Cyanobacterial ClpC/HSP100 Protein Displays Intrinsic Chaperone Activity*§

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HSP100 proteins are molecular chaperones that belong to the broader family of AAA+ proteins (ATPases associated with a variety of cellular activities) known to promote protein unfolding, disassembly of protein complexes and translocation of proteins across membranes. The ClpC form of HSP100 is an essential, highly conserved, constitutively expressed protein in cyanobacteria and plant chloroplasts, and yet little is known regarding its specific activity as a molecular chaperone. To address this point, ClpC from the cyanobacterium Synechococcus elongatus (SyClpC) was purified using an Escherichia coli-based overexpression system. Recombinant SyClpC showed basal ATPase activity, similar to that of other types of HSP100 protein in non-photosynthetic organisms but different to ClpC in Bacillus subtilis. SyClpC also displayed distinct intrinsic chaperone activity in vitro, first by preventing aggregation of unfolded polypeptides and second by resolubilizing and refolding aggregated proteins into their native structures. The refolding activity of SyClpC was enhanced 3-fold in the presence of the B. subtilis ClpC adaptor protein MecA. Overall, the distinctive ClpC protein in photosynthetic organisms indeed functions as an independent molecular chaperone, and it is so far unique among HSP100 proteins in having both “holding” and disaggregate chaperone activities without the need of other chaperones or adaptor proteins.

HSP100 is a highly conserved family of molecular chaperones that consists of several subtypes: ClpA-E and L (Class I), and ClpX-Y (Class II). Despite variations in size and gene structure, Class I proteins all have two distinct ATP-binding domains, also known as AAA modules (3). ClpA is found only in Gram-negative eubacteria like Escherichia coli, ClpB in most eubacteria and eukaryotes, ClpC in cyanobacteria, plants, and most Gram-positive eubacteria, ClpD exclusively in plants, and ClpE and ClpL in certain Gram-positive eubacteria. Class II proteins have only a single AAA module. ClpX is found in all eubacteria and eukaryotes, whereas ClpY is restricted to certain bacteria (3). In general, HSP100 chaperones have a protein unfolding activity dependent on ATP hydrolysis. ClpB promotes protein disaggregation by extracting aggregated proteins by unfolding and then delivering them to the DnaK chaperone system for refolding (4, 5). As components of the Clp protease, E. coli ClpA and ClpX unfold targeted native protein substrates and translocate them into the proteolytic ClpP chamber for degradation (6, 7). A similar mode of action is shared by ClpY (HslU), transferring unfolded protein substrates into the adjacent ClpQ (HslV) proteolytic core (8).

Although Clp proteins are found throughout nature, those in photosynthetic organisms are by far the most numerous and diverse (9). At least 23 distinct Clp proteins exist in the genome of the plant Arabidopsis thaliana, while 10 exist in the cyanobacterium Synechococcus elongatus (10). Multiple paralogs also occur for many of the different types of Clp proteins, as exemplified by the proteolytic subunits, with three ClpP paralogs in Synechococcus and six in A. thaliana (11, 12). Of these many Clp proteins, the constitutive ClpC protein is one of the most functionally important, being essential for cell viability in both cyanobacteria and plants (13, 14). It is also highly conserved, with the chloroplast-localized ClpC protein being ca. 90% similar to the orthologs in cyanobacteria (15). By analogy to other HSP100 proteins, ClpC in photobionts is believed to act as a chaperone both independently and as a regulatory subunit within Clp proteases, although as yet little experimental evidence supports this assumption. Many of the features for ClpC in photobionts differ markedly from the orthologs in Gram-positive bacteria. In Bacillus subtilis, ClpC is not essential for normal growth (16, 17) and is instead a general stress protein critical for growth at high temperatures, as well as being necessary for certain developmental processes such as genetic competence and sporulation (17). The chaperone activity of B. subtilis ClpC (BsClpC) relies on specific adaptor proteins.

