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ClpS, a Substrate Modulator of the ClpAP Machine

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Summary

In the bacterial cytosol, ATP-dependent protein degradation is performed by several different chaperone-protease pairs, including ClpAP. The mechanism by which these machines specifically recognize substrates remains unclear. Here, we report the identification of a ClpA cofactor from *Escherichia coli*, ClpS, which directly influences the ClpAP machine by binding to the N-terminal domain of the chaperone ClpA. The degradation of ClpAP substrates, both SsrA-tagged proteins and ClpA itself, is specifically inhibited by ClpS. In contrast, ClpS enhanced ClpA recognition of two heat-aggregated proteins in vitro and, consequently, the ClpAP-mediated disaggregation and degradation of these substrates. We conclude that ClpS modifies ClpA substrate specificity, potentially redirecting degradation by ClpAP toward aggregated proteins.

Introduction

In bacteria, proteolysis is required not only for quality control of proteins but also for important regulatory processes such as the heat shock response and the cell cycle (for review, see Gottesman, 1999; Wickner et al., 1999). To control these processes, protein degradation in the cytosol of *Escherichia coli* is carried out by at least five different adenosine 5′-triphosphate (ATP)-dependent proteases (ClpAP, ClpXP, HslUV (ClpYQ), Lon, and FtsH). These proteolytic machines are generally composed of a peptidase that forms two hexa- or heptameric rings stacked back-to-back (ClpP, HslV) (Wang et al., 1997; Sousa et al., 2000; Bochtler et al., 2000) in association with a hexameric ring-shaped AAA+ protein (ClpA, ClpX, HslU) (Sousa et al., 2000; Bochtler et al., 2000; Neuwald et al., 1999). In some cases (Lon and FtsH), the chaperone and peptidase components are located on a single polypeptide. ClpA is composed of four domains, an N-terminal domain of unknown function and a C-terminal domain involved in substrate recognition (Smith et al., 1999), separated by two nucleotide binding domains (NBDs). Although binding of ATP is sufficient to promote hexamer assembly, ATP hydrolysis is required for substrate unfolding (Wickner et al., 1994; Weber-Ban et al., 1999) and translocation into the active site of the associated peptidase, ClpP (Reid et al., 2001). ClpP is a unique peptidase that interacts with two distinct unfoldases, ClpA and ClpX. Since both ClpA and ClpX are able to recognize different substrates, ClpP is responsible for the specific degradation of a broad range of substrates.

A key question regarding these proteases is the nature and the mechanism of their substrate specificity. ClpAP has been implicated in the degradation of a variety of different proteins, some with amino-terminal degradation signals, such as artificial substrates of the N-end rule (Tobias et al., 1991), the P1 phage encoded RepA (Hoskins et al., 2000), ClpA itself (Gottesman et al., 1990), and others with carboxy-terminal signal sequences such as SsrA-tagged proteins produced upon stalling of translation (Keiler et al., 1996; Gottesman et al., 1998). ClpXP has also been implicated in the degradation of a number of different proteins including phage λ O protein (Gottesman et al., 1993; Wojtkowiak et al., 1993), the trans-degradation of UmuD′ when presented in an UmuD/D′ heterodimer (Gonzalez et al., 2000), and SsrA-tagged proteins (Gottesman et al., 1998). Although indirect data, as judged by λc17 immunity, suggests that the ClpAP machine contributes to degradation of SsrA-tagged substrates (Gottesman et al., 1998), it appears, by directly measuring the amount of SsrA-tagged proteins in different *clp* strains, that only ClpXP actively degrades these substrates in vivo, since SsrA-tagged proteins are stable in a *clpX* null mutant while they are degraded with identical kinetics in wild-type and *clpA* mutant strains (Gottesman et al., 1998). Nevertheless, in vitro, both ClpAP and ClpXP have been shown to degrade SsrA-tagged proteins similarly (Gottesman et al., 1998).

What is the basis of these differences in specificity? In several cases, AAA+ proteins utilize adaptor proteins to expand or modify their substrate specificity. For example, ClpX cooperates with two such adaptor proteins. RssB is a factor specific for targeting degradation of σ^S (Muffler et al., 1996; Pratt and Silhavy, 1996; Zhou et al., 2001) while SspB (Levchenko et al., 2000) enhances the degradation of SsrA-tagged substrates by ClpXP (Flynn et al., 2001). In *Bacillus subtilis*, the protease ClpCP also employs an adaptor protein, MecA, for the efficient degradation of specific proteins that regulate the development of competence (Turgay et al., 1998). These cofactors are not limited only to bacterial members of the AAA+ superfamily. The eukaryotic chaperone, p97, also uses several unrelated adaptor proteins (p47, Udf1p, and Npl4) to promote membrane fusion (Kondo et al., 1997), ubiquitin-dependent protein degradation (Meyer et al., 2000), and nuclear transport (Meyer et al., 2000), respectively. In one case, the adaptor protein p47 was shown to stabilize the N domain of its partner, p97 (Rouiller et al., 2000).

Here, we describe the identification of a ClpA-specific

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adaptor, ClpS, which inhibits degradation not only of SsrA-tagged proteins but also of ClpA itself. Consistently, ClpS binding to the N domain of ClpA displaces a prebound SsrA-tagged substrate. We observe in vitro that ClpS stabilizes the interaction of ClpA with heat-aggregated malate dehydrogenase (agg-MDH), and in the presence of ClpP, the machine enhances the degradation of two different aggregated proteins. The presence of ClpS in vivo may therefore explain why ClpA does not degrade SsrA-tagged substrates in this setting, and, thus, the ClpAPS complex may be involved in the degradation of a different class of proteins, which either aggregate or resemble the aggregates acted on in vitro.

Results

clpS Encodes a Highly Conserved Protein that Forms an ATP-Dependent Complex with ClpA

Upon examination of the *E. coli* genomic DNA sequence, we noticed that a small uncharacterized open reading frame (*yljA*, referred to here as *clpS*) was located immediately upstream of *clpA* (Figure 1A). This gene arrangement was conserved in all bacterial species containing *clpA* with the exception of *Deinococcus radiodurans*, in which the putative operon was interrupted by another gene. In *E. coli*, *clpS* encodes a 106 amino acid protein that contains a highly conserved core motif (Figure 1B). Since in many cases operons encode proteins that functionally cooperate, we speculated that ClpS may act together with ClpA to modulate ClpAP activity, as has been shown for several AAA+ proteins. Interestingly, ClpS homologs are not limited to bacterial species but were also found in all plant species sequenced to date. Since ClpA has yet to be identified in plant species, we speculate that plant ClpS homologs may cooperate with ClpC, as the ClpC specific adaptor protein, MecA, found in *B. subtilis* (Turgay et al., 1997) is also absent from current plant genomic sequences.

We first determined the endogenous levels of ClpS in *E. coli*. ClpS was overexpressed in *E. coli* and purified to homogeneity. Using an affinity-purified anti-ClpS antibody and known amounts of purified ClpS, we estimated there to be ~400 molecules of ClpS per cell in MC4100 (Figure 1C), approximately four times the number of ClpA hexamers (~100) found in the same *E. coli* strain. To demonstrate a physical interaction between ClpA and ClpS, we used gel filtration (Figure 1D). In the absence of ClpA, ClpS elutes from Superose12 at 31 min, indicating that ClpS is either monomeric or dimeric under these conditions (Figure 1D, upper panel filled triangles). The addition of ClpA (in the absence of ATP) resulted in a shift of the ClpS peak (26 min; Figure 1D, upper panel open circles) toward the elution peak of ClpA (24 min; Figure 1D, lower panel open circles) although the presence of ClpS did not alter the migration of ClpA (data not shown). The addition of ATP to ClpA and ClpS resulted in the coelution of both proteins (18 min; Figure 1D, upper and lower panels, respectively, filled circles), suggesting that ATP stabilizes the interaction between ClpA and ClpS.

