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Biochemistry

HslV–HslU: A novel ATP-dependent protease complex in *Escherichia coli* related to the eukaryotic proteasome

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ABSTRACT **We have isolated a new type of ATP-dependent protease from *Escherichia coli*. It is the product of the heat-shock locus *hslVU* that encodes two proteins: HslV, a 19-kDa protein similar to proteasome β subunits, and HslU, a 50-kDa protein related to the ATPase ClpX. In the presence of ATP, the protease hydrolyzes rapidly the fluorogenic peptide Z-Gly-Gly-Leu-AMC and very slowly certain other chymotrypsin substrates. This activity increased 10-fold in *E. coli* expressing heat-shock proteins constitutively and 100-fold in cells expressing HslV and HslU from a high copy plasmid. Although HslV and HslU could be coimmunoprecipitated from cell extracts of both strains with an anti-HslV antibody, these two components were readily separated by various types of chromatography. ATP stimulated peptidase activity up to 150-fold, whereas other nucleoside triphosphates, a nonhydrolyzable ATP analog, ADP, or AMP had no effect. Peptidase activity was blocked by the anti-HslV antibody and by several types of inhibitors of the eukaryotic proteasome (a threonine protease) but not by inhibitors of other classes of proteases. Unlike eukaryotic proteasomes, the HslVU protease lacked tryptic-like and peptidyl-glutamyl-peptidase activities. Electron micrographs reveal ring-shaped particles similar to *en face* images of the 20S proteasome or the ClpAP protease. Thus, HslV and HslU appear to form a complex in which ATP hydrolysis by HslU is essential for peptide hydrolysis by the proteasome-like component HslV.**

Proteasomes are multicatalytic proteolytic complexes present in both the nucleus and cytosol of eukaryotic cells (1). The 26S form of the proteasome catalyzes the degradation of ubiquitin-conjugated proteins (2–5), and thus it plays a key role in many cellular processes, including progression through the cell cycle (6, 7), removal of abnormal proteins, and antigen presentation (8). The proteolytic core of the 26S complex is the 20S (700 kDa) proteasome particle, which consists of four seven-membered rings. The subunits of the 20S proteasome fall into two families (9, 10): the α-type forms the two outer rings, and β-type, which contain the active sites, forms the two inner rings of the complex.

Proteasomes were thought to exist exclusively in eukaryotes and certain archaebacteria (11). However, 20S proteasomes were recently discovered in the actinomycete *Rhodococcus* (12), and in the *Escherichia coli* genome sequencing project, a novel heat-shock locus (*hslVU*) was discovered that encodes a 19-kDa protein (HslV) (13), whose sequence is similar to β-type proteasome subunits. This discovery of proteasome-related genes was surprising, because several groups had failed to observe a structure in *E. coli* resembling the proteasome or proteins resembling ubiquitin. The *hslV* gene is cotranscribed with the adjacent *hslU* gene, which codes for a 50-kDa protein containing one ATP/GTP binding motif (13). Although both HslV and HslU have close homologs in other bacteria (14), HslU is also related to the ClpX ATPase in *E. coli* (about 50% identity) (13), which is essential for the degradation of certain proteins by the ClpP protease (15). These findings raise the possibility that HslV and HslU function together as an ATP-dependent protease.

In *E. coli*, heat-shock genes are transcribed by the RNA polymerase holoenzyme containing either of the alternative sigma factors, σ^{32} or σ^{24} (16, 17). One major group of proteins induced in *E. coli* during heat-shock are the molecular chaperones that catalyze the refolding of heat-damaged proteins; another group of up-regulated proteins are proteases that catalyze the selective degradation of such abnormal proteins (18). In *E. coli*, these proteases include the ATP-dependent proteases La (Lon) (19) and ClpP (Ti) (20) and the periplasmic protease HtrA (DegP) (21). The *hslVU* promoter perfectly matches the consensus of promoters recognized by σ^{32}, and the transcription of this operon increases on heat-shock (13). Therefore, it seemed likely that HslV and HslU may also be involved in the degradation of damaged polypeptides during heat-shock.

The aim of this study was to test whether *hslV* encodes a proteolytic enzyme similar to the eukaryotic proteasome and whether its function is regulated by the HslU ATPase. We demonstrate here a novel type of protease that requires ATP hydrolysis for peptidase function and contains both HslV and HslU proteins.

MATERIAL AND METHODS

Strains, Plasmids, Media, and Culture Conditions. Strain SG12051 (C600, *lon*510, *clp*P1::Cm^r), kindly provided by S. Gottesman (National Cancer Institute, Bethesda, MD), was transformed with plasmids pUHE211-1 (*rpo*H, Ap^r) and pDMI1 (*lac*I, Km^r) by electroporation to yield strain HSL32. BL21(DE3) cells (Stratagene) were transformed with plasmid pGEM-HSL (*hslVU*, Ap^r), which carries the *hslVU* operon under its own promoter (strain B405). The *hslVU* operon was amplified by PCR using genomic DNA form *E. coli* K12. The PCR fragment, containing the *hslVU* operon and an additional 120 bp upstream of the translational start of *hslV* and 146 bp downstream of the translational termination of *hslU*, was cloned into pGEM-T vector (Promega) (pGEM-HSL).

