THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 36, Issue of September 4, pp. 22929–22935, 1998

Printed in U.S.A.

Effects of the Cys Mutations on Structure and Function of the ATP-dependent HslVU Protease in *Escherichia coli*

THE CYS[287] TO VAL MUTATION IN HslU UNCOUPLES THE ATP-DEPENDENT PROTEOLYSIS BY HslVU FROM ATP HYDROLYSIS*

(Received for publication, May 20, 1998)

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To define the role of the Cys residues in the ATP-dependent HslVU protease, mutagenesis was performed to replace either Cys[261] or Cys[287] in HslU with Val and Cys[159] in HslV with Ser or Ala. Whereas HslU/C261V could hydrolyze ATP and support the ATP-dependent proteolytic activity of HslV as well as the wild-type HslU, HslU/C287V could not hydrolyze ATP. Nevertheless, HslU/C287V could support the HslV-mediated proteolysis by forming the HslVU complex in the presence of ATP but not its absence, indicating that ATP binding but not its hydrolysis is essential for proteolysis. Whereas treatment of *N*-ethylmaleimide (NEM) resulted in dissociation of the oligomeric HslU into monomers, the C261V mutation, but not C287V, prevented the NEM effect. These results suggest that Cys[261] is involved in oligomerization and that Cys[287] is related to the ATPase function of HslU. NEM also dissociated the dodecameric HslV into monomers, and this effect could be prevented by either the C159S or C159A mutation, suggesting the involvement of Cys[159] in oligomerization of HslV. Moreover, either mutation abolished both the basal and HslU-activated proteolytic activity of HslV and its ability to activate the HslU ATPase and to form the HslVU complex, indicating that Cys[159] is essential for the proteolytic activity of HslV and its interaction with HslU. These results suggest that the Cys residues play an important role in maintaining the structure and function of the HslVU protease.

A fundamental feature of intracellular protein degradation is its requirement for metabolic energy (1, 2). In eukaryotic cells, ATP is required for ubiquitin conjugation and for function of the 26S proteasome, which constitutes the primary site for degradation of most cell proteins (3–5). The catalytic core of the 26S proteasome is the 20S proteasome, which contains multiple peptidase activities (6, 7) and the function of which is regulated by a 19S component, which associates with the termini of the 20S core (8) and has multiple ATPase activities (9).

Although *Escherichia coli* and other bacteria lack the ubiquitin-proteasome pathway, they contain a number of ATP-dependent proteases (1, 2, 10–15). Rapid degradation of many abnormal polypeptides and various regulatory proteins requires protease La (Lon), a heat-shock protein composed of four identical 87-kDa subunits. This homopolymer exhibits both ATP-dependent proteolysis and ATPase activity (16, 17). Another type of ATP-dependent protease, Ti (ClpAP), consists of two different multimeric components, both of which are required for proteolysis (18). ClpA, which is composed of 84-kDa subunits, contains ATP-hydrolyzing sites, whereas ClpP, which is composed of 21-kDa subunits, is a serine protease. ClpA is a member of a family of highly conserved polypeptides, present in both prokaryotic and eukaryotic organisms (19). This family contains two regions of particularly high homology, each of which contains a consensus sequence for ATP binding (20). In fact, the ClpP peptidase can function together with one of the homologous ATPases, ClpX, in degradation of certain regulatory proteins (21–24).

Recently, we described a new type of ATP-dependent protease in *E. coli*, the product of the *hslVU* operon, which encodes two heat-shock proteins: a 19-kDa HslV and a 50-kDa HslU (25). HslV harbors the peptidase activity, whereas HslU provides an essential ATPase activity, both of which can function together as a novel two-component protease (26–28). HslU itself has ATPase activity, whereas HslV by itself is a weak protease that slowly degrades certain hydrophobic peptides, such as *N*-carbobenzoxy (Cbz)[1]-Gly-Gly-Leu-7-amido-4-methylcoumarin (AMC), and polypeptides, including insulin B-chain and casein (27, 29). In the reconstituted enzyme, HslU markedly stimulates the proteolytic activity of HslV (more than 20-fold), whereas HslV increases the rate of ATP hydrolysis by HslU severalfold.

The primary sequence of HslV is similar to that of certain β-type subunits of the 20S proteasomes from eukaryotes, certain eubacteria, and the archaebacterium *Thermoplasma acidophilum* (30). In the 20S proteasomes, the N-terminal Thr residues of the mature β-type subunits serve as the catalytic site for proteolysis (30, 31). Like the β-type subunits of the enzyme, HslV has two such conserved N-terminal Thr residues (30). Furthermore, deletion of the N-terminal Thr or its replacement by Val completely abolishes the proteolytic activity of the HslVU protease (32). In addition, it is inhibited by

* This work was supported by grants from Korea Science and Engineering Foundation through the Research Center for Cell Differentiation and the Korea Ministry of Education (to C. H. C.) and from CREST, Japan Science and Technology Corporation (to K. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Cbz, carbobenzoxy; AMC, 7-amido-4-methyl coumarin; NEM, *N*-ethylmaleimide; HPLC, high pressure liquid chromatography.

lactacystin, a *Streptomyces* metabolite, which covalently modifies the N-terminal Thr residue on certain β-type subunits of the 20S proteasome (26, 27, 29, 31). Therefore, the HslVU protease appears to function through a similar proteolytic mechanism as the mammalian and archaebacterial proteasomes, even though HslVU does not contain α-type proteasome subunits.

All of the ATP-dependent proteases in *E. coli* described above show protein-activated ATPase activity. Moreover, inhibitors of the ATPase activity also prevent the protein degradation by the enzymes. For example, *N*-ethylmaleimide (NEM) inhibits ATP hydrolysis by HslU, and the NEM-treated HslU is unable to support the HslV-mediated degradation of both polypeptide and small peptide substrates (27, 32). Therefore, it has been suggested that hydrolysis of both ATP and protein substrates occurs in a tightly coupled process. However, it has recently been revealed that β,γ-imido-ATP and ATPγS, both of which are nonhydrolyzable ATP analogs, can support the proteolytic activity of the HslVU protease (33, 34). These findings suggest that proteolysis by HslVU can be uncoupled from ATP cleavage.

