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HslVU ATP-dependent Protease Utilizes Maximally Six among Twelve Threonine Active Sites during Proteolysis*[S]

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HslVU is a bacterial ATP-dependent protease distantly related to eukaryotic proteasomes consisting of hexameric HslU ATPase and dodecameric HslV protease. As a homolog of the 20 S proteasome β-subunits, HslV also uses the N-terminal threonine as the active site residue. However, unlike the proteasome that has only 6 active sites among the 14 β-subunits, HslV has 12 active sites that could potentially contribute to proteolytic activity. Here, by using a series of HslV dodecamers containing different numbers of active sites, we demonstrate that like the proteasome, HslV with only ~6 active sites is sufficient to support full catalytic activity. However, a further reduction of the number of active sites leads to a proportional decrease in activity. Using proteasome inhibitors, we also demonstrate that substrate-mediated stabilization of the HslV-HslU interaction remains unchanged until the number of the active sites is decreased to ~6 but is gradually compromised upon further reduction. These results with a mathematical model suggest HslVU utilizes no more than 6 active sites at any given time, presumably because of the action of HslU. These results also suggest that each ATP-bound HslU subunit activates one HslV subunit and that substrate bound to the HslV active site stimulates the HslU ATPase activity by stabilizing the HslV-HslU interaction. We propose this mechanism plays an important role in supporting complete degradation of substrates while preventing wasteful ATP hydrolysis in the resting state by controlling the interaction between HslV and HslU through the catalytic engagement of the proteolytic active sites.

ATP-dependent proteases are cellular machines that play essential roles in the controlled turnover of regulatory proteins and the clearance of damaged proteins. They harness chemical energy from ATP hydrolysis, converting it into mechanical force to unfold protein substrates and translocate them into a proteolytic chamber for degradation. These chambers of ATP-dependent proteases sequester proteolytic active sites from the cytosol, thus preventing uncontrolled access of cytosolic proteins to the active sites.

HslVU is one of the two-component ATP-dependent proteases in bacteria, consisting of HslV protease and HslU ATPase (1–3). HslV is a homolog of the β-subunit of 20 S proteasome. It forms a barrel-shaped dodecameric complex by stacking two hexameric rings of identical HslV subunits, and each of the HslV subunits contains an N-terminal Thr (Thr[1]) active site for proteolysis (4–8). The hexameric HslU ATPase, which belongs to the AAA+ family of ATPases, binds to either one or both ends of HslV dodecamer to form an HslVU complex (9–11). In the HslVU complex, the central pores of HslU and HslV are aligned, so that HslU transfers substrate polypeptide chains through the pores into the inner proteolytic chamber of HslV.

ATP binding and its subsequent hydrolysis by HslU are essential for unfolding protein substrates. It also plays essential roles in controlling the proteolytic function of HslV and the interaction between HslV and HslU (7, 12–15). HslV alone shows a very weak peptidase activity toward carbobenzoxy-Gly-Gly-Leu-7-amido-4-methyl coumarin (Z-GGL-AMC),[4] a small fluorogenic peptide substrate, but its activity increases 1–2 orders of magnitude when it binds to HslU in the presence of ATP (13, 14). Because ATPγS, a nonhydrolyzable ATP analog, stimulates the peptidase activity of HslVU even more dramatically, the activation of the HslV active sites by HslU requires ATP binding to HslU but not its hydrolysis. On the other hand, degradation of native protein substrates, such as SulA, strictly depends on ATP hydrolysis by HslU, because unfolding of proteins is necessary for their movement into and subsequent degradation in the inner proteolytic chamber of HslV (16). Chemical cross-linking analyses have shown that ATP-bound HslU interacts with HslV to form the HslVU complex, but ADP-bound HslU does not, suggesting a dynamic interaction between HslU and HslV during the ATP hydrolysis cycle (15). Therefore, an unresolved issue is how the HslVU complex is maintained during a complete proteolytic cycle of protein substrates, which would require multiple rounds of ATP hydrolysis.

Recently, we have shown that proteasome inhibitors, such as lactacystin or 4-hydroxy-5-iodo-3-nitrophenylacetyl-leucyl-leucyl-leucyl-vinylsulfone (NLVS) bind to HslV in the presence of HslU and ATP and that binding of these inhibitors to the Thr[1] residues dramatically increases the interaction between

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[4] The abbreviations used are: Z-GGL-AMC, carbobenzoxy-Gly-Gly-Leu-7-amido-4-methyl coumarin; ATPγS, adenosine 5′-*O*-(thiotriphosphate); NLVS, 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone; NTA, nitrilotriacetic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

HslV and HslU (17). Significantly, the stability of inhibitor-bound HslVU complexes is no longer influenced by the presence or absence of ATP. Assuming that the inhibitors mimic the binding of a substrate to the active site, these findings provide a mechanism for the maintenance of stable HslVU complexes when HslVU is engaged in substrate degradation. In addition, deletion of the Thr^1 residues (T1Δ) also causes a dramatic increase in the HslV-HslU interaction even in the absence of ATP (17). Thus, it is clear that the Thr^1 active sites are involved in the tight interaction of HslV with HslU, in addition to the catalytic role in peptide bond cleavage.

Although ATP-dependent protease machines typically contain multiple proteolytic active sites in their proteolytic chamber, little is known about how their active sites are coordinated and utilized during proteolysis. 20 S proteasomes from archaea and some bacteria generally have 14 active sites from two stacked heptameric rings of β-subunits. In contrast, eukaryotic 20 S proteasomes contain only 6 catalytically active subunits among the 14 β-subunits (18–20). Although HslV has 12 identical active sites, it is not clear whether all of 12 active sites are necessary for efficient substrate degradation or only some of them are sufficient. Previously, we were able to produce mixed HslV dodecamers containing both mutant (T1Δ) and wild-type subunits within the same complex in different ratios, and these dodecamers provided us a hint that only half of the HslV active sites might be sufficient for the full proteolytic activity (17). However, the evidence remains inconclusive because the T1Δ mutant subunit causes an unexpected, dramatic increase in the affinity between HslV and HslU, which could compensate for the loss of catalytic activity.

In the present study, we demonstrate that among the 12 Thr^1 residues in HslV, only ~6 of them participate in the hydrolysis of substrates as well as in the stable interaction between HslV and HslU. This conclusion is based on our findings that in the presence of HslU and ATP, increasing the inactive T1A subunits up to ~6 in a dodecamer causes little or no effect on the proteolytic activity of HslV toward all tested substrates and on proteasome inhibitor-mediated stabilization of the HslV-HslU interaction. However, a further increase gradually decreases the proteolytic activity and impairs the stable HslV-HslU interaction as well. This partial utilization of the active sites seems linked to the function of HslU; when a synthetic C-terminal peptide of HslU was used as an allosteric activator of HslV in place of the full-length HslU, the proteolytic activity of HslV declined linearly as the number of the T1A subunits was increased. Our data also suggest that each ATP-bound HslU subunit conformationally communicates with one HslV subunit, and catalytic engagement of the HslV active sites stabilizes the HslV-HslU interaction to support efficient degradation of substrates.

