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Purification and Characterization of the Heat Shock Proteins HslV and HslU That Form a New ATP-dependent Protease in *Escherichia coli**

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Soon Ji Yoo‡, Jae Hong Seol‡, Dong Hun Shin‡, Markus Rohrwild§, Man-Sik Kang‡, Keiji Tanaka¶, Alfred L. Goldberg§, and Chin Ha Chung‡‖

From the ‡Department of Molecular Biology and Research Center for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea, the §Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, and ¶Institute for Enzyme Research, Tokushima University, Tokushima 770, Japan

The *hslVU* operon in *Escherichia coli* encodes two heat shock proteins, HslV, a 19-kDa protein homologous to β-type subunits of the 20 S proteasomes, and HslU, a 50-kDa protein related to the ATPase ClpX. We have recently shown that HslV and HslU can function together as a novel ATP-dependent protease, the HslVU protease. We have now purified both proteins to apparent homogeneity from extracts of *E. coli* carrying the *hslVU* operon on a multicopy plasmid. HslU by itself cleaved ATP, and pure HslV is a weak peptidase degrading certain hydrophobic peptides. HslU dramatically stimulated peptide hydrolysis by HslV when ATP is present. With a 1:4 molar ratio of HslV to HslU, approximately a 200-fold increase in peptide hydrolysis was observed. HslV stimulated the ATPase activity of HslU 2–4-fold, but had little influence on the affinity of HslU to ATP. The nonhydrolyzable ATP analog, β,γ-methylene-ATP, did not support peptide hydrolysis. Other nucleotides (CTP, dATP) that were slowly hydrolyzed by HslU allowed some peptide hydrolysis. Therefore, ATP cleavage appears essential for the HslV activity. Upon gel filtration on a Sephacryl S-300 column, HslV behaved as a 250-kDa oligomer (*i.e.* 12–14 subunits), and HslU behaved as a 100-kDa protein (*i.e.* a dimer) in the absence of ATP, but as a 450-kDa multimer (8–10 subunits) in its presence. Therefore ATP appears necessary for oligomerization of HslU. Thus the HslVU protease appears to be a two-component protease in which HslV harbors the peptidase activity, while HslU provides an essential ATPase activity.

One of the most intriguing feature of protein breakdown in eukaryotes as well as in prokaryotic cells is the requirement of metabolic energy (1, 2). In eukaryotes, the 26 S proteasome is the major ATP-dependent protease responsible for selective degradation of proteins with aberrant structures and many short-lived proteins important in metabolic regulation and cell cycle progression (3–6) and for generation of peptides present on major histocompatibility class I molecules (7–9). The 20 S proteasome, which consists of 14 different subunits of 21–32 kDa, forms the catalytic core of the 26 S proteasome (10, 11), and its activity is regulated by a 19 S regulatory component, which associates with the termini of the 20 S core (12) and has ATPase activity (13).

A number of ATP-dependent proteases have also been identified in *Escherichia coli* (1, 2, 14–18), including protease La (Lon), a heat shock protein consisting of four identical 87-kDa subunits. The ATP-dependent protease Ti (Clp) consists of two different multimeric components, both of which are required for proteolysis. Component A (ClpA), which is composed of 84-kDa subunits, contains ATP-hydrolyzing sites, while component P (ClpP), which is composed of 21-kDa subunits, is a serine protease. When isolated, ClpA shows protein-activated ATPase activity, which in the reconstituted ClpAP complex is linked to protein breakdown. ClpA is a member of a family of highly conserved polypeptides, present in both prokaryotic and eukaryotic organisms (19). This family of proteins is further divided into at least five subfamilies, ClpA, ClpB, ClpC, ClpX, and ClpY (20, 21). The ClpA/B/C family have two regions of particularly high homology, each of which contains a consensus sequence for ATP binding (22). ClpX and ClpY contain a single consensus ATP binding-site sequence, which is very similar to those found in the ClpA/B/C family of proteins (21). ClpX can combine with ClpP to form an additional type of ATP-dependent protease that is capable of degrading several regulatory proteins (21, 23–25). *clpX* is the second gene of the *clpPX* operon, which is under heat shock regulation (21).

Blattner and co-workers (26, 27) have recently identified 26 new heat shock genes in *E. coli*, termed *hsl* genes. Of these, the *hslVU* operon has been shown to specify proteins of 19 kDa (HslV) and 50 kDa (HslU). The primary sequence of HslV has been shown to be similar to that of certain β-type subunits of the 20 S proteasomes from eukaryotes, the archaebacterium *Thermoplasma acidophilum* (28), and certain bacteria. In particular, the N terminus of HslV contains two adjacent Thr residues, and the Thr residue at the N terminus of mature β-type subunits of the 20 S proteasome has been demonstrated to be crucial for the its proteolytic activity (29). On the other hand, HslU is very similar to a *Pasteurella haemolytica* protein, LapA of unknown function, which belongs to the ClpY subfamily (21, 27).

