Mutational analysis of the ATP-binding site in HslU, the ATPase component of HslVU protease in *Escherichia coli*

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Abstract HslU is the ATPase component of the ATP-dependent HslVU protease in *Escherichia coli*. To gain an insight into the structure and function of HslU, site-directed mutagenesis was performed to generate a mutation in the ATP-binding site of the ATPase (i.e., to replace the Lys^{63} with Thr). Unlike the wild-type HslU, the mutant form (referred to as HslU/K63T) could not hydrolyze ATP or support the ATP-dependent hydrolysis of *N*-carbobenzoxy-Gly-Gly-Leu-7-amido-4-methyl coumarin by HslV. The wild-type HslU (a mixture of monomer and dimer) formed a multimer containing 6–8 subunits in the presence of either ATP or ADP, indicating that ATP-binding, but not its hydrolysis, is required for oligomerization of HslU. However, HslU/K63T remained as a monomer whether or not the adenine nucleotides were present. Furthermore, ATP or ADP could protect HslU, but not HslU/K63T, from degradation by trypsin. These results suggest that the mutation in the ATP-binding site results in prevention of the binding of the adenine nucleotides to HslU and hence in impairment of both oligomerization and ATPase function of HslU.

Key words: ATP-dependent protease; ATPase; HslVU; ClpAP; *Escherichia coli*

1. Introduction

The ATP-dependent protease Ti (ClpAP) in *Escherichia coli* consists of two different multimeric components, both of which are required for proteolysis [1–4]. ClpA is an ATPase, while ClpP contains serine-active sites for proteolysis. When isolated, ClpA behaves as a trimer of 84-kDa subunits in the absence of ATP but as a hexamer in its presence [5,6]. On the other hand, ClpP comprises two stacks of heptameric rings of 21-kDa subunits [7]. ClpA is a member of a family of highly conserved proteins that have two regions of particularly high homology, each of which contains a consensus sequence for an adenine nucleotide binding [8,9]. Both of the ATP-binding regions are characterized by the presence of Gly-X_2-Gly-X-Gly-Lys-Thr motifs, of which the Lys residue interacts with one of the phosphoryl group of the bound nucleotide [10,11]. Upon mutational analysis of the Lys residues, it has demonstrated that the first ATP-binding site is responsible for oligomerization of ClpA and the second is essential for its ATPase function [12,13].

Blattner and coworkers [14,15] have identified 26 new heat shock genes in *E. coli*, termed *hsl* genes. Of these, the *hslVU* operon has been shown to specify proteins of 19 kDa (HslV) and 50 kDa (HslU) [15]. The primary sequence of HslV has been shown to be similar to that of certain β-type subunits of the 20S proteasomes from eukaryotes, the archaebacterium *Thermoplasma acidophilum* [16], and certain bacteria. Moreover, we have recently shown that HslV and HslU comprise a new type of ATP-dependent protease in *E. coli*, which degrades the fluorogenic peptide, *N*-carbobenzoxy (Cbz)-Gly-Gly-Leu-7-amido-4-methyl coumarin (AMC) in the presence of ATP [17,18]. HslV has a putative, catalytic Thr residue at its N-terminal region and behaves as a multimer consisting of 12–14 subunits. On the other hand, HslU containing the ATP-binding site motif [15] behaves as a monomer or dimer in the absence of ATP but as a multimer consisting of 6–8 subunits in its presence [18,19]. Unlike the ClpA family, HslU has only one ATP-binding site [18], suggesting that the single ATP-binding site is responsible for both oligomerization and ATPase function of HslU.

In an attempt to determine the structural and functional relationship of HslU, we generated a mutant form of HslU, in which the Lys^{63} residue in the ATP-binding site was replaced by Thr, and examined the effects of the mutation on the ATPase activity of HslU, on its ability to activate the HslV-mediated peptide hydrolysis, and on oligomerization of its subunits. We also examined the effect of the adenine nucleotides on protection of the HslU proteins against digestion by trypsin.