EXPERIMENTAL PROCEDURES

Materials—The enzymes necessary for DNA cloning were purchased from New England Biolabs, Stratagene, and Takara. Z-GGL-AMC was purchased from Bachem. Lactacystin and NLVS were obtained from Cayman Chemical and Calbiochem, respectively. Other reagents were purchased from Sigma, unless otherwise indicated.

Vectors for production of mixed dodecamers consisting of HslV and T1A subunits were constructed, as described previously, by sequential insertions of *hslV* genes (restriction fragments of pV-1 or pVH-1) into the poly linker site of pBR-PL (see Fig. 2). Thr^1-to-Ala mutation in the vectors was generated by site-directed mutagenesis (QuikChange; Stratagene). All of the mutations were confirmed by DNA sequencing.

Protein Expression and Purification—To express HslV mixed dodecamers, BW25113 Δ*hslVU*::*kan* cells (17) harboring appropriate vectors were grown overnight at 37 °C in Luria broth supplemented with ampicillin. The proteins were purified by using Ni^{2+}-nitrillotriacetic acid (NTA)-agarose columns as described (17). Purified proteins were dialyzed against 20 mM Tris-HCl buffer (pH 7.8) containing 100 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol and stored at −70 °C for further use. HslU, HslV, and maltose-binding protein-fused SulA were purified as described (3, 14). Protein concentration was measured by the Bradford method using bovine serum albumin as a standard.

NTA Pull-down Analysis—Reaction mixtures (0.5 ml) that have HslU (150 nM) and HslV-His (75 nM) in 50 mM HEPES buffer (pH 8) containing 150 mM NaCl, 5% glycerol, and 0.04% Triton X-100 were incubated at 4 °C for 1 h in the presence of 5 mM $MgCl_2$ and 2 mM ATP. After incubation, the mixtures were added with 10 μl of 1 M imidazole and 20 μl of NTA resins and rocked at 4 °C for 1 h. The resins were washed four times with 0.5 ml of 50 mM HEPES buffer (pH 8) containing 300 mM NaCl, 5 mM $MgCl_2$, 60 mM imidazole, 5% glycerol, 0.04% Triton X-100, and 2 mM ATP. Proteins bound to NTA resins were eluted by SDS sampling buffer, subjected to SDS-PAGE, and stained with Coomassie Blue R-250.

Assays—ATP hydrolysis was measured by using an enzyme-coupled assay (21). HslU (0.2 μM) and HslV (0.2 μM) in 100 mM Tris-HCl buffer (pH 8) containing 150 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, and 0.5 mM EDTA were incubated at 37 °C with 2 mM ATP, 3 mM phosphoenolpyruvate, 0.5 mM NADH, 20 units/ml of pyruvate kinase, and 20 units/ml of lactic dehydrogenase. Absorbance at 340 nm was continuously recorded using a spectrophotometer (Ultrospec2000; GE Healthcare) equipped with a temperature controller. The rate of ATP hydrolysis was calculated from a slope within a linear range, based on the extinction coefficient of NADH ($\epsilon_{340\ nm} = 6.22 \times 10^3$).

Peptide hydrolysis was assayed by incubation of HslU (10 nM) and HslV (5 nM) with 0.1 mM Z-GGL-AMC in 100 mM Tris-HCl buffer (pH 8) containing 5 mM $MgCl_2$, 0.5 mM EDTA, and 2 mM ATP at 37 °C. Fluorescence (λ_{ex} = 355 nm, λ_{em} = 460 nm) of released AMC was continuously measured with a fluorometer (FluoStar; BMG) equipped with a temperature controller. The rate of peptide hydrolysis was then calculated from the slope within a linear range.

The degradation of α-casein and Arc proteins was assayed by incubation of HslU (0.4 μM) and HslV (0.4 μM) at 37 °C for appropriate periods with 100 mM Tris-HCl buffer (pH 8) containing 5 mM $MgCl_2$, 2 mM ATP, 20 mM phosphocreatine, and 10 units/ml of creatine kinase. For assaying the degradation of SulA, maltose-binding protein-fused SulA (2 μM) was incubated with 10 units/ml of Factor Xa, HslU (0.4 μM), 2 mM ATP,

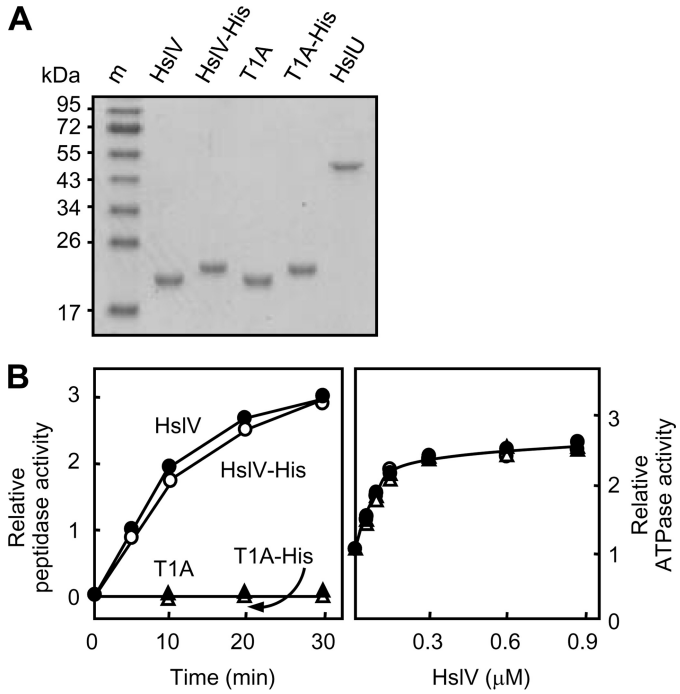

FIGURE 1. **Effect of Thr[1]-to-Ala mutation in HslV on peptide and ATP hydrolysis.** *A*, purified HslV, T1A, and their His_6-tagged proteins and HslU were subjected to SDS-PAGE on 12% slab gels followed by staining with Coomassie Blue R-250. Each lane contained 2 μg of protein. The *lane m* indicates size markers. *B*, peptide hydrolysis was assayed by the incubation of HslU (10 nM), 0.1 mM Z-GGL-AMC, and 2 mM ATP with 5 nM HslV (●), HslV-His (○), T1A (▲), or T1A-His (△) for various periods. The peptidase activity seen with HslV by incubation for 5 min was expressed as 1.0, and the others were as its relative values (*left panel*). ATP hydrolysis was assayed by incubating HslU (0.2 μM) and increasing amounts of HslV (●), HslV-His (○), T1A (▲), and T1A-His (△) proteins for 10 min. The ATPase activity seen with HslU alone was expressed as 1.0, and the others were expressed as its relative values (*right panel*).

20 mM phosphocreatine, and 10 units/ml of creatine kinase for 30 min at 37 °C and then with HslV (0.4 μM) for the next 1 h. The reactions were stopped by treatment with SDS sampling buffer. The samples were then subjected to SDS-PAGE followed by staining with Coomassie Blue R-250. For assaying the hydrolysis of Arc proteins, SDS-PAGE was performed using Tris-Tricine buffer and discontinuous 4, 10, and 16% slab gels to improve the resolution (22). For others, SDS-PAGE was routinely performed using 12% gels (23).