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known as MecA, which both target specific protein substrates to BsClpC and activate its ATPase activity (18–20). The need for such adaptors also occurs for other HSP100 proteins. In E. coli, ClpX requires RssB to target the starvation sigma factor σE for degradation (21), as well as the SspB adaptor for enhancing the binding and degradation of SsrA-tagged polypeptides (22). Similarly in E. coli, the adaptor ClpS modulates the substrate specificity of ClpA (23).

Given the current dearth of information regarding the chaperone activity of ClpC in photosynthetic organisms, we purified the full-length ClpC from the model cyanobacterium Synechococcus elongatus (SyClpC) using an E. coli-based overexpression system. The purified recombinant protein exhibited significant basal ATPase activity, as well as functioned as a molecular chaperone without the need of additional chaperones or adaptor proteins. SyClpC was also shown to stabilize unfolded polypeptides and prevent their aggregation, as well as resolubilize protein aggregates and restore their native enzymatic activity.

**EXPERIMENTAL PROCEDURES**

**Purification of SyClpC—**The Synechococcus clpC gene (2520 bp) was amplified from genomic DNA with the high fidelity Pfu DNA polymerase (Stratagene) using primers with Spal and PstI restriction sites for directional cloning. The digested clpC gene was ligated into pLMI104 (IMPACT, New England Biolabs) in-frame to the N-terminal His6-vector PQE-80L (Qiagen) in-frame and ligated into the N-terminal His6-vector PQE-80L (Qiagen) in-frame to the 3’ end of the vector PQE-80L (Qiagen) in-frame. The digested PCR product was ligated into the N-terminal His6-vector PQE-80L (Qiagen) in-frame to the 3’ end of the His-tag. The ligation product was transformed into TOP10F’ cells (Invitrogen) according to the manufacturer’s protocol, with the clpS1 insert being sequenced to verify its integrity. Cells expressing the His-tagged ClpS1 protein (SyClpS1) were grown as described above. IPTG was added (0.4 mM) and cultures left for 5 h at 16°C to induce protein expression. Afterward, cells were pelleted by centrifugation (5000 × g, 15 min, 4°C), resuspended in buffer D (20 mM sodium phosphate, pH 7.6, 500 mM NaCl, 40 mM imidazole, 5 mM DTT) and ruptured in a French Press (1000 atm). The soluble cell extract was collected after centrifugation (14,000 × g, 30 min, 4°C) and then loaded onto a 5-Ml HisTrap HP column (Amersham Biosciences). After washing the column with buffer D, SyClpS1 was eluted with buffer E (20 mM sodium phosphate, pH 7.5, 500 mM NaCl, 50 mM imidazole, 5 mM DTT). SyClpS1 was further purified by gel filtration chromatography (16/60 Superdex column, Amersham Biosciences) using buffer F (20 mM sodium phosphate, pH 7.5, 300 mM NaCl, 3 mM DTT). The SyClpS1 fraction was detected as described above and the purified protein stored in 20 mM sodium phosphate, pH 7.5, 300 mM NaCl, 3 mM DTT, and 10% glycerol. The protein concentration was determined using the BCA method (Pierce), with the concentration of SyClpS1 used in each assay based on a monomeric conformation.

**CD Spectra of SyClpS1—**CD spectra of native and denatured SyClpS1 (10 μM) in buffer (20 mM sodium phosphate, pH 7.5, 300 mM NaCl, 3 mM DTT) were analyzed in a Jasco J-715 spectropolarimeter. For the denatured sample, SyClpS1 was heated at 47°C for 30 min and then cooled to room temperature prior to the analysis. A CD spectrum over the range of 240 to 220 nm was performed on duplicate samples for both native and denatured SyClpS1.

**ATPase Assay—**SyClpS1 ATPase activity was determined using a Malachite Green Assay (25). Reactions were done at 37°C in 50 μl buffer (40 mM Tris-Cl, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mM DTT) with 0.5 μM SyClpS1. Unless stated otherwise, 5 mM ATP was also included in the assays. When testing the effect of varying pH, MES buffer was used at pH 6.5 and 6.0. Proteins tested for their possible stimulatory effect on ClpC ATPase activity were present at 1 μM. Release of inorganic phosphate was monitored spectrophotometrically at 650 nm (UV-120-02 spectrophotometer, Shimadzu). Standards were made by dissolving KH2PO4 in buffer and diluting to a range of 1–12 nmol of inorganic phosphate in 50 μl. All reactions were carried out at least in triplicate.