ClpS Inhibits ClpAP-Mediated Degradation of SsrA-Tagged Substrates

Since the in vitro degradation of SsrA-tagged proteins by ClpAP has been clearly demonstrated, we chose to elucidate the functional role of the ATP-dependent association between ClpA and ClpS by studying the degradation of two SsrA-tagged substrates, SsrA-tagged green fluorescent protein (GFP-SsrA) and a 23 residue synthetic peptide containing the SsrA sequence at the C terminus. The ClpAP-mediated degradation of GFP-SsrA was examined by following GFP fluorescence (Figure 2A) and by analyzing protein amounts on SDS-PAGE (Figure 2B) in the presence and absence of ClpS. The addition of ClpS inhibited the degradation of GFP-SsrA by ClpAP (Figure 2A, filled circles). Complete inhibition of ClpAP-mediated GFP-SsrA degradation required six ClpS monomers per ClpA hexamer (data not shown). By comparison, six monomer equivalents of SspB per ClpA hexamer resulted in only a 3- to 4-fold reduction in the rate of GFP-SsrA degradation by ClpAP (Figure 2A, open diamonds). In contrast, ClpXP-mediated degradation of GFP-SsrA was unchanged by the addition of six monomer equivalents of ClpS (Figure 2C, filled diamonds) and dramatically enhanced by 10-fold less SspB (Figure 2C, open diamonds). Together, these results suggest that each adaptor protein uses a distinct mechanism to modulate the activity of their AAA+ partner with respect to SsrA-tagged substrates. The ClpS-directed inhibition of ClpAP-mediated but not ClpXP-mediated GFP-SsrA degradation is indicative of a specific interaction between ClpA and ClpS and not between ClpS and the substrate. In contrast, SspB influences both ClpAP- and ClpXP-mediated degradation of SsrA-tagged proteins, suggesting a specific interaction with the SsrA-tagged substrate, as was recently elucidated (Flynn et al., 2001). When saturating amounts of ClpS were added after initiation of the degradation reaction (at 150 s), further ClpAP-mediated degradation of the substrate was immediately prevented (Figure 2A, filled diamonds), indicating that ClpS can efficiently prevent the binding of new substrate molecules to ClpA and suggesting that it may also trigger the release of prebound substrates.

To quantify the effect of ClpS on ClpAP-mediated degradation, we determined the half-life of degradation of a 23 residue peptide containing the 11 amino acid SsrA sequence at its carboxy terminus (Figure 2D). As a control, we also examined the effect of ClpS on ClpXP-mediated degradation of the same peptide (Figure 2D). While ClpS inhibited the rate of ClpAP-mediated degradation of the SsrA-peptide by approximately 15-fold, the rate of ClpXP-mediated degradation was essentially unchanged by ClpS. These data confirm that ClpS specifically inhibits ClpAP without affecting ClpXP-mediated degradation of SsrA-tagged substrates.

ClpS Inhibits ClpP-Mediated Autodegradation of ClpA

Over a decade ago, Gottesman et al. (1990) identified ClpA itself as the first substrate of ClpAP-mediated degradation in vitro and in vivo. While examining the effect of ClpS on the degradation of GFP-SsrA, we noticed not only that GFP-SsrA was stabilized by the addition

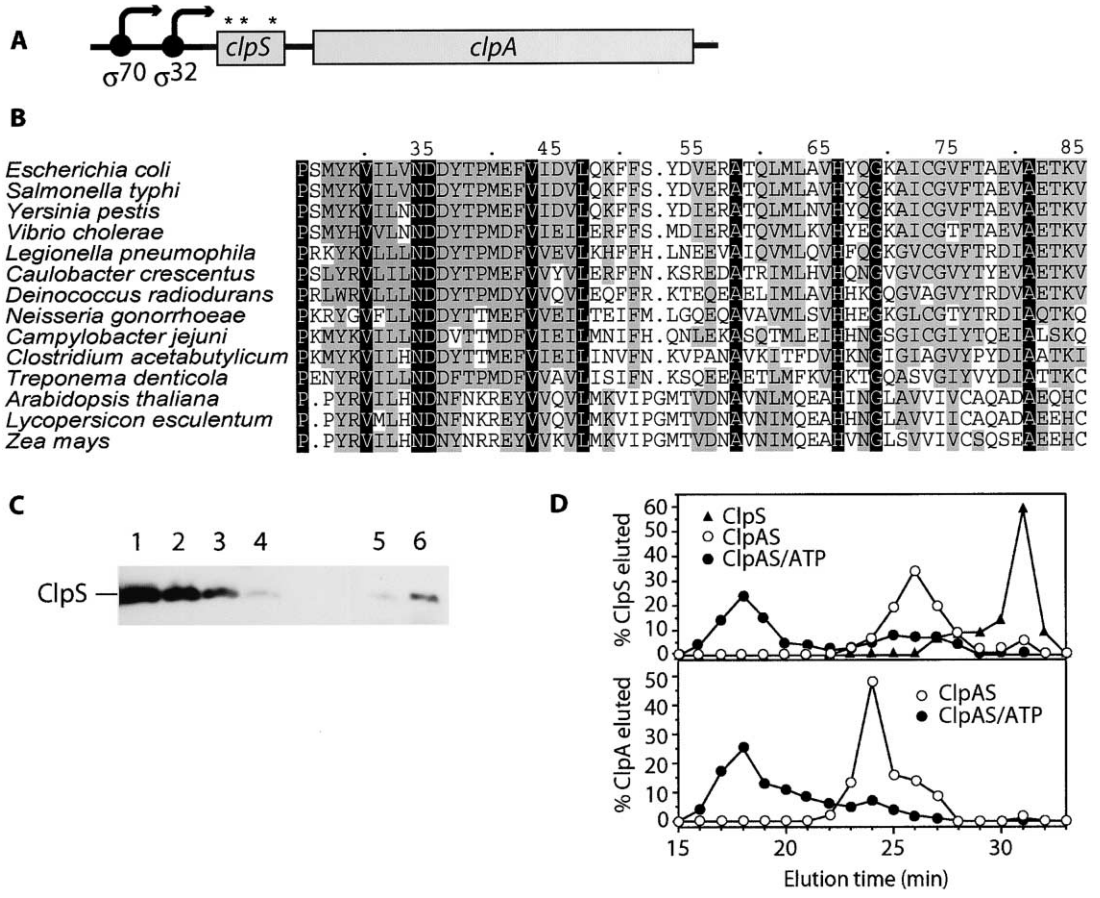

Figure 1. The *clpSA* Operon Encodes a Highly Conserved Protein (ClpS), which Interacts with ClpA in an ATP-Dependent Manner

(A) Cartoon of the *E. coli clpSA* operon. A putative ORF (*yljA,* shown here as *clpS*) is located upstream of the *clpA* gene. The relative position of the σ^{70}- and σ^{32}-dependent promoters as published in the genome sequence are shown. Start points of *clpA* transcription (Gottesman et al., 1990) are indicated by an asterisk.

