Determination of the cleavage sites in SulA, a cell division inhibitor, by the ATP-dependent HslVU protease from *Escherichia coli*

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Abstract HslVU is an ATP-dependent protease from *Escherichia coli* and known to degrade SulA, a cell division inhibitor, both in vivo and in vitro, like the ATP-dependent protease Lon. In this study, the cleavage specificity of HslVU toward SulA was investigated. The enzyme was shown to produce 58 peptides with various sizes (3–31 residues), not following the 'molecular ruler' model. Cleavage occurred at 39 peptide bonds preferentially after Leu in an ATP-dependent manner and in a processive fashion. Interestingly, the central and C-terminal regions of SulA, which are known to be important for the function of SulA, such as inhibition of cell division and molecular interaction with certain other proteins, were shown to be preferentially cleaved by HslVU, as well as by Lon, despite the fact that the peptide bond specificities of the two enzymes were distinct from each other.

Key words: ATP-dependent protease; Cleavage specificity; HslVU; SulA

1. Introduction

HslVU (also called HslUV or ClpYQ) encoded in the *hslVU* operon of *Escherichia coli* is an ATP-dependent oligomeric protease composed of the 19-kDa HslV peptidase and 50-kDa HslU ATPase [1,2]. HslV is homologous to certain β-type subunits of the 20S proteasome and forms a dodecamer of two back-to-back hexameric rings with the twelve active sites inside the central cavity [3]. HslU is a member of the Clp/Hsp100 family of molecular chaperones and forms a hexameric rings bound to either one or both HslV ends [4,5]. Since HslVU is induced by heat shock [6], the enzyme seems likely to be involved in the degradation of damaged polypeptides, like other ATP-dependent proteases [7]. On the other hand, the enzyme was shown to degrade several short-lived regulatory proteins including SulA [8,9], which is transcriptionally induced in a process called the SOS response and inhibits cell division by preventing self-assembly of FtsZ [10–12]. The degradation of SulA in vivo, however, seems to be predominantly due to the activity of another ATP-dependent protease, Lon, and HslVU appears to act as a backup for Lon, since SulA is accumulated in an *E. coli lon* mutant in the SOS response to inhibit the cell division, which is suppressed by overexpression of HslVU [8]. We have recently shown that Lon preferentially degrades certain sites of SulA with an apparent consensus sequence, Leu-Ser, in the central and C-terminal regions, which are important for the function of SulA, such as inhibition of cell division and interaction with Lon. The degradation by Lon should apparently contribute to rapid and accurate downregulation of the function of SulA [13]. Therefore, it is interesting to elucidate the mode of cleavage of SulA by HslVU to compare the results with those by Lon.

In this study, we investigated the cleavage specificity of HslVU toward SulA. The results indicate that the enzyme cleaves several sites in SulA with marked differences in the rate and/or site of cleavage as compared with Lon. Despite the differences, HslVU was shown to degrade preferentially the functionally important regions of SulA like Lon.

2. Materials and methods

2.1. Preparation of HslVU and SulA

HslV was cloned into the *Nde*I–*Xho*I site of pET21a(+) (Novagen) with a C-terminal Leu-Glu-His-His-His-His-His-His sequence and expressed in *E. coli* BL21(DE3) strain. Cells were collected after a 2-h induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C, resuspended in buffer A (20 mM Tris–Cl, 0.5 M NaCl, pH 7.9) containing 5 mM imidazole and lysed by sonication. The lysate was clarified by ultracentrifugation and applied to a His-Bind® column (Novagen). HslV was eluted by buffer A containing 1 M imidazole after washing the column with 10 column volumes of buffer A containing 60 mM imidazole, and used for further experiments. HslU was cloned into the *Nde*I–*Eco*RI site of pTYB12 (New England Biolabs) with the intein tag and an additional Ala-Gly-His sequence at the N-terminus, and expressed in *E. coli* BL21(DE3) strain. Cells were collected after an 18-h induction with 0.4 mM IPTG at 15°C, resuspended in buffer B (20 mM Tris–Cl, 0.5 M NaCl, 1 mM EDTA, pH 8.0) containing 1 mM phenylmethanesulfonyl fluoride and lysed by sonication. The lysate was clarified by ultracentrifugation and applied to a chitin beads column (New England Biolabs). The column was washed by 10 column volumes of buffer B and flushed by 3 column volumes of buffer B containing 50 mM dithiothreitol. After incubation for 40 h at 4°C, HslU with the N-terminal three additional residues released from the intein tag was eluted by buffer B containing 50 mM dithiothreitol. The protein fractions were pooled, supplemented with 5 mM EDTA and applied to a PD10 column (Amersham). HslU eluted by buffer B was concentrated and used for further experiments. SulA was prepared as described in [13].