We have recently shown that HslV and HslU comprise a new type of ATP-dependent protease in *E. coli*, which degrades the fluorogenic peptide *N*-carbobenzoxy (Cbz)[1]-Gly-Gly-Leu-7-amido-4-methyl coumarin (AMC) in the presence of ATP (30). Furthermore, an antibody against HslV precipitates HslV together

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‖ To whom correspondence should be addressed. Tel.: 82-2-880-6693; Fax: 82-2-872-1993.

[1] The abbreviations used are: Cbz, carbobenzoxy; Suc, succinyl; AMC, 7-amido-4-methyl coumarin; DTT, dithiothreitol.

with HslU from crude extracts. Thus these proteins probably function as a proteolytic complex *in vivo*, although additional factors may also be required for protease activity. In order to characterize further the enzymological properties of HslV and HslU, we cloned the *hslVU* operon and purified each of its products from an *E. coli* strain that contains the operon on a multicopy plasmid. We report here the purification of HslV and HslU proteins and the reconstitution of ATP-dependent peptidase activity. These studies clearly demonstrate that HslU is an ATPase essential for peptide hydrolysis by the purified HslV, and that HslV influences the ATPase function of HslU.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotide primers were synthesized using an automated DNA synthesizer (Applied Biosystem, model 384A) and purified by gel electrophoresis as recommended by the manufacturer. All reagents for the polymerase chain reaction, including *Taq* polymerase, were purchased from Boehringer Mannheim. Peptide substrates were obtained from BACHEM Feinchemikalien AG, Switzerland, and Peptide Institute Inc., Japan. All other reagents were purchased from Sigma, unless otherwise indicated.

Cloning of hslVU—The *hslVU* operon was cloned by the polymerase chain reaction amplification method. Nucleotide sequences of the primers used for the amplification were based on the data of Blattner and co-workers (27): 5′-CTGCATTCGGCTCGCCGCCGGG-3′ (*i.e.* 120 bp upstream of the translational start site of *hslV*) and 5′-GCAGCAAAGGCGAGGGGGAGGG-3′ (146 base pairs downstream of the translational termination site of *hslU*). Reaction mixtures for the polymerase chain reaction amplification (0.1 ml) contained 0.5 μg of the *E. coli* K12 genomic DNA, 0.5 unit of *Taq* polymerase, 1 × polymerase chain reaction buffer, 0.2 mM each of deoxynucleotide triphosphates, and 50 pmol of the primers. The reactions were carried out for 30 cycles using a DNA Thermal Cycler (Perkin-Elmer) and generated a 2.2-kilobase pair DNA fragment. The resulting fragments were then ligated into pGEM-T vector (Promega).

Preparation of Crude Extracts—The *E. coli* XL2-Blue cells (Stratagene) harboring pGEM-T/HslVU were grown in Luria broth at 37 °C overnight and kept frozen at −70 °C until use. The frozen cells (about 40 g) were thawed and resuspended in 50 ml of 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 6.5) containing 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), and 10% (v/v) glycerol (referred to as buffer A). The cells were then disrupted with a French press at 14,000 p.s.i. and centrifuged at 100,000 × *g* for 3 h. The supernatants were dialyzed against the same buffer and referred to as crude extracts.

Immunoblot Analysis—*E. coli* were grown in Luria broth until an A_{600} of 0.7. The cells were lysed in 2% SDS and subjected to electrophoresis on 13% (w/v) polyacrylamide slab gels containing SDS and 2-mercaptoethanol (31). Proteins were transferred onto nitrocellulose membranes and incubated with antiserum raised against the purified HslV or HslU. The membranes were then stained with alkaline phosphatase conjugated to anti-rabbit IgGs (32). The antisera against the Hsl proteins were prepared by injecting each protein into albino rabbits.

Assays—Peptide hydrolysis was assayed as described previously (33) using Cbz-Gly-Gly-Leu-AMC as the substrate. Reaction mixtures (0.1 ml) contained the peptide (0.1 mM) and appropriate amounts of the purified HslV and HslU or HslV alone in 0.1 M Tris-HCl, pH 8, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 1 mM EDTA. Incubations were performed for 10–40 min at 37 °C and stopped by adding 0.1 ml of 1% (w/v) SDS and 0.8 ml of 0.1 M sodium borate, pH 9.1. The release of AMC were then measured. ATP hydrolysis was assayed by incubating the similar reaction mixtures at 37 °C. After incubation, 0.1 ml of 1% SDS were added to the samples, and the phosphate released was determined as described elsewhere (34). Proteins were assayed by their absorbance at 280 nm or by the method of Bradford (35) using bovine albumin as a standard.