2. Materials and methods

2.1. Mutagenesis

The pGEM-T vector carrying the *hslVU* operon (called pGEM-T/HslVU) was constructed as described previously [18]. The 1.3 kb *Pst*I fragment from pGEM-T/HslVU was inserted into the pALTER-1 mutagenesis vector (Promega). Site-directed mutagenesis was then carried out using an Altered Sites II In Vitro Mutagenesis System (Promega). A mutagenic oligonucleotide, 5′-GGTGTCGGTACCACTGAAATCG-3′, was designed to replace Lys^{63} of HslU with Thr and to generate a new restriction site for facilitating mutant isolation. The mutated nucleotides are indicated by bold letters, and the newly created restriction site for *Kpn*I is underlined. The 1 kb DNA fragment carrying the mutation was cut out by digestion with *Not*I and *Nru*I, and ligated into pGEM-T/HslVU that had also been digested with the same enzymes. The resulting plasmid was transformed into *E. coli* strain XL2 Blue, and the mutant form of HslU was purified from the cells as described previously [18].

2.2. Assays

Peptide hydrolysis was assayed as described [17,18] using Cbz-Gly-Gly-Leu-AMC as the substrate. Reaction mixtures (0.1 ml) contained the peptide (0.1 mM) and appropriate amounts of the purified HslV and HslU in 0.1 M Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol (DTT) and 1 mM EDTA. Incubations were performed for 10 min at 37°C, and stopped by adding 0.1 ml of 1% (w/v)

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sodium dodecyl sulfate (SDS) and 0.8 ml of 0.1 M sodium borate, pH 9.1. The release of AMC was then measured. ATP hydrolysis was assayed by incubating the similar reaction mixtures at 37°C but in the absence of HslV and the peptide. After incubation, 0.2 ml of 1% SDS were added to the samples, and the phosphate released was determined as described [20]. Proteins were quantified by their absorbance at 280 nm or by the method of Bradford [21] using bovine serum albumin as a standard.

2.3. Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS and 2-mercaptoethanol was performed as described by Laemmli [22] or using Tris-Tricine buffer as described by Schägger and von Jagow [23]. The discontinuous slab gels contained 4, 10, and 16% (w/v) polyacrylamide. The sample buffer contained 150 mM Tris-HCl (pH 6.8), 1.5% SDS, 2% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue, and 7% glycerol. After electrophoresis, the gels were stained with Coomassie Blue R-250.

3. Results and discussion

3.1. Effect of the K63T mutation on the ATPase activity of HslU and its ability to support the HslV-mediated peptide cleavage

In order to investigate the importance of the ATP-binding site for HslU function, Lys^{63} was substituted by Thr by site-directed mutagenesis. The mutant form of HslU (HslU/K63T) was then purified as described previously [18]. Unlike the wild-type HslU that was purified to apparent homogeneity (Fig. 1, lane a), HslU/K63T contained a number of minor polypeptide bands (shown by arrowheads in Fig. 1, lane b). Upon immunoblot analysis using the antibody raised against the wild-type HslU, all of the minor bands strongly interacted with the antibody in addition to the 50-kDa HslU/K63T (data not shown). Thus, it appears likely that the minor bands are generated from HslU/K63T by limited proteolysis in vivo or during purification.

To determine the effect of the mutation on the function of HslU, the purified proteins were assayed for their ability to hydrolyze ATP and to support the HslV-mediated hydrolysis of Cbz-Gly-Gly-Leu-AMC. As shown in Table 1, HslU/K63T was not at all capable of cleaving ATP. It could neither support the peptide hydrolysis by HslV. We also examined whether HslU/K63T may have an influence on the function of the wild-type HslU by incubating the mixture of the HslU proteins. However, it showed little or no effect on the ATPase activity of HslU or its ability to support the hydrolysis of Cbz-Gly-Gly-Leu-AMC by HslV. Thus, the Lys residue in the ATP-binding site must be critical for the ATPase function of HslU. These results also provide a genetic evidence that

Table 1
Effects of the K63T mutation on the ATP hydrolysis by HslU and the peptide hydrolysis by HslVU

Additions	ATP cleavage (nmol/h)	Hydrolysis of Cbz-GGL-AMC (pmol/min)
Wild-type	10.5	120
K63T	0.1	2
Wild-type plus K63T	10.6	119

For ATP hydrolysis, 4 μg of the wild-type HslU or HslU/K63T or both were incubated for 1 h at 37°C. For the hydrolysis of Cbz-Gly-Gly-Leu-AMC, 0.1 μg of HslV and 0.4 μg of HslU or HslU/K63T or both were incubated for 10 min at 37°C. Assays were then performed as described under Section 2.