RESULTS

Effect of Thr[1]-to-Ala Mutation in HslV on Peptide and ATP Hydrolysis—To investigate the effects of gradual inactivation of the 12 Thr^1 active sites in an HslV dodecamer on peptide and ATP hydrolysis by HslVU, we first generated a catalytically inactive mutant of HslV (T1A) in which Thr^1 is replaced by Ala. We chose to mutate Thr^1 to alanine because this amino acid is the structurally closest to threonine but lacks the catalytically essential hydroxyl group. To facilitate the purification of T1A as well as wild-type HslV (shortly, HslV), a poly histidine tag (His_6) was attached to the C terminus, and the resulting proteins are referred to as T1A-His and HslV-His, respectively. HslV proteins (HslV, T1A, HslV-His, and T1A-His) were then purified to apparent homogeneity (Fig. 1*A*). In accord with our previous report (17), both HslV and HslV-His cleaved the Z-GGL-AMC at a similar rate in the presence of HslU and ATP (Fig. 1*B*, *left panel*). In addition, HslV-His could also degrade various protein substrates, such as α-casein, Arc repressor, and SulA, as well as HslV (data not shown), indicating that the C-terminal His tag does not interfere with the activity of HslV. On the other hand, as expected, neither T1A nor T1A-His was able to hydrolyze Z-GGL-AMC. However, both T1A and T1A-His could stimulate the basal ATPase activity of HslU to a similar extent as HslV (Fig. 1*B*, *right panel*). These results suggest that the T1A mutation mimics a proteolytically inactive state of HslV without perturbing intrinsic interactions between HslU and HslV.

Generation of Mixed Dodecamers Having Varied Numbers of T1A and HslV Subunits—We have recently developed a protocol that can produce mixed dodecamers having different numbers of T1Δ subunits in place of HslV subunits (17). By using the same approach, we generated mixed dodecamers consisting of increasing numbers of T1A subunits in place of HslV subunits (Fig. 2*A*). Briefly, this protocol utilizes the fact that the efficiency of translation initiation in *Escherichia coli* varies depending on the start codon of the gene, and the order of efficiency is ATG > GTG > TTG (24, 25). We constructed a series of vectors having multiple *hslV* and/or *t1A* genes with different combinations of the start codons to control the relative expression level of each subunit (Fig. 2, *B* and *C*). These constructs enable the co-expression of HslV and T1A subunits and their co-assembly into a dodecamer *in vivo* in a random configuration. We expressed these constructs in Δ*hslVU*::*kan* cells and purified mixed dodecamers. The average molar ratios between T1A-His and HslV subunits in purified dodecamers could be determined by densitometry on Coomassie-stained gels, because their motilities differ on SDS-PAGE. By this approach, we obtained six different mixed dodecamers containing increasing numbers (*i.e.* from ~2 to 10) of T1A subunits (Fig. 2*D*).

Effects of Increasing the Numbers of T1A Subunits in an HslV Dodecamer on Peptide Hydrolysis—To determine the effect of gradual replacement of HslV subunits by T1A subunits in an HslV dodecamer on peptide hydrolysis, each of the purified mixed dodecamers were incubated with Z-GGL-AMC in the presence of HslU and ATP. Peptide hydrolysis by mixed dodecamers increased linearly with the time, similar to that by HslV dodecamers (Fig. 3*A*). Remarkably, the rate of peptide hydrolysis by the mixed dodecamers having up to on average ~6 inactive T1A subunits (indicated by *trace a–c*) was nearly identical to that by HslV dodecamers (W_{12}). However, a further increase in the number of T1A-His subunits led to a proportional decrease in the rate of peptide hydrolysis, as revealed by plotting the observed rates of peptide hydrolysis against the average number of HslV subunits per dodecamer (Fig. 3*B*). These results suggest that approximately half of the 12 Thr^1 active sites of HslV are sufficient for exhibiting its full peptidase activity, but a further reduction in the number of Thr^1 residues leads to a decrease in peptide hydrolysis, presumably because of a lack of the active sites.

Recently, we have shown the deletion of Thr^1 caused a dramatic increase in affinity between HslV and HslU (17). Additionally, increasing the number of T1Δ subunits in a dodecamer led to a proportional increase in the affinity between HslV and

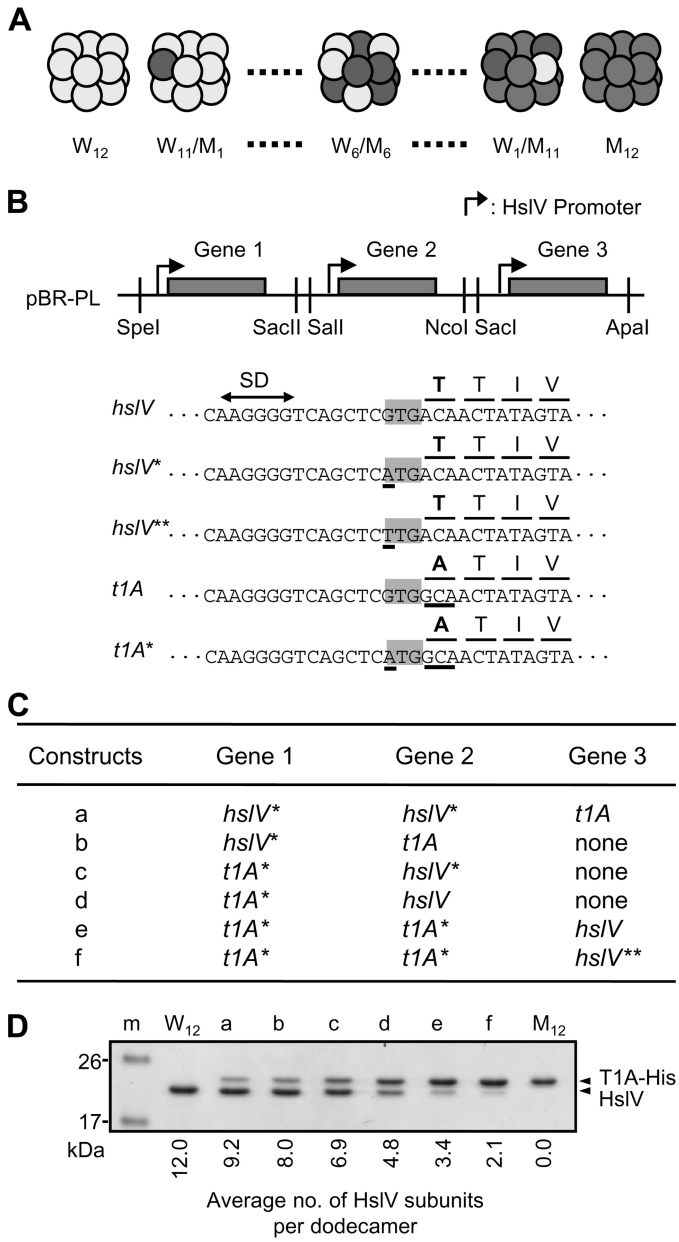

FIGURE 2. **Generation of mixed dodecamers having varied numbers of HslV and T1A subunits.** *A*, schematic diagram shows mixed dodecamers consisting of increasing numbers of wild-type HslV (*W*) and mutant T1A subunits (*M*). *B*, each of the *hslV* gene segments contains its original promoter and Shine-Dalgano (*SD*) sequence. *Underlining* indicates the nucleotides mutated from the original sequences to generate different start codons and T1A. *C*, vector constructs (*lines a–f*) used for *in vivo* generation of mixed dodecamers are listed. The start codons used in the constructs were GTG in *hslV*, ATG in *hslV**, TTG in *hslV***, GTG in *t1A*, and ATG in *t1A**. *D*, 2 μg each of purified HslV (W_{12}), T1A (M_{12}), and mixed dodecamers (*lines a–f*) was subjected to SDS-PAGE followed by staining with Coomassie Blue R-250. The intensity of each band was scanned by using a densitometer for the estimation of the number of HslV subunits (indicated by *numerals* below the *gel panel*) in each dodecamer. *Lane m* indicates size markers.