For the expression of glutathione *S*-transferase (GST)-fusion proteins, the *hslV* and *hslU* genes were PCR amplified separately using λ phage 18-126 DNA bearing the *hslVU* operon, kindly provided by F. Blattner (University of Wisconsin–Madison), and cloned into the vector pGEX-2T (Pharmacia). Vector pV106 (GST-HslV) and pU206 (GST-HslU) were electroporated into *E. coli* C600 cells. GST-fusion proteins were purified from strains C106 and C206 using the GST Purification Module (Pharmacia). To obtain antibodies, purified GST-HslV and GST-HslU proteins were injected into rabbits. Polyclonal anti-HslV and anti-HslU antibodies were then affinity purified using the GST-fusions as ligands, and depleted of anti-GST antibodies using a GST column.

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Abbreviations: hsl, heat-shock locus; GST, glutathione *S*-transferase.

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All strains were grown at 37°C in Luria–Bertani broth if not mentioned otherwise. When necessary, the medium was supplemented with ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), or kanamycin (25 μg/ml). For strains HSL32, C106, and C206, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM to induce expression of σ^{32} or the GST fusion proteins during exponential growth.

Immunoprecipitation. Cells were grown in minimal medium supplemented with essential amino acids, thiamine, and 0.5% glucose. At mid-log phase, 50 μCi (1 Ci = 37 GBq) per ml culture of TranS-Label (ICN) was added in the presence of 50 μM nonradiolabeled methionine for 2 hr. Cells were harvested and sonicated in 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, and 1 mM ATP (buffer A). After removal of debris, immunoprecipitation of the crude lysate was performed under nondenaturing conditions in buffer A plus 1% Nonidet P-40. Extracts were incubated with anti-HslV antibody for 1 hr on ice, followed by the addition of BSA-coated protein A-sepharose beads for an additional hour under gentle rocking at 4°C. The precipitates were then washed repeatedly at room temperature for 1 hr either in buffer A or buffer A plus 150 mM NaCl and 0.2% SDS. Finally, the beads were boiled in SDS/PAGE sample buffer and analyzed by 15% SDS/PAGE. Bands were detected by autoradiography.

Peptidase Assays. Enzyme samples were preincubated at room temperature for 30 min in 25 mM Tris (pH 7.6), 5 mM $MgCl_2$, and 1 mM DTT in the presence or absence of 1 mM ATP before addition of the peptide substrate. The peptidase reaction (100 μl) was initiated by the addition of the fluorogenic peptide (Bachem) to a concentration of 100-200 μM and incubated at 37°C. At different times, the reaction was terminated by the addition of 900 μl of 1% SDS, and the fluorescence of the reaction products was measured. Protein concentration of enzyme samples was determined by the method of Bradford (22).

Purification of HslV and HslU Proteins from Strain B405. Cells were grown in a 10 liter fermenter or large-scale shaker culture. All subsequent purification steps were performed at 4°C. Harvested cells (20–30 g) were washed with homogenization buffer (50 mM Tris/5 mM $MgCl_2$/1 mM DTT/1 mM ATP, pH 7.6), resuspended in 35 ml of the same buffer, and sonicated. The homogenate was centrifuged at 20,000 × *g* for 1 hr to remove debris, and the supernatant was ultracentrifuged at 100,000 × *g* for 1 hr. PEG 8000 was added to the supernatant (crude lysate) to a final concentration of 5%, and the mixture was incubated for 30 min with gentle rocking. After centrifugation at 25,000 × *g* for 15 min, the PEG pellet (10–12% of the total protein in the crude lysate) was resuspended in buffer B (10 mM potassium phosphate/5 mM $MgCl_2$/1 mM DTT/1 mM ATP/10% glycerol, pH 7.2) and centrifuged at 25,000 × *g* for 5 min to remove insoluble material. Protein (32 mg) was loaded onto a 5-ml CHT-II hydroxyapatite column (Bio-Rad) equilibrated with buffer B. Bound proteins were eluted using a 10-300 mM potassium phosphate linear gradient. Fractions of 0.5 ml were collected and assayed for proteolytic activity against Z-Gly-Gly-Leu-AMC in the presence of ATP. The active fractions, which eluted at approximately 160 mM, were pooled and concentrated by centrifugation in a Filtron 100. Concentrated sample (2 mg) was loaded onto a Superose 6 HR column (1 × 30 cm, Pharmacia) equilibrated with buffer C (25 mM Tris/5 mM $MgCl_2$/1 mM DTT/1 mM ATP/50 mM KCl/10% glycerol, pH 7.6). Fractions of 0.5 ml were collected and 1 μl aliquots of each fraction were added to a 100 μl peptidase assay to measure activity in the presence and absence of ATP. To determine ATP-independent activity samples were preincubated with apyrase (5 units per ml) to remove ATP before assaying peptidase activity

Electron Microscopy. Specimens for electron microscopy were prepared by diluting protein samples to a final concentration of 10 μg/ml in buffer C. After dilution, samples were preincubated for 30 min at room temperature, and 5-μl aliquots were placed on freshly glow-discharged carbon-coated grids for 1 min, after which excess buffer was blotted off with filter paper. Specimens were stained with 1% (wt/vol) uranyl acetate for 1 min and excess stain was blotted off. Grids were allowed to dry, and specimens were viewed in a JEOL 1200 EX or JEOL 100 CX electron microscope operating at 80 kV. Micrographs were recorded on Kodak Electron Microscopy Film 4489 emulsion at a nominal magnification of ×53,000.

