Regulation of Sula cleavage by Lon protease by the C-terminal amino acid of Sula, histidine

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SulA protein, a cell division inhibitor in *Escherichia coli*, is degraded by Lon protease. The C-terminal eight residues of SulA have been shown to be recognized by Lon; however, it remains to be elucidated which amino acid in the C-terminus of SulA is critical for the recognition of SulA by Lon. To clarify this point, we constructed mutants of SulA with changes in the C-terminal residues, and examined the accumulation and stability of the resulting mutant SulA proteins *in vivo*. Substitution of the extreme C-terminal histidine residue with another amino acid led to marked accumulation and high stability of SulA in *lon*⁺ cells. A SulA mutant in which the C-terminal eight residues were deleted (SulAC161) showed high accumulation and stability, but the

addition of histidine to the C-terminus of SulAC161 (SulAC161 + H) made it labile. Similarly, SulAC161 + H fused to maltose-binding protein (MBP–SulAC161 + H) formed a tight complex with and was degraded rapidly by Lon *in vitro*. Histidine competitively inhibited the degradation of MBP–SulA by Lon, while other amino acids did not. These results suggest that the histidine residue at the extreme C-terminus of SulA is recognized specifically by Lon, leading to a high-affinity interaction between SulA and Lon.

Key words: amino acid substitution, ATPase, ATP-dependent protease, maltose-binding protein (MBP).

INTRODUCTION

Exposure of *Escherichia coli* to agents or conditions that damage DNA results in the induction of more than 20 genes, which include those encoding DNA repair enzymes [1]. This response is called the SOS response, and the response-induced genes are called the SOS genes. One of these genes, *sulA*, encodes a cell-division inhibitor (SulA), which prevents the distribution of damaged DNA into daughter cells until DNA repair processes are completed [2,3].

SulA is an 18 kDa protein composed of 169 amino acid residues, and is highly conserved among enterobacteria species [4]. When DNA damage has occurred, SulA is produced and interacts directly with an essential cell-division protein, FtsZ [5,6]. The FtsZ associated with SulA is not able to polymerize to form an FtsZ-ring through GTP hydrolysis [7], and as a consequence cell division ceases. The re-starting of cell division is known to be regulated by two systems; one comprises transcriptional regulation leading to termination of the SOS response after DNA replication [6,8], and the other involves post-translational regulation through ATP-dependent proteases, such as ClpYQ (HslUV) and Lon protease [8–10,12].

ClpYQ is a two-chain enzyme, which is composed of ClpY, the regulatory subunit with ATPase and substrate-binding activities, and ClpP, the catalytic subunit with peptidase activity (similar to ClpAP or ClpXP). ClpP is a 19 kDa polypeptide and forms a hexameric or heptameric ring, and ClpY is a 50 kDa polypeptide which forms a six-membered ring. These two rings assemble to form a complex that acts as an ATP-dependent protease [13–17]. On the other hand, Lon is a single-chain enzyme of molecular mass 87 kDa, comprising ATPase, substrate-binding and pep-

tidase regions, and is known to form a homo-oligomer [18,19]. ClpYQ and Lon are also induced by heat shock, and are able to degrade misreading and abnormal proteins [13,15,20,21]. In addition, these proteases are produced under permissive conditions and catalyse the hydrolysis of some physiologically important proteins [10–12,21–25]. However, it remains to be determined how these proteases interact with substrates at the molecular level.

Accumulating evidence indicates that ATP-dependent proteases, such as Clp, FtsH and Lon, interact with the N- or C-terminal residues of the corresponding substrates, and that this interaction is necessary for the initiation of degradation [26–29]. We reported previously that the C-terminal eight amino acid residues of SulA are necessary for interaction with and degradation by Lon, but are distinct from the site that is cleaved by Lon [30,31]. To determine the most important of these residues for the interaction with Lon, we constructed substitution mutants of SulA with regard to the C-terminus. In this paper, we report that the presence of histidine at the C-terminal end of SulA is critical for the interaction with Lon and the subsequent degradation of SulA.