HslU and to the activation of the HslU ATPase. Although a pure T1A dodecamer could stimulate the HslU ATPase activity to the same extent as a wild-type HslV dodecamer (Fig. 1*B*), we examined whether increasing the number of T1A subunits in a dodecamer might influence the ability of the remaining HslV subunits to stimulate the HslU ATPase activity. Each of the mixed dodecamers stimulated the ATPase activity of HslU almost to the same extent seen with the wild-type HslV dodecamer, regardless of the number of T1A subunits (Fig. 3*C*), suggesting that T1A subunits in mixed dodecamers interact with HslU as well as wild-type HslV subunits. To confirm this finding, each of mixed dodecamers was incubated with HslU followed by NTA pull-down analysis. The amounts of HslU co-precipitated with mixed dodecamers were the same, regardless of the number of T1A-His subunits in a dodecamer (Fig. 3*D*). These results indicate that the observed effect on peptide hydrolysis caused by increasing the number of T1A subunits in a dodecamer is not due to the influence of T1A subunits on the intrinsic affinity between HslV and HslU.

Effects of Increasing Numbers of T1A Subunits in an HslV Dodecamer on Protein Degradation—We next examined the effect of increasing the numbers of T1A subunits in a dodecamer on protein breakdown by using two different types of substrates: SulA and Arc as a model for folded proteins and α-casein and Arc/I37A as a model for unfolded protein (26). SulA is a bacterial cell division inhibitor protein (27, 28), and Arc is a bacteriophage P22 protein that functions as a transcriptional repressor (29). Arc normally folds as a dimer, but the I37A mutation (Arc/I37A) causes it to behave as a molten globule by disrupting the dimerization (30, 31). Both SulA and Arc require an energy-dependent unfolding step for their translocation into and subsequent degradation in the inner chamber of HslV (30). On the other hand, hydrolysis of α-casein and Arc/I37A by HslVU does not strictly depend on ATP hydrolysis; these substrates can be degraded in the presence of ATPγS, a nonhydrolyzable ATP analog, although less efficiently than with ATP (Table 1).

As shown in Fig. 4, similar to peptide hydrolysis, the activity of mixed dodecamers having up to ~6 inactive T1A-His subunits (indicated by *lanes a–c*) were nearly the same as that of an HslV dodecamers (W_{12}) toward all of the tested protein substrates. Upon a further increase in the number of T1A-His subunits (*lanes d–f*), the activity decreased linearly. These results suggest that approximately half of the 12 active sites are sufficient for the full proteolytic activity regardless of the substrate (*i.e.* peptides, unfolded polypeptides, or native folded proteins). A possible explanation for these observations would be that HslVU utilizes a maximum of ~6 active sites at any given time.

Effects of Increasing the Numbers of T1A Subunits on the Inhibitor-mediated Increase in the Interaction of HslV with HslU—We have recently shown that in the presence of ATP, proteasome inhibitors, such as lactacystin and NLVS, markedly increase the interaction between HslV and HslU and cause the activation of the HslU ATPase (17). Considering that the binding of the inhibitors (particularly of peptide inhibitors) to the Thr[1] active sites likely mimics the substrate-bound state of HslV, we have suggested that substrate binding stabilizes the HslVU complex and that this stabilization might be required for substrate unfolding by HslU and translocation into the inner chamber of HslV (17).

Because T1A does not have the catalytic hydroxyl group that is required for inhibitors to form a covalent bond to HslV, it is unlikely that the inhibitors bind stably to the active sites of T1A. However, we cannot exclude the possibility that the inhibitors still bind noncovalently to the active sites of T1A subunits and

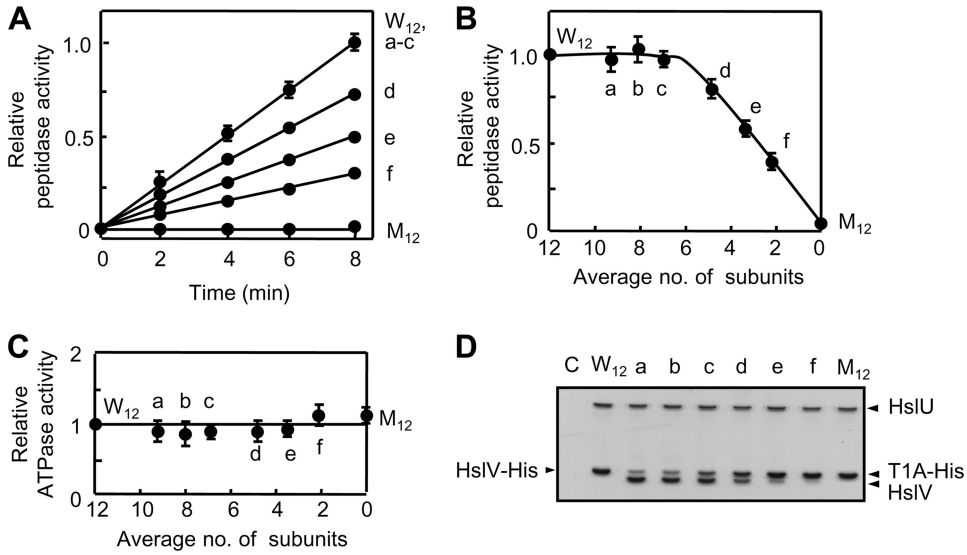

FIGURE 3. **Effects of the increasing numbers of T1A subunits in an HslV dodecamer on peptide hydrolysis.** *A*, 5 nM each of HslV (W_{12}), T1A (M_{12}), and mixed dodecamers (*lines a-f*) was incubated with HslU (10 nM) in the presence of 2 mM ATP and 0.1 mM Z-GGL-AMC at 37 °C for various periods. The peptidase activity seen with HslV dodecamer (W_{12}) by 8 min of incubation was expressed as 1.0, and the others were as its relative values. *B*, the observed rates of peptide hydrolysis in *A* were plotted against the numbers of HslV subunits in a dodecamer. *C*, ATP hydrolysis was assayed by incubation of HslU (0.2 μM) with 0.2 μM each of HslV, T1A, and mixed dodecamers. The ATPase activity seen with HslV dodecamer (W_{12}) was expressed as 1.0, and the others were expressed as its relative values. *D*, 75 nM each of HslV, T1A-His, and mixed dodecamers was incubated with HslU (150 nM), 5 mM $MgCl_2$, and 2 mM ATP at 4 °C for 1 h. The samples were then subjected to NTA pull-down analysis as described under "Experimental Procedures." For the control experiment (*lane C*), the incubation and pull-down analysis were performed in the absence of HslV.

