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The HslU ATPase acts as a molecular chaperone in prevention of aggregation of SulA, an inhibitor of cell division in *Escherichia coli*

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Abstract HslVU is an ATP-dependent protease consisting of two multimeric components: the HslU ATPase and the HslV peptidase. SulA, which is an inhibitor of cell division and has high tendency of aggregation, is degraded by HslVU protease. Here we show that HslU plays a role not only as a regulatory component for the HslV-mediated proteolysis but also as a molecular chaperone. Purified HslU prevented aggregation of SulA in a concentration-dependent fashion. This chaperone activity required oligomerization of HslU subunits, which could be achieved by ATP-binding or in the presence of high HslU protein concentrations. *hsl* mutation reduced the SulA-mediated inhibition of cell growth and this effect could be reversed upon overproduction of HslU, suggesting that HslU promotes the ability of SulA to block cell growth through its chaperone function. Thus, HslU appears to have two antagonistic functions: one as a chaperone for promotion of the ability of SulA in cell growth inhibition by preventing SulA aggregation and the other as the regulatory component for elimination of SulA by supporting the HslV-mediated degradation.

Key words: Chaperone; HslU ATPase; ATP-dependent HslVU protease; SulA; Cell division

1. Introduction

Chaperones and proteases are expected to share common characteristics because their substrates are similar, although they carry out antagonistic reactions. In addition, it has been implicated that ATP-dependent proteases have intrinsic chaperone activity, because initial steps in energy-dependent protein degradation are similar to those of chaperone-dependent protein folding. Evidence is accumulating that the ATPase components of ATP-dependent proteases indeed function as a molecular chaperone [1,2]. ClpA alone functions as a molecular chaperone, like DnaK and DnaJ, in the in vitro activation of the plasmid P1 RepA replication initiator protein by converting inactive dimers to active monomers [3,4]. ClpA also functions as a regulatory component for supporting the proteolytic activity of ClpP, a peptidase that alone can rapidly cleave small peptides, but absolutely requires ATP hydrolysis by ClpA for degradation of large polypeptide substrates [5]. Similar to ClpA, ClpX functions as a chaperone in protection of λO from heat inactivation in vitro [6], disassembly of MuA transposase–DNA intermediates during the replication and transposition of bacteriophage Mu DNA [7–9] and activation of the plasmid RK2 DNA replication initiator protein, TrfA, by dissociating dimers into monomers [10]. Also, ClpX, as an alternate regulatory component for ClpP, can support the degradation of certain regulatory proteins, such as λO protein [11,12] and the bacteriophage Mu vir repressor [7].

Another *Escherichia coli* ATP-dependent protease, which was most recently described, is the HslVU protease consisting of two heat-shock proteins: a 19-kDa HslV (ClpQ) and a 50-kDa HslU (ClpY) [13–15]. HslV harbors the peptidase activity, whereas HslU provides an essential ATPase activity, both of which can function together as a novel two-component protease. HslU itself has ATPase activity, markedly stimulates the proteolytic activity of HslV (more than 20-fold) and targets large proteins for degradation by HslV, which alone is unable to degrade proteins. Moreover, it has recently been shown that in addition to Lon, HslVU participates in the hydrolysis of SulA in vivo as well as in vitro [16–18].

The SulA protein of *E. coli* is encoded by the SOS-inducible *sulA* gene, which is also called *sfiA*. The target for SulA is the essential cell division protein FtsZ, which is a GTPase and plays a critical role in the initiation of cell division [19–21]. Hydrophobic interactions have been implicated in the complex between FtsZ and SulA [22]. In addition, the SulA protein is aggregated and precipitated in vivo as well as in vitro [23,24] and therefore, its purification in soluble form has been facilitated by a genetic manipulation to fuse maltose-binding protein (MBP) to SulA. This tendency of SulA to easily aggregate raised a possibility that its stability requires a chaperone function, such as preventing aggregation.

