Mutagenesis of two N-terminal Thr and five Ser residues in HslV, the proteolytic component of the ATP-dependent HslVU protease

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Received 28 April 1997; revised version received 26 May 1997

Abstract HslVU in *E. coli* is a new type of ATP-dependent protease consisting of two heat shock proteins: the HslU ATPase and the HslV peptidase that has two repeated Thr residues at its N terminus, like certain β-type subunit of the 20S proteasomes. To gain an insight into the catalytic mechanism of HslV, site-directed mutagenesis was performed to replace each of the Thr residues with Ser or Val and to delete the first or both Thr. Also each of the five internal Ser residues in HslV were replaced with Ala. The results obtained by the mutational analysis revealed that the N-terminal Thr acts as the active site nucleophile and that certain Ser residues, particularly Ser^{124} and Ser^{172}, also contribute to the peptide hydrolysis by the HslVU protease. The mutational studies also revealed that both Thr, Ser^{103}, and Ser^{172}, but not Ser^{124}, are involved in the interaction of HslV with HslU and hence in the activation of HslU ATPase as well as in the HslVU complex formation.

Key words: ATP-dependent protease; ATPase; Heat shock protein; HslVU; 20S proteasome; *Escherichia coli*

1. Introduction

E. coli contains a number of ATP-dependent proteases, including proteases La (Lon) and Ti (ClpAP) [1–3]. In addition, we have recently described a new type of ATP-dependent protease, the product of the *hslVU* operon, which encodes two heat-shock proteins, HslV and HslU [4–7]. While HslU consisting of 6–7 subunits of 50 kDa provides an ATPase activity, HslV that has 12 subunits of 19 kDa harbors the peptidase activity, such as against *N*-carbobenzoxy (Cbz)-Gly-Gly-Leu-7-amido-4-methylcoumarin (AMC). In the reconstituted enzyme, HslU markedly stimulates the peptide hydrolysis by HslV (up to 50-fold), while HslV increases the rate of ATP hydrolysis by HslU several-fold [6].

The primary sequence of HslV has been shown to be similar to that of certain β-type subunit of the 20S proteasomes from eucaryotes, and archaebacterium *Thermoplasma acidophilum* [8]. The 20S proteasome of eucaryotes was initially characterized as a multicatalytic protease with chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase activities [9] and, on the basis of inhibitor studies, had been proposed to contain up to five distinct proteolytic components [10]. The lack of sequence similarity to other proteases [11,12] and the inconclusive nature of inhibitor studies [13–15] had prevented the assignment of the proteasome to any of the known protease families, but its sensitivity to 3,4-dichloroisocoumarin and to peptide aldehydes had suggested that it might be an unusual Ser or Cys protease. However, it has subsequently been demonstrated that archaebacterial β-subunit and certain mammalian β-type subunits of the 20S proteasome are comprising a new family of Thr protease upon covalent modification of the N-terminal Thr residue using lactacystin, a *Streptomyces* metabolite, and upon mutational analysis on the amino acid [8,16].

Like the 20S proteasomes, HslV has two conserved Thr residues at its N terminus [4,8]. Furthermore, lactacystin has been shown to partially inhibit the peptidase activity of the HslVU protease [5,6]. In addition, the enzyme is also sensitive to peptide aldehyde inhibitors of the 20S proteasome, such as Cbz-Leu-Leu-norleucinal or acetyl-Leu-Leu-norvalinal [5]. Therefore, it has been suggested that the HslVU protease may function through a similar proteolytic mechanism as the 20S proteasomes, although HslVU does not contain α-type proteasome subunits [6,8].

In order to clarify further the role of N-terminal Thr residues in catalytic function of HslV, we generated various mutant forms of HslV, in which either of two Thr residues was replaced with Ser or Val and the first or both Thr were deleted. Site-directed mutagenesis was also performed to replace each of five Ser residues with Ala to examine whether any of the mutations may influence on the peptidase activity of HslV and/or on its ability to stimulate the ATPase activity of HslU.

2. Materials and methods

2.1. Mutagenesis

The pGEM-T vector carrying the *hslVU* operon (named pGEM-T/HslVU) was constructed as described previously [6]. The *SalI-NruI* fragment (1 kb) carrying the coding region for HslV only was cut out from pGEM-T/HslVU and ligated to the Bluescript KS^+ vector. The resulting plasmid was referred to as pBS/HslV.