TABLE 1

Hydrolysis of protein substrates by HslVU in the presence of ATP or ATPγS

HslV (0.4 μM) and HslU (0.4 μM) were incubated with α-casein, Arc, or monomeric Arc/I37A in the presence of 2 mM ATP or 0.5 mM ATPγS at 37 °C for 60, 10, and 5 min, respectively. The samples were then subjected to SDS-PAGE followed by staining with Coomassie Blue R-250. The extents of protein degradation were then determined by scanning the gel bands using a densitometer. Similar data were obtained by three independent experiments.

Proteins (concentrations)	Degradation	
	ATP	ATPγS
	%	
α-Casein (5 μM)	68.8	35.0
Arc (10 μM)	23.9	0.1
Arc/I37A (10 μM)	72.6	23.7

thereby affect the interaction between HslV and HslU. To address this possibility, we first examined whether lactacystin and NLVS influence the ability of T1A dodecamer to stimulate the ATPase activity of HslU. As previously reported (17), treatment with lactacystin or NLVS led to a dramatic increase in HslV-stimulated ATPase activity of HslU (Fig. 5*A*). In contrast to HslV, the proteasome inhibitors caused little or no effect on the T1A-stimulated ATPase activity of HslU. It also had no effect on the ATPase activity of HslU itself. These results suggest that the inhibitors cannot induce the formation of a stable complex between T1A and HslU, most likely because of the inability of T1A to bind the inhibitors.

Next, we extended the ATPase assays to a series of mixed dodecamers. Strikingly, lactacystin- or NLVS-mediated increase in the ATPase activity of HslU remained unaltered until the number of T1A subunits was increased to on average ~6/dodecamer and sharply declined upon further increase (Fig. 5*B*). We also tested the effects of the inhibitors on the interaction between HslU and mixed dodecamers by NTA pull-down experiments. Similarly to the ATPase assay results, treatment with either of the inhibitors led to a dramatic increase in the amount of HslU co-precipitated with HslV-His, and this increase remained unchanged until the number of inactive T1A-His subunits reached on average ~6/dodecamer (Fig. 5, *C* and *D*, points *a–c*). A further increase in the number of T1A-His subunits (*points d–f*) led to a sharp decrease in the amounts of co-precipitated HslU, finally reaching the level seen with HslV or T1A dodecamers in the absence of inhibitor. These results indicate that the ability of inhibitor-bound HslV to form a stable complex with HslU is highly correlated with its ability to activate HslU ATPase and that approximately half of the 12 active sites of HslV are sufficient for maintaining a stable HslVU complex when they are occupied by inhibitors.

Effects of Increasing the Numbers of T1A Subunits on Peptide and Protein Degradation by C10-peptide-activated HslV—Of interest are the findings that virtually the same number of active sites (*i.e.* ~6) are necessary and sufficient for the full activity of HslVU in several different assays. These results indicate that when HslVU is hydrolyzing substrates, a maximum of ~6 active sites in a dodecamer are catalytically active at any given moment. This limited utilization of the active sites might be attributed to a possible intrinsic property of HslV. However, considering that HslU dramatically affects the proteolytic activity of HslV in a nucleotide-dependent manner, it is more likely that the limitation originates from HslU rather than HslV itself.

To uncouple the activity of HslV from the allosteric control by HslU, we took advantage of a synthetic C-terminal peptide of HslU (termed C10-peptide). The C10-peptide alone can activate the HslV active sites to some extent by binding to the HslV-HslV subunit interfaces, and this allows HslV to hydrolyze peptides and unfolded proteins in the absence of HslU and ATP (32, 33). Therefore, if HslU limits the number of the HslV active sites on their simultaneous utilization, using the C10-peptide instead of HslU may allow unrestricted utilization of all of the available active sites regardless of their number in a dodecamer. To test this possibility, we first compared the activities of mixed dodecamers against Z-GGL-AMC in the presence of C10-peptide with those with HslU. In contrast to the activity pattern seen with HslU, the rates of peptide hydrolysis continuously decreased as the number of T1A subunits increased (Fig. 6*A*). Hydrolysis of α-casein or monomeric Arc/I37A in the presence of the C10-peptide also showed a continued decrease as the number of T1A subunits increased (Fig. 6, *B* and *C*), in contrast to those seen with HslU (Fig. 4). These

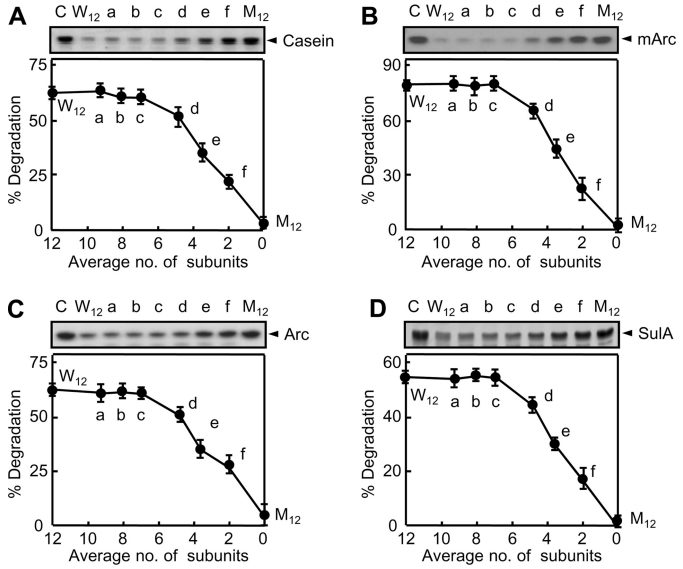

FIGURE 4. **Effects of increasing numbers of T1A subunits in an HslV dodecamer on protein degradation.** *A*, α-casein (5 μM) was incubated with HslU (0.4 μM), 2 mM ATP, 20 mM phosphocreatine, 10 units/ml of creatine kinase, and 5 mM $MgCl_2$ in the absence (*C*) and presence of 0.4 μM each of HslV (W_{12}), T1A (M_{12}), or mixed dodecamers (*lanes a–f*) at 37 °C for 1 h. The samples were then subjected to SDS-PAGE followed by staining with Coomassie Blue R-250 (*upper panel*). Extents of casein degradation, which were determined by scanning the gel bands using a densitometer, were plotted against the numbers of HslV subunits in a dodecamer (*lower panel*). *B* and *C*, experiments were performed as in *A*, but by incubation with monomeric Arc/I37A (indicated by *mArc*) or wild-type Arc (10 μM) for 15 min, respectively. *D*, maltose-binding protein-fused SulA (2 μM) was incubated with 10 units/ml of FXa, HslU (0.4 μM), 2 mM ATP, 20 mM phosphocreatine, and 10 units/ml of creatine kinase for 30 min at 37 °C. The samples were then further incubated with HslV (0.4 μM) for the next 1 h for assaying the degradation SulA.