(B) Multiple sequence alignment of the conserved core of bacterial and plant ClpS homologs. Identical amino acids found in all species are boxed in black, and similar residues found in the majority of species are boxed in gray. Amino acid numbering corresponds to the sequence of ClpS from *E. coli*.

(C) The amount of ClpS in wild-type *E. coli* (MC4100) was compared with known amounts of purified ClpS following immunoblotting with an affinity-purified anti-ClpS antibody. Lanes 1–4: purified ClpS. Lane 1, 34 ng; lane 2, 17 ng; lane 3, 8.5 ng; lane 4, 3.4 ng. Lanes 5–6: MC4100 *E. coli*. Lane 5, 31 μg total protein; lane 6, 62 μg total protein.

(D) Gel filtration elution profiles of ClpS (upper panel) alone (filled triangles) in the presence of ClpA (open circles) or ClpA and ATP (filled circles), and gel filtration elution profiles of ClpA (lower panel) in the presence of ClpS without ATP (open circles) or with ATP (filled circles).

of ClpS but also that the autodegradation of ClpA itself was inhibited (Figure 2B). This prompted us to examine the effect of ClpS, in the absence of a competing substrate, on the ClpP-mediated in vitro degradation of ClpA. In the absence of ClpS, ClpA was degraded by ClpP in an ATP-dependent manner with a half-life of ~10–15 min. However, under the same conditions, in the presence of saturating amounts of ClpS, the half-life of ClpA was increased more than 100-fold (Figure 3A, upper panel). Moreover, the inhibition of ClpA autodegradation was not a result of substrate competition by ClpS, as ClpS also remained stable throughout the course of the experiment (Figure 3A, lower panel). To determine the optimal ClpS/ClpA ratio required for efficient protection of ClpA, we quantified the amount of ClpA remaining after incubation with ClpP, ATP, and increasing amounts of ClpS (Figure 3B). Degradation of ClpA was blocked completely at a ratio of one ClpS monomer per ClpA monomer, equivalent to the binding of six ClpS monomers to a ClpA hexamer.

To examine the in vivo role of ClpS with respect to ClpA stability, we determined the half-life of ClpA in wild-type cells, *clpS*-deleted cells, and ClpS-overexpressing cells. To ensure that deletion of *clpS* did not affect ClpA synthesis, we also monitored the absolute levels of ClpA in WT and Δ*clpS* cells (data not shown). The Δ*clpS* strain was produced by deleting the entire *clpS* open reading frame in the chromosome of MC4100 cells (Datsenko and Wanner, 2000). The loss of the *clpS* gene was verified by PCR and immunoblotting using affinity-purified anti-ClpS antibodies. In exponentially growing cells, translation was blocked by the addition of spectino-

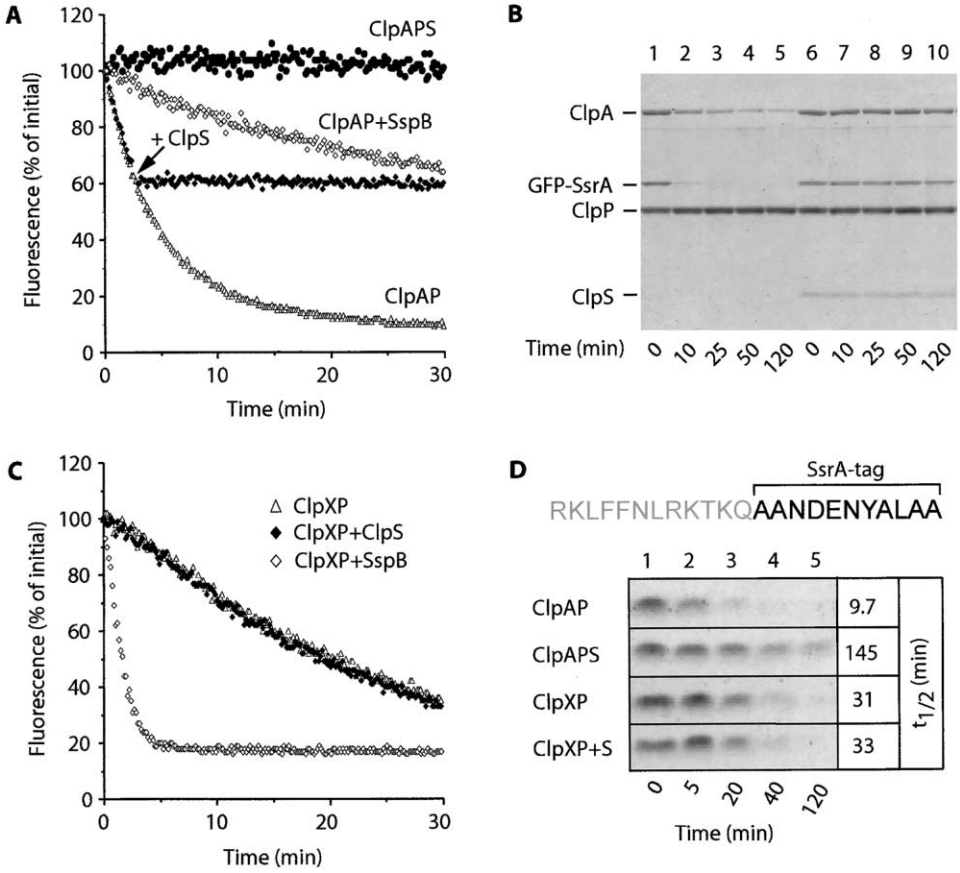

Figure 2. ClpS Specifically Blocks ClpAP-Mediated Degradation of SsrA-Tagged Substrates

(A) ClpAP-mediated degradation of GFP-SsrA was monitored by fluorescence at 510 nm (excitation wavelength was 400 nm) in the absence of additional components (open triangles), with the addition of 1 μM ClpS at t = 0 min (filled circles) or t = 2.5 min (filled diamonds) or with the addition of 1 μM SspB at t = 0 min (open diamonds).

(B) Time course of ClpAP-mediated GFP-SsrA degradation in the absence (lanes 1–5) and presence (lanes 6–10) of 0.5 μM ClpS. Reactions contained 1.3 μM GFP-SsrA and 1 μM ClpAP. Proteins were visualized by Coomassie blue staining.

(C) ClpXP-mediated degradation of GFP-SsrA was monitored by fluorescence (as described in [A]) in the absence of additional components (open triangles), in the presence of 1 μM ClpS at t = 0 min (filled diamonds), or in the presence of 0.1 μM SspB at t = 0 min (open diamonds).

(D) Time course of SsrA-peptide (50 mM) degradation by ClpAP and ClpXP in the absence or presence of ClpS (1 μM). Reactions contained 1 μM ClpP together with either 1 μM ClpA or 1 μM ClpX. All reactions were carried out at 30°C for the period indicated in the presence of an ATP regeneration system and started by the addition of 2 mM ATP. After incubation, peptide amounts were determined by quantification of the Coomassie-stained band (using the MacBAS software) following separation by Tris-Tricine SDS-PAGE. The half-life ($t_{1/2}$) of degradation was determined from the average of three independent experiments.

mycin, and the levels of ClpA were monitored using ClpA-specific antisera. In wild-type *E. coli* cells, the ClpA half-life was greater than 2 hr (Figure 3C, open circles), similar to published data (Gottesman et al., 1990), while in Δ*clpS* cells, the half-life of ClpA was reduced more than 2-fold to ~1 hr (Figure 3C, filled circles). In contrast, ClpA levels were completely stabilized in ClpS-overexpressing cells, suggesting that the levels of ClpS in vivo are limiting, at least with respect to ClpA stability.