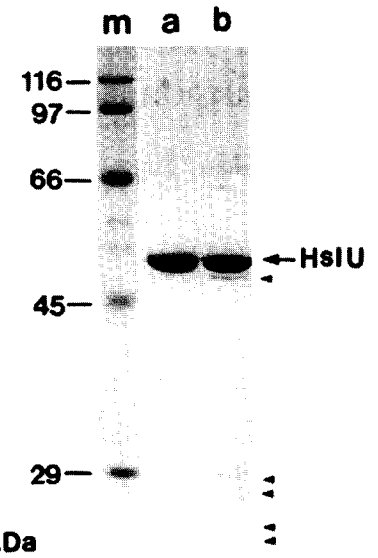

Fig. 1. PAGE of HslU and HslU/K63T. The purified HslU (lane a) and HslU/K63T (lane b) (8 μg each) were electrophoresed on a 12% (w/v) polyacrylamide slab gel containing SDS and 2-mercaptoethanol. Lane m indicates the size markers, and the arrowheads show the degradation products of the 50-kDa HslU/K63T.

ATP hydrolysis is essential for the hydrolysis of the peptide substrate by the HslVU protease.

We have previously shown that protease Ti (ClpAP) can hydrolyze small peptide substrates, such as N-succinyl-Leu-Tyr-AMC, in the absence of ATP [24]. In fact, the proteolytic component ClpP alone cleaves the substrate to a similar extent even in the absence of ClpA, although both ClpA and ATP are absolutely required for protein degradation. Thus, the catalytic mechanism and ATP requirement for small peptide hydrolysis by the HslVU protease seem to be distinct from those by the ClpAP protease.

3.2. Effect of the K63T mutation on oligomerization of HslU

In order to determine whether the mutation on the ATP-binding site may also influence the oligomeric nature of HslU, HslU/K63T was subjected to gel filtration on a Sephacryl S-300 column in the absence and presence of ATP. The purified HslU/K63T consistently ran as a 50-kDa protein whether or not ATP was present (Fig. 2B), suggesting that it remains as a monomeric form. On the other hand, the wild-type HslU, which eluted in the column fractions corresponding to 50–100 kDa in the absence of ATP, formed a multimer of 350–450 kDa in its presence (Fig. 2A). In addition, the wild-type HslU, but not HslU/K63T (data not shown), was found to oligomerize into a multimer in the presence of ADP as well as ATP (Fig. 2A). These results indicate that oligomerization of HslU requires the binding of ATP but not its hydrolysis. These findings also suggest that the mutation in the ATP-binding site may result in dramatic reduction of the affinity of HslU to ATP or ADP or in complete impairment of their binding.

To clarify further whether HslU/K63T indeed has a lower affinity to ATP or ADP than the wild-type HslU or cannot bind to the nucleotides, we compared the susceptibility of the proteins to trypsin in the absence and presence of the adenine nucleotides. As shown in Fig. 3A, ATP and ADP (1 mM)