MATERIALS AND METHODS

Materials

Chemically synthesized oligonucleotides were obtained from Sawady Technology (Tokyo, Japan). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA, U.S.A.). T4 polynucleotide kinase and a DNA ligation kit were obtained from Takara (Kyoto, Japan). *Pfu* DNA polymerase was obtained from Promega (Madison, WI, U.S.A.). Amino acids and

Abbreviations used: IPTG, isopropyl β -D-1-thiogalactopyranoside; LB, Luria–Bertani; MBP, maltose-binding protein; $S_{0.5}$, substrate concentration giving 50% of maximal activity; I_{50} , concentration of inhibitor at which 50% activity remains; see Figure 1 for nomenclature of SulA mutant proteins.

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Figure 1 C-terminal amino acid sequences of native SulA and of its substitution and deletion mutants

(A) Amino acid substitutions or deletions in SulA. Bold-face letters show substituted amino acids. SulAC161 + H indicates the addition of histidine to the extreme C-terminus of SulAC161. (B) SulA deletion mutants fused to MBP. The hatched box (codons 40–87) is the FtsZ-interaction region [5,34]; the solid box (codons 162–169) is the Lon-recognition region [30,31], and the stippled box is MBP. These mutants were constructed by PCR using synthetic primers carrying mutant sequences. (C) Synthesized oligopeptide comprising the C-terminal eight residues of SulA (SA8) and its mutants.

imizazole were obtained from Wako (Osaka, Japan). Chemically synthesized oligopeptides (see Figure 1C) were obtained as described previously [31].

Bacterial strains

E. coli strain DH5 α was used for the purification of proteins, and strains AB1157 (wild type) and JK405 (carrying *lon::Tn10*) were used for the characterization of mutant *sulA* plasmids, as described previously [9,31].

Plasmid construction

The construction of plasmids pB10a, pSulA5 (native *sulA*), pSulAC161, pMAL-p2, pMAL-p2-SulA and pMAL-p2-SulAC161 was performed as described previously [31]. For *in vivo* experiments, mutant *sulA* genes (Figures 1A and 1B) were constructed by PCR using pSulA5 as a template DNA and oligonucleotide primers carrying mutated sequences. The genes were subcloned between the *Xho*I and *Hind*III sites of pB10a. The primers used for the construction of amino acid substitution mutants and deletion mutants were as follows. The upstream primer used was 5'-AA CCT CGA GGG GCT GGA TTG ATT ATG TAC-3' (the *Xho*I site is shown in bold face and the first codon of *sulA* is underlined). As a downstream primer, 5'-A TTA

AGC TTA ATG ATA CAA ATT AGA GTG AAT TTT TAG-3' was used for wild-type *sulA*, and substitution of nucleic acids was carried out for the corresponding mutant *sulA* genes (the *Hind*III site is shown in bold face, and the stop codon of *sulA* is underlined). For the preparation of substituted or deleted mutants of SulA fused to maltose-binding protein (MBP), the *sulA* genes were amplified by PCR, treated with T4 polynucleotide kinase, digested with *Hind*III, and then subcloned between the *Xmn*I and *Hind*III sites of pMAL-p2. As an upstream primer, 5'-ATG TAC ACT TCA GGC TAT GCA C-3' was used (the first codon of *sulA* is underlined). As downstream primers, corresponding primers for the mutated *sulA* were used.

Growth medium

All cultures were carried out in Luria-Bertani (LB) medium (Difco yeast extract 5 g/l, Difco tryptone 10 g/l and NaCl 5 g/l). This medium was solidified with 15 g/l Difco agar for the preparation of LB agar plates.

Assay of the inhibitory effect of SulA on cell division

An overnight culture of AB1157 (*lon*⁺) or JK405 (*lon*⁻) cells harbouring a *sulA* mutant subcloned into pB10a was streaked horizontally on an LB agar plate containing 50 μ g/ml ampicillin

using a cotton ear pick, and then a piece of filter paper (3 mm × 80 mm), on to which 0.05 M isopropyl β -D-1-thiogalactopyranoside (IPTG) had been absorbed, was placed on the plate.