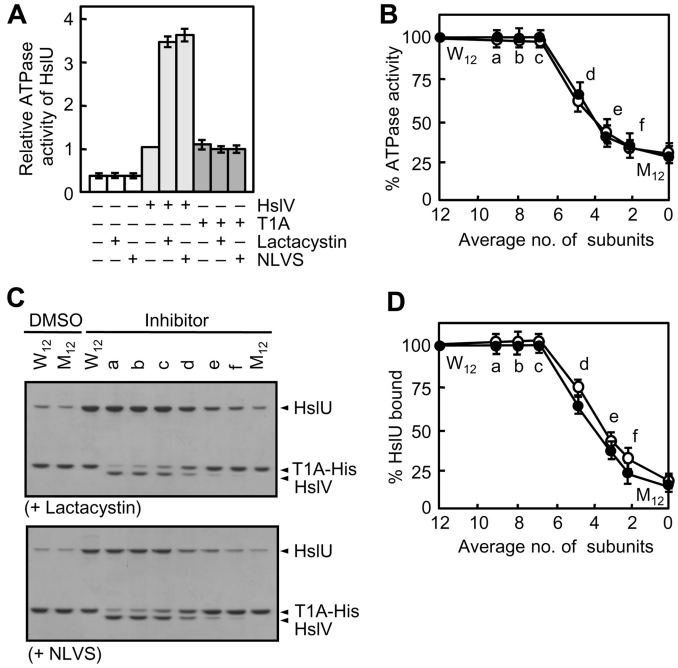

FIGURE 5. **Effects of increasing numbers of T1A subunits on proteasome inhibitor-mediated increase in the interaction between HslV and HslU.** *A*, HslU (0.2 μM) was incubated for 30 min at 37 °C with HslV or T1A dodecamers (0.2 μM) in the absence and presence of 20 μM lactacystin or 20 μM NLVS. The ATPase activity of HslU seen with HslV dodecamer was expressed as 1.0, and the others were expressed as its relative values. *B*, 0.2 μM each of HslV, T1A, and mixed dodecamers was incubated with HslU (0.2 μM) and 2 mM ATP in the presence of 20 μM lactacystin and 20 μM NLVS for 30 min at 37 °C. The ATPase activity of HslU seen by incubation with HslV in the presence of lactacystin (○) or NLVS (●) was expressed as 100%, and the others were expressed as its relative values. *C*, 75 nM each of HslV (W_{12}), T1A (M_{12}), and mixed dodecamers (*a–f*) was incubated with HslU (150 nM) and 2 mM ATP in the presence of 20 μM lactacystin (*upper panel*) and 20 μM NLVS (*lower panel*) at 4 °C for 1 h. The sample mixtures were then subjected to NTA pull-down analysis. *D*, the intensities of the HslU bands in *C* were determined by using a densitometer and plotted against the numbers of HslV subunits in a dodecamer. The intensity of the HslU band seen by incubation of HslV dodecamer (W_{12}) with lactacystin (○) or NLVS (●) was expressed as 100%, and the others were expressed as its relative values.

results suggest that in the absence of HslU, all 12 active sites may participate in the peptide bond cleavage. Thus, it appears that in the HslVU complex, HslU somehow restricts the full utilization of 12 active sites.

Mathematical Models for Stochastically Assembled HslV Dodecamers—Our results of mixed dodecamers strongly suggest that HslVU utilizes only some of its proteolytic active sites at any given moment. Because the activity of HslVU decreases only when the number of the active sites is reduced to less than on average ~6, simultaneous use of the active sites might be limited to ~6/HslVU complex. However, the stochastic nature of mixed dodecamers used in this study does not allow us to make a simple conclusion. For example, purified HslV proteins containing on average 6 wild-type HslV subunits/dodecamer should also include some other complexes with different numbers of HslV subunits. In addition, the distribution of HslV subunits and T1A subunits in two hexameric rings of dodecamers is not always uniform; in an extreme case, some dodecamers may contain all 6 HslV subunits in the first ring and all 6 T1A subunits in the second ring. These dodecamers would contribute differently to the overall proteolytic activity of a mixed population.

In an effort to address this issue, we designed a mathematical model (see supplemental material, Model A), by which we can predict the proteolytic activity of stochastically assembled dodecamers, and compared it with our experimental data. For the sake of simplicity, we initially assumed that there is no catalytic cooperativity either among HslV subunits in the hexamer or between two HslV hexamers. We also assumed that two HslV hexamers in the dodecamer contribute equally and independently to the proteolytic activity, which is in fact not necessarily the case (see "Discussion"). Using this model, we derived a set of curves presenting the average number of wild-type subunits/dodecamer *versus* predicted proteolytic activity (Fig. 7). Each curve represents each of the individual cases where we assume that the simultaneous utilization of the active site is limited to certain numbers. The comparison between these curves and the experimental data indicates that the data fit well with the model, which assumes a hexameric ring of HslV uses maximally ~3 active sites (~6/dodecamer) at any given time.

Despite a decent fit between the model and the data, we cannot completely exclude other possible scenarios because of simplifications we used for the mathematical model. A major concern might be potential cooperativity among HslV subunits (in addition to that with HslU subunits) in a hexameric ring and/or between two hexameric rings of HslV. For example, one might consider that an HslV hexameric ring would not

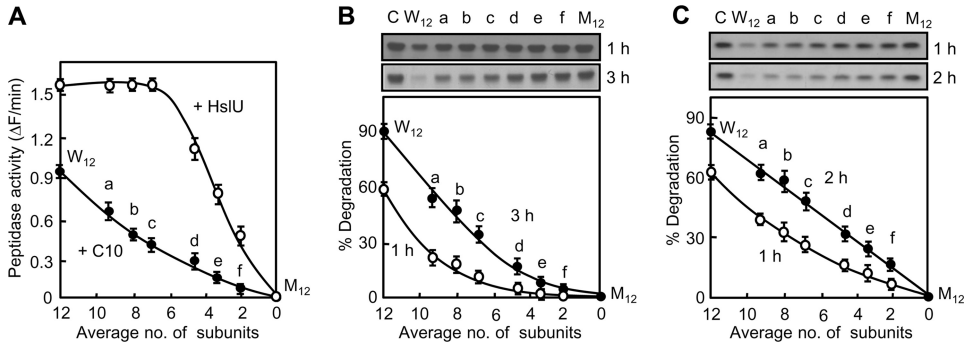

FIGURE 6. **Effects of increasing numbers of T1A subunits on peptide and α-casein degradation by C10-peptide-activated HslV.** *A*, 5 nM each of HslV (W_{12}), T1A (M_{12}), and mixed dodecamers (*points a–f*) was incubated with 30 μM C10-peptide (●) or 10 nM HslU (○) for 10 min at 37 °C and then with 0.1 mM Z-GGL-AMC for the next 10 min. The peptidase activity seen with HslV dodecamer (W_{12}) was expressed as 1.0, and the others were expressed as its relative values. *B*, 0.4 μM each of HslV, T1A, and mixed dodecamers was incubated with 30 μM C10-peptide for 10 min at 37 °C and then with 5 μM α-casein for the next 1 (○) and 3 h (●). After incubation, the samples were subjected to SDS-PAGE followed by staining with Coomassie Blue R-250 (*upper panels*). The extents of casein degradation, which were determined by scanning the gel bands using a densitometer, were then plotted against the numbers of HslV subunits in a dodecamer (*lower panel*). *C*, experiments were carried out as in *B*, but by incubation of monomeric Arc/I37A (10 μM) for 1 (○) and 2 h (●).