Site-directed mutations were created by the PCR method, which consists of two sequential PCRs [17], using pBS/HslV as the template. The primary PCR reactions were carried out using mutagenic primers and T7 or T3 primer. Reaction mixtures contained 10 ng of the template plasmid, 0.5 unit of *Taq* polymerase, 1×PCR buffer, 0.2 mM each of dNTPs, and 20 pmol of the primers. The reactions were performed for 30 cycles using a DNA Thermal Cycler (Perkin-Elmer). Prior to the secondary PCRs, the same amounts (100 ng each) of primary PCR fragments were mixed and subjected to annealing between the fragments by denaturation-renaturation reactions. The DNAs with recessed 3′-OH ends were extended by Klenow and used as the templates for the secondary PCRs. After the secondary PCR reaction, the mutated fragments were ligated into pGEM-T vector (Promega). Deletions or exchanges of the nucleotides by mutagenesis were confirmed by DNA sequencing. The resulting plasmids were transformed into *E. coli* strain XL2 Blue, and all of the mutant

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PII S0014-5793(97)00742-4

forms of HslV were purified from the cells to apparent homogeneity as described previously [6].

2.2. Assays

Peptide hydrolysis was assayed as described [6] 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, and 1 mM EDTA. Incubations were performed for 10 min at 37°C, and stopped by adding 0.1 ml of 1% (w/v) 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 [6]. After incubation, 0.2 ml of 1% SDS were added to the samples, and the phosphate released was determined as described [18]. Proteins were quantified by their absorbance at 280 nm or by the method of Bradford [19] using bovine serum albumin as a standard.

3. Results and discussion

3.1. Effects of N-terminal Thr mutations on peptide hydrolysis

HslV by itself is a weak peptidase, but its activity can be dramatically activated by HslU in the presence of ATP [6]. In order to determine the effects of the N-terminal Thr mutations in HslV on the hydrolysis of Cbz-Gly-Gly-Leu-AMC, the mutant forms of HslV were incubated with the peptide substrate in the absence and presence of HslU and ATP. As shown in Fig. 1A, replacement of the first or second Thr residue by either Ser or Val strongly impaired the peptidase activity of HslV alone. While the T1V mutation completely abolished the activity, the others reduced it by 70–80%. Similar extents of reduction in the peptide hydrolysis was observed when the same assays were performed in the presence of HslU and ATP, except for T1S (Fig. 1B). The HslV protein carrying the T1S mutation hydrolyzed the peptide nearly as rapid as the wild-type HslV in the presence of HslU and ATP, despite the finding that the same mutation resulted in about 70% decrease in the peptidase activity of HslV alone. Although it is totally unknown how the interaction of ATP-bound HslU with HslV can overcome the catalytic constraint imposed by the T1S mutation, these results suggest that the N-terminal Thr primarily contribute to the peptidase function of HslV.

To clarify further the role of the N-terminal Thr residue, the first (TΔ1) or both Thr (TΔ1,2) in HslV were deleted and the resulting mutant proteins were assayed for the peptide hydrolysis as above. Whether or not HslU and ATP are present, the peptidase activity was almost completely eliminated (Table 1). Upon similar mutational approach, the N-terminal Thr in β-subunit of the *Thermoplasma* proteasome has been demonstrated to act as an active site nucleophile [8]. Thus, the N-terminal Thr in HslV also appears to function as a catalytic site, although it is apparent that the second Thr somehow influence on the peptidase activity.

Table 1
Effect of deletion of the N-terminal Thr residues in HslV on the hydrolysis of Cbz-Gly-Gly-Leu-AMC

HslV	% Relative activity	
	-HslU	+HslU
Wild-type	100	100
TΔ1	1	3
TΔ1,2	0	1

When the peptide hydrolysis was assayed in the absence of HslU, reaction mixtures containing of 2 μg of HslV or its mutant forms and 0.1 mM Cbz-Gly-Gly-Leu-AMC were incubated 30 min at 37°C. Assays were also performed in the presence of HslU (0.3 μg) by incubating 0.1 μg of HslV or its mutant forms and 1 mM ATP for 10 min. The activities seen with the wild-type HslV in the presence and absence of HslU were expressed as 100%, and the others were as their relative values.