RESULTS

Expression of HslV and HslU—*E. coli* cells harboring pGEM-T/HslVU were grown overnight, lysed in 2% SDS, and electrophoresed on 13% (w/v) polyacrylamide slab gels containing SDS and 2-mercaptoethanol. Two major bands representing 19-kDa (HslV) and 50-kDa (HslU) polypeptides were detected in cells carrying the recombinant plasmid, but not in cells containing only the vector (Fig. 1*A*). Moreover, antibodies raised against the purified HslV and HslU (see below) strongly interacted

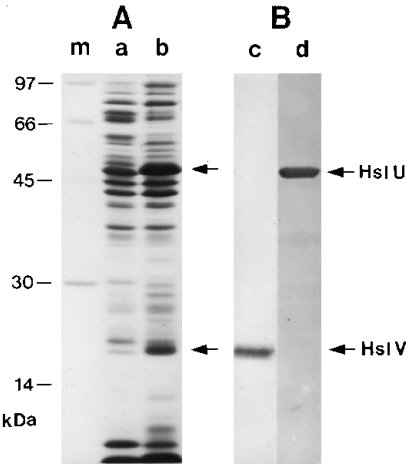

FIG. 1. **Expression of HslV and HslU.** *A*, the multicopy plasmid carrying the *hslVU* operon (pGEM-T/HslVU) (*lane b*) or the pGEM-T vector only (*lane a*) was transformed into *E. coli* XL2-Blue. The cells were grown to mid-log phase, lysed in 2% SDS, and electrophoresed in duplicate on 13% polyacrylamide slab gels containing SDS and 2-mercaptoethanol. Proteins in the gels were visualized by staining with Coomassie Blue R-250. *Lane m* shows the size markers. *B*, the same cell lysate for *lane b* was electrophoresed as above and subjected to immunoblot analysis using antiserum raised against the purified HslV (*lane c*) or HslU (*lane d*).

with these proteins in cell lysates (Fig. 1*B*), and therefore identified the two overproduced protein as HslV and HslU. Neither of these antibodies cross-reacted with purified protease La, or the ClpA or ClpP components of protease Ti (data not shown). These results confirm that both proteins are encoded from the *hslVU* operon and that they are cotranscribed as previously suggested (27).

Purification of HslV and HslU—To purify HslV and HslU, the crude extracts (3.5 g) from 40 g of the *E. coli* cells harboring pGEM-T/HslVU were loaded onto a phosphocellulose column (2.5 × 15 cm) equilibrated with buffer A. After collecting the flow-through fraction, the column was washed extensively with buffer A, and the proteins bound to the column were eluted with the same buffer containing 0.4 M phosphate. Since extracts of *E. coli* contain various unrelated peptidases and ATPases, HslV and HslU proteins were identified during purification by SDS-PAGE and staining with Coomassie Blue R-250. HslU was recovered in the flow-through and HslV in the 0.4 M phosphate eluate (data not shown).

For purification of HslV, the proteins eluted with 0.4 M phosphate were dialyzed against buffer A and loaded onto a hydroxylapatite column (1 × 8 cm) equilibrated with the same buffer. After washing the column, the bound proteins were eluted with a linear gradient of 0.05–0.5 M phosphate. The fractions containing the 19-kDa HslV protein were pooled, dialyzed against 20 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, and 10% glycerol (buffer B), and applied to a DEAE-Sepharose column (1.5 × 6 cm) equilibrated with the same buffer. Proteins bound to the column were eluted with a linear gradient of 0–0.3 M NaCl. The HslV-containing fractions were pooled, concentrated by ultrafiltration using a YM30 membrane (Amicon), and chromatographed on a Sephacryl S-300 column (2 × 80 cm) equilibrated with buffer B containing 0.1 M NaCl (Fig. 2*A*). Polyacrylamide gel electrophoresis of the column fractions reveals that HslV was purified to apparent homogeneity (Fig. 2*B*).

Recently, we have demonstrated that the HslV/HslU proteins could be coimmunoprecipitated by an antibody against HslV, and that these proteins hydrolyzed the fluorogenic peptide Cbz-Gly-Gly-Leu-AMC in an ATP-dependent fashion (30). Since the primary sequence of HslV is related to that of the

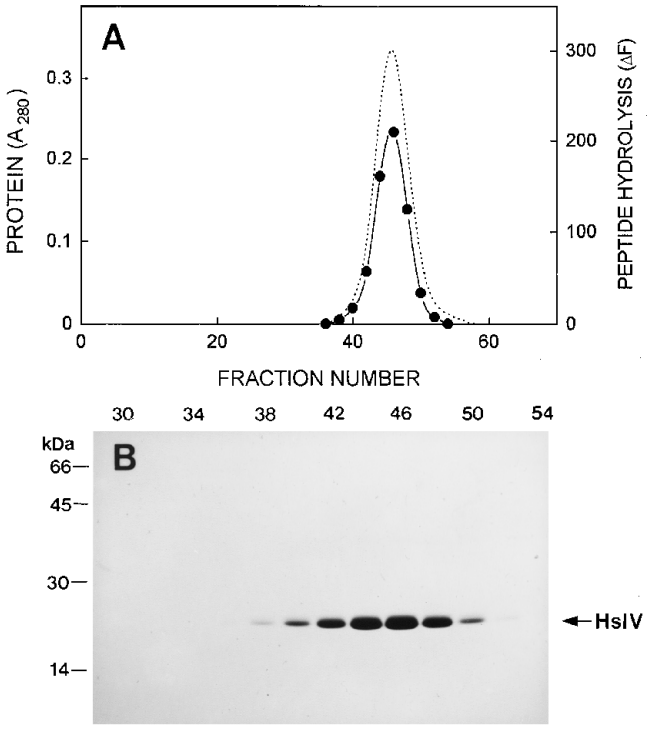

FIG. 2. **Elution of HslV from a Sephacryl S-300 column.** The proteins (18 mg) obtained from DEAE-Sepharose column were loaded on a Sephacryl S-300 column (2 × 80 cm) equilibrated with buffer B containing 0.1 M NaCl. Fractions of 2 ml were collected, and aliquots of them were assayed for the hydrolysis of Cbz-Gly-Gly-Leu-AMC (*A*) or electrophoresed as described in the legend to Fig. 1 (*B*). The *closed circles* indicate the peptide hydrolysis, and the *dotted line* shows the protein profile.