2.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis

SulA (15 μg) was incubated at 37°C with 3 μg of HslV and 12 μg of HslU in 25 μl of 50 mM Tris–Cl, pH 8.0, containing 15 mM $MgCl_2$, with or without 4 mM ATP. At appropriate intervals, a 3-μl aliquot was withdrawn and the reaction was stopped by adding 3 μl of the SDS–PAGE sample buffer. These samples were subjected to SDS–

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PAGE (15% gel) and proteins were detected by the Coomassie brilliant blue R250 staining.

2.3. Identification of the cleavage sites

To determine the cleavage specificity toward SulA, 50 μg of SulA3-169 was incubated with 8 μg of HslV and 16 μg of HslU in 250 μl of 50 mM Tris–Cl, pH 8.0, containing 15 mM $MgCl_2$, with or without 4 mM ATP at 37°C for 15 h and the reaction was stopped by addition of 35 μl of 50% trichloroacetic acid. The reaction mixture was then centrifuged and 100 μl of the supernatant was applied to a high-performance liquid chromatography (HPLC) apparatus (1100 series, Agilent Technologies) with a TSKgel-ODS-120T column (150×2.2 mm, Tosoh) eluted with a gradient of acetonitrile (0–60% in 60 min), connected to an LCQ™ DUO mass spectrometer (ThermoQuest). The amino acid sequences of the product peptides were determined from the spectra of the original and fragmented ions by using an Xcalibur Bioworks 1.0 software installed in the apparatus, as described in [13–15]. The yields of peptides were calculated from the absorbance at 215 nm divided by the number of the peptide bonds. To determine the cleavage specificity toward glucagon, 10 μg of glucagon (Sigma) was incubated with 3 μg of HslV and 6 μg of HslU in 100 μl of 100 mM Tris–Cl, pH 8.0, containing 10 mM $MgCl_2$ and 1 mM EDTA, with or without 1 mM ATP at 37°C for 2 h. The reaction mixture was analyzed as described above.

3. Results

3.1. SDS–PAGE analysis of the degradation of SulA by HslVU

The degradation profile of SulA by HslVU was investigated using SDS–PAGE analysis. Fig. 1 shows that the band of SulA almost disappeared after 5 h incubation with the enzyme in the presence of ATP under the conditions used, while intermediate protein bands, with a molecular mass at least over 10 kDa, were not detected. The degradation rate of SulA by the present enzyme was much lower than that by Lon under similar conditions [13]. On the other hand, SulA was scarcely degraded by HslVU in the absence of ATP.

3.2. Determination of the sites of cleavage in SulA by HslVU

After incubation of SulA with HslVU for 12 h in the presence of ATP, 58 peptide peaks were observed upon HPLC (Fig. 2A) and their amino acid sequences were determined from the mass spectra of the original and fragmented ions (Fig. 2B). The sizes of the peptides ranged from 3 to 31 (average ± S.D., 10.2 ± 6.5). Fig. 2B shows the 39 cleavage sites deduced from the sequences and the hydrolysis rate estimated from the yields of peptides. Remarkably, the top seven major cleavage sites included Ala80-Ser81 (yield of peptide, 100%), Ala150-Ser151 (79%), Leu54-Gln55 (76%), Ile163-His164 (70%), Leu67-Thr68 (60%), Leu49-Leu50 (46%) and Leu65-Trp66 (44%), and they are located at the central and C-terminal regions. On the other hand, for the region of residues

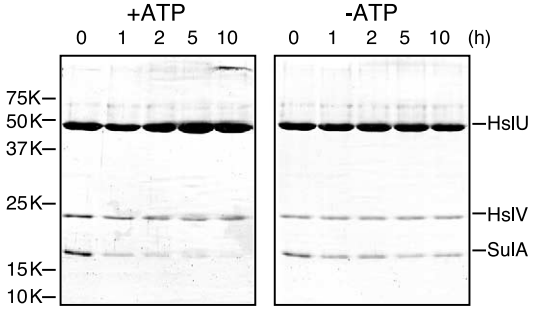

Fig. 1. SDS–PAGE analysis showing the susceptibility of SulA toward HslVU.