Binding of ClpS to the N-Terminal Region of ClpA Results in Release of SsrA-Tagged Substrates

Since a fusion protein of the first 40 residues of ClpA to β-galactosidase was shown to be rapidly degraded in a ClpA-dependent manner (Gottesman et al., 1990), and the addition of ClpS inhibited ClpA degradation in the presence of ClpP, we conjectured that ClpS may modulate ClpA activity through binding to the N terminus. To elucidate the role played by the N-terminal region of ClpA with respect to ClpS binding, we removed the first 143 residues of ClpA (ΔN-ClpA) and monitored the degradation of GFP-SsrA by fluorescence. Although deletion of the N terminus of ClpA reduced the rate of ClpP-mediated degradation of GFP-SsrA by ~90% (Figure 4A, compare filled diamonds and filled circles), degradation of GFP-SsrA was still permitted. This suggests that the N-terminal region of ClpA plays an important but not essential role in either the binding or unfolding of the substrate. Strikingly, the addition of ClpS to ΔN-ClpA did not affect the rate of ClpP-mediated degradation of the SsrA-tagged substrate (Figure 4A, open triangles), suggesting that ClpS modulates ClpAP activity through binding to the N-terminal region of ClpA. To confirm that the N-terminal region was indeed the

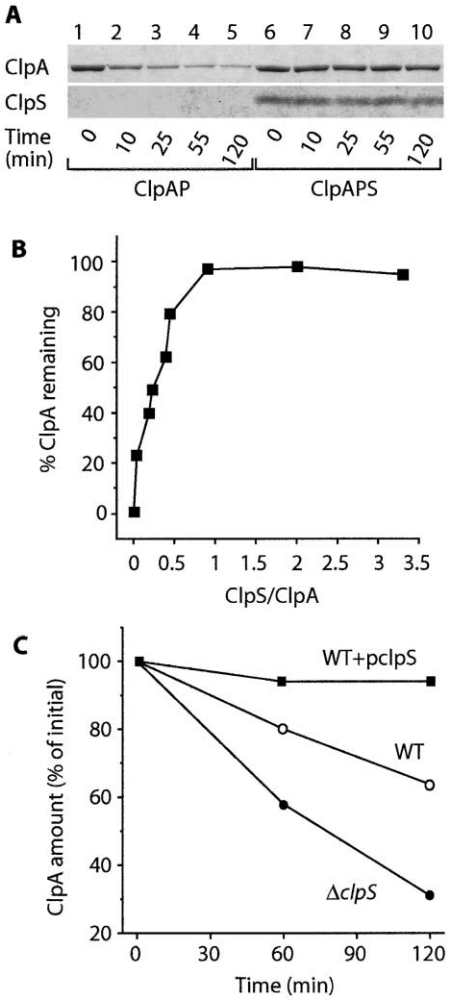

Figure 3. ClpS Inhibits ClpP-Mediated Degradation of ClpA Both In Vitro and In Vivo

(A) Inhibition of ClpA autodegradation by ClpS. ClpA (0.5 μM) was incubated with ClpP (0.5 μM), 2 mM ATP together with an ATP regenerating system at 37°C in the absence (lanes 1–5) or presence (lanes 6–10) of 1 μM ClpS for the indicated period. Samples were separated by Tris-Tricine SDS-PAGE and the proteins visualized by Coomassie blue staining.

(B) Determination of the optimal ClpS/ClpA ratio required to completely inhibit ClpA autodegradation. ClpA (0.5 μM) was incubated together with ClpP (0.5 μM), 2 mM ATP, and an ATP regenerating system at 37°C for 180 min in the presence of increasing amounts of ClpS. The amount of ClpA remaining was determined by quantification, using the MacBAS software, of the Coomassie-stained protein band at the indicated ClpS/ClpA ratios.

(C) ClpA levels were monitored using an anti-ClpA antibody in wild-type (MC4100) cells (open circles), Δ*clpS* cells (filled circles), and ClpS-overexpressing cells (filled squares) after the addition of Spectinomycin to block further protein synthesis. The amount of ClpA was determined by quantification of the immunoreactive band (using the MacBAS software).

site of ClpS interaction, the degradation of GFP-SsrA by ClpAP and ClpAPS was monitored in the presence of a stable N-terminal fragment of ClpA (N domain). The N domain, comprised of residues 1–161, forms an independent structural domain (Lo et al., 2001). In the presence of substoichiometric amounts of ClpS, partial proteolysis of the substrate proceeds (Figure 4B, open diamonds). The addition of an 8-fold excess of the N domain of ClpA partially reverses the ClpS-mediated inhibition of GFP-SsrA degradation (Figure 4B, closed diamonds). The same amount of the N domain, however, did not alter the degradation of GFP-SsrA by ClpAP in the absence of ClpS (Figure 4B, closed triangles). Together, these data suggest that the N domain alone does not inhibit the ClpAP degradation reaction by binding to the substrate but rather through a specific interaction with ClpS. Therefore, ClpS mediates its effect through binding to the N domain of ClpA.

We next tested whether ClpS exerts its inhibitory effect by preventing the binding of substrate. Some substrate proteins, such as the DNA binding domain of λ repressor, bearing an SsrA-tag (λ-SsrA), can form a stable binary complex with ClpA (Reid et al., 2001). By fluorescein maleimide labeling a single cysteine residue engineered into λ-SsrA, we could monitor substrate binding to ClpA through an increase in fluorescence anisotropy when ATPγS was added (Figure 4C). In contrast, no such rise in the anisotropy was observed upon ATPγS addition in the presence of ClpS (Figure 4D, lower trace), indicating that ClpS prevented a stable interaction between the substrate and the ClpA hexamer. We next asked whether addition of ClpS could dissociate an already formed λ-SsrA-ClpA complex. As a control, the addition of a 10-fold excess of unlabeled λ-SsrA to the preformed complex did not alter the anisotropy (data not shown). However, upon addition of ClpS to the binary complex, the anisotropy rapidly dropped (Figure 4D, upper trace), indicating that ClpS triggered dissociation of the SsrA-tagged substrate from the ClpA hexamer.

ClpS Enhances the Degradation of Heat-Aggregated MDH and Luciferase

From the preceding data, it remained unclear whether ClpS was a general inhibitor of ClpAP activity or, alternatively, modulated the binding repertoire of ClpA. In an attempt to address this question, we searched in vitro for substrates that could be recognized by ClpA in a ClpS-dependent manner and then compared the degradation of these substrates by either ClpAP or ClpAPS.

Since it has been shown that ClpB cooperates with the DnaK chaperone system (KJE) to disaggregate protein aggregates (Motohashi et al., 1999; Goloubinoff et al., 1999; Mogk et al., 1999), we speculated that ClpA (a close relative of ClpB) might also cooperate with the KJE system to mediate protein disaggregation. Alternatively, ClpA might combine with ClpS to form a separate system for the dissociation of aggregated proteins.

