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

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Jung Wook Lee†1,2, Eunyong Park†1,2, Min Sun Jeong‡, Young Joo Jeon§, Soo Hyun Eom§, Jae Hong Seol‡, and Chin Ha Chung‡1,3

From the ‡School of Biological Sciences, Seoul National University, Seoul 151-742, Korea and the §Department of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea

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 (Thr1) 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 carboxbenzoxyl-Gly-Gly-Leu-7-amido-4-methyl coumarin (Z-GGL-AMC), 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 ATPyS, 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-nitrophénylacetyl-leucyl-leucyl-4-methylcoumarin (NLVS) bind to HslV in the presence of HslU and ATP and that binding of these inhibitors to the Thr1 residues dramatically increases the interaction between

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.

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1 Both authors contributed equally to this work.
2 Recipients of a fellowship from the BK21 Program.
3 To whom correspondence may be addressed. Tel.: 82-2-880-6693; Fax: 82-2-871-9193; E-mail: chchung@snu.ac.kr.

4 The abbreviations used are: Z-GGL-AMC, carboxbenzoxyl-Gly-Gly-Leu-7-amido-4-methyl coumarin; ATPyS, adenosine 5’-O-(thiotriphosphate); NLVS, 4-hydroxy-5-iodo-3-nitrophénylacetyl-Leu-Leu-Leu-vinylsulfone; NTA, nitrilotriacetic acid; Tricine, N-(2-hydroxyethyl)glycine.
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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 (T1\(\Delta\)) 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 contain multiple proteolytic active sites in their proteolytic chamber. This conclusion is based on our findings that in the presence of HslU and ATP, increasing the inactive T1A subunits up to 33476 of substrates as well as in the stable interaction between HslV and HslU. This evidence remains inconclusive because the T1\(\Delta\) 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 (QuickChange; Stratagene). All of the mutations were confirmed by DNA sequencing.

**Protein Expression and Purification**—To express HslV mixed dodecamers, BW25113 ΔhslVU::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\(^2+\)-nitrilotriacetic 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 mM 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 100 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 lactate 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 (ε\(_{340 \text{ nm}}\) = 6.22 × 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\(_{\text{em}}\) = 355 nm, λ\(_{\text{ex}}\) = 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,
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We chose to mutate Thr1 to alanine because this amino acid is essential hydroxyl group. To facilitate the purification of T1A leads to a decrease in peptide hydrolysis, presumably because of a lack of the active sites.

Recently, we have shown the deletion of Thr¹ 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 ATP.
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HsI and to the activation of the HsI ATPase. Although a pure T1A dodecamer could stimulate the HsI ATPase activity almost to the same extent seen with the wild-type HsI dodecamer, regardless of the number of T1A subunits (Fig. 3C), suggesting that T1A subunits in mixed dodecamers interact with HsI as well as wild-type HsI subunits. To confirm this finding, each of mixed dodecamers was incubated with HsI followed by NTA pull-down analysis. The amounts of HsI co-precipitated with mixed dodecamers were the same, regardless of the number of T1A-His subunits in a dodecamer (Fig. 3D). 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 HsI and HsIU.

**Effects of Increasing Numbers of T1A Subunits in an HsI 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 \( \alpha \)-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 HsI (30). On the other hand, hydrolysis of \( \alpha \)-casein and Arc/I37A by HsI does not strictly depend on ATP hydrolysis; these substrates can be degraded in the presence of ATP\( \gamma \)S, 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 \( \sim 6 \) inactive T1A-His subunits (indicated by *lanes a–c*) were nearly the same as that of an HsI dodecamer (\( W_{12} \)) toward all of the tested protein substrates. Upon a further increase in the number of T1A-His subunits (\( W_{12} \)), 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 HsI utilizes a maximum of \( \sim 6 \) active sites at any given time.

**Effects of Increasing the Numbers of T1A Subunits on the Inhibitor-mediated Increase in the Interaction of HsI with HsIU**—We have recently shown that in the presence of ATP, proteasome inhibitors, such as lactacystin and NLVS, markedly increase the interaction between HsI and HsIU and cause the activation of the HsIATPase (17). Considering that the binding of the inhibitors (particularly of peptide inhibitors) to the Thr\(^3\) active sites likely mimics the substrate-bound state of HsI, we have suggested that substrate binding stabilizes the HsI complex and that this stabilization might be required for substrate unfolding by HsI and translocation into the inner chamber of HsI (17).

Because T1A does not have the catalytic hydroxyl group that is required for inhibitors to form a covalent bond to HsI, 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...
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. 6A). 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
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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 MgCl2 in the absence (C) and presence of 0.4 μM each of HslV (W12), T1A (M12), or mixed dodecamers (lanes α–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.

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
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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,V,V). 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,V,V complex, only the HslU-complexed HslV ring reacts with NLVS inhibitors, whereas the uncleaved complexed distal ring does not (14). Therefore, it seems more proper to
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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 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 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 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 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...
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 Thr1 residue. Although archaeal 20 S proteasomes have 14 identical Thr1 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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