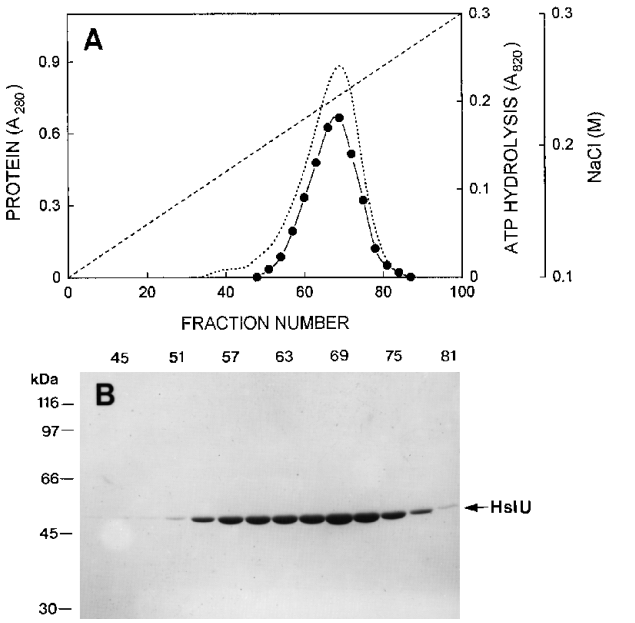

FIG. 3. **Elution of HslU from a DEAE-Sepharose column.** The proteins (93 mg) obtained from Sephacryl S-300 column were loaded on a DEAE-Sepharose column (1.5 × 10 cm) equilibrated with buffer B containing 0.1 M NaCl. After washing the column, proteins bound to the column were eluted with a linear gradient of 0.1–0.3 M NaCl. Fractions of 1 ml were collected, and aliquots were assayed for ATP hydrolysis (*A*) or electrophoresed on a 10% polyacrylamide slab gels containing SDS and 2-mercaptoethanol (*B*). The *closed circles* indicate the ATP hydrolysis, and the *dotted line* shows the protein profile.

catalytic β-type subunits of the 20 S proteasomes (27, 28), we examined whether purified HslV protein is capable of cleaving any peptide substrate of the 20 S proteasome. Purified HslV alone cleaved Cbz-Gly-Gly-Leu-AMC (Fig. 2*A*), although to a much lesser extent than in the presence of the purified HslU (see below). In addition, the extent of peptide hydrolysis was proportional to the protein content in each fraction from the Sephacryl S-300 column (Fig. 2*A*). The fractions containing HslV from the Sephacryl S-300 column were pooled and kept frozen at −70 °C.

For purification of HslU, the flow-through fraction from the initial phosphocellulose column was dialyzed against buffer B and loaded onto a DEAE-cellulose column (2 × 10 cm) equilibrated with the same buffer. After washing the column with buffer B containing 0.15 M NaCl, proteins were eluted with a linear gradient of 0.15–0.3 M NaCl. The fractions containing HslU were pooled, diluted 1:1 with buffer B, and applied to a heparin-agarose column (1.5 × 10 cm) equilibrated with buffer B. Proteins bound to the column were eluted with a linear gradient of 0.2–0.5 M NaCl. The HslU-containing fractions were pooled, concentrated by ultrafiltration using a YM30 membrane, and chromatographed on a Sephacryl S-300 column (2 × 80 cm) equilibrated with buffer-B containing 0.1 M NaCl. The fractions containing the 50-kDa HslU protein were pooled and applied to a DEAE-Sepharose column (1.5 × 10 cm) equilibrated with the same buffer. After washing the column, the bound proteins were eluted with a linear gradient of 0.1–0.3 M NaCl, and aliquots were electrophoresed as above (Fig. 3*A*). Polyacrylamide gel electrophoresis suggested that HslU was purified to apparent homogeneity (Fig. 3*B*). Since HslU contains an ATP-binding motif (27), we examined whether the purified protein exhibits ATPase activity. The extent of ATP hydrolysis was proportional to the HslU content in each fraction (Fig. 3*A*). The fractions containing HslU were pooled and kept frozen at −70 °C. From 40 g of *E. coli* carrying the *hslVU* operon on a multicopy plasmid (*i.e.* on the pGEM-T vector), we obtained approximately 8 mg of purified HslV and 60 mg of purified HslU. The proteins were stable for at least 1 month, when kept at −70 °C in the presence of 20% glycerol and 1 mM DTT. Storage at 4 °C in the absence of DTT or repeated freezing and thawing rapidly inactivated both proteins.