To test these possibilities, we first investigated the ability of ClpA to interact with agg-MDH in the presence or absence of ClpS (Figure 5A). In these experiments, preformed MDH aggregates generated at 47°C were incubated with Clp proteins, and aggregated or aggregate-associated proteins were separated from soluble proteins by centrifugation. As a control, we also compared the distribution of ClpA and ClpS in the presence of native MDH. In the presence of native MDH, ClpA was identified almost exclusively in the soluble fraction (Figure 5A, lane 1) and this distribution was unchanged by addition of ClpS (Figure 5A, lane 3). In contrast, when

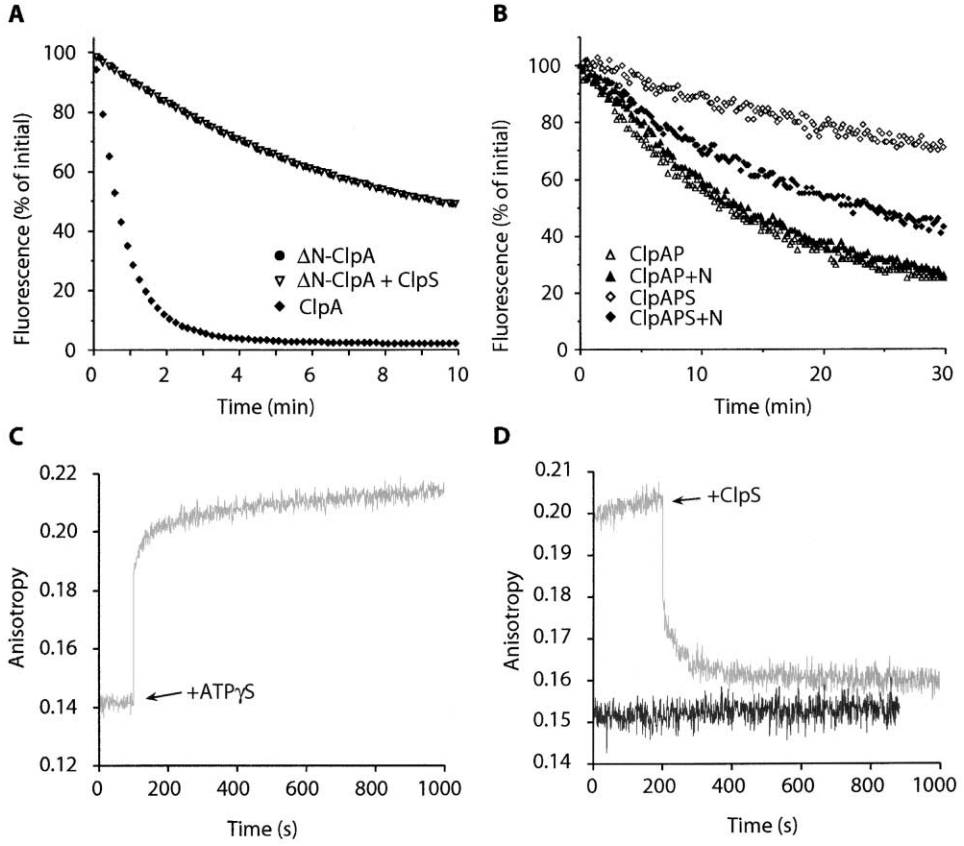

Figure 4. ClpS Triggers Release of Bound SsrA-Tagged Substrates through Interaction with the N Domain of ClpA

(A) The degradation of GFP-SsrA (4 μM) by ClpP (14 μM) in the presence of ΔN-ClpA (6 μM) was monitored by fluorescence (as described in Figure 2A) in the absence (filled circles) and presence (open triangles) of 6 μM ClpS. For comparison, the ClpP-mediated degradation of GFP-SsrA was measured in the presence of the same concentration of wild-type ClpA (filled diamonds).

(B) ClpAP-mediated degradation of GFP-SsrA was monitored in the absence (open triangles) and presence (filled triangles) of 4 μM N domain. In the presence of substoichiometric amounts of ClpS (0.4 μM), the fluorescence of GFP-SsrA by ClpAP (0.5 μM) was monitored in the absence (open diamonds) and presence (filled diamonds) of 4 μM N domain.

(C) ClpS acts at the level of substrate recognition. Fluorescence anisotropy time course of a fluorescein-labeled, SsrA-tagged substrate (fl-λ-SsrA). Binding of fl-λ-SsrA to ClpA is dependent upon assembly of ClpA hexamers by the nucleotide analog ATPγS (1 mM). ClpA (8.4 μM) was incubated with fl-λ-SsrA (0.2 μM).

(D) Binding of fl-λ-SsrA to hexameric ClpA is inhibited by ClpS. ClpA (8.4 μM), fl-λ-SsrA (0.2 μM), and ClpS (2 μM) were incubated initially (lower trace) and ATPγS (1 mM) was added. Alternatively, ClpA, fl-λ-SsrA, and ATPγS were incubated to form stable complex (anisotropy = 0.2, upper trace), and ClpS (2 μM) was added where indicated (t = 200 s). This subsaturating amount of ClpS was able to disrupt the otherwise stable interaction between ClpA and substrate.

agg-MDH was incubated with ClpA, only in the presence of ClpS was 45% of ClpA identified in the pellet fraction (Figure 5A, lane 8), suggesting that ClpS either stabilizes an existing binding activity between ClpA and agg-MDH or modulates the binding specificity of ClpA. Furthermore under these conditions, in the presence of native (Figure 5B, lanes 1 and 2) or heat-aggregated MDH (Figure 5B, lanes 3 and 4), the distribution of ClpS remained unchanged, suggesting that ClpS alone does not stably interact with heat-aggregated substrates; however, a transient interaction with the aggregate cannot be excluded.

Next, we tested the ability of ClpAP to disaggregate and degrade two different heat-aggregated proteins (agg-MDH and aggregated luciferase [agg-luci]). Using light scattering and SDS-PAGE, we monitored the effect of ClpS on ClpAP-mediated disaggregation and degradation of these proteins. In the absence of protease, the light scattering intensity of both aggregated proteins remained constant, indicating that the aggregates were stable (data not shown). Although ClpS was required for the efficient ClpAP-mediated disaggregation of both aggregated proteins, ClpS was essential only for the disaggregation of agg-luci (Figure 5D). In contrast, agg-MDH could be partially disaggregated by ClpAP alone, but the addition of ClpS improved the rate of disaggregation by approximately 2-fold (Figure 5C). Moreover, not only was the rate of ClpAP-mediated disaggregation enhanced in the presence of ClpS, but in contrast with ClpAP incubation where the aggregated proteins were essentially stable, both aggregated proteins were now efficiently degraded in the presence of ClpS (Figures 5E and Figure 5F). These data suggest that, in this case, ClpS does not radically alter ClpA substrate specificity as was demonstrated for SsrA-tagged substrates but instead modulates ClpA-mediated binding to aggregated proteins.