Western blotting analysis of SulA *in vivo*

An overnight culture of AB1157 or JK405 cells harbouring a *sulA* mutant subcloned into pB10a was diluted 100-fold with 10 ml of LB medium containing 50 μ g/ml ampicillin and incubated at 37 °C until the mid-exponential phase, and then 1 mM IPTG was added. After incubation at 37 °C for 30 min, 200 μ g/ml chloramphenicol was added, and then 1 ml aliquots were collected at intervals during the re-incubation and mixed with 10% trichloroacetic acid. Each resultant precipitate was washed with acetone, and then suspended in 50 μ l of a urea solution comprising 8 M urea, 1 mM dithiothreitol and 1 mM EDTA in 50 mM Tris/HCl, pH 8.0. Samples containing equivalent amounts of protein were mixed with 10 μ l of SDS sample buffer (composed of 10% SDS, 38% glycerol, 9.3% dithiothreitol and 0.012% Bromophenol Blue in 0.35 M Tris/HCl, pH 6.8), and then subjected to electrophoresis on 5–20% (w/v) polyacrylamide SDS-slab gels (PAGEL[®]; ATTO, Tokyo, Japan) and transferred to PVDF membranes (Immobilon P[®]; Millipore). SulA on the membranes was detected with polyclonal anti-SulA antibodies [31] and a Phototope-HRP Western blot detection kit (New England BioLabs), and the reaction products were quantified using the NIH image program.

Protein purification

The Lon protease and mutant SulA proteins fused to MBP were purified as described previously [31,32].

Degradation of MBP fusion proteins by Lon protease *in vitro*

A concentration of 0.25 μ M MBP fusion protein (MBP–SulA, MBP–SulAH169W, MBP–SulAH169N, MBP–SulAH169D, MBP–SulAH169K, MBP–SulAC168, MBP–SulAC161 or MBP–SulAC161+H; see Figure 1B) was mixed with 0.25 μ M Lon and 4 mM ATP in 200 μ l of buffer A (i.e. 7.5 mM MgCl₂ in 50 mM Tris/HCl, pH 8.0), and 25 μ l aliquots were collected at intervals during the re-incubation and then mixed with 5 μ l of SDS sample buffer. To analyse the kinetics of degradation of MBP fusion proteins by Lon protease, 0.2–5.0 μ M fusion protein was incubated with 0.05 μ M Lon and 4 mM ATP in buffer A at 37 °C for 30 min, and then SDS sample buffer was added to the reaction mixtures. These samples were boiled at 95 °C for 10 min and then subjected to SDS/PAGE. The gels were stained with Coomassie Brilliant Blue R250, destained and dried as transparent films.

ATPase activity of Lon

MBP–SulA, MBP–SulAC168, MBP–SulAC161 or MBP–SulAC161+H (2 μ M) was incubated with 0.2 μ M purified Lon protease and 4 mM [γ -³²P]ATP (specific radioactivity 5 μ Ci/ μ mol) in 50 μ l of buffer A at 37 °C for 1 h. The reaction was stopped by adding 250 μ l of ice-cold PBS without bivalent cations but containing 6% Norit SX-3 (Wako) and 0.3% Dextran T500 (Pharmacia), and then the mixture was allowed to stand on ice for 30 min, followed by centrifugation at 11 300 *g* for 5 min at 4 °C. The release of [³²P]P_i was measured by counting the radioactivity in 100 μ l of the resultant supernatant in ACS-II scintillant (Amersham) using an Aloka liquid-scintillation counter (LSC-3100II; Aloka, Tokyo, Japan).

Complex formation between MBP fusion proteins and Lon protease

MBP–SulA, MBP–SulAH169W, MBP–SulAH169N, MBP–SulAH169D, MBP–SulAH169K, MBP–SulAC168, MBP–SulAC161 or MBP–SulAC161+H (0.1 μ M) was incubated with 0.4 μ M Lon protease in 0.2 ml of buffer A at 37 °C for 5 min, and then 50 μ l of amylose resin was added to the mixture. After incubation with gentle shaking at room temperature for 10 min, the resin was precipitated by rapid centrifugation at 3000 *g* for 5 min at 4 °C and washed five times with 50 μ l of buffer A. The proteins bound to the resin were eluted with 30 μ l of 10 mM maltose in buffer A, and then mixed with SDS sample buffer and boiled. The eluate was analysed by SDS/PAGE and subsequent Coomassie Brilliant Blue staining as described above.