Whereas HslV has a single Cys residue in its C-terminal region, HslU contains two Cys residues in the second half of the ATP binding domain (25). In order to define the role of the Cys residues of HslU in ATP hydrolysis and to clarify further the inhibitory effect of NEM, site-directed mutagenesis was carried out to replace either the 261st or the 287th residue (Cys) in HslU with Val. We also replaced the 159th residue (Cys) in HslV with Ser or Ala to examine the effects of the mutations on the proteolytic function of the HslVU protease. In addition, we determined the cleavage specificity of the enzyme using insulin B-chain as a model substrate.

EXPERIMENTAL PROCEDURES

Materials—All reagents for the site-directed mutagenesis and restriction endonucleases were purchased from Promega. Peptide substrates were obtained from Bachem Feinchemikalien AG, Bubendorf, Switzerland. All other reagents were purchased from Sigma, unless otherwise indicated. HslV, HslU, and their mutant forms were purified as described previously (27).

Mutagenesis—The pGEM-T vector carrying the *hslVU* operon (named pGEM-T/HslVU) and the pBluescript KS^+ vector containing the coding region for HslV only (named pBS/HslV) were constructed as described previously (27, 32). Site-directed mutations were created by the PCR method, which consists of two sequential PCRs, using pGEM-T/HslVU for HslU and pBS/HslV for HslV as the templates.

The primary PCRs were carried out using mutagenic primers, which were designed to replace Cys^{159} of HslV with Ala or Ser and Cys^{261} or Cys^{287} of HslU with Val (Fig. 1). Reaction mixtures contained 10 ng of the template plasmids, 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 (0.1 μg 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 using a Klenow fragment and used as the templates for the secondary PCRs. After the secondary PCR, the mutated fragments were ligated into pGEM-T vector. For HslU mutants, the resulting plasmids were digested with *Nru*I and *Bgl*II, followed by ligation of the restriction fragments into the pGEM-T/HslVU plasmids that had also been digested with the same enzymes. The resulting plasmids were transformed into *E. coli* strain XL2 Blue. Substitutions of the nucleotides by mutagenesis were confirmed by DNA sequencing.

Assays—Protein breakdown was assayed using ^{125}I-labeled insulin B-chain as the substrate as described previously (35). Radioiodination of insulin B-chain was performed as described previously (35, 36). Reaction mixtures (0.1 ml) contained 10 μg of insulin B-chain and appropriate amounts of the purified HslV and HslU proteins in 0.1 M Tris-HCl (pH 8), 10 mM $MgCl_2$, 0.25–2.5 mM ATP, 1 mM dithiothreitol, and 1 mM EDTA. Incubations were performed for 1 h at 37 °C. After incubation, radioactivity released into trichloroacetic acid-soluble form (final concentration, 10% (w/v)) was determined using a gamma counter. Peptide hydrolysis was assayed using Cbz-Gly-Gly-Leu-AMC (0.1 mM) as the substrate by incubating the similar reaction mixtures for 10 min at 37 °C but containing only 1/10 the amount of the HslV and HslU proteins (27).

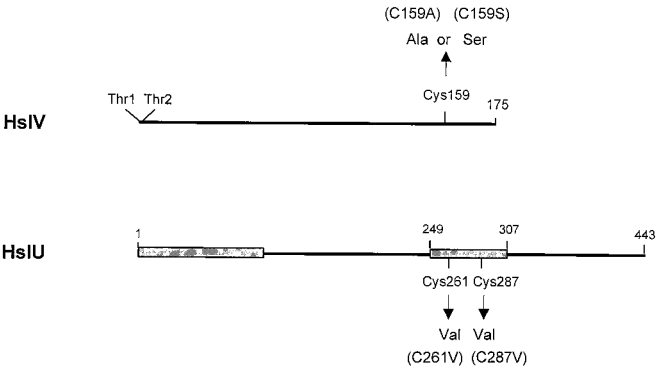

FIG. 1. **Schematic presentation of the primary structures of HslV and HslU.** The Cys residues subjected to mutagenesis are indicated. The *gray boxes* in HslU indicate the ATP binding domain, which is homologous to that in the HSP100/Clp family.

ATP hydrolysis was assayed by incubating the similar reaction mixtures at 37 °C but in the absence of the peptide or protein substrate. After incubation, 0.2 ml of 1% SDS (w/v) was added to the samples, and the phosphate released was determined as described (37). Proteins were quantified by their absorbance at 280 nm or by the method of Bradford (38) using bovine serum albumin as a standard.

Cross-linking Analysis—Aliquots (4 μg each) of the HslU or HslV proteins or their mixture were incubated 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 and absence of ATP in a total volume of 0.1 ml (34). After incubations for 20 min at 37 °C, the samples were mixed with 30 μl of 0.75 M Tris-HCl (pH 6.8) containing 7.5% SDS and 10% (v/v) 2-mercaptoethanol. They were then subjected to polyacrylamide gel electrophoresis on 4–8% (w/v) gradient slab gels containing SDS and 2-mercaptoethanol (39). Proteins in the gels were then visualized by silver staining.

