ClpP1, and ClpR was also detected in the membrane fraction, suggesting these proteins have a membrane-bound population in addition to their more abundant soluble one. The adaptor protein ClpS2 was also associated to the membrane, but it appeared more evenly distributed between the two fractions, indicating a relatively large membrane-bound population (Fig. 1).

To better understand the distribution of Clp proteins within Synechococcus, the two viable knock-out strains, ΔclpP1 and ΔclpP2, were also included in the fraction study. As shown in Fig. 1, the loss of either ClpP1 or ClpP2 did not significantly change the localization of most Clp proteins. Inactivation of ClpP1 caused an almost complete down-regulation of ClpP2 but a 3-fold increase in ClpP3 and ClpR, consistent with earlier observations (21). It is interesting to note, however, that ClpP3 no longer was localized only in the soluble fraction, and traces (10%) were also present at the membrane. The amount of ClpR in the membrane was also greatly increased in abundance compared with wild type (Fig. 1), though had a similar distribution with 20% of the total in the membrane. The molecular chaperone ClpX was more abundant in ΔclpP1, again as previously reported (21) but it remained exclusively a soluble protein. In comparison, ClpC levels remained relatively unchanged in both soluble and membrane fractions. Similarly, the levels and dis-
distribution of ClpS1 and ClpS2 did not change in the ΔclpP1 strain relative to the wild type (Fig. 1).

In contrast to the ΔclpP1 strain, less dramatic alterations were observed in ΔclpP2. The most notable change was in ClpP1 distribution, in which a greater proportion of ClpP1 protein was associated to the membranes in the absence of soluble ClpP2 (Fig. 1). In comparison, levels of ClpP3 and ClpR remained relatively unchanged in the soluble fraction, although a small amount of both proteins was evident in the membrane fraction but much less so compared with that in the ΔclpP1 strain. Levels of ClpC and ClpX remained relatively unchanged in ΔclpP2 as previously described (21), with a slight increase of ClpX protein. No significant change also occurred in the amount or distribution of the two ClpS adaptors in the absence of ClpP2.

Soluble Proteolytic Core Complexes—In *E. coli* a single homogeneous Clp proteolytic core exists, consisting of a double heptameric ring of ClpP (11). Similarly, both algae and land plants have only one Clp proteolytic core in chloroplasts, but one which is composed of multiple ClpP and ClpR subunits (24, 25). Given the many ClpP/R proteins in cyanobacteria, we next analyzed soluble cell extracts from *Synechococcus* by native-PAGE and immunoblotting using the various Clp-specific antibodies to determine the number and composition of the Clp proteolytic core. As shown in Fig. 2, two different-sized complexes were found in the wild type. ClpP1 and ClpP2 were both found in a complex of ~140 kDa while ClpP3 and ClpR were together in a single complex of ~270 kDa. Given that each ClpP/R protein has a different mass (ClpP1, 22 kDa; ClpP2, 23.5 kDa; ClpP3, 21 kDa; ClpR, 28 kDa; 21), and taking into account the arrangement of the *E. coli* ClpP core, the 140-kDa complex appears likely to be a single mixed heptameric ring of ClpP1 and ClpP2, while the 270-kDa complex would be a core structure consisting of both ClpP3 and ClpR.

In both the mutant strains the 270-kDa complex of ClpP3 and ClpR was essentially unaffected by the loss of either ClpP1 or ClpP2 (Fig. 2). In contrast, the 140-kDa ClpP1/P2 complex was completely absent in the ΔclpP1 strain, again inferring that the stability of ClpP2 relies on the presence of ClpP1. Similarly, although a complex containing ClpP1 was detectable in ΔclpP2, the signal was relatively diffuse, suggesting that the stable oligomerization of ClpP1 also needs ClpP2.

To confirm the Clp protein composition of both the 140- and 270-kDa complexes, we next performed a second dimension under denaturing conditions with the native complexes extracted from wild-type cells (Fig. 3). There are several advantages of using such a native/denaturing-PAGE method. When proteins are in their native folded state the epitopes recognized by the respective antibodies are sometimes hidden and enclosed within the protein conformation. Therefore antibodies that usually recognize proteins with ease under denaturing electrophoretic conditions may be unable to recognize their target proteins under native gel conditions. Moreover, the technique in this case ensures each antibody recognizes the correct Clp protein because each has a different mass which is easily separated on large gels. As shown in Fig. 3, the 140-kDa complex was confirmed to be composed of ClpP1 and ClpP2, while the 270-kDa complex consisted of ClpP3 and ClpR.