**Prevention of Aggregation with Heat-denatured Luciferase—**To determine the ability of ClpC to prevent aggregation, SyClpC (1.2 μM) and ATP or ATPγS (2 mM) were preheated in buffer (50 mM Tris-Cl, pH 7.5, 150 mM KCl, 20 mM MgCl2, 2 mM DTT) for 5 min at 43°C and then used to dilute luciferase to 0.1 μM final concentration. Control reactions were also done with only preheated buffer or with SyClpC in the absence of ATP. Aggregation of luciferase at 44°C was detected by increasing turbidity at 575 nm (PerkinElmer Life Sciences LS55B spectrophotometer).

**Interaction of SyClpC with Aggregated MDH—**2 μM MDH was heat-denatured in MDH buffer (50 mM Tris-Cl, pH 7.5, 150 mM KCl, 20 mM MgCl2, 2 mM DTT) and diluted to a final concentration of 1 μM in the presence of SyClpC (1 μM) with or without ATPγS (2 mM). The same procedure was performed with native MDH as control. Reactions were incubated for 5 min at room temperature and centrifuged (16,000 × g, 30 min, 4°C). The supernatant was removed and the pellet resuspended in sample buffer and analyzed by SDS-PAGE.

**Surface Plasmon Resonance Analysis—**All surface plasmon resonance studies were performed using a BLAcore X (BLAcore AB). His-tagged SyClpS1 or B. subtilis MecA was captured on the surface of a sensor chip nitrilotriacetic acid by metal chelation via Ni2+ -activated nitrilotriacetic acid, yielding a 2000 response units of immobilized pro-
tein. With a flow rate of 20 μl min⁻¹ eluent buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 50 μM EDTA, 0.005% polysorbate 20), 1 μM SyClpC or control proteins (EcClpB, BsClpC, casein, and BSA) were passed over the chip to test for interaction with the immobilized protein. The affinity between SyClpC and SyClpS1 was determined by passing different SyClpC concentrations, ranging from 125 nM to 1 μM, over the immobilized protein. After each run the chip was regenerated with buffer (10 mM HEPES, pH 8.3, 150 mM NaCl, 350 mM EDTA, 0.005% polysorbate 20) and recharged with 500 μM Ni²⁺.

MDH and Luciferase Disaggregation and Refolding—Disaggregation of aggregated MDH was done by first denaturing 2 μM MDH in MDH buffer at 47 °C for 30 min. The heat-aggregated MDH was diluted to 1 μM either alone or in the presence of SyClpC, SyClpC, and MecA (0.5 μM) or SyClpC and SyClpS1 (0.5 μM). As a control, SyClpC was substituted with the E. coli ClpB (1 μM) and KJE proteins (1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE). All reactions were performed with an ATP-regenerating system (2 mM ATP, 4 mM phosphoenolpyruvate, 20 ng ml⁻¹ pyruvate kinase). Disaggregation of MDH was measured at 30 °C by decreasing turbidity at 600 nm. For the MDH disaggregation/refolding assays, MDH (2 μM) was pre-aggregated at 47 °C for 30 min and then diluted to 1 μM either alone or with SyClpC, SyClpC and MecA (0.5 μM) or SyClpC and SyClpS1 (0.5 μM). As a control, SyClpC was substituted with the E. coli ClpB (1 μM). All reactions included the ATP regenerating system as detailed above. MDH activity was measured as described previously (20).

For the luciferase assays, luciferase was diluted in MDH buffer to 100 nM, denatured at 43 °C for 15 min to form aggregates, and then diluted to 50 nM either alone or with SyClpC, SyClpC, and MecA (0.5 μM) or SyClpC and SyClpS1 (0.5 μM). All reactions included the ATP-regenerating system as detailed above. As a control, SyClpC was substituted with the E. coli ClpB (1 μM). Reactions were incubated over time at 30 °C. Luciferase activity was determined as described previously (20).