Determination of Cleavage Specificity—Oxidized insulin B-chain (10 μg) was digested with the purified HslV (2 μg) and HslU (8 μg) proteins for 2 h at 37 °C in the presence of 1 mM ATP. The cleavage products (0.2 ml each) were analyzed by reverse phase HPLC (LC10AS, Shimadzu) using a Bondsphere column (2.1 × 150 mm, Waters). The peptides were eluted with an acetonitrile gradient using solution A, containing 0.1% (v/v) trifluoroacetic acid, and solution B, containing 0.09% trifluoroacetic acid in 80% (v/v) acetonitrile. With solution B, elution was carried out for 0–60 min at 2–37.5%, 60–90 min at 37.5–75%, and 90–105 min at 75–98% acetonitrile. Elution of the peptides was monitored by their absorbance at 214 nm. Amino acid sequences were then determined by Edman degradation using a peptide sequencer (model 491, Perkin-Elmer) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Voyager RP, PerSeptive Biosystems).

RESULTS

Effects of the Cys Mutations on the Ability of HslU in ATP Hydrolysis and in Supporting HslV-mediated Proteolysis—In order to determine the effects of Cys mutations on the ATPase activity of HslU, site-directed mutagenesis was performed to replace either Cys^{261} or Cys^{287} in HslU with Val (Fig. 1). The resulting proteins were purified to apparent homogeneity and are referred to as HslU/C261V and HslU/C287V, respectively (Fig. 2). We then examined the effects of Cys mutations on the ATPase activity of HslU. Fig. 3*A* shows that the rate of ATP hydrolysis by HslU/C261V is nearly identical to that of the wild-type HslU. In contrast, HslU/C287V was not capable of hydrolyzing ATP at all. Prolonged incubation with excess of the mutant protein (*e.g.*, incubation for 4 h with 20 μg of HslU/C287V) did not show any ATP-cleaving activity either (data not shown). HslV has been shown to stimulate ATP hydrolysis by the wild-type HslU 2–4-fold (27). To examine whether HslV can also activate the ATPase activity of the mutant forms, ATP hydrolysis was assayed in the presence of increasing amounts of HslV. As shown in Fig. 3*B*, the HslV-mediated increase in

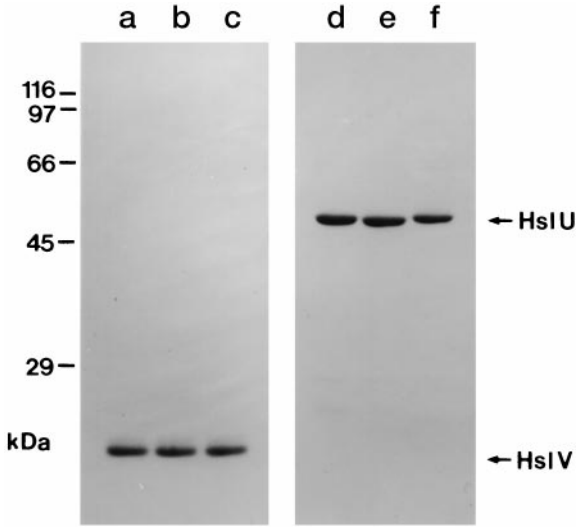

FIG. 2. **Polyacrylamide gel electrophoresis of the purified HslV and HslU and their mutant forms.** The purified HslV (*lane a*), HslV/C159A (*lane b*), HslV/C159S (*lane c*), HslU (*lane d*), HslU/C261V (*lane e*), and HslU/C287V (*lane f*) were electrophoresed in 13% (w/v) slab gels containing SDS and 2-mercaptoethanol. The gels were then stained with Coomassie Blue R250. Each lane contains 5 μg of protein.

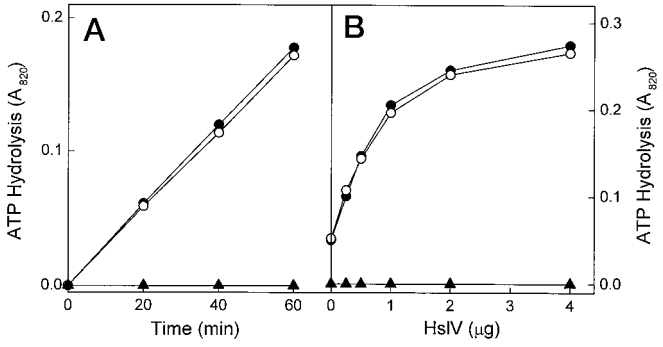

FIG. 3. **Hydrolysis of ATP by HslU and its mutant forms.** *A*, ATP hydrolysis was assayed by incubating 4 μg of HslU (●), HslU/C261V (○), or HslU/C287V (▲) and 1 mM ATP for various periods at 37 °C. *B*, assays were performed as above, except that 1 μg of the HslU proteins was incubated for 1 h in the presence of increasing amounts of HslV.

the ATPase activity of HslU/C261V occurred in parallel with that of the wild-type HslU. However, ATP hydrolysis by HslU/C287V was not evident even in the presence of HslV. Thus, Cys^{287} but not Cys^{261} appears to be involved in the ATPase function of HslU.

In order to determine the ability of HslU and its mutant forms in supporting the HslV-mediated proteolysis, HslV was incubated with increasing amounts of the HslU proteins. Fig. 4*A* shows that HslU/C261V supports the hydrolysis of ^{125}I-labeled insulin B-chain by HslV as well as the wild-type HslU. Surprisingly, HslU/C287V, which is unable to hydrolyze ATP, could also support the HslV-mediated proteolysis in the presence of ATP, although to extents much less than those seen with HslU or HslU/C261V at low protein concentrations. Without ATP, however, only the basal activity of HslV could be seen whether or not HslU and its mutant forms were present (data not shown). Similar data were obtained for the hydrolysis of Cbz-Gly-Gly-Leu-AMC (data not shown). These results indicate that ATP binding but not its hydrolysis is essential for the proteolytic function of the HslVU protease and further support the previous suggestion that ATP hydrolysis can be uncoupled from cleavage of peptide bonds by HslVU (33, 34).