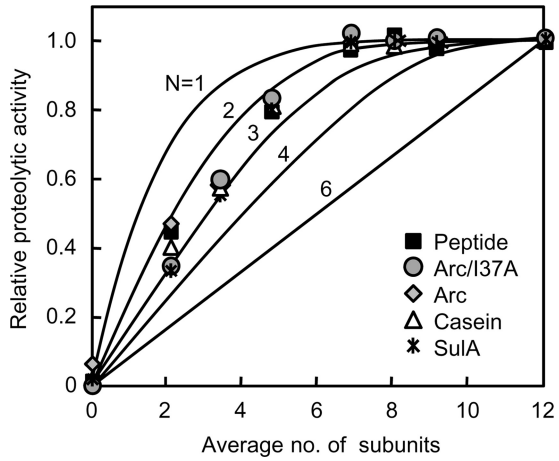

FIGURE 7. **Comparison of the proteolytic activities predicted by a mathematical model with the experimental data.** A mathematical model was devised to predict the proteolytic activity of stochastically assembled HslV dodecamers (see supplemental materials for details) and compared with the experimental data. In the model, cooperativity among HslV subunits was ignored. The letter *N* indicates the number of active sites/hexameric HslV ring assumed to be required for the full proteolytic activity of the ring.

display any proteolytic activity if the number of wild-type subunits in a ring is less than a certain number (possibly because of catalytic cooperativity among subunits in a hexameric ring). In another case, HslV dodecamers might exhibit the same proteolytic activity as long as they contain a same number of active sites; this would be true, although unlikely (see "Discussion"), if the binding of an HslU hexamer can activate both rings in an HslV dodecamer. To address this issue, we also included the all-or-none-fashion cooperativities in the mathematical model (see supplemental material, models B and C). Although we found these cooperativities affect the model curves to some extents, our experimental data still fit well with the model if assumed utilization of active sites is limited to either 2/hexamer (supplemental Fig. S1*A*) or ~6/dodecamer (supplemental Fig. S1*B*). Thus, taken together, we conclude that HslVU utilizes no more than 6 active sites at any given time. Although further studies are necessary to verify such allosteric cooperativities, the true nature of HslVU might include some intermediate cooperativity among subunits.

DISCUSSION

In the present study, we demonstrated that in the presence of HslU and ATP, ~6 of 12 active sites of HslV are sufficient for its full proteolytic activity. This finding was obtained by using mixed HslV dodecamers that have an increasing number of inactive T1A subunits in place of wild-type HslV subunits. The proteolytic activity of mixed dodecamers with up to on average 6 T1A subunits was nearly identical to that of a wild-type HslV dodecamer, regardless of the substrate tested (*i.e.* Z-GGL-AMC peptide, unfolded proteins (α-casein and monomeric Arc/I37A), or folded proteins (Arc and SulA). Upon a further increase in the number of T1A subunits, the activity toward all tested substrates decreased linearly. Moreover, the same number of active sites was required in different assay conditions; the number was not affected by varying the substrate concentrations or the molar ratio of HslU to HslV, although these concentrations altered overall proteolytic rates (supplemental Fig. S2). Thus, it is unlikely that constant proteolytic activity observed for dodecamers with more than 6 active sites is caused by diffusion limitation of substrates into the HslV proteolytic chamber. Therefore, the phenomenon would be explained if approximately half of the 12 active sites are able to participate in substrate hydrolysis at the same time, whereas the other half remains catalytically dormant.

To verify this idea, we devised mathematical models with different assumptions. Indeed, the models well agree with the data if we assume only ~6 of the 12 active sites of HslV (or up to 3/hexameric ring) are sufficient for exerting its full catalytic activity. Thus, our results strongly support the partial utilization of the HslV active sites. Because of the limited effects of tested cooperativities on the model, however, the data did not allow us to draw a definitive conclusion on whether this number is determined by the number of active sites (up to 3) per hexameric ring or the total number (~6) of active sites in a dodecamer. The latter case would be more relevant if an HslU hexamer can activate the proteolytic activity of the distal ring of HslV as well as the proximal ring in an asymmetric HslVU complex ($U_6V_6V_6$). However, we disfavor this scenario, because the activation of an HslV subunit probably requires direct binding of the C-terminal tail of an adjacent HslU subunit and because hexameric HslU and dodecameric HslV require a molar ratio of approximately 2:1 for optimal activity (14). More importantly, the cooperative interaction between two HslV rings is also argued against by the finding that in a crystal structure of an asymmetric $U_6V_6V_6$ complex, only the HslU-complexed HslV ring reacts with NLVS inhibitors, whereas the uncomplexed distal ring does not (14). Therefore, it seems more proper to

conclude that an HslV hexamer is limited to use a maximum of 3 active sites at any given time.

The partial utilization of the active sites appears to be associated with the function of HslU. When the C10-peptide instead of HslU was used as an allosteric activator to mixed dodecamers, we observed that both the peptidolytic and proteolytic activities declined linearly as the number of T1A subunits increases throughout the entire range of T1A-to-wild-type HslV subunit ratios. This finding indicates that all existing Thr^1 sites in a dodecamer equally participate in substrate binding and hydrolysis when they are bound to the C10-peptides (33). On the other hand, when an HslU hexamer docks to a hexameric ring of HslV, HslU may not simultaneously activate all 6 of the Thr^1 sites of the neighboring ring of HslV, possibly because of restrictions in conformational motions.

Considering the tight linkage between the nucleotide-binding states of HslU and the proteolytic activity of HslV, the restricted utilization of the HslV active sites is likely associated with the way HslU binds and hydrolyzes ATP. The peptidase activity of HslV, which is very weak by itself, is dramatically stimulated by HslU, and this stimulation strictly requires the presence of ATP or ATPγS. Because ADP does not allow HslU to stimulate the peptidase activity of HslV at all, only the ATP-bound HslU appears capable of exerting allosteric activation of HslV. In addition, as shown by x-ray crystal structures of HslVU, the binding of ATP to HslU is conformationally linked with the insertion of its C-terminal tails into the HslV-HslV subunit interfaces (7, 34). Therefore, utilization of the HslV active sites might depend on how many ATP molecules in an HslU hexamer bind at any given moment. By using a mutant defective in ATP hydrolysis, both HslU and ClpX (a similar ATPase in the ClpXP protease complex) have been shown to bind a maximum of three or four molecules of ATP/hexamer (35). In case of the ClpX hexamer, individual ClpX subunits have also been shown to function in a random and independent manner, arguing against sequential and concerted ATP hydrolysis models for this enzyme (35, 36). Considering the close architectural similarities between HslU and ClpX, it is likely that the HslU ATPase operates in a similar manner. Therefore, our observations on the partial utilization of the active sites during proteolysis may be explained by the fact that an HslU hexamer binds only ~3 ATP molecules at a time, assuming that ATP binding to an HslU subunit is allosterically linked to the proteolytic activation of one contacting the HslV subunit. This notion is further supported by the report that binding of ATP to HslU is noncooperative (37). A little discrepancy in the numbers could be due to the fact that in our system we used wild-type HslU, which continuously hydrolyzes ATP.