Oligomeric Nature of HslV and HslU—In order to determine the sizes of HslV and HslU, each of the purified proteins was subjected to gel filtration chromatography on a Sephacryl S-300 column (1 × 40 cm) equilibrated with buffer B containing 0.1 M NaCl. Since HslU interacts with ATP, chromatography was performed in the presence or absence of ATP. The purified HslV consistently ran as a 250-kDa complex whether or not ATP was present (Fig. 4*A*); thus, it appears to be a homopolymer consisting 12–14 subunits. In the absence of ATP, HslU behaved as a 100-kDa protein, which suggests a dimer of 50-kDa subunits, and with ATP it behaved as a 450-kDa protein, which suggests a multimer containing 8–10 subunits (Fig. 4*B*). These results indicate that ATP plays an important role in the oligomerization of HslU.

Mutual Activation of HslV and HslU—Since purified HslV by itself hydrolyzes Cbz-Gly-Gly-Leu-AMC, we first studied the effect of HslU on the peptidase activity of HslV. As shown in Fig. 5*A*, HslU dramatically stimulated peptide hydrolysis in the presence of ATP, but not in its absence. Moreover, the extent of this stimulation depended on the concentration of HslU (Fig. 5*B*). In the presence of 0.1 μg of HslV, the extent of peptide hydrolysis increased linearly with increasing the amount of HslU up to 0.8 μg (*i.e.* to an 1:8 mass ratio of HslV to HslU). This ratio is equivalent to a molar ratio of about 1:4, considering the molecular masses of HslV (250 kDa) and HslU (450 kDa) under nondenaturing conditions. Because approximately 50% of the added peptide was cleaved at the time of incubation, the substrate became limiting under these latter

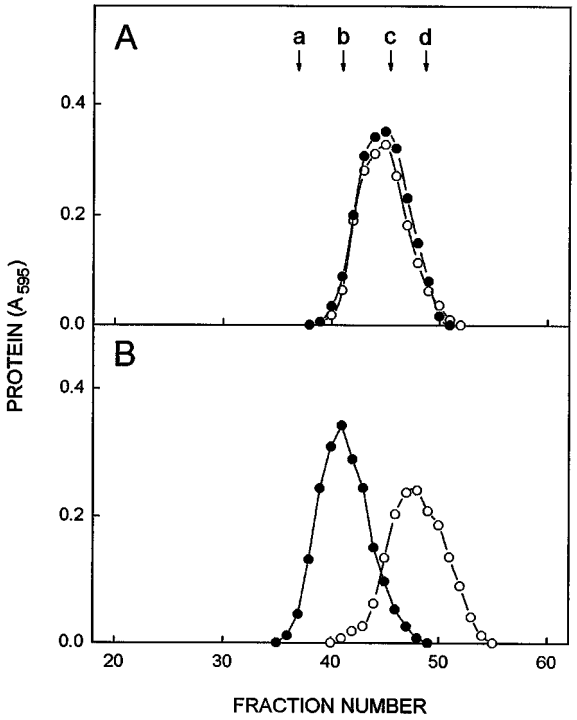

FIG. 4. **Estimation of the sizes of HslV and HslU by gel filtration chromatography on a Sephacryl S-300 column.** The purified HslV (*A*) or HslU (*B*) (0.2 mg each) was chromatographed on the gel filtration column (1 × 40 cm) equilibrated with buffer B containing 0.1 M NaCl in the presence (●) and absence (○) of 1 mM ATP. Fractions of 0.5 ml were collected at a flow rate of 10 ml/h. Aliquots of the fractions were then assayed for protein by the dye-binding method of Bradford (35). The *arrows* show the size markers: *lane a*, thyroglobulin (669 kDa); *lane b*, apoferritin (443 kDa); *lane c*, β-amylase (200 kDa); *lane d*, bovine serum albumin (66 kDa).

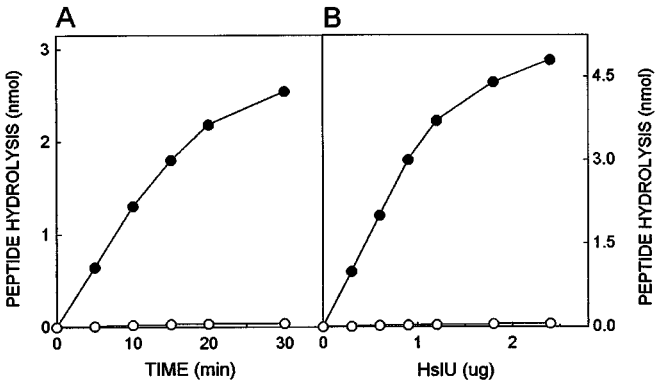

FIG. 5. **Effect of HslU on the hydrolysis of Cbz-Gly-Gly-Leu-AMC by HslV.** *A*, the peptide hydrolysis was assayed by incubating 0.1 μg of HslV, 0.1 mM Cbz-Gly-Gly-Leu-AMC, and 1 mM ATP in the absence (○) and presence (●) of 0.4 μg of HslU for various periods at 37 °C. *B*, the assays were also performed as above but by incubating 0.1 μg of HslV for 10 min in the presence of increasing amounts of HslU.

conditions. Increasing peptide concentration was impossible, since the peptide precipitates at concentrations above 0.1 mM. Thus we were not able to determine the K_m for the peptide or the optimal ratio of HslV and HslU for complex formation. Therefore further studies of the hydrolysis of ATP or the peptide were carried out in the presence of HslV and HslU at a molar ratio of 1:2 unless otherwise indicated, since the peptidase activity of HslV alone could not be detected when the amount of HslV was decreased any further.