Because the smaller size of the 140-kDa ClpP1/P2 complex likely corresponded to a single heptameric ring, it appeared that a larger intact ClpP1/P2 proteolytic core was relatively unstable under the electrophoretic conditions used in our study. In general, protein complexes can become destabilized for a variety of reasons and if the interactions between subunits are not strong, certain conditions can cause them to break down more easily than others. Conditions in size exclusion chromatography are often milder than most PAGE systems, and so we used this method to separate the different soluble Clp proteolytic core complexes from wild-type cultures. Immunoblotting of the
Cyanobacterial Clp Proteases in Vivo

In the absence of the (consistent with a ClpC hexamer). In comparison, ClpX was detected in a single elution peak corresponding to 290 kDa, the same predicted hexameric complex as observed by native-PAGE (Fig. 6).

In contrast, ClpX was observed in a 290-kDa complex which corresponds to a hexamer of the 49-kDa ClpX monomer. Although ClpC and ClpX were both more abundant in the ΔclpP1 strain relative to the wild type and unchanged in ΔclpP2 as previously reported (21), their oligomeric state was unaffected in the two mutant strains. As with the Clp core complexes, we also resolved the HSP100 proteins from wild-type cultures by native/denaturing-PAGE, but found no additional complexes, confirming that each detected complex by native-PAGE consisted of the respective monomeric protein (data not shown).

Given that *Synechococcus* ClpC was found only as a dimer by native-PAGE, we next tested whether gel filtration might reveal a higher molecular mass complex for this HSP100 protein. Separating soluble proteins by size exclusion chromatography as for the core complexes identified ClpC protein in several fractions, ranging in size from 90 to 1500 kDa. Quantification of ClpC content in each fraction from replicate experiments revealed two maximum peaks: one at ~200 kDa (matching the ClpC dimer detected by native-PAGE), the other at ~580 kDa (consistent with a ClpC hexamer). In comparison, ClpX was found at a single elution peak corresponding to 290 kDa, the same predicted hexameric complex as observed by native-PAGE (Fig. 6).

In addition to the HSP100s the two adaptor paralogs ClpS1 (13 kDa) and ClpS2 (16 kDa) were also analyzed. Neither ClpS1 nor ClpS2 were found using our native-PAGE systems (data not shown) in which the molecular mass cutoff was ~60 kDa. Using size exclusion chromatography, however, ClpS1 was detected in a single elution peak of 70–80 kDa, which based on the size of the ClpS1 monomer would correspond to a hexamer complex. In contrast, ClpS2 was found in two elution peaks, one of ~100 kDa that would correspond to a ClpS2 hexamer, and the
other at 800–900 kDa similar to the elution profile for ClpC in that size range (Fig. 6).

Association between HSP100s and Other Clp Proteins—Intact Clp proteases are notoriously difficult to detect by techniques such as native-PAGE and column chromatography, presumably because of the relatively transient nature of the interaction between HSP100 chaperone partner and the proteolytic core complex; an obstacle also experienced in this study. However, one approach that has proven successful in revealing the association of certain HSP100 proteins to a Clp proteolytic core has been immunoprecipitation (35). Because of this, we used the antibodies specific for either *Synechococcus* ClpC or ClpX to immunoprecipitate both HSP100 proteins from wild-type soluble protein extracts and analyzed these to determine which if any other Clp protein(s) co-precipitated. As shown in Fig. 7, the ClpC antibody not only immunoprecipitated ClpC but also co-precipitated ClpP1, ClpP3, ClpR, ClpS1, and ClpS2. This suggests that ClpC is the chaperone partner for the soluble ClpP3/R core and can interact with either of the ClpS paralogs. In comparison, the ClpX antibody only co-precipitated ClpP1 and ClpP2 along with ClpX, suggesting that this HSP100 protein functions as the chaperone partner for the soluble ClpP1/P2 proteolytic core.