RESULTS

Overexpression of SyClpC—SyClpC was purified using an E. coli overexpression system. Upon induction with IPTG, SyClpC was expressed as a C-terminal fusion protein of 147 kDa with the Sce VMA1 intein and CBD (Fig. 1). After cell lysis, the soluble fusion protein was bound to a chitin affinity column and washed extensively to remove nonspecific protein contamination. SyClpC (87 kDa) was then eluted after cleavage of the fusion protein under reducing conditions, with the intein/CBD partner remaining bound to the column. Subsequent purification of SyClpC by ion-exchange and gel filtration chromatography removed
the remaining protein contaminants (Fig. 1). According to gel filtration chromatography, SyClpC is a dimer in the absence of nucleotide but with the addition of ATP and casein forms a hexamer (data not shown) similar to that of other HSP100 proteins (26–30).

**SyClpC Displays Basal ATPase Activity**—To determine whether SyClpC has basal ATPase activity, we used an assay that measures the release of free inorganic phosphate when ATP is hydrolyzed to ADP. SyClpC exhibited increasing ATPase activity with increasing substrate concentration (Fig. 2). Interestingly, the ATPase activity showed apparent biphasic kinetics, with the first phase having a $K_m$ of 2.05 mM. Tests of other parameters revealed optimal ATP hydrolysis by SyClpC between 37 and 45 °C and within pH 7.0 to 8.0 (Fig. 2, B and C). SyClpC activity was dependent exclusively on ATP and not supported by other nucleotides. It was also stimulated ~2-fold by addition of casein, but not BSA. Strong product inhibition was also found, with ADP demonstrating a concentration dependent effect on ATP hydrolysis (Fig. 2D).

**SyClpC Prevents Formation of Protein Aggregates**—Certain chaperones like GroEL and ClpA in *E. coli* hold denatured proteins in a non-aggregated state (31, 32). To elucidate whether SyClpC also has such a function, luciferase was heat-denatured in the presence of SyClpC and ATPγS and analyzed for inhibition of aggregation. Luciferase is a highly heat-labile protein in the absence of SyClpC and quickly aggregated at high temperature (Fig. 3). Addition of SyClpC without ATP had no effect on the heat-induced aggregation of luciferase (data not shown). However, addition of SyClpC with ATP greatly reduced the heat-induced aggregation of luciferase, indicating SyClpC can stabilize denatured proteins. With the non-hydrolyzable analog ATPγS, SyClpC protected essentially all luciferase from aggregation (data not shown). Substituting SyClpC with BSA failed to prevent luciferase aggregation, indicating that SyClpC acts specifically as a "holder" chaperone.

**SyClpC Interacts with Aggregated Proteins**—Another role performed by certain chaperones is to bind aggregated proteins and then initiate their resolubilization. SyClpC was tested for such activity by using pre-formed protein aggregates in the form of heat-aggregated MDH. SyClpC was incubated for 5 min with aggregated MDH in the presence or absence of ATPγS and then centrifuged to pellet all insoluble proteins. The pelleted fractions were analyzed by SDS-PAGE (Fig. 4A). Only in the presence of ATPγS (lane 2) was a large proportion of SyClpC present in the pelleted fraction along with aggregated MDH, demonstrating the nucleotide dependence of SyClpC binding to aggregated proteins. In contrast, substituting aggregated with native MDH resulted in no SyClpC or MDH in the pelleted fraction (lane 3), indicating SyClpC binds specifically to the aggregated MDH.
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ClpC Exhibits Disaggregation Activity in Vitro—Because of the association between SyClpC and aggregated MDH, we next examined whether SyClpC could resolubilize protein aggregates. For this assay, the ability of SyClpC to resolubilize heat-aggregated MDH in the presence of ATP was determined by aggregate turbidity. As a control, we included the E. coli ClpB and DnaK/DnaJ/GrpE (KJE) chaperone system, which is well known for its disaggregation activity in vitro (4). SyClpC alone exhibited only minor disaggregation activity in comparison to the ClpB/KJE system (Fig. 4B). This disaggregation activity of SyClpC, however, was significant when compared with the control of MDH alone (Fig. 4C). Given that BsClpC requires the MecA adaptor to attain disaggregation activity (20), we next tested whether MecA could also stimulate SyClpC disaggregation activity. As previously observed (20), MecA alone had no disaggregation activity (data not shown). However, in the presence of MecA, SyClpC did indeed exhibit enhanced disaggregation activity but not to the same extent as the ClpB/KJE system (Fig. 4B).