Effects of the Cys Mutations on the Affinity of HslU to ATP and Interaction with HslV—The HslVU protease shows a maximal proteolytic activity against insulin B-chain or casein when HslV and HslU are incubated at a mass ratio of 1:4, which is approximately equivalent to a molar ratio of 1:2 (29, 40). To achieve the maximal activity with HslU/C287V, however, an at least 2-fold higher amount of the protein was required as compared with HslU or HslU/C261V (*i.e.*, at a mass ratio of 1:8; see Fig. 4*A*). This finding raised a possibility that the C287V mutation results in reduction of the affinity of the HslU protein for ATP and hence in the decrease in the affinity between the HslU subunits and/or between the HslU and HslV oligomers. To test this possibility, HslV was incubated with HslU/C287V at a fixed mass ratio of 1:4 and in the presence of increasing concentrations of ATP. As shown in Fig. 4*B*, the ATP concentration required for the half-maximal activity in the presence of HslU/C287V was at least 5-fold higher than that with the wild-type HslU or HslU/C261V. Thus, it appears that the requirement of high amounts of HslU/C287V in supporting the HslV-mediated proteolysis is due to its decreased affinity for ATP.

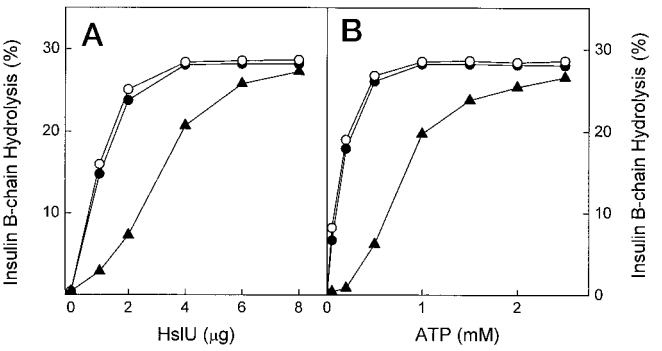

FIG. 4. **Ability of HslU and its mutant forms to support the proteolytic activity of HslV.** *A*, degradation of ^{125}I-labeled insulin B-chain (10 μg) was assayed by incubating 1 μg of HslV and 1 mM ATP for 1 h in the presence of increasing amounts of HslU (●), HslU/C261V (○), or HslU/C287V (▲). The radioactivity released into acid-soluble products was then measured and expressed as a percentage of the total radioactive protein added to the reaction mixtures. *B*, proteolysis was assayed as above, except that 1 μg of HslV and 4 μg of the HslU proteins were incubated in the presence of increasing concentrations of ATP.

In order to determine whether the reduced affinity of HslU/C287V for ATP indeed influences the interaction between the HslU subunits and/or between the HslU and HslV oligomers, a cross-linking analysis was performed by incubation of HslU/C287V with glutaraldehyde in the presence of 0.5 or 2 mM ATP. As a control, HslU and HslU/C261V were also subjected to the same cross-linking analysis. In the presence of 0.5 mM ATP, both HslU and HslU/C261V formed an oligomeric complex of 300–350 kDa, which is likely to represent a hexameric or heptameric form of HslU, whereas more than 60% of HslU/C287V remained as a monomer of about 50 kDa (Fig. 5*A*). On the other hand, all of the HslU proteins were cross-linked to the high molecular mass, oligomeric form in the presence of 2 mM ATP. When the same experiment was performed in the presence of HslV, the amounts of the HslVU complex formed by incubation of HslU/C287V and HslV in the presence of 0.5 mM ATP was much less than that seen with 2 mM ATP (Fig. 5*B*). Moreover, the amounts of the HslVU complex formed by incubation of HslU/C287V and HslV in the presence of two different ATP concentrations were approximately proportional to the amounts of the mutant HslU oligomers formed in the absence of HslV. On the other hand, HslU/C261V formed the complex with HslV nearly as well as the wild-type HslU at both ATP concentrations. Thus, it appears that the reduced ability of HslU/C287V in supporting the HslV-mediated proteolysis is due to its reduced ability in oligomerization at low ATP concentrations but not to any defect in the interaction between the

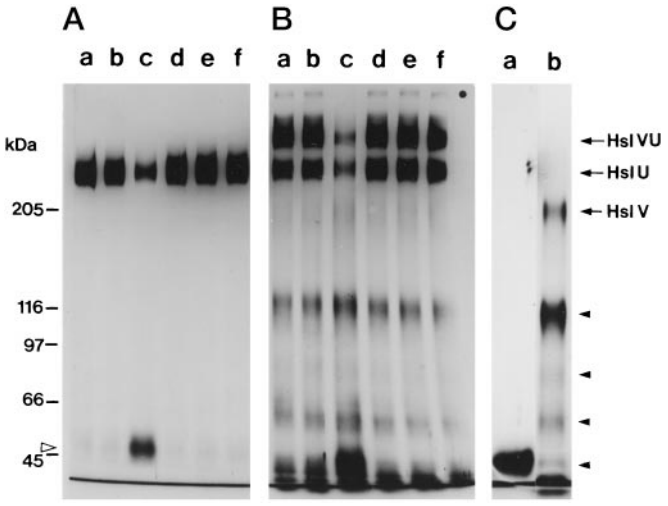

FIG. 5. **Cross-linking analysis for the assembly of the HslVU complex by the purified HslV and HslU/C261V or HslU/C287V.** *A*, aliquots (4 μg each) of HslU (*lanes a* and *d*), HslU/C261V (*lanes b* and *e*), or HslU/C287V (*lanes c* and *f*) were incubated with 0.4% glutaraldehyde for 20 min at 37 °C in the presence of 0.5 mM (*lanes a–c*) or 2 mM ATP (*lanes d–f*). The samples were then electrophoresed under denaturing conditions followed by silver staining. The *open arrowhead* indicates the HslU monomer. *B*, experiments were performed as above but in the presence of 4 μg of HslV. The *dot* may represent two HslU oligomers flanked by a HslV dodecamer. *C*, as a control, 4 μg of HslU (*lane a*) and HslV (*lane b*) by themselves were also analyzed, but in the absence of ATP. The *closed arrowheads* indicate the cross-linked intermediates of HslV: from *top* to *bottom*, hexamer, tetramer, trimer, and dimer, respectively.