So far, there is no direct evidence for the chaperone function of HslU, except that HslU is a member of the HSP100/Clp family [25], most of which are known to function as chaperones [3,8]. In the present studies, therefore, we examined whether HslU has a chaperone function in preventing aggregation of SulA. We also examined the effects of *hsl* mutation and *lon*/*hsl* double mutation on the growth of *E. coli* under conditions that SulA was overexpressed, in an attempt to determine the role of HslU chaperone in regulation of cell division.

2. Materials and methods

2.1. Bacterial strains and plasmids

MC1000 (wild-type) and its derivative strains, MC1000L (lon::tet), MC1000H (hslVU::kan) and MC1000LH (lon::tet, hslVU::kan), which had been constructed by P1 transduction [17], were used as hosts for in vivo experiments. pSulA5 containing the wild-type *sulA* gene under control of the lacUV5 promoter was transformed into the

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four isogenic strains as described previously [17]. For the expression of HslU alone, we first constructed a pACYC vector carrying the lac promoter followed by a DNA fragment containing a multicloning site. The *Eco*RI and *Hin*dIII sites in the multicloning site of Bluescript II SK$^+$ were eliminated by site-directed mutagenesis. The DNA fragment containing lac promoter and the mutated multicloning site in the Bluescript vector was cloned by PCR using primers, which had been designed to carry *Eco*RI and *Hin*dIII sites. The 0.2-kb *Eco*RI/*Hin*dIII fragment was then ligated into pACUb-M-β-gal [26,27] that had been treated with the same restriction enzymes, and the resulting plasmid was referred to as pAC1. To generate a DNA fragment containing only the coding sequence for HslU, PCR was performed using hslVU operon as template and two primers that had been designed to carry *Sac*I and *Kpn*I sites. The *Sac*I/*Kpn*I fragment was ligated into pAC1 that had been treated with the same enzymes. The resulting plasmid containing the hslU gene under control of the lac promoter was designated pAC1/HslU. This pAC1/HslU plasmid was transformed into MC1000 and its derivative strains carrying pSulA5 plasmid. pET-3Z$^+$ [28] were transformed into BL21(DE3) cells for overproduction of FtsZ.

2.2. Proteins

Purified HslV, HslU, HslU/K63T and MBP-SulA were prepared as described previously [15,17]. FtsZ was purified from BL21 carrying pET-3Z$^+$ as described [29]. Factor Xa (FXa), which can specifically cleave the linker region between MBP and SulA, was obtained from New England Biolabs. Protein concentration was determined as described [30] using bovine serum albumin as a standard.

2.3. Assay for aggregation of SulA

Reaction mixtures (a final volume of 0.2 ml) contained 120 μg (equivalent to 10 μM) of MBP-SulA, 3 μg of FXa and increasing amounts of HslU in 0.1 M Tris–HCl buffer (pH 8) containing 10 mM $MgCl_2$, 1 mM EDTA and 1 mM dithiothreitol (DTT). The samples were incubated for 150 min at 25°C in the absence or presence of 4 mM ATP. During the incubation period, aggregation of SulA was monitored by measuring the apparent absorption due to scattering at 360 nm with a 10 nm path length in a spectrophotometer.

2.4. Cross-linking analysis

Increasing amounts of HslU were incubated for 20 min at 37°C in 0.1 M HEPES buffer (pH 7.5) containing 10 mM $MgCl_2$, 1 mM EDTA and 0.4% (v/v) glutaraldehyde in the presence or absence of 1 mM ATP. After incubation, the samples were subjected to SDS–PAGE on 4–8% gradient slab gels [31]. Proteins in the gels were then visualized by silver staining.