Purification of SyClpS1—Although MecA increased SyClpC disaggregation activity in vitro, no obvious MecA ortholog exists in cyanobacteria or higher plants. However, orthologs to another adaptor protein, ClpS, do exist in photosynthetic organisms (33). In E. coli, ClpS redirects the substrate specificity of ClpA toward protein aggregates (23). Because of this, we purified the Synechococcus ortholog, SyClpS1, most homologous to E. coli and plant chloroplast ClpS. SyClpS1 (14 kDa) was overexpressed in E. coli with an N-terminal His-tag (Fig. 5A). Following induction in E. coli, the soluble SyClpS1 protein was purified to homogeneity by sequential affinity and gel filtration chromatography (Fig. 5A). According to the gel filtration chromatography, SyClpS1 is a monomeric protein similar to the ClpS homolog in E. coli (34, 35). CD spectra analysis of native and denatured SyClpS1 showed that the recombinant protein had distinct three-dimensional structure (data not shown) and thus is likely to be in a folded state.

To determine whether SyClpS1 affects the chaperone activity of SyClpC, we first tested for any physical interaction between the SyClpS1 and SyClpC proteins. Using surface plasmon resonance measurements, increasing concentrations of purified SyClpC were passed over immobilized SyClpS1, giving a change of the refractory index indicative of strong binding (Fig. 5B). When tested with immobilized MecA, addition of SyClpC produced a similar response (data not shown), again demonstrating a strong association between the two proteins. Replacing SyClpC with other HSP100 proteins (i.e. BsClpC and EcClpB) or the control proteins BSA and casein in the same assays gave no discernible change of the refractory index with SyClpS1 (Fig. 5C), indicating the specificity of the SyClpC interaction to SyClpS1. We next examined whether the binding of SyClpS1 could influence SyClpC ATPase activity. Addition of SyClpS1 gave no change in SyClpC ATPase activity, whereas adding MecA stimulated this activity ~3-fold. Moreover, SyClpS1 had no effect on the holder chaperone activity of SyClpC as shown in Fig. 3 (data not shown).

SyClpC Disaggregation and Refolding Activity Is Unaffected by SyClpS1—SyClpS1 was next tested for its effect on the disaggregation of heat-aggregated MDH by SyClpC. Addition of SyClpS1, however, failed to noticeably increase the low level of MDH disaggregation by SyClpC as previously observed (data not shown). Given the low basal level of SyClpC resolubilization activity as measured by the turbidity assay, we next used more sensitive assays of protein disaggregation/refolding based on the restoration of MDH and luciferase enzymatic activity after heat-aggregation (Fig. 6). For both aggregated MDH and luciferase alone, no discernable reactivation of activity was observed throughout the time course of the assay. Addition of E. coli ClpB alone also failed to confer any obvious MDH or luciferase activity, confirming the dependence of ClpB on the KJE chaperones for disaggregation/refolding activity (4). In contrast, addition of SyClpC restored a significant proportion of activity to both the aggregated MDH and luciferase (Fig. 6), confirming the low level of disaggregation activity by SyClpC as measured pre-
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We have demonstrated that SyClpC functions as a molecular chaperone, the first such evidence for this type of HSP100 protein from photosynthetic organisms. ClpC proteins in cyanobacteria and plants share many characteristics, most notably their structural conservation, necessity for cell viability, and constitutive, non-stress-inducible expression (13, 15). Although long believed to act as a chaperone, little was known prior to this study regarding such specific activity. In contrast, the functional roles of the less similar type of ClpC in Gram-positive bacteria are better understood. In these bacteria, ClpC is a general stress protein required for growth at high temperatures and stress tolerance (16, 17, 36). It also plays a central role in various developmental processes such as genetic competence and sporulation (17) and for virulence in several pathogens (37, 38).