**DISCUSSION**

In this study we have shown that two distinct Clp proteolytic cores are present in the cyanobacterium *Synechococcus*. This is unlike any other organism reported to date where only one core has been found per organelle or per prokaryote. In the model Clp protease from *E. coli* only one ClpP subunit exists and therefore the core is homogeneous with two heptameric rings of ClpP stacked on one another (11). However even when more than one ClpP subunit is present, the cores tend to become heterogeneous in nature and include all ClpP and, if present, ClpR subunits within a single core. This is true in *Arabidopsis* where, despite having five ClpP and four ClpR isomers present within the chloroplast, all nine of these subunits are found in a single core (24). Additionally in chloroplasts of *Chlamydomonas reinhardtii* a single mixed Clp complex is found composed of four ClpP and five ClpR subunits (25). We also show that each of the Clp proteolytic cores in *Synechococcus* associate to a different HSP100 chaperone, with ClpP3/R binding to ClpC, and ClpP1/P2 with ClpX. Along with ClpC interacting with both adaptor proteins ClpS1 and ClpS2, these results indicate that several distinctive Clp proteases likely exist in cyanobacteria (Fig. 8).

**A Soluble ClpX-ClpP1/P2 Protease**—ClpP1 and ClpP2 together form a complex of ~300 kDa as revealed by column chromatography. Given the size of each ClpP monomer and in analogy to the ClpP core complex in *E. coli*, the most likely identity of this 300-kDa complex is a ClpP1/P2 proteolytic core consisting of two heptameric rings. Not only is the core complex a mixture of ClpP1 and ClpP2 but also the individual 140-kDa heptameric rings are mixed as shown by native-PAGE. The fact that the intact core complex dissociated during separation by native-PAGE suggests the interaction between ClpP1 and ClpP2 within each ring is more stable than that between the two rings themselves.

Several previous observations had earlier hinted toward the presence of a mixed ClpP1/P2 core in *Synechococcus*. Firstly, both ClpP1 and ClpP2 are relatively low abundant proteins in
the wild type under normal growth conditions, and they are the only two possible components of a Clp protease in *Synechococcus* that are non-essential for cell viability (21, 26). Both proteins are also inducible by the same types of stress conditions including high light, cold, and supplemental UV-B irradiation (21, 36). Moreover, as confirmed in this study, inactivation of *clpP1* in *Synechococcus* causes the almost complete loss of ClpP2 without affecting *clpP2* gene expression (21), indicating the dependence of ClpP2 protein stability on ClpP1. Indeed, given the lack of any detectable ClpP2 complex in the absence of ClpP1, this infers all ClpP2 in wild-type *Synechococcus* exists in the mixed ClpP1/P2 core complex identified in this study.

In this study we also reveal that ClpX is the HSP100 partner for the ClpP1/P2 proteolytic core. The fact that *Synechococcus* ClpX forms a stable hexameric complex is consistent with the stability of the ClpX ortholog in *E. coli* (12). That ClpX is the chaperone partner for the ClpP1/P2 core is also not surprising given the organization of the relevant *clp* genes in cyanobacteria. As for the *clp* gene in most eubacteria, *Synechococcus* *clpP2* is in an operon with *clpX*, with *clpP2* positioned just upstream of *clpX* (21). The two *clp* genes are typically expressed both as monocistronic and polycistronic messages, with the proportion of both mRNA types varying between different eubacteria and on different environmental conditions (5, 21, 37). The selection pressure to separate expression of *clpX* from that of *clpP* is almost certainly related to how important the role of ClpX as an independent chaperone is in each organism. In *Synechococcus*, the constitutive role of ClpX as a chaperone is apparently essential for cell viability (21), consistent with the minor amount of polycistronic *clpP2/X* mRNA under normal growth conditions and that loss of ClpP2 as in the *ΔclpP2* strain produces no down-regulation of ClpX (21). Moreover, it is likely that monocistronic expression of *clpP2*, but not that of *clpX* increases during stresses such as those shown to induce ClpP2 protein content such as high light and low temperatures. Such an increase in monocistronic expression occurs for the *clpP* ortholog in *E. coli* during carbon starvation (38). The stress induction of both ClpP1 and ClpP2 also suggests that the proteolytic action of a ClpX-ClpP1/P2 complex is important for *Synechococcus* under certain adverse growth conditions.