2.5. Degradation of MBP-SulA and insulin B-chain by HslVU

Incubations were carried out at 37°C in the reaction mixtures (0.1 ml) containing 0.1 M Tris–HCl (pH 8), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 5 μg MBP-SulA, 0.5 μg HslV, 2 μg HslU and increasing amounts of FtsZ. After incubation, the reaction was stopped by adding 30 μl of 0.75 M Tris–HCl (pH 6.8) containing 7.5% SDS and 10% 2-mercaptoethanol. They were then subjected to SDS–PAGE on 13% slab gels [31]. The gels were stained with Coomassie blue R-250. Hydrolysis of ^{125}I-labeled insulin B-chain (10 μg) was assayed by incubating similar reaction mixtures but containing 1 μg HslV and 4 μg HslU for 1 h at 37°C. After incubation, radioactivity released into trichloroacetic acid-soluble form (10% final, w/v) was determined using a gamma counter.

3. Results

3.1. HslU suppresses aggregation of SulA in vitro

To investigate whether HslU can function as a chaperone like ClpA and ClpX, purified HslU was incubated with MBP-SulA and FXa in the absence or presence of ATP. Since SulA is known to precipitate when released from MBP-SulA [23] and since FXa does not cleave HslU (data not shown), the ability of HslU in prevention of aggregation of SulA could be monitored by measuring the apparent absorbance due to light scattering at 360 nm. Remarkably, HslU prevented aggregation of SulA in a concentration-dependent manner (Fig. 1A,B). Of particular interest was the finding that HslU suppressed aggregation of SulA even in the absence of ATP, although this effect could markedly be increased by ATP at low concentrations of HslU (e.g. 0.12 μM). Furthermore, ATPγS or ADP could also support the ability of HslU in prevention of SulA aggregation nearly as well as ATP at all of the HslU concentrations tested (data not shown). On the other hand, incubation with an excess of other proteins, such as carbonic anhydrase, showed little or no effect on aggregation of SulA whether or not ATP was present (Fig. 1B, dotted line). These results indicate that HslU is capable of preventing aggregation of SulA and its ATPase activity is not essential for the chaperone function.

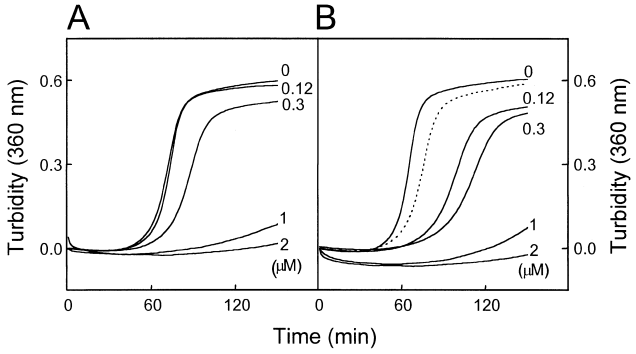

Fig. 1. Effects of HslU on aggregation of SulA and cleavage of MBP-SulA by FXa. MBP-SulA (10 μM) was incubated with FXa (3 μg) and increasing concentrations of purified HslU in the absence (A) or presence of 1 mM ATP (B) for various periods at 25°C in a total volume of 0.2 ml. Light scattering, which is the indicative of SulA aggregation, was measured as the apparent absorbance at 360 nm. The dotted line indicates carbonic anhydrase (2 μM), which was added in place of HslU as a control.

We also examined whether HslU could solubilize previously formed SulA aggregates. MBP-SulA was treated with FXa for 2 h and then incubated with 2 μM HslU. Under this condition, the intensity of light scattering remained the same as that seen without HslU (data not shown). Thus, it appears that HslU is not capable of solubilizing the already formed aggregates of SulA.

3.2. Requirement of HslU oligomerization for its chaperone function

Purified HslU at low concentrations behaves as a monomer or dimer in the absence of ATP, but forms a hexamer or heptamer in its presence [32–34]. HslU also behaves as an oligomer in the presence of ATPγS or ADP, although neither can support the HslV-mediated degradation of SulA [17]. Therefore, we suspected whether HslU at high concentrations might be able to form an oligomer even in the absence of ATP and hence to suppress aggregation of SulA. To test this possibility, increasing amounts of HslU were subjected to cross-linking analysis using glutaraldehyde in the absence or presence of ATP. As shown in Fig. 2A, HslU could form an oligomer without ATP at high concentrations of the protein but not at low concentrations. Moreover, the ability of HslU in preventing aggregation of SulA was closely correlated with the amount of the HslU oligomer formed in the absence and presence of ATP (see Figs. 1 and 2). These results strongly suggest that oligomerization of HslU, but not ATP hydrolysis,