Although the rate of disaggregation and the subse-

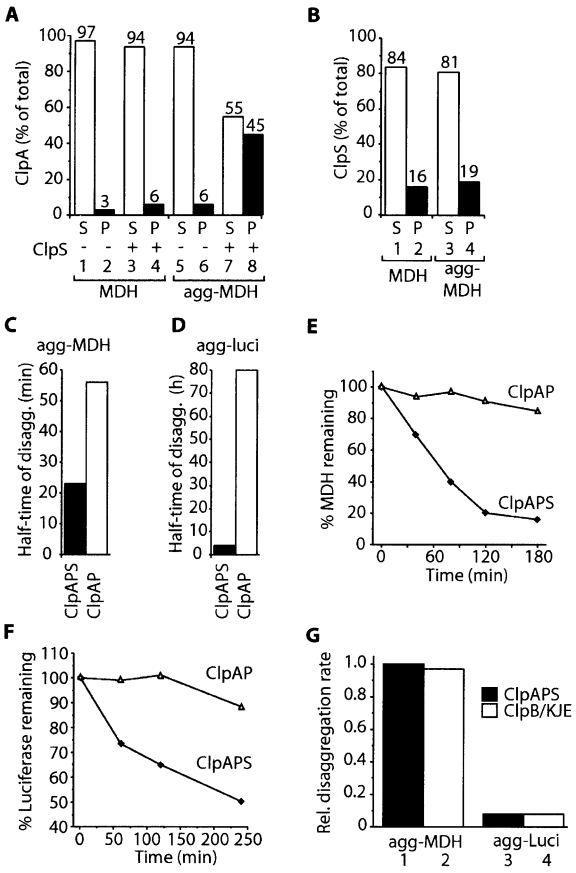

Figure 5. ClpS Stimulates ClpAP Binding and Degradation of Model-Aggregated Proteins

(A) ClpA (0.6 μM) and 2 mM ATPγS were incubated for 5 min at room temperature with native MDH (lanes 1–4) or agg-MDH (lanes 5–8) in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3, 4, 7, and 8) of ClpS (0.6 μM). Following centrifugation (30 min at 16,000 g), soluble proteins (S) and pelletable proteins (P) were separated by SDS-PAGE and the amount of ClpA was determined by quantification of the Coomassie-stained protein band using the MacBAS software.

(B) ClpS (0.6 μM) was incubated for 5 min at room temperature with native MDH (lanes 1 and 2) or agg-MDH (lanes 3 and 4). Following centrifugation (30 min at 16,000 g) soluble proteins (S) and pelletable proteins (P) were separated by SDS-PAGE, and the amount of ClpS was determined by quantification of the Coomassie-stained protein band using the MacBAS software.

(C) The half-time of agg-MDH disaggregation by ClpAP (0.5 μM) was determined from the change in the light scattering signal (excitation and emission wavelength 550 nm) in the absence (white bar) and presence (black bar) of 0.5 μM ClpS. In the absence of ClpAP, no change in the light scattering signal was observed (data not shown).

(D) The half-time of ClpAP-mediated agg-luci disaggregation was determined in the absence (white bar) and the presence (black bar) of 0.5 μM ClpS as described in (C).

(E) Degradation of agg-MDH by ClpAP (open triangles) and ClpAPS (filled diamonds) as described in (C) was monitored by quantification of the Coomassie blue-stained protein band using the MacBAS software.

(F) Degradation of agg-luci was determined as described in (E).

(G) The relative rates of disaggregation of agg-MDH (lanes 1 and 2) and agg-luci (lanes 3 and 4) by ClpAPS (black bar, lanes 1 and 3) and ClpB/KJE (white bar, lanes 2 and 4) were compared. All rates were normalized relative to the rate of ClpAPS-mediated disaggregation of agg-MDH.

quent degradation of aggregated proteins by ClpAPS were slow in comparison to ClpAP-mediated degradation of GFP-SsrA, the disaggregation rate by ClpAPS was comparable to that achieved by the bi-chaperone system ClpB/KJE using the same substrates (Figure 5G). Moreover, the rates of degradation by ClpAPS and the rates of refolding by ClpB/KJE of both model substrates were also equivalent (data not shown). Interestingly, the disaggregation of agg-MDH by ClpAPS (Figure 5C) occurred more rapidly than the ClpAPS-mediated degradation (Figure 5E), suggesting that under these conditions disaggregation of agg-MDH is not strictly coupled to degradation. In agreement with this suggestion, we observed some disaggregation but no degradation of agg-MDH by a proteolytically inactive ClpAP complex (data not shown). In contrast, the disaggregation (Figure 5D) and degradation (Figure 5F) of agg-luci by ClpAPS appear to be kinetically coupled, as the rate of disaggregation and degradation are similar, suggesting that the disaggregation and degradation reactions may, in this case, occur simultaneously.

Although the disaggregation rates of both systems (ClpAPS and ClpB/KJE) are similar in vitro, we noticed when we analyzed Δ*clpA* and Δ*clpS* cells that in comparison to wild-type cells, the amount of protein aggregation was not significantly increased (data not shown). In contrast, extensive protein aggregation occurred in Δ*clpB* cells, suggesting that ClpB/KJE-mediated disaggregation and refolding of aggregates predominate in *E. coli* cells, thereby masking the effect of deleting either *clpA* or *clpS*. This suggested that although both the ClpAPS and ClpB/KJE systems are equally efficient in vitro, the in vivo role of the ClpB/KJE system for refolding is more important than that of the ClpAPS system for the potential disaggregation and degradation of aggregated proteins. To account for the apparent discrepancy between the in vitro and in vivo effectiveness of ClpAPS-mediated disaggregation, it seemed possible that the ClpB/KJE system may successfully compete with the ClpAPS machine for substrate binding. To address this possibility, we compared the extent and stability of protein aggregates in different bacterial strains. Although the extent of protein aggregation was not increased in the Δ*clpB*Δ*clpS* double mutant as compared to the Δ*clpB* single mutant (Figure 6A, compare lanes 2 and 4), we noticed a small but significant increase in the stability of a selection of aggregated proteins (Figure 6A, p1, p2, and p3) in the Δ*clpB*Δ*clpS* double mutant (Figure 6B) consistent with a secondary role for ClpAPS in the removal of aggregated proteins in vivo. To confirm that the ClpB/KJE-mediated refolding reaction could inhibit the ClpAPS-mediated disaggregation and degradation of aggregated proteins, we measured the extent of agg-MDH disaggregation by ClpAPS in vitro in the presence of increasing amounts of either ClpB or the DnaK chaperone system (KJE). Although the addition of equimolar amounts of ClpB inhibited ClpAPS-mediated disaggregation (data not shown), we could not exclude that this inhibition resulted from the nonphysiological formation of mixed oligomers between ClpA and ClpB. To exclude the possibility of mixed oligomer formation, we examined the effect of the DnaK chaperone system on ClpAPS-mediated disaggregation. With only a 2-fold excess of DnaK over ClpA, ClpAPS-mediated disaggregation was inhibited by more than 80% (Figure 6C).

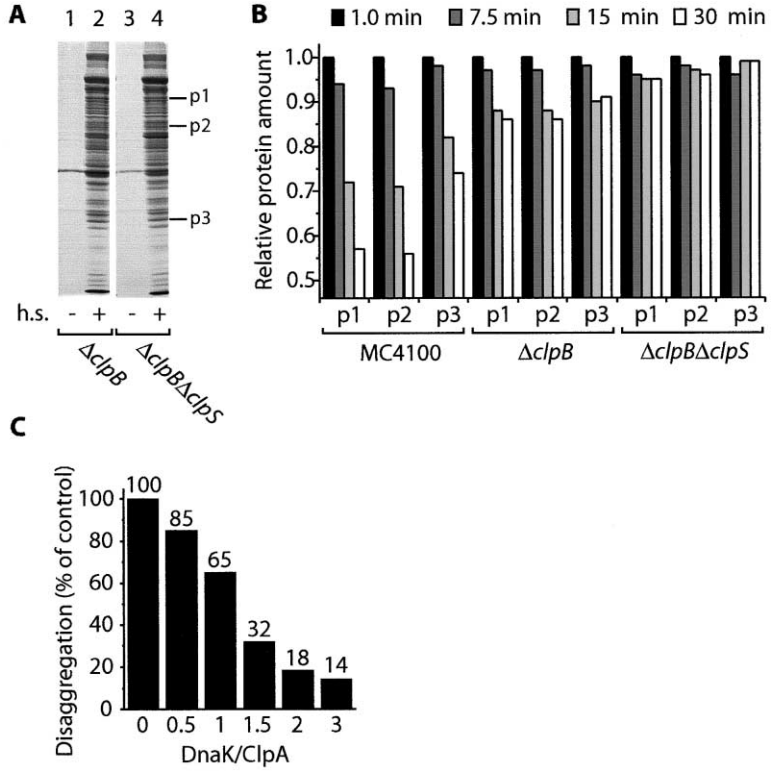

Figure 6. Functional Dominance of the ClpB/KJE System over ClpAPS-Mediated Protein Disaggregation In Vitro and In Vivo