We have typically viewed the allosteric interactions between HslU and HslV as a conformational communication from the ATP-bound state of HslU to the active sites in HslV. However, it is also possible that the effects occur in the opposite direction; the catalytic states of the HslV active sites may affect the ATP binding and/or ATP cleaving activity of HslU and the interaction between HslV and HslU. Because structured protein substrates need to be unfolded and threaded into the inner chamber of HslV prior to their complete hydrolysis, the maintenance of a stable HslVU complex is critically required to prevent disengagement of partially degraded proteins during the proteolytic processes. We proposed this model based on our recent observations that covalent modification of the Thr^1 residues in HslV by proteasome inhibitors markedly stabilizes the HslVU complex, which otherwise might be highly transient (17). Furthermore, in the presence of the inhibitors, we also observed a dramatic increase in the ATPase activity of HslU, which would facilitate the substrate unfolding. These findings suggest that the inhibitor-bound state of the active site, which likely mimics the acyl-intermediate state during proteolysis, enhances the association of HslV with HslU as well as the ATP hydrolysis rate of HslU.

The results in Fig. 5 give additional quantitative information on the involvement of the active sites in maintaining the stable HslVU complex. Using a series of mixed dodecamers of which different numbers of the active sites can be occupied by the inhibitors, we show that increasing the number of the inhibitors bound to the active sites causes a gradual enhancement of the interaction between HslV and HslU as well as the ATPase activity of HslU. Remarkably, these enhancements reach a plateau after the number of Thr^1 residues in a dodecamer is increased to approximately on average of 6 and stay constant despite a further increase. These results suggest that the interaction between HslV and HslU becomes maximally stable when ~6 of the 12 active sites are occupied by the inhibitors or possibly also by substrates. It is unclear, however, whether in mixed dodecamers with more than 6 active sites, the inhibitors bind to all of the available active sites or only to a maximum of 6 active sites. The latter case would be possible if an HslU hexamer can activate only half of HslV subunits in the contacting hexameric ring of HslV, and those activated subunits are permanently fixed as "activated" by the inhibitor modification. Finally, it is noteworthy that even though the NLVS and lactacystin can induce a tight binding between HslV and HslU, this process requires the presence of ATP in the reactions (38, 39). Therefore, it is unlikely that a substrate first binds to the active site before the allosteric activation by HslU, and then this leads to a tight binding between HslV and HslU; rather, it is more likely that there exists a concerted conformational linkage among the catalytic activation of the active sites, a tight binding between HslU and HslV, and subsequent stimulation of the ATPase activity.

Taken together, we propose a revised model for how the active sites of HslV monitor their engagement in proteolytic events and control the interaction between HslV and HslU (Fig. 8). In this model, HslVU stays in a transient complex in its resting state; this enables lower ATP consumption when it is not engaged in proteolysis. When HslU recognizes a protein substrate, it begins to unfold that protein substrate, and a basal interaction between HslU and HslV probably allows HslU to thread the partially unfolded polypeptide into the inner chamber HslV. As the HslV active sites start to engage in cleaving the polypeptide chain, the interaction between HslV and HslU becomes tighter through a conformational communication from the active sites to HslU. This tight binding should be enhanced further by an increase in the number of the active sites that are engaged in proteolytic reactions until it reaches ~6. At the same time, the tight binding between HslV and HslU

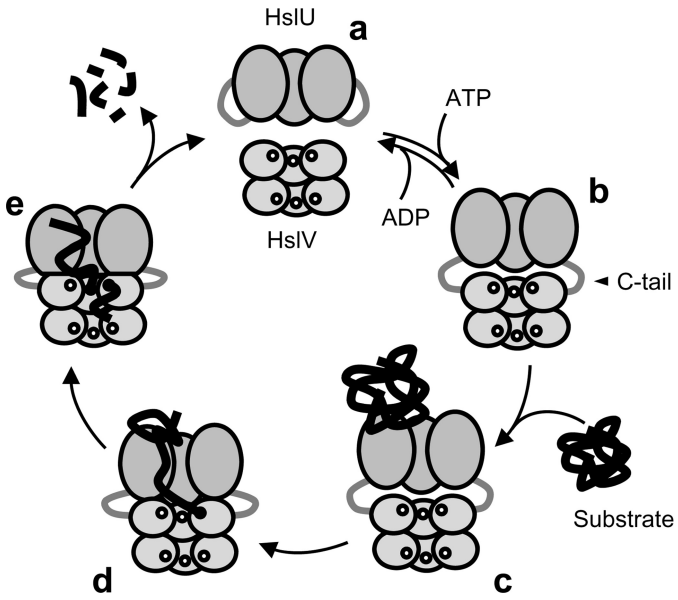

FIGURE 8. **A model for a proteolytic cycle by HslVU protease.** ATP binding may induce the movement of C-terminal tails (*C-tail*, indicated by *gray lines*) from the interfaces between HslU subunits (*a*) toward the HslV-HslV subunit interfaces (*b*), leading to intrinsic basal interaction between HslV and HslU and thus to the formation of a transient or weak HslVU complex. Upon binding of a structured protein substrate (*c*), HslU begins to unfold and translocate it to the inner chamber of HslV (*d*). The engagement of Thr^1 active sites (indicated by *small circles* in HslV) by the substrate induces a marked increase in the affinity between HslV and HslU, thus forming a tight HslVU complex and processive cleavage of peptide bonds (*d* and *e*). During this process, extensive ATP hydrolysis facilitates unfolding of protein substrates, which, however, no longer affects the affinity of HslU to HslV. Upon completion of a proteolytic cycle (*i.e.* as cleaved products leave the HslV chamber), the HslVU complex may dissociate to HslV and HslU (*a*) or be directly converted a weakly associated HslVU complex (*b*). Note that for the sake of simplicity, the diagram shows the vertically dissected half of HslVU, of which the HslU bound only to one end of HslV.

would accompany the stimulation of ATP hydrolysis by HslU, thus promoting the unfolding of the protein substrate. Upon the completion of a proteolytic cycle, HslVU would turn back to its basal state or dissociate to HslV and HslU. This active site-directed switching between a transient interaction and a tight binding of HslV with HslU would help minimize wasteful ATP hydrolysis during the resting state, while supporting high processivity for proteolysis when it is engaged with substrates. Interestingly, the inhibitor-mediated stabilization of ATP-dependent protease complexes has also been observed in eukaryotic proteasomes (40) and bacterial ClpXP (41). Therefore, it is possible that a similar mechanism exists for these machines.

Finally, it is interesting that in many organisms, proteasomes contain catalytically inactive β-subunits with noncanonical N termini in place of the functional Thr^1 residue. Although archaeal 20 S proteasomes have 14 identical Thr^1 active sites, many archaea also have additional, distantly related β-type subunits, and some of them are catalytically inactive (42). Furthermore, in eukaryotic 20 S proteasomes, only 6 N-terminal Thr residues are catalytically active among the 14 β-subunits (18–20). This number correlates well with the number of HslV active sites of that can be replaced by a catalytically inactive form without any alteration in the activity of the HslVU complex. Thus, it seems possible that during evolution, some of β-subunits became catalytically inactive while gaining other, regulatory functions, probably because such a partial loss of active sites does not result in the deterioration of proteolytic activity.

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