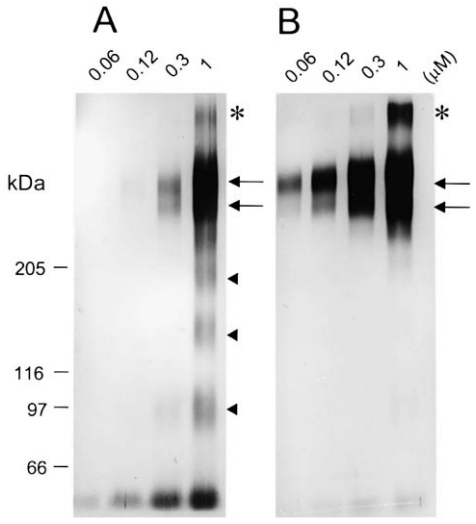

Fig. 2. Cross-linking analysis for the concentration-dependent oligomerization of HslU. Cross-linking experiments were performed by incubation of increasing concentrations of HslU with 0.4% glutaraldehyde in the absence (A) or presence of 1 mM ATP (B) as described under Section 2. The numerals on top of the gels indicate the concentration of HslU. The arrows indicate heptameric and hexameric forms of HslU, and the arrowheads show various intermediate forms. The asterisk indicates the band corresponding to two HslU hexamers (or heptamers) cross-linked.

is essential for its chaperone function in prevention of SulA aggregation.

We have previously shown that a HslU mutant (HslU/K63T), in which Lys^{63} was replaced by Thr, is not capable of binding ATP or forming oligomers [33]. In order to clarify further whether oligomerization of HslU is essential for its chaperone function, purified HslU/K63T was assayed for its ability to prevent aggregation of SulA by incubation with MBP-SulA and FXa as above. As shown in Fig. 3A, HslU/K63T (2 μM), unlike its wild-type protein, could not suppress SulA aggregation at all. Cross-linking analysis confirmed that HslU/K63T could not oligomerize into either a hexamer or heptamer, although some intermediate forms, such as dimers, trimers and tetramers, were generated upon increasing the concentration of the mutant protein (Fig. 3B). These results indicate that oligomerization of HslU is essential for its chaperone function in prevention of SulA aggregation.

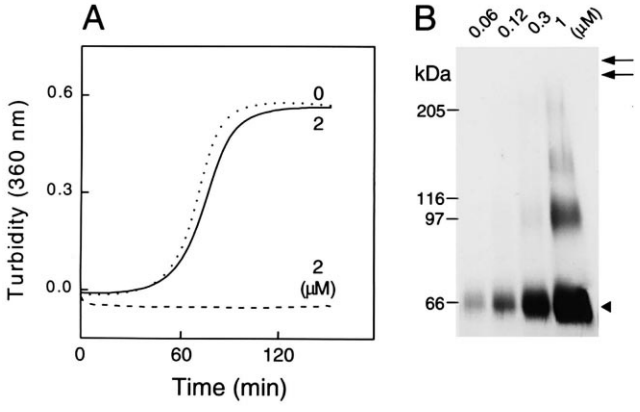

Fig. 3. Effect of HslU/K63T, which cannot form oligomer, on SulA aggregation. MBP-SulA (10 μM) was incubated with FXa (3 μg) in the absence (dotted line) or presence of 2 μM HslU/K63T (solid line) or its wild-type protein (slashed line) for various periods at 25°C (A). Cross-linking analyses were performed as in Fig. 2 but with increasing amounts of HslU/K63T (B). The arrows indicate the positions where the wild-type HslU hexamer or heptamer should migrate. The arrowhead indicates the HslU/K63T monomer.