(A) Aggregated proteins were isolated, as described by Mogk et al. (1999), from Δ*clpB* (lanes 1 and 2) and Δ*clpB*Δ*clpS* (lanes 3 and 4) cells before and after a 30 min heat shock (h.s.) at 45°C.

(B) The stability of three selected aggregation-prone proteins (p1, p2, and p3) was determined in different bacterial strains by quantification of the individual protein bands at four time points (1, 7.5, 15, and 30 min) following heat shock.

(C) The yield of disaggregation of agg-MDH by ClpAPS (as determined by light scattering after 180 min) was determined in the presence of increasing amounts of the DnaK chaperone system. For all reactions, the ratio of DnaK:DnaJ:GrpE (10:2:1) remained constant.

Under normal growth conditions, there are ~500 ClpB hexamers and ~9900 DnaK molecules per cell (Mogk et al., 1999). This represents approximately a 5-fold and 90-fold excess of active ClpB and DnaK molecules, respectively, over ClpA hexamers. Together, these findings suggest that the normal levels of DnaK (and ClpB) in the cell would be sufficient to substantially inhibit most ClpAPS-mediated degradation of aggregated proteins, thereby favoring refolding and hence recycling of aggregated proteins in preference to their removal by ClpAPS-mediated degradation.

The ClpAPS Complex Forms a Working Proteolytic Machine

Although model-aggregated proteins were degraded by ClpAP in a ClpS-dependent manner, it remained unclear if these substrates were indeed degraded by the ClpAPS complex or rather were first disaggregated by a ClpAS complex and subsequently degraded by a ClpAP complex lacking ClpS. This situation might arise, for example, if the addition of ClpS resulted in the dissociation of the ClpAP complex. In order to resolve this point, we analyzed the competitive degradation of agg-MDH and GFP-SsrA by either ClpAP or ClpAPS. If degradation of an aggregated substrate required a two-step process in which ClpAS disaggregation preceded ClpAP-mediated degradation, this would imply that free ClpAP would exist in the presence of ClpS, and hence significant degradation of GFP-SsrA could occur in the presence of ClpS. In the absence of ClpS, GFP-SsrA is rapidly degraded by ClpAP (Figure 7A) with a half-life of approximately 5 min (Figure 2A), and only in the presence of ClpS was ClpAP activity redirected toward degradation of agg-MDH (Figure 7A). Importantly, in the presence of ClpS, GFP-SsrA remains stable for at least 210 min (Figure 7A, lane 12), indicating that little or no free ClpAP complex remains in the presence of equimolar amounts of ClpS. This provides further evidence that ClpS does not dissociate the ClpAP complex but instead modulates the specificity of ClpA.

To confirm that ClpS does not dissociate the ClpAP complex, we examined the complexes using native PAGE and monitored the presence of each protein in the complex using specific antisera. If ClpS were to disrupt the ClpAP complex, then one would also expect to identify a smaller ClpA or ClpAS complex. Analysis of the native PAGE clearly shows that ClpS did not disrupt the ClpAP complex (Figure 7B), as all three components (ClpA, ClpP, and ClpS) were located in a high molecular weight complex with an apparent molecular weight consistent with an asymmetric ClpAP "bullet-like" complex (Figure 7B, lanes 2, 4, and 6). Furthermore, in the presence of ClpS, not only was there a small but significant shift in the migration of both ClpA (Figure 7B, lane 4) and ClpP (Figure 7B, lane 6) consistent with the addition of several, possibly six, ClpS monomers to the ClpAP complex, but more importantly there was no sign of a lower molecular weight form of ClpA in the presence of ClpS (Figure 7B, lane 4). Hence, it is unlikely that ClpS acts as a chaperone switch to dissociate ClpA from the ClpAP complex; rather, it modulates ClpA specificity, specifically inhibiting the degradation of SsrA-tagged proteins and facilitating the degradation of model-aggregated substrates such as agg-MDH and agg-luci.

Discussion

We report the functional characterization of an evolutionarily conserved adaptor protein, ClpS, which modulates ClpA specificity upon binding, thereby altering activity of the ClpAP machine. ClpS interacts with ClpA

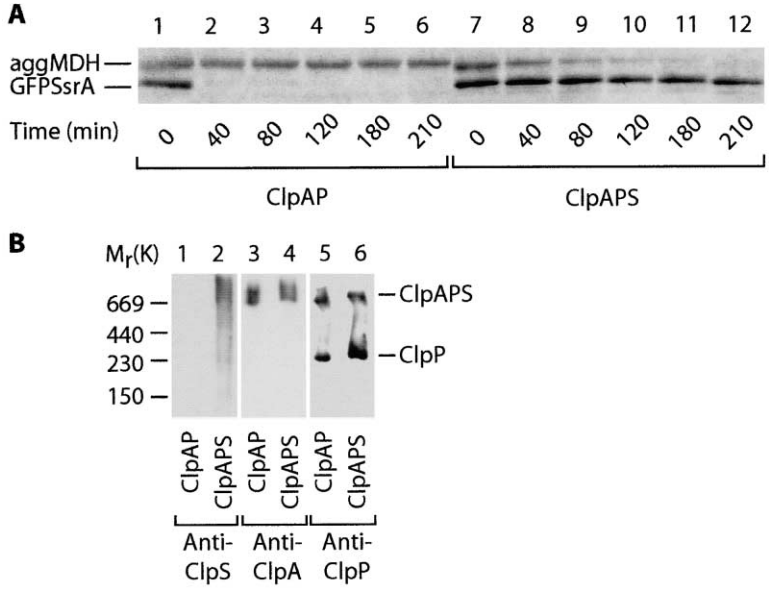

Figure 7. ClpAPS Form a Functional Complex with Altered Substrate Specificity without Blocking ClpP Activity

(A) A mixture of 1 μM GFP-SsrA and 1 μM agg-MDH was incubated together with ClpAP (0.5 μM) in the absence or presence of 0.4 μM ClpS at 37°C for varying periods. After incubation, samples were separated by 15% SDS-PAGE and proteins were visualized by Coomassie blue staining.