We then examined the effect of HslV on the ATPase activity

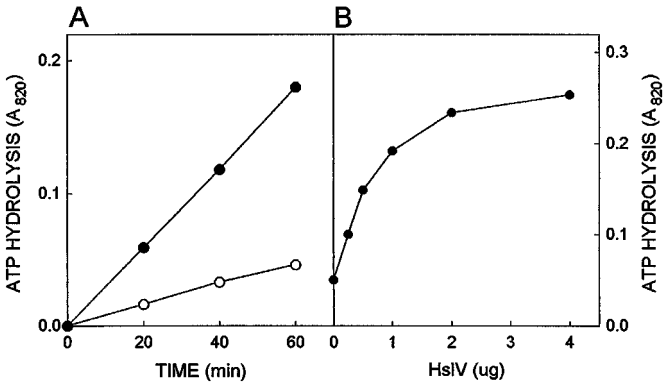

FIG. 6. **Effect of HslV on the ATPase activity of HslU.** *A*, ATP hydrolysis was assayed by incubating 1 μg of HslU and 1 mM ATP in the absence (○) and presence (●) of 1 μg of HslV for various periods at 37 °C. *B*, the assays were also performed as above but by incubating HslU for 1 h in the presence of increasing amounts of HslV.

of HslU. In the presence of HslV and HslU at a molar ratio of 1:4, HslV had little or no effect on the ATP hydrolysis by HslU (data not shown and see Fig. 6*B*). However, at a ratio of 2:1, HslV increased the rate of ATP hydrolysis about 3-fold (Fig. 6*A*). In addition, the stimulatory effect of HslV was dependent on its concentration (Fig. 6*B*). To define more precisely the stimulatory effect of HslV, the rate of ATP hydrolysis by HslU was determined in the presence of HslV and increasing concentrations of ATP, although the extent of activation of the peptide-degrading activity of HslV by HslU and ATP at the 2:1 ratio is far less than that at 1:4. Using double reciprocal plots of the data, the K_m for ATP was similar in the presence of HslV (0.28 mM) and in its absence (0.31 mM) (Fig. 7). However, the V_{max} for ATP hydrolysis by HslU was increased from 57 to 213 pmol/min upon addition of HslV. The peptide substrate of HslV, Cbz-Gly-Gly-Leu-AMC, had little or no effect on the ATPase activity of HslU, whether or not HslV was present (data not shown).

Hydrolysis of Various Peptides and Nucleotides—To determine the peptide bond-cleavage specificity of the reconstituted protease, a variety of fluorogenic peptides were tested for hydrolysis by incubation of HslV and HslU (the HslVU protease) in the presence of ATP. Cbz-Gly-Gly-Leu-AMC was cleaved by the HslVU protease at a rate of about 1550 pmol/min. *N*-Succinyl (Suc)-Leu-Leu-Val-Tyr-AMC, which is commonly used as a substrate for assaying the chymotrypsin-like activity of the 20 S proteasome, was also hydrolyzed by the HslVU protease but at a rate of about 60 pmol/min. ATP also stimulated the hydrolysis of the latter peptide but only about 3-fold. However, the HslVU protease showed little or no activity against other hydrophobic peptides, including Suc-Leu-Tyr-AMC, Suc-Ala-Ala-Phe-AMC or Suc-Gly-Gly-Phe-methoxynaphthylamide, or against basic or acidic peptides (data not shown) (30).

The effects of various nucleotides were compared to characterize further the ATPase activity of the reconstituted HslVU protease and to test whether only hydrolyzed nucleotide is essential to activate the enzyme's peptidase activity. As shown in Table I, only the nucleotides that were hydrolyzed could activate the hydrolysis of Cbz-Gly-Gly-Leu-AMC. dATP, CTP, and α,β-methylene ATP were hydrolyzed 15–35% as rapidly as ATP and supported peptide cleavage at 15–40% the rate of ATP. In contrast, GTP and UTP were not hydrolyzed and did not stimulate peptide hydrolysis. ADP and AMP were not substrates for HslU and did not activate the peptide cleavage by HslVU protease. Furthermore, a nonhydrolyzable ATP analog, β,γ-methylene ATP, did not support peptide hydrolysis. These results indicate that the HslVU protease requires ATP hydrol-

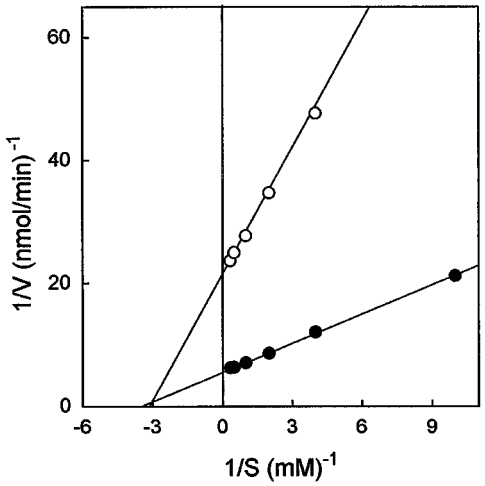

FIG. 7. **Determination of kinetic parameters for ATP hydrolysis by HslU in the presence and absence of HslV.** ATP hydrolysis was assayed by incubating 1 μg of HslU and increasing amounts of ATP in the absence (○) and presence (●) of 1 μg of HslV for 1 h at 37 °C.