ATP-bound HslU/C287V and HslV oligomers.

Effect of NEM on the ATPase Activity of HslU and Its Oligomerization—We have previously shown that pretreatment of NEM blocks not only the ATPase activity of HslU but also its ability to support the HslV-mediated proteolysis, and we therefore suggested that hydrolysis of both ATP and peptide bonds occur in a tightly linked process (27). This result is contradictory to the present findings that the HslV-HslU/C287V complex can hydrolyze protein and peptide substrates without ATP hydrolysis and to the previous findings that nonhydrolyzable ATP analogs can support the proteolysis by HslVU (33, 34). In order to clarify the discrepancy, we first compared the effect of NEM on the ATPase activity of HslU/C261V with that of the wild-type HslU. The HslU proteins were treated with 1 mM NEM for 30 min at 4 °C. The resulting samples were incubated with 3 mM dithiothreitol to inactivate unreacted NEM and then assayed for ATP hydrolysis in the presence and absence of HslV. Table I (Experiment I) shows that both the basal and HslV-stimulated ATPase activities of the wild-type HslU were strongly inhibited by NEM to a similar extent (*i.e.*, by about 80%) in accord with our previous report (29). In contrast, the same NEM treatment resulted in less than 15% reduction of the ATPase activities of HslU/C261V. Similar data were obtained when assayed for the hydrolysis of insulin B-chain with and without the treatment of NEM (Table I, Experiment II). Furthermore, upon the NEM treatment, the ability of HslU/C287V to support the insulin B-chain degradation by HslV was inhibited to an extent similar to that seen with the wild-type HslU. Thus, it appears that, of the two Cys residues, Cys^{261} is the major site that is responsible for the NEM-mediated inhibition of ATP hydrolysis by HslU and for the HslU-activated proteolytic activity of HslV. On the other hand, Cys^{287} may be buried inside the HslU protein and therefore not be easily accessible to the sulfhydryl blocking reagent. However, we cannot exclude the possibility that Cys^{287} may also be covalently modified by NEM and that the modified HslU protein is still capable of hydrolyzing ATP, although this possibility is much less likely because HslU/C287V lacks the ATPase activity.

TABLE I

Effect of NEM on the ATPase activities of HsIU and HsIU/C261V and on their ability to support the proteolysis by HsIV

Aliquots (4 μg each) of HsIU, HsIU/C261V, and HsIU/C287V were incubated with 1 mM NEM for 1 h at 4 °C. After incubation, 3 mM dithiothreitol was added to the mixtures, and the mixtures were further incubated for 10 min at the same temperature. The samples were then assayed for ATP hydrolysis (Experiment I) and for degradation of ^{125}I-labeled insulin B-chain by incubation with 2 mM ATP and 1 μg of HsIV for the next 30 min at 37 °C. The activity seen with HsIU only was expressed as 100%, and the others are expressed relative to that value.

Proteins	Relative Activity	
	Without NEM	With NEM
	%	
Experiment I: for ATP hydrolysis		
HsIU	100	20
HsIU/C261V	94	82
HsIU plus HsIV	286	55
HsIU/C261V plus HsIV	275	239
Experiment II: for Hydrolysis of insulin B-chain		
HsIU plus HsIV	100	17
HsIU/C261V plus HsIV	104	91
HsIU/C287V plus HsIV	89	15

HslU is a hexamer or heptamer in the presence of ATP, but it behaves as monomer or dimer in its absence (27, 40–42). Because oligomerization of HslU is essential for its interaction with HslV and hence for supporting the proteolytic activity of HslV, it appeared that NEM may influence oligomerization of HslU rather than blocking its catalytic function. To test this possibility, the wild-type HslU and its mutant forms were incubated with NEM for 30 min at 4 °C and then subjected to gel filtration on a Superose-6 column. Upon NEM treatment, both HslU and HslU/C287V dissociated into monomeric forms even in the presence of ATP (Fig. 6, *A* and *C*). On the other hand, HslU/C261V remained as an oligomer whether or not NEM was treated (Fig. 6*B*). These results indicate that inhibitory effect of NEM on protein breakdown is mediated by dissociation of oligomeric HslU into monomers and therefore by blocking the formation of the HslVU complex. These results also confirm that NEM interacts with Cys^{261} and the C261V mutation prevents the binding of the reagent. However, it still remains uncertain whether Cys^{287} is not accessible to NEM or the NEM binding at this site has little influence on oligomerization of HslU.

Effect of the Cys Mutation on the Proteolytic Activity of HslV and Its Interaction with HslU—Because HslV contains a single Cys residue at the position 159, we also examined the effect of the Cys mutation on the catalytic function of HslV by substitution of Cys^{159} with Ala or Ser (see Fig. 1). The resulting proteins were purified to apparent homogeneity and are referred to as HslV/C159A and HslV/C159S, respectively (see Fig. 2). As shown in Fig. 7*A*, either the C159A or C159S mutation in HslV completely abolished the ATP-dependent proteolytic activity of the HslVU protease against ^{125}I-labeled insulin B-chain. HslV is a weak protease, which alone also can degrade the polypeptide, although to extents much less than those seen in the presence of HslU and ATP (27, 29). The same mutations also abolished the basal proteolytic activity of HslV (data not shown). Similar data were obtained when the same experiments were performed using Cbz-Gly-Gly-Leu-AMC as the substrate (data not shown). Thus, Cys^{159} appears to be critical for the catalytic function of HslV.