Effects of C-terminal peptides of SulA and of various amino acids on the degradation of MBP–SulA by Lon protease

L-Proline, L-serine, L-asparagine, L-glutamine, L-lysine, L-histidine, D-histidine or imidazole (50 mM) was preincubated with 0.25 μ M Lon protease in 50 μ l of reaction buffer B (7.5 mM MgCl₂ in 100 mM Tris/HCl, pH 8.0) at room temperature for 5 min, and then 0.25 μ M MBP–SulA and 4 mM ATP were added to start the ATP-dependent proteolysis. After incubation for 30 min at 37 °C, the reaction was stopped with SDS sample buffer and the degradation of MBP–SulA was analysed by SDS/PAGE as described above. To determine the *K_i* values for synthesized oligopeptides or L-histidine with regard to the degradation of MBP–SulA by Lon protease, 0.1–0.5 mM oligopeptide or 10–50 mM histidine was mixed with 0.05 μ M Lon protease in buffer B, and then 0.2–1.0 μ M MBP–SulA and 4 mM ATP were added to the reaction mixture. After incubation for 30 min at 37 °C, degraded MBP–SulA was analysed by SDS/PAGE as described above.

RESULTS

The extreme C-terminal histidine residue of SulA regulates its sensitivity to Lon protease

We showed previously that the C-terminal eight amino acid residues of SulA are necessary for its degradation by Lon protease [31]. To determine which amino acid among the C-terminal residues is most important for the susceptibility of SulA to Lon, we constructed C-terminal alanine-substituted mutants of *sulA* (substitutions: isoleucine-163, I163A; histidine-164, H164A; leucine-167, L167A; histidine-169, H169A) under the control of the *lacUV5* promoter (Figure 1A), and the resulting plasmids were transfected into *lon*⁺ and *lon*[−] cells. These strains were streaked horizontally on to LB agar plates containing ampicillin, and then a piece of filter paper on to which 0.05 M IPTG had been absorbed was placed vertically on each plate. After incubation at 37 °C overnight, the width of the cell growth inhibition zone under the filter paper was measured. In *lon*[−] cells, native SulA and all of the C-terminal substitution mutants strongly inhibited cell growth; the width of the inhibitory zone was approx. 9 mm. In *lon*⁺ cells, however, mutants I163A, H164A and L167A showed a smaller inhibitory effect (width of the inhibitory zone < 1 mm) than native SulA (1 mm). H169A alone showed a high inhibitory effect similar to that of C161 (4 mm), which is a mutant SulA lacking all of the C-terminal eight amino acids.

In another experiment, we prepared another series of mutants, in which His-169 was replaced with other amino acids (see Figure 1A). These substitutions of residue 169 of SulA resulted in an inhibitory effect (inhibitory zone approx. 4 mm), similar to that

Figure 2 Accumulation and stability of C-terminal mutants of Sula *in vivo*

lon⁺ and *lon*⁻ cells carrying plasmids containing C-terminal mutants of *sulA* were grown at 37 °C to mid-exponential phase in LB medium containing ampicillin, and then 1 mM IPTG was added. After incubation for 30 min, samples were taken and precipitated with trichloroacetic acid (A), or 200 µg/ml chloramphenicol was added to the cultures and then aliquots were taken at 0, 2, 4, 8, 16 and 32 min during further incubation at 37 °C and precipitated with trichloroacetic acid (B). The Sula mutant proteins in the precipitates were analysed by Western blotting, as described in the Materials and methods section. (C) Amounts of Sula (●), SulaH169A (◇), SulaH169W (▲), SulaH169N (■), SulaH169D (×), SulaH169R (+), SulaC168 (○), SulaC161 (△) and SulaC161+H (□) in *lon*⁺ (left panel) and *lon*⁻ (right panel) cells were quantified with the NIH image program.

of H169A, in *lon*⁺ cells. Furthermore, we constructed a deletion mutant in which the C-terminal histidine was deleted (C168), one in which the C-terminal five residues were deleted (C164) to leave histidine as the C-terminal residue, and one in which histidine was added at the C-terminus of C161 (C161+H) (see Figure 1A), and streaked the resulting mutant strains on to plates. In *lon*⁻ cells, there was little difference between these mutants and native Sula, all of which exhibited high toxicity (inhibitory zone 9 mm). In *lon*⁺ cells, however, C168 showed high toxicity (4 mm), similar to His-169 substitution mutants and C161, while C164 and C161+H showed low toxicity (1 mm), like native Sula. These results suggest that the presence of histidine at the extreme C-terminus is necessary for Sula to be recognized and cleaved by Lon protease.