**Soluble ClpC-ClpP3/R Proteases**—In addition to the ClpP1/P2 core complex, we have also revealed a complex between ClpP3/R that almost certainly corresponds to another soluble Clp proteolytic core. Like for ClpP1 and ClpP2, the presence of ClpP3 and ClpR in the same core complex in *Synechococcus* is consistent with earlier observations. The *clpP3* and *clpR* genes are located together in a bicistronic operon and expressed both poly- and monocistronically (21). ClpP3 and ClpR are also primarily constitutive proteins, both of which are essential for cell viability (21). The 270-kDa ClpP3/R complex appears more stable than the ClpP1/P2 one, as it remained intact when resolved by both native-PAGE and column chromatography. Recently, we have purified a recombinant *Synechococcus* ClpP3/R complex by co-expression in *E. coli* that has the same size of the native ClpP3/R complex (i.e. 270 kDa) and exhibits proteolytic activity in vitro when complexed to recombinant *Synechococcus* ClpC. Dissociation of the recombinant core complex to single rings has also revealed both ClpP3 and ClpR are mixed within each ring,5 similar to that for the ClpP1/P2 core described above. Together, this supports the identity of the native ClpP3/R complex observed in this study as another soluble Clp proteolytic core. Besides being soluble, the ClpP3/R complex is the main constitutive Clp proteolytic core in *Synechococcus*. This essential complex would appear to be homologous to the stromal Clp proteolytic complex in chloroplasts of higher plants. The stromal Clp core complex in *Arabidopsis* consists of a mixture of ClpP and ClpR subunits (24), and it is necessary for chloroplast development and function (39–43). Substrates for the chloroplast Clp protease have also recently been identified, all of which perform various housekeeping roles such as in protein synthesis, folding and quality control maturation, and RNA maturation (42). Of the cyanobacterial orthologs, ClpP3 is most similar to the plastomic ClpP1 in plants (21). Given the endosymbiotic origin of the chloroplast in higher plants, it is plausible that the cyanobacterial ClpP3/R proteolytic core is the ancestor to the stromal Clp core complex in higher plants, with the added complexity of the chloroplast core complex arising later from gene transfer and duplication.

In this study we have shown that *Synechococcus* ClpC associates to the ClpP3/R core complex and thus it is the likely HSP100 chaperone partner (Fig. 8). Again this matches the situation for the chloroplast Clp protease, in which ClpC has been demonstrated also using immunoprecipitation to bind components of the mixed ClpP/R proteolytic core (30, 35, 44). Although visualized primarily as a dimer, *Synechococcus* ClpC likely acts as a homogeneous hexamer as does *E. coli* ClpA and other members of the HSP100 family (12, 45, 46). Indeed, recombinant *Synechococcus* ClpC does form a hexamer in vitro in the presence of ATP (47). Both *Synechococcus* and chloroplast ClpC (48) appear to form relatively stable dimers as do most other larger HSP100 proteins, but upon the addition of

5 F. I. Andersson, M. Sharon, E. Pojidaeva, A. V. Diemand, J. Schelin, T. M. Stanne, M. Beuttler, S. Witt, W. Baumeister, C. V. Robinson, B. Bukau, A. Mogk, and A. K. Clarke, manuscript in preparation.
ATP they oligomerize into the active hexameric conformation (49). The stability of the hexameric structure, however, appears relatively weak compared with that of the dimer, especially for the ClpC class of HSP100. This would explain the difficulty in this study to resolve native Synechococcus ClpC hexamers by native-PAGE and column chromatography.

ClpS Adapters Associate to ClpC—The adaptor protein ClpS in E. coli binds to the N terminus of ClpA and thereby alters its substrate specificity (15). Association of ClpS is necessary for the ClpAP protease to degrade N-end rule substrates, with ClpS binding directly to N-terminal destabilizing residues within the target protein (16). Similarly, the two Synechococcus orthologs, ClpS1 and ClpS2, both bind in vivo to the cyanobacterial ClpA ortholog, ClpC, presumably changing its substrate specificity. The two cyanobacterial ClpS paralogs contain the amino acids Tyr37 and Glu41 also being important (16). When crucial for binding to N-end rule substrates, with the additional ClpC in vitro ClpS1 and ClpS2, both bind to the cyanobacterial ClpA ortholog, ClpC, presumably changing its substrate specificity. These motifs in ClpS1 are conserved with those in E. coli ClpS, the motif Asp35–36 is important for the association of E. coli ClpS to ClpA (i.e. Glu79 and Glu82, 16). Association of recombinant Synechococcus ClpS1 to ClpC in vitro had been demonstrated earlier (47) although it did not interfere with the chaperone activity of ClpC toward the model protein substrates used in those assays. More recent studies have now shown that recombinant ClpS1 functions like E. coli ClpS by targeting N-end rule substrates to the ClpP3/R proteolytic core when complexed to ClpC. Interestingly, ClpS1 was found in a soluble complex in vivo corresponding to the size of a hexamer, an oligomer that might associate directly to the active ClpC hexamer. In E. coli six ClpS subunits bind to six ClpA subunits, but as yet no evidence exists for a ClpS hexamer on its own. Of course it remains possible that a substantial amount of ClpS1 is monomeric as the ortholog in E. coli (50), and thus was unable to be detected by the techniques used in this study. Certainly the high molecular mass complexes for ClpS1 would appear less stable since they were only detected by the less harsh separation method of size exclusion column chromatography.