3.3. HslU promotes the SulA-mediated cell growth inhibition

SulA is a cell division inhibitor and its constitutive expression blocks colony formation of *E. coli* through binding of SulA to and subsequent inhibition of the FtsZ GTPase, which promotes septum formation for cell division [35]. If indeed HslU functions in vivo as a chaperone in prevention of aggregation of SulA, the cells carrying *hsl* mutation would produce a less amount of soluble, functional SulA and hence could survive better than the wild-type cells under conditions that SulA is induced for overproduction. To test this possibility, *hsl*, *lon* and *lon/hsl* mutants as well as the wild-type cells were transformed with pSulA5. They were then grown overnight on a LB-agar plate streaked with IPTG in the middle. Fig. 4A shows that the inhibitory effect of SulA on the growth of *hsl* mutant cells was significantly decreased as compared to that of the wild-type cells. On the other hand, cell growth was more severely inhibited in *lon/hsl* double mutant cells than in a single *lon* mutant. The HslVU protease has recently been shown to degrade SulA in vivo as well as in vitro [16–18]. In addition, Lon is known to rapidly degrade SulA under both conditions [23,36]. Since HslU functions not only as a chaperone in prevention of SulA aggregation but also as a

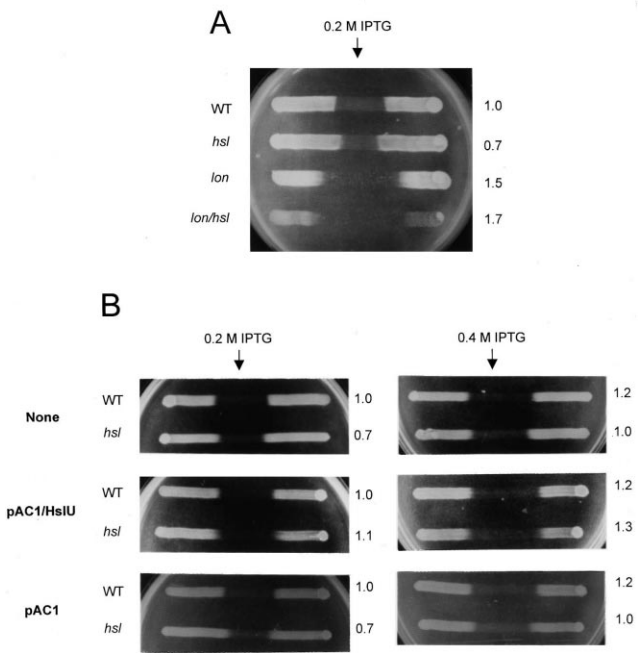

Fig. 4. Effects of *hsl* and/or *lon* mutations on cell growth. The wild-type cells (wt) and *hsl*, *lon* or *lon/hsl* mutants were transformed with pSulA5 alone (A). The wild-type cells and *hsl* null mutant cells carrying pSulA5 were also co-transformed with pAC1/HslU or pAC1 (B). They were cross-streaked against 15 μl of IPTG of the indicated concentrations on LB plates containing 100 μg/ml ampicillin or both 100 μg/ml ampicillin and 34 μg/ml chloramphenicol for the cells harboring pAC1/HslU or pAC1. They were then incubated at 37°C overnight. The numerals shown on the right side of each panel indicate relative widths of the cleared zone. The width of the cleared zone seen with the wild-type cells in (A) was expressed as 1.0 and the others were as its relative values. Similar results were obtained at at least five independent trials of the same experiments.