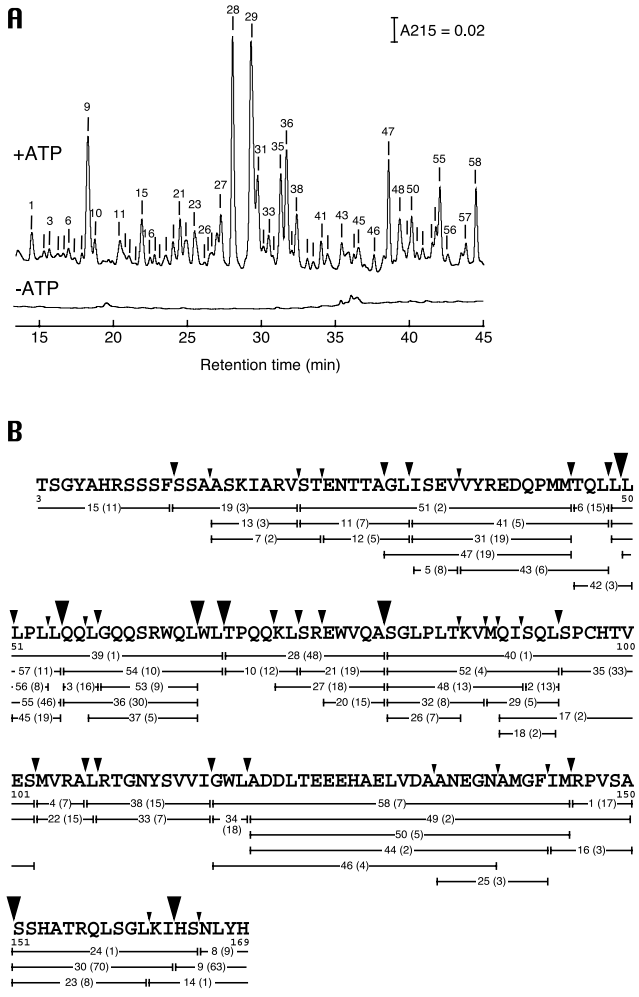

Fig. 2. The cleavage of SulA by HslVU. A: Separation of the degradation products by HPLC. SulA was incubated with HslVU in the presence or absence of ATP as described in Section 2. B: The cleavage sites of SulA after incubation in the presence of ATP. The peak numbers are arranged in the order of the retention time. The amino acid sequence is shown using the one-letter code for amino acids. Residues 1–2 were lost through the preparation of SulA as described in [13]. The number for each peptide stands for the peak number, and that in parentheses the estimated percentage yield of each peptide. Large, medium and small arrowheads indicate the major, medium and minor cleavage sites, respectively (see Table 1).

117–150, the recoveries of the product peptides were markedly low and their sizes were relatively large (average ± S.D., 16.7 ± 11.0). As for the amino acid specificity, the P_1 positions were occupied preferentially by Leu (14 sites) and other hydrophobic residues (17 sites) including Ala, Ile, Met, Val and Phe, while the P_1' positions were occupied by as many as 15 different residues (Table 1). On the other hand, no cleavage was observed after incubation of SulA with HslVU protease for 12 h in the absence of ATP (Fig. 2A).