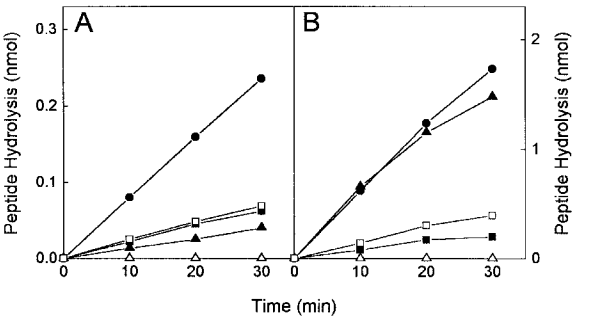

Fig. 1. Hydrolysis of Cbz-Gly-Gly-Leu-AMC by the HslV proteins carrying mutations in two N-terminal Thr residues. (A) Reaction mixtures containing 2 μg of the wild-type HslV (●) or the protein carrying the T1S (▲), T1V (△), T2S (■) or T2V (□) mutation and 0.1 mM Cbz-Gly-Gly-Leu-AMC were incubated for various periods at 37°C. (B) The peptide hydrolysis was assayed as above but by incubating 0.1 μg of the wild-type HslV or its mutant forms in the presence of 0.3 μg of HslU and 1 mM ATP.

3.2. Effects of Ser mutations on peptide hydrolysis

Individual mutation of all Ser residues in β-subunit of the *Thermoplasma* proteasome has been shown to exert little or no effect on the proteolytic activity [20]. In order to determine whether it also is the case for HslV, each of the five Ser residues in the peptidase was replaced with Ala. The mutant proteins were then assayed for the hydrolysis of Cbz-Gly-Gly-Leu-AMC in the presence and absence of HslU and ATP. Surprisingly, the S124A and S172A mutations almost completely abolished the peptidase activity, while the S103A mutation resulted in about 50% reduction of it (Table 2). On the other hand, the S5A and S143A mutations showed little or no effect on the peptide hydrolysis. In addition, the extents of reduction in the peptide hydrolysis by the mutant forms of HslV in the presence of HslU and ATP were very similar to those seen by the HslV proteins by themselves.

Seemüller et al. [8] have pointed out the presence of an additional highly conserved sequence, the Gly-Ser-Gly motif, in both eukaryotic and eubacterial β-type subunits, in which the Ser residue may also act as a potential nucleophile, but replacement of the Ser residue in the *Thermoplasma* β-type subunit with Ala was found to exert little or no effect on

Table 2
Effect of replacement of the Ser residues with Ala in HslV on the hydrolysis of Cbz-Gly-Gly-Leu-AMC

HslV	% Relative activity	
	-HslU	+HslU
Wild-type	100	100
S5A	119	124
S103A	50	50
S124A	2	3
S143A	101	95
S172A	1	1

Assays were performed as in Table 1 but using the HslV proteins, in which each of the five Ser residues was replaced with Ala.