(B) ClpAPS forms a high molecular weight complex. ClpA, ClpP, and ATPγS were incubated in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of ClpS for 10 min at room temperature before separation by 4%–15% native PAGE. Following Western transfer, proteins were immunodecorated with Anti-ClpS (lanes 1 and 2), Anti-ClpA (lanes 3 and 4), and Anti-ClpP (lanes 5 and 6) antibodies.

both in the presence and absence of ATP, although a stable interaction is only formed in the presence of ATP (Figure 1). Furthermore, ClpS binding to ClpA does not trigger release of ClpA from the ClpAP complex (Figure 7B). In contrast, a stable ClpAPS complex is formed which exhibits an altered substrate binding specificity. The binding of ClpS to the N domain of ClpA results in negative regulation of SsrA-tagged substrate degradation. In particular, binding of ClpS triggered the immediate release of a bound SsrA-tagged substrate, preventing its degradation by ClpAP (Figure 4). Thus, the negative regulatory action of ClpS lies at the level of substrate binding. The ClpS-mediated inhibition of SsrA-tagged substrate degradation is specific for the ClpAP machine, as ClpS does not affect the degradation of SsrA-tagged substrates by ClpXP (Figure 2). In contrast, SspB is a specific modulator of SsrA-tagged protein degradation, enhancing ClpXP-mediated degradation and inhibiting ClpAP-mediated degradation of the same substrate. These findings resolve inconsistencies between in vitro data where ClpAP-mediated degradation of SsrA-tagged proteins is observed (Gottesman et al., 1998) and direct in vivo data where such degradation is not observed; instead, SsrA-tagged proteins are principally degraded by ClpXP complexes (Gottesman et al., 1998). That is, the presence of SspB favors the transfer of SsrA-tagged substrates to ClpX and the presence of ClpS disfavors the transfer to ClpA, offering an explanation of the specificity exhibited in vivo.

ClpS also prevents the ClpP-mediated autodegradation of ClpA (Figure 3). Since the N-terminal residues of ClpA have been implicated in the degradation of ClpA (Gottesman et al., 1990), we propose that ClpA monomers are the *trans* target for degradation by the ClpAP machine and binding of ClpS to the N-terminal domain of ClpA blocks recognition of the N-terminal degradation sequence by ClpA hexamers. However, when associated with the hexamer, ClpS may also serve a different role, altering ClpA substrate specificity. Only in concert with ClpS can ClpAP efficiently degrade two heat-aggregated proteins tested in vitro (Figure 5). Although the in vitro disaggregation of model protein aggregates by the ClpAPS machine is as efficient as the ClpB/KJE bi-chaperone system, the ClpB/KJE system predominates in vivo. An in vitro reconstruction suggested that the DnaK chaperone system would efficiently compete with ClpAPS-mediated disaggregation of heat-aggregated proteins. We speculate that the disaggregation activity of the ClpAPS machine observed here in vitro may reflect its action on specific, as yet unidentified, substrates of ClpAPS. We propose that dominance of the DnaK system over ClpAPS-mediated disaggregation reflects a cellular strategy where the reactivation of aggregated proteins by the ClpB/KJE system occurs in preference to their ClpAPS-dependent degradation. The in vivo dissection of such a complicated network will be an important challenge for future studies.

Although there are currently a handful of examples in which the substrate binding properties of AAA+ proteins are modified by specific cofactors, only one example is known in which the AAA+ protein (p97) and the adaptor (p47) form a complex (Rouiller et al., 2000). In contrast, all other known adaptor proteins, including SspB, RssB, and MecA, interact directly with their substrate, thereby redirecting the substrate to the AAA+ component of the system. Furthermore, all adaptor proteins studied to date have been shown to positively regulate AAA+ substrate binding. In contrast, we propose that ClpS, through interaction with ClpA, has distinct effects on substrate binding, redirecting ClpA activity away from degradation of SsrA-tagged proteins and toward degradation of a subset of specific aggregated or oligomeric proteins. From our data, we suggest two possible molecular mechanisms of action by ClpS: either ClpA undergoes a conformational change upon ClpS binding, concealing one class of binding site and revealing others, or alternatively, ClpA contains two separate substrate binding regions, one of which is sterically blocked through ClpS binding. Structural studies will provide the key to understanding both the mode of ClpS binding and the mechanism responsible for the switch in ClpA substrate specificity.

Experimental Procedures

Proteins

ClpS, ClpA, ClpA N domain(1–161), ClpP, ClpX, SspB, and GFP-SsrA were overexpressed from an IPTG-inducible plasmid (pUHE21-2fdΔ12). ΔN-ClpA lacking the first 143 amino acids of ClpA was overproduced from a T7 expression plasmid. ClpS was precipitated from a clarified *E. coli* lysate by the addition of ammonium sulfate (35% saturated). After centrifugation, the pellet was dissolved in Buffer A (20 mM Tris-HCl [pH 8.8], 50 mM KCl, 1 mM DTT, and 10% glycerol) and extensively dialyzed in the same buffer before being applied to a MonoQ ion-exchange column equilibrated in Buffer A. The peak fractions, eluted with a 50 mM to 800 mM KCl gradient, were applied to a Superdex75 column equilibrated in Buffer C (20 mM HEPES [pH 7.5], 150 mM KCl, 1 mM DTT, and 10% glycerol). ClpA, ClpP, and ClpX were purified from the clarified lysates also using a combination of ion-exchange chromatography and gel filtration. His-tagged GPF-SsrA, ClpA N domain(1–161), and SspB were purified by nickel-NTA-agarose chromatography according to the manufacturer's instructions. All proteins were >95% pure as assessed by Coomassie-stained SDS-PAGE. Pig heart malate dehydrogenase (MDH) was purchased from Roche. Protein concentrations were determined with the Bio-Rad Bradford assay system using BSA as a standard. Protein concentrations refer to the protomer.

Deletion of *clpS*

Since *clpS* is located upstream of *clpA* in a putative operon, we used a new technique to knock out genes in *E. coli* (Datsenko and Wanner, 2000) which permits the subsequent removal of the selection cassette to exclude possible polar effects on the downstream gene. The appropriate primers were used to amplify the *cat* gene from pKD3 and introduce short flanking regions onto the *cat* gene, which complemented the sequence up- and downstream of *clpS*. The deletion mutant was tested simultaneously for the loss of *clpS* and the insertion of the *cat* cassette by amplification of specific fragments from the mutant DNA. The deletion was confirmed by immunoblotting of wild-type *E. coli* (MC4100) and Δ*clpS* cell extracts using an immunospecific affinity-purified anti-ClpS antibody.

Antibodies

Anti-ClpA antibodies were raised in rabbits and serum used without further purification. Rabbit anti-ClpS antibodies were affinity purified using ClpS coupled to CNBr-activated Sepharose.

Spectroscopy Measurements and Biochemical Assays

GFP fluorescence (excitation 400 nm and emission 510 nm) and light scattering (excitation and emission 550 nm) measurements were made on a Perkin-Elmer luminescence spectrometer LS50B. Fluorescence measurement reactions were carried out as described (Weber-Ban et al., 1999). Anisotropy measurements were carried out as described (Reid et al., 2001). For light scattering, MDH and Luciferase were aggregated as described (Goloubinoff et al., 1999; Schröder et al., 1993), and measurements were carried out at 30°C in the presence of 0.5 μM ClpA and 0.5 μM ClpP. All reactions incubated with Clp protein mixtures (>30 min) contained an ATP-regenerating system comprising 20 ng/ml pyruvate kinase and 4 mM phosphoenol pyruvate. The in vivo half-life of ClpA was determined by monitoring ClpA levels in different *E. coli* strains, after blocking further translation by the addition of spectinomycin (to a final concentration of 100 μg/ml; Gottesman et al., 1998). For the ClpS-overexpressing strain, protein expression was induced 90 min prior to the addition of spectinomycin. Samples taken at the different time points were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane before immunoblotting using anti-ClpA antibodies.

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