RESULTS

HslV and HslU Form a Complex with Proteolytic Activity. To demonstrate the existence of the putative HslVU protease, we initially used *E. coli* strain SG12051, which lacks the two major ATP-dependent proteases La and ClpP. To stimulate the expression of the heat-shock proteins HslV and HslU at 37°C, σ^{32} was expressed in this strain (HSL32) from an inducible plasmid under the control of the *lac* promoter. Induction of σ^{32} with IPTG led to an approximately 10-fold increase in the content of HslV and HslU proteins (Fig. 1*A*, lanes 1 and 2). Crude lysates of this strain were assayed for activity against fluorogenic peptide substrates of the proteasome. The peptide Z-Gly-Gly-Leu-AMC was only hydrolyzed in the presence of ATP. This activity was about 10-fold greater after induction of σ^{32}, than in the parental strain not carrying the σ^{32} plasmid (Fig. 1*B*). Thus, HslV and HslU or possibly

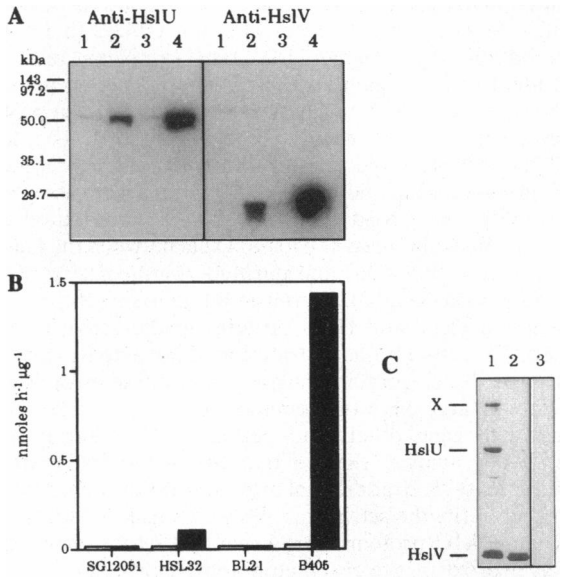

FIG. 1. (*A*) Western blot analysis of crude lysates of strains SG12051 (lanes 1), HSL32 (lanes 2), BL21 (lanes 3), and B405 (lanes 4) using anti-HslU and anti-HslV antibodies. Equal amounts of total protein per lane were subjected to 12% SDS/PAGE and transferred to nitrocellulose. Membranes were incubated with anti-HslV (1:5000) and anti-HslU (1:2500) antibodies followed by ^{125}I-labeled protein A, and bands were detected by autoradiography. Induction of σ^{32} overexpression increases protein levels of HslV and HslU about 10-fold (HSL32) and expression of HslV and HslU from a high copy plasmid (B405) increased protein levels of both proteins at least 100-fold, as determined by a PhosphorImager (Molecular Dynamics). (*B*) Peptidase activity of crude lysates of strains overexpressing σ^{32} or HslV and HslU. Increased ATP-dependent peptidase activity against Z-Gly-Gly-Leu-AMC correlates with increased protein levels of HslV and HslU (Fig. 1*A*). Solid bars represent ATP-dependent activity and open bars represent ATP-independent activity. Activity was defined as μmol of peptide substrate hydrolyzed by 1 mg of protein in crude lysates per hour. (*C*) Immunoprecipitation of HslV and HslU proteins from strain B405. HslU was coimmunoprecipitated in the presence of ATP using an anti-HslV antibody (lane 1) but not with preimmune serum under the same conditions (lane 3). A high salt wash (150 mM NaCl, 0.02% SDS) significantly reduced the amount of coimmunoprecipitated HslU (lane 2). X indicates an unidentified protein.

some other heat-shock protein, but no known ATP-dependent protease, appears responsible for this activity.

To prove that the increased activity is due to HslV/HslU and to simplify enzyme purification, we also studied strain B405, which carries the *hslVU* operon under its own promoter on a high copy plasmid (pGEM-HSL). In this strain, HslV and HslU levels were about 100-fold higher (Fig. 1*A*, lanes 3 and 4) and ATP-dependent peptidase activity in crude lysates was about 140-fold greater than in the parental BL21 cells that lacked the plasmid (Fig. 1*B*). Moreover, an antibody against HslV inhibited this proteolytic activity (see below).

To test whether HslV and HslU form a complex that is responsible for this activity, we used an affinity purified antibody against HslV. Even though this antibody did not cross-react with HslU (data not shown), it precipitated both HslV and HslU (identified with an affinity purified HslU antibody) from radiolabeled cell extracts of strain B405 (Fig. 1*C*, lane 1). Similar results were obtained with strain HSL32. Extensive washing of the immunoprecipitate with 150 mM NaCl and 0.02% SDS released HslU from the the HslV-antibody complex (Fig. 1*C*, lane 2). In certain experiments, an unidentified protein of 80-90 kDa was also coimmunoprecipitated (Fig. 1*C*, lane 1), suggesting that at least one additional protein may also associate with HslV or HslU. Together, these findings indicate that HslV and HslU form a complex that is responsible for the observed peptidase activity.

Isolation of the HslVU Protease. To isolate this protease, we initially subjected crude lysates of strain HSL32 to 5% PEG precipitation. Appreciable HslV/HslU antigens and ATP-dependent activity against Z-Gly-Gly-Leu-AMC were recovered in the precipitate, whereas over 80% of the cell proteins remained in the supernatant (data not shown). The resuspended pellet was subjected to gel filtration in the presence of ATP on Superose 6, and the eluted fractions were assayed for this activity. One broad peak was obtained that eluted at an apparent molecular mass of 650–700 kDa between the markers thyroglobulin (669 kDa) and purified *Thermoplasma* proteasome (700 kDa) (Fig. 2). Western blot analysis confirmed the presence of HslV and HslU proteins in the active fractions (Fig. 2). The rather broad distribution of this activity and of the HslV and HslU proteins suggest that these proteins can associate in large, heterogeneous complexes either with themselves, with each other, and perhaps with other proteins. SDS/PAGE analysis showed that the active fractions contained at least 15-20 additional proteins (not shown). Attempts to further purify the active complex were unsuccessful because HslV and HslU proteins were separated by ion-exchange as well as hydroxyapatite chromatography. This behavior is similar to that of protease ClpAP or ClpXP, whose two loosely associated components (ClpA or ClpX, and ClpP) are readily separated by such procedures (23).