To determine whether the Cys mutations also influence the ability of HslV to stimulate the ATPase activity of HslU, ATP

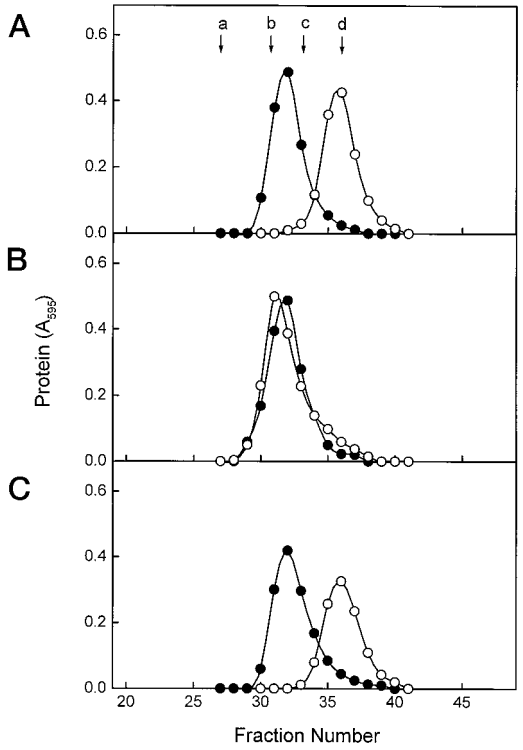

FIG. 6. **Effect of NEM on oligomerization of HslU and its mutant proteins.** The purified HslU (*A*), HslU/C261V (*B*), or HslU/C287V (*C*) (0.2 mg each) was incubated in the absence (●) and presence of 5 mM NEM (○) for 30 min at 4 °C. After incubation, 2 mM ATP was added, and the mixtures were further incubated for 10 min at 4 °C. Each of the samples was then chromatographed on a Superose-6 column (1 × 30 cm) that had been equilibrated with 20 mM Tris-HCl (pH 7.8) buffer containing 1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ATP, and 10% glycerol. The size markers used were as follows: *a*, thyroglobulin (669 kDa); *b*, ferritin (440 kDa); *c*, aldolase (153 kDa); *d*, ovalbumin (45 kDa).

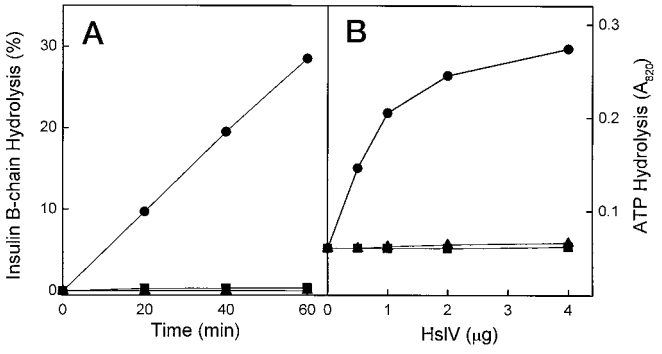

FIG. 7. **Effect of the C159S mutation on the ability of HslV in proteolysis and in activation of ATP hydrolysis by HslU.** *A*, degradation of ^{125}I-labeled insulin B-chain (10 μg) was assayed by incubating 1 μg of HslV (●), HslV/C159A (▲), or HslV/C159S (■), 4 μg of HslU, and 1 mM ATP for various periods at 37 °C. *B*, ATP hydrolysis was assayed by incubating 4 μg of HslU and 1 mM ATP in the presence of increasing amounts of HslV (●), HslV/C159A (▲), or HslV/C159S (■).

hydrolysis by HslU was assayed by incubation with increasing amounts of HslV/C159A or HslV/C159S. Unlike the wild-type HslV, both of the mutant proteins could not stimulate the ATPase activity of HslU (Fig. 7*B*), suggesting that they cannot interact with HslU. In order to clarify further whether Cys^{159} in HslV is required for the interaction between HslV and HslU and hence for the assembly of the HslVU complex, a cross-linking analysis was performed by incubation of HslV and its mutant forms with glutaraldehyde in the presence and absence of HslU (Fig. 8). Like the wild-type HslV, HslV/C159A alone

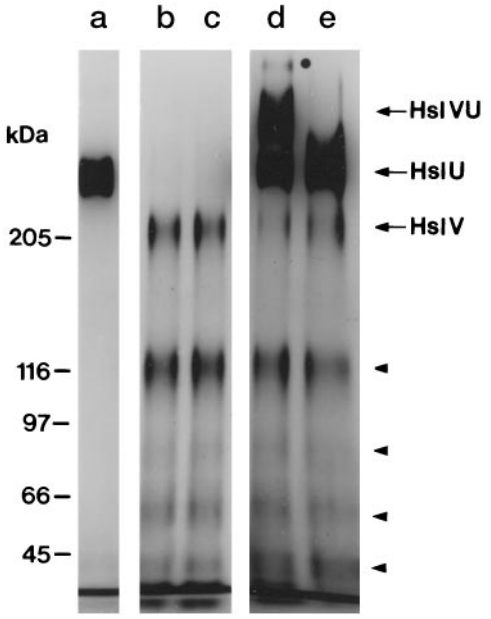

FIG. 8. **Cross-linking analysis for the assembly of the HslVU complex by HslV or HslV/C159S and HslU.** Aliquots (4 μg each) of HslV (*lanes b* and *d*) or HslV/C159S (*lanes c* and *e*) were incubated with 0.4% glutaraldehyde and 1 mM ATP for 20 min at 37 °C in the absence (*lanes b* and *c*) and presence (*lanes d* and *e*) of HslU. After incubation, the samples were electrophoresed under denaturing conditions followed by silver staining. As a control, HslU (4 μg) was also treated as above (*lane a*). The *arrowheads* indicate the cross-linked intermediates of HslV.

could form various cross-linked intermediates as well as dodecamers. However, it could not form the HslVU complex in the presence of HslU and ATP, unlike the wild-type HslV. Similar data were obtained with HslV/C159S (data not shown). These results indicate that Cys^{159} in HslV is essential for the interaction with HslU and hence for the formation of the HslVU complex.