As for ClpS1, ClpS2 was also detected in a larger native structure that would correspond to a hexameric complex. The fact that the putative ClpS2 hexamer is larger than the one for ClpS1 (due to ClpS2 monomer being larger than ClpS1) also suggests that the two ClpS adaptor proteins do not form mixed complexes. Interestingly, ClpS2 was also found in even larger sized oligomers, following the same fractionation profile of ClpC, suggesting that a certain amount of soluble ClpS2 hexamers complex with ClpC hexamers. Since ClpS2 appears to be the more abundant ClpS paralog in Synechococcus under normal growth conditions, it would seem that more ClpS2 than ClpS1 associates to ClpC constitutively. We are now investigating whether levels of ClpS1 might increase under less favorable growth conditions as experienced during different stresses.

Overall, the existence of two separate ClpS adaptors, each able to associate to ClpC raises the possibility of distinct subsets of protein substrates specific for ClpC, and thereby for the ClpC-ClpP3/R protease. Although yet to be determined, it appears likely that the two ClpS adaptors also target different protein substrates. In the E. coli ClpS, the motif Asp35–36 is crucial for binding to N-end rule substrates, with the additional amino acids Tyr37 and Glu41 also being important (16). When comparing the Synechococcus orthologs, both have the important double Asp motifs but differ in the associated amino acids (i.e. ClpS1: Phe37 and Glu41, ClpS2: Val37 and Gln41). Given that these motifs in ClpS1 are conserved with those in E. coli ClpS is consistent with our recent observation that ClpS1 also targets the same N-end rule model substrates. Because of these differences, however, it is possible that three types of the soluble ClpC-ClpP3/R protease exists in Synechococcus (i.e. ClpC-ClpP3/R, ClpC/S1-ClpP3/R, and ClpC/S2 - ClpP3/R), each with distinct protein substrates as defined by the associated ClpS paralog or lack thereof (Fig. 8).

A Role for Membrane-bound Clp Proteins in Cyanobacteria—Although all Clp proteins in Synechococcus are soluble, several are also associated to the internal membrane network. Given the types of membrane-bound Clp proteins: ClpC, ClpP1, ClpP2, ClpR, and ClpS2; it is plausible that an additional type of Clp protease exists in Synechococcus besides the two soluble ones already discussed. In the case of ClpP1, the fact that loss of ClpP2 in the ΔclpP2 strain has little effect on ClpP1 content further infers an additional role for ClpP1 independent of the soluble ClpP1/P2 proteolytic core. Interestingly, the soluble ClpP1 protein in the ΔclpP2 strain was detected only as a poorly resolved complex matching the size of a single heptameric ring, suggesting ClpP1 alone is unable to oligomerize into a stable heptamer or further into a proteolytic core complex as shown by size exclusion column chromatography. Instead, loss of ClpP2 causes a greater proportion of ClpP1 to associate to the membrane, supporting the additional involvement of ClpP1 in a membrane-bound Clp protease.

Given the heterogeneous composition of the two soluble Clp proteolytic cores, the presence of both ClpP1 and ClpR on the membranes suggests they form a mixed ClpP1/R core complex. Indeed, the existence of a ClpP1/R mixed proteolytic core could be one explanation for why in the absence of ClpP1 (as in ΔclpP1) a significant proportion of the normally soluble ClpP3 binds to the membrane, almost certainly complexed with ClpR. The need for extra cellular ClpP1 and ClpR protein relative to their soluble partner subunits (ClpP2 and ClpP3, respectively) is consistent with the expression of clpP1 (a monocistronic gene, 26) being unlinked to clpP2, and that the clpP1-clpP3 operon is transcribed both mono- and polycistronically (21). ClpC would be the HSP100 chaperone partner for such a putative membrane-bound ClpP1/R core, with the ClpS2 adaptor binding to ClpC under certain circumstances and changing its substrate specificity.