Accumulation and stability of C-terminal mutants of Sula *in vivo*

To confirm the specific role of the C-terminal histidine, we investigated the accumulation and stability of various Sula mutant proteins in *lon*⁺ or *lon*⁻ cells by Western blot analysis using anti-Sula antibodies. Figure 2(A) shows the accumulation of Sula mutant proteins (I163A, H164A, L167A, H169A, H169D, H169E, H169K, H169R, C168, C164, C161 and C161+H) in *lon*⁺ or *lon*⁻ cells after induction with 1 mM IPTG for 30 min at 37 °C. In *lon*⁻ cells, accumulation of the mutant proteins was ~90–130% of that of native Sula (results not shown). In *lon*⁺ cells, on the other hand, the accumulation of Sula mutant proteins substituted at His-169 (H169A, H169D, H169E, H169K and H169R) or of Sula deletion mutants (C168 and C161) was similar to the accumulation of native Sula in *lon*⁻ cells. However, the accumulation in *lon*⁺ cells of mutant Sula proteins in which histidine was present at a new C-terminal end

(C164), or histidine was added to the C161 deletion mutant (C161+H), was low, similar to that of native Sula (less than 10% of that of native Sula in *lon*⁻ cells).

To study the stability of the mutant Sula proteins in cells, cells containing mutant *sulA* plasmids were collected at various times during re-incubation in the presence of 200 µg/ml chloramphenicol after IPTG induction for 30 min, and the amounts of the various Sula mutant proteins (H169A, H169W, H169N, H169R, H169D, C168, C161 and C161+H) were determined (Figures 2B and 2C). In *lon*⁻ cells, the Sula mutants were as stable as native Sula, with half-lives of 8–32 min or more. Similar results were obtained with other constructed Sula mutants (results not shown). In contrast, native Sula and the C161+H mutant were very unstable in *lon*⁺ cells, with half-lives of < 2 min. Similar results were obtained indicating the rapid breakdown of mutant C164 in *lon*⁺ cells (not shown). However, all of the Sula His-169 substitution mutants and the deletion mutant lacking His-169 (C168) were all highly stable, with half-lives of ~5–15 min in *lon*⁺ cells. These results strongly suggest an important role for the C-terminal histidine in the recognition and subsequent breakdown of Sula by Lon protease.

Degradation of C-terminal Sula mutants by Lon protease *in vitro*

We next examined the degradation of C-terminal Sula mutants by Lon protease *in vitro* after purification of the mutants fused to MBP from the periplasmic fraction of *E. coli*; purification of non-fused Sula from the cytosol was not possible. MBP-Sula, MBP-SulaH169W, MBP-SulaH169N, MBP-SulaH169D, MBP-SulaH169K, MBP-SulaC168, MBP-SulaC161 and MBP-SulaC161+H (Figure 1B) were each purified by affinity column chromatography on an amylose resin, and then mixed

Figure 3 Degradation by Lon protease *in vitro* of native SulA and its mutants fused to MBP

(A) MBP-fused protein (0.25μ M) was mixed with buffer A (see the Materials and methods section) containing 0.25μ M Lon protease and 4 mM ATP, and then aliquots were taken at 0, 15, 30, 60, 90 and 120 min during incubation at 37°C , followed by SDS/PAGE analysis and Coomassie Brilliant Blue staining. (B) The amounts of MBP-fused SulA or its mutants remaining were determined with the NIH image program: ●, MBP-SulA; ▲, MBP-SulAH169W; ■, MBP-SulAH169N; ×, MBP-SulAH169D; +, MBP-SulAH169K; ○, MBP-SulAC168; △, MBP-SulAC161; □, MBP-SulAC161+H. (C) Kinetics of the degradation of MBP-fused SulA and its mutants by Lon protease. Aliquots of 0.2 – 5.0μ M MBP-SulA (●), MBP-SulAC168 (○), MBP-SulAC161 (△) or MBP-SulAC161+H (□) were incubated with 0.05μ M Lon and 4 mM ATP at 37°C for 30 min. The degraded proteins were analysed by SDS/PAGE and with the NIH image program.