All HSP100 proteins display intrinsic ATPase activity, but the features of this activity vary between different subtypes. The basal ATPase activity of SyClpC was similar to that for E. coli ClpA and ClpB (39, 40) but higher than for the yeast ClpB ortholog, Hsp104 (41). Other characteristics of SyClpC such as its nucleotide specificity, casein stimulation, and sensitivity to ADP inhibition were also comparable with E. coli and yeast HSP100 proteins. The optimal temperature and pH conditions for SyClpC ATPase activity also correlated well to those most suitable for phototrophic growth of S. elongatus. The biphasic kinetics of the SyClpC ATPase activity was less consistent with those reported for other HSP100 proteins, although such kinetics can be seen for E. coli ClpA (40). One possible explanation for this kinetic profile was that the two AAA modules in SyClpC possess different ATP affinities. However, a $K_{m}$ value could only be calculated for the first phase, whereas the ATP affinity of the second phase would appear too low for the in vivo concentration of ATP (i.e., 2–5 mM) to contribute to ATP hydrolysis. A more likely explanation is that the SyClpC AAA modules function like those in the yeast Hsp104 protein, with one module having an allosteric effect on the kinetics of ATP hydrolysis by the other (42). The high basal ATPase activity of SyClpC contrasts significantly with that of BsClpC, which relies on binding to the adaptor MecA (18, 20). The fact that MecA stimulated ATP hydrolysis by SyClpC suggests it interacts with the cyanobacterial ortholog as it does with BsClpC. Thus, although no structural MecA ortholog exists in cyanobacteria and plants, and that SyClpC has basal ATPase activity unlike BsClpC, it remains possible that an unknown adaptor for ClpC functionally equivalent to MecA exists in photobionts.

SyClpC functions as a holder chaperone in preventing unfolded polypeptides from aggregating into insoluble particles. A similar protein stabilization role has been reported for E. coli ClpA (31), ClpB (43), and the KJE system (32). This ability by SyClpC again differs from BsClpC, which displays only very weak protein stabilization activity in the absence or presence of MecA (20). The ability of SyClpC to act as a holder chaperone may well contribute to its constitutive importance in vivo as well as that of the closely related ClpC orthologs in plants (13, 14). Indeed, it is not difficult to envisage the necessity for chaperones like ClpC within the dynamic proteinaceous environments of cyanobacteria and chloroplasts. ClpC likely functions in part as a housekeeping chaperone in vivo, protecting unfolded, newly synthesized (or recently imported as in chloroplasts) polypeptides from aggregation. Such a role is particularly crucial for polypeptides destined for multimeric complexes, many of which exist in these organisms such as the photosystems and Rubisco. Large multidomain proteins in general are known to follow a relatively slow folding pathway (44) and hence are more aggregation-prone. As a consequence, ClpC could help stabilize such polypeptides prior to their correct folding and thus prevent extensive aggregation and subsequent loss of function. Such a chaperone role for

4 A. K. Clarke, unpublished data.
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ClpC would also explain its association in chloroplasts to the protein import machinery localized within the envelop membranes. Most nuclear-encoded proteins destined for chloroplasts are imported in an unfolded state through the Tic-Toc machinery. ClpC binds to Tic110 close to the exit point (45) and thus likely receives the unfolded polypeptides immediately following import. As a holder chaperone, ClpC could maintain the newly imported polypeptides in a stable unfolded state, minimizing their potential to aggregate, and then promote the correct folding process in concert with the KJE orthologs. ClpB facilitates the initial disaggregation step in this recovery pathway (4, 48), making it essential during severe stresses like heat shock when protein aggregation increases greatly. Despite this, the ability to solubilize and refold aggregate proteins is not a common feature in the HSP100 family. Indeed, to our knowledge SyClpC is the first HSP100 protein to exhibit both disaggregation and refolding activity without the need of other chaperones or adaptor proteins. E. coli ClpA has no disaggregation activity without ClpS (23, 31) nor does E. coli ClpB and BsClpC without the KJE system (4) and MecA (20), respectively. An ability to recognize and resolubilize protein aggregates as well as refold them into native structures is again consistent with SyClpC acting as a housekeeping chaperone. High constitutive levels of ClpC in both cyanobacteria and chloroplasts would not only help prevent protein aggregation but also aid in reactivating misfolded or otherwise damaged polypeptides. Low levels of such damaged proteins would continuously arise from the many processes that function inside these protein rich compartments. ClpC chaperone activities would also help buffer the impact of stresses that increase the probability of protein denaturation, misfolding, and aggregation. Stress induction of ClpB and DnaK chaperones in both cyanobacteria and chloroplasts (49, 50) would complement ClpC activity and rescue the excess of damaged polypeptides unable to be prevented by ClpC. The importance of ClpB in Synechococcus and other organisms during heat stress has been well documented (49, 51). Interestingly, loss of SyClpB doubles the normal level of SyClpC during heat stress (49), which in light of the SyClpC chaperone activities reported here would be consistent with a compensatory response.