Efforts to isolate the active protease were more successful using strain B405 carrying HslV and HslU on a high copy plasmid. Crude cell extracts were subjected to 5% PEG precipitation, and the resuspended pellet was loaded on a hydroxyapatite column. One large peak of peptidase activity was obtained, and the active fractions were concentrated and subjected to gel filtration chromatography on Superose 6. A sharp peak of peptidase activity was found that contained only two major proteins on SDS/PAGE. These bands were confirmed to be HslV and HslU by Western blot analysis (Fig. 3). Most of the HslV and HslU proteins eluted broadly in two distinct but overlapping peaks, and ATP-dependent peptidase activity was detected mainly in those fractions containing large amounts of both HslV and HslU. In these fractions, ATP stimulated Z-Gly-Gly-Leu-AMC hydrolysis up to 150-fold (Fig. 3). HslV was clearly necessary for this proteolytic activity because it could be inhibited by the anti-HslV antibody in a concentration-dependent manner. However, the antibody

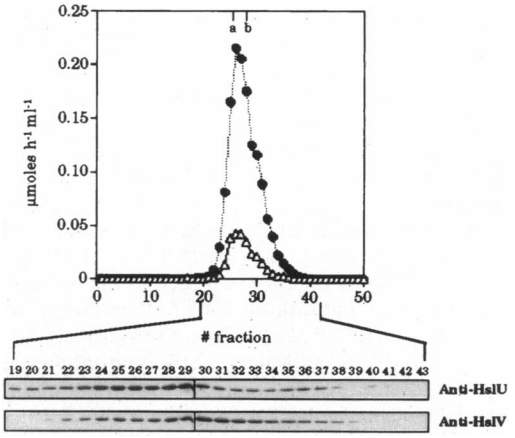

FIG. 2. Isolation of HslVU protease from strain HSL32. Harvested cells were homogenated in 50 mM triethanolamine (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 10% glycerol, and sonicated. Crude lysates were subjected to 5% PEG precipitation and the pellet was resuspended in the buffer A. Sample (2 mg) was loaded onto a Superose 6 HR column (Pharmacia) equilibrated with 25 mM Tris (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP. Markers: a, thyroglobulin (669 kDa); b, *Thermoplasma* proteasome (700 kDa). Fractions were assayed for peptidase activity in the presence (●) and absence of ATP (△). Activity was defined as μmol of peptide substrate hydrolyzed by 1 ml of fraction per hour. Note that samples were not preincubated in the presence of apyrase, which leads to an overestimation of ATP-independent peptidase activity against Z-Gly-Gly-Leu-AMC. Active fractions (20 μl) were analyzed by 12% SDS/PAGE and transferred to nitrocellulose. Membranes were incubated with anti-HslV (1:5000) and anti-HslU (1:2500) antibodies followed by alkaline phosphatase-conjugated anti-rabbit-IgG (Promega, 1:7500) and developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

against HslU (or against ClpP or protease La) or preimmune serum had no inhibitory effect (Table 1).

On gel filtration in the presence of ATP (Fig. 3), both HslV and HslU appear to form homopolymers. Although the HslV monomer is 19 kDa, the fractions with the highest HslV content (fractions 30 and 31) eluted at a molecular weight of ≈280-320 kDa, which suggests a complex of 14–16 subunits (perhaps two rings with seven subunits). The HslU monomer is 50 kDa, but in the presence of ATP, the fraction with the highest HslU content (fraction 29) eluted at an apparent molecular mass of 480-520 kDa, which suggests a complex of at least 10 subunits.

Properties of the HslVU Protease. Using the Superose fractions, which had the highest ATP-dependent peptidase activity (Fig. 3), we studied the ability of different nucleotides to support proteolysis. As was found with crude lysates from strain HSL32, the purified HslVU protease hydrolyzes the peptide Z-Gly-Gly-Leu-AMC only in the presence of ATP. CTP, GTP, UTP, ADP, AMP, and the nonhydrolyzable ATP analog AMP-PNP did not support peptidase activity (Table 2). Thus, this activity appeared to require ATP-hydrolysis. Interestingly, when added together with ATP at equimolar concentrations (1 mM), ADP caused a marked inhibition of the activation by ATP, but AMP had no such effect.

To define the nature of the proteolytic mechanism, we studied the effects of various protease inhibitors on Z-Gly-Gly-Leu-AMC hydrolysis. Several types of inhibitors that block the mammalian proteasome inhibited the HslVU protease, including the peptide aldehydes Z-Leu-Leu-norleucinal (MG132) and acetyl-Leu-Leu-norleucinal (calpain inhibitor I). This activity was also inhibited by 3,4-dichloroisocoumarin and, to a lesser extent, by lactacystin (Table 3). With longer incubation, the degree of inhibition by lactacystin increased, reaching 35% after 45 min (data not shown). In mammalian or archaebacterial proteasomes, these agents modify the N-