with Lon protease and ATP, and incubated at 37°C . MBP-SulA was digested rapidly by Lon, but the substituted SulA proteins MBP-SulAC168 and MBP-SulAC161 were degraded more slowly (Figures 3A and 3B). For kinetic analysis, we defined the

Figure 4 Effects of variants of SulA on the ATPase activity of Lon

Reaction mixtures containing 0.2μ M purified Lon protease (as the monomer) and 4 mM [γ - ^{32}P]ATP in the absence or presence of 2.0μ M MBP-LacZ α , MBP-SulA, MBP-SulAC168, MBP-SulAC161 or MBP-SulAC161+H were incubated at 37°C for 1 h, and then the radioactivity of [^{32}P]P_i released from [γ - ^{32}P]ATP was measured as described in the Materials and methods section. The means $\pm$ S.D. for three independent experiments are shown.

$S_{0.5}$ value as the substrate concentration giving 50% activity. The sensitivity of MBP-SulAC161+H to Lon protease was similar to that of MBP-SulA, with $S_{0.5}$ values of approx. 1.0μ M, while those of both MBP-SulAC168 and MBP-SulAC161 seemed to be higher (Figure 3C). These results support those obtained in the *in vivo* experiments, suggesting that the C-terminal histidine of SulA determines the high affinity of the interaction between SulA and Lon protease.

ATPase activity of Lon protease in the presence of C-terminal SulA mutants

Lon protease exhibits ATPase activity, and it is known that this activity increases in the presence of a substrate. We investigated whether a C-terminal mutant of SulA increases the ATPase activity of Lon protease to a different extent than native SulA. Lon protease and ATP were incubated at 37°C for 1 h in the absence or presence of MBP-LacZ α (negative control), MBP-SulA, MBP-SulAC168, MBP-SulAC161 or MBP-SulAC161+H, and then liberated [^{32}P]P_i was measured. In the presence of MBP-SulA, the hydrolysis of ATP increased approx. 2.5-fold, while MBP-LacZ α , MBP-SulAC168 or MBP-SulAC161 did not increase the ATPase activity significantly (Figure 4). However, MBP-SulAC161+H increased the hydrolysis of ATP by Lon protease to almost the same extent as did native MBP-SulA. These results suggest that the presence of the C-terminal histidine is also necessary for SulA to stimulate the ATPase activity of Lon protease.

Interaction between Lon protease and C-terminal SulA mutants

The results in Figure 3(C) suggest that the C-terminal histidine of SulA determines the high affinity of the interaction between SulA and Lon protease. To investigate this possibility further, we examined whether or not a C-terminal SulA mutant forms a complex with Lon protease. MBP-SulA or a mutant protein was

Figure 5 Complex formation between Lon and variants of Sula

MBP-SulA, MBP-SulAH169W, MBP-SulAH169N, MBP-SulAH169D, MBP-SulAH169K, MBP-SulAC168, MBP-SulAC161 or MBP-SulAC161 + H ($0.1 \mu\text{M}$) was incubated with $0.4 \mu\text{M}$ Lon protease without ATP in a $200 \mu\text{l}$ reaction mixture at 37°C for 5 min, and then $50 \mu\text{l}$ of amylose resin was added to the mixture. After incubation with gentle shaking at room temperature for 10 min, the resin was washed with the reaction buffer, and then proteins adsorbed to the resin were eluted with 10 mM maltose. The eluate was analysed by SDS/PAGE and subsequent Coomassie Brilliant Blue staining as described in the Materials and methods section (A), and Lon protease co-eluted with MBP-fused proteins was quantified with the NIH image program (B). Protein sizes are shown on the left (where K denotes kDa), and the position of each protein is indicated on the right.

mixed with Lon protease, and then amylose resin was added to the mixture. If Lon forms a complex with a MBP-SulA mutant, Lon should be eluted together with the mutant. Figure 5 shows that Lon protease was co-eluted with native MBP-SulA and with MBP-SulAC161 + H, but hardly at all with other Sula mutant proteins. These results suggest that the presence of histidine at the extreme C-terminus of Sula is indispensable for stable complex-formation between Sula and Lon protease.