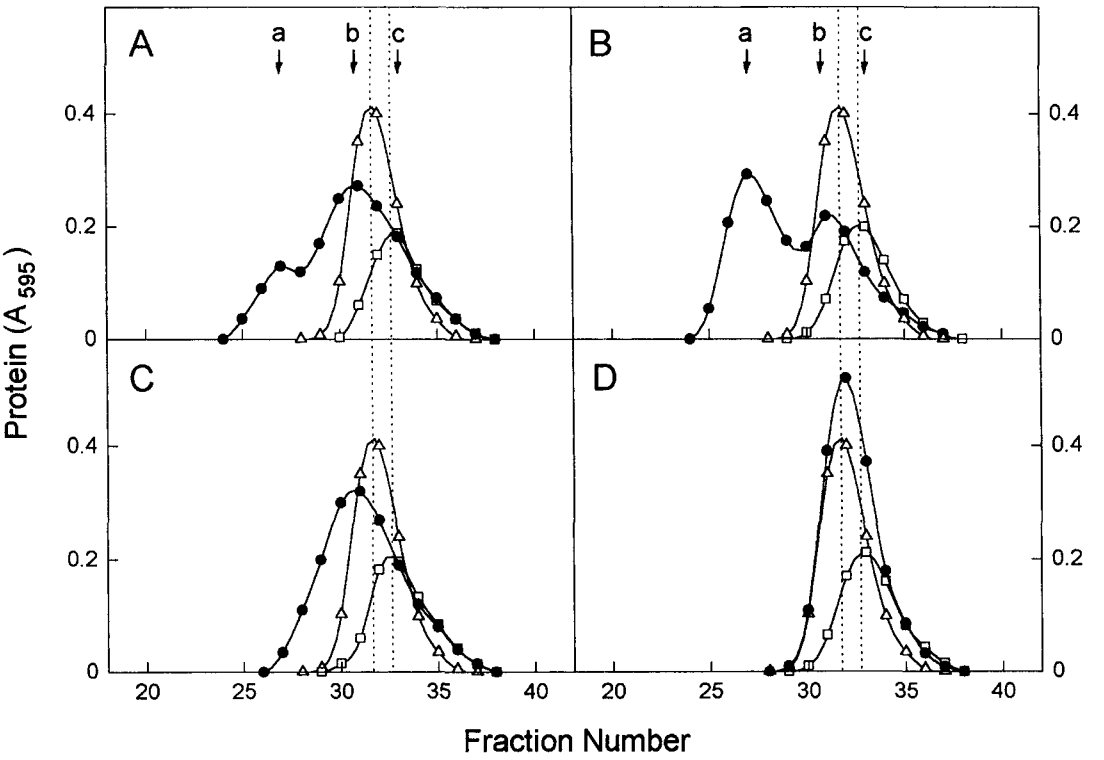

Fig. 2. Effects of Thr or Ser mutations on the interaction between HslV and HslU. The mixtures (●) of 150 μg of HslU and 50 μg of the wild-type (A) or the HslV proteins carrying the TΔ1 (B), S103A (C), and S172A mutations (D) were incubated on ice for 10 min and then subjected to gel filtration on a Superose-6 column (1×30 cm) equilibrated with 50 mM Tris-HCl (pH 7.8) buffer containing 0.2 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 25 mM $MgCl_2$, 2 mM ATP, and 10% (w/v) glycerol as described [22]. The HslV proteins by themselves (△) and HslU alone (□) were also chromatographed as above. Fractions of 0.5 ml were collected, and aliquots of them were assayed for proteins using the dye-binding method [19]. The size markers used are: a, thyroglobulin (669 kDa); b, apoferritin (443 kDa); c, catalase (232 kDa). The dotted lines indicate the positions where the peaks of HslU (left) and HslV (right) by themselves were eluted.

the proteolytic activity [20]. In *E. coli* HslV, Ser^{124} corresponds to the potential nucleophile Ser residue in the motif. And the S124A mutation was found to completely eliminate the peptidase activity of HslV whether or not HslU and ATP were present (see Table 2), unlike the archaebacterial proteasome. Therefore, it appears possible that Ser^{124} may also participate in the catalytic function of HslV. However, it is also possible that the amino acid together with the certain other Ser residues, such as Ser^{103} and Ser^{172}, are close to or conformationally coupled with the active site or the substrate-binding site in HslV rather than direct participation in catalytic function.

3.3. *Effects of HslV and its mutant forms on ATP hydrolysis by HslU*

HslU alone hydrolyzes ATP, and its activity can be stimulated 2- to 4-fold by HslV [6]. To determine whether the Thr or Ser mutations may also influence on the ability of HslV to stimulate the ATPase activity of HslU, each of HslV and its mutant forms was incubated with HslU and ATP. Interestingly, the ability of the most mutant proteins to activate the ATPase activity of HslU was closely correlated their capacity to hydrolyze the peptide in the presence of HslU and ATP (Table 3), as if the peptidase function of HslV is required for activation of the ATP hydrolysis by HslU. However, the HslV protein carrying the S124A mutation, which is unable to hydrolyze Cbz-Gly-Gly-Leu-AMC, stimulated the ATPase activity of HslU nearly as well as the wild-type HslU. Furthermore, the HslV protein lacking the N-terminal Thr, which neither can cleave the peptide, stimulated it much more effectively than the wild-type protein. Under the assay conditions, Cbz-Gly-Gly-Leu-AMC showed little of no effect on the ATPase activity of HslU whether or not HslV is present (data not shown). These results suggest that Thr^{2}, Ser^{103}, and Ser^{172} in HslV may involve in the interaction with HslU, although the structural or functional basis for the effects of the Thr and Ser mutations in HslV on the ATPase activity of HslU is unclear.