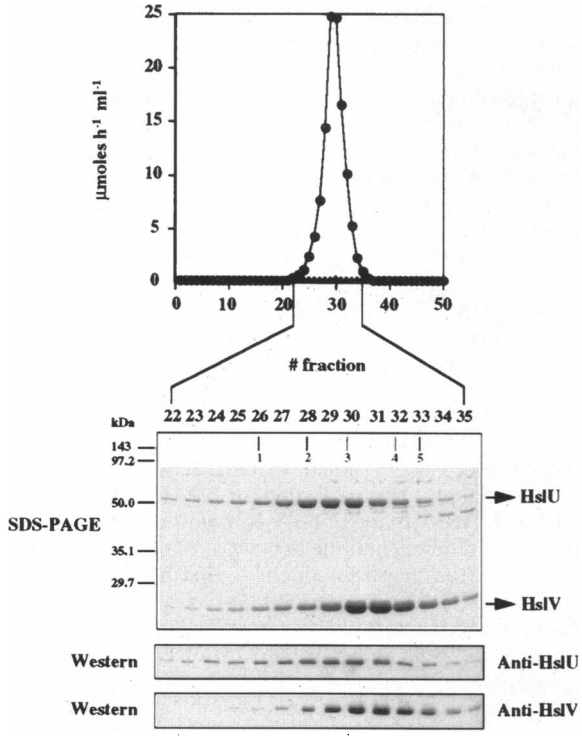

FIG. 3. Purification of HslV and HslU using strain B405. Peptidase activity of Superose 6 fractions against Z-Gly-Gly-Leu-AMC in the presence (●) and absence of ATP (△). Markers: 1, thyroglobulin (669 kDa); 2, *Thermoplasma* proteasome (700 kDa); 3, apoferritin (443 kDa); 4, β-amylase (200 kDa); 5, alcohol dehydrogenase (150 kDa). Active fractions (4 μl) were analyzed by 12% SDS/PAGE and Western blotting.

terminal threonine. Therefore, we tested whether the N terminus of HslV is a threonine. N-terminal sequencing of the purified protein using a ProSequencer 6600 (MilliGen) demonstrated that the N-terminal methionine was missing and that the protein starts with a threonine (data not shown). Therefore, the threonine hydroxyl could serve as a nucleophile and be accessible for modification by 3,4-dichloroisocoumarin and lactacystin. *N*-ethylmaleimide also caused a significant inhibition of this activity, which suggests that a sulfhydryl group is essential for the peptidase or ATPase activity. By contrast, a variety of inhibitors of metalloproteases (bestatin, *O*-phenanthroline) and serine or sulfhydryl proteases (chymostatin, leupeptin, E-64) had no effect and phenylmethylsulfonyl fluoride showed little inhibition. Similar data were obtained using crude lysates and fractions obtained after gel filtration from strain HSL32 (data not shown).

Several observations suggest that the HslVU protease is a new ATP-dependent protease. The activity was found in strain HSL32, which lacks ClpP and protease La, and thus it was not due to contaminations by these proteases. Moreover, protease La did also not elute close to the peak of peptidase activity (Fig. 3) isolated from strain B405 as judged by Western blot analysis (data not shown). Furthermore, purified protease La is not able to hydrolyze Z-Gly-Gly-Leu-AMC, and agents that inhibit protease La were not able to inhibit the HslVU protease (data not shown). Similarly, purified ClpAP is not able to hydrolyze Z-Gly-Gly-Leu-AMC in an ATP-dependent manner (data not shown), and the HslVU protease does not hydrolyze Suc-Leu-Tyr-AMC, which is the preferred peptide substrate of the ClpAP protease (24).

Although the HslVU protease degraded Z-Gly-Gly-Leu-AMC rapidly, it hydrolyzed Suc-Ala-Ala-Phe-AMC and Suc-Leu-Leu-Val-Tyr-AMC very slowly, which are rapidly hydrolyzed by chymotrypsin and proteasomes from *Thermoplasma* (11, 25) or *Rhodococcus* (12) (Table 4). In addition, ATP activated Z-Gly-Gly-Leu-AMC hydrolysis up to 150-fold and stimulated cleavage of Suc-Ala-Ala-Phe-AMC and Suc-Leu-Leu-Val-Tyr-AMC only 3- to 5-fold (data not shown). No cleavage of

Table 1. Inhibition of ATP-dependent peptidase activity by anti-HslV antibodies

Antibody	Dilution	% remaining activity
No antibody		100
Anti-HslV	1:1000	55
	1:500	16
	1:100	2
Anti-HslU	1:1000	80
	1:500	92
	1:100	92
Preimmune serum	1:100	106
Anti-ClpP	1:100	114
Anti-Lon	1:100	120

An enzyme sample, 0.4 μg of Superose fraction no. 30 (Fig. 3), was incubated 30 min at room temperature in the presence of ATP and different antibodies. After preincubation, Z-Gly-Gly-Leu-AMC was added to a final concentration of 200 μM and the mixture was incubated at 37°C for 15 min before reaction was stopped and fluorescence was measured. For affinity-purified anti-HslV and anti-HslU antibodies, 1:100 is 24 μg/ml final concentration. For other polyclonal antisera, 1:100 is 0.6 mg/ml.

Table 2. Nucleotide requirement for the hydrolysis of Z-Gly-Gly-Leu-AMC by the HslVU protease

Addition	Concentration	% activity
ATP	1 mM	100
CTP	1 mM	2
GTP	1 mM	2
UTP	1 mM	3
AMP	1 mM	5
AMP + ATP	1 mM (each)	85
ADP	1 mM	2
ADP + ATP	1 mM (each)	5
AMP-PNP	1 mM	4
None		3
Apyrase	5 units per ml	0.7
EDTA	10 mM	0.2

An enzyme sample, 0.4 μg of Superose fraction no. 30 (Fig. 3), was incubated 30 min at room temperature in the presence of ATP, CTP, GTP, UTP, AMP, ADP, AMP-PNP, apyrase, or EDTA before addition of peptide substrate. The mixture was incubated with 200 μM peptide at 37°C for 15 min before reaction was stopped and fluorescence was measured.