Effects of various amino acids on the degradation of MBP-SulA by Lon protease

In a previous study, we showed that a synthesized oligopeptide comprising the C-terminal eight residues of Sula (SA8) interacted directly with Lon and competitively inhibited the degradation of MBP-SulA by Lon protease [31]. Table 1 shows the I_{50} values (i.e. concentrations of inhibitor at which 50% activity remains) for the inhibition by SA8 and SA8H164A (a peptide in which the C-terminal histidine at position 164 is replaced with alanine; Figure 1C) of the degradation of MBP-SulA by Lon protease *in vitro*. The I_{50} values of SA8 and SA8H164A were 0.30 ± 0.08 and $1.13 \pm 0.57 \text{ mM}$ respectively, but that of SA8H169A (a peptide in which the C-terminal histidine at position 169 is replaced with

Table 1 I_{50} values of the C-terminal peptides of Sula and histidine for the degradation of MBP-SulA by Lon protease

Values are given as means $\pm$ S.D. for four independent experiment at 0.1, 0.2, 0.5 at $1.0 \mu\text{M}$ MBP-SulA.

Inhibitor	I_{50} (mM)
SA8	0.30 ± 0.08
SA8H164A	1.13 ± 0.57
SA8H169A	nd*
L-Histidine	33.5 ± 30.9
D-Histidine	nd†
Imidazole	nd†

* nd, not detected. When $0.05 \mu\text{M}$ Lon was incubated in the reaction mixture with $0.2 \mu\text{M}$ MBP-SulA and $500 \mu\text{M}$ SA8H169A for 30 min at 37°C , the amount of MBP-SulA degraded did not change.

† nd, not detected. When $0.25 \mu\text{M}$ Lon was incubated in the reaction mixture with $0.25 \mu\text{M}$ MBP-SulA and 50 mM D-histidine or imidazole for 30 min at 37°C , the amount of degraded MBP-SulA did not change.

alanine; Figure 1C) could not be determined. These results indicate that Lon recognizes the histidine residues among the C-terminal eight residues of Sula, distinguishing His-164 from His-169, and that the C-terminal His-169 mainly determines the affinity of Sula for Lon.

To elucidate the specific role of histidine among amino acids, we next examined whether histidine alone inhibits the degradation of Sula. L-Histidine at 50 mM inhibited the degradation of MBP-SulA by Lon protease by approx. 90% (Figures 6A and 6B). However, other amino acids, except lysine, did not inhibit degradation at all. Inhibition of the degradation of MBP-SulA by L-histidine was competitive, with an I_{50} value of $33.5 \pm 30.9 \text{ mM}$ (Figure 6C and Table 1). However, the I_{50} values of D-histidine and imidazole, a molecule that comprises the structure of the side chain of histidine, could not be determined. These results suggest that the histidine residue at the extreme C-terminus of Sula is recognized specifically by Lon, leading to a high-affinity interaction between Sula and Lon.

DISCUSSION

Lon protease of *Escherichia coli* hydrolyses a number of important proteins, such as λN , RcsA, CcdA, UmuD, StpA and Sula [8,22–25,33]. However, the interaction between Lon and these substrates has not been analysed closely at the molecular level. Here we report that the presence of histidine at the extreme C-terminus of Sula is indispensable for the degradation of Sula by Lon protease (Figures 2 and 3), and that the C-terminal histidine of Sula determines the high affinity of the interaction between Sula and Lon (Figures 3C and 5). There seem to be two possibilities for the role of the C-terminal histidine of Sula and the mechanism by which it determines the high affinity of Sula for Lon. The first is that the C-terminal histidine is involved in maintenance of the entire structure of Sula in the correct conformation, which also regulates other functions of Lon. This possibility, however, is unlikely, because some C-terminal substitution or deletion mutants of Sula inhibited the division of *lon*[−] cells, and MBP-SulAC168 interacted with purified FtsZ *in vitro* (results not shown), suggesting that the C-terminal histidine does not regulate the entire structure or function of Sula. Moreover, trypsin digestion resulted in similar fragment patterns for degraded MBP-SulA and MBP-SulAC168, implying that the C-terminal histidine does not regulate the general conformation