TABLE I
Hydrolysis of various nucleotides and their ability to support the hydrolysis of Cbz-Gly-Gly-Leu-AMC by HslV/HslU

The hydrolysis of various nucleotides was assayed by incubating 0.5 μg of HslV and 2 μg of HslU in the presence of various nucleotides (1 mM). Incubations were performed for 1 h at 37 °C. The cleavage of Cbz-Gly-Gly-Leu-AMC was also assayed as above but one-fifth of the proteins and 0.1 mM peptide were incubated for 10 min instead of 1 h. The activities seen with ATP were expressed as 100% and the others were as its relative values.

Nucleotides	Relative activity for	
	Nucleotide hydrolysis	Peptide hydrolysis
	%	
ATP	100	100
dATP	33	36
ADP	4	2
AMP	1	0
Ap(CH_2)pp[a]	18	26
App(CH_2)p[b]	0	0
GTP	3	1
CTP	20	16
UTP	7	5

[a] Ap(CH_2)pp, α,β-methylene adenosine 5′-triphosphate.
[b] App(CH_2)p, β,γ-methylene adenosine 5′-triphosphate.

ysis by HslU for peptidase function and that the nucleotide hydrolysis correlate with peptide cleavage.

Effects of Various Protease Inhibitors—To characterize further the catalytic mechanism of the HslVU protease, the effects of a variety of site-specific reagents and protease inhibitors were examined. Preincubation of HslV with high concentrations of the serine protease inhibitors, phenylmethylsulfonyl fluoride (2 mM) or diisopropyl fluorophosphate (10 mM), inhibited the ATP-dependent hydrolysis of Cbz-Gly-Gly-Leu-AMC by about 70%. Preincubation of HslV with a sulfhydryl-blocking agent, *N*-ethylmaleimide, followed by inactivation of the inhibitor with DTT, also resulted in the inhibition of the peptide hydrolysis. HslV contains one cysteine residue; HslU has two (27). Therefore, we also studied the effect of *N*-ethylmaleimide on the ATPase activity of HslU and its ability to activate the peptidase function of HslV. As shown in Fig. 8, *N*-ethylmaleimide caused a concentration-dependent inhibition of both activities to a similar extent. None of the other inhibitors tested had any effect on the ATPase activity of HslU, whether or not HslV was present (data not shown). Thus, the free sulfhydryl residues in HslU, as well as in HslV, seem to play an important

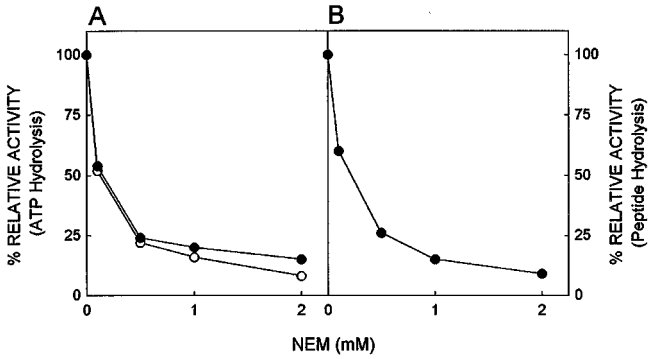

FIG. 8. **Effect of *N*-ethylmaleimide on the hydrolysis of ATP and Cbz-Gly-Gly-Leu-AMC.** *A*, for ATP hydrolysis, 2 μg of HslU were incubated with increasing amounts of *N*-ethylmaleimide for 5 min at 37 °C. After the incubation, the mixtures were added with 3 mM DTT and 1 mM ATP and further incubated for the next 1 h in the absence (○) and presence (●) of 0.5 μg of HslV. *B*, for peptide hydrolysis, incubations were performed as above, but for 10 min with one-fifth of the amounts of the Hsl proteins. The activities seen without *N*-ethylmaleimide were expressed as 100%, and the others were as their relative values.

role in the function of the protein and hence in the ATP-stimulated peptide hydrolysis.