Table 3. Inhibitors of the ATP-dependent peptidase activity of the HslVU protease

Inhibitor	Concentration	% inhibition
Z-Leu-Leu-norleucinal (MG132)	10 μM	97
acetyl-Leu-Leu-norleucinal	10 μM	90
3,4-Dichloroisocoumarin	200 μM	50
Lactacystin	100 μM	24
N-ethylmaleimide	100 μM	62
Bestatin	10 μM	0
Chymostatin	100 μM	0
E 64	1 mM	0
Leupeptin	10 μM	0
O-phenanthroline	100 μM	0
Phenylmethylsulfonyl fluoride	1 mM	10

An enzyme sample, 0.4 μg of Superose fraction no. 30 (Fig. 3), was preincubated 30 min at room temperature in the presence of various inhibitors and ATP before adding Z-Gly-Gly-Leu-AMC to a final concentration of 200 μM. The mixture was incubated 15 min at 37°C before the reaction was stopped, and fluorescence was measured.

Table 4. ATP-dependent hydrolysis of synthetic peptide substrates by the HslVU protease

Substrate	μmol $h^{-1} \cdot mg^{-1}$
Hydrophobic substrates	
Z-Gly-Gly-Leu-AMC	76.6
Suc-Ala-Ala-Phe-AMC	0.6
Suc-Leu-Leu-Val-Tyr-AMC	0.2
Bz-Arg-Gly-Phe-Phe-Leu-MNA	0
Glt-Ala-Ala-Ala-MNA	0
Suc-Phe-Leu-Phe-βNA	0
Suc-Leu-Tyr-AMC	0
Z-Gly-Pro-AMC	0
Acidic substrates	
Z-Leu-Leu-Glu-MNA	0
Ac-Tyr-Val-Ala-Asp-AMC	0
Basic substrates	
Boc-Leu-Arg-Arg-AMC	0
Boc-Gly-Lys-Arg-AMC	0
Bz-Phe-Val-Arg-AMC	0
Z-Gly-Gly-Arg-AMC	0
Z-Arg-AMC	0

Superose fraction no. 30 (Fig. 3) was tested for its ability to hydrolyze various peptide substrates. Activity was defined as μmoles peptide substrate hydrolyzed by 1 mg of enzyme per hour. AMC, 7-amido-4-methylcoumarin; Boc, *t*-butyloxycarbonyl; Bz, benzoyl; Glt, glutaryl; MNA, 4-methoxy-β-naphthylamide; βNA, β-naphthylamide; Suc, succinyl; Z, benzyloxycarbonyl.

various acidic or basic peptide substrates, which are cleaved by mammalian proteasomes, could be detected (Table 4).

After negative staining, electron micrographs of active fractions containing only HslV and HslU revealed a homogeneous population of ring-shaped particles with an approximate diameter of 11 nm (Fig. 4). The particles appear to have 6- to 7-fold rotational symmetry and appear very similar to top views of the 20S proteasome (10) and of ClpP and ClpA (26). In addition to the ring-shaped particles, occasional larger structures were evident which could be side images of two rings stacked along the axis of symmetry. These structures may represent side views of HslV or HslU and multiple stacked rings may represent the HslVU complex.

DISCUSSION

The new ATP-dependent protease from *E. coli* is related to the proteasome β-subunits based on sequence similarities with HslV and its apparent mode of action. Interestingly, the HslVU protease does not contain subunits similar to the proteasome α-type subunits. Although no additional component was required for activity, we cannot rule out definitely the existence of proteins resembling α-subunits in *E. coli*. *hslV* and *hslU* genes are also found in *Haemophilus influenzae* (27), and the recent sequencing of its complete genome made it possible for us to search for such α-type sequences. No such gene was found in *H. influenzae*. In archaebacteria and yeast, α-subunits are necessary for the formation of the β-rings, and they appear to limit the access of cytosolic proteins to the proteolytic sites on the β-subunits (28). Presumably, HslV forms such rings spontaneously or these functions of the α-rings are provided by HslU.

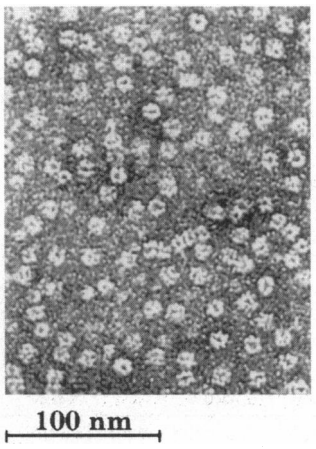

FIG. 4. Electron micrographs of the HslVU protease. Samples containing HslV and HslU were negatively stained with uranyl acetate. (Bar = 100 nm.)