Figure 6 Effects of various amino acids on the degradation of MBP-SulA by Lon protease

(A) Proline, serine, asparagine, glutamine, lysine or histidine (50 mM) was preincubated with 0.25 μ M Lon protease in 50 μ l of buffer B (7.5 mM MgCl₂ in 100 mM Tris/HCl, pH 8.0) at room temperature for 5 min, and then 0.25 μ M MBP-SulA and 4 mM ATP were added to start the ATP-dependent proteolysis. After incubation for 30 min at 37 °C, the reaction was stopped and the degradation of MBP-SulA was analysed by SDS/PAGE. Protein sizes are shown on the left (where K denotes kDa). (B) The results were analysed quantitatively by means of the NIH image program. The means $\pm$ S.D. of three independent experiments are shown. (C) Inhibition of the degradation of MBP-SulA by histidine. Purified Lon (0.05 μ M) was incubated with 0–50 mM histidine in buffer B, and then various concentrations of MBP-SulA (●, 0.2 μ M; ▲, 0.3 μ M; ■, 0.5 μ M; ○, 1.0 μ M) and 4 mM ATP were added to the reaction mixtures. After incubation for 30 min at 37 °C, the degraded MBP-SulA was analysed by SDS/PAGE and with the NIH image program.

of SulA (results not shown). The other possibility is that the C-terminal histidine is recognized specifically by Lon at this site. Figure 6 shows that L-histidine itself specifically and competitively inhibited the degradation of MBP-SulA by Lon, but that D-histidine or imidazole did not (Table 1). These results suggest that the C-terminal histidine of SulA is strictly recognized

by Lon through stereospecific discrimination between the L- and D-forms, and not merely through an interaction with the imidazole moiety of histidine. Consequently, we conclude that the C-terminal histidine of SulA is specifically recognized by Lon protease, leading to a strong interaction between SulA and Lon.

The affinity of SulA for Lon protease seems to be greater than in the case of other substrates of Lon, such as casein, N protein, insulin B chain and CcdA, although the conditions for the reaction and the methods for measuring the kinetics of degradation of these substrates in previous studies were different from ours [22,23,31]. Why must SulA have histidine at the C-terminal end? What is the significance of the C-terminal histidine of SulA in cell physiology? The answers might be found in the function of this protein as an inhibitor of cell division. SulA, as a cell-division inhibitor, is very important in preventing the distribution of damaged DNA. Excessive accumulation of SulA, however, is toxic with regard to cell viability. Therefore SulA seems to be preferentially trapped and more rapidly degraded by Lon than other substrates.

There are many proteins with histidine at their C-terminal ends in the cell. It seems possible that Lon protease could interact with and degrade these proteins. Our previous study showed that MBP fused to the C-terminal residues of SulA interacted with Lon protease *in vitro* [30]. This fusion protein, however, was not digested by Lon *in vitro*, and LacZ fused to the C-terminal eight residues of SulA was stable in *lon*⁺ cells [30]. These results indicate that the C-terminal histidine leads to an interaction between Lon and the substrate, but that this interaction is not sufficient for degradation by Lon, implying a requirement for some other domain that is recognized as a substrate.

In conclusion, we have shown that the extreme C-terminal histidine residue of SulA is the most important amino acid for the recognition and degradation of this protein by Lon protease. This is a novel finding, as there have been no previous reports concerning such a role for the C-terminal amino acid of a substrate in the determination of its susceptibility to the corresponding protease. Other ATP-dependent proteases, such as Clp and FtsH, recognize the N- and/or C-terminal residue(s) of a substrate [26–28], but the most important residue or the key amino acid determining substrate degradation has not been reported. ClpXP and ClpAP degrade proteins to which C-terminal peptide tails have been added by means of the SsrA-tagging system; the C-termini of these proteins bear non-polar amino acid residues, but do not consist of a single specific amino acid [27]. Compared with these reports, the results of our present study seem to provide a novel clue for understanding the molecular interaction between a protease and its substrate. This information might be useful in the analysis of other mechanisms underlying substrate recognition by the relevant proteases, and also for clarifying the post-translational regulation of energy-dependent proteolysis under physiological conditions.

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