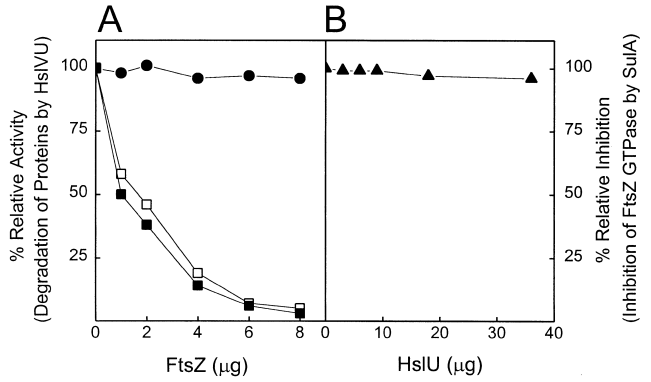

Fig. 5. Experimental analysis for comparing the affinity of SulA to FtsZ with that to HslU. (A) MBP-SulA (5 μg) was incubated with HslV (0.5 μg), HslU (2 μg) and increasing amounts of FtsZ for 2 h at 37°C in the absence (□) or presence (■) of 1 mM GTP, followed by SDS–PAGE. The gels were stained with Coomassie blue R-250 and the band corresponding to SulA was quantified using a Phosphoimager (Fuji). As a control, ^{125}I-labeled insulin B-chain (10 μg) was incubated as substrate in place of MBP-SulA (●). Proteolysis was then assayed as described in Section 2. The activity of HslVU seen in the absence of FtsZ was expressed as 100% and the others were as its relative values. (B) MBP-SulA (15 μg) was incubated with increasing amounts of HslU for 10 min at 37°C in 50 mM HEPES (pH 7.2) buffer containing 50 mM KCl, 10 mM $MgCl_2$, 1 mM ATP and 1 mM DTT. After incubation, the samples were added with FtsZ (16 μg) and 1 mM [γ-^{32}P]GTP (0.2 mCi/mmol, 0.2 μCi/ml) and further incubated at 37°C for 60 min [21]. After incubation, the reaction was terminated and the release of ^{32}Pi was determined as previously described [43]. The activity of FtsZ seen in the absence of HslU was expressed as 100% and the others were as its relative values.

regulatory component for the degradation of SulA by HslV, it appears that overproduction of SulA causes more severe inhibition of cell growth in the absence of both Lon and HslVU than in the presence of either of the enzymes.

In order to determine whether the HslU chaperone is indeed related to the SulA-mediated cell growth inhibition, the wild-type cells and *hsl* mutants were transformed with both pSulA5 and pAC1/HslU. They were then subjected to the cell growth inhibition assay as above but at two different concentrations of IPTG. Upon co-transformation of pAC1/HslU, growth inhibition in *hsl* mutants increased to a level comparable to that seen in the wild-type cells at each IPTG concentration (Fig. 4B). On the other hand, co-transformation of pAC1 vector alone showed little or no effect on the growth of respective cells. These results strongly suggest that HslU is capable of preventing aggregation of SulA by functioning as a chaperone in vivo and hence increasing the ability of SulA in blocking cell division.

3.4. Choice of SulA for interaction with HslU or FtsZ

For cell growth inhibition by SulA, it is essential for the inhibitory protein to bind to FtsZ. Therefore, SulA bound to HslU needs to be released from the chaperone for interaction with FtsZ or directly interact with FtsZ to form a HslU/SulA/FtsZ ternary complex. To test this possibility, we first examined the effect of increasing concentrations of purified FtsZ on degradation of MBP-SulA by the HslVU protease. Since FtsZ has GTPase activity that is essential for Z-ring formation for promotion of cell division [37], we performed the experiment in the absence and presence of GTP. As shown in Fig. 5A, FtsZ inhibited the degradation of MBP-SulA by HslVU in a concentration-dependent manner whether or not GTP was present. However, FtsZ at all concentrations showed little or no effect on the degradation of insulin B-chain by HslVU. In addition, FtsZ was not hydrolyzed at all by the protease whether or not GTP was present (data not shown), indicating that it does not interact with HslU. These results suggest that SulA has a much higher affinity to FtsZ than to HslU and its binding site for HslU may be sterically hindered by the tight interaction between SulA and FtsZ.

SulA is known to inhibit the GTPase activity of FtsZ and hence block its Z-ring formation [29,35]. In order to determine whether HslU competes with FtsZ for binding to SulA, we assayed the ability of SulA to inhibit GTP hydrolysis by FtsZ in the presence of increasing concentrations of HslU. Little or no effect of HslU on the inhibitory activity of SulA was evident at all of the concentrations tested (Fig. 5B). These results again suggest that SulA has a much higher affinity to FtsZ than to HslU and therefore can be easily transferred from the HslU/SulA complex to FtsZ for its inhibitory function on cell division.