DNA sequences similar to *hslV* and also to β-type proteasome genes have been reported in *Pasteurella haemolytica* and *Bacillus subtilis* (14), as well as in *H. influenzae* (27). From their DNA sequences, all these HslV proteins contain, following the initiating methionine, a conserved N-terminal threonine, whose hydroxyl group could serve as the active-site nucleophile in a proteolytic mechanism similar to that of the eukaryotic and archaebacterial proteasome β-subunits (29–31). We have shown that the initial methionine of HslV is removed *in vivo* exposing a N-terminal threonine, and the activity of the HslVU protease is inhibited by the peptide aldehyde inhibitors Z-Leu-Leu-norleucinal (MG132) and acetyl-Leu-Leu-norleucinal (calpain inhibitor 1), which are reversible transition-state inhibitors of the proteasome, as well as serine and cysteine proteases. In the *Thermoplasma* proteasome, the N-terminal threonine was found to form a hemiacetal intermediate with the aldehyde residue of the acetyl-Leu-Leu-norleucinal (29). The peptidase activity of HslVU was also inhibited by 3,4-dichloroisocoumarin and, to a lesser extent, by lactacystin. This inhibition by lactacystin increased with time, which suggests a covalent reaction between the inhibitor and enzyme. Lactacystin has been shown to react selectively with the N-terminal threonine of subunit X of the mammalian proteasome, but not to inhibit serine or cysteine proteases (30). Similarly, 3,4-dichloroisocoumarin becomes linked to the N-terminal threonine of β-type subunits of the *Thermoplasma* proteasome in a time-dependent fashion (T. Akopian and A.L.G., unpublished work). By contrast, phenylmethylsulfonyl fluoride, an inhibitor of serine proteases, had little effect on HslVU activity, and E64, a potent inhibitor of cysteine proteases, or inhibitors of metalloproteases, such as *O*-phenanthroline or bestatin, had no effect. Like other β-type proteasome sequences, HslV contains a Gly-Ser-Gly motif. However, the Ser in this motif does not act as a nucleophile in the catalytic mechanism, because its elimination by site-directed mutagenesis did not affect the proteolytic activity in *Thermoplasma* (31). Because this is the only highly conserved Ser in the HslV sequence, it seems unlikely that HslV is a serine protease. Taken together, these data indicate that the HslVU protease, like other proteasomes, has a threonine-dependent catalytic mechanism.

In related studies, we have demonstrated that the HslVU complex hydrolyzes ATP at significant rates. This function must be provided by HslU, because purified GST-HslU (used to obtain anti-HslU antibodies) by itself exhibits ATPase activity (unpublished results). In principle, *N*-ethylmaleimide may inhibit the ATP-dependent peptidase activity by reacting with the sulfhydryl group on the one cysteine in HslV or the two cysteines in HslU. We have found that the purified ATPase HslU is sensitive to *N*-ethylmaleimide (unpublished results) as are other ATPases, such as p97 (32).

ATP hydrolysis seems to play a different role in the function of HslVU than in other known ATP-dependent proteases. Unlike HslVU, protease La requires ATP binding and not hydrolysis for peptidase activity, i.e., nonhydrolyzable ATP analogs fully support peptide hydrolysis by protease La (33). For ClpP, neither ATP binding nor hydrolysis is essential for the cleavage of small peptides, but ATP hydrolysis allows degradation of larger peptides or proteins (34). Our data indicate that under the present conditions, ATP hydrolysis is an absolute requirement for the peptidase function of the HslVU protease.

Possibly, ATP consumption is required for formation of the active enzyme or is linked to peptide hydrolysis, or for both processes, as is true of protease ClpAP and the 26S proteasome. However, more rigorous analysis is required to define more precisely the nucleotide requirement of this new enzyme in the presence of different types of substrates.

One other interesting feature of the HslVU protease is that ADP is an inhibitor of peptide hydrolysis, and at equivalent concentrations, shows a dominant inhibitory effect over ATP. ADP (but not AMP) is also a potent inhibitor of protease La (35), which *in vivo* is maintained in an inactive state with an ADP bound to each subunit, until the binding of a protein substrate allosterically releases the ADP and activates proteolysis (36). Future studies should clarify whether ADP might act in a similar fashion to control the HslVU protease.

The coimmunoprecipitation of HslV and HslU from crude cell extracts using an anti-HslV antibody indicates that they associate with each other *in vivo*, although this association appears to be weak even in the presence of ATP. Both subunits appear necessary for activity and, in related studies, we have been able to reconstitute this ATP-dependent peptidase activity from pure HslV and HslU proteins (unpublished results). HslV and HslU are readily separated by various chromatographic methods, as is the ClpAP protease complex, which also dissociates rapidly into its components (ClpA and ClpP) (23). Similarly, ClpP and ClpX cannot be purified as a complex. Interestingly, we were not able to isolate a high molecular weight form (>700 kDa) of the activity from the strain expressing HslV and HslU on the high copy plasmid (B405), as we could from strain HSL32 that overexpresses σ^{32}. Possibly, some additional factor or some modification of HslV or HslU promotes the formation or stability of the active complex.

In *E. coli*, *B. subtilis*, and *H. influenzae*, the *hslV* gene occurs in an operon with a *hsl*U-related sequence. The *hslU* gene is highly conserved among these various gram positive and gram negative bacteria, and it is also related to *clp*X. Unlike ClpA HslU has only one ATP-binding site and shows no obvious sequence similarity to the ATPases of the eukaryotic 26S proteasome, which are related to the ATP-dependent metalloprotease FtsH in *E. coli* (37). The structural organization of the *E. coli* ClpAP protease and the 26S proteasome appear similar in several respects and may function in a similar ATP-dependent fashion (26). However, ClpAP protease does not appear to have been an evolutionary precursor of the 26S proteasome because ClpP lacks any sequence similarity to the β-type proteasome subunits and, unlike HslV, is a serine protease (38).