What role such a membrane-bound Clp protease would have in cyanobacteria is still unclear. Attempts to resolve membrane-bound Clp complexes using blue native-PAGE failed to detect proteins larger than the monomers (data not shown). Recently, however, we have shown by crosslinking studies that in wild-type Synechococcus ClpP1, ClpR and ClpC, but not ClpS2 bind to phycobilisomes (PBS), a large (~5000 kDa) multiprotein complex that functions as the main light-harvesting array in cyanobacteria.6 This association suggests that a membrane-bound ClpC-ClpP1/R protease might target parts or all of the PBS for degradation under certain conditions. The addi-

6 A. Tryggvesson, K. Barker-Åström, P. Gustafsson, and A. K. Clarke, manuscript in preparation.
Cyanobacterial Clp Proteases in Vivo

The Clp proteolytic core associates with a different HSP100 partner, ClpX with ClpP3/R. In the case of ClpC, two ClpS adaptors also associate, opening the possibility of separate sub-sets of protein substrates for the ClpC containing proteases. We also raise the possibility of a third, membrane-bound Clp protease consisting of another type of proteolytic core distinct from the two soluble ones. Overall, the existence of distinctive Clp proteolytic core complexes in cyanobacteria is an unusual characteristic in comparison to Clp proteases in other eubacteria and in the organelles of eukaryotes. Indeed, given the diversity of Clp proteases in cyanobacteria, one of the key questions remaining is identifying the different protein substrates targeted by each, a challenge we are now investigating.

Acknowledgment—We thank Anna Sokolenco for technical help.