4. Discussion

The present studies have demonstrated that HslU, the ATPase component of the ATP-dependent HslVU protease, has a chaperone function in prevention of aggregation of SulA, an inhibitor protein of *E. coli* cell division. It has recently been shown that the HslVU protease, in addition to Lon, degrades the SulA proteins in vivo as well as in vitro [16–18]. Thus, the HslU ATPase appears to have two antagonistic functions: one as a chaperone for promotion of the ability of SulA in cell growth inhibition by preventing SulA aggregation, and the other as the regulatory component for elimination of SulA by supporting the HslV-mediated degradation.

Of interest is the finding that under in vitro conditions ATP itself was not needed for the chaperone activity of HslU as long as it is kept in oligomeric form, such as in the presence of ADP or high concentrations of the protein. Moreover, a HslU mutant (HslU/K63T), which is unable to bind to the adenine nucleotides or form either hexamer or heptamer due to the substitution of Lys^{63} in the consensus ATP-binding motif [33] by Thr, was not capable of preventing SulA aggregation. Therefore, oligomerization, but not ATP-binding per se, appears essential for in vitro interaction of HslU with SulA and hence for protection of the inhibitor protein from self-aggregation. It has been reported that the chaperone function of the ClpX ATPase in protecting λO replication activity from heat inactivation does not require ATP, although it can be stimulated by the nucleotide [6]. In addition, it has been shown that a significant amount of isolated ClpX forms an oligomer in the absence of ATP, although the nucleotide promotes its oligomerization [38]. However, the ATPase activity of ClpX as well as of ClpA is essential for their additional, important chaperone function in disassembly and release of bound target proteins, such as the Mu transposase and RepA, respectively [3,9].

It is of note that, unlike for the HslU/SulA complex formation, the binding of HslU with ATP is essential for the degradation of SulA by HslV, since the ADP-bound form of HslU cannot interact with HslV [39]. We have recently demonstrated that ATP hydrolysis is absolutely required for the degradation of SulA by the HslVU protease [17], but not

for that of casein, insulin B-chain or *N*-canbobenzoxy-Gly-Gly-Leu-7-amide-4-methyl-coumarin. ATPγS, a non-hydrolyzable ATP analog, has been shown to support the hydrolysis of the latter substrates by HslVU even better than ATP, indicating that ATP-binding but not its hydrolysis is required for the proteolysis [39]. Moreover, a mutant form of HslU (HslU/C287V), in which Cys^{287} was replaced by Val and therefore cannot cleave ATP, could support the HslV-mediated proteolysis of the polypeptides by forming the HslVU complex in the presence of ATP but not in its absence [40]. In contrast, the degradation of SulA could not at all be seen upon incubation of HslV with ATP-bound HslU/C287V or with ATPγS-bound HslU [17]. Furthermore, nucleotide specificity of HslU seems to be strictly limited to ATP, since no other nucleotide triphosphate (not even dATP) could support the degradation of SulA by HslVU. Therefore, it appears that ATP hydrolysis by HslU is required for unfolding of aggregation-prone SulA and subsequent channeling it into the inner chamber of HslV, where the proteolytic active site is located [41]. On the other hand, this ATP hydrolysis-dependent chaperone function of HslU seems not required for the HslV-mediated degradation of casein, insulin B-chain or small peptides, which lack appreciable secondary structure. In fact, Lon has been shown to degrade the unfolded form of CcdA, but not its native form, without ATP hydrolysis [42].

In summary, we have shown that HslU has a chaperone function in prevention of aggregation of SulA in vivo as well as in vitro. We also suggest that HslU, as a component of HslVU protease, also has ATP hydrolysis-dependent chaperone activity in unfolding of SulA for degradation by HslV.

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