Effects of NEM on Oligomerization of HslV—HslV is a dodecamer of 19-kDa subunits, consisting of two hexameric rings (27, 40–43). In order to determine whether NEM may also influence the oligomeric nature of HslV, the sulfhydryl blocking reagent was incubated with HslV or its mutant forms for 30 min at 4 °C. The samples were then subjected to gel filtration on a Sephacryl S-300 column. Upon NEM treatment, the wild-type HslV protein was eluted in the fractions corresponding to about 20 kDa (Fig. 9*A*), indicating that the NEM treatment results in complete dissociation of the dodecameric complex (about 250 kDa) into monomers. In addition, this NEM-treated, monomeric HslV showed little or no activity against insulin B-chain or Cbz-Gly-Gly-Leu-AMC whether or not HslU is present (data not shown). However, the HslV protein carrying either the C159A or the C159S mutation behaved as a dodecamer whether or not NEM was treated (Fig. 9*B*; data not shown), indicating that Cys^{159} is the site for modification by NEM. These results suggest that Cys^{159} is involved in oligomerization of HslV, and introduction of a bulky alkyl group to the residue prevents the formation of the HslV dodecamer.

Peptide Bond Specificity of HslVU Protease—In order to determine the cleavage specificity, insulin B-chain was incubated with the HslVU protease and ATP for 2 h. Upon reverse-phase HPLC of the reaction products on a C_{18} column, five peptide peaks were detected by absorbance at 214 nm (Fig. 10*A*). The cleavage sites on insulin B-chain were then determined by mass spectrometric analysis of each peptide peak. From the estimated molecular masses of the peptides and from the known amino acid sequence of insulin B-chain, the cleavage

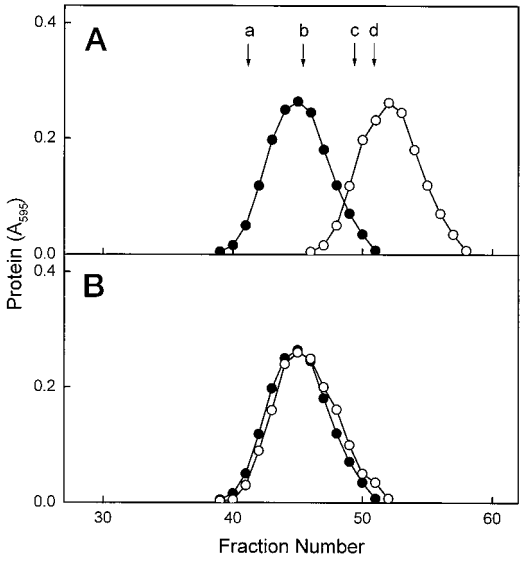

FIG. 9. **Effect of NEM on oligomerization of HslV and HslV/C159S.** Aliquots (50 μg each) of the purified HslV (*A*) and HslV/C159A (*B*) were incubated in the absence (●) and presence (○) of 5 mM NEM for 30 min at 4 °C. Each of the samples was then subjected to gel filtration on a Sephacryl S-300 column (1 × 47 cm) that had been equilibrated with 20 mM Tris-HCl (pH 7.8) buffer containing 1 mM EDTA, 5 mM $MgCl_2$, and 10%(w/v) glycerol. Proteins eluted from the column were assayed by the dye binding method (38). The size markers used were as follows: *a*, apoferritin (443 kDa); *b*, catalase (232 kDa); *c*, bovine serum albumin (66 kDa); *d*, carbonic anhydrase (29 kDa).

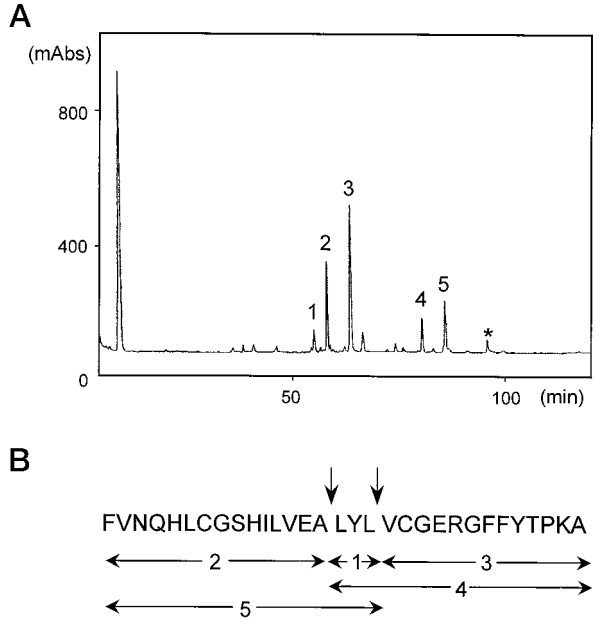

FIG. 10. **Determination of the cleavage sites in insulin B-chain.** Oxidized insulin B-chain (10 μg) was digested with the purified HslV (2 μg) and HslU (8 μg) for 2 h at 37 °C in the presence of 1 mM ATP. The cleavage products were then separated (*A*), and their amino acid sequences were determined (*B*) as described under "Experimental Procedures." The *asterisk* indicates the position at which intact insulin B-chain was eluted. The cleavage sites are indicated by the *vertical arrows*.

sites in the polypeptide were deduced. Fig. 10*B* summarizes the amino acid sequences of the peptides and the cleavage sites by the HslVU protease. These results indicate that the HslVU protease preferentially cleaves on the carboxyl side of certain hydrophobic amino acids in insulin B-chain (*i.e.* the peptide bonds of Leu^{7}-Val^{8}, Ala^{11}-Leu^{12}, Tyr^{14}-Leu^{15}, and Leu^{18}-Val^{19}). Nearly identical data were obtained when HslV was incubated with HslU/C287A and ATP or with the wild-type HslU and ATPγS (data not shown). Thus, ATP hydrolysis does not seem to influence the cleavage specificity of the HslVU protease.