REFERENCES

1. Vierstra, R. D. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 385–410
2. Anderson, B., and Aro, E. M. (1997) Physiol. Plant. 100, 783–793
3. Adam, Z. (2000) Biochimie (Paris) 82, 647–654
4. Herskho, A., Heller, H., Elias, S., and Ciechanover, A. (1983) J. Biol. Chem. 258, 8206–8214
5. Gottesman, S., Clark, W. P., Creyc, L. V. D., and Maurizi, M. R. (1993) J. Biol. Chem. 268, 22618–22626
6. Tomyosay, T., Yamanaka, K., Murata, K., Suzuki, T., Boulouc, P., Kato, A., Niki, H., Hiraga, S., and Ogura, T. (1993) J. Bacteriol. 175, 1352–1357
7. Fu, G. K., Smith, M. J., and Markvit, D. M. (1997) J. Biol. Chem. 272, 524–538
8. Kim, K. I., Park, S. C., Kang, S. H., Cheong, G. W., and Chung, C. H. (1999) J. Mol. Biol. 294, 1363–1374
9. Becker, G., Klauke, K., and Henge-Aronis, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6439–6444
10. Henge-Aronis, R. (2002) Curr. Opin. Microbiol. 5, 591–595
11. Wang, J. M., Hartling, J. A., and Flanagan, J. M. (1997) Cell 91, 447–456
12. Grimaud, R., Kessel, M., Beuron, F., Steven, A. C., and Maurizi, M. R. (1998) J. Biol. Chem. 273, 12476–12481
13. Schirmer, E. C., Glover, J. R., Singer, M. A., and Lindquist, S. (1996) Trends Biochem. Sci. 21, 289–296
14. Gottesman, S., Maurizi, M. R., and Wickner, S. (1997) Cell 91, 435–438
15. Dougan, D. A., Reid, B. G., Horwich, A. L., and Bukau, B. (2002) Mol. Cell 9, 673–683
16. Ehrb, A., Schmidt, R., Bornewann, T., Schneider-Mergener, J., Mogk, A., Zahn, R., Dougan, D. A., and Bukau, B. (2006) Nature 439, 753–756
17. Herrmann, R. G. (1997) in Eukaryotic Protein Synthesis (Schenk, H. E. A., Herrmann, R. G., Jeon, K. W., Mülner, N. E., and Schwemmler, W., eds), pp. 73–118, Springer, Berlin Heidelberg New York
18. Martin, W., and Herrmann, R. (1998) Plant Physiol. 118, 9–17
19. Martin, W., Stoebe, B., Goremkyn, Hansmann, S., Hasegawa, M., and Kowallik, K. V. (1998) Nature 393, 162–165
20. Sokoleno, A., Poiradeva, E., Zinchenko, V., Panichkin, V., Glaser, V. M., Herrmann, R. G., and Shestakov, S. V. (2002) Curr. Genet. 41, 291–310
21. Schelin, J., Lindmark, F., and Clarke, A. K. (2002) Microbiol. 148, 2255–2265
22. Clarke, A. K. (1999) Ann. Bot. 83, 593–599
23. Adam, Z., Adamska, I., Nakabayashi, K., Ostertzer, O., Haussuhl, K., Manuell, A., Zheng, B., Vallon, O., Rodermel, S. R., Shinozaki, K., and Clarke, A. K. (2001) Plant Physiol. 125, 1912–1918
24. Pellet, J. B., Ytterberg, J., Liberis, D. A., Roestoff, P., and van Wijk, K. J. (2001) J. Biol. Chem. 276, 16318–16327
25. Majeran, W., Friso, G., van Wijk, K. J., and Vallon, O. (2005) FEBS J. 272, 5558–5571
26. Clarke, A. K., Schelin, J., and Porankiewicz, J. (1998) Plant Mol. Biol. 37, 791–801
27. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) J. Gen. Microbiol. 111, 1–61
28. Campbell, D., Zhou, G., Gustafsson, P., Oquist, G., and Clarke, A. K. (1995) EMBO J. 14, 5457–5466
29. Myers, J., Graham, J., and Wang, R. T. (1980) Plant Physiol. 66, 1144–1149
30. Sokoleno, A., Lerbs-Mache, S., Altschmied, L., and Herrmann, R. G. (1998) Planta 207, 286–295
31. Poiradeva, E., Zinchenko, V. V., Shestakov, S. V., and Sokoleno, A. (2004) J. Bacteriol. 186, 3991–3999
32. Clarke, A. K., and Critchley, C. (1992) Plant Physiol. 100, 2081–2089
33. Porankiewicz, J., and Clarke, A. K. (1997) J. Bacteriol. 179, 5111–5117
34. Maurizi, M. R. (1992) Experientia 48, 178–201
35. Halperin, T., Ostertzer, O., and Adam, Z. (2001) Planta 213, 614–619
36. Porankiewicz, J., Schelin, J., and Clarke, A. K. (1998) Mol. Microbiol. 29, 275–283
37. Yoo, S. J., Seol, J. H., Kang, M. S., Ha, D. B., and Chung, C. H. (1994) Biochem. Biophys. Res. 203, 798–804
38. Li, C., Tao, Y. P., and Simon, L. D. (2000) J. Bacteriol. 182, 6630–6637
39. Kuroda, H. and Maliga, P. (2003) Nature 425, 86–89
40. Zheng, B., Macdonald, T. M., Sutinen, S., Hurry, V., and Clarke, A. K. (2006) Planta 224, 1103–1115
41. Sjögren, L. L. E., Stanne, T. M., Zheng, B., Sutinen, B., and Clarke, A. K. (2006) Plant Cell 18, 2635–2649
42. Rudella, A., Friso, G., Alonso, J. M., Ecker, J. R., and van Wijk, K. J. (2006) Plant Cell 18, 1704–1721
43. Koussetzvity, S., Stanne, T. M., Peto, C. A., Giap, T., Sjögren, L. L. E., Zhao, Y., Clarke, A. K., and Chory, J. (2007) J. Mol. Biol. 63, 85–96
44. Desimone, M., Weiss-Wichert, W., Wagner, E., Altenfeld, U., and Johann-ningmeier, U. (1997) Bot. Acta 110, 234–239
45. Zolkiwski, M. (1999) J. Biol. Chem. 274, 28083–28086
46. Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G. P., Bartunik, H. D., and Huber, R. (2000) Nature 403, 800–805
47. Andersson, F. I., Blakytny, R., Kirstein, J., Turgay, K., Bukau, B., Mogk, A., and Clarke, A. K. (2005) J. Biol. Chem. 281, 5468–5475
48. Pellet, 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
49. Maurizi, M. R. (1991) Biochem. Soc. Trans. 19, 719–723
50. Zeth, K., Ravelly, R. B., Paal, K., Cusack, S., Bukau, B., and Dougan, D. A. (2002) Nat. Struct. Biol. 9, 906–911