A somewhat surprising outcome of this study was the stimulation of SyClpC disaggregation and refolding activities by MecA. As an adaptor, MecA recognizes and targets protein substrates to BsClpC. It was first identified from a screen for repressors of competence development (52) and has since been shown to recognize ComK, a transcription factor that regulates the development of competence in B. subtilis (53). MecA also targets unfolded and aggregated proteins to BsClpC and is necessary for BsClpC to exhibit the disaggregation and refolding activity displayed by SyClpC alone (20). At least one other adaptor protein in B. subtilis interacts with ClpC. YpbH is a MecA paralog that induces ClpC ATPase activity like MecA (18) but targets different substrates to ClpC (19, 20). Because of the co-existence of MecA and ClpC in all sequenced genomes of Gram-positive bacteria, it has been proposed ClpC relies on MecA to select protein substrates and thus become functionally active (20). In this regard, the ClpC in photobionts again differs from the orthologs in Gram-positive bacteria. Not only does SyClpC display protein disaggregation and refolding activity alone, but no MecA orthologs exist in the sequenced genomes of cyanobacteria and plants. The stimulatory effect of MecA on SyClpC chaperone activity observed in vitro, however, raises the possibility that a functionally equivalent adaptor might exist in photosynthetic organisms. A likely candidate for such an adaptor protein was ClpS, for which orthologs in both cyanobacteria and plants have been identified (32). In E. coli, ClpS can stably complex to ClpA and thereby alter its protein substrate specificity. Binding of ClpS prevents ClpA from recognizing SsrA-tagged polypeptides and promotes the binding of protein aggregates (23). Amino acids important for the ClpA-S interaction have been identified (i.e. Glu79, Glu82, and Lys36 in ClpS; Glu28, Glu38, Thr31, and Arg46 in ClpA; Ref. 35). Most of these critical amino acids are conserved in the cyanobacterial orthologs (Glu79 and Glu82 in SyClpS1; Glu28, Thr31, and Arg46 in SyClpC), consistent with the strong interaction between SyClpC and SyClpS1 observed in this study. Despite these characteristics, the cyanobacterial ortholog was unable to stimulate the ATPase activity of SyClpC or its disaggregation and refolding activity as was MecA. This suggests that although SyClpS1 strongly binds to SyClpC, it is not necessary for the in vitro chaperone activities displayed by SyClpC in this study. Thus, as adaptor proteins for ClpC, ClpS and MecA would appear to function differently from each other, at least in cyanobacteria and most likely in chloroplasts. The most obvious explanation for this discrepancy is that the smaller ClpS protein in photobionts acts more to simply direct the binding specificity of ClpC to certain protein targets but is not needed to activate ClpC chaperone activity, while MecA is crucial for both roles in Gram-positive bacteria. The target proteins defined by ClpS and MecA would also differ. Besides the differences in substrate recognition shown in this study, MecA is known to target proteins such as ComK and ComS to ClpC in vivo (53), substrates for which no orthologs have yet been identified in cyanobacteria and plants.

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Cyanobacterial ClpC Molecular Chaperone Activity