DISCUSSION

The present studies have demonstrated that the Cys residues in both HslV and HslU play important roles in maintaining the structure and function of the HslVU protease. The single Cys residue at position 159 of HslV appears to be involved in oligomerization of its subunits, interaction with HslU for assembly of the HslVU protease, and the proteolytic function of the enzyme complex. Treatment of NEM resulted in complete dissociation of the dodecameric HslV into monomers. However, involvement of Cys^{159} in the HslV oligomerization seems to be indirect, because its substitution with Ala or Ser, which abolishes the NEM effect, did not interfere with the HslV oligomer formation. Perhaps the Cys residue is located close to the contact region of one HslV subunit to the contiguous subunit, and the introduction of the bulky alkyl group to the sulfhydryl residue upon NEM treatment may sterically interfere with the subunit-subunit interaction.

Despite the finding that the replacement of Cys^{159} with Ala or Ser shows no effect on the oligomeric nature of HslV, the HslV protein carrying either of the mutations was unable to stimulate the ATPase activity of HslU or to form the HslVU complex. Therefore, it appears that Cys^{159}, particularly its sulfhydryl group, plays an important role in the interaction with HslU. This Cys residue also appears to be important for the catalytic function of HslV, because the same mutation abolished both the basal and HslU-activated proteolysis by HslV. However, whether HslU and ATP are present or not, HslV was not sensitive to other inhibitors of the cysteine proteases, such as leupeptin or E64. Thus, the mechanism for involvement of Cys^{159} in the catalytic function of the HslVU protease remains unknown. Perhaps it is located close to the active site Thr residue of HslV and thereby provides an atmosphere that is appropriate for the catalytic function of the N-terminal Thr residue. In fact, it has recently been demonstrated by x-ray structural analysis that Cys^{159} in HslV is proximal to the Thr residue (43).

The two Cys residues in HslU appear to also play critical roles in maintaining the structure and function of the HslVU protease. Of these, Cys^{261} of HslU seems to be involved in oligomerization of its subunits, because treatment of NEM, even in the presence of ATP, resulted in complete dissociation of the hexameric or heptameric HslU into a monomer. Similar to the case of HslV, however, involvement of the Cys residue in the HslU oligomerization seems to be indirect, because its substitution with Val, which eliminates the NEM effect, did not interfere with the ATP-dependent oligomer formation.

Of particular interest is the finding that the HslU protein carrying the Cys^{287} to Val substitution is not capable of hydrolyzing ATP at all but still can support the proteolytic activity of HslV. Therefore, it seems clear that ATP binding but not its hydrolysis is essential for the proteolytic function of the HslVU protease, unlike that of other ATP-dependent proteases, including protease La and protease Ti. However, unlike the C287V mutation, the HslU mutant proteins carrying either Ser or Ala in place of Val, which we have also generated using the same mutagenic approach, could hydrolyze ATP and support the HslV-mediated proteolysis nearly as well as the wild-type HslU (data not shown). Therefore, the size and/or hydrophobicity of the side chain of amino acid 287 appears to be critical for the ATP-cleaving reaction by HslU. Possibly, introduction of the relatively bulky side chain of Val at position 287 may alter the geometry of ATP bound to the Walker A type ATP binding region, as evidenced by reduction in the affinity to ATP, and lead to impairment of ATP cleavage reaction by HslU.

What is the role of ATP hydrolysis by the HslVU protease? In the first place, ATP hydrolysis may be required for the chaperone function of HslU. This assumption is based on the fact that HslU is a member of HSP100/Clp family (44), most of which are known to also function as chaperones (45, 46). In addition, similar to ClpA and protease La, of which the protein-activated ATPase activities are essential for the chaperone activities (45, 47), HslU harbors the ATPase activity that can be activated by proteins (29). However, no evidence exists at present for the chaperone function of HslU.

The second possibility is that ATP hydrolysis by the HslVU protease may be required for unfolding of its protein substrates for channeling into the central cavity of HslV, where the Thr active site is located (43), as well as for exposing the scissile bonds, as is the case for protease Ti and the 26S proteasome (48–51). However, the HslVU protease has been shown to degrade insulin B-chain and casein even more rapidly in the presence of ATPγS, a nonhydrolyzable ATP analog, than with ATP (34). Moreover, the HslU mutant protein lacking the ATPase activity can support the proteolytic activity of HslV nearly as well as the wild-type HslU when ATP is present above 2 mM. Nevertheless, the possibility cannot be ruled out, because the polypeptide substrates used in this experiment are known to lack any appreciable secondary structure. In fact, protease La has been demonstrated to degrade the unfolded form of CcdA, but not its native form, without ATP hydrolysis (52).

Finally, ATP hydrolysis may play an important role in the recycling of the HslVU protease in the process of protein breakdown. It has previously been demonstrated that the ADP-bound form of HslU, although it can form an oligomer, is unable to interact with HslV to generate an active form of the HslVU protease complex (34). In addition, ADP has a much higher affinity for HslU than ATP. Therefore, after cleaving the scissile bonds in a protein substrate, ATP hydrolysis and subsequent generation ADP may promote the dissociation of the HslVU protease into the HslU and HslV oligomers so as to be available for the next round of proteolytic attack. In this possibility, protein substrates are assumed to alter the conformation of HslU in such a way to increase the affinity for ATP to replace the bound ADP. In fact, the affinity of ATP for protease La has been reported to increase upon binding of protein substrates (17). Therefore, when protein substrates are no longer available, HslU may remain as an ADP-bond form, which cannot interact with HslV. This manner may prevent excess or inappropriate degradation of normal cell proteins.

Acknowledgment—We are grateful to Dr. Dong Eun Park for helpful comments and discussion.

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