3.3. Cleavage of model peptides by HslVU

The enzyme was shown to cleave five peptide bonds of glucagon (Fig. 3) in the presence of ATP. The Met29-Asn30 bond was cleaved most rapidly. In the absence of ATP, the cleavage rate was approximately 10% of that in the presence of ATP, although the cleavage specificity was not changed. Similarly, the enzyme was shown to degrade oxidized insulin

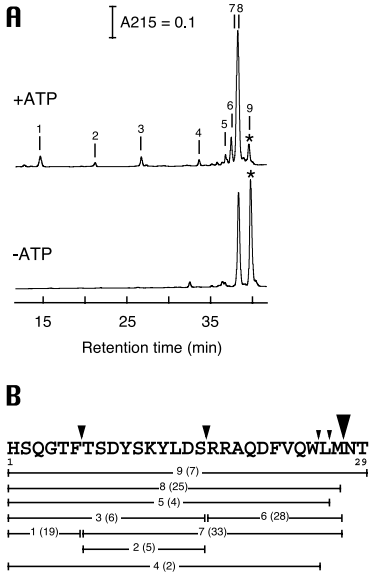

Fig. 3. The cleavage of glucagon by HslVU. A: Separation of the degradation products by HPLC. Glucagon was incubated with HslVU in the presence or absence of ATP as described in Section 2. Stars indicate peaks of the original peptide. B: The cleavage sites of glucagon after incubation in the presence of ATP. The amino acid sequence is shown using the one-letter code for amino acids. The number for each peptide stands for the peak number, and that in parentheses the estimated percentage yield of each peptide. Large, medium and small arrowheads indicate the major (86% hydrolysis), medium (24–38%) and minor (2–4%) cleavage sites.

B chain in the absence of ATP at a rate approximately 10% of that in the presence of ATP (data not shown), and the cleavage specificity was the same as in the presence of ATP (major sites of cleavage: Ala15-Leu16 and Leu18-Val19). The peptide bond specificity toward oxidized insulin B chain is the same as that reported previously by Yoo et al. [16].

4. Discussion

In this study, we investigated the cleavage specificity of HslVU toward SulA. The cleavage specificity of HslVU has been reported for several fluorogenic peptides [1,2] and oxidized insulin B chain [16], but not so far for the physiological protein substrates. Thus, this study is thought to provide fundamental insight into the cleavage mechanism of the enzyme toward such substrates under physiological conditions. Moreover, SulA is not only a physiological substrate of the present enzyme but also that of another ATP-dependent protease, Lon, whose cleavage specificity toward SulA has been reported in our previous paper [13]. Therefore, we are now able to compare their cleavage mechanisms.

HslVU was shown by the SDS–PAGE analysis to degrade SulA in an ATP-dependent manner, but at much a slower rate than Lon (Fig. 1). This is consistent with the possibility that HslVU seems to act as a backup for Lon as described in Section 1. It is worthwhile to note that the recognition mechanisms of the two enzymes for SulA have been reported to be rather different from each other; the C-terminal region of SulA was shown to be essential for the interaction with Lon [17], but not with HslVU [18]. This may be related to the difference in the relative cleavage rates of the respective, susceptible sites in SulA between the two enzymes, though further detailed study is necessary for clarifying this point. On the other hand, no intermediate protein band was observed in the SDS–PAGE analysis, as in the case of Lon, indicating that HslVU degrades the substrate in a processive fashion without release of polypeptide intermediates, like other ATP-dependent proteases [19–21].

In the presence of ATP, the peptide fragments from the central and C-terminal regions were obtained in much higher yields than those from the other regions, and the major cleavage sites, such as Ala80-Ser81, Ala150-Ser151, Leu54-Gln55, Ile163-His164, Leu67-Thr68, Leu49-Leu50 and Leu65-Trp66, were shown to be located in these regions. This indicates that these regions were rapidly processed by the enzyme to release the product peptides. It is notable that these regions are important for the function of SulA, rich in the secondary structures and well corresponding to those preferentially cleaved by Lon [13]. The central regions were reported to be important for the activity of SulA as a cell division inhibitor and presumably for constituting a critical surface for interaction with FtsZ, an essential cell division protein [22]. Moreover, the locations of the residues essential for the inhibitory activity (Arg62, Leu67, Trp77 and Lys87) are highly corresponding to