Table 3
Effects of HslV and its mutant forms on ATP hydrolysis by HslU

HslV	ATP hydrolysis by HslU (nmol/h)	Relative activity (%)
None	3.2	100
Wild-type	7.7	241
T1S	7.1	222
T1V	3.2	100
T2S	4.2	131
T2V	4.7	147
TΔ1	28.9	903
TΔ1,2	3.3	105
S5A	7.8	243
S103A	5.1	159
S124A	7.6	228
S143A	6.5	203
S172A	3.1	97

ATP hydrolysis was assayed by incubating 2 μg of HslU and 1 mM ATP at 37°C for 1 h in the absence and presence of 0.7 μg of the wild-type HslV or its mutant forms as described in Section 2.

3.4. Effects of Thr or Ser mutations on interaction between HslV and HslU

The isolated HslU behaves as a monomer or dimer of 50-kDa subunits in the absence of ATP but as a hexamer or heptamer (i.e. 300–350 kDa) in its presence [6,21–23]. On the other hand, HslV behaves as a dodecamer of 19-kDa subunits (240 kDa) whether or not ATP is present. In order to determine the effects of the Thr or Ser mutations on the interaction of HslV with HslU (i.e. on the formation of the HslV/HslU complex), we first analyzed the behavior of the HslV mutant proteins on a Superose-6 column. None of the mutations showed any effect on the chromatographic behavior (i.e. on the oligomeric nature) of HslV (data not shown), indicating that the mutations did not alter the gross conformation of the peptidase.

We then incubated HslV or its mutant forms with HslU at a molar ratio of 1:2, at which condition the maximal peptidase activity was achieved [6], in the presence of ATP for 10 min on ice. After incubation, the mixtures were chromatographed on the same gel filtration column. A significant portion of the wild-type HslV and HslU was eluted in the fractions corresponding to the molecular mass of 500–600 kDa (Fig. 2A), indicating that the two proteins form a complex. Noteworthy is the finding that the ability of HslV mutant proteins to form a complex with HslU is tightly correlated with their capacity to stimulate the ATP hydrolysis of HslU. While the HslV proteins carrying the T1V, TΔ1,2, and S172S mutations, which are unable to stimulate the ATPase activity, could not form a complex with HslU (Fig. 2D and data not shown), the HslV protein carrying the TΔ1 mutation, which activate the ATP hydrolysis much more effectively than the wild-type HslV, showed a higher tendency to form a complex with HslU (Fig. 2B). Likewise, the HslV proteins carrying the T1S and S124A mutations, which stimulate the ATPase activity of HslU nearly as well as the wild-type HslV, formed a complex with HslU to an extent similar to that seen with the wild-type HslV (Fig. 2A and data not shown), while those carrying the T2S, T2V, and S103A, which have a reduced ability to stimulate the ATPase activity, formed a complex to a lesser extent (Fig. 2C and data not shown). These results suggest that the extents of reduction in the ability of the HslV mutant proteins in the activation of ATP hydrolysis may reflect the degree of interaction of the specific amino acids in HslV with HslU in the HslVU complexes.

In this regard, the N-terminal Thr or Ser^{124} in HslV does not seem to directly involve in the interaction with HslU, since replacement of either residue with Ser or Ala, respectively, shows little or no effect on the ability to stimulate the ATP hydrolysis by HslU or to form a complex with the ATPase. Thus, it appears that Thr^{2}, Ser^{103} and Ser^{172} participate in the interaction of HslV with HslU, perhaps by residing at the interface between HslV and HslU in their complex. However, noteworthy is the finding that the HslV protein lacking the N-terminal Thr can activate the ATP hydrolysis by HslU and form a complex with the ATPase much better than the wild-type HslV. Therefore, the N-terminal Thr seems to provide a steric constraint on the interaction of Thr^{2} with HslU in addition to its role in the catalytic function of HslV. X-ray crystallographic analysis of the HslVU complex should clarify the structural and functional role of the Thr and Ser residues in HslV.

Acknowledgements: This work was supported by grants from the Korea Science and Engineering Foundation through Research Center Cell Differentiation and the Ministry of Education.

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