Gel filtration data and electron micrographs suggest that the structure of the HslVU protease is similar to that of the ClpAP protease, in which ClpP is composed of two rings of seven subunits that are flanked by complexes of ClpA consisting of rings of six subunits (26). The limited electron microscopic findings and gel filtration data would be consistent with HslV forming two seven-membered rings, similar to the β-type proteasome subunits or to ClpP, and with HslU forming a large complex of at least 10 subunits. Because HslV is related to β-type proteasome subunits, and HslU is an ATPase similar to ClpX, the HslVU protease thus appears to exhibit properties intermediate between those of the ClpAP family of proteases and the 26S proteasome. Earlier studies of the simple 20S proteasome in *Thermoplasma acidophilum* have greatly contributed to our knowledge about the structure and mechanism of the eukaryotic proteasome. Similarly, careful study of the mechanisms of this novel bacterial protease complex should serve as a useful model system to further elucidate how ATPases may regulate the 20S proteasome within the 26S complex and to expand our understanding of the evolution of the proteasome.

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1. Peters, J. M. (1994) *Trends Biochem. Sci.* **19,** 377–382.
2. Waxman, L., Fagan, J. & Goldberg, A. L. (1987) *J. Biol. Chem.* **262,** 2451–2457.
3. Hough, R., Pratt, G. & Rechsteiner, M. (1987) *J. Biol. Chem.* **262,** 8303–8313.
4. Ganoth, O., Leshinsky, E., Eytan, E. & Hershko, A. (1988) *J. Biol. Chem.* **263,** 12412–12419.
5. Hershko, A. & Ciechanover, A. (1992) *Annu. Rev. Biochem.* **61,** 761–807.
6. Glotzer, M., Murray, A. W. & Kirschner, M. (1991) *Nature (London)* **349,** 132–138.
7. Ghislain, M., Udvardy, A. & Mann, C. (1993) *Nature (London)* **366,** 358–362.
8. Gaczynska, M., Rock, K. L. & Goldberg, A. L. (1993) *Enzyme Protein* **47,** 354–369.
9. Tanahashi, N., Tsurumi, C., Tamura, T. & Tanaka, K. (1993) *Enzyme Protein* **47,** 241–251.
10. Lupas, A., Koster, A. J. & Baumeister, W. (1993) *Enzyme Protein* **47,** 252–273.
11. Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl, R. & Baumeister, W. (1989) *FEBS Lett.* **251,** 125–131.
12. Tamura, T., Nagy, I., Lupas, A., Lottspeich, F., Cejka, Z., Schoofs, G., Tanaka, K., De Mot, R. & Baumeister, W. (1995) *Curr. Biol.* **5,** 766–774.
13. Shuang-En, C., Burland, V., Plunkett, G., Daniels, D. L. & Blattner, F. R. (1993) *Gene* **134,** 1–6.
14. Lupas, A., Zwickl, P. & Baumeister, W. (1994) *Trends Biochem. Sci.* **19,** 533–4.
15. Wojtkowiak, D., Georgopoulos, C. & Zylicz, M. (1993) *J. Biol. Chem.* **268,** 22609–22617.
16. Grossman, A. D., Erickson, J. W. & Gross, C. A. (1984) *Cell* **38,** 383–390.
17. Raina, S., Missiakas, D. & Georgopoulos, C. (1995) *EMBO J.* **14,** 1043–1055.
18. Goldberg, A. (1992) *Eur. J. Biochem.* **203,** 9–23.
19. Goff, S., Casson, L. & Goldberg, A. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6647–6651.
20. Kroh, H. & Simon, L. (1990) *J. Bacteriol.* **172,** 6026–6034.
21. Lipinska, B., Zylicz, M. & Georgopoulos, C. (1990) *J. Bacteriol.* **172,** 1791–1797.
22. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
23. Maurizi, M. R., Thompson, M. W., Singh, S. K. & Kim, S.-H. (1994) *Methods Enzymol.* **244,** 314–331.
24. Woo, K. M., Chung, W. J., Ha, B. D., Goldberg, A. L. & Chung, C. H. (1989) *J. Biol. Chem.* **264,** 2088–2091.
25. Dahlmann, B., Kuehn, L., Grziwa, A., Zwickl, P. & Baumeister, W. (1992) *Eur. J. Biochem.* **208,** 789–797.
26. Kessel, M., Maurizi, M. R., Kim, B., Kocsis, E., Trus, B. L., Singh, S. K. & Steven, A. C. (1995) *J. Mol. Biol.* **250,** 587–594.
27. Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., *et al.* (1995) *Science* **269,** 496–511.
28. Zwickl, P., Kleinz, J. & Baumeister, W. (1994) *Nat. Struct. Biol.* **1,** 765–770.
29. Löwe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W. & Huber, R. (1995) *Science* **268,** 533–539.
30. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J. & Schreiber, S. L. (1995) *Science* **268,** 726–731.
31. Seemüller, E., Lupas, A., Stock, D., Löwe, J., Huber, R. & Baumeister, W. (1995) *Science* **268,** 579–582.
32. Peters, J. M., Walsh, M. J. & Franke, W. W. (1990) *EMBO J.* **9,** 1757–1767.
33. Goldberg, A. L. & Waxman, L. (1984) *J. Biol. Chem.* **260,** 12029–12034.
34. Hwang, B. J., Woo, K. M., Goldberg, A. L. & Chung, C. H. (1988) *J. Biol. Chem.* **263,** 8727–8734.
35. Menon, A. S. & Goldberg, A. L. (1987) *J. Biol. Chem.* **262,** 14921–14928.
36. Menon, A. S. & Goldberg, A. L. (1987) *J. Biol. Chem.* **262,** 14929–14934.
37. Confalonieri, F. & Duguet, M. (1995) *BioEssays* **17,** 636–650.
38. Maurizi, M. R., Clark, W. P., Kim, S. H. & Gottesman, S. (1990) *J. Biol. Chem.* **265,** 12546–12552.