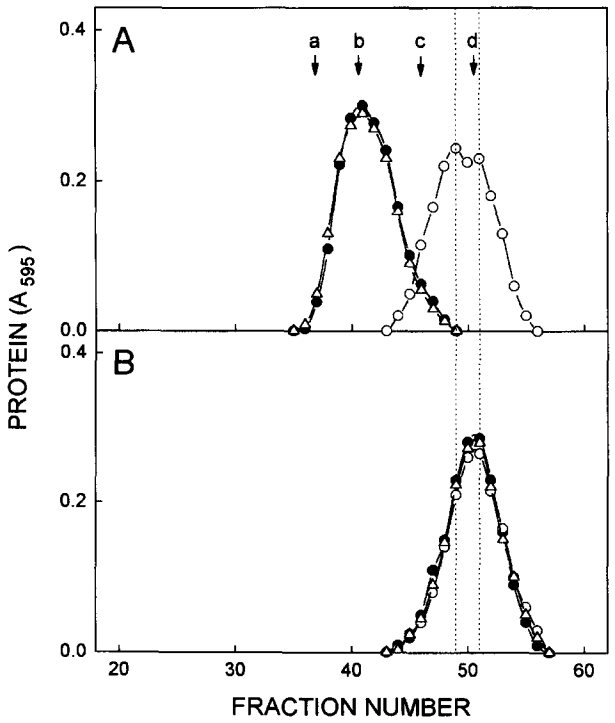

Fig. 2. Effect of ATP or ADP on oligomerization of HslU and HslU/K63T. The purified HslU (A) and HslU/K63T (B) (0.1 mg in 0.5 ml) were incubated at 4°C for 10 min in the absence (○) or presence of 1 mM ATP (●) or ADP (△). After incubation, each protein sample was subjected to gel filtration on a Sephacryl S-300 column (1×47 cm) equilibrated with 20 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 0.1 M NaCl, and 10% (v/v) glycerol. For the samples incubated with ATP or ADP, the gel filtration column was also equilibrated with either of the nucleotides (1 mM). Fractions of 0.5 ml were collected at a flow rate of 12 ml/h, and aliquots of them were assayed for protein as described by Bradford [21]. The arrows show the size markers: a, thyroglobulin (669 kDa); b, apoferritin (443 kDa); c, β-amylase (200 kDa); d, bovine serum albumin (66 kDa).

could protect the wild-type HslU from digestion by trypsin. The nonhydrolyzable ATP analog, β,γ-methylene-ATP, but not AMP, could also prevent the trypsin digestion (data not shown). In contrast, HslU/K63T was rapidly degraded by trypsin whether or not the adenine nucleotides were present (Fig. 3B). Moreover, increase in the concentration of ATP or ADP up to 10 mM did not show any protection effect on the degradation of HslU/K63T by trypsin (data not shown). Therefore, it is likely that the mutation in the ATP-binding site of HslU abolishes the binding of the adenine nucleotides to HslU and hence the mutant protein is no longer able to form a multimer or to cleave ATP. Since the wild-type HslU, but not HslU/K63T, forms a multimer in the presence of ATP or ADP, these results also suggest that the multimeric HslU complex is more resistant to degradation by trypsin than monomeric or dimeric form.

3.3. ATP concentration-dependent oligomerization of HslU

In an attempt to determine the optimal concentration of ATP that is required for oligomerization of HslU, we first examined the effect of increasing concentrations of ATP on its ability to protect the protein from digestion by trypsin. As shown in Fig. 4A, the protection effect increased upon raising the concentration of ATP and reached to a maximum at 20–50 μM, although a significant amount of HslU was still degraded by trypsin. We then examined the effect of increasing concentrations of ATP on oligomerization of HslU using a Sephacryl S-300. The size of HslU increased upon raising the ATP concentration, and majority of the protein migrated as a multimeric complex with the size of 350–450 kDa at 50 μM or above (Fig. 2A and 4B). Thus, it appears that HslU does not form a discrete intermediate(s) but forms oligomers containing various number of 50-kDa subunits depending on ATP concentration.

ClpA, the ATPase component of protease Ti (ClpAP), contains two distinct ATP-binding sites [8,9]. Upon mutational analysis of ClpA (i.e., by replacing the Lys residue in either of the two ATP-binding motifs with Thr), we have recently demonstrated that the first site is responsible for oligomerization and the second is essential for ATPase function [13]. HslU, unlike ClpA, has only one ATP-binding site, suggesting that the single site is involved in oligomerization of its subunits as well as in ATPase function. In addition, the K_m value for ATP for its cleavage by HslU has been shown to be about 0.3 mM [18]. It is noteworthy that the concentration of ATP required for oligomerization of HslU is much lower than the K_m for ATP hydrolysis. Although we cannot estimate the K_d for HslU oligomerization since dissociation–association of HslU does not seem to be a simple one-step reaction, it can clearly be observed that majority of the HslU subunits form a multimeric complex at 50 μM ATP. Therefore, the ATPase function of HslU may occur in two sequential processes: the single ATP-binding site is first involved in the formation of a multimeric HslU complex at relatively low concentrations of ATP and then in providing the bound nucleotide for ATP-cleavage reaction, that should occur at the nearby catalytic site requir-