DISCUSSION

The purpose of this work was to gain further insight into the subunit composition and mode of action of the recently discovered HslVU protease in *E. coli* (30). We have purified its two components, the heat shock proteins HslV and HslU, to apparent homogeneity and proven that HslU is an ATPase, while HslV by itself is a weak peptidase that degraded certain hydrophobic peptides, such as Cbz-Gly-Gly-Leu-AMC. HslU markedly activated peptide hydrolysis by HslV, while HslV increased the rate of ATP hydrolysis by HslU. Furthermore, ATP hydrolysis appears to be tightly coupled to the cleavage of the fluorogenic peptide, since these two processes were roughly proportional to one another with different nucleotides, and the nonhydrolyzable ATP analog, β,γ-methylene ATP did not support peptide cleavage. The *in vitro* reconstitution of ATP-dependent peptidase activity from purified HslV and HslU proteins clearly demonstrates that the HslVU protease is a new type of two-component protease.

It is noteworthy that the magnitude of the stimulation of peptide hydrolysis by ATP was dependent on the amount of HslV added (*i.e.* the molar ratio of HslU over HslV). For example, ATP stimulated peptide hydrolysis about 50-fold with HslV and HslU at a ratio of 1:1 and more than 200-fold at a ratio of 1:4. With the purified HslV and HslU, we unfortunately could not determine the stoichiometric ratio that may give a maximal, ATP-stimulated peptidase activity. These findings suggest that the affinity of HslV for HslU is rather low and that the active complex dissociates readily. Accordingly, no high molecular weight complex was formed when mixtures of purified HslV and HslU were incubated and subjected to gel filtration on a Sephacryl S-300 or Superose-6 column in the presence of ATP (data not shown). Direct evidence that HslV and HslU form a complex came from related experiments, where we were able to show that HslV and HslU can be co-immunoprecipitated from cell extracts, although these two components readily dissociate during purification (30).

ATP appears to play a distinct role in the function of the HslVU protease from that in protease Ti (ClpAP). We have previously shown that ClpP, which is the proteolytic component of protease Ti, reduces ATP hydrolysis by the enzyme's ATP-binding component, ClpA, in the absence of protein substrates, but stimulates its ATPase activity in their presence

(36). By contrast, the peptide substrates of HslV had little or no effect on the ATPase activity of HslU. However, protein substrates of HslV have not yet been identified, and they may also stimulate ATP hydrolysis as they do with ClpA, ClpB ATPase, and protease La. It is noteworthy that ATP hydrolysis is essential for activation of peptide hydrolysis by the HslVU protease, unlike protease Ti, which does not require either ATP binding or cleavage for hydrolysis of small fluorogenic peptide substrates (33). Therefore, the role of ATP in peptide hydrolysis by the HslVU protease is likely to be distinct from that of protease Ti.

Interestingly, purified HslU, which is dimeric in the absence of ATP, forms a multimer containing 8–10 subunits of 50 kDa when ATP is present. We have previously shown that the trimeric ClpA becomes a hexamer of 84-kDa subunits upon treatment of ATP (37). Therefore, ATP appears to play a similar role in oligomerization of the ATPase components of both bacterial ATP-dependent proteases, although the multimeric nature of ClpX, which is an alternative ATPase component of protease Ti, is not yet known. Unlike ClpA, which contains two ATP binding sites, HslU and ClpX have only one site for nucleotide binding. By mutational analysis of *clpA*, we have recently demonstrated that the first ATP-binding site is responsible for oligomerization, while the second site is essential for the ATPase function (38). Effects of mutations in the ATP-binding site of HslU on oligomerization and ATP hydrolysis are under investigation.

It has recently been shown, using electron microscopic studies, that ClpP consists of two layers of seven-membered rings (39). Our preliminary electron microscopic studies of fractions containing both HslV and HslU proteins revealed ring-shaped structure very similar to images of ClpP, ClpA, or the top view of the 20 S proteasome and larger structure which appear to be side images of two stacked rings (30). Furthermore, the primary sequence of HslV is similar to those of certain β-type subunits of the 20 S proteasome from eukaryotes and from the archaebacterium *T. acidophilum* (28). The N-terminal Thr residues of the mature β-type subunits serve as the catalytic site for proteolysis and are covalently linked to lactacystin (29), a *Streptomyces* metabolite. Like the β-type subunits of the 20 S proteasome, HslV has two such conserved N termini Thr residues (28). Accordingly, the peptidase activity of HslV in the presence of HslU and ATP is highly sensitive to peptide aldehyde inhibitors of the 20 S proteasome (40), such as Cbz-Ile-Glu-(*O-t*-butyl)-Ala-leucinal and Cbz-Leu-Leu-norvalinal, and to lactacystin, which specifically interacts with the N-terminal Thr residue of one subunit of the 20 S proteasome (29), although to a lesser extent (data not shown) (30). Supporting these findings, N-terminal sequencing of the purified HslV has revealed that the N-terminal Met is removed, and that the subsequent two Thr residues is not blocked (30). Thus, its hydroxyl group can presumably function as a nucleophile. Therefore we conclude that the HslVU protease is a novel type of protease in the eubacterium *E. coli*, and that its catalytic mechanism is likely to be similar to that of the mammalian and archaebacterial proteasomes, although HslVU does not contain α-type proteasome subunits. The sequence similarity of HslV to β-type proteasome subunits, and HslU to ClpX, and the fact that the HslVU protease is a two-component system, such as ClpAP and ClpXP, suggest that the properties of the HslVU protease are intermediate between the eukaryotic proteasomes and the Clp family of proteases.

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