Table 1
Cleavage sites of SulA by HslVU

Major (>40% hydrolysis)	Medium (10–40% hydrolysis)	Minor (<10% hydrolysis)
Ala80-Ser81	Met45-Thr46	Val23-Ser24
Ala150-Ser151	Ser102-Met103	Ser165-Asn166
Leu54-Gln55	Leu94-Ser95	Val36-Val37
Ile163-His164	Leu32-Ile33	Leu53-Leu54
Leu67-Thr68	Ile116-Gly117	Val88-Met89
Leu49-Leu50	Leu48-Leu49	Leu161-Lys162
Leu65-Trp66	Ala30-Gly31	Thr86-Lys87
	Leu50-Leu51	Ala16-Ala17
	Leu73-Leu74	Thr25-Glu26
	Gln71-Lys72	Gln56-Leu57
	Leu119-Ala120	Phe143-Ile144
	Met145-Arg146	Met89-Gln90
	Leu57-Gly58	Asn139-Ala140
	Arg75-Glu76	Ala134-Ala135
	Ala106-Leu107	Ile91-Ser92
	Leu107-Arg108	
	Phe13-Ser14	

The sites of cleavage are shown in the decreasing order of the estimated extents of cleavage.

those of the preferential cleavage sites. Thus, preferential cleavage in this region should apparently contribute to the rapid and accurate downregulation of SulA. On the other hand, the C-terminal region was shown to be essential for the recognition and degradation by Lon as described in [17], though it remains unknown whether the region is important for the recognition or degradation by HslVU. On the other hand, for the region of residues 117–150, recoveries of the product peptides were markedly low and their sizes were relatively high, unlike the case of Lon. Interestingly, this region corresponds well to that essential for the interaction with HslU (residues 125–148) [18]. Thus, the interaction might somehow contribute to such a phenomenon in the region. The region other than the central or C-terminal regions was cleaved to only minor extents, which is, therefore, thought to contribute substantially little to abolishment of the SulA function by disruption of the tertiary structure.

The relationship between the size and recovery of the product peptides is similar to that reported for mammalian and archaeal proteasomes; the abundance of the product peptides decreases with increasing chain length [23,24]. The diversity in the size of the peptides produced (3–31 residues) by the present enzyme, as well as that of proteasome, cannot be accounted for by the 'molecular ruler' model, in which the product peptides are generated by the cooperative action of two adjacent active sites, the distance between which is thought to determine the product sizes (seven or eight residues long) [25]. Among the 58 product peptides, 20 peptides were shorter than seven residues and 30 peptides were longer than eight residues (Fig. 2B), suggesting that a longer peptide fragment once generated by the action of one of the active sites diffuses within the central cavity of the enzyme to be cleaved further by the action of the same or other active sites. Probably, the sizes of the product peptides may be simply determined by the time that a fragment stays within the enzyme molecule. On the other hand, 34 product peptides (60% in number) are of less than 10 residues, suggesting that peptides processed to such a short size are easily released from the enzyme.

In the absence of ATP, cleavage of SulA by HslVU was observed neither in the SDS–PAGE nor HPLC analysis, though the enzyme was shown to cleave glucagon and oxidized insulin B chain without ATP at a rate of approximately 10% of that with ATP. Thus, the protease activity of HslVU, especially toward a protein substrate with a high molecular weight, seems to be more highly dependent on ATP than that of Lon, which was shown to degrade the N- and C-terminal regions of SulA, although a little, in the absence of ATP under similar conditions [13].

As for the amino acid side chain specificity toward SulA and model peptides, it is a significant feature that the P_1 positions were preferentially occupied by hydrophobic residues, especially Leu, though all sites with such residues were not cleaved. On the other hand, no other clear-cut consensus residues or sequences of residues were found around the cleavage sites. Thus, cleavage specificity of the enzyme does not seem to be simply due to the primary structure of the substrate. Such an amino acid side chain preference of HslVU is rather similar to that of Lon; however, the cleavage sites were significantly different from each other. Especially, it is notable that the Leu-Ser bond is preferred by Lon, but scarcely by HslVU.

It is surprising that the present enzyme cleaves preferentially the central and C-terminal regions of SulA, as well as Lon, despite the differences in the rate and sites of cleavage and length of the product peptides. Such a preference apparently contributing to rapid and accurate downregulation of the function of the physiological substrate should provide a novel insight into the physiological significance of degradation of intracellular proteins by ATP-dependent proteases.

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