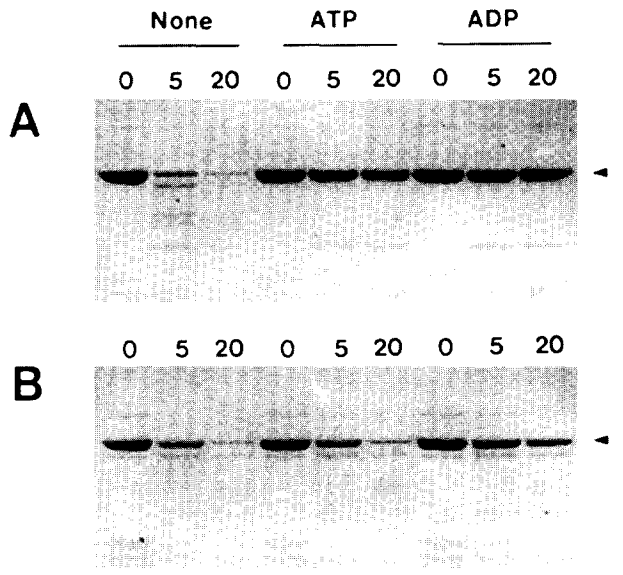

Fig. 3. Effect of ATP or ADP on the degradation of HslU and HslU/K63T by trypsin. Reaction mixtures (0.1 ml) containing 8 μg of the purified HslU (A) and HslU/K63T (B) in 0.1 M Tris-HCl (pH 8), 1 mM DTT, 1 mM EDTA, 10 mM $MgCl_2$, and 5 mM $CaCl_2$ were incubated with 50 ng of trypsin for various periods at 37°C in the presence and absence of ATP or ADP. After incubation, the samples were electrophoresed on a discontinuous slab gel under denaturing conditions as described under Section 2. The numerals shown on the top of the gels indicate the incubation period, and arrowheads show the 50-kDa HslU proteins.

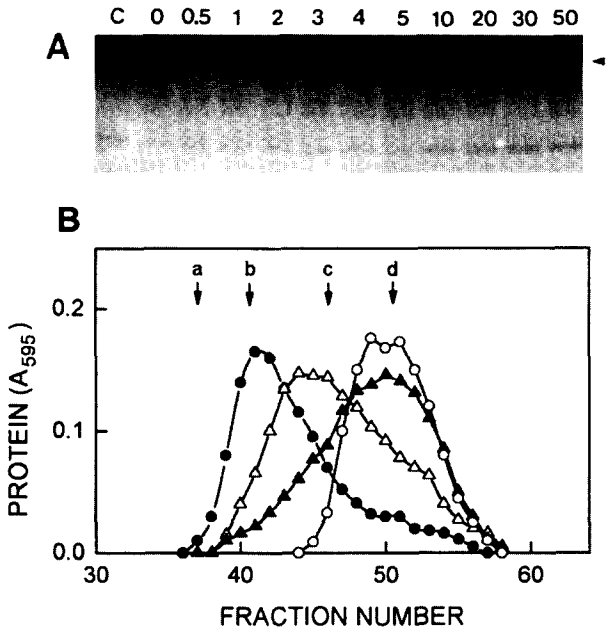

Fig. 4. Effect of increasing concentrations of ATP on the trypsin-mediated degradation of HslU and on the oligomerization of its subunits. A: Reaction mixtures were prepared as in Fig. 3 but in the presence of increasing concentrations of ATP. The samples were incubated at 37°C for 10 min followed by gel electrophoresis in a discontinuous slab gel. The numerals on the top of the gel indicate the ATP concentrations, and the arrowheads show the 50-kDa HslU proteins. B: The HslU proteins (0.1 mg in 0.5 ml) were incubated at 4°C for 10 min in the absence (○) and presence of 5 (▲), 20 (△), and 50 μM ATP (●). The samples were then subjected to gel filtration on a Sephacryl S-300 column as in Fig. 2. The size markers shown by arrows were also the same those used in Fig. 2.

ing additional affinity to ATP. However, this possibility requires an assumption that only the multimeric form of HslU can hydrolyze ATP, since the monomeric HslU/K63T is not capable of hydrolyzing the adenine nucleotide.

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